Fish Diseases and Disorders, Volume 1: Protozoan and Metazoan Infections Second Edition

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Fish Diseases and Disorders, Volume 1: Protozoan and Metazoan Infections Second Edition

Edited by

P.T.K. Woo

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Preface to the Second Edition

Worldwide consumption of fish and fish products has continued to escalate in the last few decades as the population increases and with the realization that fish is an excellent protein. The aquaculture industry is now the single fastest-growing food production process in the world, this is partly because fish is also less expensive to produce. Since the publication of the first edition of this trilogy on 'fish diseases and disorders', a tremendous volume of research has been conducted on parasites, especially those that cause morbidity and mortality in fish. This is reflected in the current enlarged edition; however, the aims, philosophy, focus, audience and format of this edition have remained unchanged.

Significant changes in coverage in this edition include the addition of three new chapters (Chapters 1, 19 and 20), and four of the original chapters have been completely rewritten and are by new authors (Chapters 5, 8, 9 and 12). The remaining chapters have been updated and revised, and many of them also have new co-authors. The fact that these changes are possible bodes well for the study of fish diseases as it indicates a very dynamic and rapidly evolving discipline with considerable expertise. This is especially evident in the identification of new areas of research, in the development of expertise in the younger generation of scientists and in the application of new technologies to better understand these pathogens and to devise strategies to minimize their impact on fish health.

On a more personal note, I am delighted to welcome our 16 new authors and co-authors, and I am confident their expertise, experience and insights into fish health will without a doubt make this a significantly better book. To our original authors, I am most grateful for their continued support and contributions.

As with the first edition I am hopeful that this edition will be useful to colleagues, and that it will also serve to highlight the relevance and importance of our discipline to the aquaculture industry.

Patrick T.K. Woo

Preface to the First Edition

Fin fish is the primary source of protein for humans in many parts of the world and this is especially true in most developing countries. The catch-fish industry has declined significantly and the decline is due to a series of factors which include over-fishing, loss of fish habitats and environmental pollution. In the past few decades numerous international agencies and national governments have encouraged and continue to encourage private industries to be involved in aquaculture or have themselves gone into intensive fish culture, usually under artificial and/or semi-artificial conditions. Disease outbreaks (infectious and non-infectious) with resulting high mortalities occur more often when fish are held under relatively crowded and confined conditions. Also, mass mortality of healthy fish may occur even under good environmental conditions when an infectious agent is accidentally introduced into the culture system.

This volume is the first of three proposed volumes on diseases and disorders of freshwater and marine fishes (fin and shellfish). It is on parasitic infections while the second and third volumes are on microbial and non-infectious diseases/disorders. No single author can hope to write these volumes with any authority, hence I have chosen well qualified, internationally recognized experts to write the chapters. Each chapter deals with a specific disease/disorder or a group of closely related diseases. The primary purpose is to produce comprehensive and authoritative reviews by experts who are actively working in the area or have contributed greatly to our understanding of the disease/disorder.

The principal audience for the books is research scientists in the aquaculture industry and universities, fish health consultants and managers of private and government fish health laboratories. The books are also appropriate for graduate and senior undergraduate students who are studying diseases of aquatic organisms. The series may also serve as reference text books for undergraduate and graduate courses in general parasitology, microbiology, environmental studies and for courses on impacts of diseases in aquaculture.

The secondary audience is research scientists with expertise in related disciplines (e.g. immunology, molecular biology) who wish to know about specific important fish disease(s) so that they may be able to initiate research programmes in their areas of expertise. I expect this secondary audience to increase as it becomes evident that fish disease is an important component of the aquaculture industry and that fish health can be used as an indicator of problems in the aquatic ecosystem.

P.T.K. Woo

1 Phylum Amoebozoa

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Introduction

Amoebic gill disease (AGD) of sea-caged Atlantic salmon, *Salmo salar* (L.), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), was first described by Munday (1985) soon after Atlantic salmon cage culture was initiated in Tasmania. *Neoparamoeba pemaquidensis*, the causative agent of AGD, is currently the most important pathogenic amoeba in fish and causes significant losses in marine-cultured salmonids (Munday *et al.*, 2001). The same species of amoeba also infects turbot (*Scophthalmus maximus*) in north-west Spain (Dykova *et al.*, 1995, 1998a). Other species of amoeba that have been isolated from fish are either secondary disease-causing agents or non-pathogenic. Species of amoeba of the genus *Paramoeba* are the primary disease-causing agents in sea urchin (*Strongylocentrotus droebachiensis*) (Jones, 1985; Jones and Scheibling, 1985) and in blue crab (*Callinectes sapidus*) (Sprague and Beckett, 1969; Johnson, 1977), causing morbidity and mortality. As AGD is by far the most economically significant amoeba infecting aquatic organisms, it is the only one that has been a subject of intense research.

AMOEBIC GILL DISEASE

Host Range and Geographical Distribution

Atlantic salmon and rainbow trout are most susceptible to AGD. Outbreaks of the disease in Atlantic salmon have been reported from Tasmania (Munday *et al.*, 1993), Ireland (Rodger and McArdle, 1996; Palmer *et al.*, 1997), France (Findlay and Munday, 1998), Spain (Rodger and McArdle, 1996) and Washington State, USA (Douglas-Helders *et al.*, 2001a). The disease has not been reported from Canada, Iceland, Scotland or Norway.

Apart from outbreaks in rainbow trout, AGD is not common in *Oncorhynchus* spp. (Munday *et al.*, 2001). New Zealand produces a greater tonnage of salmonid products than Tasmania; however, under very similar environmental conditions, only a few minor outbreaks of AGD have been diagnosed in the chinook salmon, *Oncorhynchus tshawytscha*. There was only one significant outbreak of the disease in the coho salmon, *Oncorhynchus kisutch*, although it was monitored in Washington State and California from 1985 to 1987

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(Kent *et al.*, 1988). AGD has also been diagnosed in sea-caged brown trout, *Salmo trutta* L., in France (Munday *et al.*, 2001).

AGD due to *N. pemaquidensis* has also emerged as a problem of turbot in north-west Spain since 1995 (Dykova *et al.*, 1995, 1998a). No such problem exists with turbot culture in France, even though the disease is present in salmonids there (Munday *et al.*, 2001). There have also been reports on AGD in European sea bass, *Dicentrarchus labrax*, and sharp-snout sea bream, *Diplodus puntazzo* (Dykova and Novoa, 2001).

Foster and Percival (1988b) identified *N. pemaquidensis* on the gills of wild, immature couta, *Thyrsites atun* (Euphrasen), caught in the vicinity of infected salmonids in Tasmania, but the parasite has not been identified on wild fish since (Dawson, 1999; Nowak *et al.*, 2000; Douglas-Helders *et al.*, 2002).

Systematics and Taxonomy

Amoeba describes an assemblage of unicellular eukaryotes that use pseudopodia for locomotion and prey capture. Amoebae are members of the phylum Amoebozoa and are characterized by amoeboid movement. Taxonomy of amoeba is based on morphological characteristics, and recently also on molecular characteristics of the single-stranded ribosomal RNA (ssrRNA) gene (Sims *et al.*, 1999). Morphological characteristics that are used to identify the aetiological agent of AGD include habitat, dimensions, nuclear characteristics, size, shape and number of pseudopodia, and surface filaments and microscales (Page, 1970, 1973, 1987; Cann and Page, 1982; Kent *et al.*, 1988; Dykova *et al.*, 2000). The parasome (*Nebenkörper*) *Perkinsiella amoeba*, a symbiotic organism that lives in the cytoplasm (Perkins and Castagna, 1971; Hollande, 1980), is also often used as a diagnostic feature (Page, 1970, 1973; Cann and Page, 1982).

N. pemaquidensis belongs to the phylum Amoebozoa, class Lobosea, subclass Gymnamoebia, order Euamoebida and family Vexilliferidae (Page, 1987). This gill-associated organism was initially placed in the genus *Paramoeba* (Roubal *et al.*, 1989), and described as *Paramoeba pemaquidensis* by Kent *et al.* (1988). Page (1987) reclassified 'naked' amoeba, and re-described the organism as *Neoparamoeba pemaquidensis.* This name is generally accepted and is used in the recent literature.

Morphology and Life Cycle

Amoebae (Fig. 1.1) freshly removed from infected gills appear as subspherical (15–40 μm diameter), transitional forms with up to 50 digitate pseudopodia (Kent *et al.*, 1988; Roubal *et al.*, 1989; Munday *et al.*, 1990; Rodger and McArdle, 1996; Dykova *et al.*, 1998a). The organism has a nucleus (≈ 5 μm diameter) and one or more parasomes (≈ 4 μm), which stain positively with Feulgen. It has an extensively flattened hyaline zone, with irregular anterior margin, and occasionally conical pseudopodia projecting ahead of the anterior margin in locomotion. The organism has a floating form with fine pseudopodia radiating from a central cell mass (Page, 1970; Cann and Page, 1982). Electron microscopy reveals numerous adherent filaments, extending 350 nm from the cell surface (Kent *et al.*, 1988; Dykova *et al.*, 1998a). These are absent in *N. pemaquidensis* from culture (Kent *et al.*, 1988; Dykova *et al.*, 2000). Amoebae from gills are significantly larger than those from cultures (Kent *et al.*, 1988; Roubal *et al.*, 1989; Dykova *et al.*, 2000).

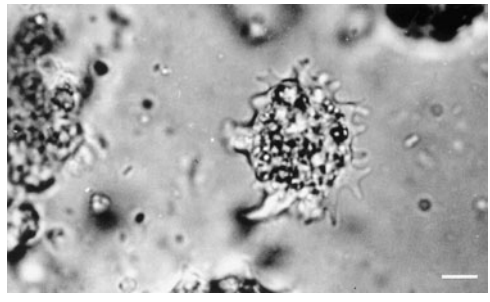


Fig. 1.1. Transitional form of *N. pemaquidensis* with digitate pseudopodia (×4740). Courtesy of V. Findlay.

In histological sections, the amoeba (Fig. 1.2) is vacuolated and frequently closely adherent to the gill epithelium (Roubal *et al.*, 1989; Munday *et al.*, 1990; Dykova *et al.*, 1995), an observation confirmed using the electron microscope (Roubal *et al.*, 1989).

Host–Parasite Relationship

Clinical and pathological features

Peak mortalities in infected Atlantic salmon smolts in Tasmania may reach 10% per week (Foster and Percival, 1988b) while those in turbot (500 g) in Spain may range between 5 and 20% over a period of 3 months, from October to December (Dykova *et al.*, 1998a).

Clinical signs include lethargy and respiratory distress, with salmonids rising to the surface of the water and displaying increased opercular movements (Kent *et al.*, 1988; Munday *et al.*, 1990; Rodger and McArdle, 1996). However, in experimental studies, Powell *et al.* (2000) were unable to confirm that AGD-infected fish actually had a measurable increase in ventilation frequency.

In Atlantic salmon, macroscopic lesions are usually multifocal patches of white to grey swollen gill tissue with associated excess mucus (Fig. 1.3; Munday *et al.*, 1990; Rodger and McArdle, 1996). These are most numerous in the dorsal portions of the gill arch (Adams, 2000; Adams and Nowak, 2001). In rainbow trout the mucoid branchitis is more diffuse (Munday *et al.*, 2001). Infected turbot have behavioural alterations, namely, reduced feeding and reverse posture (ventral side up), gills covered with excess mucus, clubbed, necrotic gill filaments with patches of greyish peripheral discoloration (Dykova *et al.*, 1998a).

Histopathological lesions in salmonids and turbot are similar, with the main lesion being hyperplasia and hypertrophy of the gill epithelium (Kent *et al.*, 1988; Roubal *et al.*, 1989; Munday *et al.*, 1990; Dykova *et al.*, 1995; Rodger and McArdle, 1996; Zilberg and Munday, 2000). The sequential development of the disease in experimentally infected Atlantic salmon was described

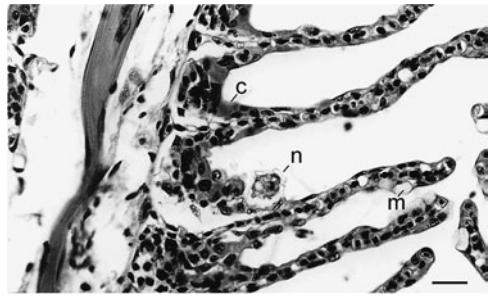


Fig. 1.2. *N. pemaquidensis* (n) attached to healthy-appearing gill lamellae at 2 DPE to fish with AGD (mucus cell, m; chloride cell, c; H & E, ×1580) (Zilberg and Munday, 2000).



Fig. 1.3. Heavy amoebic gill disease infection in Atlantic salmon, showing discrete and coalesced patches (Munday *et al.*, 2001).

by Zilberg and Munday (2000). Briefly, at 2 days post-exposure (DPE) to AGD-infected fish, *N. pemaquidensis* were seen on the gills, which were otherwise normal (Fig. 1.2). By 4 DPE, multifocal hyperplasia and lamellar fusion appeared, involving up to 15 gill lamellae per foci by 7 DPE. Epithelial cells were both hyperplastic and hypertrophic and the tissues were spongiotic (Fig. 1.4). *N. pemaquidensis* was more abundant, associated mainly with the hyperplastic epithelium and, in some instances, was sloughed off with sheets of branchial tissue (Fig. 1.5). Also, a marked increase in mucus cells was noted. At 28 DPE, epithelial hyperplasia and lamellar fusion were extensive, with numerous associated *N. pemaquidensis* (Fig. 1.5). Occasionally interlamellar vesicles developed, often containing amoebae and inflammatory infiltrates in the supporting tissues

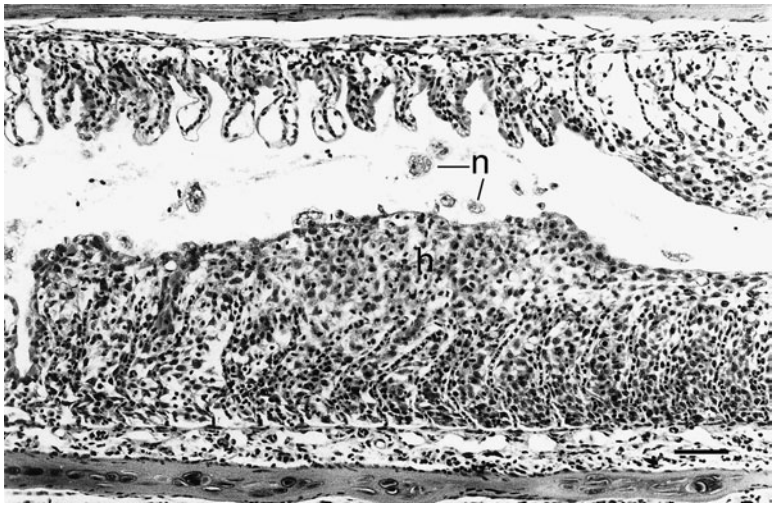


Fig. 1.4. Hyperplastic and oedematous gill tissue (h) at 14 DPE to fish with AGD. *N. pemaquidensis* (n) can be seen between gill filaments, both attached to and detached from the epithelium (H & E, ×480) (Zilberg and Munday, 2000).

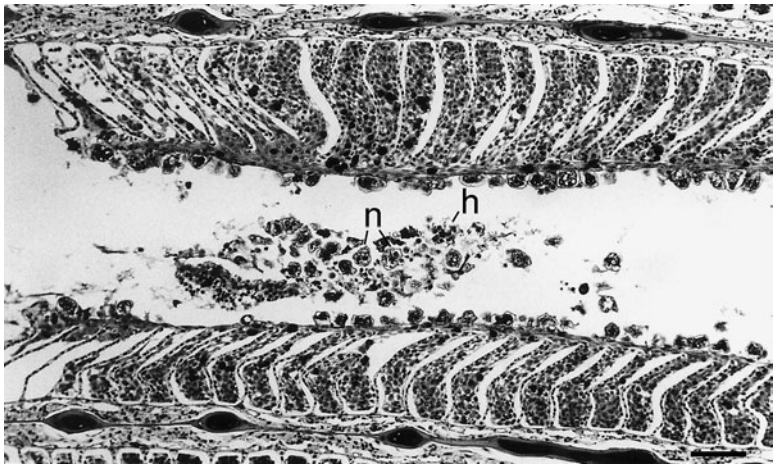


Fig. 1.5. Extensive hyperplasia and associated *N. pemaquidensis* on gills of fish at 28 DPE to fish with AGD. *N. pemaquidensis* (n) both attached to the epithelium and associated with exfoliated hyperplastic cells (h) (×400) (Zilberg and Munday, 2000).

(Munday *et al.*, 1990; Dykova *et al.*, 1995, 1998a). *Neoparamoeba pemaquidensis* is confined to the gill surface, and there is no evidence of histopathological changes in internal organs (Kent *et al.*, 1988; Zilberg and Munday, 2000).

The crucial metabolic perturbations associated with AGD are still unclear. Severely affected fish have elevated blood sodium levels (Munday *et al.*, 1990) and respiratory acidosis (Powell *et al.*, 2000). But these abnormalities are not widespread or severe enough to explain the clinical signs.

Pathogenesis

N. pemaquidensis is capable of colonizing the normal gill epithelium (Zilberg and Munday, 2000), probably by lectin/glycoconjugate

bonds. It is likely that the glycoconjugate is galactose or *N*-acetylgalactosamine (Findlay, 2001). This mechanism may also be involved in the apparent affinity of *N. pemaquidensis* for hyperplastic gill epithelium (Munday, *et al.*, 1990; Nowak and Munday, 1994; Dykova, *et al.*, 1995). A vicious cycle seems to develop whereby the infection leads to the production of hyperplastic gill epithelium, which attracts more amoebae, and so on, until the fish dies, host immunity intervenes and/or the environmental conditions become less suitable for the pathogen. This will be discussed further below under 'Epizootiology'.

Epizootiology

There is some evidence from experimental studies that *N. pemaquidensis* becomes more virulent with sequential passage through naïve hosts (Findlay *et al.*, 2000). The minimum infectious dose for Atlantic salmon in an experimental setting is about 230 *N. pemaquidensis* per litre of water (Zilberg *et al.*, 2001). A single smolt with AGD may have several hundred thousands of amoebae (D. Zilberg and B.L. Munday, 1999, Tasmania, unpublished); consequently the danger posed by a cage of infected fish is tremendous. Even dead fish have live *N. pemaquidensis* for up to 3 days *post mortem* (Douglas-Helders *et al.*, 2000b) so the danger does not cease with the demise of the fish.

Atlantic salmon and rainbow trout appear to be the most susceptible salmonids. There is good field and experimental evidence that previously exposed fish acquire a degree of resistance to reinfection after a freshwater bath (Foster and Percival, 1988a; Findlay *et al.*, 1995; Findlay and Munday, 1998). However, this is only relative and can be overcome by a large challenge dose. Minor hyperplastic lesions on the gills are associated with some increase in severity of AGD (Nowak and Munday, 1994; Zilberg and Munday, 2000) but are probably not significant in deciding the eventual outcome. Severe gill lesions from jellyfish damage and clubbing and necrosis gill syndrome are sometimes rapidly colonized by *N. pemaquidensis* with drastic consequences (J. Handlinger, 2000, personal communication).

Clinical AGD is always associated with water temperatures coupled with salinity. In general, outbreaks in salmonids have only occurred at water temperatures between 12 and 20°C (Kent *et al.*, 1988; Munday *et al.*, 1990; Rodger and McArdle, 1996) although amoebae are found on the gills at 9–10°C (Munday *et al.*, 1990; Clark and Nowak, 1999; Douglas-Helders *et al.*, 2001c). In experimental infections the severity of the disease can be effectively manipulated within the range of 12–14°C (Findlay, 2001) but at 16–18°C it is very difficult to control (Akhlaghi *et al.*, 1996; B.L. Munday, 1994, unpublished observation). Similarly, the number of *N. pemaquidensis* in the water within the vicinity of the cages is elevated during the summer (Douglas-Helders *et al.*, 2002).

The situation in relation to salinity is intriguing. All reported long-term infections in salmonids have been associated with high salinity (≥ 32 p.p.t.) (Foster and Percival, 1988b; Munday *et al.*, 1990; Rodger and McArdle, 1996; Clark and Nowak, 1999). In contrast, AGD in turbot has occurred at a constant salinity of 22 p.p.t., a salinity at which amoebae disappear from the gills of infected rainbow trout within 24 h (D. Zilberg and B.L. Munday, 1999, unpublished).

Neoparamoeba Page, 1987 is the gynamoeba (naked amoeba) most frequently isolated from marine habitats. Individual species, including *N. pemaquidensis*, have wide distribution throughout the world, and are found both in open waters and in sediments (Page, 1970, 1973; Cann and Page, 1982). This may explain the occurrence of the disease around the world. Similarly, *N. pemaquidensis* was identified in sediments from fish farming areas and also away from fish farms in Tasmania (Crosbie *et al.*, 2002; Nowak *et al.*, 2002). This finding provides confirmation that the protozoan is also widespread in the marine environment around Tasmania.

N. pemaquidensis was found on the biofouling that naturally develops on salmon nets in cage cultures, but the contribution of amoeba on the biofouling is still questioned

(Tan *et al.*, 2002). Non-fouled nets and nets treated with anti-foulants have more *N. pemaquidensis* on them than untreated nets (Foster and Percival, 1988b; Tan *et al.*, 2000) and increase the prevalence of AGD (Douglas-Helders *et al.*, 2003a). A possible explanation for this is the increased load of bacteria on non-fouled nets, which is a major food source for *N. pemaquidensis*.

The disease is spread in the water. *N. pemaquidensis* has been detected in waters over 1 km from cages stocked with infected fish (Douglas-Helders *et al.*, 2002). The parasite can survive in the water and maintain its infectivity for at least 14 days (Douglas-Helders *et al.*, 2003b).

Immunity

Atlantic salmon can develop resistance to AGD. Infected fish maintained in fresh water for 4 weeks were parasite-free and resistant to experimental reinfection (Findlay *et al.*, 1995; Findlay and Munday, 1998). Two freshwater baths (current treatment for the disease) also produced similar resistance in laboratory trials (Findlay and Munday, 1998). The situation is apparently different under field conditions as occasionally up to eight bath treatments are required during a single growth cycle in sea cages (Mitchell, 2001). Serum antibodies have been identified in previously infected fish, but these do not correlate with resistance to the disease (Findlay *et al.*, 1995). Antibodies against *N. pemaquidensis* do not alter its ability to induce AGD (Zilberg and Munday, 2001b). This suggests that a non-specific or acquired cellular immune response, or a combination of both, may be responsible for the acquired resistance. Thus far, all efforts to immunize fish against AGD using different exposure regimes to *N. pemaquidensis* antigens have failed (Akhlaghi *et al.*, 1996; Zilberg and Munday, 2001a).

In Vitro Culture and Propagation

Gill-associated *N. pemaquidensis* grows on seawater agar seeded with numerous species of live and autoclaved bacteria (Page, 1973; Cann and Page, 1982; Kent *et al.*, 1988; Munday *et al.*, 1990; Dykova *et al.*, 1998a, 2000). Optimal growth occurs in a salinity of 15 p.p.t. and at 20°C (Kent *et al.*, 1988).

Despite a number of attempts, it has not been possible to produce AGD in fish with cultured organisms (Kent *et al.*, 1988; Howard *et al.*, 1993; Findlay, 2001). AGD has only been achieved by cohabiting naïve fish with infected fish (Howard *et al.*, 1993; Akhlaghi *et al.*, 1996; Findlay, 2001) or by exposing them to isolated *N. pemaquidensis* from infected gills (Zilberg *et al.*, 2001).

Nutrition and Physiology

N. pemaquidensis feeds on bacteria by putting out short pseudopodia, forming a 'food cup' around a bacterium, and also by engulfing bacteria by local invagination of the anterior margin of the hyaline zone (Page, 1970). Partially digested bacteria have been observed in food vacuoles (Cann and Page, 1982) and in the cytoplasm of *N. pemaquidensis* from fish gills (Roubal *et al.*, 1989; Dykova *et al.*, 1998a).

N. pemaquidensis reproduces by binary fusion, with accompanying division of nucleus and parasome. Divisions of the nucleus and parasome are not simultaneous, but each daughter cell has its own parasome (Page, 1970).

Diagnosis of Infection

It is important to distinguish between AGD and the presence of *N. pemaquidensis* with no or inconsequential gill pathology. For the aquaculturist the former is the crucial diagnosis, whereas clinicians and particularly researchers have an interest in both these aspects of the diagnosis. On-farm diagnosis is done by counting the typical mucoid patches on the gills (Alexander, 1991). While this has been shown to be highly correlated with the presence of *N. pemaquidensis* in experimental infections on Atlantic salmon (Zilberg and Munday,

2000; Findlay, 2001), the association is inconsistent in the field (Clark and Nowak, 1999). In turbot, grossly visible lesions are not necessarily present in infected fish (Dykova and Novoa, 2001).

Several diagnostic methods are available for the confirmation of a clinical diagnosis. Wet mounts taken from fish gills can be stained using an immunofluorescent antibody test (IFAT) (Howard and Carson, 1993) or with Quick Dip® (Fronine Pty Ltd, Riverstone, New South Wales, Australia) (Zilberg *et al.*, 1999). The latter is somewhat problematic in that amoebae can be difficult to differentiate from gill epithelial cells. A dot-blot technique has also been developed (Douglas-Helders *et al.*, 2000a, 2001a). According to Douglas-Helders *et al.* (2001a), the test appears to be sensitive and specific, and is suited for mass screening of fish.

In a more elaborate method, concentrating mucus washed off with seawater and applying a drop of the loose pellet on a coverslip in a wet chamber for 30 min allows trophozoites to attach to the coverslip (Dykova and Novoa, 2001). Trophozoites can be directly observed under a light microscope, or fixed with Davidson's fixative and stained with haematoxylin and eosin (H & E), Feulgen reaction and DNA staining.

Gill samples can also be examined using histological methods. In experimental infections, the amoebae are seen attaching to the gill epithelium at 2 DPE – this is the earliest of any method to confirm an infection (Zilberg and Munday, 2000). IFAT is positive in 7 DPE (Zilberg and Munday, 2000). A specific PCR has been developed; it identifies the parasite with 95% certainty (Wong and Elliot, 2000; Elliot *et al.*, 2001), and is suitable for use with fish gills, sea water and biofouling.

Polyclonal antibodies developed against the Tasmanian isolate (Howard and Carson, 1993) react with organisms present on fish in Ireland (Rodger and McArdle, 1996), New Zealand and France (T.S. Howard and J. Carson, 1999, Tasmania, personal communications). According to PCR assays, organisms recovered from AGD on salmonids in Australia, Ireland and Washington State and from turbot in Spain are identical. However, as the American and Spanish organisms can survive in much lower salinities than the Australian *N. pemaquidensis*, it appears that the assay does not distinguish between biovars with different physiological characteristics.

Prevention and Control

Treatment

The most effective and frequently used treatment is freshwater baths. Briefly, fish are bathed in fresh water close to zero salinity for 2–6 h (Foster and Percival, 1988a). According to Munday *et al.* (1990), a freshwater bath has three effects; it reduces the number of amoebae on the gills, removes seawater-stable mucus and reduces any hypernatraemia which may develop.

After a 2–6 h, normal, on-farm, freshwater bath, up to 27% of the amoebae remain viable and these organisms are capable of initiating AGD under experimental conditions (Clark *et al.*, 2000; Findlay, 2001). A freshwater bath, however, dramatically reduces the number of gill lesions (Parsons *et al.*, 2001). Thus, killing the amoebae on the gills seems to be less important in comparison to returning the gill epithelium to normality as a result of the treatment. In fact, removal of mucus from the gills seems to be the most important function of a freshwater bath, as this process removes amoebae mechanically in a manner similar to natural exfoliation of gill mucus (Fig. 1.5; Zilberg and Munday, 2000). In the short term, it potentially alleviates the physiological perturbations produced by AGD (Powell *et al.*, 2000; Powell and Nowak, 2001). The efficiency of a freshwater bath is improved when soft water is used (Roberts and Powell, 2002). This is probably due to enhanced removal of less viscous mucus, as binding with $CaCO_3$ increases mucus viscosity. It is probable, but unproven, that the replacement mucus is rich in such immune components as lysozyme, which help to inhibit recolonization of the gills by *N. pemaquidensis.*

As only severely affected fish have hypernatraemia (Findlay, 2001), reversal of this change is likely to be of only minor importance.

A large number of other treatments have been tried. The chemicals that have some effects on pre-existing AGD are narasin ((4*S*)-4-methylsalinomycin)) (Cameron, 1992), levamisole (1-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole) (Zilberg *et al.*, 2000), chlorine dioxide and chloramine-T (Powell and Clark, 2001; Powell *et al.*, 2002). However, none is as effective and safe as freshwater baths.

Control

Control of AGD on salmonids in Tasmania has revolved mainly around the timing of freshwater baths, some of which are regarded as prophylactic (Douglas-Helders *et al.*, 2001b), and some farmers avoid the problem entirely or partially by utilizing low-salinity sites for all or part of the marine culture phase.

The Tasmanian industry has kept multiple classes of stock (spring smolts, out-of-season smolts, etc.) on a single site. Although no objective data are available, the apparent increase in virulence of *N. pemaquidensis* with serial passage under experimental conditions suggests that the 'all-in all-out' strategy may be more desirable. In this strategy, once smolts are stocked at a site they are grown and harvested, and only then can the next stocking be carried out. Similarly, increased biomass at individual lease sites inevitably reduces distances between cages, thereby rendering more likely the transfer of infection from cages with clinically affected fish to other cages. Undoubtedly, the effects of management strategies on the occurrence and severity of AGD will require more intensive investigation in the future.

Although chinook salmon are more difficult to culture than Atlantic salmon and rainbow trout, there could be merit in having at least a proportion of these fish at a site if their apparent relative resistance to AGD can be confirmed.

Summary and Conclusions

Amoebic gill disease due to *N. pemaquidensis* is a major constraint on salmonid mariculture in Tasmania. It is also a cause of intermittent, serious, disease outbreak in Atlantic salmon in Ireland, France and North America and an emerging problem in turbot culture in Spain. For salmonids, treatment with freshwater baths is the only proven therapy but this is stressful for the fish. In view of the relatively low salinity at which AGD occurs in turbot, it is doubtful that freshwater baths will be as successful as with salmonids.

Wild fish in Tasmania are not known to be susceptible to the disease, although they have small numbers of *N. pemaquidensis* on their gills. It is yet to be determined if such 'carrier' fish are a source of *N. pemaquidensis* that can produce AGD in cage-cultured salmonids.

Research to date has not shown that immunization is likely to be a useful prophylactic measure, although the use of extracellular products as vaccine candidates has not been explored.

There is an acute lack of information related to the effects of such managerial practices as keeping fish of different ages at one site and increasing overall stocking levels at individual sites on disease outbreaks.

Future Research Directions

Host resistance and immune factors

1. It is anomalous that nothing is known about the effects, if any, of a freshwater bath on the immune status of salmonids with AGD. Studies to elucidate this may well provide information, which will enable better use of immunomodulators.

2. Research on possible immune responses to extracellular products may lead to development of protective vaccines, which has been elusive to date.

3. Identification of lectin/receptor complexes responsible for *N. pemaquidensis* colonizing the gills may lead to the

Table 1.1. Different species of amoeba that were reported in the literature, isolated from different species of aquatic organisms around the world.

Species	Host	Organ	Geographic location	Reference
Acanthamoeba sp.	Perch (*Perca fluviatilis*)	Brain, liver,	Czech Republic	Dykova *et al*., 1999b
	Silver, white bream (*Blicca bjoerkna*)	Spleen		
	Gymnocephalus cernuus	Brain		
	Chub (*Leuciscus cephalus*)	Brain, kidney		
	Rutilus rutilus	Kidney		
	Sheat-fish (*Silurus glanis*)	Spleen		
A. polyphaga	White sucker (*Catostomus commersoni*) and common shiner (*Notropis cornutus*)	Intestine	New York	Franke and Mackiewicz, 1982
	Blue tilapia (*Tilapia aurea*),[1] largemouth bass (*Micropterus salmoides*), striped bass (*Morone saxatilis*), channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*), rainbow trout (*Salmo gairdneri*), carp (*Cyprinus carpio*)	Gills, urinary bladder, spleen, gall bladder, blood	South-eastern USA	Taylor, 1977
Cochlipodium sp.[2]	Rainbow trout	Gills	West Virginia, USA	Noble *et al*., 1997
C. minus	Perch	Liver, kidney, brain, spleen, gills	Czech Republic	Dykova *et al*., 1998b
Flaballula citata[2]	Turbot (*Scophthalmus maximus*)	Gills		Dykova *et al*., 1999a
F. calkinsi[2]	Turbot	Gills		
Hartmannella sp.	White sucker (*Catostomus commersoni*), common shiner (*Notropis cornutus*)	Intestine	New York	Franke and Mackiewicz, 1982
H. vermiformis[3]	*Tilapia nilotica* (*Oreochromis niloticus*)	Kidney	Czech Republic (farm)	Dykova *et al*., 1997
Naegleria sp.	*Tilapia nilotica*	Gills	Alabama, USA	Taylor, 1977
	Perch	Spleen, kidney	Czech Republic	Dykova *et al*., 2001
	Bream	Brain		
	Rainbow trout	Gills		
N. australinesis	Catfish (hybrid)	Brain	Thailand (farm)	Dykova *et al*., 2001
N. gruberi	White sucker (*Catostomus commersoni*), common shiner (*Notropis cornutus*)	Intestine	New York	Franke and Mackiewicz, 1982
Paramoeba invadens[1]	Sea urchin	Nerve, water vascular tissue	Nova Scotia, Canada	Jones, 1985; Jones and Scheibling, 1985

Continued

Table 1.1. *Continued*. Different species of amoeba that were reported in the literature, isolated from different species of aquatic organisms around the world.

Species	Host	Organ	Geographic location	Reference
P. perniciosa[1]	Blue crab	Connective tissue, haemal spaces, blood vessels	Maryland and Virginia, USA	Sprague and Beckett, 1969; Johnson, 1977
Platyamoeba sp.[2]	Turbot	Gills	Spain	Leiro *et al*., 1998
P. longae[2]	Turbot	Gills	Spain	Dykova *et al*., 1999a
P. murchelanoi[2]				
P. weinsteini[2]				
P. douversi[2]				
P. leei[2]				
P. plurinucleolus[2]				
Rosculus ithacus	*Tilapia nilotica*	Kidney	Czech Republic (farm)	Dykova *et al*., 1997
Saccamoeba sp.	Atlantic salmon (*Salmo salar*)	Gills	Czech Republic	Dykova *et al*., 2002
Thecamoeba sp.[2]	Rainbow trout	Gills	Oregon, USA	Sawyer *et al*., 1975
T. hoffmani[2]	Rainbow trout, coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tschawytscha*)	?	Michigan, USA	Sawyer *et al*., 1974
Vahlkampfia sp.	Blue tilapia, largemouth bass, brown trout (*Salmo trutta*)	Gills, skin mucus	South-eastern USA	Taylor, 1977
Vannella caledonica,[2]	Turbot	Gills	Spain	Dykova *et al*., 1999a
V. septentrionalis,[2]				
V. anglica[2]				
V. platypodia	Goldfish (*Carassius auratus*) Chub (*Leuciscus cephalus*)	Kidney Brain	Czech Republic	Dykova *et al*., 1998b
Vexillifera expectata	Perch	Liver	Czech Republic	Dykova *et al*., 1998c
Unidentified[2]	European catfish (*Silurus glanis*)	Gills, kidney, liver, spleen, intestine	Germany	Nash *et al*., 1988
Unidentified[2]	Arctic charr (*Salvelinus alpinus*)	Gills	Canada	Speare, 1999
Unidentified[2]	Rainbow trout	Gills	West Virginia, USA	Bullock *et al*., 1994

[1]Isolation was associated with a disease outbreak.
[2]Secondary pathogen.
[3]Pathogenicity was experimentally proven.

production of vaccines targeting key lectins involved in adherence.

4. As has been accomplished for other diseases, selective breeding for resistance to AGD may be productive. The present system of using ever-increasing numbers of freshwater baths ensures the continued susceptibility of salmonid stocks.

5. The apparent inherent resistance of chinook salmon to AGD needs to be studied more closely.

Parasite factors

1. More research is required to identify differences (apart from pathogenicity) between *N. pemaquidensis* isolated from 'carrier' fish and from fish with AGD, and to identify virulence factor(s) in the pathogen.

2. Since Padilla-Vaca *et al.* (1999) have shown that the glycoconjugate profile of the bacteria used as the nutritional lawn for *Entamoeba histolytica* is crucial in determining the pathogenicity of the organism, it is important to conduct similar studies for *N. pemaquidensis.*

3. More studies are required to understand the salinity effects on strains of *N. pemaquidensis.*

Management factors

1. The effects of introducing sequential populations of naïve fish into a single production site need to be monitored.

2. The use of epidemiological modelling to forecast the potential effects of increasing fish and/or cage numbers at any one site needs to be examined closely.

OTHER AMOEBAE

There is information in the literature regarding the isolation and identification of a large number of different species of amoeba from aquatic organisms. Some findings are incidental, unassociated with specific disease conditions, and others are associated with morbidity and mortality. Species of *Paramoeba* infect internal organs and cause morbidity and mortality in sea urchin (Jones, 1985; Jones and Scheibling, 1985) and in blue crab (Sprague and Beckett, 1969; Johnson, 1977; Table 1.1). In fish, *N. pemaquidensis* is the only amoeba that was confirmed to be a primary disease-causing agent. Several species of amoeba can cause disease as secondary pathogens, following a primary gill infection that is caused by bacteria (Bullock *et al.*, 1994; Noble *et al.*, 1997) or by *N. pemaquidensis* (Dykova *et al.*, 1999a). Some of the amoebae associated with disease conditions seem generally to be free-living, and change their mode of life under conditions that are unfavourable to the fish (except for *N. pemaquidensis*), and some are probably a part of the normally associated fauna. Table 1.1 summarizes much of the published literature on amoebae associated with aquatic organisms. Their effect as disease-causing agents, in many of the reported cases, is yet to be determined.

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2 Phylum Dinoflagellata

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Introduction/Economic Importance

Dinoflagellates are commonly found in aquatic ecosystems. They are important primary producers and consumers, as well as endosymbionts in many invertebrates (Taylor, 1987; Fensome *et al*., 1993). Many dinoflagellates produce ichthyotoxins, which have caused mass mortalities in wild and cultured fish (Noga, 1998; Rensel and Whyte, 2003). About 140 of the approximately 2000 known living species are parasites, most being parasites of invertebrates (Drebes, 1984). Five genera have been reported as fish parasites: *Amyloodinium, Piscinoodinium, Crepidoodinium, Ichthyodinium* and *Oodinioides*. One unclassified dinoflagellate has also been described (Buckland-Nicks and Reimchen, 1995). Only *Amyloodinium*, *Piscinoodium* and *Ichthyodinium* are of any known or potential economic significance. While there is evidence that the toxic dinoflagellate *Pfiesteria* can also exhibit parasitic tendencies (Vogelbein *et al*., 2002), it is not a typical parasite and thus will not be discussed in this review.

Amyloodinium

Amyloodinium ocellatum is the most common and important dinoflagellate parasitizing fish (Becker, 1977; Lawler, 1980; Lauckner, 1984; Noga, 1996). It causes serious morbidity and mortality in brackish and marine warmwater food fish at aquaculture facilities worldwide (Table 2.1) and is often considered the most consequential pathogen of marine fish (Paperna *et al*., 1981). Outbreaks can occur extremely rapidly, resulting in 100% mortality within a few days. *A. ocellatum* is also a major problem in aquarium fish (Nigrelli, 1936; Brown and Hovasse, 1946; Hojgaard, 1962; Dempster, 1972; Lawler, 1977b), including both public aquaria and hobbyist tanks. It has also very rarely caused natural epidemics, best documented in fish of the Salton Sea, a hypersaline inland lake in eastern California, USA (Kuperman *et al*., 2001).

Piscinoodinium

Piscinoodinium is morphologically and developmentally similar to *Amyloodinium* and causes a highly similar disease in tropical freshwater fish. Most reports of the parasite have been in fish held in aquaria (Jacobs, 1946; Schäperclaus, 1951; Reichenbache-Klinke, 1955; Geus, 1960; van Duijn, 1973; Lom and Schubert, 1983) but it has more recently been recognized as a commercial

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Table 2.1. Amyloodiniosis infections reported in food fish.[1, 2]

Geographical location	Hosts	References
Gulf of Mexico (Mississippi, Texas, Louisiana, Florida, USA)	Striped bass (*Morone saxatilis*) Redfish (*Sciaenops ocellata*) Grey mullet (*Mugil cephalus*) Pompano (*Trachinotus carolinus*)	Lawler (1980), Landsberg *et al.* (1994)
Atlantic Ocean (North Carolina, USA)	Hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) Summer flounder (*Paralichthys dentatus*)	Noga *et al.* (1991), E Noga, 1995, unpublished data
Atlantic Ocean (South Carolina, USA)	Hybrid striped bass Southern flounder Redfish	Jenkins *et al.* (1998), Smith *et al.* (1999) Sandifer *et al.* (1993)
Atlantic Ocean (Florida, USA)	Southern flounder (*Paralichthys lethostigma*)	Benetti *et al.* (2001)
Atlantic Ocean (Florida Keys, USA)	Snapper (*Lutjanus* sp.)	S. Citino (Florida, 1994, personal communication)
Pacific Ocean (Mexico)	Bullseye puffer (*Spheroides annulatus*)	Fajer-Ávila *et al.* (2003)
Caribbean Sea (Martinique, French West Indies)	European sea bass (*Dicentrarchus labrax*)	Gallet de Saint-Aurin (1987)
Caribbean Sea (Isla de Margarita, Venezuela)	Pompano (*Trachinotus goodei, T. carolinus*)	Gaspar (1987)
Mediterranean Sea (Spain)	European sea bass	Alvarez-Pellitero *et al.* (1993)
Mediterranean Sea (Italy)	European sea bass Sharp-snout sea bream (*Puntazzo puntazzo*)	Ghittino *et al.* (1980), Giavenni (1988), Rigos *et al.* (1998)
Mediterranean Sea (Sète, France)	Sea bream (*Sparus aurata*)	Paperna and Baudin Laurencin (1979)

Continued

Table 2.1. *Continued*. Amyloodiniosis infections reported in food fish.

Geographical location	Hosts	References
Mediterranean Sea (Israel)	European sea bass Sea bream	A. Colorni (Israel, 1994, unpublished data)
Mediterranean Sea (Sicily, Italy)	Yellowtail (*Seriola dumerili*)	Aiello and D'Alba (1986)
Adriatic Sea (Italy)	European sea bass	Ghittino (personal communication cited in Paperna, 1980)
Aegean Sea (Turkey)	Sea bream European sea bass	Tareen (unpublished data cited in Tareen, 1986)
Red Sea (Eilat, Israel)	Sea bream European sea bass Mullet (Mugilidae)	Paperna *et al.* (1981)
Red Sea (Eilat, Israel)	Tilapia (*Oreochromis mossambicus*)	A. Colorni (Israel, 1994, unpublished data)
Persian Gulf (Kuwait)	Sobaity sea bream (*Acanthopagrus cuvieri*)	Tareen (1986)
Pacific Ocean (Hawaii, USA)	Pacific threadfin (*Polydactylus sexfilis*)	Montgomery-Brock *et al.* (2001)
Pacific Ocean (Iloilo, Philippines)	Grey mullet	Baticados and Quinitio (1984)
Pacific Ocean (Taiwan)	Ayu (*Plecoglossus altivelis*)	Chien and Huang (1993)
Pacific Ocean (Australia)	Mulloway (*Argyrosomus japonicus*)	Fielder and Bardsley (1999)
Pacific Ocean (Thailand)	Barramundi (*Lates calcarifer*)	Chonchuenchob *et al.* (1987)

[1]This list includes reported infections only where the contagion was identified in a local source or was introduced into local waters with the fish. It does not include numerous other reports from captive aquarium fishes worldwide.
[2]Note that almost all cases were diagnosed as *A. ocellatum* via light microscopy; ultrastructural confirmation was not performed in most instances.

aquaculture pathogen as well (Shaharom-Harrison *et al.*, 1990; Ramesh *et al.*, 2000).

Ichthyodinium

Ichthyodinium chabelardi parasitizes the developing eggs of Atlantic sardines (*Sardina pilchardus*) and a somewhat similar parasite has been observed in other coldwater marine fish (Hollande and Cachon, 1952, 1953; Pederson and Koie, 1994; Stratoudakis *et al.*, 2000). Infection rates can be very high, with as much as half or more sampled eggs being infected (Meneses and Re, 1991; Meneses *et al.*, 2003). Such high prevalences have led to speculation that this parasite might be a significant and unrecognized cause of larval mortality.

Host Range

Amyloodinium

A. ocellatum is one of the few fish parasites that can infest both elasmobranchs and teleosts (Lawler, 1980) and almost all fish that live within its ecological range are susceptible to infestation. *Amyloodinium* has continued to be one of the most serious impediments to warmwater mariculture, with well over 100 species known to be susceptible. Even freshwater fish, such as centrarchids (*Lepomis*), tilapia (*Tilapia*) or guppies (*Poecilia reticulata*), are highly susceptible to infestation when they are in saline waters (Lawler, 1980; Noga and Bower, 1987; Kuperman and Matey, 1999; Table 2.1). In one instance, it has been observed to even infect a monogenean worm, another fish ectoparasite (Colorni, 1994), attesting to its cosmopolitan feeding habits.

Piscinoodinium

Many tropical fish are susceptible to *Piscinoodinium*, with anabantids (Siamese fighting fish, gouramis), cyprinids (goldfish, barbs) and cyprinodontids (killifish) frequently affected (Noga, 1996). Tilapia have also experienced epidemics (Ramesh *et al.*, 2000). Temperate species (e.g. common carp (*Cyprinus carpio*), tench (*Tinca tinca*)) and larval amphibians (*Amblystoma mexicanum*, *Rana temporaria* and *Rana arvalis*) are also susceptible (Geus, 1960) but reports from amphibians are extremely rare.

Crepidoodinium

Crepidoodinium cyprinodontum, which closely resembles *Amyloodinium* and *Piscinoodinium*, was reported in the late 1960s on some estuarine cyprinodontid fishes in the Gulf of Mexico and the Atlantic Ocean near Virginia and North Carolina, USA. Reported hosts included various killifish: *Fundulus majalis*, *Fundulus heteroclitus*, *Fundulus similis*, *Fundulus luciae*, *Lucania parva* and *Cyprinodon variegatus* (Lawler, 1967, 1968; Williams, 1972). *Crepidoodinium australe* (Lom *et al.*, 1993) was identified from sand whiting (*Silago ciliata*), an estuarine fish in New South Wales, Australia. Both parasites caused relatively minor damage to their hosts and were not fatal.

Ichthyodinium

Ichthyodinium chabelardi has been observed in Atlantic sardine populations in the Mediterranean Sea, Adriatic Sea and north-east Atlantic Ocean. Morphologically similar parasites have been reported in a number of other coldwater marine fish, most notably Atlantic mackerel (*Scomber scombrus*), sea bream (*Sparus aurata*), blue whiting (*Micromesistius poutassou*) and anchovy (*Engraulus encrasicolus*) (Stratoudakis *et al.*, 2000). A similar organism has also been identified in eggs and larvae of Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) from waters around Denmark (Baltic Sea, etc.) (Pedersen and Koie, 1994).

Oodinioides

This parasite was reported on freshwater and marine aquarium fish. Little is known

about its importance, because it has not been reported since its original description (Reichenbache-Klinke, 1970). The absence of proof that this is a dinoflagellate and the lack of further reports since its initial description make this taxon highly questionable (Lom, 1981).

Unclassified dinoflagellate

This parasitic dinoflagellate has only been identified on fish from a single lake on the Charlotte Islands off the coast of British Columbia, Canada (Reimchen and Buckland-Nicks, 1989). It primarily infests stickleback (*Gasterosteus aculeatus*), but has also been observed on salmonids (*Oncorhynchus kisutch* and *Salvelinus malma*) in that lake. Interestingly, despite often severe infestations, no apparent harm to its hosts has been observed.

Taxonomy/Systematics

Effective control of parasitic dinoflagellates requires a thorough understanding of their epidemiology, including host and geographical range, as well as other factors affecting transmission. These factors can only be discerned via a thorough understanding of the phylogenetic relationships among the different parasite isolates. Such studies may also lead to more effective methods for diagnosis of specific pathogens.

Having characteristics of both plants and animals, dinoflagellates are classified as both zoological and botanical taxa. Our review will use the botanical nomenclature, since the most recent taxonomic studies of these parasites have followed this classification scheme. Two groups within the phylum Dinoflagellida have fish-parasitic species: the class Blastodiniphyceae, order Blastodiniales, family Oodiniaceae (having the genera *Amyloodinium*, *Piscinoodinium* and *Crepidoodinium*); and the class Syndiniophyceae, order Syndiniales, family Syndinidae (having the genus *Ichthyodinium*) (Fensome *et al.*, 1993). There is increasing evidence that, even within the Oodiniaceae, parasitic dinoflagellates may be a polyphyletic group, which are evolutionarily derived from different ancestors (Cachon and Cachon, 1987; R.W. Litaker, M.G. Levy and E.J. Noga, unpublished data). The reader should refer to Cachon and Cachon (1987) and Fensome *et al.* (1993) for the most recent overviews of parasitic dinoflagellate taxonomy.

Oodinid dinoflagellates

Oodinids presently include the ectoparasites *Amyloodinium* Brown and Hovasse, 1946 (*A. ocellatum* (Brown, 1931) Brown and Hovasse, 1946), *Piscinoodinium* Lom, 1981 (*P. pillulare* (Schäperclaus, 1951) Lom 1981), *P. limneticum* (Jacobs, 1946) Lom, 1981 and *Crepidoodinium* Lom and Lawler, 1981 (*C. virginicum* Lom, 1981, *C. australe* Lom, Rohde and Dykova, 1993).

Differentiation of the fish-parasitic oodinids has traditionally been based primarily upon the morphology of the trophont, especially the type of host attachment and the mode of nutrition (Lom, 1981) (see 'Parasite Morphology/Life Cycle' and Table 2.2). Fish-pathogenic oodinids were originally classified in the genus *Oodinium*; however, important differences recognized by Brown and Hovasse (1946) and Lom and colleagues (Lom, 1981) justified their separation into different genera. Ongoing studies suggest that further separation of these taxa might be warranted.

Several undescribed oodinid-like ectoparasites have been reported from fish (Overstreet, 1968; Lom and Lawler, 1973; Paperna and Zwerner, 1976). Differences in environmental tolerances (see 'Parasite Morphology/Life Cycle'), as well as morphological differences (dinospore morphology, presence/absence of a stigma or 'pigmented eyespot', etc.), also suggest that undescribed species or strains might exist (see below). However, further research is needed in this area.

Amyloodinium

Some have proposed that the dinospore might have taxonomic usefulness. In the

Table 2.2. Key differentiating criteria of ectoparasitic dinoflagellates infecting fish.

Genus	Host cytopathology	Penetration of holdfasts into cytoplasm	Stomopode	Clove-like bodies	Stigma	Chloroplasts	Food vacuoles	Lytic bodies	Mucocysts	Starch granules
Amyloodinium	Severe	Deep	Y	Y	Y	N	Y	Y	Y	Y
Piscinoodinium	Moderate	Moderate	N	N	N?[1]	Y	N	N	Y	Y
Crepidoodinium	Mild	None	N	N	N	Y	N	N	Y	Y

[1]*P. pillulare* dinospores have been reported by Hirschmann and Partsch (1953) as having a stigma. However, others have not observed a stigma in either *P. pillulare* (Lom, 1981) or *P. limneticum* (Jacobs, 1946).

original genus description of *Amyloodinium*, dinospores were elongate (Brown, 1934; Brown and Hovasse, 1946), while other isolates described later had anteroposteriorly compressed dinospores (Lawler, 1980; Noga *et al.*, 1991). However, all recent descriptions of *Amyloodinium*, including isolates from the Gulf of Mexico, Atlantic Ocean, Mediterranean Sea and Red Sea, have displayed only anteroposteriorly compressed dinospores (Lawler, 1980; Noga *et al.*, 1991; Landsberg *et al.*, 1994; M.G. Levy, M.F. Poore, A. Colorni, E.J. Noga and R.W. Litaker, unpublished data), making the usefulness of this feature uncertain. A more significant finding is that *Amyloodinium* dinospores have been presumed to be 'naked' (i.e. not having plates or 'armour'), making them of the gymnodinoid type. However, improved ultrastructural techniques led to the discovery that dinospores collected from redfish (*Sciaenops ocellatus*) and presumably originating from the Gulf of Mexico, Florida, USA, and parasites collected from an aquarium in Florida having an amyloodiniosis outbreak both had the same type of delicate plates. A partial characterization of the Kofoidian plate tabulation pattern, which is a very important taxonomic feature in armoured dinoflagellates, was consistent with placement in the free-living order Peridiniales, subclass Peridiniphycidae, class Dinophyceae (Landsberg *et al.*, 1994).

Recent molecular analysis of the small subunit ribosomal RNA (SSU-rRNA) gene of *A. ocellatum* isolate DC-1 (Noga, 1987) also suggests a possible phylogenetic relationship of *Amyloodinium* to the subclass Peridiniphycidae (Litaker *et al.*, 1999). The current molecular data, however, are insufficient to unequivocally confirm the placement of *Amyloodinium* in the Peridiniphycidae. What the molecular data do indicate unequivocally is that *Amyloodinium* and the recently discovered *Pfiesteria* group are primitive dinoflagellates (R.W. Litaker, M.G. Levy, M.F. Poore, A. Colorni and E.J. Noga, unpublished data). The *Pfiesteria* group currently includes the putatively toxic algae *P. piscicida* and *P. shumwayae,* as well as species tentatively classified as 'cryptoperidiniopsisoid', 'Lucy' and 'Shepherd's crook' spp. (Steidinger *et al.*, 2001). Interestingly, while not a classical parasite, *Pfiesteria* has been shown to be able to prey upon host tissues using a peduncle (Burkholder *et al.*, 1992; Vogelbein *et al.*, 2002), which might be structurally and functionally analogous to the stomopode that *Amyloodinium* uses to feed on host tissues.

In terms of interspecific relationships, further analysis of the SSU-rRNA gene, including the internal transcribed spacer (ITS) region, of three different geographical isolates of *Amyloodinium* (DC-1, Gulf of Mexico (Florida) and Red Sea), showed > 99% sequence identity, strongly suggesting that these isolates might be the same species (Levy *et al.*, in preparation).

Piscinoodinium

There are two described species of *Piscinoodium*. *Piscinoodinium* (= *Oodinium*) *limneticum* was the first species described, and was identified from North American aquarium fish (Jacobs, 1946). Without prior knowledge of this report, Schaperclaus (1951) described a second species, *P.* (= *Oodinium*) *pillulare*, from European aquarium fish. Detailed ultrastructural studies by Lom and Schubert (1983) of what they considered to be *O. pillulare* led to the transfer of this species to the genus *Piscinoodinium*. However, since the original species descriptions of both *P. limneticum* and *P. pillulare* only included light microscopy, the actual species studied by Lom and its relationship to other 'freshwater *Oodinium*' parasites is unclear.

For example, in striking contrast to the molecular phylogenetic findings with *Amyloodinium* that support its placement in the class Blastodiniphyceae, data recently obtained from the SSU-rRNA gene of an isolate that has many typical features of *Piscinoodinium* (i.e. typical trophont infecting skin of freshwater tropical fish) place this organism in the class Dinophyceae, subclass Gymnodiniphycidae. This makes it closely related to such dinoflagellates as the symbiont *Symbiodinium pilosum* and the free-living *Gymnodinium simplex*. In addition, its ultrastructural features do not

appear to correspond to published descriptions of *Piscinoodinium* (M.G. Levy and R.W. Litaker, unpublished data). These molecular and morphological data suggest that the taxonomic status of this genus (and possibly that of *Crepidoodinium*) requires significant revision.

Syndinid dinoflagellates

The syndinids include intracellular parasites and those that inhabit cavities. *Ichthyodinium* Hollande and Cachon, 1953 (*I. chabelardi* Hollande and Cachon, 1953) was classified by Taylor (1987) as a member of the subclass Syndiniophycidae, order Syndiniales, family Syndiniaceae. However, molecular studies will be needed to confirm its taxonomic placement and determine whether one or several species are involved.

Unclassified dinoflagellate

The stickleback parasite from British Columbia has not yet been classified. Interestingly, this parasite has only been found in a single lake in British Columbia and it has been suggested that it might have co-evolved with its highly geographically restricted stickleback host (Buckland-Nicks and Reimchen, 1995).

Parasite Morphology/Life Cycle

All fish-parasitic dinoflagellates are highly adapted to a parasitic mode of existence, and the trophozoite stage bears little resemblance to free-living dinoflagellates. It is only during the disseminative (dinospore) stage that typical dinoflagellate morphology is apparent (Fig. 2.1).

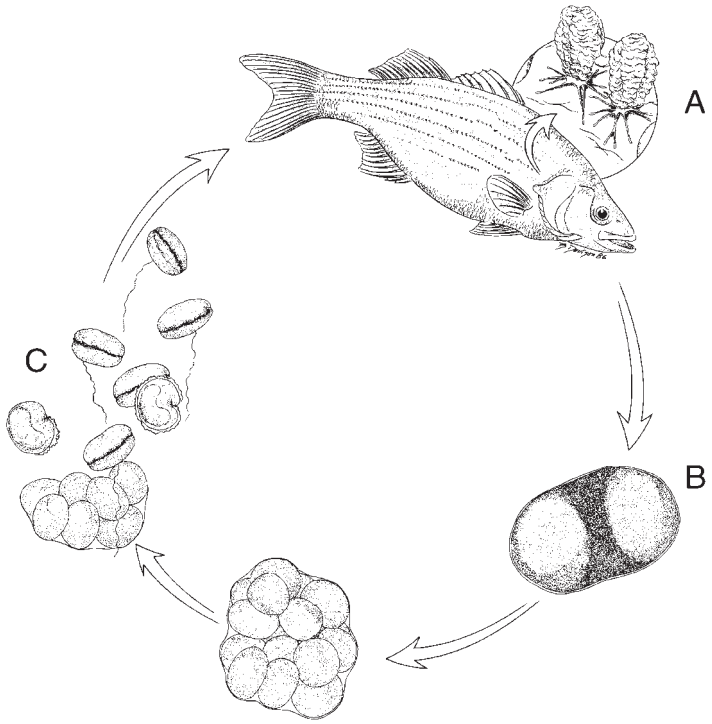


Fig. 2.1. Life cycle of oodinid parasites of fish (from Noga, 1987; courtesy of *Science*).
(A) Parasitic trophont that feeds on the skin and gill epithelium. (B) Encysted tomont stage that divides to produce free-swimming infective dinospores (C).

Oodinids

Amyloodinium, *Piscinoodinium* and *Crepidoodinium* have the same life cycle (Fig. 2.1). They feed as sessile, sac-like trophozoites (trophonts) on the skin or gill epithelium (Figs 2.2, 2.3). Trophonts are bounded by a cell wall with a thin, resistant, pellicular envelope. Beneath the cell membrane is a layer of flat amphiesmal vesicles, as is found in free-living dinoflagellates (Dodge and Crawford, 1970). Various organelles, including chloroplasts, mitochondria, Golgi apparatus, trichocysts and mucocysts, may be present.

Trophonts have a prominent stalk (Fig. 2.4), a cytoplasmic evagination with holdfasts that anchors the parasite to the host and in some cases absorbs nutrients. The stalk is a characteristic of oodinid (and blastodinid) dinoflagellates. After feeding, the trophont detaches and withdraws its stalk, forms a reproductive cyst (tomont) and divides asexually several times (Fig. 2.2B). After the last asexual division, motile forms (dinospores; also referred to as gymnospores or zoospores) are released, which are capable of infecting a new host. This type of reproduction is termed palintomy. Dinospores have a girdle, sulcus and two flagella (Fig. 2.3B). Recently, it has been shown that *Amylooodinium* dinospores also have a peduncle (which might form the trophont's stomopode) as well as an associated rhizoid-like complex (Landsberg *et al.*, 1994), previously only known in the trophont. Dinospores are fairly uniform in size and the number of dinospores produced per tomont is roughly proportional to the size of the trophont.

Amyloodinium

LIFE CYCLE AND MORPHOLOGY. Light microscopic aspects of the life cycle have been described in detail (Brown, 1934; Nigrelli, 1936; Brown and Hovasse, 1946). The trophont is pear-shaped to ovoid and up to 350 µm long (Figs 2.2, 2.3). Trophonts are reported to have a cellulose wall consisting of a theca with amphiesmal plates. Thecal armour is complete except for a gap at the base of the trophont. An osmophilic ring encircles the basal region, and an attachment plate bears numerous filiform rhizoids (Fig. 2.4), which exit through the break in the theca. The rhizoids are enlarged at their bases, which serves to anchor them deep in the host cell. A 30 µm stomopode is associated with the attachment plate. It passes from deep within the trophont and exits proximal to the sulcal fold. This organelle, which probably functions in feeding, contains two types of inclusions: 'lytic bodies' and 'clove-like structures', both believed to aid digestion. Starch granules and food vacuoles containing host cell cytoplasm are scattered throughout the cytoplasm, and chloroplasts are absent (Lom and Lawler, 1973).

After feeding, the trophont detaches from the host, retracts its rhizoids and becomes a tomont. Divisions within a common cyst wall produce up to 256 dinospores (Brown, 1934; Nigrelli, 1936). Dinospores are 8–13.5 µm long by 10–12.5 µm wide. Brown (1934) and Nigrelli (1936) described *A. ocellatum* as having gymnodinoid (barrel-shaped) dinospores, but all later studies only described isolates with anteroposteriorly compressed dinospores (Lawler, 1980; Noga *et al.*, 1991; Landsberg *et al.*, 1994; Fig. 2.3). A red stigma, which accounts for the name 'ocellatum', is present throughout the life cycle.

ECOLOGICAL FACTORS AFFECTING GROWTH. *Amyloodinium* is a warmwater parasite and is more pathogenic at higher temperatures (Paperna, 1980; Kuperman *et al.*, 2001); thus, in more temperate regions, it is only a problem in warmer months (Noga *et al.*, 1991; Kuperman and Matey, 1999). In Red Sea isolates, where environmental tolerances have been best studied, the optimal temperature for tomont division and sporulation ranges from 23 to 27°C. Completion of tomont division is limited to 16–30°C (Paperna, 1984) and infections do not occur at less than 17°C (A. Colorni, Israel, personal communication). Strain DC-1 tomonts held at 15°C stop dividing after four divisions; however, even after 17 weeks' exposure to 15°C, these tomonts can

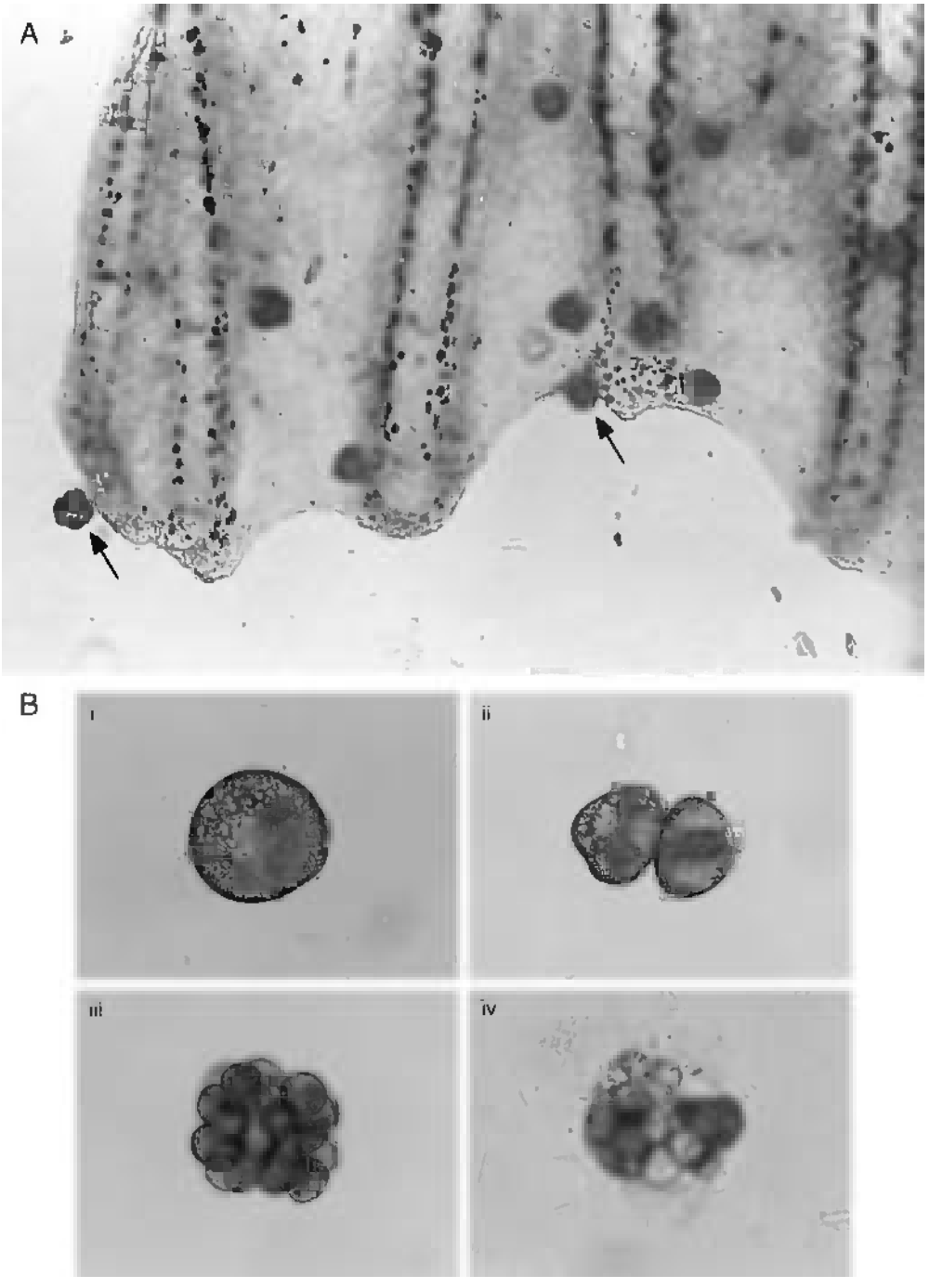


Fig. 2.2. Light micrographs of *Amyloodinium* (A) Trophonts (arrows) on a damselfish (*Dacyllus* sp.) fin. (B) Tomonts in various stages of development: (i) single cell (just differentiated from trophont); (ii) two-cell; (iii) nearly mature; and (iv) mature, with some dinospores already having exited the tomont (photos courtesy of A. Colorni).

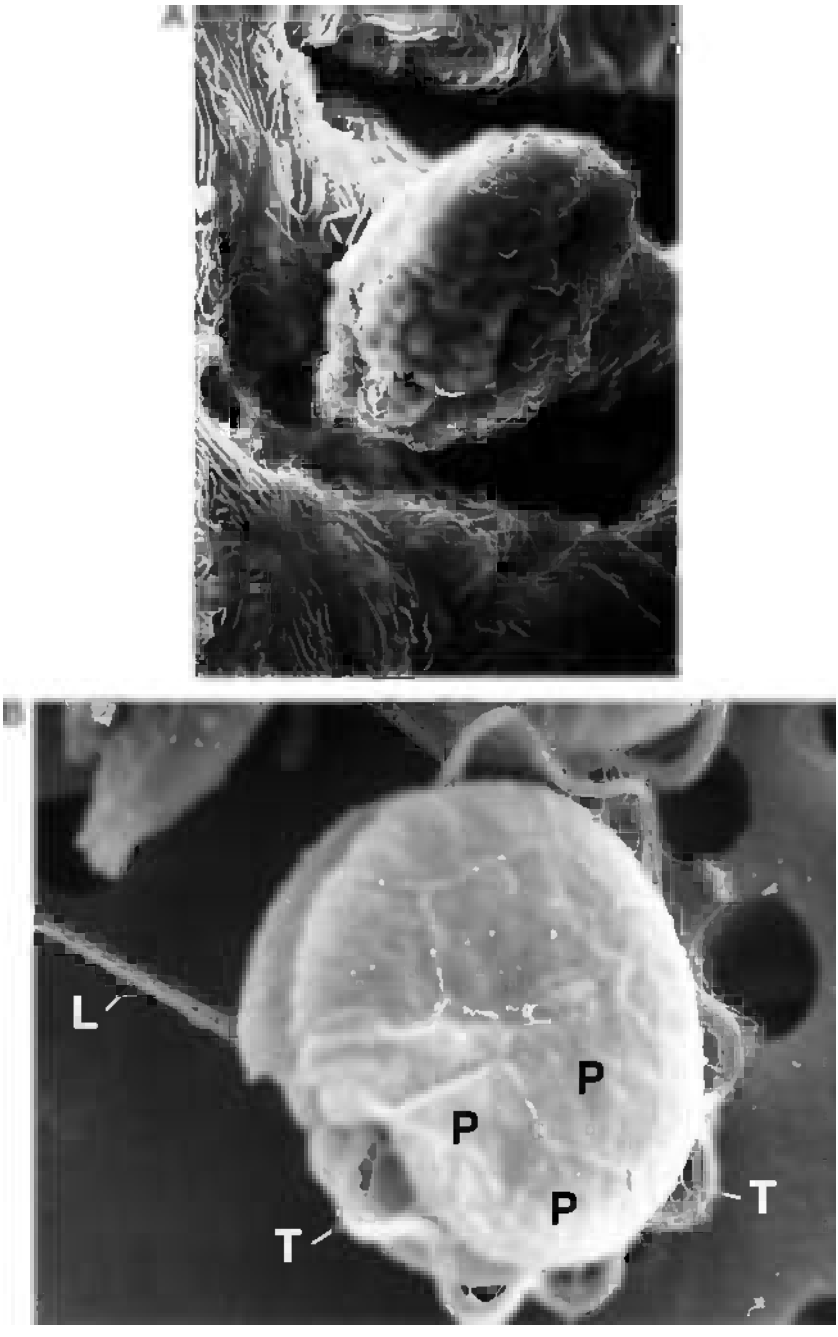


Fig. 2.3. Scanning electron micrographs of *Amyloodinium*: (A) A trophont attached to gill. (B) A dinospore from a Gulf of Mexico isolate, showing armour or plates. Representative plates are labelled (P). Note the dinospore's antero-posterior compression ('hamburger' shape). L, longitudinal flagellum; T, transverse flagellum (from Landsberg *et al.*, 1994; courtesy of *Diseases of Aquatic Organisms*).

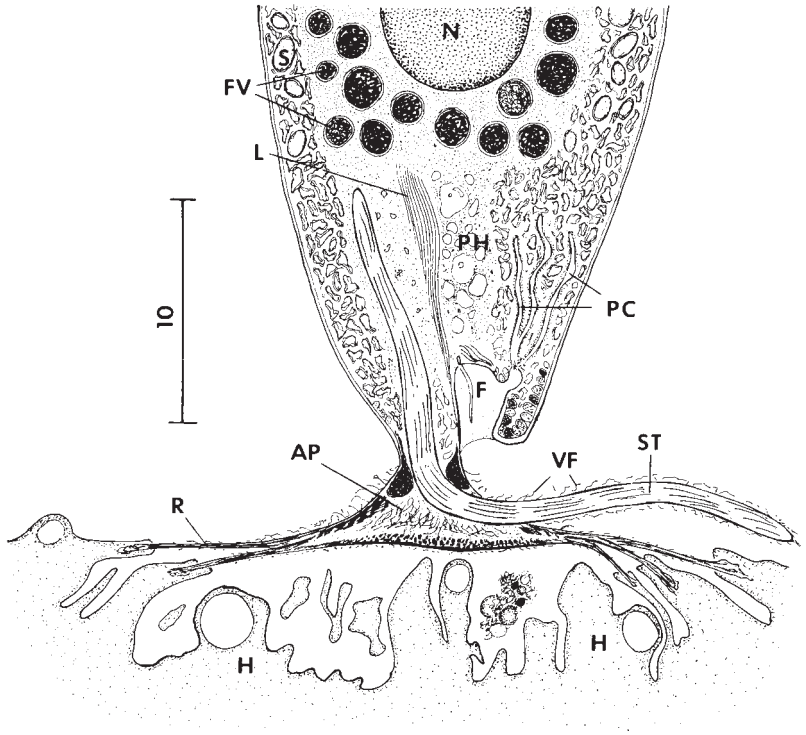


Fig. 2.4. Host attachment mechanism of *Amyloodinium*. Scale is in microns. H, host cell; AP, attachment plate; R, rhizoid; ST, stomopode tube; VF, velum-like pellicular folds; F, flagellum; PC, pusular canal; L, fibrillar ledge; FV, food vacuole; N, nucleus; PH, phagoplasm. (From Lom and Lawler, 1973; courtesy of *Protistologica*).

produce dinospores when returned to 25°C (C.E. Bower, Connecticut, personal communication).

Temperature also affects salinity tolerance, which narrows as one deviates further from the optimal temperature range. Effective tomont division and sporulation of Red Sea isolates occurred at up to 50 psu (practical salinity units). The minimum effective salinity varied from 12 to 20 psu, depending upon the isolate (Paperna, 1984). Amyloodiniosis severity in larval mulloway (*Argyrosomus japonicus*), an estuarine sciaenid cultured in New South Wales, Australia, was also closely related to salinity, but infection could occur (albeit at low levels) at 5 psu; peak levels were at 20 psu, with declining levels to 35 psu (Fielder and Bardsley, 1999). Hojgaard (1962) also reported that isolates in Danish public aquaria were inhibited by 10 psu. However, *Amyloodinium* in the Salton Sea was most pathogenic when temperatures were very high (39–41°C), even though the salinity was also very high (46 psu) (Kuperman and Matey 1999; Kuperman *et al.*, 2001). Frequent outbreaks have been reported in some oligohaline waters as well (McIlwain, 1976), with epidemics occurring at salinities as low as 2.8 psu in Gulf of Mexico estuaries (Lawler, 1977b). The reason for such differences in salinity tolerance are unknown, but the salinity of the Red Sea usually fluctuates very little (around 40 psu), whereas salinity in estuarine regions varies greatly, depending upon freshwater runoff.

Other risk factors have not been well studied, although low dissolved oxygen has been associated with some epidemics (Sandifer *et al.*, 1993; Kuperman *et al.*, 2001). Bacteria and protozoa inhibit the

development of *Amyloodinium* (Brown, 1934; Bower *et al.*, 1987; Noga and Bower, 1987) by delaying dinospore emergence and killing sporulating tomonts before dinospores emerge (Brown, 1934; Bower *et al.*, 1987; Oestmann and Lewis, 1995). It is uncertain whether this inhibition is due to a direct toxic effect or environmental changes (e.g. hypoxia/anoxia would be most likely to occur in contaminated cultures).

Piscinoodinium

LIFE CYCLE AND MORPHOLOGY. The trophont is a yellow-green to brown, pyriform or sac-like cell, up to 140 μm long (Schäperclaus, 1951). They are almost round when mature and appear to possess a rudimentary sulcus (Lom and Schubert, 1983). A short stalk with an attachment disc extends from the base (Fig. 2.5) and thin nail-like holdfasts (rhizocysts) radiate from the stalk. Head parts of the rhizocysts are inverted in separate compartments (rhizothecas) in the sole of the disc while their shafts are firmly embedded in host cell cytoplasm (Fig. 2.5). While rhizocysts have a very intimate attachment to the host cell, they are much less aggressive than the rhizoids of *Amyloodinium* (Fig. 2.4).

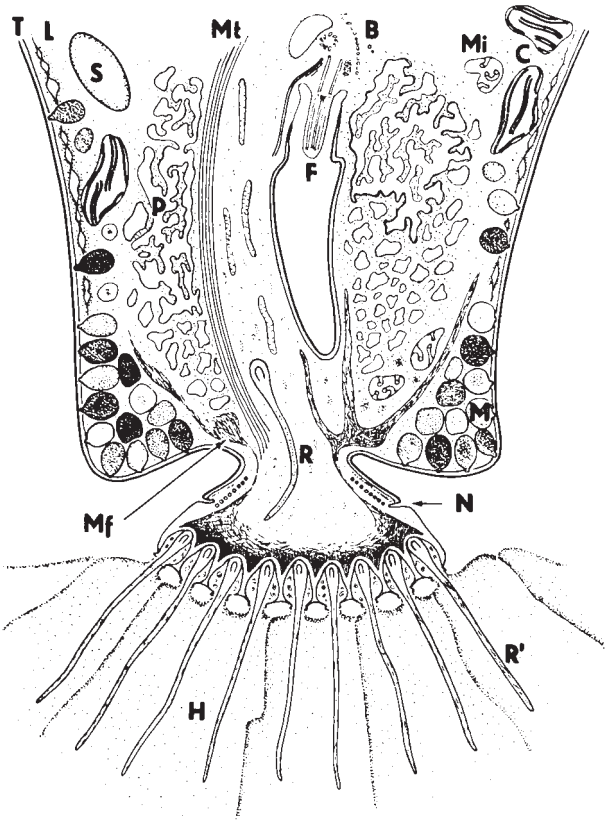


Fig. 2.5. Host attachment mechanism of *Piscinoodinium*. H, host cell; R, rhizocyst supposedly migrating into the attachment disc; R', rhizocyst 'in position' within the rhizotheca, embedded into the host cell cytoplasm; MF, microfibrillar strands converging to form a perinema-like ring around the 'neck' of the attachment disc; N, notch on the upper surface of the attachment disc; above it, the theca is subtended by a circular ribbon of microtubules and an electron-dense substance; C, chloroplast; S, starch grains; Mi, mitochondrion; Mt, microtubular ribbons along the zone of special cytoplasm extending from nucleus into the attachment disc; B, complex of basal bodies; F, flagellum; T, theca; L, subthecal lacunae; P, pusular system. (From Lom, 1981; courtesy of *Folia Parasitologica [Praha]*).

The theca covers the entire cell except for the area of the attachment disc. The periphery of the cell contains disc-shaped chloroplasts and numerous starch granules. Up to 256 dinospores (10–19 μm long × 8–15 μm wide in *P. limneticum*) are produced from each tomont. *Piscinoodinium pillulare* is reported to possess a red stigma that is absent in *P. limneticum* (Schaperclaus, 1991).

ECOLOGICAL FACTORS AFFECTING GROWTH. The life cycle can be completed in 10–14 days under optimal conditions. Optimal temperature for *P. pillulare* is 23–25°C, with sporulation requiring 50–70 h for an average-sized tomont. At 15–17°C, sporulation requires 11 days (van Duijn, 1973). Optimal conditions are probably similar for *P. limneticum*. Under crowded conditions or in stagnant water, sporulation is inhibited and smaller dinospores are produced. Lower temperature slows the life cycle and results in larger dinospores (Jacobs, 1946). Mass mortalities in tank-reared fish have been associated with decreasing temperatures (dropping from 30°C to 21°C) (Ramesh *et al.*, 2000). Shaharom-Harrison *et al.* (1990) also observed epidemics with either increasing or decreasing temperatures in pond-cultured fish. Decreasing temperature is a common stressor in fish and can lead to development of a number of opportunistic infections, probably due to temperature-mediated immunosuppression.

Crepidoodinium

LIFE CYCLE AND MORPHOLOGY. The trophonts of *C. cyprinodontum* are large and somewhat flattened, measuring up to 670 μm long by 130 μm wide (Lom and Lawler, 1973), while those of *C. australe* are even larger and very flattened (leaf-like), measuring up to 820 μm long by 235 μm wide (Lom *et al.*, 1993). Both species have been observed only on the gills. Trophonts have well-developed chloroplasts (giving them a bright green appearance), spongy cytoplasm and abundant starch granules. The holdfast organelle consists of numerous attachment projections and rhizoids (Fig. 2.6) formed by cytoplasmic stems evaginated through thecal openings. The finger-like rhizoids are attached only superficially to the host cell membrane, which is modified at the point of contact. The attachment resembles the cell junction of epithelial cells. Penetration of host cells has not been observed. Up to 2048 dinospores are produced from a single tomont.

Syndinids

Ichthyodinium

LIFE CYCLE AND MORPHOLOGY. *I. chabelardi* parasitizes the vitelline (yolk) sac of sardines, where it rapidly multiplies, filling the yolk sac with parasites; it causes larval death by depleting energy reserves and rupturing the yolk sac after hatching. Sardine eggs are mainly infected during colder months, with peak prevalences in January–February. Up to 33% of all sampled eggs have been infected in some surveys (Meneses and Re, 1991). Infections are inapparent until closure of the blastopore occurs in the developing embryo (Meneses and Re, 1991). In mackerel, where a similar parasite occurs, it can be seen somewhat earlier in development (Meneses *et al.*, 2003; www.plank.oupjournals.org).

The life cycle is quite complicated (Fig. 2.7). The earliest detectable parasitic stage in the yolk sac is the 'primordial schizont' (Figs 2.7, 2.8), which begins as a spherical, uninucleated trophont, ~ 8–15 μm in diameter; it undergoes multiple nuclear divisions to form a large (100–200 μm), multinucleated plasmodium. The primordial schizont then cleaves into several uninucleate, elongated 'secondary schizonts', which undergo further divisions. In the final stage, 'last generation schizonts' are produced, which resemble the early primordial schizonts. In the advanced stages, parasites fill the yolk sac (Fig. 2.8). After the host dies, the freed parasite divides once or twice more in the water. The resultant free-swimming dinospores survive several days in culture but, under laboratory conditions, are not infective to sardine eggs by

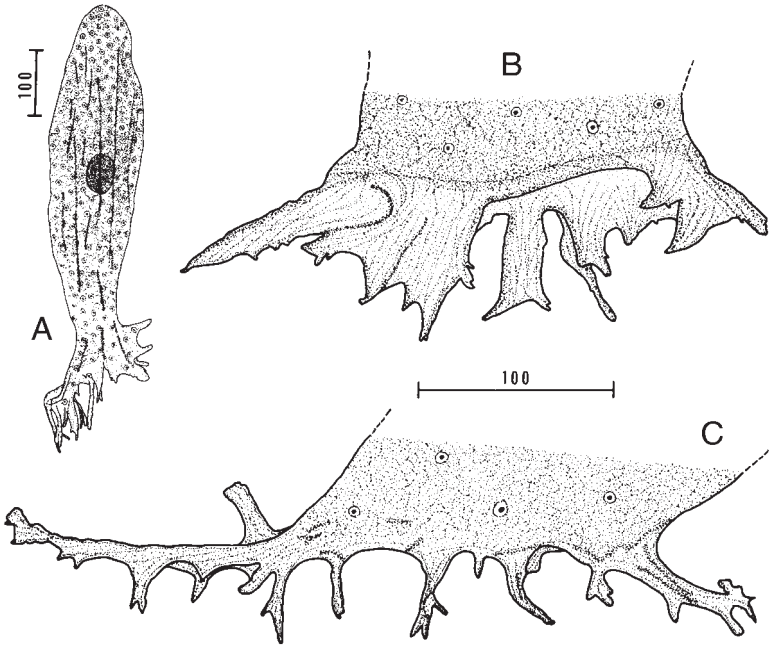


Fig. 2.6. Morphology of *Crepidoodinium*. Scales are in microns (from Lom and Lawler, 1973; courtesy of *Protistologica*). (A) Trophont with a rounded nucleus and the flattened holdfast organelle ramified into numerous lobose or finger-like projections from which the small attachment rhizoids arise. (B) and (C) Enlarged holdfast portions of the trophonts; only the tips of small rhizoids emanating from these major and minor branches contact the host epithelial cells.

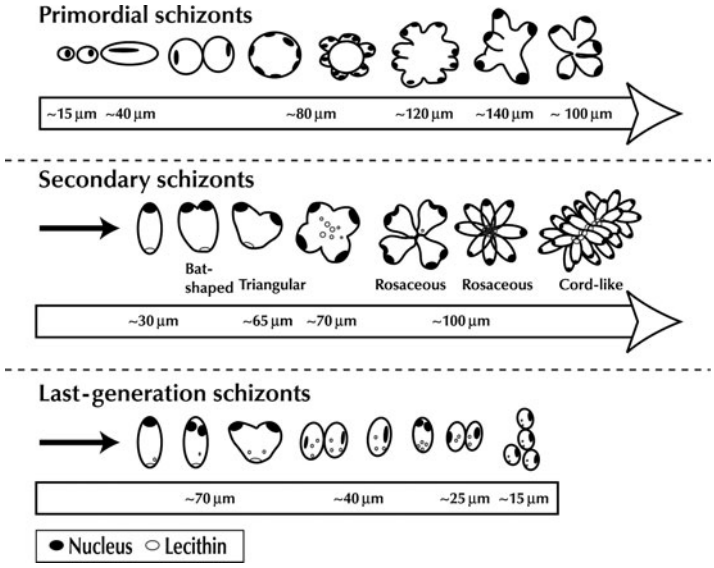


Fig. 2.7. *Ichthyodinium* developmental stages in Atlantic mackerel. (From Meneses *et al.*, 2003; courtesy of *Journal of Plankton Research*.)

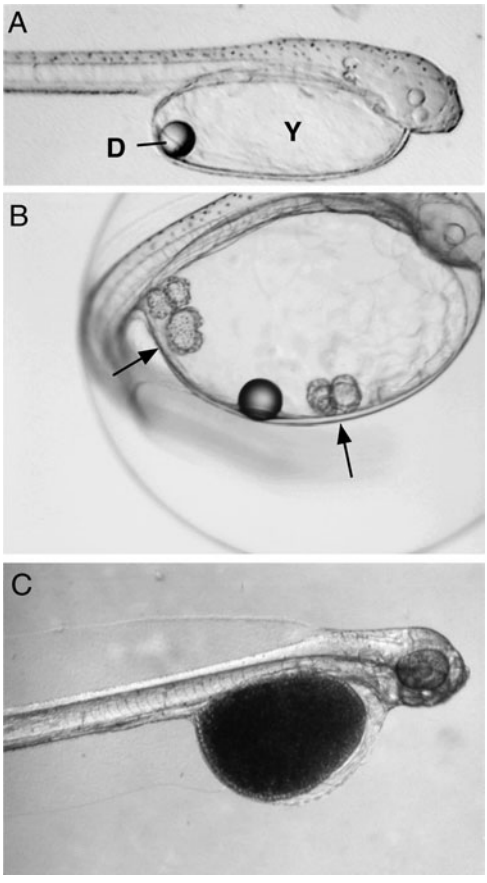


Fig. 2.8. Atlantic sardine embryo and larva infected with *Ichthyodinium*. (A) Normal larva. Y, yolk sac, D, oil droplet. (Photo courtesy of I. Meneses.) (B) Early stage infection of embryonating egg having several primordial schizonts in the yolk sac (arrows). (Photo courtesy of I. Meneses.) (C) Advanced infection of larva, with parasites filling the yolk sac. (Photo courtesy of I. Meneses and Y. Stratoudakis) (from Stratoudakis *et al.*, 2000; courtesy of *Journal of Fish Biology*).

either exposure to eggs in water or direct inoculation of dinospores into the yolk mass (Hollande and Cachon, 1953).

Unclassified dinoflagellate

LIFE CYCLE AND MORPHOLOGY. The life cycle of the stickleback parasite from British Columbia has been reported to be extremely complex and unusual, with a number of developmental stages and life forms. These include a trophont on the skin or gills, a 'vegetative cyst' (also on the skin or gills), several amoeboid stages that develop from germinating vegetative cysts, a 'resting cyst' that occurs off the fish, and a dinospore (presumably the disseminative stage). A typical dinoflagellate nucleus (dinokaryon) was observed in the vegetative cyst and dinospore stages, whereas a typical eukaryotic nucleus was reported for the amoeboid stages. Dinospore and vegetative cyst stages contained chloroplasts, whereas the amoeboid stages, which were observed on less than five occasions, lacked chloroplasts. Since observations of all putative developmental stages were observed in non-axenic cultures, and most of the stage transformations were not observed or documented, the possibility that some of the reported life cycle stages might be unrelated contaminants must be eliminated. For example, based upon observations of non-axenic cultures, it was initially proposed that the dinoflagellate *P. piscicida* had over 24 stages, incuding multiple amoeboid forms, in its life cycle (Burkholder and Glasgow, 1997). However, subsequent studies using highly specific molecular probes indicated that *P. piscicida* actually has a very simple life cycle and that the 'amoeboid stages' in *P. piscicida* cultures are true amoeba (i.e. contaminants introduced on the fish which were used as a food source for *P. piscicida* or from elsewhere) (Litaker *et al.*, 2002).

Host–Parasite Relationships

Amyloodinium

Amyloodinium causes amyloodiniosis, marine velvet disease, marine *Oodinium* disease and oodiniosis.

Clinical signs/pathology

Clinical signs of amyloodiniosis include anorexia, depression, dyspnoea (swimming

near the water surface with laboured breathing) and pruritis (Brown, 1934; Brown and Hovasse, 1946; Lawler, 1977a, b; Noga, 1996). The gills are usually the primary site of infestation, but heavy infestations may also involve the skin, fins and eyes. Paperna (1980) found that the skin was the primary site of infestation in sea bream (*S. aurata*) larvae. Heavily infested skin may have a dusty appearance ('velvet disease'), but this is not a common finding and fish often die without obvious gross skin lesions. Young fish appear to be most susceptible, although there are few hard data in this area.

Trophonts may also occur on the pseudobranch and in the branchial cavity and nasal passages (Lawler, 1980). Although tomonts have been reported in the oesophagus, stomach and intestines (Brown, 1934; Hojgaard, 1962), they probably developed elsewhere and were subsequently swallowed. Cheung *et al.* (1981) reported a case of presumptive amyloodiniosis in the viscera of a porkfish (*Anisostremus virginicus*). However, only tomont-like structures were seen and there was no strong morphological evidence that this organism was *Amyloodinium*.

A single trophont can feed on multiple epithelial cells simultaneously (Paperna, 1980). This is at least partly responsible for the extensive damage caused by the parasite. The hydrodynamic forces exerted on the trophont, which projects into the water when the fish swims, may exacerbate damage. Trophonts can also move slowly back and forth while attached (Noga, 1987), also facilitating host cell fragmentation.

Mild infestations (e.g. one or two trophonts per gill filament) cause little pathology. However, heavy infestations (up to 200 trophonts per gill filament) cause serious gill hyperplasia, inflammation, haemorrhage and necrosis. Death is usually attributed to anoxia and can occur within 12 h with an especially heavy infestation (Lawler, 1980). In contrast, acute mortalities are sometimes associated with apparently mild infestations, suggesting that hypoxia may not always be the cause of death. Osmoregulatory impairment and secondary microbial infections due to severe epithelial damage may also be important causes of debilitation and death.

Paperna (1984) felt that the serious gill pathology associated with many *A. ocellatum* infestations was more severe than the parasitosis would indicate. He speculated that *A. ocellatum*, like some other dinoflagellates, may produce an exotoxin responsible for some of the hyperplasia and necrosis. This remains to be proven, but, in cell culture, *A. ocellatum* trophonts produce a very localized area of cell necrosis and this appears to be associated with the feeding activity of the parasite and not toxin production (Noga, 1987).

Innate immunity

Although most fish species in *Amyloodinium*-infested waters are susceptible, certain factors clearly limit the ability of *Amyloodinium* to proliferate, as feral fish usually have very few parasites (Lawler, 1980). Except in very rare instances (Overstreet, 1993; Kuperman *et al.*, 2001), epidemics have only been reported in the stressful confines of aquaculture (Table 2.1), even though there is often very high prevalence in feral fish (Lawler, 1980). Some fish species are naturally resistant to infestation. These include killifish (*Fundulus grandis*), American eel (*Anguilla rostrata*), molly (*Poecilia latipinna*) and others belonging to seven families. Resistant species are generally those which produce thick mucus or can tolerate low oxygen levels, presumably due to their greater ability to withstand attack or feeding on gill tissue by the parasite (Lawler, 1977b). In some instances, older fish appear to be more resistant. Quantitative comparisons of susceptibility also showed that clownfish (*Amphiprion ocellaris*) are more susceptible than striped bass (*Morone saxatilis*), which are more susceptible than Mozambique tilapia (*Oreochromis mossambicus*) (C.E. Bower, 1988, Connecticut, personal communication).

Host factors must play an important role in host–parasite interactions. Fish-parasitic oodinids feed exclusively on or within the epithelial tissues of the skin or gills. Thus, all host–parasite interactions (i.e. host

recognition, defensive mechanisms responsible for protecting against these pathogens, etc.) are located in the mucus or on/in epithelial cells and extracellular fluid of the epithelium. Serum from blue tilapia (*Oreochromis aureus*) with no previous exposure to *Amyloodinium* has anti-*Amyloodinium* activity in a cell culture infectivity assay. As little as 1.25% blue tilapia serum is parasiticidal and 10% is completely inhibitory (Landsberg *et al.*, 1992). Heating the serum to 47°C for 20 min or 56°C for 30 min, as well as treating it with carageenan or zymosan, suggested that complement-like activity was responsible for at least part of the activity.

Amyloodinium is also highly sensitive to natural, host-produced antibiotics, known as histone-like proteins (HLPs). The HLPs are small (~ 13–21 kDa), basic proteins that are related to histones H2B and H1; they are present in high concentrations in the skin and gills of hybrid striped bass and other fish (Noga *et al.*, 2001). A comparison of the antibiotic activity in mucus versus epidermis compartments of the skin of hybrid striped bass suggested that the majority of antibiotic (including HLP-1) activity resided in the epidermis, although some activity was present in the mucus. The concentrations at which HLPs were lethal to *Amyloodinium* were well within the range in which these compounds are present in fish tissues; thus they probably play an important role in protecting fish against this parasite. Interestingly, HLPs are highly lethal to trophonts but have no effect on dinospores (Noga *et al.*, 2001). Both the ability of the parasite to infect host cells and the ability to grow and differentiate after infection were severely inhibited. This preferential toxicity for trophonts is highly unusual, since virtually all drugs act against the dinospore (infective stage) and trophonts are typically highly resistant to therapy (see 'Prevention and Control'). In contrast, magainin 2, a peptide antibiotic produced in the skin of the African clawed frog (*Xenopus laevis*), was equally toxic to both trophonts and dinospores (Noga *et al.*, 2001).

In addition to direct killing of *Amyloodinium* trophonts, there was evidence that both HLP-1 and magainin 2 caused severe developmental abnormalities, including delayed development, in both the trophont and the tomont. The deleterious effects were manifested in what appeared to be 'delayed mortality', where normal-appearing parasites would die later in development (Noga *et al.*, 2002). These data suggest that normal, non-immune skin and gill tissues contain potent defences against protozoan ectoparasites and that the effects of these defences may extend beyond their transient interactions with the parasites, which has important implications for this host–parasite relationship.

Acquired immunity

At present, there are no practical vaccines available for treatment of any fish-parasitic protozoa, including dinoflagellates (Woo, 1996). However, recent studies have identified important defensive mechanisms that might be used to specifically enhance protection from these pathogens. Early studies provided anecdotal evidence that fish recovering from amyloodiniosis were resistant to reinfestation (Lawler, 1977b, 1980; Paperna, 1980). Smith *et al.* (1993) then showed that serum from fish immunized with dinospores could agglutinate living dinospores and kill *Amyloodinium* in cell culture. Immunized fish also mounted an antibody response that was detectable via enzyme-linked immunosorbent assay (ELISA). Using DC-1 isolate dinospores as the capture antigen, this ELISA detected anti-*Amyloodinium* serum antibody in blue tilapia immunized with the DC-1 isolate (Smith *et al.*, 1993) and in sea bream immunized with a Red Sea *Amyloodinium* isolate (Noga *et al.*, 1992). Using this same ELISA, anti-*Amyloodinium* serum antibody was also detected in hybrid striped bass (*M. saxatilis* × *Morone chrysops*) that had recovered from a spontaneous amyloodiniosis outbreak on a North Carolina fish farm (Smith *et al.*, 1994). These data suggest that fish can mount a significant antibody response with both experimental and natural challenges and that there was considerable cross-reactivity between these three

Amyloodinium isolates. The latter supports the molecular data indicating that *Amyloodinium* might be a very homogeneous taxon (see 'Taxonomy/Systematics'). Using tomont antigen, Cecchini *et al.* (2001) also detected anti-*Amyloodinium* antibody in cultured European sea bass (*Dicentrarchus labrax*) that had recovered from an amyloodiniosis outbreak.

Subsequent studies have shown that *Amyloodinium*-infested fish can develop protective resistance following experimental challenge. Following weekly sublethal challenges with *Amyloodinium*, tomato clownfish (*Amphiprion frenatus*) developed significant immunity to infection in about 1 month. This protection was long-lived (at least 6 months) and appeared to be directed against the trophont (Cobb *et al.*, 1998a). Protection was associated with an antibody response as measured by ELISA. The reaction of immune fish serum against dinospore and trophont antigens in Western blots suggested the presence of both shared and stage-specific antigens (Cobb *et al.*, 1998b). Immune serum also reacted with trophonts and dinospores in an indirect fluorescent antibody test. There was a suggestion that immunity could also be passively transferred to naïve fish (Cobb *et al.*, 1998b). Local antibody was hypothesized to be more important than serum antibody since protection lasted long after serum antibody was undetectable via ELISA.

Piscinoodinium

Piscinoodinium causes freshwater velvet, rust disease, gold dust disease, pillularis disease and freshwater *Oodinium* disease.

Clinical signs/pathology

Piscinoodinium infests the skin and gills (Fig. 2.9). Clinical signs are similar to those of amyloodiniosis, except that fish can withstand much heavier infestations. It is most pathogenic for young fish, which may die within 1–2 weeks, while older fish may live for months. Heavy infestations produce

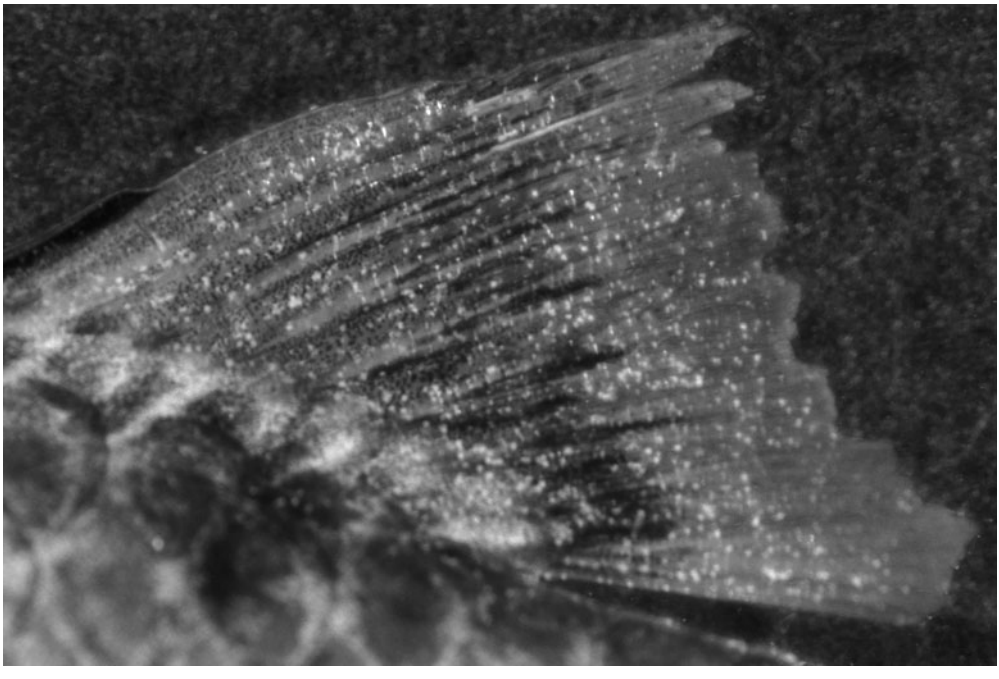


Fig. 2.9. *Piscinoodinium* infestation of the dorsal fin of a tiger barb (*Barbus*). Each refractile white focus is a parasite.

a yellow or rusty sheen to the skin. There is excess mucus, darkening of the skin, dyspnoea, anorexia and/or depression (Jacobs, 1946; van Duijn, 1973; Shaharom-Harrison *et al.*, 1990). Skin ulcers (Shaharom-Harrison *et al.*, 1990) and tattered, sloughing epithelium (Schaperclaus, 1951) are seen in some cases. Rhizocysts anchor the trophont to its host and damage the epithelium. This damage may be exacerbated by the hydrodynamic forces exerted on the trophont projecting into the water moving past the fish. Cellular damage is thought to attract parasites because trophonts typically cluster in damaged areas.

Gill histopathology ranges from separation of the respiratory epithelium to severe hyperplasia of the entire filament. Filament degeneration and necrosis may occur. Skin infestations can cause deep, focal erosions of the epithelium (Ferraz and Sommerville, 1998). Besides skin and gills, trophonts may also infest the oral cavity and the pharynx (Ramesh *et al.*, 2000). Some parasites in either skin or gills may become almost entirely covered by hyperplastic epithelium (van Duijn, 1973; Shaharom-Harrison *et al.*, 1990; Ferraz and Sommerville, 1998), probably due to the chronic irritation caused by infestation. Early reports suggested that some 'encysted' parasites may even sporulate (Geus, 1960), but others have observed dead parasites in the epithelial cysts (Ferraz and Sommerville, 1998), suggesting that this is a successful protective host response. Outbreaks may be precipitated by stress (e.g. poor water quality or unfavourable temperatures). There is some evidence that recovered fish may be resistant to reinfection (Jacobs, 1946).

Unclassified dinoflagellate

Clinical signs/pathology

The stickleback parasite from British Columbia induces an extremely severe epidermal hyperplasia on the skin, resulting in a grossly visible, white 'gelatinous coating' that can cover a large area of the body (Reimchen and Buckland-Nicks, 1989). This proliferative host response eventually encloses the parasites. The parasites and their host response are most prevalent in mid-summer, and are not detectable during winter. Interestingly, the vegetative cyst, not the trophont, is the life stage almost exclusively observed on fish. However, trophonts of other parasitic dinoflagellates such as *Amyloodinium* will rapidly form tomonts when disturbed. Thus the observance of only vegetative cysts due to rapid transformation prior to fixation is a possibility.

In Vitro Culture

Amyloodinium

Amyloodinium can be maintained *in vivo* by serial exposure of infective dinospores to susceptible hosts (Lawler, 1980; Bower *et al.*, 1987), but this is very labour-intensive and requires considerable space for holding fish. Noga and Bower (1987) developed a method of passaging the parasite on gnotobiotic fish larvae. Individual tomonts were decontaminated by repeated passage through several baths of sterile saline with antibiotics (Noga, 1992). Guppies (*P. reticulata*) were then successfully infected with germ-free dinospores. The parasites completed at least one full life cycle on the host fish and were serially passaged up to six times.

In some cases, the parasites on gnotobiotic larvae continued to grow and reproduce long after the host had died. This suggests that factors other than death of the host per se are responsible for the parasite normally leaving the host. Rapid exodus from a dead host is common among other fish ectoparasites (Hoffmann, 1967). *Amyloodinium* survives for several days after its host dies so long as the tissues that support its growth are nourished (Noga and Bower, 1987).

Organ cultures of fish larvae also supported parasite growth (Noga and Bower, 1987) and *A. ocellatum* readily propagates on a fish gill cell line (G1B cells, derived from walking catfish (*Clarias batrachus*),

American Type Culture Collection No. CRL-2536) maintained in a 1 : 1 mixture of cell culture medium and artificial sea water (Noga, 1987). One isolate (DC-1) has been continuously propagated *in vitro* for over 15 years and hundreds of cell culture passages (E.J. Noga, unpublished data). Cell-cultured parasites are similar morphologically and developmentally to those produced *in vivo* (Noga, 1987) and can readily kill fish (Cobb *et al.*, 1998a). Isolates from the Gulf and Atlantic coasts of the USA, the Red Sea and the Mediterranean Sea have also been continuously cultured *in vitro* for years using the same method (Noga *et al.*, 1991; M.G. Levy, A. Colorni, A. Diamant and E.J. Noga, unpublished data).

Amyloodinium can also be propagated in a highly defined medium called IO2/HBSS, consisting of a 1 : 1 mixture of artificial sea water and Hank's balanced salt solution. Fetal bovine serum (which is non-toxic to *Amyloodinium* at a 5% concentration) or other additives are not needed (Noga, 1989). Media of less than 300 mosmol did not support parasite survival (Noga, 1989). The effects of mineral composition on parasite survival indicate that ionic strength (i.e. salinity) is only one determinant of survival (see 'Parasite Nutrition and Physiology').

The G1B cell culture system can be used to quantitatively evaluate the infectivity of *Amyloodinium* dinospores (Noga, 1992). Exposure to anti-parasite drugs or immune components produces a reproducible, dose-dependent inhibition of infectivity (Noga, 1987; Smith *et al.*, 1993; Noga *et al.*, 2001). It is a much more sensitive indicator of parasite survival than measuring inhibition of motility (Noga, 1987; Noga *et al.*, 2001) and allows the quantitative evaluation of infectivity in a way which is analogous to colony counts of bacteria.

Because of the problems they encountered with establishing a Gulf of Mexico *Amyloodinium* isolate from redfish, Oestmann and Lewis (1995) developed a method for decontaminating large numbers of tomonts by first exposing the parasites to a brief osmotic shock, followed by density gradient centrifugation using Percoll; further antibiotic treatment resulted in consistently microbe-free preparations. Because they were unable to propagate this *Amyloodinium* isolate using a monolayer of redfish dorsal fin cells (RDFCs), they designed an alternative method that involved the production of RDFC aggregates, which allowed completion of the parasite's life cycle *in vitro* (Oestmann and Lewis, 1996a).

Parasite Nutrition and Physiology

Amyloodinium

The mechanism by which *Amyloodinium* feeds on host cells is unclear. A single trophont can damage and kill several host cells (Lom and Lawler, 1973; Noga, 1987). This probably accounts for the severe injury inflicted on the host by trophonts. Rhizoids anchor the parasite to the host cells, but probably do not absorb nutrients (Lom and Lawler, 1973). The stomopode may be a source of digestive enzymes that are injected into host cells or it may serve as a feeding tentacle that gathers fragments of cells severed by the pulling motion of the rhizoids. This is supported by the observation of the constant twisting motion of the trophont (Noga, 1987), which may facilitate the severance of the host cell fragments.

There is *in vitro* evidence that *A. ocellatum* probably derives all its nutrition from host cells. It grows as well in a medium with only mineral salts, phosphate and glucose as it does in a medium with additional trace minerals, vitamins and amino acids (Noga, 1989). It is likely that absorption of nutrients from the water, as with free-living dinoflagellates, is of minimal importance. Further studies are needed, however, to substantiate this hypothesis.

Mineral composition has an important influence on the growth and survival of *A. ocellatum in vitro* (Noga, 1989). Mineral composition had different effects on various life stages. Media with K^+, Mg^{2+}, Ca^{2+} and SO_4^{2-} content were best for continued growth. Media that enhanced dinospore differentiation to trophonts would not support continued survival. Certain mineral ions

(possibly K^+, Mg^{2+}, Ca^{2+}, and SO_4^{2-}) inhibit dinospore differentiation while stimulating development of other stages (Noga, 1989). In magnesium-deficient sea water, trophonts grow to normal size but fail to divide and eventually die (Oestmann and Lewis, 1996a). The effect of mineral composition on parasite growth and development suggests that mineral fluxes occur even while the parasite is attached to the host. Piscine intracellular fluid differs considerably from that of sea water with respect to concentrations of ions such as K^+ and Na^+. Hence the parasite may need to regulate by ionic exchange with the environment (Noga, 1989).

Other dinoflagellates

It has been hypothesized that the presence of chloroplasts and lack of food vacuoles suggest that *Piscinoodinium* can derive nutrition from photosynthesis (Lom and Schubert, 1983). The extensive ramifying nature of the attachment organelle implies that some nutrition is also obtained from the host, probably by osmotrophy.

In *Crepidoodinium*, the minute rhizoids contact but do not penetrate enough of an area of the host cell membrane for osmotrophy to supply much, if any, nutrition (Lom *et al.*, 1993). Thus *Crepidoodinium* is more appropriately termed an ectocommensal rather than a parasite. It is an autotroph that probably uses the fish primarily as an attachment site.

Diagnosis of Infections

Classical methods

Gross skin infestations of oodinids are most easily seen on dark-coloured fish. Skin parasites are best observed using indirect illumination, such as by shining a flashlight on top of the fish in a darkened room. Observing fish against a dark background also helps. While presumptive diagnosis of oodinid infestation may sometimes be made from the gross clinical appearance (e.g. 'velvet'), microscopic identification of trophonts or tomonts is required for definitive diagnosis. If fish are small, they can be restrained in a dish of water, and eyes, skin and fins examined under a dissecting microscope (Fig. 2.9). Lifting the operculum allows examination of the gills. Trophonts are removed by gently brushing or scraping the skin or gills, followed by microscopic examination of the sediment, which contains detached parasites. Snips of gill are also removed from living or recently dead fish for examination (Lawler, 1977b, 1980; Noga, 1996).

Freshwater dips dislodge marine dinoflagellates and are especially useful for small fish. Fish are placed in a beaker of fresh water for 1–3 min. After 15–20 min, tomonts settle to the bottom of the beaker. Saltwater baths can be used to dislodge *Piscinoodinium*. They are detected using a dissecting or inverted microscope (Bower *et al.*, 1987). Fish are best examined while still living or soon after death, as parasites detach shortly after host death. Interestingly, the kinetoplastid flagellate parasite *Ichthyobodo* is detached from fish by treatment with tricaine anaesthetic in poorly buffered water (Callahan and Noga, 2002). Whether tricaine has the same effect on oodinid dinoflagellates is unknown.

Immunological and molecular methods

Fish that are recovering from spontaneous *Amyloodinium* infestation or that have been experimentally exposed to parasite antigen may produce serum antibody that is detectable by ELISA or dinospore agglutination assay (Smith *et al.*, 1992; Cobb *et al.*, 1998a, b; Cecchini *et al.*, 2001). Such assays might be useful for monitoring levels of protection in susceptible populations, since elevated antibody titres have been associated with resistance (Cobb *et al.*, 1998a, b).

Sequencing of the SSU-rRNA genes from three geographical isolates of *A. ocellatum* (DC-1, Gulf of Mexico (Florida) and Red Sea) revealed > 99% sequence identity (Levy *et al.*, in preparation). Using these sequences, consensus *Amyloodinium*-specific oligonucleotide primers were developed for

a PCR assay. The assay could detect as few as 10 dinospores/ml of water when processed using the MoBio® Soil Extraction Kit (MoBio, Inc., Solana Beach, California, USA) followed by PCR. The assay was also specific for *Amyloodinium*, potentially allowing for highly sensitive monitoring of pathogen load in susceptible populations.

Prevention and Control

Amyloodinium

The economic importance of warmwater mariculture has created an effort towards the development of methods for the prophylaxis and treatment of amyloodiniosis. *Amyloodinium* has a very rapid reproductive rate and can complete its life cycle in less than 1 week under optimal conditions; thus prompt treatment is imperative to prevent it from quickly overwhelming a susceptible fish population. The free-swimming dinospore is susceptible to chemotherapy (Lawler, 1980; Paperna, 1984), but trophonts and tomonts are relatively resistant, making eradication difficult. For example, tomonts tolerate copper concentrations that are over ten times the levels that are toxic to dinospores (Paperna, 1984). Even when tomonts are inhibited from dividing, they can often resume dividing when returned to untreated water (Paperna *et al.*, 1981). Thus periodic examination for reinfestation after treatment is advisable.

Copper is most widely used for treatment (Bower, 1983; Cardeilhac and Whitaker, 1988). The free copper ion is the active component and free copper must be maintained at 0.12–0.15 mg/l for 10–14 days to control epidemics. Higher concentrations of free copper should be avoided because they are toxic to fish. Copper levels needed to treat amyloodiniosis are also toxic to most invertebrates and algae. Free copper ions are unstable in sea water and thus copper levels should be monitored closely, and adjusted as needed. Chelating agents (e.g. citrate or ethylenediamine tetra-acetic acid (EDTA)) increase the stability of copper in water but can be more difficult to monitor (Noga, 1996).

C. Bower (Connecticut, personal communication) discovered that the antimalarial chloroquine diphosphate is very safe and effective in treating amyloodiniosis. Experimentally infested clownfish (*A. ocellaris*) were free of *A. ocellatum* infestation after a 10-day exposure to a single waterborne treatment of 5–10 mg/l chloroquine diphosphate. Chloroquine has no effect on tomont division, but kills dinospores immediately upon their excystment. This concentration is non-toxic to fish, but is highly toxic to micro- and macroalgae and to various invertebrates (C.E. Bower, Connecticut, personal communication) and thus cannot be used in reef tanks, at least as a waterborne formulation. The pharmacokinetics of orally administered chloroquine in cultured red drum, *S. ocellatus*, suggests that it shows promise as an oral medication (Lewis *et al.*, 1988). However, chloroquine is very expensive and is not likely to be approved for food fish.

Many other agents have shown limited success against amyloodiniosis. Flush treatment with 100–200 mg/l formalin for 6–9 h caused trophonts to detach from the fish, but tomonts resumed division after the removal of the formalin (Paperna, 1984). Nitrofurazone (50 mg/l), Furanace® (2.5 mg/l), acriflavin (6 mg/l) or malachite green (0.5 mg/l) also halted tomont division or killed this stage over a 4–17-day treatment period, but parasite development often resumed after the drug was removed from the water.

Long-term baths with chlorotetracycline, tetracycline, acetic acid and potassium permanganate were ineffective (Lawler, 1977a). The herbicides simazine, endothall or diuron did not control amyloodiniosis, but the antiseptic benzalkonium chloride is reportedly effective (Johnson, 1984).

Polyether ionophorous antibiotics adversely affect osmotic regulation in parasites and it was hypothesized by Oestmann and Lewis (1996b) that use of one such agent, lasalocid, might be effective against *Amyloodinium*, which has a highly effective osmoregulatory capacity. *In vitro* treatment

of tomonts with its water-soluble salt, 3,*N*-methylglucamine lasalocid, completely inhibited dinospore emergence at concentrations as low as 0.01 mg/l. Treatment of infestations on red drum fry for 24 h with 0.10 mg/l reduced gill infestation intensity by 80% and none of the fish treated with 1 mg/l had any signs of infestation. The cost effectiveness of these dosages was not discussed, although it is encouraging that lasalocid is currently used for treating coccidiosis in other food animals.

Amyloodinium tolerates wide temperature and salinity ranges, making environmental control difficult (Paperna, 1984). Lowering the temperature to 15°C arrests the disease process, but this is almost never feasible. Lowering salinity delays but does not prevent infestations (Barbaro and Francescon, 1985), unless fish are placed in fresh water. A short freshwater bath of up to 5 min dislodges most but not all trophonts (Kingsford, 1975; Lawler, 1977b).

A novel biocontrol strategy was proposed by Oestmann *et al.* (1995), based upon the observation that larval brine shrimp (*Artemia salina*) readily prey on dinospores. All dinospores were eliminated after 8 h in an *in vitro* assay at a ratio of 8 nauplii/ml and 10,000 dinospores/ml (1 nauplius/1250 dinospores). Adding nauplii prior to experimental challenge of fish also reduced, but did not eliminate, the infestation.

The most successful medical approaches have involved repeated treatments, often followed by removal of the fish to a clean (uncontaminated) tank. For example, treatment of juvenile bullseye puffers (*Sphoeroides annulatus*) in sea water with 51 mg/l formalin for 1 h or 4 mg/l formalin for 7 h significantly reduced *Amyloodinium* load on the skin and gills. Reinfestation occurred after 15 days but was controlled by repeating the treatment (Fajer-Ávila *et al.*, 2003).

Laboratory treatment with hydrogen peroxide at either 75 or 150 mg/l eliminated *Amyloodinium* trophonts from Pacific threadfin (*Polydactylus sexfilis*) without fish mortality (Montgomery-Brock *et al.*, 2001). In a field trial, two 75 mg/l treatments 6 days apart, followed by moving the fish to a clean tank, reduced parasites to undetectable levels. However, fish treated with a 300 mg/l dose died, suggesting that this drug has a relatively low therapeutic index. Treatment of Pacific threadfin with a 5 min freshwater bath followed by transfer to a clean tank, repeated three times every 3 days, was also effective (Ostrowski and Molnar, 1998).

It is likely that repeatedly reducing the parasite burden with sequential treatments is allowing time for an acquired immune response to develop, which helps to clear the infection (see 'Host–Parasite Relationships').

In terms of prevention, the risk of introducing infectious dinospores into an aquaculture system may be reduced by disinfection of incoming water, such as by ultraviolet irradiation (Lawler, 1977b). Ageing of water beyond the survival time of dinospores and quarantine of new fish for at least 20 days are additional measures that may reduce, but not eliminate, the risk of parasite introduction. Dinospores remain infective for at least 6 days at 26°C (Bower *et al.*, 1987) and one must allow time for all tomonts to sporulate. To reduce epidemics in wild-caught southern flounder, fish were treated prophylactically and also placed in low-salinity (0–1 psu) water for at least 1 week (Smith *et al.*, 1999).

Other dinoflagellates

Heating water to 33–34°C reportedly controls *Piscinoodinium* infestations (Untergasser, 1989), but some aquarium fish cannot tolerate such high temperatures. However, for all treatments, the temperature should be raised to the parasite's optimum (~ 25°C) to ensure that the susceptible stage (dinospore) is rapidly exposed to the treatment. Temperature should be raised no more than 1°C/h.

The safest and most effective treatment for piscinoodiniosis is salt (about 1 teaspoon NaCl or artificial seawater salt per 5 gallons of water) for 10–14 days. For infestations requiring more immediate treatment, a 35 g/l NaCl dip for 1–3 min dislodges trophonts. Immersion for 3–5 days in 7 g/l NaCl

combined with 40 mg/l potassium permanganate is reportedly curative (van Duijn, 1973). However, some tropical aquarium fish cannot tolerate such high salt concentrations, and levels of potassium permanganate that exceed 2 mg/l of active ingredient are not considered safe for fish (Plumb, 1979). Exposure of *Piscinoodinium*-infested matrinxa (*Brycon cephalus*) to 6 psu NaCl for 96 h significantly reduced the parasite load on the transported fish and was apparently well tolerated by this stenohaline freshwater fish (Carneiro *et al.*, 2002).

Immersion for 10 days in methylene blue (3 mg/l) or acriflavine (10 mg/l) is reportedly curative (Jacobs, 1946; van Duijn, 1973). Higher dosages of methylene blue are recommended in organically rich water. However, methylene blue is toxic to nitrifying bacteria (Collins *et al.*, 1975) and many aquatic plants (van Duijn, 1973). While *Piscinoodinium* is susceptible to copper (van Duijn, 1973), copper is unsafe for fish when used in waters having less than 50 mg/l alkalinity.

Reducing lighting to inhibit autotrophy has been advocated during drug treatment (van Duijn, 1973) but its effectiveness is unproven. Dinospores remain infective for up to 48 h (Jacobs, 1946; van Duijn, 1973). *Piscinoodinium* has been observed asymptomatically on a number of fish, including goldfish (*Carassius auratus*), common carp, Siamese fighting fish (*Betta splendens*) and gourami (*Colisa* sp.) (Thilakaratne *et al.*, 2003), suggesting that strict quarantine of susceptible fish is needed to prevent inadvertent spread. Attempts to treat other parasitic dinoflagellates have not been reported.

Summary and Recommended Research

Increasingly, natural fisheries have reached or surpassed their sustainability, and thus the gap between fish supplies and consumer demand must be met by aquaculture. Within the stressful confines of aquaculture, parasitic dinoflagellates exert their greatest impact. More effective methods are especially needed to control disease caused by *Amyloodinium*, the most important parasitic dinoflagellate affecting fish. It is very difficult to eliminate the infestation and, with the increasing regulations on the use of drugs in aquaculture, it is necessary to optimize the application of currently approved drugs, as well as try other approaches. One alternative to drugs is environmental manipulation, but a better understanding of environmental conditions that affect parasite growth and survival is needed, as well as a means for the feasible utilization of these data in commercial applications. The strong evidence for a protective immune response against *Amyloodinium* holds great promise for the eventual development of protective vaccines. Likewise, the identification of potent non-specific defences has the potential to allow broad-spectrum protection.

Parasitic dinoflagellates are a polyphyletic group and molecular studies are needed to clarify the taxonomic relationships among various taxa at all levels, from major taxonomic groups to intraspecific relationships. These data are also needed to provide highly specific and sensitive diagnoses for effective biosecurity and management of infestations/infections in culture. This will also help to avoid the unwanted introduction and spread of exotic isolates and would be especially useful for detecting latent carriers, which are the most likely reservoirs for parasitic dinoflagellate infestations/infections. Molecular studies will also be critical to understanding the taxonomic relationships and biology of *Ichthyodinium*, which appears to affect a large number of important feral fish species.

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3 Diplomonadida (Phylum Parabasalia) and Kinetoplastea (Phylum Euglenozoa)

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Introduction

Members of the order Diplomonadida and class Kinetoplastea are flagellated protozoans that are generally found in the digestive tract and blood/body fluids of vertebrates (fishes, amphibians, reptiles, birds and mammals). In addition, some species are ectoparasitic and are found on the body surface and/or gills of fishes.

Diplomonads (Figs 3.1 and 3.2) have bilateral symmetry, and each parasite has two karyomastigonts. A karyomastigont consists of one to four flagella, of which one is recurrent, and an axial structure of microtubules and accessory filaments (Lee, 1985). Most of these parasites are extracellular in the intestinal tract, and the active trophozoites multiply by binary fission. They usually form cysts, which are passed into the external environment with faeces, but cysts have not been found in some species. Transmission from host to host is direct, and infection normally occurs via the ingestion of cysts or trophozoites.

Kinetoplastids (Figs 3.7, 3.12 and 3.33) have one or two flagella, an axoneme and a paraxial rod, which arise from a flagellar pit (Vickerman, 1976a). This parasite has a mitochondrion that contains DNA, and it usually extends the length of the body (Vickerman, 1977). The kinetoplast, which is part of the mitochondrial system, stains reddish with Romanovsky stains. The mitochondrion is mainly concerned with aerobic respiration (Vickerman, 1976b). Most species are extracellular parasites in animals, and they are in the blood/tissue fluids, digestive tract and internal organs of their vertebrate and invertebrate hosts. Ectoparasitic kinetoplastids and those associated with the digestive tract have direct transmissions, while the haematozoic parasites are usually transmitted by bloodsucking invertebrates.

Many diplomonads and kinetoplastids cause disease in their vertebrate hosts. Some (e.g. *Trypanosoma cruzi* in humans, *Trypanosoma vivax* in cattle, *Hexamita meleagridis* in turkeys) are very well studied because of their medical and/or economic importance; however, relatively less is known about those that cause disease in fish, amphibians and reptiles. This review is focused on the diplomonads (*Hexamita* and *Spironucleus*) and kinetoplastids (*Ichthyobodo*, *Cryptobia* and *Trypanosoma*) that are known to cause disease in freshwater and marine fishes.

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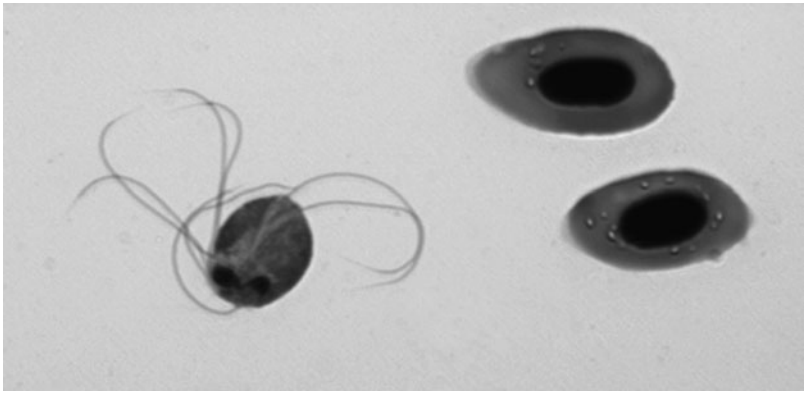


Fig. 3.1. Trophozoites (light microscope) of *Spironucleus* from the blood of an experimentally infected chinook salmon (× 1000) (Guo and Woo, unpublished).

Hexamitosis and Spironucleosis

Introduction

Hexamita and *Spironucleus* (collectively called hexamitids in the current review) are frequently reported in the digestive tract of fishes, and sometimes also in internal organs and tissues. These parasites have direct life cycles, they are difficult to distinguish under the light microscope and some species are associated with morbidity and mortality (diseases known as hexamitosis, spironucleosis and possibly 'hole-in-the-head' disease) in salmonids, cyprinids and ornamental aquarium fishes. Although the gross pathology and histopathology of the disease(s) (e.g. spironucleosis) have been described in some detail, the mechanism(s) of the disease(s) has not been studied or elucidated, and subclinical infections are often reported in the field. Sporadic outbreaks of fatal systemic spironucleosis have occurred in cyprinids (Molnar, 1974), in eels (Einszporn-Orecka, 1979), in tropical aquarium fishes (Becker, 1977; Ferguson and Moccia, 1980; Gratzek, 1988; O'Brien *et al.*, 1993), in Atlantic salmon (Mo *et al.*, 1990; Poppe *et al.*, 1992) and in chinook salmon (Kent, 1992; Kent *et al.*, 1992). The disease was initially referred to as systemic hexamitosis because it was thought the pathogen belonged to the genus *Hexamita*. Factors that precipitated these outbreaks are not known, and hence these parasites remain potential problems for the aquaculture industry.

Geographical distribution and host range

Hexamitids are presumed to have a very wide host range and geographical distribution. These parasites are in wild, farmed and aquarium fishes in cold, temperate and warm waters. They have been reported in Acipenseridae, Anguillidae, Catostomidae, Centarchidae, Cyprinidae, Cyprinodontidae, Gadidae, Gasterosteidae, Mugilidae, Percichthyidae, Percidae, Salmonidae, Siganidae and Sparidae. Arthur *et al.* (1976) indicated that prevalence of these parasites varies greatly in fishes and even in fish between adjacent geographical localities.

Hexamita salmonis has been reported in fishes in North America, Europe and Asia. It is usually found in freshwater fishes but it has also been reported from marine fishes (Becker, 1977; Mo *et al.*, 1990; Buchmann *et al.*, 1995). The disease is most commonly found in fingerlings, although yearlings and smolts may also show clinical signs of the infection. Outbreaks of the disease are usually sporadic in aquaculture facilities, and they have seldom been reported in wild fishes (Allison, 1963; Uzmann and Hayduk, 1963; Becker, 1977; Mo *et al.*, 1990).

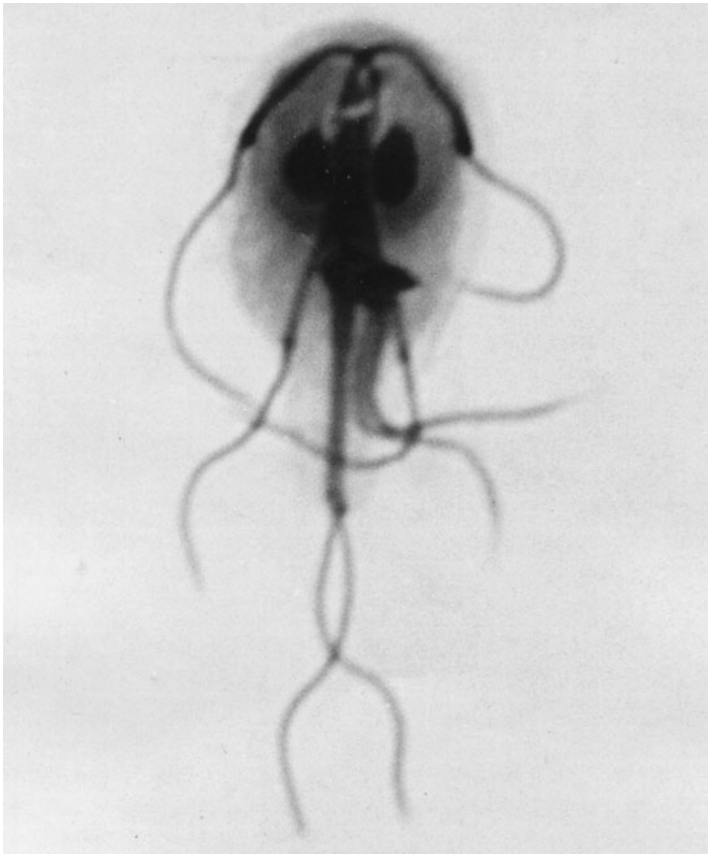


Fig. 3.2. Trophozoite (light microscope) of *Giardia microti* from the intestine of a naturally infected meadow vole (*Microtus pennsylvanicus*) from Guelph, Ontario, Canada (× 1150; original).

Spironucleus vortens is in angelfish (*Pterophyllum scalare*) in Florida, USA (Poynton *et al.*, 1995), in discus (*Symphysodon discus*) and angelfish in the UK (Paull and Matthews, 2001) and in ide (*Leuciscus idus*) in Norway (Sterud and Poynton, 2002). It is presumed to be the causative agent in 'hole-in-the-head' disease in cichlids. The parasite is also known as 'discus parasite' as it is often found in discus fish (Gratzek, 1988).

Spironucleus are known to be pathogenic and cause disease in salmonids maintained in sea cages. *Spironucleus barkhanus* has been isolated from Atlantic salmon (*Salmo salar*), grayling (*Thymallus thymallus*) and Arctic charr (*Salvelinus alpinus*) in Norway (Mo *et al.*, 1990; Poppe *et al.*, 1992; Sterud *et al.*, 1998, 2003), while the hexamitid in chinook salmon (*Oncorhynchus tshawytscha*) in sea cages on the Pacific coast in Canada (Kent *et al.*, 1992) is believed to be different.

Systematics and taxonomic position

Members of the order Diplomonadida have a double set of organelles and bilateral symmetry (Lee, 1985; Vickerman, 1990; Figs 3.1 and 3.2), and those that are known to cause disease in vertebrates include *Hexamita*, *Spironucleus*, *Octomitus* and *Giardia*.

The hexamitids (e.g. *Hexamita*, *Spironucleus*) are presumed to have evolved from *Enteromonas* (Brugerolle, 1975; Siddall *et al.*, 1992), which has a single karyomastigont. Parasitism is believed to have

proceeded from *Trepomonas* to *Hexamita*, to *Spironucleus*, to *Octomitus* and finally to *Giardia*. *Trepomonas* and *Hexamita* can live in rich organic waters or as intestinal parasites, while the remaining three genera are all parasitic. There is a gradual reduction in the size of the cytostome from *Trepomonas* to *Hexamita* and to *Spironucleus* (Fig. 3.1), and the organelle is absent in *Octomitus* and *Giardia*. *Giardia* (Fig. 3.2) is the only diplomonad that has a ventral disc, which is used for attachment to intestinal cells.

It is generally accepted that *Hexamita* has an elongated to spherical body and spherical nuclei, while a pyriform body and elongated nuclei are characteristic of *Spironucleus* (Becker, 1977; Lee, 1985; Poynton and Sterud, 2002). However, the shape of the body may be distorted in air-dried smears, as was shown with *Giardia* (Grant and Woo, 1978). A less unambiguous ultrastructural feature is the position of the anterior kinetosomes (flagellar bases), and it is one of the features used to distinguish *Hexamita* from *Spironucleus* (Lee *et al.*, 1985). Also, the two recurrent flagella in their canals (also sometimes referred to as flagellar pockets) are located between the nuclei in *Spironucleus* while those in *Hexamita* are on the surface of the nuclei (Brugerolle and Lee, 2000).

The taxonomy of hexamitids in fishes needs further careful study because most species descriptions are only based on morphological features as seen under the light microscope. Electron microscopy (both scanning and transmission) is useful but it also has limitations. The use of biological features for species differentiation has been employed successfully with *Trypanosoma* and *Cryptobia* (Woo, 1994) and it may also be a useful approach with *Spironucleus*. These include host specificity of the parasite as determined in cross-transmission studies and the use of other biological characteristics (e.g. antigenic, culture characteristics) to help delineate species of haemoflagellates in fishes, especially those parasites that are morphologically similar to each other (Bower and Woo, 1977; Jones and Woo, 1991, 1992; Woo and Thomas, 1991).

There are at least 20 nominal species of *Hexamita* and ten species of *Spironucleus* (Vickerman, 1990), and a few more species of *Spironucleus* have been described since the review by Vickerman. Hexamitids from the intestinal tract of salmonids are usually assumed to be *H. salmonis*, and this assumption should be tested under rigid experimental conditions. Currently, three species of hexamitids (*H. salmonis*, *S. vortens* and *S. barkhanus*) and perhaps an undescribed species of *Spironucleus* are known to cause disease in fishes, and they are the focus in the current review.

H. salmonis has two sac-like nuclei, which taper at the anterior end. Also, its kinetosomal complexes are anteromedial, and its recurrent flagella between the two nuclei exit the body posteriorly (Moore, 1922). Also, in *H. salmonis* the cytostome openings are caudal and at the flattened posterior end of the body, but in *Spironucleus* they are posterolateral and the body may taper to a caudal projection (Brugerolle *et al.*, 1973; Becker, 1977; Kulda and Nohynkova, 1978; Lee, 1985).

Kent *et al.* (1992) has suggested that *H. salmonis* may actually be a *Spironucleus*; and Sterud *et al.* (1997) rightly pointed out that the transfer of *H. salmonis* to the genus *Spironucleus* should be done after 'comprehensive morphological, scanning and transmission electron microscope studies of flagellates from the type host and locality (brook trout, from Bath, New York)'. However, Poynton *et al.* (2004) prematurely made the transfer based on the study of a hexamitid isolated from juvenile rainbow trout from a farm in Northern Ireland (Ferguson, 1979). It should be noted that the main focus of the initial report (Ferguson, 1979) was to investigate the high mortality of juvenile rainbow trout in the fish farm. Ferguson (1979) presumed that the intestinal flagellate was *H. salmonis*, although no pathological lesions (Ferguson, 1989) were detected in the intestine of infected fish. His presumptive identification of the parasite was probably based on the fish host (a salmonid) and on the successful use of furazolidone to treat the infection and to reduce fish mortality. There is little doubt

that the Poynton *et al.* (2004) study indicates that the hexamitid from rainbow trout in Northern Ireland (Ferguson, 1979) is a *Spironucleus*, but is it *H. salmonis*? As indicated by Sterud *et al.* (1997), a very important factor in a systematic study of this type is that the parasite should be from the type host and type locality of *H. salmonis*. This obviously was not done, and it is another reason why the proposal made by Poynton *et al.* (2004) is not used in this review.

Based on gene sequences (e.g. genes for elongation factor 1 alpha, α-tubulin) Keeling and Doolittle (1996, 1997) suggested that the two hexamitids isolated from the systemic disease in salmonids (Mo *et al.*, 1990; Kent *et al.*, 1992; Poppe *et al.*, 1992) are not *Spironucleus*. This suggestion has not been accepted by other workers (e.g. Sterud *et al.*, 1998, 2003; Guo and Woo, 2004a,b). Keeling and Doolittle (1996, 1997) also indicated that the two isolates from salmonids, one now described as *S. barkhanus* (Sterud *et al.*, 1997) and the other perhaps being an undescribed species or subspecies of *Spironucleus* (F.C. Guo and P.T.K. Woo, unpublished), belong to a single species. This latter parasite is currently under study and will be referred to as the 'chinook *Spironucleus*' in the current discussion.

Parasite morphology, host specificity and life cycle

Morphology

Live trophozoites of *Hexamita* and *Spironucleus* vary from elongated to nearly spherical and the two genera are usually difficult to separate. The pyriform trophozoites become more spherical before they divide. The lengths of live organisms may reach 20 μm and the organisms have bilateral symmetry. Each side has a nucleus and four flagella and this is the karyomastigont. The four kinetostomes of each karyomastigont are arranged in two pairs, with the two anterior kinetostomes designated as K1 and K2 while the two posterior ones are K3 and R (R is associated with the recurrent flagellum). The six anterior flagella are for locomotion, while the two recurrent flagella pass through the body and emerge as free posterior flagella (Lee, 1985). Flagella lengths are about 1.5–2 times the length of the body.

The nuclei of *Hexamita* are oval or spherical and are apposed at their flattened median portions, whereas those in *Spironucleus* are pyriform and S-shaped and are adjacent at their narrow anterior ends (Brugerolle *et al.*, 1973; Brugerolle, 1974; Kulda and Nohynkova, 1978). The two recurrent flagella in their canals run between the nuclei in *Spironucleus*, while in *Hexamita* they are on the surface of the nuclei (Brugerolle and Lee, 2000). The apposed kinetosomal complexes in *Hexamita* are anterolateral near each nucleus, while those in *Spironucleus* are anteromedial and near the extreme anterior, with the recurrent flagellum passing on the internal side of the nuclei (Brugerolle *et al.*, 1973; Brugerolle, 1974; Lee, 1985). The kinetosomal pocket in the nucleus is shallow in *Hexamita*, while it is deep in *Spironucleus* (Siddall *et al.*, 1992).

Diplomonad cysts are usually oval in shape, and each cyst usually contains two recently divided flagellates. Consequently, the cyst will have four nuclei, a flagellar sheath, partially disassembled microtubular ribbons, a striated rootlet fibre and glycogen rosettes (Brugerolle *et al.*, 1973; Januschka *et al.*, 1988; Vickerman, 1990).

Fixed and stained trophozoites of *H. salmonis* measure 8–14 μm in length and 6–10 μm in width (Kulda and Lom, 1964a), while the oval or round cysts measure 7 × 10 μm. Many cysts contain two flagellates (Becker, 1977).

Live *S. vortens* trophozoites are pyriform in shape and measure 12.5–20.5 μm in length and 5.0–11.2 μm in width. Protargol-stained specimens are much smaller and they are 5.5–9.9 μm in length and 3.3–4.8 μm in width. The body (under scanning electron microscope (SEM)) is adorned with compound lateral ridges, which counter-cross at the posterior end, where the recurrent flagella emerge (Poynton *et al.*, 1995).

Live *S. barkhanus* trophozoites vary from pear-shaped to nearly spherical, with body length 15.6 (11.0–20.0) μm and width 9.4 (6.0–14.0) μm (under light

microscope). Trophozoites prepared for SEM are smaller, with average length 7.6 (5.7–9.0) µm and width 5.2 (4.4–6.3) µm. The six flagella exit anterolaterally, while the two recurrent flagella emerge posteromedially from cytostome openings, which are surrounded by crescent-shaped ridges (under SEM). The body is not adorned (Sterud *et al.*, 1997).

Briefly, the two elongated sac-like nuclei of *S. barkhanus* taper at their anterior ends (under transmission electron microscope (TEM)), and the two kinetosomal complexes are in deep depressions close to the anterior tips of the nuclei. The recurrent flagella are located between the two nuclei and exit posteromedially through two cytostomes. Each cytostome is partially surrounded by a striated lamina, which originates from the kinetosomal area. More ultrastructure details of the organism are in Sterud *et al.* (1997).

Live trophozoites of the 'chinook *Spironucleus*' from experimentally infected chinook salmon are about the same size as *S. barkhanus*, and in Leishman–Giemsa stained smears the nuclei in some specimens appear tear-drop-shaped (Fig. 3.1). SEM specimens show that the two posterior cytostomes are surrounded by crescent-shaped ridges and the body is not adorned (Kent *et al.*, 1992). The 'chinook *Spironucleus*' infects chinook salmon and may cause transitory infection in Atlantic salmon; *S. barkhanus* from Atlantic salmon does not infect chinook salmon (Kent *et al.*, 1992; F.C. Guo and P.T.K. Woo, unpublished). This is one indication that they are different; however, further comparative studies of the biology of the two organisms are needed.

Host specificity

Although hexamitids are common in fishes, little is known about their actual host specificity as determined in cross-transmission studies. Some species are presumed to be not host-specific as they have been reported from fishes that are not closely related. Hexamitids in the intestinal tract of salmonid fishes are usually assumed to be *H. salmonis*; hence the parasite has been reported in North America, Europe and Asia. It usually occurs in freshwater fishes but it has also been reported in marine fishes (e.g. Becker, 1977; Mo *et al.*, 1990; Buchmann *et al.*, 1995*).* Similarly, *S. vortens* described from a warmwater fish (*P. scalare*) in Florida, USA (Poynton *et al.*, 1995), has also been reported from a coldwater fish (*L. idus*) in Norway (Sterud and Poynton, 2002). These are reports of natural infections and should be confirmed with well-controlled experimental studies.

Spironucleus elegans from cichlids does not readily infect salmonids (Kulda and Lom, 1964a,b). However, amphibians are presumed to be a source of infection for fish since angelfish can acquire *S. elegans* from newt rectal fluid (Kulda and Lom, 1964a). Kent *et al.* (1992) showed that the 'chinook *Spironucleus*' from systemic infection in chinook did not infect Atlantic salmon. Cross-transmission studies (Guo and Woo, 'unpublished') using the 'chinook *Spironucleus*' and *S. barkhanus*, and hatchery raised salmon showed that most Atlantic salmon could not be infected by the 'chinook *Spironucleus'* – both parasites used in the study were the initial outbreaks of the disease.

Life cycle

Trophozoites of hexamitids are usually found extracellularly in the lumen of the intestine, in internal organs or in lesions on the body surface (Nigrelli and Hafter, 1947; Paperna and Overstreet, 1981; Mo *et al.*, 1990; Poynton and Morrison, 1990; Kent, 1992; Kent *et al.*, 1992). However, *S. barkhanus*, which causes systemic disease in Atlantic salmon, also has an intracellular stage, and the significance of this is not known. The parasite is in host cells in capillaries and sinusoids of the liver, spleen and head kidneys (Sterud *et al.*, 2003).

Infection is generally believed to occur via the ingestion of cysts or trophozoites; however, the rectal route of infection has also been suggested (Moore, 1922; Kulda and Lom, 1964a,b; McElwin and Post, 1968; Becker, 1977; Poynton and Morrison, 1990;

Kent *et al.*, 1992). In intestinal infections, cysts and trophozoites are normally voided with the faeces. Kent *et al.* (1992) did not find cysts in chinook salmon with systemic infections and they suggested that transmission was via trophozoites. It was assumed that the mucus and/or faecal material from the fish protected the trophozoites from osmotic stress in the aquatic environment. Poppe *et al.* (1992) also suggested that the trophozoites in skin lesions on heavily infected Atlantic salmon might be a source of infection.

S. barkhanus has two distinct developmental phases in experimentally infected Atlantic salmon (Guo and Woo, 2004a). The parasites were in the blood 1–8 weeks after infection (blood phase; Fig. 3.3) before they disappeared from the blood and were confined to the internal organs (spleen, liver, etc.), eye socket, muscles and in lesions on the body surface (tissue phase). Fish mortality occurred during both the blood and tissue phases of the disease, and there were no detectable differences in susceptibility to disease and mortality in three full-sib families of Atlantic salmon (Guo and Woo, 2004a).

Kent *et al.* (1992) suggested that systemic spironucleosis in chinook salmon held in sea cages was transmitted directly from infected to uninfected fish and this was probably through exposure of uninfected fish to ascitic fluid and tissues of infected fish. Guo and Woo (2004a) demonstrated direct transmission of *S. barkhanus* from infected to uninfected Atlantic salmon when fish were held in the same tank. Parasites were detected in the blood of fish about 9 weeks after the start of the cohabitation study with experimentally infected fish.

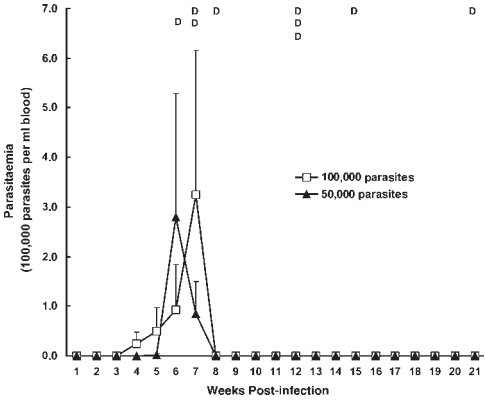


Fig. 3.3. Course of *Spironucleus barkhanus* infection (blood phase of the disease) in experimentally infected Atlantic salmon (from Guo and Woo, 2004a; courtesy of *Diseases of Aquatic Organisms*). D, a dead fish.

Host–parasite relationships

Intestinal infections

Trophozoites are commonly found in the lumen of the intestine, while cysts are often not seen. Trophozoites of *H. salmonis* are usually in the upper intestine and pyloric region, and they are also sometimes in the gall bladder and rectum. In heavy infections they may occur throughout the intestinal tract (Moore, 1922; Becker, 1977; Uldal and Buchmann, 1996). *S. elegans* is normally in the lower intestine and rectum (Kulda and Lom, 1964a; Becker, 1977), while *Spironucleus torosa* seems to be confined to the rectum of codfish (Poynton and Morrison, 1990). Parasites that are morphologically similar to *S. torosa* have been found in the rectum of marine codfish (Alexeieff, 1910).

It is presumed that some hexamitids in trout can only get established if the intestine is not physiologically normal (Kulda and Lom, 1964b). It has also been suggested that some subclinical infections may become problematic if the immune system of the infected fish is depressed; this may result in increased parasitaemias, which may develop into systemic infections (Bassleer, 1983).

Systemic infections

Systemic hexamitid infections have been reported in cyprinids (Molnar, 1974), eels (Einszporn-Orecka, 1979), salmonids (Mo *et al.*, 1990; Kent, 1992; Kent *et al.*, 1992; Poppe *et al.*, 1992) and tropical aquarium freshwater fishes (Becker, 1977; Ferguson and Moccia, 1980; Gratzek, 1988; O'Brien *et al.*, 1993), with trophozoites in the blood, gall bladder, heart, kidney, liver, spleen, eye, brain, muscles, mesentery and abdominal

cavity. There are also reports of hexamitids in cranial skeletal tissues (Ferguson, 1989) and in pustules on the skin of infected cichlids (Nigrelli and Hafter, 1947).

Factors that promote the development of systemic infections are not known, nor have the mechanisms of the disease been elucidated. It was suggested that invasion of the intestinal epithelium and dissemination of the infection might occur if the immune system of the fish host was compromised. Also, necrotic changes and lesions in the intestine, such as those caused by other parasites (e.g. acanthocephalans), might provide easy access to the blood, and the blood disseminated the parasite to other organs (Molnar, 1974; Becker, 1977; Einszporn-Orecka, 1979; Ferguson and Moccia, 1980). Invasion of intact intestinal epithelium was not assumed to be the route of systemic infection in naturally infected Atlantic salmon (Poppe *et al.*, 1992). It was suggested that the infection was acquired when the fish were in fresh water and the disease was triggered by stress associated with transportation or smoltification. Some extra-intestinal infections were also accompanied by secondary infections, and the latter might be more pathogenic (Bassleer, 1983).

As indicated earlier, *S. barkhanus* in experimentally infected Atlantic salmon has two distinct developmental phases (blood and tissue phases). The parasite multiplies by binary fission in the blood soon after infection, and is detectable in increasing numbers for the first 2–4 weeks, after which the numbers decline to an undetectable level and this marks the end of the blood phase of the disease (Fig. 3.3). Fish mortality occurs in both the blood and tissue phases of the disease (Guo and Woo, 2004a).

It is suggested here that pathogenic hexamitids secrete a histolytic enzyme(s) which is an important contributing factor to the development of clinical disease in systemic disease (e.g. anaemia, lesions) and fish mortality. This virulent factor may be similar to the metalloprotease secreted by *Cryptobia salmositica*, a pathogenic flagellate that causes salmonid cryptobiosis (see later section). The secreted protease will allow the hexamitid to cause the anaemia, and will produce lesions in internal organs and large ulcers on the body surface (Fig. 3.4) of infected fish. Also, direct transmission studies of the two pathogens ('chinook *Spironucleus*' and *S. barkhanus*) via cohabitation of infected and uninfected fish (Kent *et al.*, 1992; Guo and Woo, 2004b) also support the hypothesis that the pathogens secrete a protease(s) which subsequently facilitates the invasion of the blood system by the pathogens to initiate the blood phase of the disease.

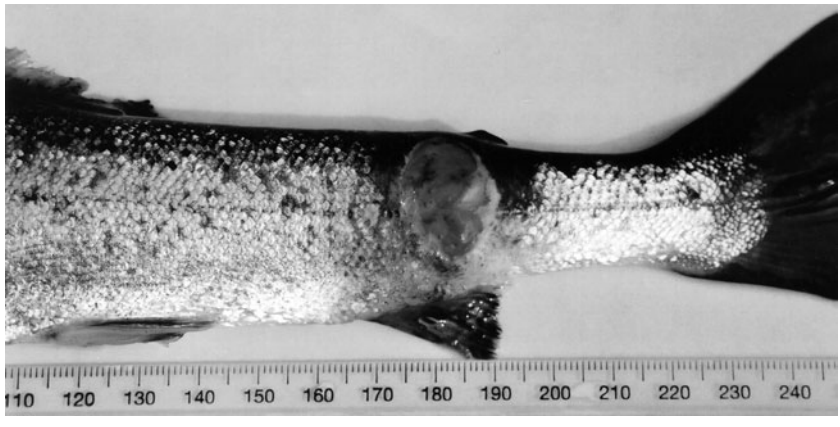


Fig. 3.4. A large ulcer on the body surface of Atlantic salmon experimentally infected with *Spironucleus barkhanus* (from Guo and Woo, 2004a; courtesy of *Diseases of Aquatic Organisms*).

Susceptibility

Families and species of fish are known to differ in their susceptibility to hexamitid infection and disease; for example, codfish (Gadidae) and porgies (Sparidae) are presumed to be susceptible to natural infections (Alexeieff, 1910), while brook trout (*Salvelinus fontinalis*) is the salmonid most susceptible to *H. salmonis* under hatchery conditions (Moore, 1922). The parasite does not cause clinical disease in coho salmon (*Oncorhynchus kisutch*), while steelhead trout (*Oncorhynchus mykiss*) suffer mortality (Uzmann *et al.*, 1965). In many freshwater tropical fishes, there are no clinical signs (presumably infected with *S. vortens*) of infections, whereas, in angelfish, discus (cichlids) and gouramis (belontiids), clinical disease is evident (Gratzek, 1988). Similarly, *S. barkhanus* is not known to cause clinical disease in naturally infected grayling and Arctic charr but causes systemic disease and mortality in Atlantic salmon (Sterud *et al.*, 1997, 1998; Guo and Woo, 2004b). However, Guo and Woo (2004a) found no significant difference in the susceptibility of three families of Atlantic salmon to experimental *S. barkhanus* infection. It is likely that some fish stocks (within a susceptible host species) are more susceptible than others, as has been shown in salmonid cryptobiosis (see later section).

Susceptibility to disease may also, in part, be related to the age of the host; for example, *H. salmonis* is not known to cause clinical disease in adult salmonids, while it may be pathogenic to younger fish (Becker, 1977). This is similar in tropical aquarium fishes (Bassleer, 1983). However, age susceptibility needs more careful laboratory studies as some adult fish in natural populations may have acquired immunity due to prior exposure(s) to the parasite as juvenile fish.

Clinical signs and pathology

In general, hexamitid infections in the intestine are usually chronic, with low numbers of parasites, but in heavy infections clinical disease may occur. Clinical signs of the disease in natural infections include anorexia, emaciation, weight loss, weakness, whirling, listlessness, abdominal distension, anaemia, faecal pseudocasts, pale stringy faeces, a red vent, exophthalmia and dark coloration (Moore, 1922; Allison, 1963; Becker, 1977; Ferguson and Moccia, 1980; Post, 1987; Andrews *et al.*, 1988; Gratzek, 1988; Mo *et al.*, 1990; Buchmann and Uldal, 1996).

Fish infected with *H. salmonis* may have anaemia, ascites, enteritis and intestinal contents that are yellowish and watery or jelly-like, with excessive amounts of mucus. The pathology associated with the infection varies from no effects (Uzmann *et al.*, 1965) to haemorrhages in the intestine (Roberts and Shepherd, 1974), catarrhal enteritis (Sano, 1970) and hepatocellular necrosis (Ferguson, 1979). Mortalities of infected juvenile rainbow trout were moderate to severe in outbreaks of the disease (e.g. Ferguson, 1979; Uldal and Buchmann, 1996). Infected rainbow trout were smaller and often had empty gastrointestinal tracts because of the anorexia (Uldal and Buchmann, 1996). Infected brook charr normally do not have clinical disease (M'Gonigle, 1940), while cyprinids with intestinal hexamitosis have enteritis, liver necrosis and serous exudates in the abdominal cavity (Molnar, 1974).

Infected aquarium tropical fish may have yellow mucus in their intestines, enteritis and enlarged and inflamed gall bladders (Molnar, 1974; Becker, 1977; Bassleer, 1983; Ferguson, 1989). High parasitaemias in the intestine are frequently associated with severe disease (Post, 1987; Andrews *et al.*, 1988). Hence, Gratzek (1988) suggested that the flagellate interfered with nutrition by competing for essential nutrients and/or by damaging the intestinal epithelium to affect digestion and absorption. The histopathology in the intestine includes gastritis and perforation with serosal granuloma in the stomach (Ferguson, 1989).

In systemic infections, the stomach of fish may be perforated, with chronic inflammations in internal organs, atrophy of renal tubules and necrosis of the kidney. These fish are usually in poor condition and die (Amlacher, 1970; Ferguson and Moccia, 1980). Flagellates are sometimes in renal peritoneal exudates in the kidney. Fish with

hole-in-the-head disease (presumed to be caused by *S. vortens*) have small hole-like lesions on the body surface which produce yellow, cheesy strings of mucus. In some fish (e.g. angelfish), these lesions may be confined to the head region, occasionally extending posteriorly along the lateral line (Bassleer, 1983; Andrews *et al.*, 1988; Paull and Matthews, 2001). Bilateral ulcers arise from large erosions in the cranial cartilage (Ferguson, 1989). Hexamitid infections in breeding angelfish may also lower the hatchability of eggs and/or cause the death of young fry (Gratzek, 1988).

Naturally infected Atlantic salmon with systemic disease may have dermatitis, with whitish discoloration of the peduncle and caudal fins. Infected post-smolts have whitish granulomatous nodules, which contain many flagellates, in the pale liver and kidneys. The most severe lesions are in large fish (4–5 kg), with additional brown abscesses in the caudal muscles. Livers and kidneys have multifocal necrosis, with oedema, congestions, haemorrhages and fibrosis. Fish with advanced disease have severe muscular degeneration, cholangiohepatitis and perihepatitis, encephalitis and meningitis (Poppe *et al.*, 1992). In experimentally infected Atlantic salmon, *S. barkhanus* causes disease with high mortality. The clinical signs include skin blisters, ulcers on the body surface (Fig. 3.4) and unilateral exophthalmia (Fig. 3.5). Gross pathologies include haemorrhaging in internal organs, splenomegaly, nodules in the liver (Fig. 3.6a), deformed (globulated) spleen (Fig. 3.6b) and granulomatous lesions in the spleen (Guo and Woo, 2004a).

Naturally infected chinook salmon with systemic infections have hypertrophic livers, which may be mottled with petechial haemorrhages and whitish, friable areas. There are also serosanguineous ascites, blood clots in the visceral cavity, enlarged spleen and kidney and petechiae throughout the skeletal muscles. Blood vessels, especially those in the liver and lower intestine, have large numbers of parasites. The liver is oedematous, congested and inflamed, while the renal interstitium is hyperplastic. The anaemia is caused partly by haemodilution and haemorrhage (Kent, 1992; Kent *et al.*, 1992).

In vitro culture and propagation of *Hexamita* and *Spironucleus*

H. salmonis was first cultured axenically at 10°C in Medium 199 supplemented with human cord serum, lactalbumin hydrolysate and antibiotics (penicillin, streptomycin and mycostatin). Cultures were supersaturated with nitrogen and maintained in a nitrogen atmosphere (Uzmann and Hayduk, 1963). More recently, the parasite was cultivated at pH 7.5 in Eagle's minimum essential medium (MEM), supplemented with fetal bovine serum (FBS), 4% bile-supplemented



Fig. 3.5. Unilateral exophthalmia in Atlantic salmon experimentally infected with *Spironucleus barkhanus* (from Guo and Woo, 2004a; courtesy of *Diseases of Aquatic Organisms*).

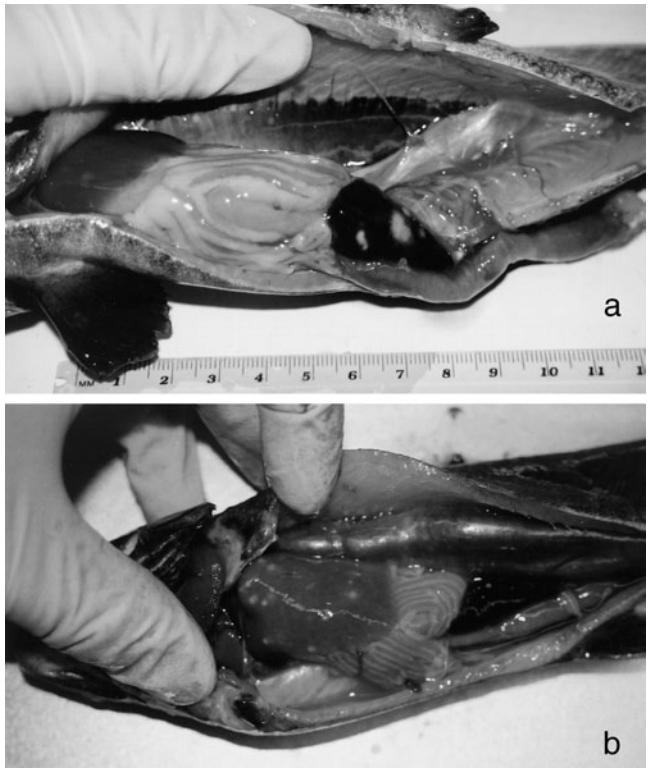


Fig. 3.6. Large nodules in the liver (a) and globulated spleen (b) in Atlantic salmon experimentally infected with *Spironucleus barkhanus* (from Guo and Woo, 2004b; courtesy of *Diseases of Aquatic Organisms*).

TYI-S-33 medium (Keister, 1983) and antibiotics. The flagellate multiplied rapidly at 10°C and multiplication was slower at 5°C. The addition of bile (30–960 mg bile per litre of medium) supported better growth than those either without bile or with a higher amount of bile (Uldal and Buchmann, 1996).

The modified TYI-S-33 medium (Keister, 1983) at 10°C supported rapid *in vitro* multiplication of *S. barkhanus* from Atlantic salmon and the 'chinook *Spironucleus*' from chinook salmon (Sterud, 1998; F.C. Guo and P.T.K. Woo, unpublished). Both parasites multiplied rapidly by binary fission. Also, isolates from muscle abscesses of Atlantic salmon and from the gall bladder of grayling were grown at 5, 10, 15 and 20°C. However, the isolate from grayling had a slightly higher optimum temperature and a higher upper temperature limit than the salmon isolate (Sterud, 1998).

Sangmaneedet and Smith (2000) also cultured *S. vortens* from the intestine of angelfish in TYI-S-33 medium. The parasite multiplied rapidly at 25, 28 and 31°C and the optimal pH ranged from 6.5–7.5. All cultures supplemented with either bovine or fish bile had lower numbers of parasites than those without bile.

Parasite nutrition

Endocytosis and exocytosis occur at the cytostomes (Brugerolle, 1974; Kulda and Nohynkova, 1978; Lee, 1985; Vickerman, 1990). In *Hexamita*, the cytostomes can dilate to ingest large bacteria; however, in *Spironucleus* only smaller bacteria and particles are ingested because of smaller cytostomes (Vickerman, 1990). Poynton and Morrison (1990) suggested that *S. torosa*

from codfish may also feed directly on the cytoplasm of intestinal cells because of the intimate association between the parasite and intestinal cells, as seen under the TEM.

Very little is known about the metabolism, biochemistry or genetics of hexamitids (Becker, 1977). The apparent absence of mitochondria, Golgi apparatus and microbodies in these parasites is presumed to indicate a rather primitive metabolic process (Kulda and Nohynkova, 1978). The main carbohydrate storage product is glycogen (Honigberg *et al.*, 1981; Vickerman, 1990), and intestinal hexamitids are either anaerobes or aerotolerant anaerobes.

Diagnosis of infection

In heavy infections the parasite is easily detected using light microscopy. Samples should be obtained from freshly killed fish, and thin smears (e.g. along the gastrointestinal tract, from the blood and imprints of internal organs) should be air-dried and fixed in alcohol before staining.

To confirm the number of flagella and the body shape which are consistent with hexamitids, living organisms should be examined in wet mount preparations (vaseline-ringed cover slips) under a light microscope using bright field or phase contrast or Normarski illumination. The addition of viscous Protoslo or methyl cellulose will slow the organisms for closer examinations (Lee *et al.*, 1985). For the flagella to stain well (Fig. 3.1), thin smears should be post-fixed in 10% buffered formalin (Woo, 1969), as was done for *Cryptobia* and *Trypanosoma* (see later sections). Also, the two nuclei stain well in post-fixed smears and organ imprints.

As indicated earlier, *S. barkhanus* has an initial blood phase after infection before the parasite disappears from the blood and is found mainly in internal organs (Guo and Woo, 2004a). The haematocrit centrifuge technique, which is very useful for detecting low numbers of *Trypanosoma* (e.g. Woo, 1969, 1970) and *Cryptobia* (e.g. Woo and Wehnert, 1983) in the blood, is also very sensitive for the detection of *Spironucleus*. The technique is especially useful soon after infection or towards the end of the blood phase of the disease (Guo and Woo, 2004b). Guo and Woo (2004b) also described a technique to quantify the numbers of parasites in internal organs during the tissue phase of the disease.

An enzyme-linked immunosorbent assay (ELISA) was developed to detect specific antibodies in the blood. This immunological technique is a modification of that for cryptobiosis (Sitja-Bobadilla and Woo, 1994) and it is especially useful for epidemiological studies where large numbers of fish have to be examined. It is also an excellent technique to detect infections during the tissue phase of the disease, when there are a very low number of parasites in the blood (Guo and Woo, 2004b).

Prevention and control

The introduction of hexamitids into fish culture facilities can be minimized by quarantine of new fish. Cleaning of filters and gravel will also help since accumulation of organic material can promote growth of hexamitids in the water column (Gratzek, 1988). Good animal husbandry and nutrition may also reduce the number and severity of outbreaks. Amphibians, which are known to harbour *S. elegans*, may be a source of infection for fish.

Post (1987) recommended treatment when the infection has about 15–30 trophozoites per microscopic field. Dimetridazole and metronidazole may be added to the feed and they are less commonly delivered as bath treatments. As far as is known, both drugs are not licensed for use on fish that are for human consumption (Gratzak, 1988; Stoskopf, 1988). An antibacterial treatment (furazolidone), usually added to the feed, has been used successfully to control hexamitids in the intestine and reduce fish mortality (Ferguson, 1979; Andrews *et al.*, 1988). The nitroimidazoles (metronidazole, benznidazole, ronidazole and secnidazole) completely eliminate intestinal *H. salmonis* infections in rainbow trout, and some non-nitroimidazoles (albendazole, aminosidine, diethylcarbamazine and nitroscanate) are just as effective. However, other

non-nitroimidazoles (e.g. amprolium, bithionol, febantel, praziquentel) have no detectable effects on the number of parasites in infected fish (Tojo and Santamarina, 1998). Dimetridazole, metronidazole and mebendazole are quite efficient in inhibiting multiplication of *S. vortens* under *in vitro* conditions (Sangmaneedet and Smith, 1999).

Ichthyobodoses

ICHTHYOBODOSIS IN FRESHWATER FISHES

Introduction

Ichthyobodosis in freshwater fishes is caused by the ectoparasite *Ichthyobodo necator*. The disease was known as costiasis after the previous name of the parasite, *Costia necatrix*, which was originally described from brown trout (*Salmo trutta*) alevins in France. The organism has a free-living stage in water (Figs 3.7 and 3.8) and a parasitic stage (Fig. 3.9), which is usually attached to dorsal fins and the tips of secondary gill lamellae (Fig. 3.10) of fish (Fish, 1940; Tavolga and Nigrelli, 1947). The free-living stage has a long and a short flagellum (Fig. 3.7); however, these flagella are not seen when the organism attaches to epithelial cells (Fig. 3.9).

Geographical distribution and host range

Ichthyobodo has worldwide distribution, is not host-specific and has a very wide pH and temperature tolerance. The parasite has been recorded on salmonids in hatcheries at temperatures between 2°C and 14°C (Urawa, 1992), on cyprinids at 2°C and 30°C (Schaperclaus, 1986) and on poecilids and tilapia at a temperature as high as 38°C (Tavolga and Nigrelli, 1947). It is an important pathogen of young salmonids and cyprinids, especially in hatcheries where fish are cultured in high numbers (e.g. Bohl, 1975; Ellis and Wootten, 1978; Bullock and Robertson, 1982; Awakura *et al.*, 1984; Rosengarten, 1985; Broderud and Poppe, 1986; Urawa, 1993, 1995, 1996).

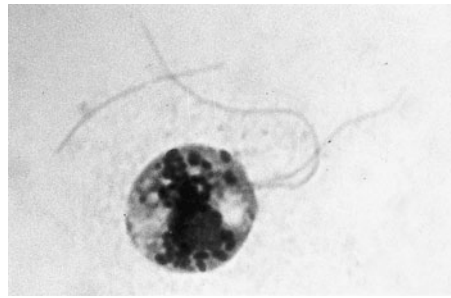


Fig. 3.7. Free-living *Ichthyobodo necator*: note the two free flagella (× 1000) (from Miyazaki *et al.*, 1986; courtesy of Dr T. Miyazaki).

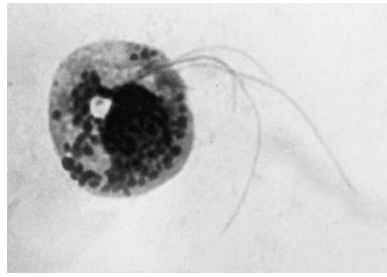


Fig. 3.8. Free-living pre-division form of *Ichthyobodo necator*: note that it is larger than the non-dividing form, and it has more than two free flagella (× 1000) (from Miyazaki *et al.*, 1986; courtesy of Dr T. Miyazaki).

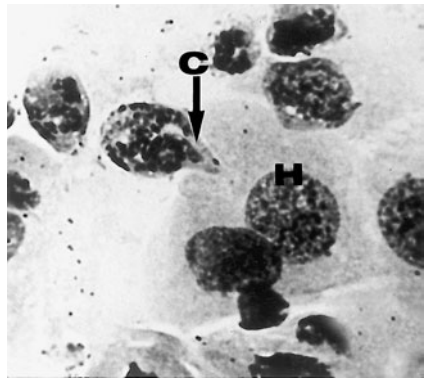


Fig. 3.9. Parasitic form of *Ichthyobodo necator* attached to a host cell: note its shape and its pointed cytostome (c) (from Miyazaki *et al.*, 1986; courtesy of Dr T. Miyazaki).

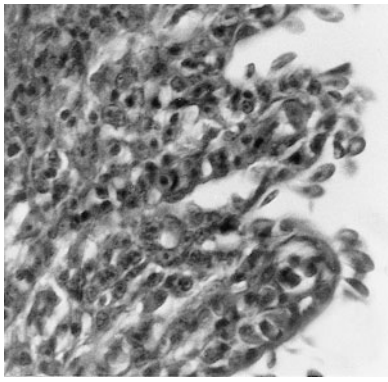


Fig. 3.10. Large numbers of *Ichthyobodo necator* attached to gill filaments: note that the gill filaments show hyperplasia of epithelial cells and fusion of the lamellae (× 480) (from Miyazaki *et al.,* 1986; courtesy of Dr T. Miyazaki).

Systematic and taxonomic position

A recent study on kinetoplastid phylogeny (Moreira *et al.*, 2004) agrees with the earlier suggestion of Callahan *et al.* (2002) that *Ichthyobodo* requires a new grouping. Hence, Moreira *et al.* (2004) proposed a new subclass (Prokinetoplastina) and a new order (Prokinetoplastida) for *Ichthyobodo*, and this classification is adopted in the current review.

Briefly, *I. necator* was first described by Henneguy as *Bodo necator* and was later transferred to the genus *Costia* by Leclerq. The generic name *Costia* was subsequently found to be already in use, and according to Joyon and Lom (1969) Pinto replaced it with *Ichthyobodo* and renamed the parasite *I. necator.* Two species of *Ichthyobodo* (*I. necator* and *I. pyriformis*) have been reported from freshwater fishes. *I. pyriformis* was described from salmonids in the USA (Davis, 1943). Body measurements of the two parasites overlap and there are no morphological characters visible under the light microscope to distinguish them. Hence, *I. pyriformis* is generally accepted as a small form of *I. necator* and is a junior synonym (e.g. Tavolga and Nigrelli, 1947; Vickerman, 1976b; Becker, 1977).

Partial small subunit ribosomal DNA (ssu-rDNA) sequences of 14 isolates from both freshwater and marine fishes indicate that *Ichthyobodo* is a complex of sibling species (Todal *et al.*, 2004). The parasites were from fishes in Norway, Japan, Singapore, South Africa and Brazil and the conclusions were that these represent eight strains or species which belong to two parasite lineages (A and B). Briefly, two of the eight *Ichthyobodo* strains or species were from fishes in Norway, and the freshwater parasite (lineage A) from the skin of Atlantic salmon parr, *S. salar*, and the three-spined stickleback, *Gasterosteus aculeatus*, was suggested to be *I. necator sensu stricto*. The *Ichthyobodo* (sublineage B1) that has an affinity for the gills of both fresh- and seawater salmon is a different species. The Atlantic cod, *Gadus morhua*, is infected with a marine species (sublineage B3) and it is different from the two on salmonids. The two parasites (sublineage B2) from freshwater cyprinids (*Cyprinus carpio* and *Carassius auratus*) from different parts of the world and the two *Ichthyobodo* isolates (sublineage B2) from unrelated North and South American freshwater fishes (*Morone* hybrid and *Apistogramma* sp.) are closely related. A more recent study (Callahan *et al.*, 2005), using an internal transcribed spacer (ITS) rRNA sequence on isolates from fishes in the USA, Japan and Greece, supports the conclusion that *Ichthyobodo* is a multispecies complex. All but one (from rainbow trout) of the isolates in the study were identified as four of the eight species suggested by Todal *et al.* (2004). The isolate from rainbow trout was considered a new species and was tentatively designated species IX. Many of these species were identified from multiple hosts, and one species was isolated from both marine and freshwater fishes (Callahan *et al.*, 2005).

Morphology and life cycle

The free-swimming form of *I. necator* is ovoid to spherical and measures 5–18 μm by 3–8 μm (Fig. 3.7). It divides by binary fission and its pre-division stage has three and four flagella (Fig. 3.8) and is larger than the

stage with two flagella. The nucleus is round to oval, and basophilic granules are often seen in the cytoplasm. There is a large basophilic body at the base of the flagella (Fish, 1940; Tavolga and Nigrelli, 1947; Vickerman, 1976b). The parasitic stage elongates and attaches to a host cell by its pointed anterior end (Fig. 3.9). Tavolga and Nigrelli (1947) suggested that the flagellate also has a saprophagous stage, which attaches to and feeds on detached cells and scales. Other workers (e.g. Bauer, 1959) had not seen such a form and were generally sceptical about its existence.

The cell membrane consists of two membranous layers with a fibrillar layer in between them (Schubert, 1966; Joyon and Lom, 1969). The nine peripheral fibrils of the flagella continue to form the kinetoplast–mitochondrion complex. The nuclear membrane also has two layers and there is a single nucleolus.

Infection occurs when free-swimming forms attach to a fish. The flagella on the free-swimming stage are presumed to be used for site selection, and the ventral flat disc is used for attachment in the parasitic form (Schubert, 1966, 1978). Both free-living and parasitic stages multiply by longitudinal binary fission (Tavolga and Nigrelli, 1947; Bauer, 1959). Free-living individuals with three to four flagella (pre-division stage) are often seen in the aquatic environment (Fig. 3.8) and they occur in large numbers when conditions are favourable, and fish become heavily infected (Fig. 3.10) 1–2 weeks after infection (e.g. Andai, 1933). The free-swimming and parasitic forms are presumed to encyst under adverse environmental conditions. The cysts in the water become an additional source of infection and contribute to the spread of the organism to new areas.

The organism has been found in the roe of spawning carp and it is presumed that fish become infected after hatching (Hlond, 1963). Other sources of infection are water supplies and feral fishes that come into a hatchery with the water. Amphibian larvae (salamander, frog) are also considered potential sources of infection (Becker, 1977).

Host–parasite relationships

Host susceptibility

Malnourished and/or juvenile fish are more severely affected than healthy adults (Robertson, 1985; Rosengarten, 1985), and high mortality (40–73%) is associated with heavy infections (e.g. Robertson, 1979; Poppe and Hastein, 1982; Awakura *et al.*, 1984; Rosengarten, 1985; Urawa, 1987). Outbreaks and infections on cyprinids become more severe when infected fish from outdoor ponds or tanks are transferred to indoor tanks (e.g. Bauer, 1959; Bauer *et al.*, 1969). Important factors that contribute to disease outbreaks include stress on the fish due to transfer and higher indoor temperature, which promotes more rapid parasite multiplication.

Swordtails (*Xiphophorus hellerii*) are very susceptible and usually die 8–14 days after infection (Tavolga and Nigrelli, 1947), while platyfish (*Platypoecilus maculates*) are less susceptible. Tilapia (*Tilapia macrocephala*) and guppies (*Poecilia reticulata*) have light infections or the infections are confined to the caudal region.

It is generally assumed that some protective immunity is acquired on recovery from an infection. Rainbow trout (*O. mykiss*) immunized with either whole *Tetrahymena thermophila* or their cilia were more resistant than naïve fish (Wolf and Markiw, 1982).

Course of infection and foci of investigation

Parasitaemias on salmonids usually peak at about 4 weeks after fish larvae start feeding and mortality is highest between 4 and 8 weeks (Robertson, 1979). Foci of infections on the fish include the cuff of skin sheltered by the operculum, pectoral and pelvic fins and the area adjacent to the dorsal fin (Robertson *et al.*, 1981; Robertson, 1985). Parasites are not normally found on the head anterior to the operculum.

Clinical signs and pathology

Fish with relatively light infections on the body may roll in the water (flashing) and rub against immersed objects or sides of the tank.

These activities are apparently responses to skin irritations caused by the parasite. However, fish with heavy infections are often listless and anorexic. Spots appear on the body surface and these fuse to form greyish films on fins and body surface. Also, the gills are usually swollen (Fish, 1940; Miyazaki *et al.*, 1986) and other clinical signs include increased mucus secretions and frayed or destroyed fins. Some infected fish may not be able to maintain an upright position or swim to the water surface (Savage, 1935; Bauer, 1959). Fish with only gill infections are listless and anorexic but do not flash or have excessive body mucus.

Goblet cells are often not seen in the epidermis and there is hyperplasia of Malpighian cells in salmonids with heavy body infections. Spongiosis, vacuolization, loss of cytoplasmic and nuclear details in the suprabasal layers may occur in the epidermis. Oedema is followed by degeneration and sloughing of the epidermis (Ellis and Wootton, 1978; Robertson *et al.*, 1981; Awakura *et al.*, 1984; Robertson, 1985). It is likely that mortality is due in part to osmoregulatory problems with resultant haemodilution (Robertson *et al.*, 1981; Robertson, 1985). The histopathologies are similar in infected plaice and flounders that are maintained in sea water (Bullock and Robertson, 1982; Cone and Wiles, 1984).

Acute hyperplasia and fusion of secondary gill lamellae are obvious in heavily infected channel catfish (Miyazaki *et al.*, 1986). Hyperplasia of epithelial cells in the interlamellar spaces is extensive, with fusion of the lamellae and marked clubbing of gill filaments (Fig. 3.11). Epithelial cells with parasites become necrotic and blood vessels collapse, with proliferation of mucus cells in clubbed filaments.

Diagnosis of infection

Clinical signs are usually used for preliminary diagnosis of the disease. The infection is confirmed by examination of mucus from gills and of the body surface for flagellates under the light microscope. The parasite is quite fragile and often ruptures during fixation and staining. Carefully prepared smears (e.g. from the body surface) are usually fixed in Shaudinn's fixative and transferred to ethyl alcohol before being stained (e.g. using iron haematoxylin, carmine, periodic acid–Schiff) (Becker, 1977). At present, there are no serological techniques that can be used to either detect the infection or determine the immunological state of fish.

Control

Flush treatment with formalin (166 ppm) for 1 h or a formalin bath (1 : 4000) for 15–30 min is well tolerated by salmon fry (Fish, 1940; Imamovic, 1986; Skrudland, 1987). Also, treatment for 1 h in a formalin bath (1 : 6000) is also very effective. Formalin treatment is not recommended if the

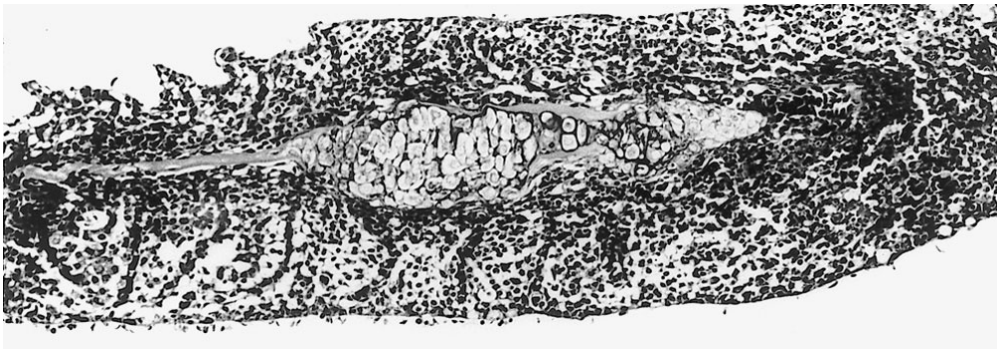


Fig. 3.11. Clubbing of gill filament due to heavy *Ichthyobodo necator* infection (from Miyazaki *et al.*, 1986; courtesy of Dr T. Miyazaki).

young fish have bacterial gill disease. It is also important to have good aeration during formalin treatment because formalin (a reducing agent) combines with the dissolved oxygen in the water to form formic acid that may kill fish (Helms, 1967). Dipping infected salmonids in dilute acetic acid (2000 ppm) to kill the parasite is also used (Hora and Pillay, 1962). Other treatments include exposing fish (usually fingerlings) to a weak solution of malachite green (1 : 300,000 to 1 : 400,000) for 40–60 min (Becker, 1977). Modifications of these treatments (in amounts and treatment periods) are in Hoffman and Meyer (1974).

Cyprinids lose their infection when exposed daily to copper sulphate (500 ppm) for 1–2 min or in sodium chloride (10,000 ppm) for 15–30 min (Osborn, 1966; Schaperclaus, 1986). More concentrated salt solutions (5%) are recommended for larger fish. A 1 h flush treatment with pyridylmercuric acetate (2 p.p.m.) also eliminates the parasite. Bithionol (25 mg/l for 3 h or on 2 consecutive days) is effective in eliminating the parasite from rainbow trout (Tojo *et al.*, 1994). The drug is not toxic to fish at the recommended dosage.

To control an outbreak, ponds with infected fish are drained and quicklime or chloride of lime is added before restocking with new or treated fish. Other useful chemicals include methylene blue, potassium permanganate, aureomycin, globucid, lysol and quinine hydrochloride (Amlacher, 1970; Schaperclaus, 1986).

The parasite multiplies rapidly between 10 and 25°C. It encysts at about 8°C and dies at temperatures above 30°C (van Duijin, 1973). Hence raising the water temperature to 32°C for 5 days is also effective against temperature-sensitive strains but this approach is not recommended for coldwater fish.

ICHTHYOBODOSIS IN MARINE FISH

Geographical distribution and host range

Ichthyobodo has been found on salmonid smolts in the sea (Ellis and Wootten, 1978; Roubal *et al.*, 1987; Bruno, 1992) and on wild plaice (*Pleuronectes platessa*) from Scotland (Bullock and Robertson, 1982; Bullock, 1985), winter flounders (*Pseudopleuronectes americanus*) from Newfoundland (Cone and Wiles, 1984) and Japanese flounders (*Paralichthys olivaceus*) at the Hokkaido Institute of Mariculture, Japan (Kusakari *et al.*, 1985).

Wood (1979) indicated that the freshwater *Ichthyobodo* survived on fish following their transfer to the marine environment. Consequently, it was assumed that the parasite on marine fish was acquired when fish were in fresh water and the parasite survived when infected fish migrated or were transferred to sea water. However, Morrison and Cone (1986) described an *Ichthyobodo* from haddock (*Melanogrammus aeglefinus*) caught 120 km from Nova Scotia, Canada, and Diamant (1987) found the parasite on common dabs (*Limanda limanda*) from the North Sea, thus suggesting the existence of purely marine species of *Ichthyobodo*. Recent studies confirmed the high salinity tolerance of the parasite, and Callahan *et al.* (2005), using molecular techniques, showed that the same species of *Ichthyobodo* occur in both marine and freshwater fishes.

Marine *Ichthyobodo*

The parasite has been found on a variety of marine fishes (Urawa *et al.*, 1998). Bruno (1992) showed that mean width and length of *Ichthyobodo* from salmonid fry in fresh water were significantly greater than those of the parasite from fish in sea water. However, Bruno's measurements of the marine parasite were lower than those from other marine fishes (Morrison and Cone, 1986; Diamant, 1987). Roubal and Bullock (1987) found differences in the attachment discs and cytostome processes between freshwater and marine *Ichthyobodo* from salmonids. According to Lamas and Bruno (1992), the cytostome process of marine *Ichthyobodo* on Atlantic salmon is smooth along its entire length, while that of *I. necator* has ridge-like projections.

I. necator from freshwater fish survived and multiplied on chums (*Oncorhynchus keta*) when infected salmon were

transferred to sea water (Urawa and Kusakari, 1990). However, there were minor morphological changes (e.g. reductions in body width and in contractile vacuoles) when the parasite was in sea water. The parasite was also found on chum fry in estuaries, and *I. necator* from chums was not infective to Japanese flounders, while the parasite from flounders was not very infective to chums.

Clinical signs and pathology

The parasite caused high mortality of juvenile Japanese flounders in a hatchery in Japan (Urawa *et al.*, 1991). The fish were maintained in canvas tanks supplied with sea water at 17°C and about 40% of 43,000 fish were infected. Large numbers of parasites were on the entire dorsal surface of infected fish, including the head. The clinical signs were loss of appetite, greyish slime on the body surface and erosion of the fins.

The histopathologies are similar to those in freshwater salmonids (Robertson *et al.*, 1981) and they include extensive hyperplasia of the epidermis with almost total depletion of goblet cells, nuclear and cytoplasmic degeneration of epithelial cells, and vacuolation and severe spongiosis in most of the epidermal layer. Also, epidermal cells lose their interdigitating cell membranes (Urawa *et al.*, 1991).



Fig. 3.12. *Cryptobia salmositica* with a red blood cell from a fish with microcytic and hypochromic anaemia: note that the red blood cell is not oval and there is reduced haemoglobin (× 1150) (from Woo, 1987a; courtesy of *Advances in Parasitology*).

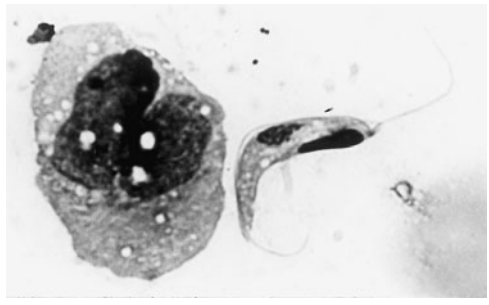


Fig. 3.13. Ectoparasitic slender form of *Cryptobia salmositica* next to an epithelial cell on the body surface of a rainbow trout 6 weeks after infection (× 1150) (from Woo and Wehnert, 1983; courtesy of *Journal of Protozoology*).

Cryptobiosis

Introduction

Cryptobia spp. (Figs 3.12 and 3.13) infect and cause diseases in many species of marine and freshwater fishes (e.g. salmonids and flatfish). The parasite may be on the body surface and/or gills (ectoparasitic), in the digestive tract or in the blood (endoparasitic) of fishes. It has worldwide distribution and there are at least 52 nominal species (Becker, 1970; Lom, 1979; Woo, 1987a). The ectoparasites are on the body surface or attached to the gills (Figs 3.14 and 3.15), while the endoparasites are either in the blood (Fig. 3.12) with an ectoparasitic stage on the body surface (Fig. 3.13) or in the digestive tract (Figs 3.16–3.18). There are also numerous reports of unidentified *Cryptobia* species. It is likely that many of the described species are not valid; however, their taxonomic status can only be settled after careful experimental studies. Also, many of these flagellates, e.g. *Cryptobia catostomi* in white suckers (*Catostomus commersoni*), are not known to cause disease (Bower and Woo, 1977; Thomas and Woo, 1992a) and such non-pathogenic species are not included in the present discussion.

Briefly, the parasite (Figs 3.12, 3.13 and 3.14) is an elongated (oval to ribbon-like)

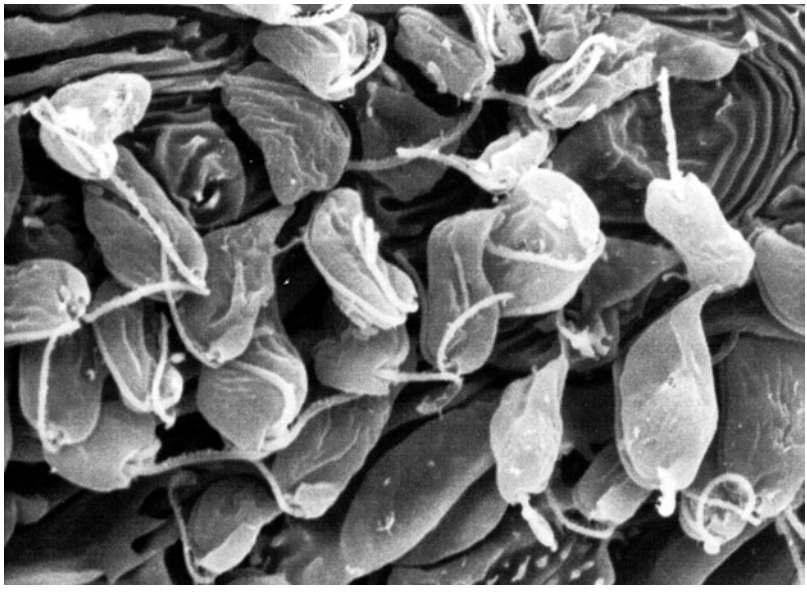


Fig. 3.14. Large numbers of *Cryptobia branchialis* (scanning electron microscope) attached to gill filaments of a naturally infected tilapia (from Kuperman *et al.*, 2002; courtesy of the late Dr B.I. Kuperman).

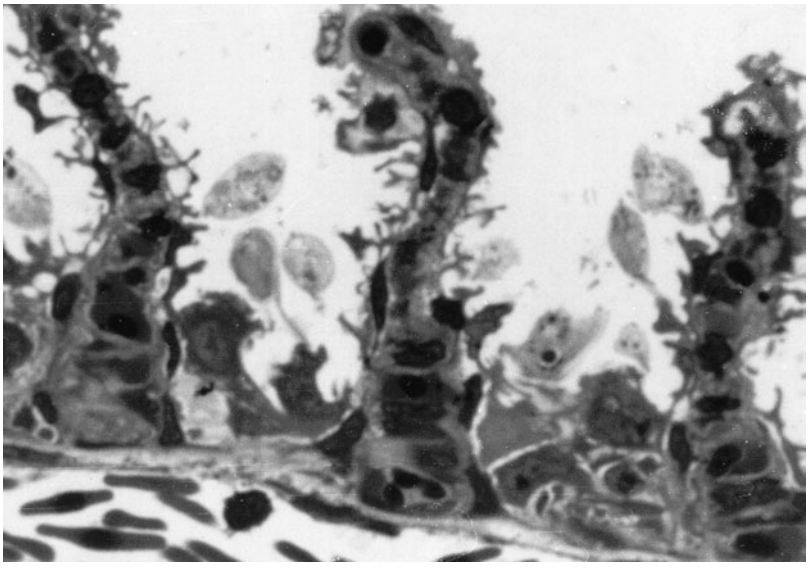


Fig. 3.15. Histological section of gill lamellae of a naturally infected tilapia to show hypertrophied gill epithelial cells with a large number of *Cryptobia branchialis* (from Kuperman *et al.*, 2002; courtesy of the late Dr B.I. Kuperman).

flagellate with two flagella, which originate from the anterior end. The shorter anterior flagellum is free, while the recurrent flagellum is attached to the body and it ends as a free flagellum at the posterior end. The oval to elongated kinetoplast is very prominent and is located at the anterior end in close proximity to the nucleus. The parasite

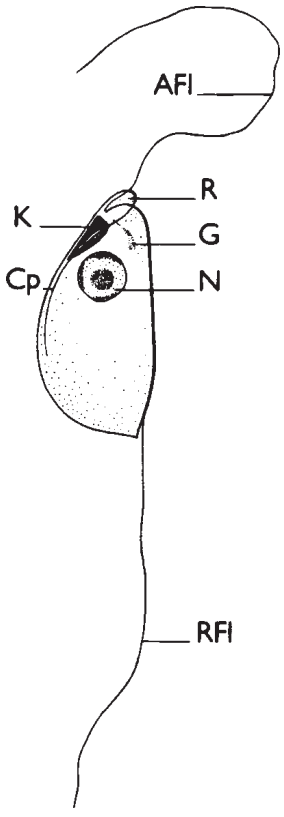


Fig. 3.16. Drawing of a free-swimming *Cryptobia iubilans*: note the short prominent rostrum (R), slender cytopharynx (Cp), Golgi complex (G), anterior flagellum (AFl), recurrent flagellum (RFl) and kinetoplast (K) (from Nohynkova, 1984a; courtesy of *Protistologica*).

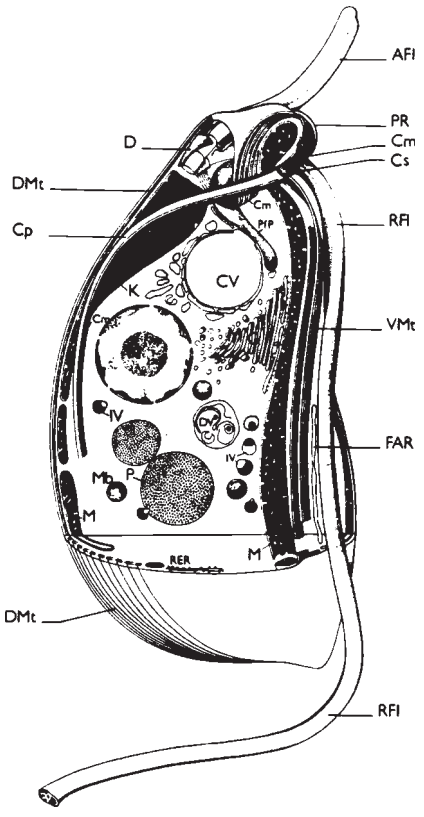


Fig. 3.17. Composite drawing of *Cryptobia iubilans* to show its principal ultrastructures at the electron microscopic level: note the microtubules (Cm), the mitochondrion (M) extending the length of the flagellate, the subpellicular microtubules (DMt), anterior flagellum (AFl), preoral ridge (PR), cytostome (Cs), recurrent flagellum (RFl), ventral subpellicular microtubules (VMt), flagellum-associated reticulum (FAR), cytopharynx (Cp), dense lamina (D), postflagellar pit (PfP), contractile vacuole (CV), kinetoplast (K), nucleus (N), Golgi complex (G), digestive vacuole (DV), inclusion vacuole (IV), polysaccharide (P), microtubules (Mb) and rough endoplasmic reticulum (RER) (from Nohynkova, 1984a; courtesy of *Protistologica*).

multiplies by longitudinal binary fission under both *in vivo* and *in vitro* conditions (Woo, 1987a).

Systematics and taxonomy

Moreira *et al.* (2004) proposed dividing the class Kinetoplastea into two new subclasses, namely the Prokinetoplastina (*Ichthyobodo*) and the Metakinetoplastina (e.g. *Cryptobia*, *Trypanosoma*). They further proposed four new orders within the Metakinetoplastina; two of these are relevant to the present discussion, the Parabodonida (e.g. *Cryptobia*) and the Trypanosomatida (e.g. *Trypanosoma*). This broad division of the group is adopted in the current review.

The genus *Cryptobia* was initially proposed for biflagellated organisms in the reproductive systems of snails (Leidy, 1846). The genus was later expanded to include other morphologically similar flagellates infecting other invertebrates and

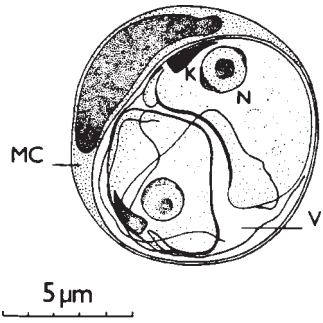


Fig. 3.18. Drawings of two intracellular *Cryptobia iubilans* within the vacuole (V) of the host macrophage-like cell (MC). K, kinetoplast; N, nucleus (from Nohynkova, 1984a; courtesy of *Protistologica*).

vertebrates (e.g. ectoparasites on gills/body surface and endoparasites in blood/digestive tract of fishes). The genus *Trypanoplasma* was proposed by Laveran and Mesnil (1901) for a biflagellated haemoflagellate in freshwater fish. Crawley (1909) synonymized *Trypanoplasma* with *Cryptobia* because of their morphological similarities. Also, numerous non-haematozoic *Cryptobia* species have been described from the body surface and intestinal tracts of aquatic organisms, many of which are from fishes (Woo, 1987a). In general, scientists in Europe (e.g. Lom, 1979; Steinhagen *et al.*, 1989a, 2000) retain *Trypanoplasma* for haematozoic species and *Cryptobia* for non-haematozoic species because they believe the differences in life cycles (haematozoic species are transmitted indirectly by leeches, while non-haematozoic species have direct transmissions) and the presence of a contractile vacuole (in ectoparasitic species) are sufficient for their separation. However, North American workers (e.g. Bower and Woo, 1977; Newman, 1978; Khan *et al.*, 2001) agreed with Crawley. Woo (e.g. 1987a, 1994, 1998, 2001, 2003) has always supported Crawley because there are biological similarities between the haematozoic and non-haematozoic parasites. For example, *C. salmositica* (Fig. 3.12) and *Cryptobia bullocki* are haematozoic parasites; however, they have an ectoparasitic phase in the mucus on the body surface of fish (Woo and Wehnert, 1983; E.M. Burreson, personal communication). Ectoparasitic forms of *C. salmositica* (Fig. 3.13) are morphologically similar to blood forms and they are infective when inoculated into fish (Woo and Wehnert, 1983). The parasite is transmitted from infected to uninfected fish when fish are allowed to mix freely in a tank or are separated by a wire screen to eliminate bodily contacts (Woo and Wehnert, 1983) or when infected and uninfected fish are brought together briefly in a dip net (Bower and Margolis, 1983). A functional contractile vacuole was seen in electron micrographs of blood forms of *C. salmositica* (Paterson and Woo, 1983). This organelle is essential for the ectoparasitic form to survive in fresh water when it is in the mucus on the body surface.

Woo (1994) divided the *Cryptobia* into two subgenera. The haematozoic and normally digenetic species (i.e. parasites with indirect life cycle) belong to the subgenus *Trypanoplasma*, while the subgenus *Cryptobia* contains the non-haematozoic monogenetic (i.e. one host) parasites. This proposal still indicates the close phylogenetic relationships between the two groups but shows that there are biological differences between them. He further suggested that non-haematozoic species (ectoparasitic on gills/body surface and endoparasitic in intestine) might warrant further division into additional subgenera because of the distinct environmental and/or physiological conditions under which they live. This, however, should only be done after careful experimental studies. The division into two subgenera indicates a close phylogenetic relationship between the two groups of organisms and yet indicates some distinctive differences. Wright *et al.* (1999) studied the ssu-rRNA gene sequence of four species of haematozoic *Cryptobia* (one from Europe and three from North America) and confirmed that 'neither tree topology, nor nucleotide differences, nor the type of RNA editing supports a generic distinctiveness for *Trypanoplasma*'. However, based on kDNA structure and ssu-rRNA gene sequences, Dolezel *et al.* (2000) concluded that

'the subgeneric status of *Trypanoplasma* within the genus *Cryptobia*, as proposed by Woo (1994), is inadequate, and that the genus *Trypanoplasma* . . . be considered valid'. Moreira *et al.* (2004) supported the suggestion that the haematozoic species be in the genus *Trypanoplasma*; however, Callahan *et al.* (2002) agreed with the conclusion of Wright *et al.* (1999). It is evident that more extensive studies (biological and molecular) of the non-haematozoic *Cryptobia*, especially those in the intestine of fishes, are required to help resolve this systematic problem.

Two hypotheses have been proposed on the origin of the haematozoic parasites in fish. These have been discussed more fully in an earlier review (Woo, 1987a). Briefly, Woo and Wehnert (1983) suggested that members of the subgenus *Trypanoplasma* arose from free-living flagellates (e.g. genus *Procryptobia*) via the ectoparasites (subgenus *Cryptobia*) on the body surface of fish. The second hypothesis (Nohynkova, 1984a) was that the haematozoic species were linked to the intestinal flagellates (subgenus *Cryptobia*). Both hypotheses agree that the haematozoic and non-haematozoic species are closely related.

Two non-haematozoic species (*Cryptobia branchialis* and *Cryptobia iubilans*) are known to cause disease, while the majority of species (e.g. *Cryptobia stilbia*, *Cryptobia dahlia*) are not pathogenic to fish. As indicated earlier, little is known about the non-haematozoic species (those that are on the body surface and in the digestive system of aquatic organisms); consequently, a comprehensive study (using molecular and biological approaches) is needed on these parasites. Woo (2003) suggested that the study would help to determine the relationships between haematozoic and non-haematozoic species and this would also help to clarify the status of *Trypanoplasma*. He also recommended that, until this study was done, it would be more prudent to assign all species to the genus *Cryptobia*. This is the approach that will be used in this review.

At least five species of *Cryptobia* are known to cause disease in fishes (Woo, 1987a), and they are the focus of this chapter. *C.* (*Cryptobia*) *branchialis* is an ectoparasite while the rest are endoparasites. *C.* (*Cryptobia*) *iubilans* is associated with the intestine while *C.* (*Trypanoplasma*) *salmositica*, *C.* (*T.*) *bullocki* and *Cryptobia* (*T.*) *borreli* (syn. *Trypanoplasma cyprini*) are haematozoic species. For convenience, the subgeneric grouping of the parasites will not be used in the following sections.

Cryptobiosis in freshwater fish

C. branchialis, *C. iubilans*, *C. salmositica* and *C. borreli* are normally parasitic on/in freshwater fishes; however, *C. branchialis* and *C. salmositica* have been found on/in fish in sea water. *C. branchialis* is an ectoparasite, while *C. iubilans* is in the digestive tract and associated internal organs. Both pathogens are transmitted directly between fish. The haematozoic *C. salmositica* is normally transmitted by blood-sucking leeches (digenetic life cycle); however, it can also be transmitted in the absence of leeches (monogenetic life cycle). As far as is known, the haematozoic *C. borreli* requires blood-sucking leeches for indirect transmission.

Geographical distribution of parasite and host range

C. branchialis (Figs 3.14 and 3.15) is ectoparasitic on the gills of a large number of freshwater fishes and has been implicated in morbidity and mortality of cultured carp, goldfish and catfish in Asia, Eastern Europe and North America (e.g. Chen, 1956; Bauer *et al.*, 1969; Naumova, 1969; Hoffman, 1978). However, *Cryptobia* with similar morphological features to those of *C. branchialis* have been found on marine and estuarine fishes in Chesapeake Bay and its tributaries, USA (Burreson and Sypek, 1981), on sea bream (*Sparus aurata*) in sea water in southern France (Blanc *et al.*, 1989) and on tilapia (*Oreochromis mossambicus*) in the Salton Sea, an inland salt lake in

southern California, USA (Kuperman *et al.*, 2002).

C. iubilans occurs extracellularly (Fig. 3.16) in the stomach, intestine, gall bladder, spleen, ovary and liver and intracellularly in macrophage-like cells (Fig. 3.18). It infects four species of freshwater aquarium cichlids (*Herichthys cyanoguttatum, Cichlasoma meeki, Cichlasoma nigrofasciatum, Cichlasoma octofasciatum*) in the former Czechoslovakia (Nohynkova, 1984a). Recently, four commercial producers of discus (*Symphysodon aequifasciatus*) in the southern USA had fish infected with the pathogen (Yanong *et al.*, 2004).

C. salmositica (Fig. 3.12) was initially described from coho salmon (*O. kisutch*) in Washington State, USA (Katz, 1951). It has since been reported in all species of Pacific *Oncorhynchus*, *S. trutta, Prosopium williamsoni*, seven species of sculpins (*Cottus* spp.), a cyprinid (*Rhinichthys cataractae*), a sucker (*Catostomus snyderi*) and a stickleback (*G. aculeatus*) (Woo, 1987a). These fishes are in freshwater streams from California to British Columbia, and in south-western Alaska (Wales and Wolf, 1955; Becker and Katz, 1965b; Katz *et al.*, 1966; Bower and Margolis, 1984b). It is most likely that the parasites in the cyprinid and in the sucker are not *C. salmositica* because other species of cyprinids and suckers cannot be experimentally infected with the parasite (Wehnert and Woo, 1980). Also, cyprinids and suckers have their own species of *Cryptobia* and they are not infective to salmonids (Woo, 1987a).

Lom (1979) suggested that the poorly described *Cryptobia makeevi* in *Oncorhynchus gorbuscha* and *O. keta* from the Amur River in East Asia might be *C. salmositica*. This indicates the need for more careful comparative morphological, developmental (e.g. in the leech vector), antigenic (e.g. using Western immunoblots) and molecular (e.g. using partial ssu-rDNA) studies. Li and Woo (1996) described a species-specific DNA probe (1.2 kilobase pairs) to distinguish *C. salmositica* from *C. bullocki*, *C. borreli* and *C. catostomi*. It is a simple and practical technique as the parasite DNA can be extracted from infected blood dried on filter paper. Cross-experimental transmissions and virulence (e.g. clinical signs of the disease) studies may also be useful in deciding the taxonomic status of *C. makeevi*.

C. borreli was described by Laveran and Mesnil (1901) from red-eye (*Leuciscus erythrophthalmus*) caught in ponds in Garches, France. The parasite has since been found in the blood and tissues of several freshwater cyprinids in Europe. It sometimes causes heavy losses in cultured carp, tench and crucian carp (Lom, 1979). The parasite is not host-specific and causes mortality in experimentally infected goldfish (*C. auratus*) and the common carp (*C. carpio*) (Dykova and Lom, 1979; Lom, 1979). After extensive morphological and cross-transmission studies, Lom (1979) concluded that the parasites from carp, goldfish and crucian carp in Europe are all *C. borreli*, and that the pathogenic *Trypanoplasma cyprini* described by Plehn (1903) from carp in Germany is a junior synonym of *C. borreli*. Comparative antigenic and biochemical studies of parasite isolates from cyprinids throughout Europe may help to confirm Lom's conclusion.

Geographical distribution and biology of leech vectors

Piscicola salmositica is the only known vector of *C. salmositica*. This freshwater leech is not host-specific and is in cold, fast-flowing rivers and streams from British Columbia to northern California (Becker and Katz, 1965a,c). Cocoons and adult *P. salmositica* are susceptible to drying and freezing, and draining areas where cocoons are deposited is a method to control leeches in hatcheries (Bower and Thompson, 1987). Adult leeches are susceptible to chlorine (Bower *et al.*, 1985), and hence the chemical can be considered for controlling leeches under certain conditions.

C. borreli develops in two freshwater leeches (*Hemiclepsis marginata* and *Piscicola geometra*). *H. marginata* is in small ponds and slow-moving streams throughout Europe and Asia (Elliott and Mann, 1979; Sawyer, 1986; Shanavas *et al.*,

1989), while *P. geometra* prefers cooler waters and occurs in fast-flowing streams and in lakes throughout Eurasia (Mann, 1961; Malecha, 1984; Sawyer, 1986).

Parasite morphology

C. branchialis (Fig. 3.22) is found on fish in fresh and salt water. Living specimens from freshwater fish have body length 14.0–23.0 μm and body width 3.5–6.0 μm, while fixed specimens are smaller: body length 9.0–18.0 μm and 2.2–4.8 μm; anterior flagellum 7.7–11.0 μm; posterior flagellum 10–15 μm; nucleus round to oval (1.2 × 1–1.7 μm). The cytoplasm contains refractile granules and the parasite attaches to host epithelium by its posterior flagellum (Chen, 1956; Kuperman *et al.*, 2002).

The parasite (fixed specimens) in young tilapia from the Salton Sea is somewhat smaller: body length 10.5 (7.5–11.6) μm; body width 4.1 (2.8–4.6) μm; anterior flagellum 8.7 (6.1–10.2) μm; recurrent flagellum 19.5 (13.8–28.2) μm. The latter extends at the posterior end as a free flagellum, which attaches the parasite to the gill epithelial cells (Kuperman *et al.*, 2002).

C. iubilans (extracellular stage; Fig. 3.16) is oval to elongated and measurements are based on fixed specimens: body length 5.5–12.5 μm; body width 3.5–5.5 μm. The anterior flagellum is 1.5–2 times its body length and the posterior free flagellum is longer than the anterior flagellum. Its triangular kinetoplast is located at the anterior end of the body and is close to its round nucleus. Its ultrastructure was studied and described in detail (Fig. 3.17) by Nohynkova (1984a).

C. salmositica (Fig. 3.12) is an elongated organism and its body measurements are based on air-dried stained blood smears: body length 14.9 (6.0–25.0) μm; body width 2.5 (1.3–4.0) μm; anterior flagellum 16.1 (6.5–27.0) μm; posterior free flagellum 9.0 (4.0–17.0) μm; kinetoplast length 2.0–9.0 μm; kinetoplast width 0.5–2.0 μm; nucleus length 1.5–3.5 μm; nucleus width 1.0–2.5 μm; ratio of anterior flagellum to body length 1.07 (0.40–1.95); ratio of posterior flagellum to body length 0.61 (0.25–1.15) and ratio of anterior flagellum to posterior free flagellum 1.97 (0.6–3.7) (Katz, 1951).

The ultrastructure of the parasite is generally similar to that of other *Cryptobia* spp. However, the blood form of *C. salmositica* has a functional contractile vacuole (with systole and diastole stages) and its lumen has electron-dense filamentous materials (Paterson and Woo, 1983). Contractile vacuoles have not been found in other haematozoic species (Vickerman, 1971; Brugerolle *et al.*, 1979). In *C. salmositica*, the vacuole is at the base of the flagellar pocket and is associated with the postflagellar pit (Paterson and Woo, 1983). A similar contractile vacuole occurs in *C. iubilans* and it is also located just posterior to the postflagellar pit and close to the Golgi complex (Fig. 3.17; Nohynkova, 1984a).

C. borreli is an elongated parasite with considerable variations in size and shape. Small and slender forms predominate during acute infections while large stumpy parasites are the principal forms in chronic infections. Measurements (Kruse *et al.*, 1989b) are based on fixed and stained specimens: body length 24.4 (± 3.0) μm; body width 4.9 (± 1.2) μm; anterior flagellum 14.6 (± 3.6) μm; posterior free flagellum 7.9 (± 2.7) μm; kinetoplast length 7.6 (± 1.6) μm; nucleus length 4.1 (± 0.86) μm; nucleus width 2.1 (± 0.47) μm; the oval nucleus is in the anterior part (near the convex margin) of the parasite. The ultrastructure of *C. borreli* is similar to that of other *Cryptobia* and it does not have a contractile vacuole (Brugerolle *et al.*, 1979).

Transmission of *Cryptobia*

Direct transmission

C. branchialis presumably are dislodged from the gills of infected fish and become free in the water column. These flagellates are brought into the gill chamber via the mouth and the organisms attach to the gill filaments, where they multiply rapidly under favourable conditions.

Cichlids (*C. nigrofasciatum*) become infected with *C. iubilans* after ingesting organs of infected fish and/or parasites in

the water as a result of regurgitation or defecation of infective materials (Nohynkova, 1984a). The parasite survives for at least 4 h at 20°C in aquarium water.

C. salmositica is not only transmitted by blood-sucking leeches (see below); it is also transmitted directly between fish. The parasite in the blood of infected juvenile sockeye (*Oncorhynchus nerka*) is released on to the body surface through a blister caused by the dissociation of connective tissues near the abdominal pore (Bower and Margolis, 1983). It is in the mucus on the body surface of rainbow trout (*Oncorhynchus mykiss*) about 6 weeks after experimental infection. Round and slender ectoparasitic forms (Fig. 3.13) are infective when injected into fish (Woo and Wehnert, 1983).

Woo and Wehnert (1983) showed that 67–80% of uninfected trout became infected if uninfected fish were allowed to mix freely with infected trout in the same tank. The infection rate was reduced to 50% if infected and uninfected fish were separated by a wire screen and with uninfected fish 'downstream' to infected fish. Biochemical studies of *C. salmositica* showed that it has two proteases, a cysteine protease (49, 60, 66 and 97 kDa) and a 200 kDa metalloprotease (Fig. 3.19). The metalloprotease is a histolytic enzyme (Fig. 3.20) and is an important virulent factor (Zuo and Woo, 1997a,d, 1998a). It is probably involved in direct transmission of the parasite by first causing lesions on the skin of infected fish so that the parasite becomes ectoparasitic. The parasite is then carried in mucus strands and it enters the recipient fish either through a lesion on the body surface or penetrates the mucus membrane of the gills or oral cavity (Woo and Wehnert, 1983).

Direct transmission was more efficient and rapid if heavily infected and uninfected juvenile sockeye were held together in a dip

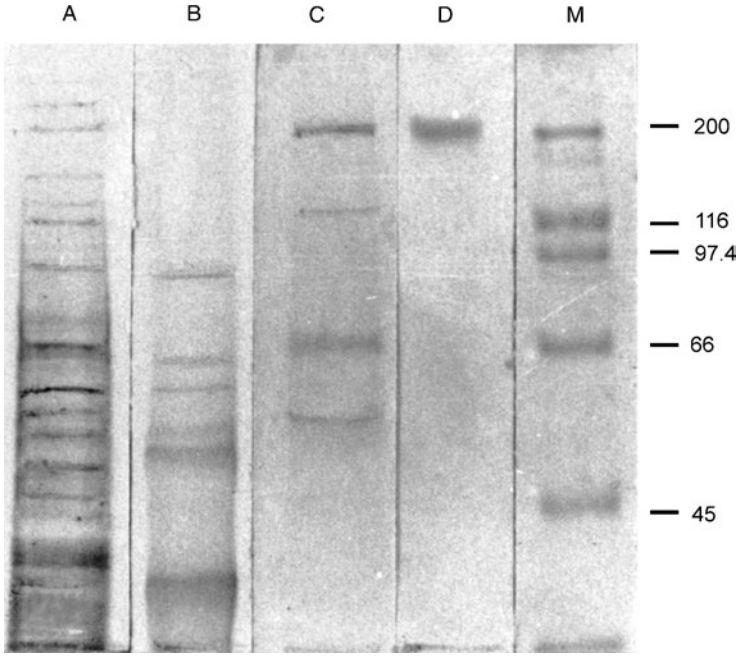


Fig. 3.19. Purified cysteine protease and metalloprotease from *Cryptobia salmositica*. Lane A, crude parasite lysate; B, partially purified cysteine protease from diethylaminoethyl (DEAE)-agarose column; C, metalloprotease from DEAE-agarose column; D, purified metalloprotease from Sephacryl S-300 column; M, molecular weight markers (kDa). (From Woo, 2003, which was modified from Zuo and Woo, 1997d; courtesy of *Diseases of Aquatic Organisms*.)

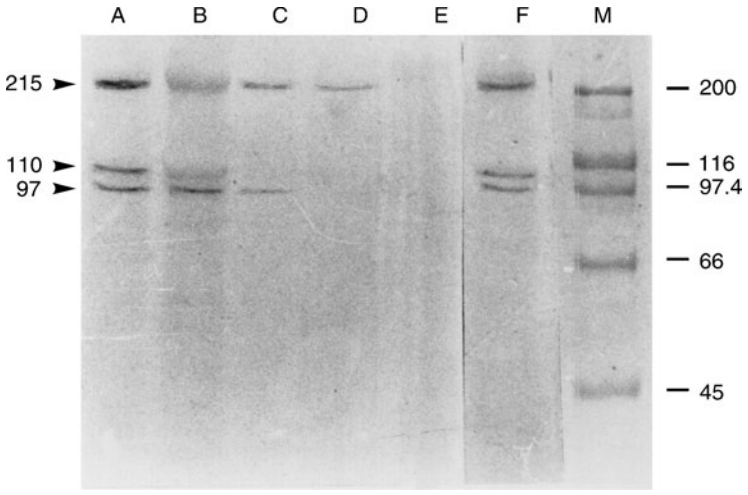


Fig. 3.20. *In vitro* degradation of collagen type V by purified metalloprotease from *Cryptobia salmositica*. Lanes A–E, collagen incubated with metalloprotease for 0, 2, 5, 6 and 8 h, respectively. Lane F, collagen + phosphate-buffered saline at 8 h. Lane M, molecular weight markers (kDa). (From Zuo and Woo, 1997d; courtesy of *Diseases of Aquatic Organisms*.)

net for brief periods out of water (Bower and Margolis, 1983). Mortality due to cryptobiosis was 64–89% in fish held in fresh water and 94% in sea water. There was field evidence to suggest that direct transmission was enhanced under hatchery conditions, in that the prevalence of the parasite in coho salmon rose from 29% to 74% after fish were transferred to another pond, while the prevalence in non-transferred fish remained the same. Bower and Margolis (1983) also suggested the increase in prevalence might be due to stress related to a relapse of the infection as a result of the transfer.

Becker and Katz (1965a) indicated that there were outbreaks of cryptobiosis in hatcheries where no leeches were found. Consequently, direct transmission of *C. salmositica*, especially in hatcheries, clearly needs more studies and further careful documentation.

Indirect transmission

In general, haematozoic *Cryptobia* multiplies in the crop of blood-sucking leeches and the parasite in the crop is infective to fish. Slender forms accumulate in large numbers either in the crop or in the proboscis sheath and these are transmitted to fish when the leech feeds.

The freshwater leech *P. salmositica* became infected with *C. salmositica* after it fed on infected fish, and transmitted the parasite when it fed again (Becker and Katz, 1965a). Large numbers of parasites were in the crops of leeches 7–8 days after an infective blood meal; however, there were no indications that flagellates were in the proboscis sheaths of infected leeches. S. Li and P.T.K. Woo (unpublished) found numerous slender *Cryptobia* in the proboscis sheaths of *P. salmositica* removed from naturally infected salmon. The isolates of *Cryptobia* from proboscis sheaths were morphologically similar to the slender ectoparasitic forms of *C. salmositica* found on the body surface of fish (Fig. 3.13) and the isolates were infective and caused disease when inoculated into trout. The development of *C. salmositica* in its leech vector needs more careful studies using laboratory-reared leeches.

The development of *C. borreli* in the leech *H. marginata* was first described by Robertson (1911). Kruse *et al.* (1989a) described its development in laboratory-reared *P. geometra*. Briefly, parasites ingested with the blood meal multiplied in the crop,

and long slender flagellates were present in large numbers in the crop 8–10 days later. All stages in the leech were infective to fish. The parasites were transmitted experimentally to the common carp by allowing infected leeches to feed on them. The development of the parasite, its mode of transmission and the lack of transovarian transmission in the leech vector are presumed to be similar to those of *C. salmositica* in *P. salmositica* (Becker and Katz, 1965a).

In vitro culture and propagation of *Cryptobia*

C. salmositica from fish was initially cultured and subcultured in Hanks' solution with 10% heat-inactivated fetal calf serum at 5 and 10°C (Woo, 1979). The parasite multiplies by longitudinal binary fission, and newly divided parasites are slender. These are morphologically similar to the newly divided parasites in the blood of fish (Woo, 1978) or on the body surface of infected fish (Woo and Wehnert, 1983). Cultures are always infective when inoculated into fish. *C. salmositica* and *C. bullocki* also multiply at 10°C in MEM with Hanks' salts (pH 7.2–7.3), 25 mM Hepes buffer, 25% heat-inactivated FBS, L-glutamine and 0.33% dextrose (Woo and Li, 1990; E.M. Burreson, personal communication). MEM is the medium of choice because *C. salmositica* multiplies rapidly and rosette colonies are present in about 5 weeks. Formation of colonies indicates a healthy culture and the peak number of parasites is about 9×10^6 parasites/ml of medium in about 6 weeks (Li and Woo, 1991a). A strain that has been in continuous culture for over 15 years is still infective to fish. It does not cause disease but confers protection when it is used as a live vaccine in fish (Woo and Li, 1990). The addition of even a minute amount of fresh plasma from sexually mature rainbow trout (male or female) significantly increases the rate of multiplication of the parasite and the final numbers of parasites in culture. Plasma from female brood fish is significantly much better in promoting parasite multiplication (peak number of parasites at about 16×10^6 parasites/ml in 7 weeks) than that from male brood fish which is about 6×10^6 parasites/ml. The factor(s) that promotes multiplication is heat labile (Currie, 2004).

C. borreli from carp was cultured at 25°C in SNB-9 diphasic blood agar medium supplemented with vitamins and antibiotics (Nohynkova, 1984b). It divided by binary fission and the parasite lost its infectivity to fish after six subcultures. A Hungarian strain cultured in SNB-9 blood agar at 20°C (Hajdu and Matskasi, 1984) was also not infective to fish (Jones *et al.*, 1993). Similarly, Peckova and Lom (1990) were also not successful in maintaining infective cultures of *C. borreli* in supplemented SNB-9 blood agar. The cultures lost their infectivity to fish after 10–14 days. S. Li and P.T.K. Woo (unpublished) successfully cultured and subcultured the parasite continuously in a cell-free medium (TDL 15 with 10% FBS, 1% goldfish serum and 17 mM Hepes). The parasite multiplied readily and was infective to goldfish and carp. Recently, Steinhagen *et al.* (2000) showed that the parasite multiplied rapidly in a mixture of Hanks' balanced salt solution (45%), L15 (22.5%), MEM (22.5%) and distilled water (10%), supplemented with 5–10% of heat-inactivated pooled carp serum. The parasite was maintained for more than 5 months (ten passages) and was infective to fish.

Glycoproteins, surface membrane and metabolism

The non-pathogenic *C. catostomi* has a distinctly different polypeptide profile from that of the two pathogenic species (*C. salmositica* and *C. bullocki*), although the three species have many polypeptides of similar molecular mass and there are antigenic similarities between them (Woo and Thomas, 1991; Verity and Woo, 1996).

As indicated earlier, *C. salmositica* was attenuated after prolonged *in vitro* culture (Woo and Li, 1990). The attenuated strain (Fig. 3.21) is smaller in size and has at least 21 polypeptide bands, and five bands (20–200 kDa) are either absent or



Fig. 3.21. Attenuated (vaccine) strain of *Cryptobia salmositica* (light microscope; × 1150) (from Woo, 2003; courtesy of *Journal of Fish Diseases*).

antigenically different from those in the original pathogenic strain (Woo and Thomas, 1991). The avirulent strain does not have the 200 kDa metalloprotease (Zuo and Woo, 1997a), and its surface is less negatively charged (~ 7.7 mV) than that of the pathogen (~ 15 mV). Major compositions of the surface membrane include sialic acid residues, phosphate groups and protein glycoconjugates and these probably contribute to the negative surface charge (Vommaro *et al.*, 1997). There are also differences in expression of surface membrane carbohydrate residues and glycoproteins between the pathogenic and attenuated strains; specifically the increased surface carbohydrate residues on the attenuated strain coincide with the loss of virulence (Feng and Woo, 2001). *C. catostomi* also has more carbohydrates on its surface membrane and is more reactive with lectins than the pathogenic *C. salmositica* (Feng and Woo, 1998d).

A monoclonal antibody (an immunoglobulin G1 (IgG1); designated mAb-001) was produced against a surface membrane epitope of *C. salmositica*. The antibody agglutinates living parasites but does not activate complement to lyse the pathogen. *In vitro* exposure of the parasite to the antibody reduces its survival and infectivity in fish (Feng and Woo, 1996b). The antibody mAb-001 inhibits parasite multiplication and respiration (Hontzeas *et al.*, 2001) and significantly reduces cysteine and metalloprotease activities under *in vitro* conditions (Zuo *et al.*, 1997). The antibody is against a 200 kDa glycoprotein, and its epitope (designated Cs-gp200) consists of carbohydrate determinants and a conformational polypeptide with internal disulphide bonds. It is a hydrophilic antigen and is secreted by the parasite (Feng and Woo, 1998a). Cs-gp200 has its asparagine-bound *N*-glycosidically linked hybrid-type carbohydrate chain with the minimum length of a chitobiose core unit. It has a phosphatidylinositol residue, which anchors the conformational polypeptide (with disulphide bonds) to the surface membrane. The molecule is extensively post-translationally modified (Feng and Woo, 1998b). Cs-gp200 has high mannose components and it appears as a doublet in the pathogenic strain and as a single band in the vaccine strain (Feng and Woo, 2001).

Another monoclonal antibody (mAb-007; an IgG3 isotype with kappa light chains) was produced against a major 47 kDa polypeptide secreted by pathogenic (*C. salmositica* and *C. bullocki*) and non-pathogenic (*C. catostomi*) species of *Cryptobia*. This antigenic polypeptide is located in the cytoplasm of the parasite and is also secreted by both the pathogenic and attenuated strains of *C. salmositica*. It can be detected readily (using an antigen-capture ELISA) in the blood of fish at 1–2 weeks after infection. The amount of antigen is higher in rainbow trout infected with the pathogenic than with the non-pathogenic strain. This, in part, may be because the pathogen multiplies readily in rainbow trout while the vaccine strain does not (Verity and Woo, 1996).

C. salmositica and *C. bullocki* consume oxygen under *in vitro* conditions, and parasite motility and oxygen consumption are significantly reduced after the parasites are exposed to sodium azide. However, pre-azide activities are restored in *C. salmositica* once the azide is removed, even after 24 h of exposure (Thomas *et al.*, 1992). In cultures the parasite produces hydrogen peroxide and some glycolytic products (i.e. lactate and pyruvate), even when it is under aerobic conditions. It is also sensitive to acidic pH; hence for large-scale culture of

C. salmositica the medium has to be adequately buffered, as the carbon dioxide it produces changes the pH of the medium (Ardelli and Woo, 2001c). If the parasite mitochondrion is damaged (e.g. exposure to the trypanocidal drug isometamidium chloride), its oxygen consumption and carbon dioxide production decrease dramatically, with concurrent and significant increase in secretions of glycolytic products (Ardelli and Woo, 2001a). This shows that the parasite can readily switch from predominantly aerobic respiration to glycolysis. *C. salmositica*, as with *C. borreli* (Opperdoes, 1988), has its Embden–Meyerhof pathway enzymes (e.g. hexokinase, fructose-6-biophosphate aldolase, triosephosphate isomerase, glucose phosphate isomerase), as well as the peroxisomal enzyme, catalase, sequestered in glycosomes (Ardelli *et al.*, 2000). *Cryptobia* and *Trypanosoma* are the only known organisms where glycolysis is compartmentalized in microbodies called glycosomes. They may also contain enzymes for peroxide metabolism, and it is presumed that the compartmentalization of enzymes facilitates glycolysis (Opperdoes, 1988).

C. salmositica does not multiply rapidly in MEM unless FBS is added to the medium. Multiplication increases with increasing amounts of FBS and reaches a peak number of approximately 9×10^6 parasites per ml of medium. Glucose is depleted from the medium and the depletion increases with increasing numbers of parasites (Li and Woo, 1991a). The optimum temperature for *in vitro* multiplication for virulent and avirulent strains is 10°C, although the avirulent strain multiples significantly faster than the pathogenic strain (Woo and Thomas, 1992). The avirulent strain has been maintained in continuous *in vitro* culture since 1989 (Woo and Li, 1990), and it is still infective, does not cause disease and is protective to fish. The pathogenic and vaccine strains have minor nutritional differences, especially in their utilization of carbohydrates. The pathogen multiplies more readily in MEM with monosaccharide supplements, specifically D (–) ribose, D (+) xylose, D (+) galactose, D (+) glucose, D (+) mannose and D (–) fructose. However, the attenuated strain does better with disaccharides, e.g. β-D (+) glucose, α-lactose, maltose and sucrose. The attenuated strain multiplies more readily than the pathogen when L-glutamine and D (–) proline are added to the medium (Ardelli and Woo, 2003). The significance of the differences in nutritional preferences between the two strains is not well understood.

Both strains (pathogenic and attenuated) of *C. salmositica* have a cysteine (thiol) protease (49, 60, 66 and 97 kDa), and the enzyme has been isolated and partially purified (Zuo and Woo, 1997a, d, 1998a). Its optimal activity is at pH 5.0, and it has many properties (e.g. substrate specificity, inhibitor sensitivity, optimal pH) similar to those of the protease from pathogenic mammalian trypanosomes (Zuo and Woo, 1998a). The cysteine protease (Fig. 3.19) is a metabolic enzyme and it has been detected in pathogenic and non-pathogenic species of haematozoic *Cryptobia* (Zuo and Woo, 1997a). Like the purified metalloprotease (Fig. 3.20), the partially purified cysteine protease has high proteolytic activities against azocasein, haemogloblin and fibrinogen and it also has high enzymatic activity against albumin (Zuo and Woo, 1998a). About 80% of its *in vitro* activity is inhibited by the monoclonal antibody mAb-001 (Zuo *et al.*, 1997). As indicated earlier, this monoclonal antibody agglutinates live parasites, and inhibits the *in vitro* multiplication and oxygen consumption of the parasite (Feng and Woo, 1996b; Hontzeas *et al.*, 2001). It is therapeutic when injected into fish with acute infections (Feng and Woo, 1997b). These studies indicate that the cysteine protease is an important metabolic enzyme and is probably involved in intracellular protein catabolism, which results in the release of amino acids for protein synthesis and parasite multiplication.

C. salmositica multiplies rapidly by binary fission in fish (Woo, 1978). Parasite loads are significantly higher in rainbow trout with high (e.g. 3.5 g/dl) than with low (e.g. 1.0 g/dl) plasma proteins (Thomas and Woo, 1990b). Also, trout on an ascorbic acid-deficient diet have lower parasitaemias than fish on an ascorbic acid-supplemented

diet (Li *et al.*, 1996). The parasite does not multiply in MEM unless supplemented with FBS, and it multiplies less readily with 10% and 15% FBS than with 25% and 30% FBS (Li and Woo, 1991a). Glucose is depleted from the medium, and its depletion increases with increased numbers of parasites. Similarly, the pathogenic *C. borreli* utilizes glucose (Meyer *et al.*, 2002) and also has its glycolytic enzymes sequestrated in glycosomes (Opperdoes *et al.*, 1988).

Diagnosis and detection of infection

Clinical signs can be used as indications of acute infections and for preliminary diagnosis. Parasites are easily detected using the wet mount technique during the acute phase of the disease. Fresh samples (excised gills and body mucus for ectoparasitic species; internal organs and contents from the digestive tract for intestinal species; and blood/ascitic fluid for haematozoic species) are collected from living fish or within minutes of death and examined under cover slips using bright-field or phase-contrast microscopy (at × 160 and × 400) for actively moving flagellates. For confirmation, air-dried smears of parasites are fixed first in 100% ethanol and then in buffered formalin, stained in Giemsa's stain and examined under a light microscope (Woo, 1969).

The clotting technique (Strout, 1962) and the haematocrit centrifuge technique (Woo, 1969; Woo and Wehnert, 1983) are used to concentrate and detect low infections of haematozoic species. These techniques are especially useful when parasite numbers are low, e.g. either before acute disease or during the chronic phase of the infection. For parasites of coldwater fish (e.g. salmonids), the haematocrit tubes with blood are centrifuged at 4–10°C for 5 min at 13,000 g (Woo and Wehnert, 1983; Bower and Margolis, 1984a). *C. salmositica* becomes very sluggish and dies if the blood is kept at about 21°C; hence it should be kept cold at all times. Other species (e.g. *C. catostomi*) can be centrifuged at about 20°C or at room temperature (Bower and Woo, 1977). After centrifugation, the junction of the plasma and packed red cell in the tube is examined under the microscope at 16 × 10 (Woo, 1969). The sensitivity of the technique is high, detecting *C. salmositica* infections when there are about 75 organisms per ml of blood (Bower and Margolis, 1984a). Its sensitivity is increased if more than one capillary tube of centrifuged blood is examined, as was shown with mammalian trypanosomes (Woo and Rogers, 1974).

The microscopic immuno-substrate-enzyme technique (MISET) and the immunofluorescent antibody technique (IFAT) are equally sensitive in detecting specific antibodies against *C. salmositica* (Woo, 1990). MISET is preferred because it does not require a fluorescent microscope and the slide can be stored for extended periods after it has been examined. An antibody ELISA is also available to detect *C. borreli* infections in carp (Jones *et al.*, 1993) and for *C. salmositica* in salmonids (Sitja-Bobadilla and Woo, 1994). Finally, Verity and Woo (1996), using an antigen-capture ELISA, detected infections in experimentally infected rainbow trout (inoculated with either the virulent or the avirulent strains of *C. salmositica*) as early as 1 week after infection. The test was negative for all fish prior to inoculation of the parasite but was consistently positive after experimental infection.

Fish–*C. branchialis* relationships

Attachment to host cells

According to Kuperman *et al.* (2002), *C. branchialis* (Figs 3.14 and 3.15) attaches to the gill epithelium of infected fish in one of two ways. The most common mode of attachment is for the membrane of the free portion of the recurrent flagellum to expand to form a ledged ridge which is in close contact with the cell membrane of the host cell (Fig. 3.22). This mechanism provides numerous interfaces with the host cell along most of the free recurrent flagellum. The second type of attachment is for the tip of the recurrent flagellum to adhere to the host cell. In both cases, the flagellum does not seem to

penetrate the host cell membrane but is only inserted into an intercellular invagination.

Clinical signs, pathology and mortality

Gills of infected fish with large numbers of *C. branchialis* are abnormally red. The body is usually covered with a copious amount of mucus and it generally darkens before the fish dies. Infected fish are anorexic and are usually close to the water surface (Bauer *et al.*, 1969; Kuperman *et al.*, 2002). The parasite destroys the epithelium of gill filaments of susceptible fish in heavy infestations, and this leads to formation of thrombi and eventually death of the fish (Chen, 1956). The parasite has been associated with mortality of cultured carp, goldfish and catfish.

There is some controversy regarding the pathogenicity of the parasite. At the ultrastructural level, Lom (1980) was unable to show that *C. branchialis* caused lesions to the gills of infected carp (*C. carpio*) from the former Czechoslovakia and fantail darter (*Etheostoma flabellare*) from the USA. His conclusions were that the parasite was not pathogenic and that mortality in infected fish was probably caused by an unknown pathogen. According to Chen (1956), the disease is more severe in young grass carp (*Ctenopharyngodon idella*) than in older fish and in other species of carp. Hence, Woo (1987a) suggested that the Lom study be repeated with young grass carp to help resolve the confusion. Also, a multitude of factors could have contributed to the lack of disease and this would include the virulence of the parasite 'strain' that was used.

Fish–*C. iubilans* relationships

Clinical signs and mortality

Cichlids infected with *C. iubilans* are lethargic and anorexic. Most infected fish died within 3 months after the onset of clinical disease (Nohynkova, 1984a). Similar clinical



Fig. 3.22. *Cryptobia branchialis* (transmission electron microscope) to show attachment of parasite recurrent flagellum to cell membrane of gill epithelium (from Kuperman *et al.*, 2002; courtesy of the late Dr B.I. Kuperman).

signs, including stunted growth and emaciation, were reported in five different outbreaks of the disease in juvenile discus fish in the USA. In one outbreak, the mortality was 93% (280 out of 300) in 1 week and it was assumed that *C. iubilans* was the primary cause of death (Yanong *et al.*, 2004). Nine of these fish had light trichodinid and monogenean infections on the gills and body surface. However, heavy infections of *Cryptobia* were in the stomach, with moderate to heavy infections of hexamitids and *Capillaria* in the intestine. Multifocal granulomas were seen in the liver, spleen, posterior kidney, stomach and intestine.

Development in fish

Nohynkova (1984a) did not indicate finding obvious perforation of the gut in infected fish. She suggested that the parasite spread from the stomach to other organs, including the spleen and ovary. It would be very challenging to elucidate the route and mechanism of spread of the parasite to other organs, especially those that are not continuous with the stomach. Perhaps the infected macrophage-like cells are responsible for transporting the parasite to other organs. This would indicate the significance of the intracellular stage in this parasite.

Using scanning electron microscopy, Yanong *et al.* (2004) demonstrated numerous *C. iubilans* on or penetrating into the gastric mucosa in naturally infected fish. They also confirmed that the parasite was in vacuoles in host cells. The identification of the parasite was based on the detection of a triangular kinetoplast and other morphological features of *C. iubilans*, such as an anterior rostrum.

Morbidity and mortality in infected discus fish seem to be related to water quality, concurrent infections with other parasites and bacteria, diet and the size and age of infected fish. Metronidazole is not effective against the parasite in fish, while bath treatments with dimetridazole (80 mg/l for 24 h and repeated daily for 3 days) or 2-amino-5-nitrothiazol (10 mg/l for 24 h and repeated for 3 days) seem to decrease the prevalence of the parasite (Yanong *et al.*, 2004).

Fish–*C. salmositica* relationships

Course of infection and clinical signs

The number of *C. salmositica* in the blood of rainbow trout sometimes has a major peak followed by a minor peak before the chronic phase of the disease (Woo, 1979; Thomas and Woo, 1990b; Ardelli and Woo, 2002). The parasite multiplies more rapidly and with higher parasitaemias in fish with higher plasma protein levels than in fish that are fed a high-protein diet. (Thomas and Woo, 1990b, 1992b; Li and Woo, 1991b).

C. salmositica multiples rapidly at 10°C in the blood of salmonids and in tissue-culture medium (Woo, 1979; Woo and Li, 1990). However, it multiplies less readily under *in vivo* (Woo *et al.*, 1983) and *in vitro* (Woo and Thomas, 1992) conditions at 5, 15 or 20°C. It divides by binary fission and the first indication of division in an infected fish is the production of two new flagella. This is followed by duplications of the kinetoplast and the nucleus. Cytokinesis begins at the posterior end and division is unequal and symmetrogenic. The parasite retains its elongated form during the division (Woo, 1978).

Clinical signs of the disease in *Oncorhynchus* spp. include exophthalmia (Fig. 3.23), splenomegaly (Fig. 3.24), general oedema, abdominal distension with ascites (Fig. 3.25), hepatomegaly, anaemia, positive antiglobulin reactions of red cells

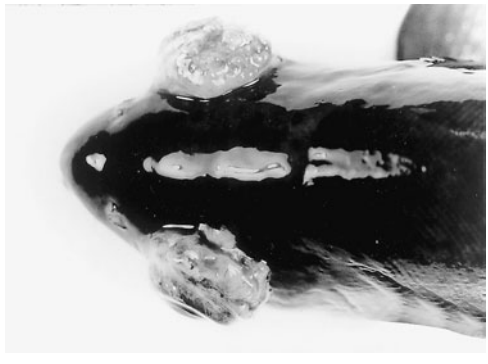


Fig. 3.23. Dorsal view of obvious exophthalmia in a rainbow trout experimentally infected with *Cryptobia salmositica* (from Woo and Poynton, 1995; courtesy of CABI, Wallingford, UK).

(Fig. 3.26) and anorexia (Woo, 1979; Thomas and Woo, 1988, 1990a, 1992b; Li and Woo, 1991b; Chin *et al.*, 2004b). Also, some fish are obviously quite emaciated. The immune system in infected fish is depressed (Jones *et al.*, 1986; Sin and Woo, 1993) and the haemolytic activity of complement is low (Thomas and Woo, 1989b). Also plasma thyroxine (T3 and T4), protein and glucose are lowered, along with depletion of liver glycogen (Laidley *et al.*, 1988). The metabolism and swimming performance of infected fish are significantly reduced (Kumaraguru *et al.*, 1995). The bioenergetic cost of the disease is considerable to infected fish. These are some of the contributing factors that retard fish growth, as there are significant reductions in food consumption, dry weight and energy gained and gross conversion efficiency in infected juvenile trout (Beamish *et al.*, 1996).

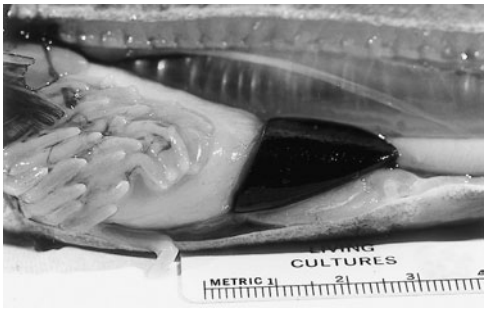


Fig. 3.24. Splenomegaly in rainbow trout with acute experimental cryptobiosis (from Woo, 1979; courtesy of *Experimental Parasitology*).

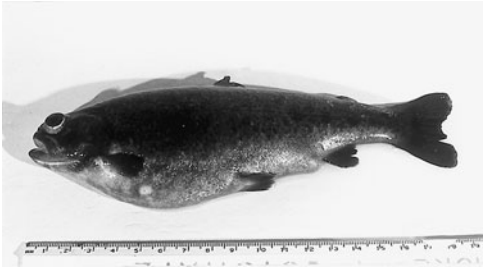


Fig. 3.25. General oedema with abdominal distension and ascites in rainbow trout with acute experimental cryptobiosis (from Woo, 1979; courtesy of *Experimental Parasitology*).

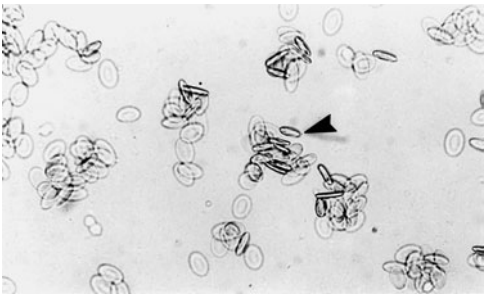


Fig. 3.26. Agglutination of red blood cells (from a rainbow trout experimentally infected with *Cryptobia salmositica*) by anti-trout immunoglobulins (positive Coombs' reaction), to demonstrate immune complexes on surface membranes of red blood cells (from Thomas and Woo, 1995; courtesy of CABI, Wallingford, UK).

The parasite is presumed not to cause disease in sculpins and they are considered the principal reservoir hosts of the parasite (Becker and Katz, 1965a, 1966). Little is known about the sculpin–parasite relationships and this should be one area of future studies. Laboratory-raised *Cryptobia*-tolerant brook charr, *S. fontinalis*, can be infected with the pathogen. Although parasitaemias are just as high as in *Oncorhynchus* spp., there are no clinical signs of the disease in infected *Salvelinus* (see later section for mechanism of protection). Hence Ardelli *et al.* (1994) suggested that charr would serve as a reservoir host for *C. salmositica* in areas on the Pacific coast where charr and *Oncorhynchus* spp. occurred together.

Pathology and mortality

The anaemia, a consistent clinical sign of the disease, is microcytic and hypochromic in rainbow trout, and its severity is directly related to parasitaemia (Woo, 1979). There are obvious lesions in haemopoietic tissues during the acute phase of the disease in juvenile rainbow trout. The severity of lesions is related to parasitaemias in the blood and to extravascular localization of the parasite (Bahmanrokh and Woo, 2001). In rainbow trout the blood components return to pre-infection values during the chronic phase of the infection (Li and Woo, 1991b). Haemodilution, splenomegaly and haemolysis are

contributing factors to the anaemia (Woo, 1979; Laidley *et al.*, 1988; Thomas and Woo, 1988). R. Arcuri and P.T.K. Woo (unpublished) showed that infected trout had significantly lower blood osmolarities from 2 weeks after infection and this was related to high parasitaemias and anaemia.

The anaemia is in part caused by haemolysis, and this is the result of secretions by living parasites and the release of parasite antigens after the parasites have been ruptured by complement-fixing antibodies. The secreted antigen (haemolysin) lyses red cells directly, while the released antigens form immune complexes with antibodies on red cells. These result in intravascular and/or extravascular haemolysis (Thomas and Woo, 1988). Red blood cells from parasitaemic trout are antiglobulin-positive or Coombs' test-positive (Fig. 3.26) and these red cells are lysed when incubated with trout complement. The antigens are also secreted by the pathogenic strain in cultures (Thomas and Woo, 1989a; Woo and Thomas, 1992).

A 200 kDa glycoprotein (metalloprotease) has been identified, isolated and purified (Fig. 3.19) from the pathogenic *C. salmositica*. The enzyme has been purified and its optimal activity is pH 7.0 (Zuo and Woo, 1997a, d, 1998a). It has high proteolytic activities against azocasein, haemoglobin, fibrinogen and gelatin, but low activity against albumin. It is inhibited by metal-chelating agents and excess of zinc ions, but activated by calcium ions (Zuo and Woo, 1997d, 1998a). The purified enzyme lyses red blood cells under *in vitro* conditions (Zuo and Woo, 2000) by digesting proteins in erythrocyte membranes (Zuo and Woo, 1997d). Consequently, it is an important contributing factor to anaemia, and is the 'haemolysin' that was earlier identified as one of the causes of anaemia (Thomas and Woo, 1988, 1989a). The metalloprotease is also collagenolytic (Fig. 3.20), and readily degrades collagen (types I, IV and V) and laminin (Zuo and Woo, 1997d). The protease is secreted by the parasite in fish (Zuo and Woo, 1997c) and in culture (Zuo and Woo, 1998a). In cultures, its secretion is significantly increased in the presence of either type I or type IV collagen and/or their breakdown products (Zuo and Woo, 1998b). Since the metalloprotease is secreted by the pathogen it contributes to the development of the disease and histopathogical lesions (see below) in infected fish. This confirms that the severity of the disease in *Oncorhynchus* spp. is directly related to the parasitaemia (Woo, 1979).

The severity of the disease, appearance of the clinical signs and mortality are also related to size of the inoculum, water temperature, diet and the genetics of the fish (e.g. Woo, 1979; Woo *et al.*, 1983; Bower and Margolis, 1985; Jones *et al.*, 1986; Li and Woo, 1991b; Chin *et al.*, 2002, 2004a). Anorexia is another consistent clinical sign of the disease and it contributes to immunodepression in infected fish during acute disease (Thomas and Woo, 1992b); however, it is also beneficial to the host because it decreases plasma protein by reducing protein intake (Li and Woo, 1991b). Reduction in plasma protein lowers parasite multiplication and hence the parasitaemia. Lower parasitaemia decreases the severity of the disease and mortality.

The anaemia and large numbers of parasites occluding small blood vessels would combine to reduce oxygen delivery to tissues and vital organs. Part of this is manifested as an increase in susceptibility of infected fish to environmental hypoxia. This would be an important contributing factor to fish mortality under some conditions, especially when dissolved oxygen is reduced as a result of overcrowding, slow water flow or algal blooms (Woo and Wehnert, 1986).

According to Putz (1972), *C. salmositica* was more pathogenic to coho salmon (*O. kisutch,* with 100% mortality) than to chinook salmon (*O. tshawytscha*) in the USA. However, Bower and Margolis (1985) found 100% mortality in chinook salmon, while coho salmon from some stocks in Canada did not die from the infection (0% mortality). The difference is probably due to the genetics of the fish. For example, sockeye salmon (*O. nerka*) from the Fulton River stock (British Columbia, Canada) suffered high mortality when injected with about 100

parasites per fish, while the Weaver Creek stock of sockeye salmon (British Columbia) had light mortality even when injected with about a million parasites per fish (Bower and Margolis, 1984a). Mortality of infected sockeye salmon is consistent within the same fish stock and with different parasite isolates (Bower and Margolis, 1985).

The histopathology includes focal haemorrhages, congestion of blood vessels, occlusion of capillaries with parasites and oedematous changes in kidney glomeruli (Putz, 1972). Bahmanrokh and Woo (2001) conducted a sequential study in experimentally infected juvenile rainbow trout and showed that the histopathology was a generalized inflammatory reaction, and lesions were in connective tissues and the reticuloendothelial systems. Lesions were seen first in the liver, gills and spleen at about 1–2 weeks post-infection (wpi). Endovasculitis and mononuclear infiltration occurred at 3 wpi and these were followed by tissue necrosis and extravascular localization of parasites at 4 wpi. Extensive necrosis of tissues was related directly to high parasitaemias and extravascular localization of flagellates. Necrosis in the liver and kidney, depletion of haematopoietic tissues and anaemia were probably responsible for mortality of fish during the acute phase of the disease. Regeneration and replacement of necrotic tissues with normal structures were noticeable in haematopoietic and reticular tissues at 7–9 wpi and these were associated with reduced parasitaemias in the blood (recovery or chronic phase of the disease).

Acid phosphatase (ACP) is also found in *C. salmositica*. It occurs both in membrane-bound and water-soluble fractions of the parasite lysate. The pathogenic strain has high total ACP activity, but this decreases on prolonged culture in MEM, and this decrease is associated with loss of virulence. Also, the membrane-bound ACP in the pathogen is more resistant to the ACP inhibitor sodium tartrate (Zuo and Woo, 1996). Compared with the pathogenic *C. salmositica*, there are also increased carbohydrate residues on the surface membrane of the non-pathogenic *C. catostomi* (Feng and Woo, 1998b) and on the attenuated strain of *C. salmositica* (Feng and Woo, 2001). It is tempting to suggest that the increased expression of carbohydrate resides on the *C. salmositica* vaccine strain and reduced ACP also contribute to its loss of virulence. Further studies are needed to more fully understand the mechanism of the disease.

Immune response

The piscine immune system is well developed (Chapters 18 and 19), and both innate and acquired (adaptive) immunity are involved in protecting fish from parasitic diseases (Woo, 1987b, 1992; Woo and Jones, 1989; Jones, 2001).

INNATE IMMUNITY. In the present discussion, absence of disease in infected fish is pathogen tolerance, while resistance to infection is pathogen resistance. Some naïve brook charr infected with *C. salmositica* do not have the disease (*Cryptobia*-tolerant charr) although their parasitaemias are as high as those in rainbow trout. Also, there are charr that cannot be infected with *C. salmositica*, and these are *Cryptobia*-resistant fish. Innate resistance to *Cryptobia* infection is inherited by progeny and controlled by a single dominant Mendelian locus (Forward *et al.*, 1995). Fresh plasma of *Cryptobia*-resistant charr lyses the parasite (lytic titres range from 1 : 2 to 1 : 4) via the alternative pathway of complement activation (Forward and Woo, 1996). Also, many other fishes (e.g. goldfish, white suckers) are also resistant to *C. salmositica* infection, and the mechanism of innate resistance in these refractory fishes is also the alternative pathway of complement activation (Wehnert and Woo, 1980). The undiluted plasma of refractory fish lyses the parasite in 30–60 min at 4°C and the lytic titres range from 1 : 4 to 1 : 8. Further studies on innate resistance (either to infection or to disease), especially in *Oncorhynchus* spp., would be rewarding because it can be exploited and it may be an alternative protective strategy against this and other pathogenic organisms (Woo, 1992).

As indicated earlier, parasitaemias in infected *Cryptobia*-tolerant brook charr are

just as high as or higher than those in *Oncorhynchus* spp.; however, they do not suffer from cryptobiosis. Hence, they may be reservoir hosts for the pathogen on the west coast of North America, where the distributions of *Oncorhynchus* and *Salvelinus* overlap (Ardelli *et al.*, 1994). Also, there are no detectable differences in the immune responses of both *Cryptobia*-tolerant and *Cryptobia*-resistant charr to other antigenic stimulations (Ardelli and Woo, 1995).

Infected *Cryptobia*-tolerant brook charr are resistant to disease because the metalloprotease secreted by *C. salmositica* is effectively neutralized by a natural antiprotease – the α_2-macroglobulin in blood. The amount of α_2-macroglobulin is higher in brook charr than in rainbow trout prior to infection, and it remains high (about 40%) during the infection, while that in trout drops to about 12% as the anaemia becomes evident (Zuo and Woo, 1997b,c). Neutralization of parasite metalloprotease by α_2-macroglobulin was demonstrated under both *in vivo* and *in vitro* conditions (Zuo and Woo, 1997b,c,d). Since *Cryptobia*-tolerant brook charr do not suffer from clinical disease, their immune system readily controls the infection and they recover more rapidly than trout from the infection. An obvious option to control cryptobiosis is to produce transgenic *Cryptobia*-tolerant salmon (Woo, 2001). It is expected that the transgenic salmon, like *Cryptobia*-tolerant brook charr, will maintain a high level of α_2-macroglobulin to neutralize the secreted metalloprotease and hence there will be reduced or no disease. This exploitation of innate resistance is a novel approach to the management of an infectious disease and is worth consideration. The strategy will probably initiate discussions on the ramifications of applying such a strategy against an infectious organism. One of the obvious advantages in this approach is that further human interventions (vaccination, chemotherapy, etc.) are not required once the transgenic animal is produced. The immune system can be relied on to effectively control the infection since there is no clinical disease in the animal.

The attenuated strain multiplied in Atlantic salmon and parasitaemia peaked at 5 weeks post-vaccination (wpv). Antibodies were detectable at 4 wpv and the antibody titre increased as the parasitaemia declined. Respiratory burst activity of circulating granulocytes (Fig. 3.27) in vaccinated Atlantic salmon was significantly higher than in unvaccinated fish, and the highest activity occurred with rising parasitaemia and the lower activity with declining parasitaemia (Fig. 3.28). There were significant increases in the proportion of granulocytes (to total leucocytes) at 4 wpv. Also, at 6 wpv the proportion of lymphocytes and monocytes increased significantly and remained elevated. These indicate both innate (respiratory burst activity and increases in numbers of granulocytes corresponding to rising parasitaemia) and acquired (antibody production and increases in the proportion of monocytes and lymphocytes corresponding to declining parasitemia) immune responses to the live vaccine (Chin and Woo, 2005).

ACQUIRED (ADAPTIVE) IMMUNITY. The attenuated *C. salmositica* produces low parasitaemia in rainbow trout, does not cause disease, circulates in the blood for at least 6 months and is protective when used as a live vaccine (Woo and Li, 1990). It is used routinely as an experimental vaccine (see below) as it has no detectable bioenergetic cost to juvenile rainbow trout (Beamish *et al.*, 1996), and it protects juvenile and adult salmonids from the pathogen (e.g. Sitja-Bobadilla and Woo, 1994; Li and Woo, 1995; Feng and Woo, 1997a, 1998c; Ardelli and Woo, 2002; Mehta and Woo, 2002).

The humoral response to the parasite is detectable in some fish at about 2 wpi (Jones and Woo, 1987; Woo, 1990; Sitja-Bobadilla and Woo, 1994; Chin *et al.*, 2004a,b). Families of Atlantic salmon with early detectable antibody production had significantly lower parasitaemias than those with delayed response, and they recovered earlier (Chin *et al.*, 2004a).

Fish that recover from the disease are protected, and their antisera contain agglutinating, neutralizing and complement-fixing antibodies (e.g. Sitja-Bobadilla and

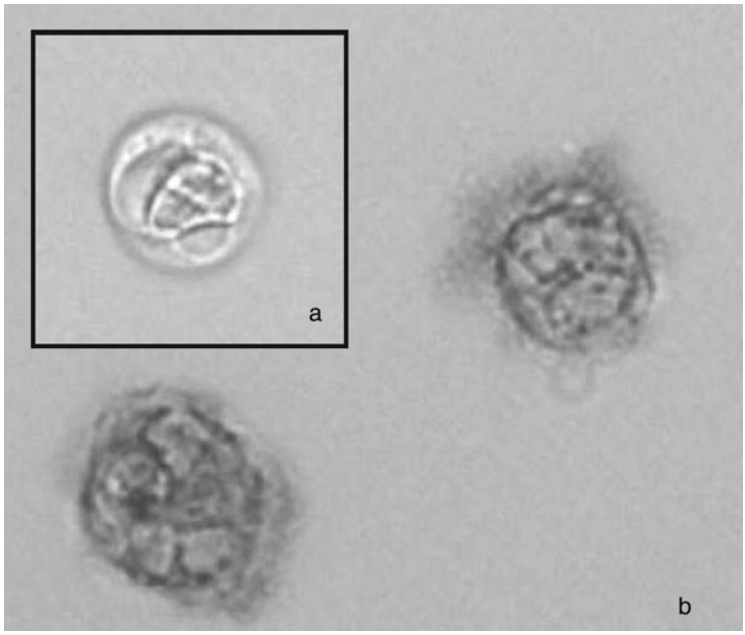


Fig. 3.27. Neutrophils from peripheral blood of Atlantic salmon (× 400): (a) one normal (inactivated) cell – absence of formazan after *in vitro* exposure to nitroblue tetrazolium (NBT) dye; (b) two activated cells – presence of formazan after *in vitro* exposure to NBT (original; courtesy of Adrian Chin).

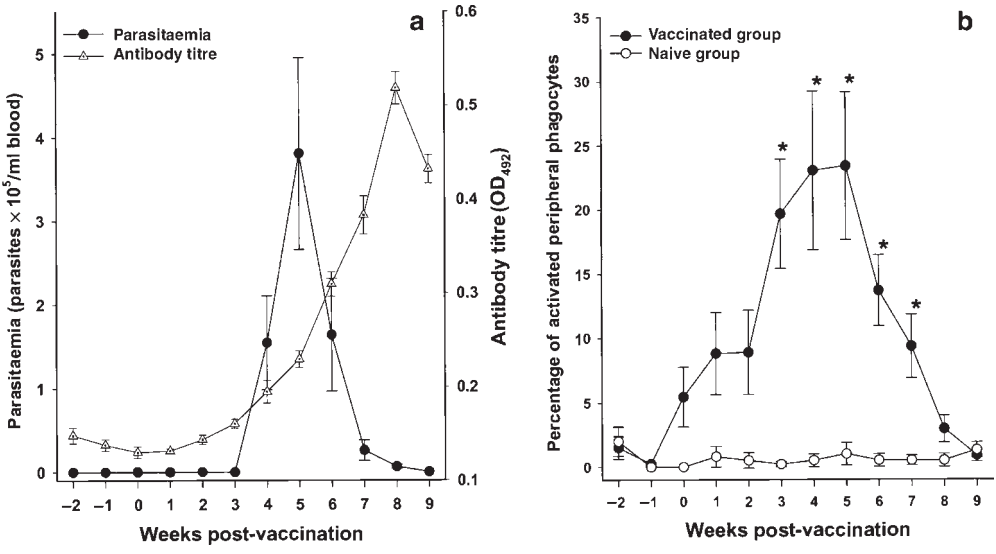


Fig. 3.28. (a) Parasitaemia (± SE) and antibody titre (OD_{492} ± SE) in Atlantic salmon after being inoculated with the live *Cryptobia salmositica* vaccine; (b) percentage of activated peripheral phagocytes (± SE) in the blood of Atlantic salmon after being vaccinated with the *Cryptobia salmositica* vaccine – an asterisk indicates a significant difference ($P < 0.05$) in percentage of activated phagocytes between vaccinated and naïve groups (from Chin and Woo, 2005; courtesy of *Parasitology Research*).

Woo, 1994; Li and Woo, 1995; Feng and Woo, 1997a, 1998c; Ardelli and Woo, 2002; Mehta and Woo, 2002). Intraperitoneal implantation of cortisol lowers antibody production and this increases parasitaemia in rainbow trout. The mortality of infected cortisol-implanted fish is higher than in infected fish or uninfected cortisol-implanted fish (Woo *et al.*, 1987). Also, both antisera from recovered fish and the monoclonal antibody mAb-001 are therapeutic and prophylactic against the parasite in fish (Feng and Woo, 1997b). Titres of complement-fixing antibodies in recovered and vaccinated fish rise significantly after *C. salmositica* challenge (e.g. Li and Woo, 1995; Ardelli and Woo, 1997, 2002; Feng and Woo, 1998c; Mehta and Woo, 2002), and this classical anamnesis response also confirms that the protection is in part due to humoral response in recovered and vaccinated fish.

As indicated earlier, mAb-001 produced against a 200 kDa epitope (Feng and Woo, 1996b) inhibits the activities of cysteine and metalloproteases (Zuo *et al.*, 1997). The antibody also inhibits parasite multiplication and oxygen consumption (Hontzeas *et al.*, 2001), agglutinates the parasite, does not fix complement, but is protective when injected into fish (Feng and Woo, 1997b).

Thymectomy in adult rainbow trout does not decrease production of complement-fixing antibody in infected and vaccinated fish in long-term study. However, it reduces protective antibody production in short-term thymectomized fish (Feng and Woo, 1997a). Parasitaemias and the production of complement-fixing antibodies in thymectomized and intact rainbow trout (injected with rabbit anti-thymocyte serum (RATS) before *Cryptobia* infection) are not significantly different from those in control fish (infected but not injected with RATS). Both groups acquire protection on recovery. RATS is not cytotoxic to B-like cells and thus the protective antigen(s) on *C. salmositica* is thymus independent (Feng and Woo, 1998c).

Peritoneal macrophages in ascites fluid of infected rainbow trout contain engulfed parasites (Woo, 1979). This was confirmed under *in vitro* conditions with macrophages from the head kidneys in both vaccinated and recovered fish (Li and Woo, 1995). Adoptive transfer of leucocytes and plasma from immune fish conferred partial protection in naïve fish (Jones and Woo, 1987). Cell-mediated responses to the parasite were demonstrated using delayed-type hypersensitivity and macrophage inhibition migration tests (Thomas and Woo, 1990a), and enumeration of lymphocyte numbers in intact and thymectomized rainbow trout (Feng and Woo, 1996a). Also, activities of circulating lymphocytes (T cells and B cells) in infected Atlantic salmon were depressed until about 4 wpi. In fish injected with the *C. salmositica* vaccine, the humoral response (i.e. B-lymphocytes) was greater than the cell-mediated response (i.e. T-lymphocytes), and this was the reverse in fish infected with the pathogen (Ardelli and Woo, 2002). Respiratory burst activities of macrophages from infected rainbow trout were significantly higher than those in vaccinated fish. However, this response from vaccinated fish rose rapidly after parasite challenge and was comparable to that in infected fish (Mehta and Woo, 2002). This is also a classical anamnesis response.

Infected fish lost their infections or there was no mortality when the water temperature was raised to 20°C (Woo *et al.*, 1983; Bower and Margolis, 1985). Woo (1987a) suggested that modification(s) of this approach might be useful for protecting fish. Bower and Evelyn (1988) confirmed that infected juvenile sockeye salmon acclimated to 20°C survived, while all infected fish maintained at 10°C died from the disease. Also, 60 of temperature-acclimatized infected fish survived a parasite challenge at 10°C, while 95 of infected naïve fish died.

Vaccine and vaccination

LIVE VACCINE. As indicated earlier, *C. salmositica* has been attenuated under *in vitro* conditions (Woo and Li, 1990). The vaccine strain (Fig. 3.21) is smaller, it has lost polypeptide bands and some of the

remaining bands are antigenically different. The vaccine strain also multiplies much more readily than the pathogenic strain in tissue-culture medium (Woo and Thomas, 1991, 1992). It was used as an experimental vaccine in many species of salmonids, and all vaccinated fish were protected from disease when challenged with the pathogen (e.g. Ardelli and Woo, 1995, 1997, 2002; Li and Woo, 1995; Staines and Woo, 1997; Feng and Woo, 1998c; Mehta and Woo, 2002). Rainbow trout vaccinated in fresh water and transferred to sea water were also protected (Li and Woo, 1997).

A single dose of the vaccine protected rainbow trout for at least 24 months. The titres of complement-fixing antibodies in vaccinated fish rose significantly after challenge. This is a classic secondary response. Under *in vitro* conditions, activated macrophages from head kidneys of vaccinated fish showed both antibody-independent and antibody-dependent cytotoxicity. Also, in the presence of antisera, the macrophages were very efficient in engulfing living parasites. They were significantly more efficient than activated macrophages with sera from naïve fish or macrophages from naïve fish with antisera (Li and Woo, 1995).

Humoral and cell-mediated immunity are important components of the protection in fish against cryptobiosis (e.g. Thomas and Woo, 1990a; Li and Woo, 1995; Feng and Woo, 1996a; Ardelli and Woo, 2002; Mehta and Woo, 2002).

DNA VACCINE. Pathogens usually change their surface epitopes to evade the immune response; consequently, a surface epitope-based vaccine generally does not protect against all isolates of the pathogen. However, an enzyme-based vaccine will probably be more protective because it is unlikely that enzymes are significantly different between isolates. Also, antibodies against an enzyme-based vaccine may inhibit the enzymatic activity in taxonomically unrelated pathogens; thus a specific but broad-spectrum vaccine. A recombinant protein of the 200 kDa metalloprotease of *C. salmositica* and a recombinant metalloprotease DNA vaccine have been produced. Trout injected with the DNA vaccine were consistently more anaemic than controls at 2–5 wpv, and agglutinating antibodies against *Cryptobia* were detected in the blood of vaccinated fish at 5–7 wpv. Also, vaccinated fish consistently had lower parasitaemias, delayed peak parasitaemia and faster recovery (Tan, 2005). Antibodies from vaccinated fish also agglutinated a taxonomically unrelated pathogen (*Spironucleus*) from chinook salmon (C.W. Tan and P.T.K. Woo, unpublished). This cross-reaction indicates that the *Cryptobia* DNA vaccine may have the potential to be a broad-spectrum vaccine. Further careful studies are required to follow up on this suggestion.

Immunodepression and anorexia

C. salmositica depresses the piscine immune system during the acute phase of the disease. Production of antibodies to sheep red cells and *Yersinia ruckeri* were depressed in infected trout (Wehnert and Woo, 1981; Jones *et al.*, 1986; Sin and Woo, 1993). Jones *et al.* (1986) showed that *Cryptobia*-infected trout exposed to *Y. ruckeri* suffered higher mortality than fish infected with either pathogen. When *Cryptobia*-infected fish that were earlier exposed to *Yersinia* were re-exposed to the bacteria, they were as susceptible as fish that did not have prior exposure to the bacterium.

Anorexia contributes to humoral immunodepression in infected trout (Thomas and Woo, 1992b); however, as indicated earlier, it is also beneficial to infected fish as it lowers the plasma proteins and subsequently the severity of the infection (Li and Woo, 1991). In infected rainbow trout, the haemolytic levels of complement are about 20% of pre-infected levels and this persists throughout the infection (Thomas and Woo, 1989b). Low complement decreases phagocytic activity and antigen presentation by macrophages and hence may contribute to immunodepression. Further studies are needed to elucidate the mechanism in immunodepression and the factors that contribute to it.

Anorexia was evident at 3 wpi in juvenile trout at 10°C and peaked at 4 wpi,

which coincided with peak parasitaemias. However, at 5°C anorexia was at 5 wpi and was most severe at 7 wpi, with peak parasitaemia at 8 wpi. Before the onset of anorexia, there were no significant correlations between mean share of meal and coefficient of variation in feeding but these became significant during anorexia. Fish on an anorexic diet (pair-fed to infected fish) responded well at 10°C to *C. salmositica* vaccine and this could partly be because of constant antigenic stimulation by the live vaccine (Chin *et al.*, 2004b) compared with earlier studies (Wehnert and Woo, 1981; Jones *et al.*, 1986; Sin and Woo, 1993).

Effects of salmonid cryptobiosis on fish production

The parasite is a lethal pathogen of salmon in semi-natural and intensive salmon culture facilities on the Pacific coast of North America (Bower and Thompson, 1987). The prevalence of the parasite in downstream migrants of Pacific salmon ranged from 3% to 21% in some streams (Becker and Katz, 1966). Experimental studies showed that infected pre-smolt salmon retained their infection when transferred to salt water and mortality was not reduced when fish were in sea water (Bower and Margolis, 1985). Consequently, the disease may be quite a significant cause of fish mortality in the sea; however, no field studies have been conducted to validate this suggestion.

There were serious outbreaks of the disease in juvenile salmon in hatcheries (fresh water) in Washington State, USA. These include three separate outbreaks in chinook salmon in three localities in 1972 and 1973 (Wood, 1979). The fish had massive numbers of *Cryptobia* in their blood, they were anaemic and lethargic and some had abdominal distension and generalized oedema. Parasites were on the body surface and in abdominal fluid in fish with acute disease. There was a substantial loss of juvenile chinook salmon, while coho salmon of the same age in the same or adjacent ponds were unaffected. More recently, P.F. Chapman (1993, personal communication) described outbreaks of the disease in chinook salmon in hatcheries in Washington State, which began in December 1992 and peaked in February 1993. Infected fish had the typical clinical signs (anaemia, splenomegaly, ascites) of the disease, and 65,000 fish were involved, with peak mortality of 0.1% per day in February. In one hatchery the mortality of chinook broodstock was about 50%. He noted that cryptobiosis had occurred in the same hatcheries in the past but was not as severe.

Cryptobiosis also causes mortality in sexually mature salmon. Significant mortalities were associated with post-spawning rainbow trout and pre-spawning chinook salmon in North America (Wales and Wolf, 1955; P.F. Chapman, 1993, personal communication) and pre-spawning pink salmon (*O. gorbuscha*) in the former Soviet Union (Makeyeva, 1956). Wales and Wolf (1955) suggested that many post-spawning trout in a hatchery in California died from a combined effect of *Cryptobia* and *Saprolegnia parasitica.* Makeyeva (1956) concluded that heavy *Cryptobia* infection was the cause of pre-spawning mortality of pink salmon in a tributary of the Amur River. She found lower numbers of parasites in fish that had spawned and assumed that the fish were infected just before spawning and hence were able to spawn successfully.

Currie (2004) showed experimentally that spawning female rainbow trout were much more susceptible (significantly higher parasitaemias) than sexually mature males. All infected females had exophthalmia, while none of the males had this clinical sign. Also, most infected females with eggs died before or shortly after spawning and none of the infected males died. Infected males initially increased milt production and sperm concentration; however, this declined as the disease progressed. There were no differences in survival and weights between progeny of uninfected females fertilized by sperm from infected and uninfected males. Further experimental studies should be conducted to better understand the conditions (e.g. susceptibility to secondary infections) that contribute to

mortality of infected fish during their reproductive state.

Some adult Pacific salmon had detectable infections as early as 5 days after they returned to fresh water (in November) in the Fraser River, British Columbia, Canada. Parasitaemias were very high in the fish at spawning, and many fish died before they spawned (Bower and Margolis, 1984b). In the Soleduck hatchery (Washington State, USA), about 50% of spring chinook salmon broodstock brought in from streams annually die from cryptobiosis (L. Peck, 1994, personal communication).

In 1996 there was a severe outbreak of cryptobiosis in Caspian salmon (*S. trutta caspius*), Ardon hatchery, Republic of North Osetia-Alania, Russia (P. Golovin, personal communication). Very little is known about this *Cryptobia* (e.g. specific identification, its vector, geographical distribution, reservoir host), other than the fact that massive numbers of parasites were in the blood and clinical signs were similar to those of cryptobiosis in *Oncorhynchus* spp. The pathogenic flagellate from Caspian salmon is morphologically similar to *C. salmositica* from Pacific salmon (P.T.K. Woo, unpublished). Cross-transmission and other studies (e.g. antigenic, immunological, molecular) would have to be conducted to better characterize and identify the parasite from Caspian salmon.

The most recent outbreak of the disease in spring chinook salmon brood stock was in a hatchery on the Rogue River in Oregon, USA (C. Banner, 2004, personal communication). Mortality due to cryptobiosis was over 50% and moribund fish had a massive number of parasites in the blood (Fig. 3.29). No leeches were found on infected fish and total mortality was similar in male and female fish.

Outbreaks of the disease caused by *C. salmositica* have also occurred in fish held in sea cages. In 1997 the parasite caused significant morbidity and mortality in smolts and pre-harvest chinook salmon in a hatchery on Vancouver Island, Canada. There was a small mortality spike (about 1%) in post-smolts in the first 10–15 weeks after transfer to salt water. Re-emergence of

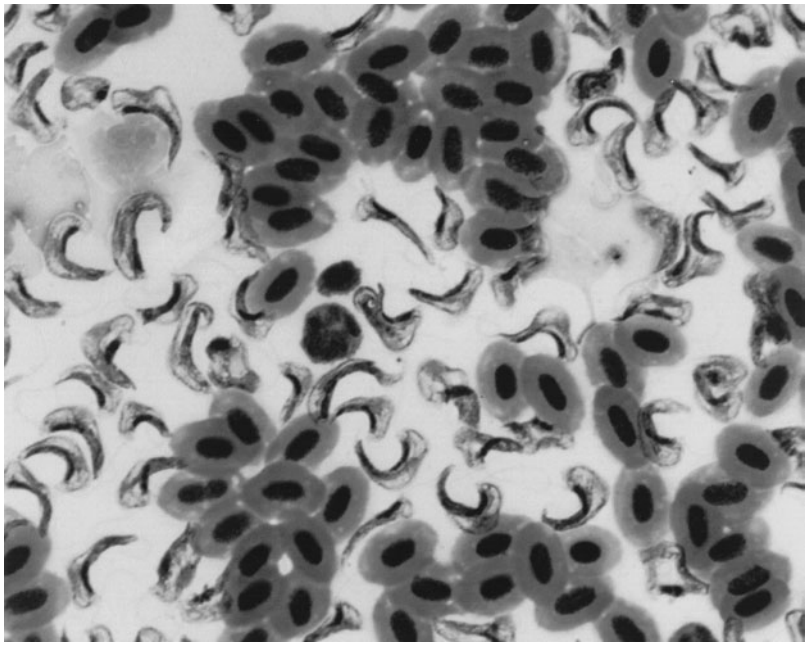


Fig. 3.29. Blood smear from a sexually mature spring chinook salmon naturally infected with *Cryptobia salmositica* (courtesy of Craig Banner, Oregon Department of Fish and Wildlife, USA).

the disease as a significant cause of morbidity and mortality occurred later in the pre-harvest fish. According to hatchery management, the outbreak was confined to fish exposed to unfiltered surface water and did not appear to be linked to handling of fish. Also, mortality seemed to be associated with age and major stressors, such as harassment by marine mammals. Another outbreak in the pre-harvest chinook salmon occurred in the same hatchery in 2001. *Cryptobia* was found in large numbers in the blood and ascites fluid of moribund fish, and clinical signs (e.g. exophthalmia, anaemia, anorexia) were evident in many fish. Fish mortality varied between cages and ranged from 3.3% to 24.9%. Briefly, the parasite was detected in the blood of some fish (while fish were in fresh water in the hatchery) before they were transferred to sea cages in August–September 1999. Parasites from moribund fish in sea cages are morphologically similar to *C. salmositica*, and cause clinical disease in experimentally infected rainbow trout. Also, the *C. salmositica* vaccine protects fish from two of these isolates (M. Eldridge and P.T.K. Woo, unpublished). It is suggested here that the outbreak in sea cages was initiated because of relapse in some infected fish (possibly due to 'stress'), and the pathogen was rapidly transmitted directly to other fish during weighing and grading when fish were brought into direct contact.

Epizootiology of salmonid cryptobiosis

The prevalence of *C. salmositica* in salmon returning to fresh water to spawn was low in September but increased to about 100% in December and January. Also, returning salmon had detectable infections within 5 days in fresh water and the longer fish were in fresh water the higher were their parasitaemias (Bower and Margolis, 1984b). These increases were assumed to be related to increased numbers of leeches in the streams in November (Becker and Katz, 1965b, 1966; Bower and Margolis, 1984b).

Becker and Katz (1966) found that the prevalence of the parasite in young salmon varied greatly (3–21%) between streams and years. Fingerlings had detectable infections in the autumn and winter. The parasite was also in downstream migrants (normally 1-year-old fish) in early spring. Wood (1979) had found *Cryptobia* in coho and chinook salmon less than 60 days old.

The torrent sculpin (*Cottus rhotheus*) is considered the principal reservoir host of the pathogen. This will have to be confirmed with laboratory studies. Becker and Katz (1966) found the parasite in 60% of sculpins between April 1961 and October 1962 in Oregon. The prevalence (27%) was lowest in small fish (< 65 mm); however, the parasitaemia was highest in small fish and it decreased in larger fish. In British Columbia the prevalence in sculpins (*Cottus aleuticus*) ranged from 8 to 95% between September 1981 and September 1982 (Bower and Margolis, 1984b). In small sculpins (< 40 mm) the prevalence was low between August and November and highest in April. This seasonal trend was seen in large sculpins (> 40 mm) and their prevalence was higher than that in small fish.

Chemotherapy and immunochemotherapy

There have been extensive studies on the chemotherapy of pathogenic mammalian trypanosomes (e.g. Peregrine, 1990); however, little is known about the effectiveness of drugs on *Cryptobia*. *Trypanosoma* and *Cryptobia* are related but many trypanocidal drugs (e.g. suramin, berenil and antrycide) have no detectable *in vitro* effects on *C. salmositica* (P.T.K. Woo, unpublished). Although a combination of antibiotics (penicillin, streptomycin and amphotericin B) affects the *in vitro* viability of *C. salmositica* in cultures, it does not seem to affect trypanosomes. However, the antibiotic combination does not have a therapeutic effect in rainbow trout with cryptobiosis (Thomas and Woo, 1991). Similar results were obtained when cultures of newly isolated *C. salmositica* were exposed to a similar combination of antibiotics. Amphotericin B is the main cryptobiocidal factor in the antibiotic combination (Chapman, 1994).

Crystal violet, a triphenylmethane dye, is trypanocidal and it is routinely added to the blood in many South American blood banks to prevent transmission of *T. cruzi* in blood transfusions (Docampo *et al.*, 1988; Docampo and Moreno, 1990). Crystal violet is also effective against *C. salmositica* under *in vitro* conditions (Ardelli and Woo, 1998). Low concentrations of the dye inhibit multiplication of *C. salmositica*, lower its infectivity to fish and cause ultrastructural lesions on mitochondrial and nuclear membranes. However, the therapeutic dose causes high fish mortality (74%) in juvenile rainbow trout (B.F. Ardelli and P.T.K. Woo, unpublished).

Isometamidium chloride (Samorin) also lyses *C. salmositica* under *in vitro* conditions. It is widely used against trypanosomiasis in domestic animals (Kinabo *et al.*, 1989), and is used as a prophylactic drug against bovine trypanosomiasis (Kinabo and Bogan, 1987). In fish, Samorin (1.0 mg/kg weight) injected intramuscularly reaches a peak level in the blood of salmon 2–3 weeks after intramuscular injection (Ardelli and Woo, 2000). The drug is therapeutic against *C. salmositica* in rainbow trout during pre- and post-clinical phases of the disease. It is not effective during acute disease as the drug 'modifies' surface epitopes of the parasite so that they are not lysed by complement-fixing antibodies (Ardelli and Woo, 1999). However, it is more effective in infected Atlantic salmon, and at a higher dose (2.5 mg/kg) the infection is eliminated in about 30% of adult fish and it significantly reduces the parasitaemias in remaining fish. Also, all infected juvenile chinook salmon treated with isometamidium (1.0 mg/kg) at 2–3 wpi survived the disease, while 100% of untreated infected fish died. The drug also has prophylactic value, and it does not affect fish growth, food consumption, blood complement levels or haematocrit values in fish (Ardelli and Woo, 2001b).

Under *in vitro* conditions the drug accumulates rapidly in the kinetoplast of the parasite and causes condensation of its kinetoplast DNA, formation of vacuoles and swelling of mitochondrial cristae (Fig. 3.30). *In vitro* oxygen consumption and carbon dioxide production decrease significantly after drug exposure (Fig. 3.31), with a very significant increase in the secretion of glycolytic products (lactate and pyruvate) as the parasite switches from aerobic respiration to glycolysis after its mitochondria are damaged by the drug (Ardelli and Woo, 2001a). Also, *in vitro* exposure to sublethal levels of the drug reduces infectivity of the parasite to fish and changes the surface glycoprotein antibody-receptor sites of the parasite. This alteration of surface epitopes explains the protection of some parasites from lysis by complement-fixing antibodies when rainbow trout with acute infections were treated with the drug (Ardelli and Woo, 1999). Further work is needed to better understand the drug–pathogen interactions.

In general, chemotherapy is selective toxicity. Woo (1995) suggested that conjugation of a drug to a parasite-specific antibody would reduce the amount of drug required for effective treatment. This approach would significantly increase the cost of treatment and it was expected that it would not be used routinely. Advantages associated with using less drug would include reduced side effects due to chemotherapy and accumulation of drug residues in host tissues. It also lowers the risk of the development of drug resistance by the pathogen. Reduced accumulation of the drug residue in host tissues is an important consideration if the fish are for consumption.

Ardelli and Woo (2001d) conjugated isometamidium to polyclonal antibodies and mAb-001. The conjugated drug is on the entire parasite while the unconjugated drug accumulates in the kinetoplast. Before drug conjugation both antibodies agglutinate living parasites but they react differently after drug conjugation. Conjugated polyclonal antibodies lyse the parasite under *in vitro* conditions but parasites are no longer agglutinated. In contrast, the conjugated mAb-001 does not lyse *C. salmositica* but agglutinates it. After *in vitro* exposure to the drug–polyclonal antibody conjugate, the

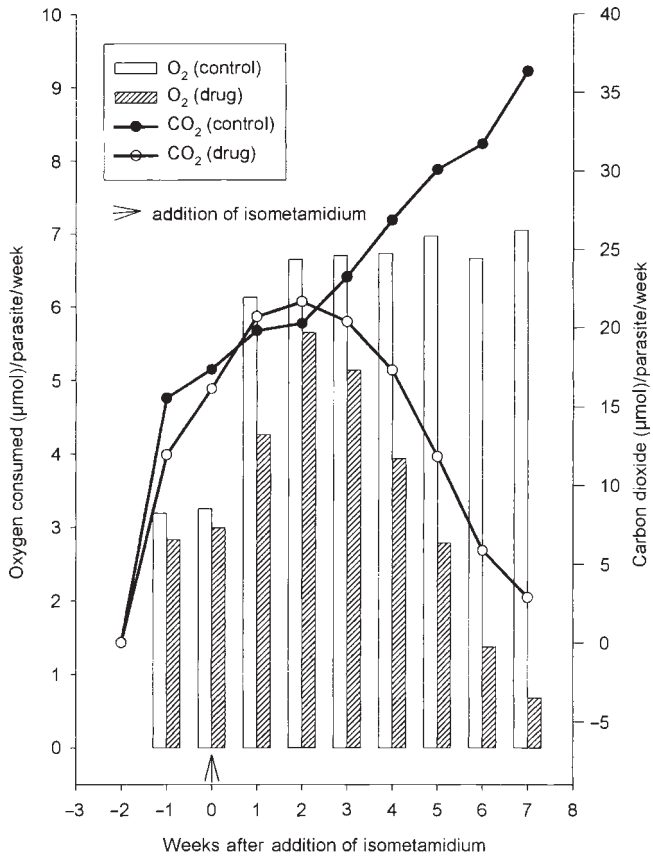


Fig. 3.30. Oxygen consumption and carbon dioxide production by *Cryptobia salmositica* after *in vitro* exposure to isometamidium chloride (from Ardelli and Woo, 2001a; courtesy of *Journal of Parasitology*).

infectivity of the parasite and subsequent parasitaemia were significantly lowered. Also, survival was higher in chinook salmon infected with the drug–antibody conjugate-exposed parasite. These results are encouraging and further studies are needed to refine the approach.

Fish–*C. borreli* relationships

Route and course of infection

The parasite multiplies rapidly after infection and this is followed by its elimination from peripheral blood (Steinhagen *et al.*, 1989a; Jones *et al.*, 1993). Water temperatures affected the length of prepatent periods, height of parasitaemias and duration of infections (Steinhagen *et al.*, 1989a), as was also shown in salmonid cryptobiosis (Woo *et al.*, 1983). Also, the *C. borreli* infection lasted 20 weeks in fish kept at 20°C and it was shortened to 12 weeks at 30°C (Steinhagen *et al.*, 1989a).

Keysselitz (1906) indicated that post-spawning cyprinids were more susceptible to the parasite than sexually mature fish. Effects of *C. borreli* on pre- and post-spawning cyprinids need to be examined more closely.

Pathology and mortality

Infected fish are anaemic (Lom, 1979; Steinhagen *et al.*, 1990; Jones *et al.*, 1993),

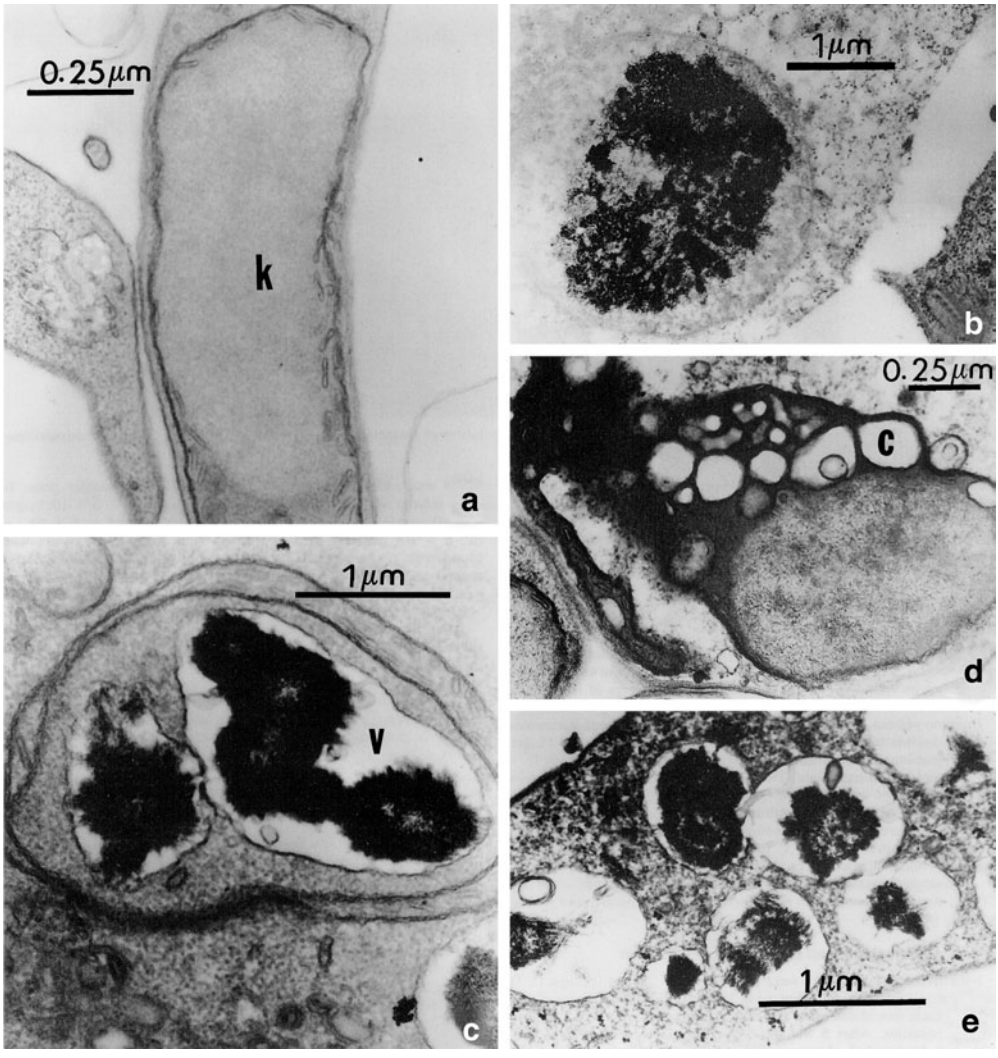


Fig. 3.31. Micro-lesions in *Cryptobia salmositica* (transmission electron microscopy) after *in vitro* exposure to isometamidium chloride: (a) normal kinetoplast – not exposed to Samorin; (b) condensation of DNA in kinetoplast after exposure to Samorin; (c) vacuole formation in kinetoplast after drug exposure; (d) swelling of cristae after exposure to Samorin; (e) vacuole formation in cytoplasm after drug exposure (V, vacuole; C, cristae; K, kinetoplast) (from Ardelli and Woo, 2001a; courtesy of *Journal of Parasitology*).

and the anaemia is associated with high parasitaemia and is absent in the blood of recovered fish. The kinetics and mechanism of the anaemia are not well studied but it is presumed that they are similar to those in salmonid cryptobiosis. The numbers of granuloblasts and granulocytes in carp increased after infection and peaked at 44 days (Steinhagen *et al.*, 1990).

Parasites are located extra- and intravascularly in goldfish. The intercellular spaces in fat cells are dilated and are often full of flagellates. Diffused degenerative changes and glomerulitis and tubulonephrosis occur in the kidneys. Also, foci of necrotic tissues are obvious in livers (Dykova and Lom, 1979; Bunnajirakul *et al.*, 2000). Cytopathological lesions are obvious in

renal tubule epithelium cells early after infection. As the infection progresses, there is loss of basilar invaginations with swelling of mitochondria and deterioration on mitochondrial internal membrane. Cells of the distal tubule are affected earlier than those of the proximal tubule. The cytological changes suggest loss of functions, which will probably lead to ionic and osmoregulatory problems (Rudat *et al.*, 2000). The infection destroys about 40% of nephric tubules, and infected fish excrete more electrolytes in their urine than uninfected carp. Uninfected carp normally excrete highly diluted urine (about 10% plasma osmolarity). As the infection progresses, the osmolarity increases to about 26% plasma osmolarity, with increased Na^+ ions (Meyer *et al.*, 2002). Consequently, destruction of excretory renal structures leads to severe effects on osmoregulation in infected carp, and this, together with a decrease in oxygen supply due to the anaemia and occlusions of capillaries with parasites, contributes to fish mortality.

Nitric oxide is detrimental to the host tissues. Phagocytic cells from organs in infected carp produced nitric oxide and this increased nitrate levels in the serum. Host survival was higher if infected carp were treated with aminoguanidine, an inducible nitric oxide synthase inhibitor (Saeij *et al.*, 2002). The high amount of nitric oxide produced by leukocytes in infected carp did not affect parasite survival but it might interfere with the coordinated defensive response of activated cells in the carp (Scharsack *et al.*, 2003a). Also, *C. borreli* was not sensitive to responses of activated neutrophils from head kidneys of infected carp. The parasite probably interfered with the production of immunological signals of these cells, and this probably resulted in immunodepression, which favoured parasite survival in infected fish (Scharsack *et al.*, 2003b).

C. borreli killed 56–80% of experimentally infected goldfish (Lom, 1979). As in *C. salmositica* infections, mortality due to *C. borreli* is also probably related to the size of the inoculum and water temperature. Age seems to be another important factor. Lom (1979) indicated that mortality was 40–80% in first-year carp and this was reduced significantly in older carp.

Immunity

INNATE IMMUNITY. Non-specific antibodies are produced soon after *C. borreli* infection, and an *in vitro* study on carp phagocytes suggests that there is a mechanism that may limit the multiplication of the parasite prior to the production of specific antibodies (Saeij *et al.*, 2003b). It seems that there is a heat-labile fraction of the pathogen which, together with CpG motifs in the DNA of *C. borreli*, induces the production of nitric oxide and also probably the inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-1β.

ACQUIRED (ADAPTIVE) IMMUNITY. Infected goldfish that survive an infection are protected (Lom, 1979). Sera from recovered fish agglutinate *C. borreli* (Scharsack *et al.*, 2004) and parasites are lysed by complement-fixing antibodies in sera from immune fish (Saeij *et al.*, 2003b; Scharsack *et al.*, 2004). Infected carp rapidly produced antibodies in the first 4 weeks of infection (Jones *et al.*, 1993), peak antibody production coincided with decline in parasitaemia and most fish recovered 8–12 weeks after infection. Recovered fish were protected from challenge. Immunosuppressive agents significantly increased parasitaemias in carp, which resulted in high fish mortalities (Steinhagen *et al.*, 1989b).

Daily handling stress increased susceptibility of carp to *C. borreli*, and *in vitro* studies showed that cortisol suppressed *Cryptobia*-induced expression of IL-1β, TNF-α, serum amyloid A and nitric oxide synthase (Saeij *et al.*, 2003a). Macrophage-depleted carp infected with the parasite did not have lethal parasitaemias but had lethal bacteraemias. Those fish that survived were protected when challenged with *C. borreli* (Saeij *et al.*, 2003c). Infected outbred carp responded with the production of specific antibodies, while the highly susceptible isogenic hybrid carp did not. This suggests

a relationship between susceptibility and antibody production (Wiegertjes *et al.*, 1995). Sera from carp that were highly susceptible to the pathogen did not have antibodies (either to agglutinate or to lyse the parasite); however, peripheral leukocytes from these carp and more resistant carp responded equally well to mitogenic stimulation (Scharsack *et al.*, 2004). The mechanism of non-responsiveness to the pathogen in highly susceptible carp needs further elucidation.

Cryptobiosis in marine fish

C. bullocki causes disease and mortality in marine fishes. The parasite is transmitted by blood-sucking leeches. It is readily cultured in MEM at 15°C and culture forms are infective to fish (E.M. Burreson, 1993, personal communication). The various diagnostic procedures developed for *C. salmositica* can easily be adapted for use with *C. bullocki*.

Geographical distribution of parasite and host range

C. bullocki was described from the blood of winter flounders (*P. americanus*) in New Hampshire, USA (Strout, 1965). It is found in estuarine and inshore fishes along the Atlantic coast of North America (from New Brunswick to Georgia) and also in the northern Gulf of Mexico (Strout, 1965; Laird and Bullock, 1969; Daily, 1978; Newman, 1978; Becker and Overstreet, 1979; Burreson and Zwerner, 1982). The parasite is common in flounders (prevalence from 70 to 100%) that use estuaries as nursery grounds. These include winter and summer flounders (*P. americanus* and *Paralichthys dentatus*) along the Atlantic coast, and southern flounder (*Paralichthys lethostigma*), hogchoker (*Trinectes maculatus*) and croaker (*Micropogonias undulatus*) on the southern Atlantic coast to the northern Gulf of Mexico. Also, the smooth flounder (*Liopsetta putnami*) in New England and hogchoker and summer flounder in Chesapeake Bay, USA, are commonly infected with the parasite.

Parasite morphology

Specimens from air-dried smears are longer and more slender than those that are wet-fixed in osmic vapour (Strout, 1965). The following description is based on stained air-dried specimens: body length 17.6 (12.5–23.1) μm; body width 2.7 (1.2–4.5) μm; anterior flagellum 13.1 (8.3–19.1) μm; posterior free flagellum 8.5 (4.4–15.7) μm; length of kinetoplast 3.3 (1.7–5.5) μm; width of kineoplast 1.1 (0.6–2.0) μm; length of nucleus 3.4 (1.8–6.8) μm; anterior of nucleus to anterior end 4.2 (0.9–6.3) μm; and anterior of kinetoplast to anterior end 1.5 (0.1–4.6) μm. The cytoplasm is alveolar and darkly stained chromatin granules are often seen in the posterior part of the body.

Geographical distribution of the leech vector

The marine leech, *Calliobdella vivida*, is the vector. It is not host specific and is found on fish in estuaries or near the coast from Newfoundland to the northern Gulf of Mexico. It is present in autumn, winter and spring (Sawyer *et al.*, 1975; Appy and Dadswell, 1981; Madill, 1988).

Life cycle and transmission of *C. bullocki*

C. bullocki rounds up at 10°C and starts to divide within 24 h in the crop of the leech. Slender forms appear about 4 days later, concentrate in the anterior part of the crop and are in the proboscis sheath a day later. Water temperatures affect the rate of development in the leech – at 20°C flagellates are in the proboscis sheath in 24 h, while their appearance is delayed to 10 days at 5°C. Infected leeches still have flagellates in their proboscis sheaths even after three consecutive feedings on uninfected fish (Burreson, 1982a).

Fish–*C. bullocki* relationships

Clinical signs and pathology

The disease develops slowly in summer flounders and clinical signs include anaemia, splenomegaly, exophthalmia, sluggishness and abdominal distension with ascites (Fig. 3.32) (Burreson, 1982b; Burreson and Zwerner, 1984). Clinical disease is evident in experimentally infected juvenile summer flounders at 5 wpi and fish are normally dead at 11 wpi.

Ascites and haemorrhaging in the ventral musculature are evident in about 5 wpi (Burreson, 1982b; Burreson and Zwerner, 1984). In acute infections, the parasite occurs extravascularly and is in haemopoietic organs and interstitial adipose tissues. Necrotic foci are in the liver, with diffuse necrosis throughout the spleen. Ulcerative and haemorrhagic lesions in the abdominal cavity, oedema, haemorrhage and necrosis of the intestine and oedema of the stomach may also be present. The parasite is also in the submucosa of the gut and liver. The glomeruli are congested and there are focal glomerular lesions in the kidneys (Newman, 1978).

Immunity

Complement-fixing antibody against the parasite was detected in experimentally and naturally infected summer flounders kept at 24°C and 10–22°C, respectively (Sypek and Burreson, 1983). Cell-mediated immunity seemed to be more important than antibodies at temperatures below 12°C. It was suggested that antibodies were responsible for the recovery in fish and the decrease in prevalence of the disease in the spring. Sypek and Howe (1985), after studying naturally infected fish, suggested that temperature would not be the only regulating factor.

A naturally infected fish that had recovered from the infection for at least 1 year was immune to challenge. Its

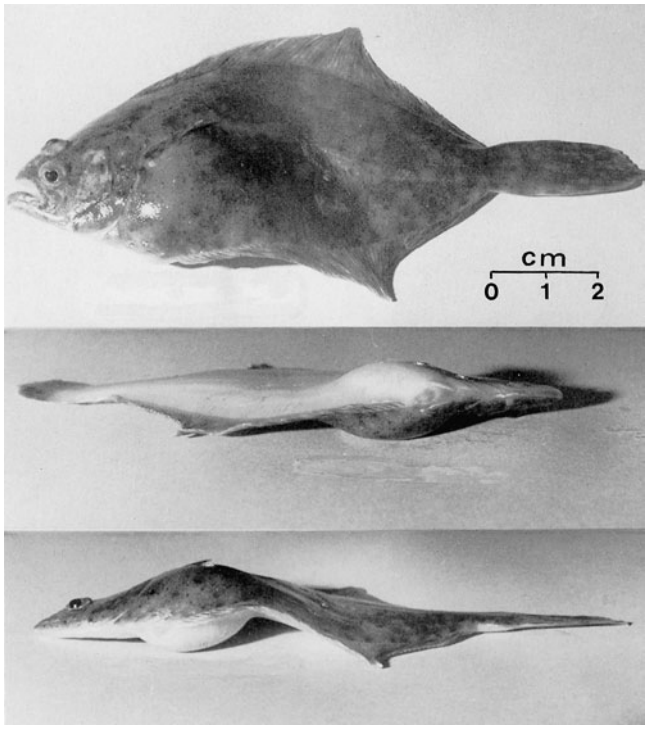


Fig. 3.32. Severe ascites in juvenile flounder experimentally infected with *Cryptobia bullocki*: three views of the same infected fish (from Burreson and Zwerner, 1984; courtesy of Dr E.M. Burreson).

antibody titre rose rapidly after challenge (Burreson and Frizzell, 1986). This indicates immunological memory and the study should be repeated with experimentally infected fish (Woo, 1987a).

Epizootiology of flatfish cryptobiosis

According to Burreson (Chapter 15), the prevalence of *C. bullocki* is low in migrating fishes in Chesapeake Bay because they are exposed to leeches for only short periods. They migrate out of the bay as leeches hatch and re-enter the bay as adult leeches are dying. However, resident fishes (hogchoker, oyster toadfish and juvenile summer flounders) have high prevalence because they are exposed to leeches throughout winter, and mortality of juvenile summer flounders is highest when the water temperature is low (0.5–1.5°C). Infected fish with ascites brought into the laboratory died within 2–6 weeks at 5°C. However, if they were maintained at 10°C and 13°C, the ascites disappeared and the fish survived the disease (Burreson and Zwerner, 1984).

Burreson (1982b) found the parasite in about 100% of juvenile summer flounders in the autumn in lower Chesapeake Bay. Dead flounders (with clinical signs) were seen and also the number of juvenile flounders caught decreased rapidly through the winter. Infected juvenile flounders from warmer waters (8°C) did not have abdominal distension with ascites. Hence low water temperature seems to be important in the development of the disease and fish mortality. The higher temperatures may enhance immunological response and thus help to control the infection. The precise role(s) temperature plays in morbidity and mortality in infected fish needs to be studied more closely.

Trypanosomiasis

Introduction

Trypanosomes usually have a free flagellum at the anterior end (Fig. 3.33). About 190 species of piscine trypanosomes have been described and host specificity of the vast majority of species is not known. Some species are monomorphic while others are pleomorphic. It is likely that many of the species are invalid; however, this can only be confirmed after careful experimental studies (Woo, 1994). Piscine trypanosomes are always transmitted by leeches and most species are not known to cause disease and/or mortality in the host; hence they will not be discussed in the present review.

TRYPANOSOMIASIS IN FRESHWATER FISH

Geographical distribution and host range

Trypanosoma danilewskyi was first described from the blood of the common carp (*C. carpio*) in Europe (Laveran and Mesnil, 1904). The parasite has since been found in carp, goldfish (*C. auratus*), tench (*Tinca tinca*) and eel (*Anguilla* sp.) in Europe (Thompson, 1908; Pavlovskii, 1964; Lom, 1973) and in *Saccobranchus fossilis* in India (Qadri, 1962). The parasite is not host specific and is infective when inoculated experimentally into *Barbus conhus*, *Danio malabaricus*, *C. commersoni*, *Notropis cornutus*, *Etheostoma caeruleum* and *Ictalurus nebulosus* (Woo and Black, 1984).

T. danilewskyi-like trypanosomes have been isolated from numerous fish species and from different localities. *In vitro* and *in vivo* studies (see below) clearly indicate that there are significant differences between the isolates, specifically in their nutritional requirements, cultural characteristics and changes in virulence (on subpassage in fish). These are some of the reasons why this author believes that it is premature to synonymize *T. danilewskyi* with *Trypanosoma carassii*, as Lom and Dykova had done.

It is suggested here that the parasite is most probably a species complex, as was shown with *Ichthyobodo* (see earlier section), and that future biological studies should help to establish the taxonomic status of the various isolates. These should include developmental (in the vector, in the fish host), antigenic (e.g.

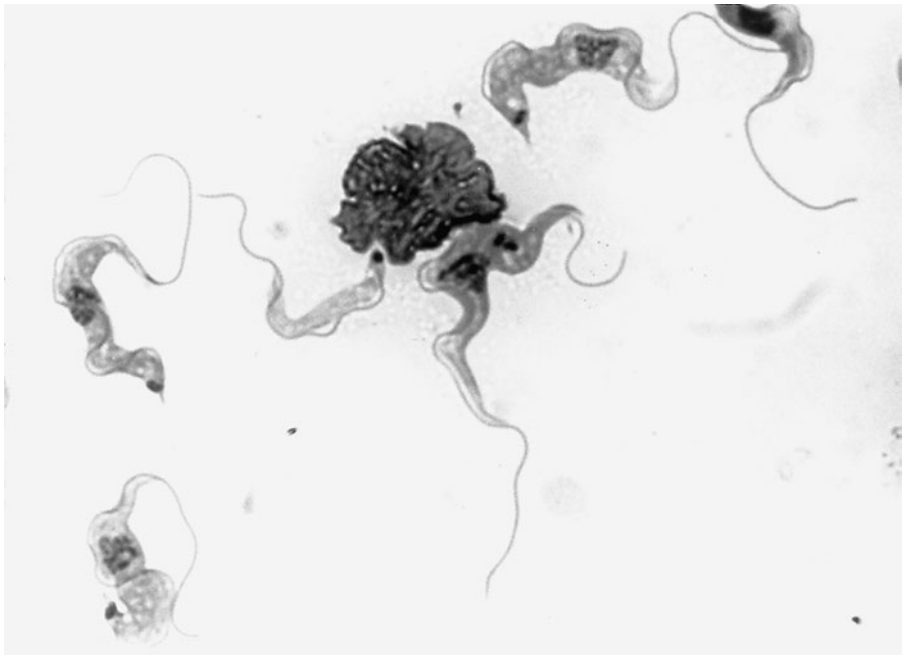


Fig. 3.33. *Trypanosoma danilewskyi* in the blood of an experimentally infected goldfish: note the monomorphic trypomastigotes, a non-nucleated abnormal form, and a pre-division stage with two kinetoplasts, two free flagella and a nucleus (× 1150) (from Woo, 1981a; courtesy of *Journal of Parasitology*).

using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblots), cross-protection (e.g. using recovered fish, complement-fixing antibodies), molecular (e.g. using ssg-rDNA) and susceptibility (e.g. using genetically known carp stocks) studies.

Morphology and multiplication

T. danilewskyi (Fig. 3.33) is a slender monomorphic trypanosome and the following measurements are based on air-dried blood smears. Specimens are from a goldfish at 4 weeks after experimental infection (Woo, 1981a). The strain was initially isolated from crucian carp (*C. auratus gibelio*) in the former Czechoslovakia by Dr J. Lom, Institute of Parasitology, Czech Republic. Morphometrics are based on 50 well-stained specimens: distance from posterior tip to kinetoplast (PK) = 1.7 μm ± 0.61 (0.6–2.5); distance from kinetoplast to middle of nucleus (KN) = 10.7 μm ± 2.69 (7.8–15.0); length of body (PA) = 21.2 μm ± 3.64 (15.6–24.9); length of free flagellum (AF) = 14.3 μm ± 2.30 (9.2–18.2); maximum body width excluding undulating membrane (BW) = 2.3 μm ± 0.40 (1.6–3.1).

Woo and Black (1984) showed that the host fish significantly affects the morphometrics of the trypanosome. The PK, AF and area of the nucleus are similar among samples from the same species of fish host but differ significantly between samples from five different host species.

The ultrastructure of piscine trypanosomes is similar to that of mammalian trypanosomes. The surface coat of *T. danilewskyi* is fibrillar and thin and is morphologically similar to the stercorarian trypanosomes of mammals. The parasite has a well-developed cytostome, which is located near the nucleus. Membrane-bound dense granules are in juxtaposition to the cytopharyngeal tube (Lom

et al., 1980; Paulin *et al.*, 1980) and the prominent nucleolus; and chromatin patches are attached to the inner nuclear membrane (Paterson and Woo, 1984).

One of the major epitopes on the surface coat of blood trypomastigotes is a mucin-like glycoprotein, which is the target of the humoral response (Lischke *et al.*, 2000). The glycoprotein has a polypeptide backbone, consisting of threonine, glycine, serine, alanine, valine and proline residues. Each polypeptide is associated with carbohydrate chains composed of about 200 monosaccharide units (galactose, *N*-acetylglucosamine, xylose, sialic acid, fucose, mannose and arabinose), which are most probably *O*-linked to hydroxyl amino acids.

The parasite multiplies rapidly by binary fission in the blood (Woo, 1981a). The first division stage is the production of a new flagellum and this is followed by division of the kinetoplast. The new flagellum flips posteriorly, and along it new cytoplasm is produced or accumulated. The nucleus divides and one daughter nucleus migrates posteriorly past the two kinetoplasts. Body division is by transverse constriction at a point between the kinetoplasts. This division process is different from the traditional longitudinal binary division of most other trypanosomes.

Development in the leech vector

After ingestion with the blood meal, *T. danilewskyi* develops in the leech *H. marginata* (Robertson, 1911; Qadri, 1962); the biology of the leech vector is in Chapter 15. Unequal division of trypomastigotes in the crop of the leech results in tadpole-like epimastigotes. Sphaeromastigotes are also present but their significance is not known. Epimastigotes divide repeatedly by binary fission, with eventual appearance of slender metatrypanosomes. These migrate and accumulate in the proboscis in about 10 days and may be present even several months after an infective blood meal. Transmission to fish is presumed to occur when infected leeches feed.

In vitro culture of the parasite

T. danilewskyi was cultured and subcultured at 25°C in diphasic blood agar. Infectivity of cultures, which contained both epimastigotes and trypomastigotes, to goldfish decreased with subcultures. The exception was the 'Ma' strain, which was isolated from goldfish. It multiplied as trypomastigotes in diphasic medium and retained its infectivity after a considerable number of *in vitro* subcultures (Lom and Suchankova, 1974).

The parasite was also cultured at 25°C with fish cells in Eagle's MEM with 10% bovine fetal or calf serum supplement (Smolikova *et al.*, 1977). It grew better with the addition of haemin (2 mg per 100 ml of medium) and did not grow in the medium without living cells. However, another strain of *T. danilewskyi* was cultured and subcultured as trypomastigotes in MEM (with Hanks's salts and L-glutamine, supplemented with 10% FBS) at 20°C without fish cells (Islam and Woo, 1992). The strain also multiplied rapidly as infective trypomastigotes in a serum-free medium (Wang and Belosevic, 1994a). It did not grow in four other media, including diphasic blood agar medium (SNB9) supplemented with vitamins (Islam and Woo, 1992), but this medium combination supported growth of another strain (Skarlato *et al.*, 1987).

The *in vitro* division process of the parasite (as trypomastigotes) is similar to that in fish (see above) and the parasite in MEM is always infective to goldfish (Islam and Woo, 1992). The parasite multiplied more readily in a medium supplemented with 10% fish serum (goldfish, carp or foil barb) than with mammalian serum (horse or fetal bovine) or no serum (Bienek and Belosevic, 1997). Secretory products of cultured macrophage or fibroblast from goldfish significantly enhanced the multiplication of *T. danilewskyi* in cultures (Bienek and Belosevic, 1999). Overath *et al.* (1998) was also successful in cultivating infective trypanosomes using a modified MEM supplement with 5% heat inactivated FBS and 5% carp serum.

Host–parasite relationships

Course of infections and clinical signs

The parasite multiplies rapidly in goldfish when they are kept at 20°C and more slowly at 30 or 10°C (Lom, 1973; Woo *et al.*, 1983; Islam and Woo, 1992). The parasitaemia increases after infection in fish maintained at 20°C and starts to decline at about 28 days after infection. These fish eventually recover from the infection and are immune to homologous challenge (Woo, 1981b; Islam and Woo, 1991a; Overath *et al.*, 1999).

The parasite causes anorexia in experimentally infected goldfish (Islam and Woo, 1991c) and this is most evident during high parasitaemia. Fish that survive the disease return to normal feeding.

Pathology and mortality

T. danilewskyi also causes anaemia in goldfish (Robertson, 1911; Dykova and Lom, 1979; Islam and Woo, 1991b). As in salmonid cryptobiosis (see earlier section), the severity of the anaemia in trypanosomiasis is directly related to the number of parasites in the blood, and it is also partly caused by a lytic factor and haemodilution (Islam and Woo, 1991b). The lytic factor(s) is secreted by living parasites and it lyses red cells in the absence of specific antibodies. It is likely that the mechanism of the anaemia in trypanosomiasis is similar to that in salmonid cryptobiosis and that the virulent factor is a protease.

Mortality of infected goldfish is related to the size of the inoculum, and a small inoculum produces low mortality (Woo, 1981b; Islam and Woo, 1991a). Infectivity and virulence of one parasite strain (isolated from carp) increased on repeated transfer in goldfish (Lom, 1979). However, the virulence of two other strains of *T. danilewskyi* (isolated from eels and tench) decreased on repeated transfer in goldfish. No explanations were provided for the difference and, since no experimental details were given, the study should be carefully repeated.

Immunity

Goldfish that have recovered from acute infections are protected on subsequent homologous challenge (Lom, 1973; Woo, 1981b; Islam and Woo, 1991a; Overath *et al.*, 1999) and plasma from these immune fish contains neutralizing antibodies (Woo, 1981b). Passive immunization with IgM purified from recovered fish confirms that the infection is controlled by antibodies (Overath *et al.*, 1999). The immunity is non-sterile as low numbers of parasites are in the blood of recovered fish. Intraperitoneal injection of corticosteroid into recovered goldfish significantly increases the number of trypanosomes in the peripheral blood of immune fish (Islam and Woo, 1991a; Overath *et al.*, 1999). Oestradiol can modulate the susceptibility of infected goldfish. Oestradiol-implanted fish had significantly higher parasitaemias and mortality than sham-operated fish. Also, mitogen-induced proliferation of circulating lymphocytes from oestradiaol-implanted fish was impaired compared with those from sham-operated fish (Wang and Belosevic, 1994b). Excretory/secretory products from *T. danilewskyi*, when injected with Freund's complete adjuvant, confer protection (Bienek *et al.*, 2002). This is encouraging and protective antigen(s) need further characterization.

As indicated earlier, cultured *T. danilewskyi* is infective to goldfish (Wang and Belosevic, 1994a) and it survives well under *in vitro* conditions in normal goldfish serum. However, if its surface proteins are removed, using trypsin, the parasite is lysed via the alternative pathway of complement activation. The trypsinized parasite regains its resistance to lysis after 24 h when cultured in a trypsin-free medium (Plouffe and Belosevic, 2004). The significance of this is not well understood.

Diagnosis of infection

During the acute phase of the disease, parasites are readily detected by examination of a drop of freshly collected blood under the light microscope. Taxonomic identification

of the parasite is best done with alcohol–formalin-fixed blood smears stained with Giemsa's stain (Woo, 1969). The haematocrit centrifuge technique (Woo, 1969) can be used to detect infections (e.g. early after infection or in the chronic phase of the infection), which are not usually detectable using the wet-mount technique. There are no serological techniques for piscine trypanosomiasis; however, serodiagnostic techniques developed for salmonid cryptobiosis (see earlier section) can easily be adapted for trypanosome infections.

TRYPANOSOMIASIS IN MARINE FISH

Geographical distribution and host range

Trypanosoma murmanensis (Fig. 3.34) was initially described from cod (*Gadus callarias*) from the Barents Sea, Murmansk, in the former USSR (Nikitin, 1927). It is commonly found in Atlantic cod (*G. morhua*) in coastal Newfoundland, Canada (Khan, 1972, 1986; Khan *et al.*, 1980a). Natural infections have also been detected in Pleuronectiformes and Perciformes (Khan, 1977a; Khan *et al.*, 1980b).

The trypanosome is not host specific (Khan, 1977a; Khan *et al.*, 1980b), and experimentally infected fishes include the American eel (*Anguilla rostrata*), Atlantic cod (*G. morhua*), tomcod (*Microgadus tomcod*), cunner (*Tautogolabrus adspersus*), striped wolffish (*Anarhichas lupus*), Vahl's eelpout (*Lycodes vahlh*), Arctic eelpout (*Lycodes reticulatus*), oceanpout (*Macrozoarces americanus*), longhorn sculpin (*Myoxocephalus octodecemspinosus*), shorthorn sculpin (*Myoxocephalus scorpius*), American plaice (*Hippoglossoides platessoides*), yellowtail flounder (*Limanda ferruginea*) and winter flounder (*P. americanus*).

Morphology and life cycle of parasite

Morphology

T. murmanensis (Fig. 3.34) is a pleomorphic trypanosome with small, intermediate and large forms (Khan, 1972, 1977a). The following description is based on 50 stained trypanosomes from naturally infected cod (Khan, 1972). The nucleus and kinetoplast are oval and six to eight longitudinal striations are present in large specimens; PK = 9.1 μm (6.0–14.0); KN = 32.9 μm (25.0–38.0); NA (distance from middle of nucleus to anterior end) = 34.6 μm (26.0–40.0); PA = 76.7 μm (57.0–92.0); AF = 6.9 μm (6.0–8.0). Measurements of the trypanosome from other naturally infected fishes are somewhat different (Khan, 1977a).

Life cycle

The parasite multiplies as amastigotes and sphaeromastigotes in the digestive tract of the marine leech *Johanssonia arctica*. The epimastigotes (presumed to have developed from sphaeromastigotes) migrate to the proboscis of the leech, where they transform into infective metatrypanosomes and these are inoculated into fish when the leech feeds (Khan, 1976). The development is completed in 62 days at 0–10°C and in 42 days at 4–6°C.

Small slender trypomastigotes appear in the blood of cod 3 days after infection.

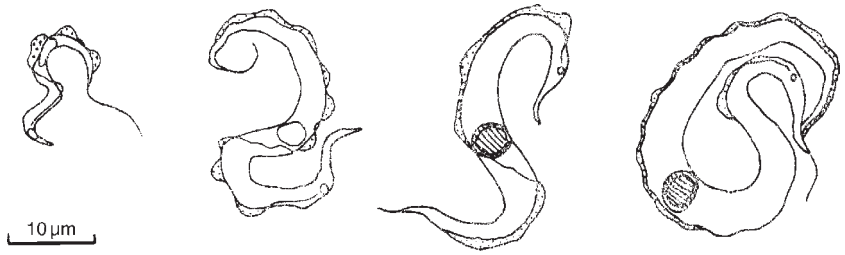


Fig. 3.34. Drawings of small, intermediate and large forms of *Trypanosoma murmanensis* from the blood of experimentally infected cod (from Khan, 1976; courtesy of *Canadian Journal of Zoology*).

They grow into large forms in 29–55 days. The pleomorphic nature of the parasite is more apparent as the parasitaemia starts to decline at about 29 days after infection (Khan, 1976).

Geographical distribution of leech vector

The vector of *T. murmanensis* is the marine leech *J. arctica*. It is commonly found in the Arctic seas and the north-western Atlantic, where the water temperature is usually at –1 to 2°C and at depths of about 165 m (Epshtein, 1962; Meyer and Khan, 1979; Khan, 1982, 1991; Sawyer, 1986).

Fish–parasite relationships

Course of infection

The parasite is not known to multiply in the fish host. Khan (1976) suggested that the slight increase in the number of trypanosomes at 3 and 10 days after infection was due to the late entry of some parasites into the general circulation.

Clinical signs and pathology

Infected fish are anaemic and lethargic, with emaciation and splenomegaly in some fish (Khan, 1985). The severity of the anaemia and decrease in plasma proteins are associated with parasitaemias (Khan, 1977b; Khan *et al.*, 1980c). The anaemia is probably associated with an inactive haemopoietic system. The condition factor and somatic indices of liver, spleen and heart are altered in experimentally infected *G. morhua*, *P. americanus*, *M. scorpius* and *M. octodecemspinosus*. Also, spleens of infected juvenile cod are congested with blood and melanomacrophage centres; and they are also more common in infected than in uninfected juvenile winter flounders (Khan, 1985).

Mortality

The parasite caused high mortality in experimentally infected cod and winter flounders kept at 0 to 1°C. Mortality ranged from about 7% in 3+-year to 65% in 0+-year cod. The mortality figures were similar in immature winter flounders (17–56%). Larger fish (presumably older) were less susceptible and adult flounders did not die from the infection (Khan, 1985).

Evidence of trypanosomiasis in naturally infected fishes

There are several trypanosomes that are presumed to cause lesions in organs and/or changes in the blood of naturally infected fish. Fish from the field are often infected with a variety of pathogens and may have also been subjected to stress factors (e.g. toxic pollutants, nutritional deficiencies). Hence, it is difficult to ascribe abnormalities to any one particular cause. At best, these field reports can only be considered as preliminary and some are briefly included here.

Neumann (1909) found inflammation of the brain, fatty degeneration in organs, anaemia and eosinophilia in skates (*Raja punctata*) that had high numbers of *Trypanosoma variabile*. According to Smirnova (1970), the number of red cells, haemogloblin contents and serum protein levels of *Trypanosoma lotae*-infected burbot (*Lota lota*) were lowered, while the number of phagocytic white cells was elevated.

Fish infected with *Trypanosoma vittati* and *Trypanosoma maguri* had low numbers of red cells and haemogloblin (Tandon and Joshi, 1973). However, the numbers of white cells and immature or abnormal red cells were higher in the infected fish. *Clarias batrachus* and *Channa punctatus* infected with *Trypanosoma batrachi* and *Trypanosoma aligaricus* had lower red cell counts and lower haemogloblin (Gupta and Gupta, 1985). The numbers of agranulocytes and granulocytes were higher in infected fishes, except for neutrophils, which were significantly lower. Blood glucose levels were lower in *C. punctatus*, *C. batrachus*, *Heteropneustes fossilis* and *Mystus seenghala* infected with trypanosomes (Tandon

and Joshi, 1974). Also, serum alkaline phosphatase and cholesterol levels were lowered in infected *Cirrhina mrigala*, *C. batrachus*, *Mastacembelus armatus*, *M. seenghala* and *Wallago attu* (Tandon and Chandra, 1977a, b).

Since naturally infected fish can have mixed infections, it is recommended that the parasite first be cloned. The cloning is done using either susceptible laboratory-reared fish (e.g. Woo, 1979) or an appropriate culture medium (e.g. Jones and Woo, 1991). The cloned parasite is then identified (Woo, 1994) and cultured. To determine pathogenicity, all three strains (field, cultured and cloned strains) should be used to experimentally infect hatchery-reared fish. This approach would satisfy Koch's postulates for determining the aetiological agent for a disease.

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4 *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum Ciliophora)

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Phylum Ciliophora – General Considerations

The phylum Ciliophora currently contains 7200 known species, but there are probably several thousand more that are as yet undiscovered (Corliss, 1979). Ciliates are found in virtually all bodies of water, ranging from small ponds or streams to open oceans. Most are free-living, but many occur as commensals or symbionts, and a few are parasites of invertebrates and vertebrates. Ciliates range in size from approximately 10 μm to 4500 μm and their body shape varies from nearly spherical to highly ovoid (Corliss, 1979). They have a rigid body form, resulting from a distinct fibrous cortex just below the surface plasma membrane. The ciliate cell has a large number of organelles and inclusions. Some distinguishing characteristics of ciliates are listed below (Corliss, 1979; Nanney, 1980):

1. Nuclear dimorphism. With few exceptions ciliates possess two nuclei, a transcriptionally active macronucleus and at least one germ-line micronucleus.
2. Cilia or compound ciliary organelles, such as cirri. These are often present in large numbers and arranged in distinct patterns. Some species have them only in one stage of the life cycle.
3. Infraciliature located below the surface of the cell. This infrastructure consists of basal bodies and a host of more or less closely associated microtubules and fibrils.
4. A cytostome, or cell mouth, is usually present. Some ciliates are commensals on the gills or skin of fishes and others (such as *Tetrahymena* spp.) are opportunistic parasites. Ciliates are often found on dead or moribund fishes. Parasitic ciliates are not common. This chapter describes the two most virulent parasitic ciliates of fishes, *Ichthyophthirius multifiliis* and *Cryptocaryon irritans*.

Introduction

I. multifiliis, Fouquet, 1876, and *C. irritans*, Brown, 1951, are pathogenic ciliates that infect fresh- and saltwater fishes, respectively. This chapter reviews what is currently known about the pathobiology of both these parasites. In each section *I. multifiliis* is discussed first, followed by *C. irritans*.

I. multifiliis, also referred to as Ich, is one of the most pathogenic protozoan parasites of fishes. There is no official record on the annual economic loss attributed to ichthyophthiriasis, even though it is considered to be a major problem in aquaculture (Hoffman, 1999). Epizootics were

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reported in China as early as the 10th century (Hines and Spira, 1974a). The first major outbreak in North America was described at the end of the 19th century (Stiles, 1894). Ich was relatively unknown in Russia before 1940, but since then it has become a serious disease of carp (Bauer, 1953). The significance of the problem will increase with the growth of aquaculture.

Concomitant with the rapid development of mariculture in the last decade, *C. irritans* has also become an increasingly important problem. Sikama first reported the parasite in Japan in 1938.

Geographical Distribution and Host Range of the Parasites

Ichthyophthirius multifiliis

Geographical distribution

Ich is a cosmopolitan parasite of fishes (Nigrelli *et al.*, 1976; Valtonen and Keränen, 1981). Infections have been reported from virtually all regions where fishes are cultured, including the tropics and sub-Arctic, as well as in feral fish populations from most continents (Nigrelli *et al.*, 1976; Valtonen and Keränen, 1981). Fouquet first described the organism in France in 1876 (Stiles, 1894). According to Stiles, Hildendorf and Paulick published observations on a fish disease caused by a ciliate (presumably *I. multifiliis*) in Hamburg, Germany, in 1869. These were the first detailed descriptions of the parasite and its life cycle. The disease was known in fishes in Europe in the Middle Ages (Hoffman, 1999), however. There is evidence that it originated in Asia as a parasite of carp (Dashu and Lien-Siang, referenced by Hines and Spira, 1974a).

The worldwide spread of the parasite was facilitated by transportation of fish by humans (Nigrelli *et al.*, 1976). Paperna (1972) reported on an outbreak in Uganda and noted that *I. multifiliis* was not found in that country in a previous study. He further indicated that a number of fish species had been imported into Uganda from the USA and Hong Kong. Wurtsbaugh and Tapia (1988) described an epidemic in fishes in Lake Titicaca on the Peru–Bolivian border. They noted that a number of fishes, salmonids and atherineds, had been introduced into Lake Titicaca and its watersheds at various times in the past.

Bragg (1991) found a higher incidence of Ich in salmonid fishes from South African rivers in areas where intensive aquaculture occurred. He suggested that aquaculture contributed to increased infections in both river and hatchery fishes. In contrast, epizootics of Ich occurred in adult sockeye salmon, *Oncorhynchus nerka*, during the 1994 and 1995 spawning seasons in the Skeena River watershed in northern British Columbia, Canada. In this case, it is suggested that resident fish were the most likely source of the parasite in the watershed because several species were found with light infections of Ich. Transmission of the parasite to anadromous sockeye salmon was probably enhanced by the high density of fish held below the spawning grounds prior to moving into the spawning area (Traxler *et al.*, 1998).

Host range

Ich appears to parasitize all freshwater fishes. There are no records of species with complete natural resistance (Ventura and Paperna, 1985). There are suggestions of variation in the degree of susceptibility between fishes, however. These variations may depend on such factors as genetic background, physiological status of the fishes, parasite strains and environmental conditions (Clayton and Price, 1988, 1994).

Epizootics in native fish populations often occur in only one species of fish. Elser (1955) reported on an outbreak in a reservoir in Maryland in which the disease affected predominantly yellow perch (*Perca flavescens*). Allison and Kelly (1963) described an epizootic in rivers of north-western Alabama. The majority of infected fishes were gizzard shad (*Dorosoma cepedianum*) and threadfin shad (*Dorosoma petenense*). In an outbreak reported in Kentucky the only fishes infected were blackstripe topminnows

(*Fundulus notatus*) (Kozel, 1976). An epizootic that occurred in Lake Titicaca on the Peru–Bolivia border primarily affected killifish (*Orestias* spp.) and the majority (93%) of dead were *O. agassii* (Wurtsbaugh and Tapia, 1988). The occurrence of epizootics in only one or a few species in a mixed fish population may not indicate genetic variation in resistance, but rather different physiological states that predispose certain individuals or groups to disease. Wurtsbaugh and Tapia (1988) observed that most of the fishes that died in the epizootic were gravid or spent adult *O. agassii*. Pickering and Christie (1980), in a study on parasite infection of brown trout (*Salmo trutta* L.), found Ich more frequently in precocious mature pre-spawning males than in immature fishes. They concluded that sexual maturation was associated with an increase in prevalence and severity of infestation with ectoparasites. Reproductive stress may also be a factor in the apparent variation in susceptibility to infection.

Nigrelli *et al*. (1976) proposed that there are physiological races of *I. multifiliis* related to the temperature tolerances of the host fishes. Thus, races of *I. multifiliis* exist that infect coldwater (7.2–10.6°C) fishes, such as salmon, and others that infect warmwater (12.8–16.1°C) tropical fishes. To date, there is no experimental evidence to substantiate or refute this idea. Fishes with wide ranges of temperature tolerance, such as carp and catfish, may be susceptible to both cold- and warmwater parasites. Ich epizootics in Arctic fishes suggest that these outbreaks occur when the water temperature reaches a moderate range. Valtonen and Keränen (1981) reported on an epizootic in a salmon hatchery in central Finland in two consecutive years when the water temperature rose to 14°C or higher.

Paperna (1980) stated that *I. multifiliis* in Europe and Asia is highly pathogenic for carp, with a preference for this species. There are no experimental data to support this speculation. Although ichthyophthiriasis often occurs in carp in Europe and Asia, this could merely be due to the fact that carp are the primary fish raised in these areas and intensive culture conditions predispose them to infection.

Seasonal fluctuations in infection

Outbreaks of *I. multifiliis* occur when conditions are favourable for rapid multiplication of the parasite. These include a suitable environment and susceptible fishes. According to McCallum (1982), fish density is not a constraint on the establishment of infection. Nevertheless, there does appear to be a requirement for a minimum number of fishes before an epizootic develops. Severe infections occur most commonly in dense populations of fishes.

A critical condition for an outbreak is water temperature. The duration of the developmental cycle of *I. multifiliis* is significantly influenced by temperature (MacLennon, 1937, 1942; Nigrelli *et al.*, 1976; Noe and Dickerson, 1995). Generally, as the temperature rises (up to 25–28°C), parasite activity increases and the life cycle is completed in a shorter time than at lower temperatures. In addition, the number of tomites in each cyst varies with the temperature of the water, which reflects the number of cell divisions (Nigrelli *et al.*, 1976).

Stress can bring about an outbreak in a fish population. Stress is a complex physiological reaction that causes the release of steroids from the adrenal glands, which in turn decreases the immune function of the host. A wide variety of factors induce stress in fishes: these include crowding, low dissolved oxygen, chemical pollutants in the water, high temperature and spawning activities.

Ichthyophthiriasis usually occurs when fishes are stressed and the water temperature is relatively warm. Epizootics arise in aquarium-reared fishes when optimum conditions for parasite development and rapid multiplication are present. The parasite reproduces when fishes are under stress from low oxygen and/or crowded conditions. Outbreaks occur in the spring as the water warms and when fishes are spawning (Elser, 1955; Allison and Kelly, 1963). This is very apparent in reports of disease in sub-Arctic areas. Ich epizootics in Finland

occurred when water temperatures were above 14°C, and stopped as soon as the temperature fell (Valtonen and Keränen, 1981).

The cyclic nature of outbreaks is also influenced by the development of immunity in fish populations. It is well documented that fishes recovered from *I. multifiliis* infections develop protective immunity (Burkart *et al.*, 1990; Matthews, 1994; Dickerson and Clark, 1996). Epizootics occur when there is a sufficiently large population of susceptible fishes. As the infection progresses, highly susceptible fishes die while those that are more resistant develop immunity. With time the majority of the surviving fishes become immune. As these conditions develop, the epizootic wanes and losses stop. The surviving population of fishes reproduces and with time the level of acquired disease resistance decreases, allowing another epizootic to occur under suitable conditions.

How does the parasite survive between outbreaks? Ich is an obligate parasite and as such requires susceptible hosts to propagate. There is no evidence of a resistant stage that allows survival during environmental changes or in the absence of fishes. Thus, it is most likely that the parasite is maintained through a low-level infection in a population. The dynamics of the host–parasite relationship during the inter-epizootic periods is poorly understood.

Temperature is an important factor in the persistence of infection in fish populations. When water temperature is low, the parasite grows and develops more slowly. The growth period of trophonts on fishes ranges from 1 week at 20°C to 20 days at 7°C (Nigrelli *et al.*, 1976; Noe and Dickerson, 1995; Aihua and Buchmann, 2001). Growth occurs at temperatures as low as 2–5°C (Bauer, 1953; Aihua and Buchmann, 2001). McCallum (1982) found no density-dependent constraints on the establishment of *I. multifiliis* infections in naïve hosts. There was, however, significant variation in the numbers of parasites found on each fish. These differences were attributed to such factors as the amount of mucus produced by individual fishes, nutritional status and stress. In a subsequent study, McCallum (1986) examined the role of host death on the reproductive potential of *I. multifiliis* in a population of susceptible fishes. He concluded that the low prevalence between epizootics was consistent with the idea that *I. multifiliis* and fish populations are regulated by parasite-induced host mortality. He developed a mathematical model that quantitatively reproduced the observed epizootic behaviour of an *I. multifiliis* infection.

Cryptocaryon irritans

Host range, geographical distribution, prevalence and seasonal fluctuations

C. irritans is a parasite of marine fishes. Sikama first described cryptocaryoniasis in Japan in 1938. The disease is now thought to occur worldwide (Sikama, 1961). The parasite is a recurring problem in marine fishes in aquaria and is becoming a concern in commercial mariculture (Colorni, 1985). It was suggested that the parasite's incidence in fishes in the USA occurred initially through the importation of fishes from Hawaii and the Indo-Pacific area (Nigrelli and Ruggieri, 1966). The parasite also occurs in marine fish populations on the east coast of the USA.

The prevalence of the parasite in native fishes in North America is not well documented. The parasite was found on a few fishes in Mission Bay, California (Wilke and Gordin, 1969). These fishes were examined at a time when there was a concomitant outbreak of cryptocaryoniasis in aquaria at the Scripps Institute of Oceanography. It was unclear whether the native fishes were naturally infected or whether they acquired the infection as a result of contamination with tomonts and theronts released from the Institute.

C. irritans is not fastidious in its host selection (Colorni, 1985). The host range does not appear to be as great as that of *I. multifiliis*, however. Fishes vary in susceptibility to infection. Elasmobranchs are generally resistant (Wilke and Gordin, 1969). Also, there are apparent differences in degrees of susceptibility in marine teleosts.

Colorni (1985) observed that, when outbreaks of *C. irritans* infections occurred in aquaria with mixed fish species, some fishes were more readily infected than others. This confirmed an earlier observation of Wilkie and Gordin in 1969. Nigrelli and Ruggieri (1966) listed 27 species of marine fishes native to the Atlantic or Indo-Pacific areas that had died of *C. irritans* infections. Wilkie and Gordin (1969) reported 93 susceptible and 19 resistant marine fishes. It was suggested that these differences might be related to the ecological niches of the various species (Wilke and Gordin, 1969).

Cryptocaryoniasis appears to be primarily a disease of fishes in tropical environments. It does not occur in water below 19°C (Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969), and it is seen most commonly in aquaria kept at 20–25°C (Nigrelli and Ruggieri, 1966). Temperature affects the development of the parasite (Cheung *et al.*, 1979). Many potentially susceptible fishes reside in cold water; these can be infected if the temperature rises above 19°C (Wilke and Gordin, 1969).

Systematics

Ichthyophthirius multifiliis

The first description of the etiologic agent of white spot disease was by Hilgendorf and Paulicki in 1869 (Stiles, 1894). They described the life cycle of the ciliate and placed it in the genus *Pantotricha*. In 1876 Fouquet published a detailed description of the organism and its life cycle. He placed the organism in a new genus (*Heterotricha*) and proposed the name *Ichthyophthirius multifiliis*. At present, *multifiliis* is the only recognized species of *Ichthyophthirius* (Lee *et al.*, 1985). Based on differences in gross nuclear morphology between isolates, however, it was suggested by Nigrelli *et al.* (1976) that subspecies could exist. Serotypic strains of *I. multifiliis* are described based on the presence of specific surface proteins, referred to as immobilization antigens (Dickerson *et al.*, 1993).

I. multifiliis is placed in class Oligohymenophora, subclass Hymenostomata, order Hymenostomatida, suborder Ophryoglenina and family Ichthyophthiridae.

Cryptocaryon irritans

Sikama described a parasitic holotrichous ciliate as the cause of a disease outbreak in marine fishes and suggested that the disease was similar to ichthyophthiriasis. He recognized that this organism was different from *I. multifiliis* and proposed the name *Ichthyophthirius marinus* (Sikama, 1961). Brown had previously described this parasitic ciliate, and proposed the name *Cryptocaryon irritans*, however (Brown, 1950). Brown published a more detailed description of the cycle of macronuclear development, which clearly distinguishes *C. irritans* from *I. multifiliis* (Brown, 1963).

Although Sikama first described cryptocaryoniasis, Kerbert may have observed the disease much earlier (Stiles, 1894).

C. irritans is placed in class Oligohymenophora, subclass Hymenostomata, order Hymenostomatida, suborder Ophryoglenina and family Ichthyophthiridae. Cheung found in a scanning electron microscopic study that the organism does not have oral accessory membranes (membranelles) similar to those of *I. multifiliis* (see below) and hence questioned the inclusion of *C. irritans* in the order Hymenostomatida (Cheung *et al.*, 1981). Colorni and Diamant noted that the similarities between *I. multifiliis* and *C. irritans* are more likely to be the result of an adaptive convergence of life histories, rather than phylogenetic proximity (Colorni and Diamant, 1993). These authors suggested a reassessment of the taxonomic status of *C. irritans* as well. Based on sequence analysis of the 18S ribosomal RNA (rRNA) gene, Wright and Colorni have proposed that the ciliate be placed in the order Prorodontida within the class Prostomatea. They proposed a new family name: Cryptocaryonidae (Wright and Colorni, 2002). Distinct biological and pathological differences have also been found between isolates of *C. irritans* taken

from fish in the Red and Mediterranean Seas (Diamant *et al.*, 1991; Colorni and Diamant, 1993).

Parasite Morphology and Life Cycle

Ichthyophthirius multifiliis

Ich is a holotrichous, histophagous ciliate. It possesses a large, reniform macronucleus and at least one small, round micronucleus (Peshkov and Tikhomirova, 1968; Hauser, 1973; Matthews, 1996). There are up to four micronuclei per theront, varying from one to four depending on temperature (Matthews, 1996). The micronuclei of ciliates are transcriptionally inactive and play a role in genetic exchange (Nanney, 1980). While micronuclear exchange occurs in some ciliates by autogamy or conjugation, others reproduce indefinitely without it (Nanney, 1980). It is possible that *I. multifiliis* undergoes autogamy or conjugation. Our own experience indicates that laboratory isolates maintained by serial passage on fishes gradually lose their infectivity and become senescent, a phenomenon that indicates the need for autogamy or sexual conjugation (Noe and Dickerson, 1995). There is also experimental evidence to suggest that *I. multifiliis* cells conjugate in the epithelium during infection (Matthews, 1996).

Ich cyclically transforms between an obligate fish-associated trophont and a free-living reproductive stage, the tomont. The theront is the infective stage of the parasite. The nomenclature used in this chapter is from standard references of ciliate taxonomy and protozoan parasitology (Corliss, 1979; Lee *et al.*, 1985; Lom and Dykova, 1992). Refer to Fig. 4.1 for a summary of the various stages involved in the life cycle. The daughter cells (tomites) of the tomont differentiate within a thin-walled cyst (see below) into free-swimming infective theronts. The theront is pyriform to fusiform in shape with a tapered posterior end (Fig. 4.2). It is about 30 μm × 50 μm in size, but this varies greatly, depending on the initial size of the tomont (MacLennon, 1942; Canella and Rocchi-Canella, 1976; Kozel, 1986; Geisslinger, 1987). The entire surface of the theront is covered with cilia, each of which measures approximately 5.0 μm in length by 0.2 μm in diameter (Kozel, 1986). A longer cilium, two to three times the length of the others, protrudes from the posterior end of the cell (Canella and Rocchi-Canella, 1976; McCartney *et al.*, 1985; Kozel, 1986; Geisslinger, 1987).

The theront continuously spins on the longitudinal axis as it swims. The caudal cilium probably serves to maintain directional stability (Matthews, 1996). The ciliate

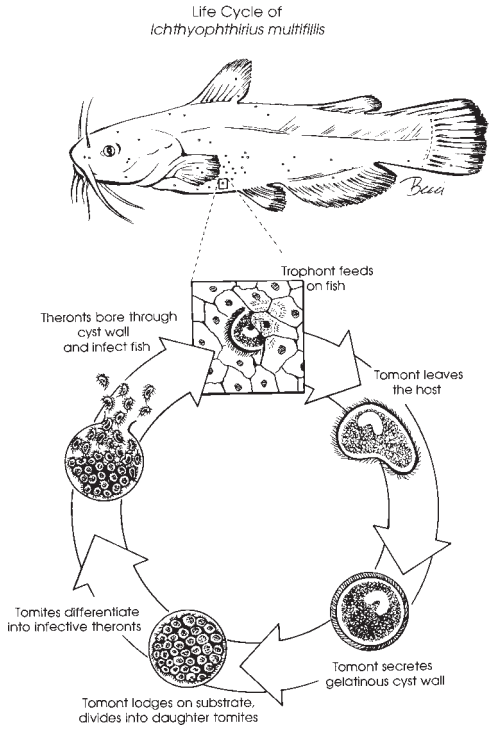


Fig. 4.1. Life cycle of *Ichthyophthirius multifiliis*. The infective theronts attach to susceptible fishes and penetrate through the surface mucus and epithelium. Trophonts feed at the basal layers of the skin and gill epithelia. Mature tomonts leave the fishes, secrete gelatinous cysts and divide to form daughter tomites. The tomites differentiate into infective theronts. The rate of development both on and off the host is influenced by temperature. The life cycle of *Cryptocaryon irritans* is similar except that developmental times are longer. See text for details.

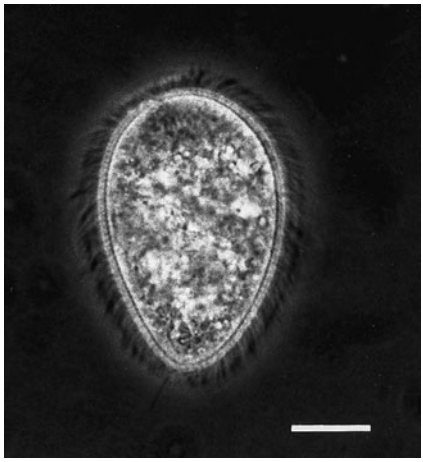


Fig. 4.2. *Ichthyophthirius multifiliis* theront. Phase-contrast micrograph of the infective stage of the parasite. Bar = 16 µm.

frequently alternates between slow cruising and intermittent bursts of speed preceded by tumbling, which often results in a random change of direction (MacLennon, 1935b; Wagner, 1960; Kozel, 1986; Matthews, 1996). The parasite is attracted towards light and is positively chemotactic to unknown substance(s) released from fishes (Lom and Cerkasova, 1974; Wahli and Meier, 1991). Theronts swim for approximately 4 days, but are probably not infective for the entire period (Parker, 1965; Lom and Cerkasova, 1974).

The theront attaches to the surface epithelium of the skin and gills and penetrates within 5 min to the basal layer (Ewing *et al.*, 1985; Kozel, 1986; Cross and Matthews, 1992; H.W. Dickerson, personal observation). Secretory mucocysts, concentrated at the apical end of the theront, are discharged as it approaches the fish (Ewing *et al.*, 1985; Ewing and Kocan, 1992). Ewing *et al.* (1985) and Ewing and Kocan (1992) postulated that the released substance plays a role in attachment. Geisslinger (1987) found that theronts appeared to stick to epithelia by means of their cilia, and saw no evidence at the scanning electron microscope (SEM) level that material released by mucocysts was involved. He suggested that theronts attach by means of specialized thigmatic cilia (Geisslinger, 1987). More work is obviously needed to clarify these observations.

The most important structure associated with penetration is the apical perforatorium (MacLennon, 1935a; Roque *et al.*, 1967; Canella and Rocchi-Canella, 1976; Ewing *et al.*, 1985). Cannella and Rocchi-Cannella (1976) described this organelle in detail at the light microscopic level. It consists of a group of apical ectoplasmic ridges, each arising between ciliary rows that converge to form a 1.5 to 2 µm protrusion at the tip of the theront. The parasite uses this structure to physically force its way through the epithelial layers (MacLennon, 1935a; Canella and Rocchi-Canella, 1976). The attachment and invasion into the epithelium by *I. multifiliis* is a very physical and active process, requiring considerable energy expenditure by the parasite.

There is considerable dispute in the literature concerning the rate of development of the cytostome (Canella and Rocchi-Canella, 1976). The small primitive buccal apparatus of the theront changes in the trophont to a vestibular cavity that is large enough to ingest relatively large cell debris (MacLennon, 1935a; Canella and Rocchi-Canella, 1976). According to MacLennon, the theront virtually eats its way into the epithelium, using a cell mouth that differentiates immediately upon entering the host. In contrast, Cannella and Rochi-Cannella contend that the cytostome only develops at 18–32 h (at a temperature of 26–27°C) after invading the host. Ewing showed an electron micrograph depicting a young trophont (40 min after infection) in gill epithelium, with necrotic cell debris inside a vestibular-like structure (Ewing *et al.*, 1985). This would tend to support MacLennon's findings that the theront develops a functional buccal apparatus soon after it enters the host. Because a cytostome is not visible in Ewing's micrograph, however, it is hard to discern whether the putative buccal apparatus is real or merely a fold in the cell membrane.

The organelle of Lieberkühn, also referred to as the 'watch glass organelle', lies in the oral region of the theront. It is a dense, ovoid, non-membrane-bound

structure overlaid by an alveolar surface thrown into five to seven ridges that face the oral cavity (Lynn *et al.*, 1991). The function of this organelle is a mystery, although it could possibly be involved in phototaxis. It exists only in the theront stage and disappears soon after the parasite colonizes a fish.

The theront penetrates to the basal layer of the epidermis (Ventura and Paperna, 1985) and undergoes differentiation and growth. Once the vestibular apparatus becomes functional, the trophont increases dramatically in size. The digestive cycle of the parasite has been divided into three main stages, based on the ultrastructure of the food vacuoles (Lobo-da-Cunha and Azevedo, 1993). The growth rate is positively correlated to increases in the ambient temperature (MacLennon, 1942; Ewing *et al.*, 1986). The parasite rotates and moves in an amoeboid fashion, using the perforatorium to scrape cells and cell debris from the edges of the lesion, which it then ingests. The ciliate's surface is continually thrown into folds as the cell rotates and moves in the epithelial tissue. The large horseshoe-shaped macronucleus is readily apparent under a light microscope. The parasite continues to grow by adding ciliary rows as well as membrane and cytoplasmic organelles (including mucocysts, contractile vacuoles and ribosomes). The single contractile vacuole of the theront multiplies to several hundred in the trophont (Canella and Rocchi-Canella, 1976; Chapman and Kern, 1983; Ewing and Kocan, 1986).

The trophont creates a tissue space in the epithelial layers as it feeds and moves. The parasite within this vesicle appears as a white spot about 1 mm in diameter. Large numbers of these are easily visible: hence the common name 'white spot disease' (Figs 4.3 and 4.4). Occasionally, several parasites occur within the same vesicle. Most researchers attribute these to theronts penetrating within the same path or comigration within the epithelium (MacLennon, 1935b; Canella and Rocchi-Canella, 1976; Matthews, 1994). Ewing *et al.* (1988), however, calculated that the number of trophonts within a particular section of epithelium increased during infection, and postulated that parasites multiply within vesicles. Definitive proof of cell multiplication on the fish requires direct demonstration of trophonts in the process of dividing; this has not yet been observed.

The duration of infection is variable and depends on different factors. These include temperature, fish species, physiological state of the host and body region in which the parasite resides (Canella and Rocchi-Canella, 1976). If an infected fish dies, the trophont leaves, possibly in response to changes in oxygen tension and the pH of the tissues. Before the parasite can survive off the fish, however, it must first attain a critical size and stage of differentiation. MacLennon (1942) found that

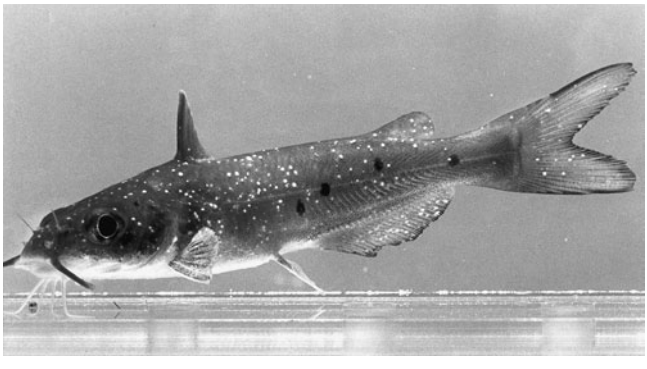


Fig. 4.3. Channel catfish fingerling (*Ictalurus punctatus*) infected with *Ichthyophthirius multifiliis*. The fish was exposed to parasites for 7 days at 23°C. Each white spot represents a single trophont.



Fig. 4.4. The same fish as in Fig. 4.3 seen at a higher magnification. Notice how the trophonts within the skin are often raised above the surface of the fish.

parasites of less than 95 µm were not viable if removed from a fish. In agreement with MacLennon, Ewing *et al.* (1986) showed that the predicted minimum diameter necessary for survival ranged between 85 and 104 µm. The time required to reach these sizes is primarily a function of the temperature and the size of the parasite when it initially infected the fish. In MacLennon's studies, trophonts attained these sizes within 2 days at 27°C (with a calculated growth rate of 5.9% per hour) and 3 days at 22°C (with a growth rate of 8.3% per hour). Ewing *et al.* (1986) found that close to 100% of the trophonts that left after 2 days at 21 and 24°C were able to survive. Parasites associated with fish for longer periods (4 days as opposed to 3) were able to exit the epithelium more quickly when the host died (Ewing and Kocan, 1987). When mature, trophonts exit the epithelium, secrete cysts, divide and produce active, infective theronts. Ewing *et al.* (1986) proposed that, before the parasite can survive outside the host, its contractile vacuole must develop so that it can respond to the osmotic changes that occur as the trophont leaves the epithelium.

Theronts injected into the abdominal cavities of channel catfish (*Ictalurus punctatus*) grow for a period of up to 3 weeks (Dickerson *et al.*, 1985). Trophonts within the peritoneal cavity eventually die without secreting a cyst or undergoing divisions, and the dead parasites became enclosed within granulomatous tissue.

The trophont is defined as a tomont as soon as it ceases to feed and extricates itself from the epithelium. This is the reproductive stage of the organism. The parasite has usually left the fish at this stage, but sometimes it remains superficially attached to the surface mucus. Secretory mucocysts of the tomont are discharged to produce a gelatinous cyst wall, in which the cell undergoes multiple binary divisions (Ewing *et al.*, 1983). The period between leaving the fish and secreting the cyst wall is influenced by temperature. At 21–23°C, the tomont swims for approximately 1 h before it secretes the sticky proteinaceous matrix. When placed in water at a temperature below 10°C, tomonts do not secrete a cyst wall or divide (H.W. Dickerson, unpublished results). When cooled cells are warmed to 21–23°C, they secrete a cyst and proceed to divide normally. Secretion of the cyst can also be delayed by placing tomonts into minimal essential cell-culture media (Wahli and Matthews, 1999).

Tomonts attach on virtually any substrate in the immediate aqueous environment. Occasionally, however, encysted tomonts are found free-floating or in the surface mucus of moribund or dead fishes. The gelatinous capsule anchors the dividing

parasite to the substrate. The matrix also appears to prevent entry of bacteria and fungi. Investigators have studied the cyst wall by light (MacLennon, 1937) and electron microscopy (Ewing *et al.*, 1983). MacLennon observed that the wall has inner and outer layers, each of which is composed of proteinaceous fibrils. Ewing *et al.* confirmed that the cyst consists of two layers: an inner homogeneous layer with the same consistency and electron density as the material found in mucocysts; and a less dense outer layer covered with bacteria and other debris. They found that the wall varied in width, with the thickest side at the point of attachment to the substrate. In some instances, secretion of the cyst wall continues after the first or second division, resulting in partitioning into chambers, within which the daughter cells divide. MacLennon found that the cyst wall could be removed following the first division without affecting the viability of the dividing organism.

Shortly after the parasite secretes the cyst wall, it begins a series of palintomic divisions. This results in 200–800 tomites. The period between secretion of the cyst and the first cell division is dependent on temperature and tomont size. At 23°C, the first division usually occurs between 30 and 75 min (Canella and Rochi-Canella, 1976; H.W. Dickerson, personal observation). At the same time, the vestibular buccal apparatus and the perforatorium are resorbed. The number and size of tomites vary and depend on the number of cell divisions, which is correlated directly with the initial size of the tomont (MacLennon, 1937; Canella and Rocchi-Canella, 1976).

Divisions are completed in 18–24 h at 23°C. The tomont divides nine to ten times to produce 516 to 1024 tomites. Tomites develop into infective theronts. Differentiation of tomites into theronts involves the acquisition of a pyriform cell shape, the development of a rudimentary buccal apparatus and the formation of a perforatorium. Food vacuoles are not evident in theronts, although small vacuoles with acid phosphatase activity have been observed (Lobo-da-Cunha and Azevedo, 1993). The first few theronts to differentiate bore their way out of the cyst, leaving a hole in the wall through which the remaining organisms leave.

Cryptocaryon irritans

C. irritans is a holotrichous ciliate that infects the surface epithelia of marine fishes. It goes through an obligate feeding stage and a fish-free reproductive stage during the course of its life history. The various forms of the parasite reach approximately the same sizes as their *Ichthyophthirius* counterparts. The infective theront (Fig. 4.5) is pyriform in shape and 25 to 60 µm in length (Brown, 1963; Nigrelli and Ruggieri, 1966; Cheung *et al.*, 1979; Colorni, 1987; Colorni

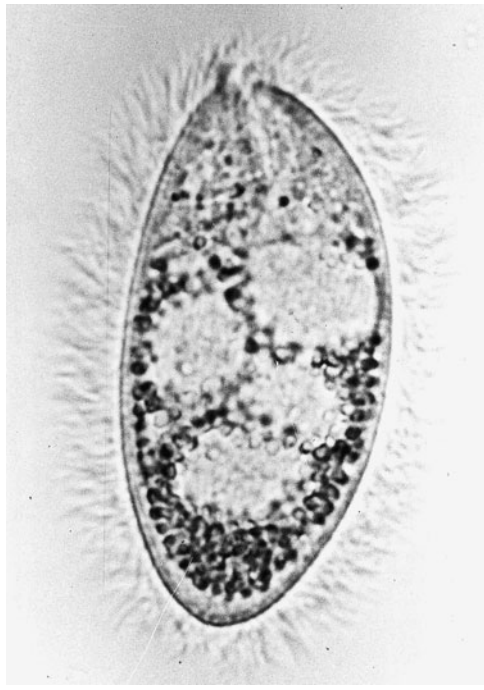


Fig. 4.5. *Cryptocaryon irritans* theront. Photomicrograph of the infective stage of the parasite. The size of the *C. irritans* theront is similar to that of *I. multifiliis* (it is shown here at a greater magnification than the theront in Fig 4.2). Photomicrograph courtesy of Dr A. Colorni, Israel Oceanographic and Limnological Research Ltd, National Center for Mariculture, PO Box 1212, Elat 881122, Israel.

and Diamant, 1993). Feeding trophonts reach diameters of 60 to 450 µm (Cheung *et al*., 1979; Colorni, 1985; Colorni and Diamant, 1993; Matthews *et al*., 1993). The mature trophont leaves the fish as a free-swimming tomont (Fig. 4.6) and secretes a cyst, in which it undergoes multiple divisions to produce 200 or more daughter tomites. Tomites differentiate into infective free-swimming theronts, which attach to and penetrate into the skin and gill epithelia of susceptible fishes.

There have been a number of studies on the morphology of *C. irritans* (Brown, 1963; Nigrelli and Ruggieri, 1966; Cheung *et al*., 1981; Colorni and Diamant, 1993; Matthews *et al*., 1993; Kesintepe, 1995). *C. irritans* has two types of nuclei, a lobed macronucleus and several smaller micronuclei (Brown, 1963; Nigrelli and Ruggieri, 1966; Colorni and Diamant, 1993). The macronucleus of the trophont has four lobes arranged into a crescent (Brown, 1963; Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969; Colorni and Diamant, 1993). Each lobe is approximately 10 µm long by 8 µm wide and contains one to two nucleoli (Colorni and Diamant, 1993). The macronuclear membrane has a trilaminar structure with many nuclear pores. The cytoplasm of the *Cryptocaryon* trophont is more opaque than that of *Ichthyophthirius*, making the nucleus of live unstained specimens more difficult to see. The four lobes of the macronucleus fuse into a continuous strand in the encysted tomont before it begins to divide (Brown, 1963; Colorni and Diamant, 1993). The tomont does not multiply by simple palintomic division, as in *Ichthyophthirius*, but instead undergoes a process of 'budding'. As first described by Brown (1963), a section of cytoplasm containing part of the macronucleus is pinched off at one end of the cell. This cytoplasmic and nuclear fragment then proceeds to divide into two cells of equal size. The process is repeated with the remaining segment of the tomont until a number of tomites of equal size are produced within the cyst. Nigrelli and Ruggieri (1966) also described the division of the tomont as being asymmetric, resulting in the initial production of a group of daughter cells at one pole followed by division of the rest of the cell.



Fig. 4.6. *Cryptocaryon irritans* tomont. Scanning electron micrograph of the reproductive stage of the parasite after leaving the fish and preceding secretion of the cyst in which it undergoes division. Bar = 25 µm.

During cell reproduction the micronuclei proliferate by mitotic division, with five to seven eventually being distributed into each of the daughter cells (Brown, 1963; Colorni and Diamant, 1993). Brown indicated that not all of the micronuclei were viable, and postulated that some disintegrated and reassociated in the process of autogamy. The time required for tomont division is longer in *Cryptocaryon* than in *Ichthyophthirius* (see below).

The buccal apparatus of the parasite differentiates after nuclear development and before the theront leaves the cyst (Brown, 1963). It consists of a single ring of cirri surrounding the oral opening, which is about 20 µm in diameter (Cheung *et al*., 1981; Colorni and Diamant, 1993). Cirri are

composed of two adjacent cilia (Kesintepe, 1995). Nigrelli and Ruggieri (1966) postulated that this stiff cirrus ring is used to burrow into the epithelium and scrape cells for ingestion. The prominent perforatorium, which is important in the infection and feeding processes of *Ichthyophthirius*, has not been described in *C. irritans*.

The buccal cavity of the trophont has a protrusile membrane with a smaller extension below it (Brown, 1963). It was suggested that the protrusile membrane functions to draw in mucus, blood and cellular debris as the parasite feeds (Nigrelli and Ruggieri, 1966). In this regard the buccal apparatus of the *C. irritans* trophont differs significantly from that of *Ichthyophthirius*.

Flask-shaped mucocysts are present, but are not differentiated into crystalline and secretory forms as described in *I. multifiliis* (Ewing and Kocan, 1992). They do not appear to contribute to cyst formation (Colorni and Diamant, 1993), but may be involved in host invasion (Colorni and Diamant, 1993; Matthews *et al.*, 1993). The encysted parasite is resistant to chemical treatment, suggesting that the cyst wall is impermeable to soluble substances in the water (Herwig, 1978). The cyst wall on average is 0.5 to 1 μm thick (Matthews *et al.*, 1993). It has a loose, fibrillar outer layer and a multilayered, compact inner wall (Kesintepe, 1995).

Cheung *et al.* (1981) published electron micrographs depicting tomonts with truncated cilia. Nigrelli and Ruggieri (1966) stated that the cilia became absorbed before the parasites secreted cyst walls. Other studies found that the cilia were shed rather than resorbed at this stage (Colorni and Diamant, 1993; Matthews *et al.*, 1993). Loss of cilia does not occur with *Ichthyophthirius*, where all stages of the parasite are ciliated, including the tomites within the cyst.

Kesintepe (1995) found contractile vacuoles to be present, a result that is in agreement with the findings of Sikama (1961). An organelle of Lieberkühn is not present in any stage of the parasite (Colorni and Diamant, 1993; Matthews *et al.*, 1993; Kesintepe, 1995). The absence of this structure raises doubts as to the classification of *Cryptocaryon* in the same suborder (Ophryoglenina) as *Ichthyophthirius*.

Development of the parasite

As with *Ichthyophthirius*, ambient temperature influences the growth and development of *Cryptocaryon*. The trophont remains on the fish for 3–7 days at optimal growth temperatures (23–30°C) (Cheung *et al.*, 1979; Colorni, 1985; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994). *C. irritans* differs from its freshwater counterpart in that it generally takes longer to reproduce and differentiate outside the fish. Secretion of the cyst is usually completed within 24 h at 25°C. Cheung *et al.* (1979) found that only 10% of tomonts held at 7°C secreted cysts. When the organisms were incubated at 25°C, the majority (66%) secreted cysts.

At 24–27°C, *Cryptocaryon* tomonts take 3–28 days to divide and produce infective theronts, with most emerging by the eighth day (Nigrelli and Ruggieri, 1966; Cheung *et al.*, 1979; Colorni, 1987; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994). *I. multifiliis* completes these divisions in 18–24 h. Tomonts divide and develop into theronts at an asynchronous rate (Colorni and Diamant, 1993; Matthews *et al.*, 1993; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994). Yoshinaga and Dickerson (1994) discovered that theronts are released from cysts with a circadian periodicity and enter the water between the hours of 0200 and 0900, regardless of the day that they leave the cyst.

Summary of the life cycle of C. irritans *and comparison with that of* I. multifiliis

In many respects the life cycle of *Cryptocaryon* is similar to that of *Ichthyophthirius* (see Fig. 4.1). A fish-associated trophont stage and a free-living reproductive tomont stage occur in both parasites. As with *I. multifiliis*, the parasitic trophont of *Cryptocaryon* remains on the fish for approximately 5–7 days at 25°C. The reproductive stage of *C. irritans*, however, usually takes much longer to develop than that of

I. multifiliis and commonly extends to 8 days at 25°C and longer at lower temperatures. Two hundred or more infective theronts emerge from each encysted tomont and each of these penetrates the host by means of specialized ciliary membranelles associated with the buccal apparatus. In contrast, *I. multifiliis* penetrates using a specialized apical structure, the perforatorium.

Host–Parasite Relationships of *I. multifiliis*

Host selection

I. multifiliis does not appear to have a predilection for any specific group of fishes, although it is believed that the organism originated as a parasite of carp (Hoffman, 1999).

There is a linear relationship between the number of theronts to which a host is exposed and the resultant parasite burden (McCallum, 1982). Theronts are positively phototactic (Lom and Cerkasova, 1974; Wahli and Meier, 1991). Wahli and Meier (1991) could not demonstrate that theronts were attracted to fish, a result in disagreement with that of Lom and Cerkasova (1974), who found that theronts were attracted by components of fish blood. Houghton (1987) observed that theronts were attracted to pieces of fish tissue in the water, suggesting a possible short-range homing mechanism.

Epizootics of *I. multifiliis* appear to occur uniformly in populations of male and female fishes. There are reports where infections occurred predominantly in one sex, however. Male guppies (*Lebistes reticulatus*) were reported to be more severely infected than females (Paperna, 1972). Similarly, in brown trout (*S. trutta*) mature males were more frequently infected than females, with the most severe infections occurring on precociously mature pre-spawning males (Pickering and Christie, 1980). In contrast, in an epizootic in Lake Titicaca, the majority of the dead and infected fish were gravid or spent female killifish (*O. agassii*) (Wurtsbaugh and Tapia, 1988). Infection may not be a function of predilection of the parasite for either sex, but rather due to the fact that infection occurs on fishes under the greatest stress. Reproductive activity is a significant stress on fishes.

Fishes exposed to *I. multifiliis* can develop protective immunity (Hines and Spira, 1974c; Burkart *et al.*, 1990); hence survivors of an epizootic are resistant to subsequent infection. Therefore, in native populations, young fishes might be more susceptible to infection than older individuals if the latter were previously exposed to the parasite. In naïve feral fish populations, all ages appear to be equally susceptible to infection.

Distribution of parasite on host

Except in very severe infections, *I. multifiliis* is not usually uniformly distributed on the body of the fish. The parasite occurs most frequently on the dorsal surface, particularly the head and fins (Hines and Spira, 1973a; Kozel, 1976).

The gills are important sites of infection. The volume of water passing through the gills increases the opportunity for attachment, and parasites interfere with gaseous exchange once infection is established. In unstressed fishes there is very little mucus covering the secondary lamella of the gills (Handy and Eddy, 1991), which may render the gills more susceptible to infection than other body sites.

Genetic susceptibility

In experimental *I. multifiliis* infections, only a portion of the infecting theronts develop into trophonts (McCallum, 1982). McCallum (1982) suggested that this variability depends on the genetic background of the host. A major host resistance factor is the production of surface mucus, which is increased in response to infection (Hines and Spira, 1974c; Ventura and Paperna, 1985). There may be genetic factors that influence the amount and composition of fish mucus.

Significant variation in susceptibility to infection occurs among fish species.

When effects due to other variables (such as time of infection and temperature) are removed, heterosis (hybrid vigor) contributes to resistance to *I. multifiliis* (Clayton and Price, 1992, 1994).

I. multifiliis is believed to have originated as a parasite of carp (Hoffman, 1999). The long host–parasite association may have led to the development of resistant strains of carp. Preliminary studies using scale patterns as genetic markers suggested that strains of carp vary in their resistance to *I. multifiliis* (Clayton and Price, 1988).

Behavioural modifications

In the early stages of disease, fishes congregate near water intakes to reduce contact with free-swimming theronts (Kabata, 1985). Fishes also 'flash' or rub their bodies against objects in reaction to skin and gill irritation caused by the theronts (Brown and Gratzek, 1980). Fishes swim more rapidly than normal and often leap out of the water. As the disease progresses, they become less active and congregate at the bottom of ponds or aquaria (Hines and Spira, 1973a). Fishes also lie near the edges of ponds, moving their gill opercula rapidly in an attempt to obtain more oxygen (Kabata, 1985). This is related to gill damage caused by the parasite. With very heavy infections, fishes become lethargic and stop feeding (Hines and Spira, 1973a).

Gross pathology – *I. multifiliis*

In very mild *I. multifiliis* infections the only detectable pathological change is the presence of a few white spots on the surface of the fishes. In more severe cases there are usually large numbers of spots on the skin (see Figs 4.3 and 4.4). Occasionally, however, *I. multifiliis* only infects the gills, with no obvious gross lesions on the body surface.

Ulcers develop in the skin of heavily infected fishes and are often sites of secondary bacterial or fungal infections. The fins become frayed due to loss of tissue between the fin rays (Hines and Spira, 1973a).

As mentioned earlier, mucus production is increased. Heavily infected carp have a thick, lumpy covering of surface mucus (Hines and Spira, 1974a). Increased production of mucus is not a unique response to *I. multifiliis*, however, as it occurs on most fishes that are exposed to irritants or skin parasites.

Infected fishes have enlarged spleens and kidneys and pale, mottled livers. Their peritoneal cavities contain fluid (Hines and Spira, 1974a). Peritoneal fluid exudation could be a secondary effect caused by opportunistic bacterial or fungal infections and/or anorexia of the fishes in the later stages of the infection. Enlarged gall bladders (a change associated with starved animals) were reported in moribund Ich-infected carp (Hines and Spira, 1974a).

Gross pathology – *C. irritans*

The gross lesions in *C. irritans* infections are similar to those seen in fishes infected with *I. multifiliis*. Petechial haemorrhages occur in the skin (Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969). Fishes produce excessive amounts of mucus in response to infection (Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969). With severe infections, skin ulcers occur with secondary *Pseudomonas* spp. bacterial infections (Nigrelli and Ruggieri, 1966).

Clinical signs

The common clinical signs of ichthyophthiriasis are the characteristic disseminated white surface lesions ('spots'). Each spot represents a developing trophont within an epithelial capsule or vesicle (see Fig. 4.4). Visible parasites develop several days after the initial attachment of theronts (Hines and Spira, 1973a; Ventura and Paperna, 1985). In cases where the infection is restricted to the gills, these are not visible.

An early physiological response to infection is an increase in surface mucus production (Hines and Spira, 1973a). Skin penetration by theronts stimulates expanded

numbers of mucus-secreting cells in the epidermis. These multiply not only in areas around the parasite, but throughout the epidermis (Hines and Spira, 1974a). In severe infections, mucus may stream off the posterior edges of the fins and tail.

A careful observer can detect infection before the development of surface lesions by noting changes in fish behaviour (see above). Initially, infected fishes swim more rapidly and rub themselves against objects. As the disease progresses, the fishes surface in order to reach dissolved surface oxygen. They become increasingly lethargic and eventually cease feeding.

In severe infections there is erosion of the epithelia, leading to ulcer formation and exposure of the deeper tissues to bacterial and fungal invasion. A common cause of secondary invasions is the fungus *Saprolegnia* spp., which appears as tufts of 'fuzz' on the skin.

The clinical signs of a *C. irritans* infection are similar to those seen in ichthyophthiriasis. Fishes develop small white cysts on their body surfaces (Wilke and Gordin, 1969; Yoshinaga and Dickerson, 1994). These lesions are often numerous, small, greyish vesicles rather than the larger white spots associated with Ich infections (Nigrelli and Ruggieri, 1966). *C. irritans* infection frequently involves the eyes and leads to corneal clouding and blindness (Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969). Marine fishes infected with *C. irritans* produce excessive amounts of surface mucus (Nigrelli and Ruggieri, 1966; Wilke and Gordin, 1969; Huff and Burns, 1981).

Histopathology – *I. multifiliis*

The nature and severity of histopathological changes seen in *I. multifiliis* infections vary greatly. This variation is influenced by such host factors as stress and nutritional status. Nevertheless, parasite load is the major factor contributing to the diverse tissue changes. In general, mild infections elicit minor cellular reactions. The extensive histopathological changes reported to occur in *I. multifiliis* infections are only seen in severe epizootics or in experimental infections with large numbers of parasites (Ventura and Paperna, 1985).

Histopathology of the skin consequent upon mild infection

In primary *I. multifiliis* infections (that is, the first exposure of fish to the parasite), there is little reaction to the penetrating theronts. After approximately 40 h, most trophonts are located next to the basement membrane. The cells between the parasite and the basement membrane become hydropic, vacuolated or necrotic, with pyknotic nuclei (Ventura and Paperna, 1985). The growing trophont gradually lifts and displaces the epithelial cell layers until it lies within an epithelial capsule that extends above the skin surface (Ventura and Paperna, 1985). The epithelial layer overlying the trophont expands to cover the parasite during this growth stage. The epithelium retains its architecture of differentiated cell populations (Ventura and Paperna, 1985). Cell damage is observed only in the cellular layers in direct contact with the developing trophont. Host cell debris can be observed in the food vacuoles of the trophont and in the spaces in the epithelial capsule around the parasite. There is evidence of haemorrhage occurring in the skin as a result of parasite invasion (Chapman, 1984; Ventura and Paperna, 1985). Large, pale-staining, alarm substance cells have been observed in the area of the developing trophont (Chapman, 1984). In mild infections only a few leukocytes are seen in the epithelium.

Histopathology of the skin consequent upon heavy infection

Heavy infections elicit a severe inflammatory reaction in the skin. Penetration by numerous theronts leads to increased epithelial cell hyperplasia. This cellular proliferation could be a defence mechanism (Ventura and Paperna, 1985). In addition to hyperplasia, there is a generalized increase in the number of mucus cells in the skin (Hines and Spira, 1974a). The epithelium in heavily infected fishes may be up to four times

its normal thickness due to proliferation of epithelial and mucus cells (Hines and Spira, 1974a).

Extensive cell necrosis and histolysis occur around trophonts developing in the hyperplastic epithelium. Empty spaces are present, along with hydropic, vacuolated and necrotic cells (Ventura and Paperna, 1985). There is congestion in the dermal lymphatics, leading to oedema and an increased epithelial infiltration of neutrophils, eosinophils and lymphocytes (Hines and Spira, 1974a; Ventura and Paperna, 1985). The outermost epithelial cells degenerate and slough off, eventually exposing the underlying basement membrane (Hines and Spira, 1974a). It was suggested that the extensive cellular reaction observed in the skin of fishes heavily infected with *I. multifiliis* is a hypersensitivity reaction (Hines and Spira, 1974a; Ventura and Paperna, 1985).

Histopathology of the gills

Theronts attach to the gills at the middle or base of the gill lamellae. The young trophonts penetrate and displace the interlamellar epithelium until they reach the basement membrane (Ventura and Paperna, 1985). Host cell debris is seen in the cytoplasmic vacuoles of the parasite. Epithelial cells migrate from the apical end of the adjacent lamellae and cover the developing trophont, producing a multilayered cap over the parasite. The cell layer over the parasite also includes mucus cells and chloride cells (Hines and Spira, 1974a; Ventura and Paperna, 1985). Epithelial cell proliferation occurs not only adjacent to the parasite but also all along the lamellae (Hines and Spira, 1974a). Mature trophonts may occupy three or four lamellae (Ventura and Paperna, 1985). Mucus cells become prominent in the proliferating epithelium and comprise up to 50% of the cells in an interlamellar area (Hines and Spira, 1974a). The respiratory epithelium, however, retains its squamous morphology during this proliferation. At this time, there is a significant increase in the number of neutrophils in the area. The amount of lymphocyte infiltration is variable (Hines and Spira, 1973b). As the infection progresses, the epithelial cells continue to proliferate and eventually fill up the interlamellar space until the lamellae are completely cornified or 'clubbed' (Hines and Spira, 1974a).

In the late stages of heavy infections, complete atrophy of the gill lamellae is seen, along with areas of necrosis of the gill filaments (Hines and Spira, 1974a).

Histopathology – *C. irritans*

There is limited information on the histopathologic changes that occur with *C. irritans* infections. From the analogies in the infective and feeding stages of *I. multifiliis* and *C. irritans*, it is reasonable to assume that the cellular changes caused by both parasites are similar. The major lesions caused by *C. irritans* are vesicles within the skin. Mucus-producing cells proliferate in the skin and gills. Epithelia of infected gills become hyperplastic and eroded in severe cases.

Clinical pathology

Although the total leukocyte numbers do not change during *I. multifiliis* infections, there is a differential shift in the various leukocyte populations. Infected fishes develop a lymphocytopenia and neutrophilia (Hines and Spira, 1973a). The number of neutrophils in circulation early in the infection may increase 5-fold with no concurrent rise in total leukocyte numbers. The shift in distribution of leukocyte cell populations is accompanied by an increase in the number of immature blast cells. It was suggested that the leukocyte changes in infected fishes are non-specific stress reactions (Hines and Spira, 1973b).

Studies on serum levels of Na^+, K^+, Mg^+ and blood urea ammonia indicated significant osmoregulatory disturbances (Hines and Spira, 1974b). In severe infections there was a marked drop in serum Na^+ and Mg^+ levels and a rise in serum K^+ levels. Blood urea-ammonia levels also increased during the course of the infection.

Immune response – *I. multifiliis*

It was recognized early that fishes surviving *I. multifiliis* outbreaks become resistant to reinfection (Bushkiel, 1910). The duration of immunity depends upon the severity of the initial infection (Bauer, 1953). A number of studies determined the period of immunity in fishes recovered from natural infections or cured by chemical treatment (Beckert and Allison, 1964; Parker, 1965; Hines and Spira, 1974c; McCallum, 1986). Immunity induced by single exposure of channel catfish to the parasite lasts for at least 3 months (Pyle, 1983). In the natural course of infection, surviving fishes eventually become free of the parasite (Valtonen and Keränen, 1981). Premunition has not been demonstrated, although a small percentage (< 5%) of infecting parasites developed in immunized carp (Cross and Matthews, 1992).

The immune responses of most fish are complex reactions involving the activation and interaction of a variety of cell populations and the production of humoral factors, including antibodies. Some host reactions are general, responding similarly no matter what parasite is encountered; others are specific, reacting only with the parasite that induced the reaction. The former responses are innate reactions, and the latter are referred to as adaptive or acquired. In *I. multifiliis* infections, both innate and adaptive reactions appear to play significant roles.

Surface mucus is the fish's first line of defence against infection. To infect, theronts must penetrate this mucus layer and burrow into the epithelium. Mucus-secreting cells increase in the skin of infected fishes (Hines and Spira, 1973a; Ventura and Paperna, 1985). Heavily parasitized fishes characteristically have a thicker mucus coat than uninfected fish. Theronts have limited energy stores, which could become depleted during penetration of the thickened mucus layers. Trapped theronts would eventually be removed from the surface with the excess mucus. In this regard, the naturally thin mucus layer of gills could be a predisposing factor for infection at this site. One report indicated that the mucus layer does not completely cover the secondary lamellae in unstressed rainbow trout (Handy and Eddy, 1991).

In addition to acting as a physical barrier, surface mucus also contains antiparasitic factors. Hines and Spira (1974c) and Wahli and Meier (1985) found that mucus and serum from immune carp and rainbow trout (*Salmo gairdneri*) immobilized trophonts *in vitro*. It was suggested that this immobilizing activity was due to the presence of antibodies and that these prevented the penetration of theronts through the mucus. Xu and Dickerson, using dot-blot assays, found that Ich-immune channel catfish have mucus antibodies against membrane antigens of *I. multifiliis* (Xu, 1995). In contrast, Cross and Matthews (1993b) were unable to demonstrate binding of carp mucus antibodies to thin sections of fixed theronts. The discrepancy in these findings is probably due to the fact that specific antibodies are present in relatively low levels in mucus (as compared with serum) and may not have been detectable in the experiments of Cross and Matthews. More recent work suggests that mucosal antibodies are protective. This work is discussed further below.

A basic innate host reaction to *I. multifiliis* is epithelial cell proliferation. In mild infections there is little reaction other than the formation of an epithelial cell capsule around the parasite itself (Ventura and Paperna, 1985). In severe or repeated infections, however, extensive epithelial cell proliferation occurs (Hines and Spira, 1974a; Ventura and Paperna, 1985). This epithelial hyperplasia could interfere with penetration of the theront. The skin of infected fishes also becomes infiltrated with neutrophils, basophils, eosinophils, eosinophilic granular cells, macrophages and lymphocytes (Ventura and Paperna, 1985; Cross and Matthews, 1993b). Increases in eosinophilic granular cells were coupled to the acquired immune response against the parasite (Cross and Matthews, 1993b). Leukocytes (granulocytes) were observed adjacent to parasite surfaces without apparent adherence. How these cells interact with the trophonts in the tissue is not well understood.

Some of the lymphocytes found in the skin of infected fishes are described as non-specific cytotoxic cells (NCC) (Graves *et al.*, 1984). In laboratory experiments, NCC isolated from the anterior kidney of channel catfish were found to be cytotoxic against deciliated *Tetrahymena pyriformis*, a free-living ciliate. This activity was blocked following incubation of isolated NCC with formalin-killed *I. multifiliis* (Graves *et al.*, 1984, 1985a,b). Based on these results, it was hypothesized that NCC also react with and kill immobilized *I. multifiliis*. It was found that the NCC were mobilized out of anterior kidneys in Ich-infected fishes and the circulating NCC had increased target-cell affinity and killing capacity (Graves *et al.*, 1985b). Also, there is a decrease in lymphocytes in circulation in Ich-infected fishes (Hines and Spira, 1973b). Theoretically, this decrease could be attributed (at least partly) to NCC passing out of circulation into the skin to interact with immobilized theronts, although this has never been tested directly.

As described previously, sera and mucus collected from infected fishes immobilize theronts *in vitro* (Hines and Spira, 1974c). This phenomenon was taken as evidence that fishes produced antibodies against the parasite. Various antigens, including theronts (Burkart *et al.*, 1990), trophonts (Areerat, 1974) and cilia (Goven *et al.*, 1981), were used to induce fishes to produce immobilizing or agglutinating factors in their sera. Immobilization and agglutination were assumed to be the result of antibody activity. Nevertheless, in early studies, the precise character of the factors was not determined. It was subsequently demonstrated that channel catfish immune to Ich produce antibodies to ciliary antigens of the parasite (Clark *et al.*, 1988). A strong positive correlation exists between antibody levels in the serum as determined by an ELISA test and the ability of the serum to agglutinate live *I. multifiliis* theronts *in vitro*. These results strongly support the conclusion that the serum anti-parasite activity is indeed antibody-mediated. Subsequent studies by Cross and Matthews (1993a) have also shown binding of fish serum antibodies to surface membranes of *I. multifiliis*.

The relationship between serum and mucus antibody levels is still under investigation (Maki and Dickerson, 2003). It was reported that a secretory form of antibody analogous to mammalian secretory antibody occurs in channel catfish (Lobb and Clem, 1981). Channel catfish are protected against *I. multifiliis* challenge following intraperitoneal injection of immobilizing murine monoclonal antibodies (mAbs) against *I. multifiliis* (Lin *et al.*, 1996). Mouse antibody immunoglobulin G1 (IgG1) was transported from the peritoneal cavity to the surface of the fish, where it reacted with the parasite. In contrast, transfer of immune fish sera or immobilizing mouse IgM mAbs did not protect against *I. multifiliis* infection, suggesting that the teleost serum tetrameric antibody does not pass as readily to the surface as the monoclonal mouse IgG (Lin *et al.*, 1996). An adaptive, protective immune response is elicited against Ich following the intraperitoneal injection of live theronts. As one might expect with a large extracellular parasite such as *I. multifiliis*, antibodies are important effectors of protective immunity (Cross, 1993; Matthews, 1994; Dickerson and Clark, 1998). Their mechanism of activity is just beginning to be elucidated. An early hypothesis was that antibodies immobilize theronts in the mucus layer of the skin (Hines and Spira, 1974c). Immobilization *in vivo* may be an indirect effect rather than a direct action of the antibodies on theront cilia. Clark *et al.* (1987) showed that immobilized theronts were surrounded by a mucus-like material, and suggested that antibody binding caused discharge of parasite mucocysts. The clumping of theronts was the result of their being trapped in this released substance. Cross (1993) demonstrated the binding of carp serum antibody to a gelatinous mucus-like material surrounding tomonts. He hypothesized that this material is responsible for immobilization *in vitro* and may form a barrier to further antibody binding. It was proposed by Ewing *et al.* (1985) that discharge of theront

mucocysts is also a means of attachment and penetration into the epithelium. Premature discharge of mucocysts could result in theronts that are unable to infect fishes even though they have penetrated the surface mucus and come into contact with the epithelium.

To directly observe the fate of live parasites on fish, Cross and Matthews (1992) placed theronts on the tails of immune or naïve carp and observed them under the microscope. They discovered that the parasites penetrated into the skin of both naïve and immune fish within minutes. Theronts were unable to remain colonized on immune fish, however, and left within 2 h. The authors argued that immunity was the result of a humoral factor due to the rapidity of the response. There was no evidence of parasites being immobilized or killed in the tissue.

Fishes have subpopulations of lymphocytes similar to those that occur in mammals (Yocum *et al.*, 1975; Lobb and Clem, 1982; Secombes *et al.*, 1982). B-lymphocytes develop into antibody-producing cells, and analogous cells are elicited in the antibody response against *I. multifiliis*. In mammals, T-lymphocytes are a well-characterized, diverse cell population involved in both regulation (e.g. TH_1 and TH_2 helper lymphocytes) and implementation (T-cytotoxic cells) of the immune response. T-cytotoxic cells react with antigens on cell surfaces and kill foreign-antigen-bearing cells. The two model reactions for T-cell activity are delayed-type hypersensitivity reactions and graft rejection. Fishes have been shown experimentally to develop delayed hypersensitivity reactions and will reject heterologous scale grafts (Hildemann, 1972; Kikuchi and Egami, 1983; Tataner and Manning, 1983). It is unlikely that fishes develop either type of response against *I. multifiliis*. Houghton and Matthews (1986, 1990, 1993) argue for a cell-mediated effector response against *I. multifiliis*, based on experiments showing that corticosteroid treatment of Ich-immune carp increases their susceptibility to infection, while serum antibody levels remain high.

Immune response – *C. irritans*

There is a dearth of information on the immune response of fishes to *C. irritans* infection. It has been suggested (without experimental evidence) that fishes maintain an immunity by premunition (Nigrelli and Ruggieri, 1966). It seems reasonable to postulate that fishes produce antibodies to *C. irritans* both in the circulation and possibly in surface mucus. These antibodies could play similar roles to those involved in protective immunity against *I. multifiliis*.

Mechanisms of disease – *I. multifiliis*

Fishes infected with small numbers of *I. multifiliis* show few signs of infection other than the development of white spots. Fishes tolerate a few parasites very well. As previously stated, this situation suggests that teleost fishes and *I. multifiliis* have had a long association, and as a result have evolved a balanced host–parasite relationship. Disease and/or deaths occur when there is a disturbance in this balanced interaction. This disruption is most frequently the result of infection with large numbers of parasites. When conditions are right (e.g. optimum temperature and sufficient number of hosts), the parasite population increases very rapidly. As a result, fishes are exposed to many parasites in a short period of time.

Infection by *I. multifiliis* triggers hyperplasia of gill epithelial cells. As a result the gill interlamellar spaces are reduced, which significantly limits the surface area available for oxygen interchange. Severe infections thus cause fishes to become oxygen-starved. In addition to being the primary site of oxygen uptake, the gills also function in both osmoregulation and excretion of nitrogenous wastes (Smith, 1929). Epithelial hyperplasia interferes with these functions as well. Infected carp have decreased levels of serum sodium and magnesium and increased levels of serum potassium (Hines and Spira, 1974b). High levels of serum urea-ammonia nitrogen and nitrogenous wastes accumulate in the blood. An additional contributing factor to ionic imbalance is the interplay

between nitrogenous waste excretion and ionic exchange. When NH_4^+ ions are excreted through the gills, Na^+ ions are taken up from the water into circulation (Maetz and Garcia-Romeu, 1964). Thus, the decrease in NH_4^+ excretion during infection contributes to lowered serum Na^+ concentrations. Increased mucus production in the gills of infected fishes interferes with osmoregulatory functions as fish mucus is relatively impermeable to water and ions (Hughes, 1970). Proliferative and degenerative changes in the skin also influence osmoregulation. The skin is a major barrier to ion loss and to water entry in fishes (Wikgren, 1953), and the subepidermal oedema and disruption of the epithelium cause ionic imbalance (Hines and Spira, 1974b). Finally, fishes with severe infections are under stress, and stress reactions have been reported to elicit serum ionic imbalances (Stanley and Colby, 1971; Wedemeyer, 1972).

Ich infections cause severe cellular reactions in the skin. Gradual desquamation, degeneration, necrosis and sloughing of the superficial epithelial cells occur in the area of the growing parasite (Ventura and Paperna, 1985). This devitalization of the skin reduces the ability of fishes to resist infections by opportunistic pathogens. These secondary infections add stress to an already badly compromized host and increase the mortalities in a population of infected fishes.

Mechanisms of disease – *Cryptocaryon irritans*

The limited body of literature on *C. irritans* does not contain any significant description of disease mechanisms. Both the life cycle of and the pathology associated with *C. irritans* are similar to those of *I. multifiliis*. Thus, it seems reasonable to assume that the mechanisms of disease would be similar for the two parasites.

Antigens of *Ichthyophthirius multifiliis*

It was observed as early as the beginning of the 20th century that fishes surviving mild outbreaks of *Ichthyophthirius* become solidly immune to reinfection (Bushkiel, 1910). It has since been documented in many reports that fishes recovered from infection are protected against subsequent attack by the parasite (Butcher, 1941; Bauer, 1953; Beckert and Allison, 1964; Parker, 1965; Hines and Spira, 1974c; Beckert, 1975; Matthews, 1994; Dickerson and Clark, 1996). The fact that fishes develop such a strong immunity against infection makes it theoretically possible to create protective vaccines for the control and prevention of disease outbreaks.

The use of controlled infections for vaccination

Fishes have been successfully immunized against *Ichthyophthirius* using controlled infections. In one study, goldfish (*Carassius auratus*) were vaccinated following exposure to 0.5 and 1.0 median lethal doses (LD_{50}s.) of infective theronts (Parker, 1965). An LD_{50} was 108 theronts per fish, with a density of 3.7 theronts per ml of water. Immunized fish were resistant to lethal challenges of eight to ten LD_{50}s. Channel catfish (*Ictalurus punctatus*) vaccinated by exposure to 20 or 40 encysted tomonts and subsequently treated with 0.1 ppm malachite green had lower mortality than non-immunized fishes when challenged with 20 tomonts per fish (Areerat, 1974). Mirror carp (*Cyprinus carpio*) were immunized following exposure to sublethal doses of tomonts (Hines and Spira, 1974c). In this study fishes recovered naturally without treatment and remained free of parasites when challenged with large numbers of tomonts (500 per fish). One hundred per cent of naïve control fishes died when exposed to tomonts or infected fish in the same system.

Live versus killed vaccines

Infection with live parasites produces a more substantial and prolonged protective immune response than vaccination with killed organisms. This is attributed to an increase in parasite mass as a result of growth on the host, excretory or secretory

products released by the live parasite during infection and the inflammatory response elicited by parasite activity.

Live vaccines usually consist of either a carefully controlled infection with a fully virulent parasite (such as those for *Ichthyophthirius* described above) or an infection with a less virulent or attenuated strain. To date, no attenuated strains of Ich have been described and the use of the virulent organism for vaccination carries the risk of inducing high mortalities in the population one is attempting to immunize. Ich cannot be easily propagated, which also makes its use as a live vaccine impractical on a large scale. Nevertheless, the intraperitoneal injection of fish with live theronts or surface exposure to sublethal numbers of parasites is currently the most effective means to immunize small numbers of fish (Dickerson *et al.*, 1985; Dickerson and Clark, 1996; Maki and Dickerson, 2003; Xu *et al.*, 2004).

Attempts have been made to immunize fishes against *Ichthyophthirius* using killed parasite preparations. In an early study, goldfish were injected intraperitoneally with freeze-thawed theronts (Parker, 1965). When challenged, the fish had fewer visible trophonts compared with non-immunized control fish. The protection was less than that afforded by vaccination with live organisms. Areerat (1974) injected channel catfish fingerlings intraperitoneally or intramuscularly with lysed tomonts with or without Freund's complete adjuvant. One hundred per cent of the immunized fishes survived infection for at least 7 days following challenge. Beckert (1975) was able to induce protection after injecting channel catfish intraperitoneally with killed trophonts mixed with Freund's complete adjuvant.

Assessment of protection

It is difficult to compare results of vaccination trials from different laboratories because experiments were conducted using different antigens, fishes and challenge procedures. One study directly compared (in the same system) the protection afforded by live vaccines and various killed parasite fractions (Burkart *et al.*, 1990). The vaccination and testing were done on channel catfish under uniform conditions. The study confirmed that controlled infection with live organisms afforded higher protection than either injection or immersion with killed parasites. Vaccination by intraperitoneal or surface infection provided complete protection when fishes were challenged 21 days later. These findings agree with other studies where it was found that fishes exposed to non-lethal doses of parasites (Hines and Spira, 1974c; Houghton and Matthews, 1990) or to lethal doses followed by treatment (Beckert and Allison, 1964; Houghton and Matthews, 1986; Clark *et al.*, 1987) were protected against further infection. In contrast, vaccination with killed cell preparations was much less effective; in most cases, all of the vaccinated groups died following challenge (see below). The differences in protection between live and killed parasites suggest either that relevant immunogens are molecules rapidly turned over on the live parasite (e.g. membrane proteins or secretory and excretory products) or they are denatured by the procedures used to treat the parasite in killed vaccines (freezing, deciliation or formalin fixation).

Theront versus trophont antigens

Protective immunity is elicited in channel catfish following exposure to live theronts (Burkart *et al.*, 1990; Dickerson and Clark, 1996; Maki and Dickerson, 2003; Xu *et al.*, 2004). Because theronts differentiate into trophonts soon after they attach to fish, it is difficult to determine whether protection is elicited by stage-specific antigens. To address this question, antigens from both theronts and trophonts were compared directly in vaccination trials (Burkart *et al.*, 1990). Channel catfish were immunized with: (i) freeze-thawed or formalin-fixed theronts; (ii) isolated theront cilia; and (iii) formalin-fixed trophonts. One hundred per cent mortality was seen in all groups except the one injected with killed trophonts, which had a mean mortality of 51% ± 38. This protection was lower than that reported

by Areerat (1974) but suggested that formalin-killed trophonts contain protective antigens. The finding in this experiment that fish injected with Ich cilia all died following challenge is in direct disagreement with a previous report where vaccination with theront cilia was purported to significantly reduce mortality (Goven *et al*., 1981). Nevertheless, in all groups inoculation with both trophont and theront antigens increased the survival period (days to death) over that of control fish. It thus appears that killed antigen preparations elicited some protection, although not enough to prevent death. More recently, Xu *et al*. (2004) demonstrated protection following immunization with killed trophonts.

Immobilization antigens

Sera from immune fishes immobilize free-swimming theronts and tomonts *in vitro* (Fig. 4.7). Hines and Spira (1974c) were the first to describe this phenomenon and they postulated that immobilization was elicited by the binding of antibodies to parasite cilia. Immobilization of theronts and trophonts by immune fish sera has been described by other researchers as well (Beckert and Allison, 1964; Parker, 1965; Areerat, 1974; Wahli and Meier, 1985; Houghton and Matthews, 1986; Clark *et al*., 1987, 1988). Clark *et al*. (1988), using dot-blot assays, demonstrated that immobilizing antisera from immune channel catfish contained antibodies that reacted specifically with ciliary membrane proteins. This was the first time that a direct correlation was shown between immobilization and the presence of antibodies against parasite antigens.

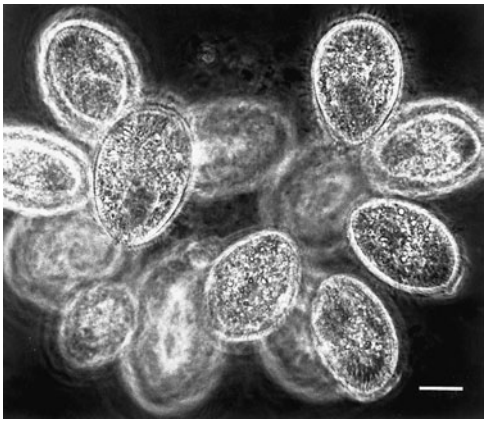


Fig. 4.7. *Ichthyophthirius multifiliis* theronts immobilized by antibody. Theronts were incubated with immobilizing mouse monoclonal antibodies for 30 min and photographed under a phase-contrast microscope. Bar = 16 µm.

The immobilization antigens (i-antigens) of *Ichthyophthirius* are glycosyl-phosphatidyl-inositol (GPI)-anchored membrane proteins ranging between 40 and 70 kDa (Dickerson *et al*., 1989, 1993; Lin and Dickerson, 1992; Clark *et al*., 2001; Clark and Forney, 2003). Analysis of these antigens following mAb affinity chromatography indicated that they share common epitopes and that immobilization is dependent on native protein conformation (Lin *et al*., 1996; Wang and Dickerson, 2002; Clark and Forney, 2003).

Hines and Spira (1974c) suggested that immobilization of parasites is responsible for protection after observing that naïve fishes remained disease-free when challenged in the presence of immune fishes. They postulated that immobilizing antibodies are released into the water from the mucus of immune fishes. The hypothesis that antibodies are directly involved in protection has been tested using mAbs in passive immunization studies (Clark *et al*., 1996; Lin *et al*., 1996). In these studies, murine mAbs that immobilize *Ichthyophthirius* were found to confer virtually complete protection against an otherwise lethal parasite challenge when injected intraperitoneally (i.p.) into naïve channel catfish. As is the case for immobilization, the protection afforded by these antibodies is serotype-specific (Lin *et al*., 1996). While these studies provide clear evidence of a role for i-antigens in protective immunity, experiments carried out by Cross and Matthews (1992) with actively immune fish generated conflicting results. When carp that had been immunized by previous exposure to *Ichthyophthirius* were re-exposed to infectious theronts, parasites readily penetrated the skin. Nevertheless, within 2 h of

exposure, ~ 80% of those initially present had disappeared with little or no trace. The conclusion was that parasites were being forced to exit fish prematurely in response to host factors (presumably antibodies) within the skin. The ability of parasites to invade and then exit the skin was clearly inconsistent with the presence of immobilizing antibodies in the tissue, and raised obvious questions regarding the role of i-antigens in forced exit and protective immunity overall. To reconcile these findings, a further series of passive immunization trials was conducted, this time using fish that were already infected with *Ichthyophthirius* (Clark *et al.*, 1996). In this case, passive transfer of immobilizing antibodies forced rapid, premature exit of parasites from the host, exactly as described by Cross and Matthews (1993b) for actively immune fish. Trophonts that exited fish in response to murine antibodies were viable and developed to form new theronts. Subsequent studies with actively immune fish showed that parasites that escape the host can directly reinfect naïve animals (Wahli and Matthews, 1999). Thus, while premature exit represents an entirely novel mechanism of humoral immunity, it may act as an evasion strategy for the parasite as well (Clark *et al.*, 1996; Clark and Forney, 2003).

Passive transfer experiments suggest that i-antigen-elicited immunity requires the production of cutaneous (mucosal) antibodies in fish (Dickerson and Clark, 1998). Of a total of eight immobilizing mAbs that gave protection in passive immunization trials, seven were IgG-class antibodies. The only mAb that failed to protect was an IgM-class antibody (Lin *et al.*, 1996). Similarly, antibodies from actively immune fish (which are tetrameric IgM-like molecules) failed to protect naïve animals following passive transfer, despite the fact that such antibodies strongly immobilize the parasite *in vitro*. Based on ELISA and *in vitro* immobilization assays, IgG class mAbs reached the serum and mucus of test animals following i.p. injection. In contrast, serum antibodies from immune fish entered the serum but not the mucus, while IgM murine mAbs entered neither compartment (Lin *et al.*, 1996). Because immobilizing antibodies are present in the skin and cutaneous mucus of actively immune fish (Xu, 1995; Xu and Klesius, 2003), these results strongly suggest that protective antibodies are produced locally in the skin. These results are entirely consistent with biochemical evidence that serum and mucus antibodies are metabolically distinct (Lobb and Clem, 1981). Whether such antibodies arise locally from lymphocytes within the skin or are produced elsewhere and enter the epithelium through some, as yet, unidentified pathway is unknown (Dickerson and Clark, 1998).

Concurrent infection and cross-immunity

Protective immunity against *Ichthyophthirius* is highly specific. Hines and Spira (1974c) noted that immune carp became infected with other ectoparasites even though they were resistant to reinfection with Ich. This observation was used to argue that immunity against Ich was an acquired specific response. It was of interest, therefore, when other researchers suggested that the ciliate *Tetrahymena* shared common antigens with *Ichthyophthirius* (Goven *et al.*, 1981), and that *Tetrahymena* cilia could be used to vaccinate fish against Ich. Subsequent work indicated that trout were also protected after bath immunization in cultures of *Tetrahymena thermophila* (Wolf and Markiw, 1982). Wolf found that fish immunized with *Tetrahymena* were resistant to both *Ichthyobodo necatrix* and Ich. It was reported that goldfish (*C. auratus*) vaccinated with *T. pyriformis* were protected against *I. multifiliis* as well as a number of other protozoan parasites (Ling *et al.*, 1993). Other labs could not repeat these findings, however (Dickerson and Clark, 1996; Matthews, 1996). Sera from carp immunized by intraperitoneal injection with live *Tetrahymena* did not immobilize Ich, and the fish were not protected against infection (Houghton *et al.*, 1992). Immobilizing sera from Ich-immune channel catfish reacted with Ich ciliary membrane proteins but not with those of *Tetrahymena* (Clark *et al.*, 1988). A possible explanation for the discrepancy is that the protection

observed in the earlier studies of Goven and Wolf was the result of non-specific innate immunity, rather than acquired immunity.

Cross protection occurs between different immobilization serotypes of *I. multifiliis* (Leff *et al.*, 1994; Jarrett, 1997). Channel catfish immunized against Ich are protected against challenge with homologous or heterologous parasite strains.

Antigens of *C. irritans*

Marine fish surviving infection with *C. irritans* become resistant to reinfection (Colorni, 1985, 1987). Preliminary results from our laboratory (H.W. Dickerson and T. Yoshinaga, unpublished results) indicate that saltwater-adapted mollies (*Poecilia latipinna*) exposed to controlled *Cryptocaryon* infection and cured by treatment with malachite green and formalin develop immunity to lethal challenge. The antigens that elicited this response were not identified.

In Vitro Culture and Propagation of the Parasites

One of the major problems encountered when working with either *Ichthyophthirius* or *Cryptocaryon* is that both organisms are obligate parasites and neither can be grown with artificial media.

Ichthyophthirius is routinely grown by serial passage on channel catfish fingerlings (Noe and Dickerson, 1995). Briefly, an infected fish is placed in a 20 l aquarium with five naïve fishes. When these fish become heavily infected, all but one are removed and placed in 5 l glass jars containing aerated water. Five naïve fish are again added to the aquarium to maintain the infection. The fish in the jars are transferred to fresh jars daily. Trophonts that leave the fish settle to the bottoms of the jars and encyst. The water is gently poured off. The attached tomonts are rinsed in sterile water and allowed to divide overnight, and the free-swimming theronts are collected by centrifugation (Dickerson *et al.*, 1989). When trophonts are needed, they are gently dislodged from the surface of heavily infected fishes and placed into ice-cold sterile water in glass centrifuge tubes. Long-term culture of the parasite can be achieved by lowering the holding temperature of infected fish. Viable and infective parasites are recovered up to 30 days after infection from channel catfish held at 10°C (Noe and Dickerson, 1995).

Placing single trophonts into 1 ml of sterile water in individual wells of 24-well plastic tissue-culture plates clones the parasites. These develop into theronts overnight at 23–25°C. Theronts derived from cloned tomonts are then used to infect susceptible channel catfish fingerlings. These fishes are placed in an aquarium containing naïve fishes.

Cryptocaryon culture is maintained in much the same way except that salt water is used and the development times are longer than those of *Ichthyophthirius*. A standard method for propagating *C. irritans* on grey mullet (*Chelon labrosus*) has been described (Burgess and Matthews, 1994), and a procedure for growing the parasite on saltwater-adapted black mollies (*P. latipinna*) is available (Yoshinaga and Dickerson, 1994).

Cryopreservation

To date, cryopreservation and retrieval of viable, infective organisms has not been possible with either parasite (Everett *et al.*, 2002). There have been several attempts with Ich, one of which succeeded in keeping tomonts viable for several months in liquid nitrogen (Beeler, 1981). Although the tomonts divided into theronts following thawing, their infectivity was not tested.

Parasite Biochemistry and Molecular Biology

Very little is known about the physiological and nutritional requirements of either *Ichthyophthirius* or *Cryptocaryon*, except that they are obligate parasites that grow only by feeding on live fishes. Attempts to

culture *Ichthyophthirius* in artificial media have not been successful (Hlond, 1967; Beckert, 1975). Survival of theronts and tomonts can be extended in selected media, however (Ekless and Matthews, 1993).

A number of early studies examined the biochemical composition of the various developmental stages of *Ichthyophthirius*. Differential staining was used to identify glycogen, nucleic acids and proteins (MacLennon, 1935a; Yottenckar and Uspenskaya, 1964; Uspenskaya and Ovchinnikova, 1966). Glycogen, lipids and proteins accumulate in the cytoplasm of trophonts during the feeding period and diminish during the development of the theront. Differences in protein patterns exist in trophonts and infective theronts when examined by SDS-PAGE (Pyle and Dawe, 1985).

Molecular biology of the i-antigens of *Ichthyophthirius*

Parasite strains collected from the wild and maintained as clonal isolates express anywhere from one to three i-antigen polypeptides with apparent relative molecular masses (M_rs) of 40–60 kDa on SDS-PAGE (Clark *et al.*, 1995; Clark and Forney, 2003). Based on immobilization with specific antisera, five i-antigen serotypes, designated A–E, are presently known (Clark *et al.*, 1995). The majority of these (ten of 12 naturally occurring field isolates) fall into the three groups A, C and D. The most common serotype, namely D, has been identified in isolates ranging from China to the USA (Lin *et al.*, 2002). Cells that express the D serotype show a single i-antigen band of ~ 52/55 kDa following SDS-PAGE and Western blotting. The 52/55 kDa band has been shown to resolve into four or more isoelectric variants on two-dimensional gels, and genes encoding at least two distinct i-antigens have now been isolated from the G5 parasite strain (a representative of serotype D) (Lin *et al.*, 2002). The two genes (designated *IAG52A[G5]* and *IAG52B[G5]*) are co-expressed in the G5 isolate (although at widely different levels), and their products share roughly 40% identity in terms of primary sequence. Apart from D, A is the best-characterized parasite i-antigen serotype to date. Natural isolates obtained from Georgia (G1) and New York (NY1) are members of this group. These isolates express either two (G1) or three (NY1) i-antigen polypeptides based on SDS-PAGE and Western blotting, with the G1 proteins migrating at ~ 48 and 60 kDa size ranges (Lin and Dickerson, 1992; Wang *et al.*, 2002). The gene for the 48 kDa antigen has been sequenced (*IAG48[G1]*), and predicts a protein that shares 44% and 57% homology with the *IAG52A[G5]* and *IAG52B[G5]* gene products, respectively (Clark *et al.*, 1999). When the predicted sequences of all three proteins are compared, they differ most in the region between adjacent -C-X_2-C- motifs (particularly in the central repeats) and are most conserved at their carboxy-termini. As suggested for the *Paramecium* antigens (Preer *et al.*, 1987), the central repeats may harbour epitopes recognized by immobilizing antibodies.

Attempts to amplify the genes for the 48 and 52/55 kDa antigens using gene-specific primers and genomic DNA from heterologous serotypes have proved unsuccessful (Clark and Forney, 2003). Furthermore, the genes themselves fail to hybridize with DNA of heterologous i-antigen serotypes in Southern blots probed under conditions of high stringency (Dickerson *et al.*, 1993). Under low stringency conditions, the same genes recognize only a limited number of bands in Southern blots of DNA from homologous or heterologous strains, indicating that only a small number of i-antigen genes exist in any given isolate. This limited repertoire most probably arose from gene duplication and genetic drift involving point mutations and/or intra- or intergenic recombination. In the free-living ciliates, cells that express a particular serotype contain multiple i-antigen genes that are expressed in a mutually exclusive fashion under different sets of environmental conditions (Doerder *et al.*, 1996). There is little evidence that this occurs in *Ichthyophthirius*, however, and i-antigen expression in *I. multifiliis* appears to be highly stable. Clonal parasite isolates grown on fish have

been shown to maintain a given serotype for > 100 generations (H.W. Dickerson and T.G. Clark, unpublished). Furthermore, changes in steady-state levels of i-antigen transcripts have not been detected when infective theronts are exposed to different temperatures (temperature change is one of the principal stimuli leading to antigenic variation in free-living species (Clark and Forney, 2003). While there is little evidence for antigenic switching in *Ichthyophthirius*, the abundance of i-antigen transcripts varies significantly in development. Transformation from the host-associated (trophont) to the infective (theront) stage is accompanied by a dramatic increase in abundance of i-antigen mRNAs, with transcript levels reaching as high as 6% of total poly-A^+ RNA (Clark *et al.*, 1992). The fact that i-antigen transcript levels increase in parallel with the infectivity of the organism bears on the functional role of these surface proteins (Clark *et al.*, 1992).

Based on sequences obtained from three cloned genes, the i-antigens of *Ichthyophthirius* share a common structure, consisting of hydrophobic N- and C-termini that direct endoplasmic reticulum translocation and GPI-anchor addition, respectively, and a series of five to six imperfect tandem repeats of ~ 80 amino acids each spanning their length (Lin *et al.*, 2002). As is true of the i-antigens of the free-living ciliates, the repeats are characterized by the presence of periodic cysteine residues, which fall into register when the repeats are aligned. The parasite antigens contain six cysteines per repeat, which occur as -C-$X_{2,3}$-C- motifs within the larger-order framework -C-$X_{19–21}$-C-X_2-C-$X_{16–47}$-C-X_2-C-$X_{20–22}$-C-X_3. The spacing of the cysteine residues is consistent with the i-antigens being metal-binding proteins (Berg and Shi, 1996; Schwabe and Klug, 1996; Clark *et al.*, 1999; Clark and Forney, 2003). The epitopes recognized by immobilizing antibodies are destroyed by sulphhydryl reducing agents (Wang, 2001), and, based on studies of the *Paramecium* i-antigens (Preer *et al.*, 1987), there is good reason to believe that some or all of the cysteine residues within the *I. multifiliis* proteins are disulphide-linked. In addition to the -C-$X_{2,3}$-C- motifs, a number of other structural elements appear to be conserved among the parasite i-antigens, most notably, CP-X-G (T/A) sequences at the start of each repeat and a KKLTSGA domain just upstream of the C-termini in each protein (Lin *et al.*, 2002). The latter element may be a recognition sequence for the transamidase that adds the GPI anchor to the C-terminal residue of the mature protein (Clark *et al.*, 2001).

Immobilization-antigen coding sequences display the same non-standard codon usage employed in other genes from hymenostomatid ciliates (including *Paramecium* and *Tetrahymena*), where UAA and UAG stop codons specify glutamine instead. The existence of 18–25 such codons in each of the three i-antigen genes sequenced precludes their expression in conventional systems without extensive *in vitro* mutagenesis.

Diagnosis of Infection

Fishes infected with either *I. multifiliis* or *C. irritans* usually develop characteristic white spots on their surface (see section on clinical signs above). In mild infections the parasites are not readily seen. The parasites are more difficult to see on fishes with lightly pigmented skin. In early stages of infection, large numbers of theronts can actually kill a fish before the parasite becomes visible and death is caused by massive damage to the gill epithelia.

As mentioned earlier (see section above on host–parasite relationships), infected fishes often have behavioural changes caused by the irritation from parasites in the skin and gills. This behaviour is non-specific, however, and can also be associated with other bacterial, fungal and protozoan diseases.

To make a definitive diagnosis of ichthyophthiriasis or cryptocaryoniasis, it is necessary to microscopically examine tissue from a gill arch, a tail fin or the body surface. The large (200 to 800 µm) ciliated trophonts are easily seen in unstained wet mounts (× 10–40 magnification). The trophont of Ich

has a distinctive horseshoe-shaped nucleus, which is a pathognomonic sign of infection. In early or very heavy infections, theronts will also be seen. They are ciliated and pyriform in shape and can easily be confused with *Tetrahymena* spp.

The diagnosis of *Cryptocaryon* is also best made from a wet mount taken from the gills, skin scraping or fin sections. The presence of a large ciliated protozoan is indicative of cryptocaryoniasis. The nucleus of *C. irritans* is crescent-shaped and lobed.

Prevention and Control of the Parasites

Disease control and prevention in an intensive fish production operation depend upon an integrated management programme. The basic elements are: prevention of exposure of fishes to the parasites; prompt identification of the disease if it occurs; treatment of infected fishes; and immunization (if possible). Prevention of disease is always more cost-effective than treatment.

Ich and *C. irritans* infections are usually introduced into fish populations by the addition of infected fishes. In freshwater fish culture, no animals should be introduced into a facility unless they have been in quarantine for 2–3 weeks (Brown and Gratzek, 1980). If possible, the quarantined fishes should be held in moderately warm water (24°C). This is the optimal temperature for the development of *I. multifiliis* and in 2–3 weeks the parasite would have gone through several infection cycles. Thus, if the fishes were carrying low levels of infection, there would be ample time for it to become apparent. The water from the quarantine facility (pond, raceway, aquarium, etc.) should not be circulated into any other fish holding facility.

If an Ich infection develops during the quarantine, the fishes should be treated. Treatments that control the free-swimming theronts break the cycle of infection by preventing reinfection. In addition, it is also possible to treat trophonts that are on the fishes (see chemotherapy section, below).

Physical treatments without the use of chemicals

The objective for the treatment of a disease is to break the cycle of infection. In *I. multifiliis* infection, the most vulnerable stage is the free-swimming theront. The trophont is located deep in the epidermis of the fish and is protected from most agents that are added to the water.

The simplest treatment is reduction or removal of theronts. The repeated transfer of fishes to different aquaria is effective. Daily transfer for 5–7 days is usually sufficient to break the cycle of infection (Brown and Gratzek, 1980; Houghton and Matthews, 1990). In large fish culture operations, dilution is used if the fishes can be placed in a raceway. A rapid flow of water is maintained for a week to reduce the number of theronts.

In closed fish culture systems where the water is recycled, exposing the water to ultraviolet light can control Ich infections. The recycled water is passed through an ultraviolet light sterilizer and exposed to 91,900 μW/cm before being cycled back into the aquaria containing fish (Gratzek *et al.*, 1983).

Theronts are killed at temperatures higher than 30°C. Maintenance of fishes at these temperatures for a week will eliminate infection. The water temperature should be raised gradually to allow the fishes to acclimate to the change. This treatment is often combined with dilution. The use of elevated temperatures to control Ich infection is obviously unsuitable for coldwater fishes.

Chemotherapy

A variety of chemicals have been used for treating *I. multifiliis*, none of which is universally successful.

Sodium chloride was one of the first to be used (Stiles, 1894), where it was reported that theronts were killed instantly in saturated salt solutions. Stiles (1894) proposed creating a salt concentration gradient for use as treatment. This can be done by placing

salt below a slotted or mesh floor in the aquarium (Cross, 1972). The fishes tend to stay in the upper portions of the aquarium. When tomonts leave the fishes, they fall into areas of high salt concentration and are killed. Various concentrations of salt (7000–20,000 ppm) have been suggested for pond treatments (Kabata, 1985). In general, salt treatment does not rapidly control the infection and often does not completely remove the parasite from the environment. The use of salt may have an additional benefit, however. Fishes infected with *I. multifiliis* have decreased serum sodium and magnesium (Hines and Spira, 1974b), and sodium in the water may help fishes maintain osmotic balance, thus reducing stress.

Formalin is a common treatment. Fishes in aquaria are treated with 25 ppm (1 ml formalin to 10 gallons of H_2O) of formalin on alternate days until the infection is cleared. Usually the water is changed on days between treatments. Pond fishes are treated with 15 to 25 ppm (5 to 8 gallons of formalin/acre foot water). Bath treatments are also used; fishes are treated with 160–250 ppm formalin for 1 h daily until mortality stops (Brown and Gratzek, 1980). Care must be taken when formalin is used in ponds in hot weather. If the formalin kills the algae in the pond, the pond may be depleted of oxygen (Cross, 1972) .

Malachite green has also been used, either alone or in combination with formalin. The zinc-free oxalate salt of malachite green is the only form of the chemical that is effective against *Ichthyophthirius*. Fish in aquaria are treated with 0.1 ppm at 3- to 4-day intervals until the infection is cleared. Carbon filters in the aquaria should be removed during treatment. Aeration must be maintained (Brown and Gratzek, 1980). Pond fishes are also treated with 0.1 ppm at 3- to 4-day intervals. Bath treatments are done at 2 ppm for 30 min. Malachite green is a suspected carcinogen and must therefore be handled with care. It is not approved for use on food fishes in the USA because of the possibility of residual contamination in tissues. When malachite is used in combination with formalin, a stock solution is prepared which consists of 14 g of malachite green dissolved in 1 gallon of formalin. Fishes in aquaria are treated by adding 1 ml of stock solution to 10 gallons of water. The treatment is repeated three times with a 3-day interval between each treatment (Brown and Gratzek, 1980). Again, aeration must be maintained, and carbon filters removed. Raceways are treated (1 ml/10 gallons) for 6 h daily and flushed after each treatment. Ponds are treated at 3- to 4-day intervals. The mixture is usually very effective, especially in treating fishes in aquaria. The incorporation of malachite green into fish food has been reported to be effective against trophonts residing on the fish (Schmahl *et al.*, 1992a, 1996). The fish must be healthy enough to eat for this treatment to work.

Another chemical that has been successfully used is potassium permanganate. This is recommended for ponds (Brown and Gratzek, 1980). The dose is 2 ppm (or less) for scaleless fishes and 2–5 ppm for scaled fishes. The effectiveness of potassium permanganate is reduced by large amounts of organic matter in the water.

A number of other chemicals have been used; these include copper sulphate (Brown and Gratzek, 1980; Straus, 1993), acriflavine, quinine, mepacrine, mercury compounds and chloramine-T (Cross, 1972). These have not been consistently effective in controlling *Ichthyophthirius* infections.

Attempts have been made to kill trophonts on infected fishes. This approach has been used to eliminate *I. multifiliis* from fishes before they are introduced to a pond, lake, river, aquarium, etc. A major problem is that the mucus and overlying epithelium prevent access of chemicals to the parasite. Three approaches – hyperosmotic shock, vacuum infiltration and surfactant immersion – were tested as a means of delivering drugs to trophonts in the skin. None of the procedures was effective (Post and Vesely, 1983). Toltrazuril was found to reach trophonts embedded in the skin of fishes (Mehlhorn *et al.*, 1988). Immersion of fishes in water containing 10 µg/ml of the drug for 4 h (repeated daily for 3 days) killed fish-associated parasites. This treatment was only effective against trophonts and did not

kill theronts. Another triazinone, HOE 092 V, formulated as a water-soluble preparation, was also effective against trophonts in carp and cardinal tetras (*Paracheirodon axelrodi*) when applied once as a medicated bath at the same concentration and exposure time as toltrazuril (Schmahl *et al.*, 1992b). Quinine has also been used as a food additive (Schmahl *et al.*, 1996).

Control of *I. multifiliis* outbreaks requires good animal husbandry and management in addition to the use of therapeutic agents. Dead fishes should be removed as soon as they are found because trophonts begin to drop off and encyst within hours. Aquaria should be scrubbed and water recycled through a diatomaceous earth filter (Brown and Gratzek, 1980). This will dislodge and remove developing cysts and theronts from the aquaria. In raceways where the water flow can be increased, the bottoms should be swept daily. This will dislodge developing cysts and many will be flushed out in the water flow. In shallow ponds, sweeping the bottom will bury some cysts while others attached to the suspended bottom material can be flushed out by increasing the water flow (Brown and Gratzek, 1980).

Aquaria, ponds and raceways should be drained, cleaned and allowed to dry after an outbreak. Drying kills the parasite. The bottom of a dried pond should be treated with lime. If ponds cannot be drained completely, the residual water should be treated with a disinfectant such as calcium hypochlorite (Brown and Gratzek, 1980).

Immunization

The ideal method to prevent infection of fishes with *I. multifiliis* is prophylactic immunization. Prevention of disease is always preferred to treatment. At the present time, there are no practical, commercially available vaccines against Ich. A variety of components of *I. multifiliis* have been used to experimentally induce immunity in fishes, but, because *I. multifiliis* is an obligate parasite that can only be collected from live fishes, large-scale production of antigenic material for vaccines is extremely difficult.

Immobilization antigens have been identified as protective immunogens (Wang and Dickerson, 2002; Wang *et al.*, 2002), and their genes have been cloned (Clark *et al.*, 1995; Lin *et al.*, 2002). A promising system for expression of these genes is the use of *T. thermophila* (Gaertig *et al.*, 1999).

A cadmium-inducible metallothionein promoter allows high-level expression of *Ichthyophthirius* i-antigen genes in *Tetrahymena*. Plasmid constructs have been created in which the coding regions of i-antigen genes IAG48[G1] and IAG52A[G5] are placed downstream of the 1.9 kb 5′ untranslated region of MTT1 (T.G. Clark, unpublished results). Plasmid replacement vectors have been introduced into *T. thermophila* strain CU522 by biolistic bombardment. This strain is sensitive to growth in taxol by virtue of a mutation in the BTU1 gene (*btu1-1-K350M*), which permits targeted gene replacement into the BTU1 locus and selection with taxol (Gaertig *et al.*, 1999). Following transformation, resulting cell lines produce large amounts of recombinant protein in the presence but not in the absence of 5 μg/ml $CdCl_2$ (H.W. Dickerson, T.G. Clark and J. Gaertig, unpublished results). The respective antigens are clearly visible on the surface of *Tetrahymena* transformants by immunofluorescence microscopy. Expression from the *IAG52A[G5]* gene is approximately 1% of total cell protein. Preliminary evidence suggests that *T. thermophila* cells expressing Ich i-antigens elicit protective immunity when injected intraperitoneally into channel catfish (Wang, 2001). Work is under way to further develop this system for the expression and delivery of Ich protective antigens.

Prevention and control – *Cryptocaryon irritans*

As with *I. multifiliis*, the control of *C. irritans* in marine fishes depends upon breaking the cycle of infection. A variety of treatments have been used to control *C. irritans*.

A simple method is separation of fishes from the encysted tomonts before they develop into infective theronts. In small

aquaria transferring the fishes to clean aquaria every 3 days can do this. The tanks are cleaned and dried between uses (Colorni, 1987). Another method is to place the fishes in cages in the open sea for at least 10 days. The cages should be several metres above the sea floor. Tomonts fall to the bottom and the released theronts are unable to reach the fishes (Colorni, 1987). In large display tanks with both fishes and invertebrates, chemical treatment is not feasible. The following method was found to be useful. A 1–2 cm layer of clean, fine sand was spread uniformly on the bottom of the tank. After 3 days, the sand was removed with a suction dredge and replaced with a new layer of sand. The procedure was repeated four times at 3-day intervals (Colorni, 1987).

Chemotherapy

Many of the same chemicals used to treat *I. multifiliis* infections have been tried against *C. irritans*. One chemical that appears to work is copper sulphate. Immersion of fishes in 0.15–0.25 ppm copper sulphate for 3–10 days is recommended (Herwig, 1978). The treatment may have to be repeated several times. Brown and Gratzek (1980) suggested that continual exposure to copper (0.15 ppm) will control *C. irritans* infections. The solubility of copper is adversely affected by calcium carbonate in the water. It was suggested that citric acid or glacial acetic acid be added to chelate the copper to keep it in solution. This is questionable since the chelated copper may not be available for activity against the parasite (Herwig, 1978).

A mixture of cupric acetate (0.42 ppm), formalin (5.26 ppm) and Tris buffer (4.8 ppm) has been an effective treatment (Nigrelli and Ruggieri, 1966). Another variation is to use copper sulphate (0.15–0.2 ppm), citric acid and methylene blue stock (1 ml of 1% solution/2.5 gallons of water) until the water is a clear blue colour (Nigrelli and Ruggieri, 1966).

Quinine derivatives are effective drugs against *C. irritans*. Quinine hydrochloride, quinine sulphate and quinacrine hydrochloride (atebrine or mepacrine) kill theronts and can be used interchangeably at 8–12 mg/gallon. Quinine hydrochloride is soluble in salt water; quinine sulphate stays effective over a wide range of water pH; and atebrine is the least expensive (Herwig, 1978). During treatment the tanks should be protected from light, which inactivates the drugs. Fishes may occasionally show toxic reactions. The clinical signs of drug toxicity include loss of equilibrium, loss of appetite and rapid movement of gill opercula. Treatment of the water with 2–4 drops per gallon of a 0.2% stock solution of potassium permanganate solution will generally counteract the toxicity (Herwig, 1978).

Exposure of the parasite to either hypo- or hypersalinity has been proposed. Colorni (1985) reported that four repeated exposures of 3 h duration in 10% sea water cleared the infection in 5–8 days. Quinine-HCl and 45% sodium chloride had a synergistic effect when used to treat *C. irritans* on red snappers (Huff and Burns, 1981). Killifish were cleared of *C. irritans* in sea water diluted 1 : 1, 1 : 2 and 1 : 3 with distilled water (Cheung *et al.*, 1979). The reduced salinity appeared to affect the trophonts on the fishes as well as the encysted tomonts. The use of hyposalinity may be limited by the ability of the fishes to tolerate the low salt concentrations. Estuary fishes are more likely to tolerate low salt treatments than open-sea or reef-dwelling fishes.

A suggested treatment is as follows: an initial 5–15 min bath in fresh water followed by quarantine in a saltwater aquarium containing atebrine at 4–6 mg/gallon. The freshwater dip is repeated on the second day and the atebrine concentration increased to 8–12 mg/gallon. This routine is repeated for a total of 5 days. The fishes should be observed for an additional 10 days after treatment (Herwig, 1978).

Summary and Conclusions

I. multifiliis and *C. irritans* are parasitic ciliates of freshwater and marine fishes, respectively. Ichthyophthiriasis and cryptocaryoniasis are significant diseases in fish culturing systems. Outbreaks produce financial losses, resulting not only from fish mortalities, but also from the cost of control

measures and treatments. There are a variety of treatments or combinations of treatments available, none of which is ideal. An important area of research is the development of effective prophylactic control methods to prevent catastrophic outbreaks.

When available, immunization is one of the most cost- and time-effective means of preventing disease. There are significant problems hindering the development of practical vaccines against *I. multifiliis* and *C. irritans*, however. One of the major obstacles is that these ciliates cannot be grown in axenic culture, which precludes the large-scale culture of organisms for preparation of vaccines. Future research should address the problem of mass production of protective antigens. One solution is the development of *in vitro* cultivation systems for *I. multifiliis* and *C. irritans*, which will require basic studies on parasite physiology and biochemistry. The current and future resources provided by genomic technologies in ciliates are sure to provide advances in this area. The genome of the free-living ciliate *T. thermophila* is currently available for comparative studies (Turkewitz *et al.*, 2002), and an *Ichthyophthirius* expression sequence tag library is under construction (T.G. Clark, unpublished). A second solution would be the use of genetic engineering for the production of recombinant protective antigens in easily cultured organisms. The free-living ciliate *T. thermophila* is a promising system for the large-scale production of heterologous genes, and has already been successfully used to express the i-antigens of *I. multifiliis* (Gaertig *et al.*, 1999).

Further research is also necessary to understand the immune response against both parasites. Although it has been evident for some time that fishes develop protective immunity against *I. multifiliis*, the effector mechanisms are only now becoming elucidated. It is necessary to study further these responses in order to take logical approaches to vaccination. For example, the common antigens responsible for cross-protection among different serotypic strains of *I. multifiliis* remain to be characterized. Also, the pathways through which antigens are presented and processed at mucosal surfaces remain to be determined. Likewise, the mechanisms of antibody production and transport to mucosal surfaces require further study.

Methods to preserve and maintain infective organisms are needed. One of the problems in immunization trials is the lack of standard challenge methods. If viable parasites could be preserved by freezing, then standard isolates would be available for these purposes. Preservation techniques would benefit other areas of research as well. Collection and cryopreservation of isolates from different geographical areas, with different fish species and at different times would allow comparative studies. This could help to resolve taxonomic questions related to a number of species of *Ichthyophthirius* and *Cryptocaryon*. Also, the question of biotypes or strains and host specificity could be investigated in more detail.

Finally, a re-examination of the classification of *Cryptocaryon* is in order due to significant differences from *Ichthyophthirius* in morphology, development and genomic sequence.

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5 Trichodinidae and Other Ciliophorans (Phylum Ciliophora)

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Introduction

In the chapter on 'Trichodinids and other ciliates', Jiri Lom (1995) mentioned that ciliophorans are amongst the most common and widely distributed symbionts of fishes, either as parasites or as commensals. We now know a little more about ciliophorans that cause disease and mortality in fish, and also have methods to control them. He also stated that many facets of their existence are in urgent need of further study, and regrettably this has not changed in the last 10 years. Trichodinids are one of the most commonly encountered symbiont groups in the aquatic environment; however, much of the diversity of this and other ciliophoran groups is virtually unknown. What is worse, the relationship of many ciliophorans to their hosts is based on mere speculation. When a ciliophoran, however inoffensive it may be, is found growing massively on a fish, it is often automatically considered a pathogen and an appropriate 'osis' is then added. Also, with the exception of *Tetrahymena*, none of these ciliophorans has been cultivated *in vivo* and their metabolism and nutritional requirements are unknown.

The aim of this chapter is to update Lom (1995), as much of it is still relevant today. We would, however, like to place our stamp on the peritrichs, especially the trichodinids, as our research interest and expertise are in this group of ciliophorans. Furthermore, we shall also provide additional information on the African ciliophorans.

The major part of this contribution is on the trichodinids, with the focus on those associated with fish. The rest of the chapter deals with the other ciliophorans, separating them into those parasites commonly associated with fish and causing pathology (obligate fish parasites), commensals which seldom cause pathology (obligate fish commensals) and lastly free-living ciliophorans associated with fish which also cause disease (facultative fish parasites). Succinct but thorough information on ciliophoran morphology and ultrastructure may be found in Lee *et al*. (1985).

Trichodinids

The order Mobilida Kahl, 1933 (class Oligohymenophorea de Puytorac *et al*., 1974; subclass Peritrichia Stein, 1859) comprises mobile ciliated organisms with conical, cylinder-shaped bodies, of which the dominant feature is an aboral adhesive disc. This suborder is represented by three families, the Urceolariidae Dujardin, 1841,

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Trichodinopsidae Kent, 1881 and Trichodinidae Raabe, 1963. The former two are associated with invertebrates and they have simple plate-like denticles. The Trichodinidae all have complex structures in the aboral adhesive disc and are epizoics (sometimes endozoics) of a broad range of aquatic invertebrate and vertebrate hosts, with seven of the ten genera associated with fish. It is both the largest family in species numbers and the best known worldwide, especially those associated with fish. Van Leewenhoek in his letter in 1703 to the *Philosophical Transactions of the Royal Society* described 'some animalcula' associated with 'green weeds growing in the water'. After describing a *Hydra*, he remarked that this animal 'had her body laden with another sort of little creatures, whose shape was flat beneath and round above'. These 'little creatures' could be none other than *Trichodina pediculus* (O.F. Muller, 1786) Ehrenberg, 1838, the first description of a representative of the order Mobilida.

The cup- to cylinder-shaped body of a trichodinid (Fig. 5.1A) is covered by a thin membranelle, the pellicle. A spiral of ciliary rows on the adoral side (adoral zone) can make from one half a turn (180°) to

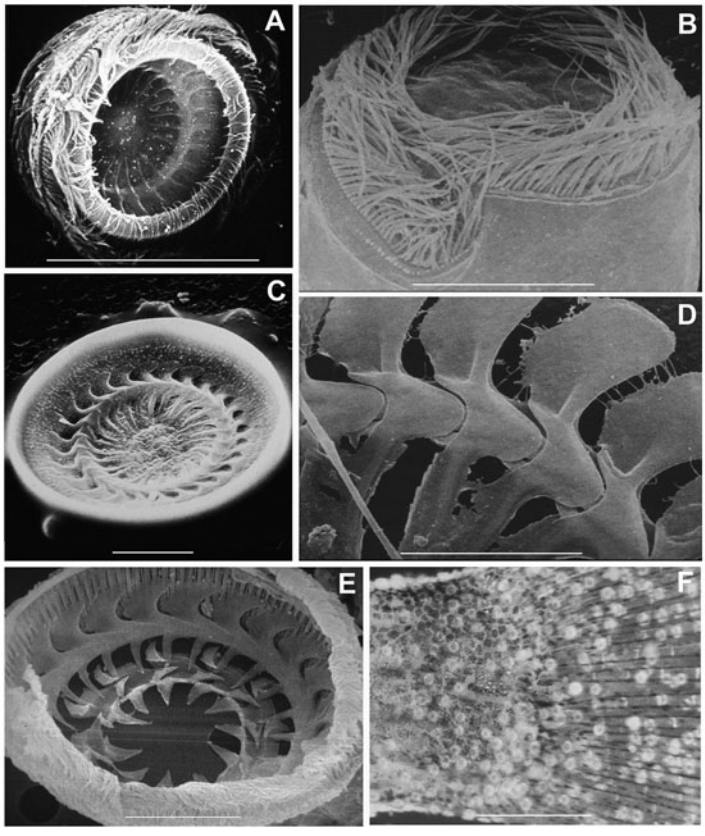


Fig. 5.1. SEM micrographs of *Trichodina heterodentata* (A–E). A. Aboral side with compound wreath of cilia used for movement. B. Oral view showing adoral spiral entering infundibulum. C. Denticle ring with radial and peripheral pins: with pellicle removed. D. Denticles after soft body has been removed, viewed aborally. E. Daughter cell after division, with old denticle ring in the process of resorption and new denticle ring already formed. Note the stronger old radial pins with thinner new pins in between. F. Young fingerling of *Oreochromis mossambicus* with a heavy infestation of trichodinids on the body. Scale bars = 20 µm (A–E), 1 mm (F). A–F originals.

three full turns around the oral surface and this organelle is used for feeding (Fig. 5.1B). The opposite side of the body contains a complex adhesive disc (Fig. 5.1C), comprising a skeletal ring. The ring consists of varying numbers of interlinking units, commonly referred to as denticles, which, although solid, consist of three distinct regions, i.e. the distal blade, a central part and a proximal ray (Figs 5.1D and 5.2A). The shape of the ray, in particular, forms the basis of differentiation between the genera, together with the structure of the adoral spiral. The most complex denticles are found within the largest genus, *Trichodina* Ehrenberg, 1838, where both the ray and the blade are well developed (Fig. 5.2A, B). The locomotory organelle consists of a compound wreath of oblique ciliary rows (Fig. 5.1A), varying between six and ten cilia per row, as well as a row of single cilia, the basal row, which is separated from the compound wreath by a septum. A third ring of cilia that is present in some species, called the marginal cilia, is located just adoral to the basal cilia. The nuclear apparatus consists of a macronucleus, normally horseshoe-shaped, very rarely ellipsoidal, as well as a small micronucleus visible in some species (Fig. 5.2C).

During binary fission, the buccal ciliature undergoes a rather intricate stomatogenic process (Lom, 1964). Division is initiated by the micronucleus, followed by a sequence of developments of both the macro- and the micronuclei. The first sign

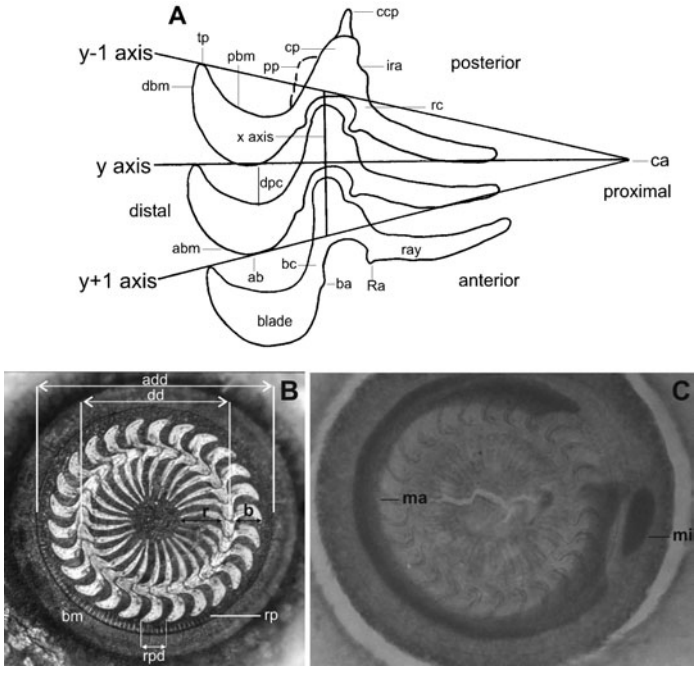


Fig. 5.2. A. Schematic drawing of denticles of *Trichodina magna* to illustrate the sequence and method of the description of denticle elements, according to the method of Van As and Basson (1989). B. Diagnostically important features in the adhesive disc of trichodinids. C. Diagnostically important features of the nuclear apparatus. Abbreviations: ab, blade apex; abm, anterior blade margin (surface); add, adhesive disc diameter; b, blade length; ba, blade apophysis; bc, blade connection; bm, border membrane with striations; ca, centre of adhesive disc; ccp, central conical part; cp, central part; dbm, distal blade margin (surface); dd, denticle ring diameter; dpc, deepest point of curve relative to apex; ira, indentation in lower central part; ma, macronucleus; mi, micronucleus; pbm, posterior blade margin (surface); pp, posterior projection; r, ray length; ra, ray apophysis; rc, ray connection; rp, radial pins; rpd, number of radial pins per denticle; tp, tangent point. B, C originals.

of division in the adhesive disc is the presence of a distinct band to the distal side of the blade, which impregnates in a different way in silver-impregnated specimens from the rest of the adhesive disc. This band has been associated with the formation of a new denticle ring and initially consists of thickened areas on each side of the radial pins adoral to the thatched band that connects the radial pins. Subsequently these areas on several radial pins will fuse to form a platelet. The adhesive disc separates into two semicircles, which then close to form two smaller discs in the daughter individuals. During division the number of denticles is reduced to half the original number. At this stage the thickened band consists of elongated platelets, clearly overlapping, which continue to extend in subsequent stages, appearing plaited. Each of these platelets will develop into a new denticle, first the central part, then the blade and lastly the ray, restoring more or less the number of denticles present in the parent individual. The old ring is gradually resorbed (Fig. 5.1E). Along with the development of the denticles, a completely new striated membrane is formed. The distal development of the blades from the platelets coincides with an extension of radial pins in this direction. In ectozoic species, such as *Trichodina heterodentata* Duncan, 1977, the extension is limited only from the central part in a distal direction, whereas in endozoic species, such as *Trichodina xenopodos* Fantham, 1924, the growth is in both directions, as the striated membrane extends from the tip of the ray to the border membrane. In young daughter cells the radial pins are newly formed, but are extensions of the original radial pins. At some stage before the complete resorption of the old denticle ring, an additional set of radial pins, each placed between existing pins, will develop (Fig. 5.1E). According to Lom (1973), these new radial pins originate from barren kinetosomes found between consecutive radial pins.

The development of the new border membrane and the complex hinge is still unknown. Coinciding with the denticle development, the nuclei undergo changes to gradually evolve into a horseshoe shape, even before resorption of the old ring is completed. The consecutive stages of development coincide with a gradual increase in the size of the cell. Conjugation has been studied in *Trichodina reticulata* Hirschmann and Partsch, 1955, where micro- and macroconjugants are found, not differing markedly in size. In the macroconjugant, a new ring of denticles is formed outside the resorbed old one and with the same number of denticle elements.

Since some trichodinid ciliophorans have been implicated in mortalities of wild as well as cultured fishes, they have been the subject of study for some considerable time. Although the first description dates back to 1838, it was only in 1958 that a standard taxonomic system, based on the description of the silver-impregnated specimens, was adopted (Fig. 5.2B). This system of Lom (1958) has more recently been expanded on by the present authors (Van As and Basson, 1989) in order to facilitate differentiation of closely related species (Fig. 5.2A). The taxonomy is largely based on the morphology of the denticles in the adhesive disc.

Various theories (Pénard, 1922; Zick, 1928; Precht, 1935; MacLennan, 1939; Davis, 1947; Richards, 1949; Haider, 1964) concerning the function of the adhesive disc and its elements have been put forward. Initially it was thought that the denticles protruded from the adhesive disc, inflicting damage on the host by their abrasive action when specimens rotate in one place, as stated by Lom (1973). This assumption was later discarded by the same author when it was shown that the entire body, including the adhesive disc, is covered by the pellicle. The view, however, prevailed that the denticle ring was in some way responsible for host damage as it formed part of the adhesive disc, which functions as a sucking cup. When trichodinids attach firmly to their host, epithelial cells are drawn into the adhesive disc. This disc, however, plays no part in feeding as the opening of the cytostome is situated on the adoral surface, with the spiral of cilia leading into the cytopharynx. These cilia create a current to facilitate transport of food particles into the cytostome. Electron microscope

studies revealed the complexity of the adhesive disc, which includes not only denticles but other elements, such as a striated membrane terminating in hinge-like structures, on which the border membrane with its radial pins articulates. All the elements within the adhesive disc are interlinked via a network of myonemes, which are attached to different parts of the body, thus providing contractile properties to the ciliophoran. Kruger *et al.* (1995) described the morphology of the denticles. Scanning electron microscope studies on this material revealed a remarkable analogy between the vertebrae in the spinal column of vertebrates (Van As and Basson, 1990).

Two distinct articulation surfaces can be seen, i.e. the apophysis of the blade and the apophysis of the ray (Fig. 5.1D). The central conical part tapers to a sharp point, fitting into the opening of the preceding denticle. The structure of these denticle parts suggests that restricted turning movements of individual denticles may be possible. As in the spinal column of vertebrates, where excessive bending in a dorsal or ventral direction is prevented by the neural spine on the dorsal side and the haemal spine on the ventral side, the blade and ray prevent distortion of the denticle ring. This implies that the adhesive disc will maintain its basic shape whilst allowing enough flexibility to attach to an uneven surface. An additional analogy between the denticle ring and the vertebral column is that the denticle ring not only serves as support for the body, but facilitates attachment of myonemes, which make body movement possible when these ciliophorans execute different movements, i.e. swimming free or attaching to the surface of its host.

The presence of internal body support in invertebrates and protists is not uncommon; even systems with articulating parts are found in some organisms. However, to our knowledge, the articulated denticle ring of trichodinids is the only example of a support system in unicellular organisms with the resemblance and analogy to the spinal column of vertebrates.

All trichodinids are essentially commensals. Ectozoic species use their host as a convenient substrate upon which they glide and to which they temporarily attach. They feed on waterborne particles and bacteria, as well as detritus particles from the fish surface. Trichodinids never occur in large numbers on a healthy fish and they also show over-dispersion under natural conditions. In a firmly attached *Trichodina*, the rim of the border membrane 'bites' into the surfaces of epithelial cells, and the surface it encircles is forcibly vaulted as by a sucker; these activities probably cause irritation to the fish. In a fish debilitated by some environmental factors or on fish larvae or young fry, the natural 'repellent ability' of the fish surface is impaired and the trichodinids proliferate (Fig. 5.1F). A massive number of trichodinids can, by their constant attachment and rotating movements, seriously damage the epithelial or epidermal cells. Under these circumstances the trichodinids behave like ectoparasites, feeding on disrupted cells and the associated bacterial growth. They may even penetrate into the gills or skin. In heavy infestations, ectozoic trichodinids may also occur in the rectum and cloaca (Richardson, 1938).

Heavily infested fish may exhibit a greyish-blue colour, formed by excessive mucus secretion and peeled epithelia, and frayed fins. The excessive epithelial growth is believed to be a protective reaction, but, at the same time, trichodinids feed on it. Debilitated fish are sluggish, swimming just beneath the water surface or near the water edge, and they cease feeding. Trichodinosis is frequently found in young fry, especially in the spring in freshwater fish stressed by harsh winter conditions and in fishes in freshwater or marine farms (Van As *et al.*, 1984).

Oldewage and Van As (1987) reported mortalities in an impoundment in South Africa where trichodinids were predominant on cichlids. The fish were stressed by high organic loads and low winter temperatures, where an abrupt cold spell resulted in annual winter kills. Heavy trichodinosis may cause losses of up to 50% of fish stocks. Growth inhibition is likely to be more common; however, this has very seldom been evaluated. Sanmartin Durán *et al.* (1991)

recorded a *Trichodina* sp. infestation on young cultured *Scophthalmus maximus* resulting in about 26% weight loss over a 12-month period.

Since the description of *T. pediculus* from *Hydra*, more than 260 species, representing ten genera, have been described. Seven of these genera are associated with fish. There are roughly 259 species from fishes, of which 178 are well-established valid species (confirmed using a silver-impregnated staining technique) from marine, estuarine and freshwater habitats. Representatives of *Trichodinella* (Raabe, 1950) Šrámek-Hušek, 1953 and *Tripartiella* Lom, 1959 are found exclusively on the gills of fishes. Some species of *Trichodina* occur on the gills, skin and fins, while many show preference for the gills and a minority live exclusively on the body surface. A small number of trichodinids (both *Trichodina* and *Paratrichodina* Lom, 1963 species) live inside the fish body, mostly in the urinary tract.

Most trichodinid species show very little host specificity (e.g. *Trichodina rectuncinata* Raabe, 1958 is on the gills of several marine fish families), while others are more host specific (e.g. *Tripartiella orthodens* Basson and Van As, 1987) (Van As and Basson, 1987). Some species, such as *Trichodina jadranica* Raabe, 1958, occur on both marine and freshwater fish hosts. Trichodinids also have a worldwide distribution, probably via transcontinental introductions of fish. Common species such as *Trichodina nigra* Lom, 1961, *Trichodina mutabilis* Kazubski and Migala, 1968, *Trichodina acuta* Lom, 1961, *T. heterodentata* Duncan, 1977 and *Trichodinella epizootica* (Raabe, 1950) are found on most families of fishes in Europe, Asia, America and Africa (Lom and Dykova, 1992).

GENUS *TRICHODINA* EHRENBERG, 1838. The denticles consist of well-developed blades, rays and central parts. The blades are flat, straight or often semicircular and the rays are either spine- or needle-shaped or rod-like and of various lengths. The central parts lack anteriorly directed projections. The adoral spiral is of various lengths, usually 360° (in a few cases only 330°) or as much as 540°.

Since the original description of the type species from *Hydra*, more than 200 species have been described from the skin, fins, gills and urinary bladder of fishes and amphibians and also from the integument of invertebrates. Of these species, only approximately 141 are considered as valid. Representatives of this genus have been reported from all continents except Antarctica. The majority of species are ectozoic.

Trichodina acuta Lom, 1961 (Fig. 5.3A) is a widely distributed freshwater species found mainly on the skin, rarely on the gills, of most families of fishes in Eurasia, North America, the Philippines and Africa. It has a clear centre in the adhesive disc (impregnates with a clear central circle). The denticles (18 to 23 in number) characteristically have strong, curved rays. The adhesive disc ranges from 35 to 60 µm and the denticle ring is from 22 to 36 µm.

Trichodina centrostrigeata Basson, Van As and Paperna, 1983 (Fig. 5.3B) is found mainly on the gills of a number of freshwater fish species, but is mostly associated with cichlids in Africa, Taiwan and the Philippines. The centre of the adhesive disc contains 11 to 16 separate centre ridges. The adhesive disc ranges from 31 to 48 µm, the denticle ring from 19 to 33 µm and the number of denticles from 23 to 30.

Trichodina compacta Van As and Basson, 1989 (Fig. 5.3C) is found on the skin and fins of several freshwater fish families in southern Africa, Israel and Taiwan, but shows a clear preference for cichlids. A clear central circle is present. The adhesive disc ranges from 29 to 49 µm and the denticle ring from 17 to 30 µm, with 15 to 21 denticles.

Trichodina fultoni Davis, 1947 (Fig. 5.3D) is a widely distributed pathogenic freshwater parasite of cyprinid, perciform, centrarchid, anguilliform and other fishes in Eurasia and North America. The clear centre in the adhesive disc has argentophilic spots. The adhesive disc ranges from 62 to 82 µm, the denticle ring from 41 to 55 µm and the number of denticles from 27 to 31. Pathogenicity at 12°C was established for

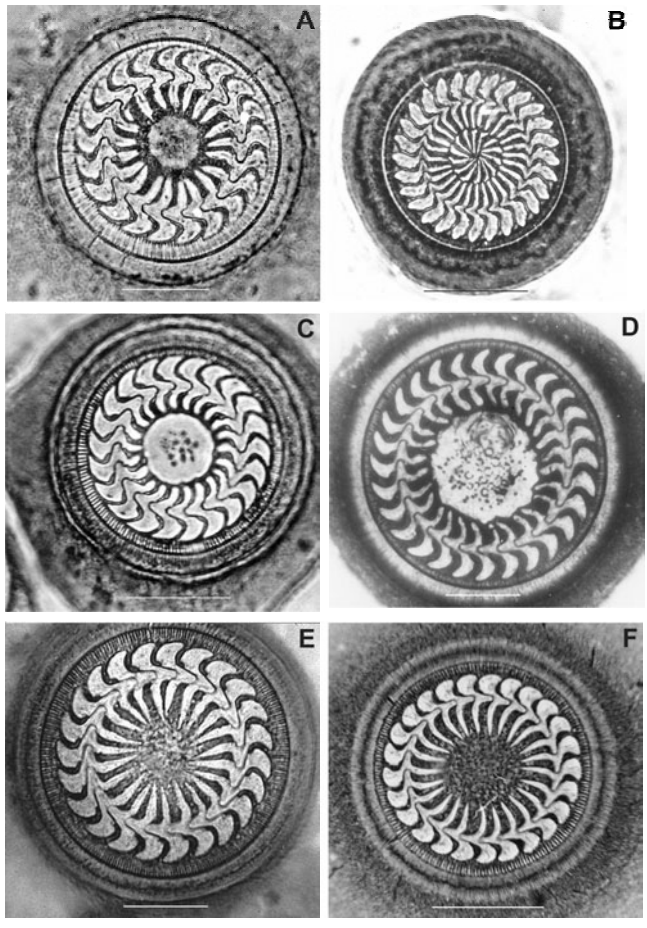


Fig. 5.3. Silver-impregnated adhesive discs of *Trichodina* species. A. *T. acuta,* B. *T. centrostrigeata,* C. *T. compacta,* D. *T. fultoni* (courtesy of Dr Lom), E. *T. heterodentata,* F. *T. maritinkae*. Scale bars = 20 µm. A–C, E, F originals.

several North American fishes and cultured eels (Hoffman and Lom, 1967; Markiewicz and Migala, 1980).

Trichodina heterodentata Duncan, 1977 (Fig. 5.3E) was originally described from cichlids in the Philippines and has since been recorded from the skin, rarely the gills, of various freshwater fish families in southern Africa, the Middle East, Taiwan and South America, showing a distinct preference for cichlids. The adhesive disc ranges from 38 to 82 µm, the denticle ring from 23 to 51 µm, and the number of denticles from 20 to 30.

Trichodina jadranica Raabe, 1958 was originally described from the gills of the marine fish *Mullus barbatus* and later from other marine fishes. It is also a common species on the gills of freshwater fishes, as well as a pathogen in eel cultures. It is rather a small ciliophoran, the adhesive disc ranging from 18 to 35 µm, with 15 to 27 denticles in the denticle ring.

Trichodina maritinkae Basson and Van As, 1991 (Fig. 5.3F) seems to be restricted to representatives of the family Clariidae. It has been reported from Africa and Taiwan. The adhesive disc ranges from 31 to 50 µm and the denticle ring from 21 to 32 µm, with 22 to 32 denticles.

Trichodina murmanica Poljanski, 1955 (Fig. 5.4A) is a marine species with a clear centre in its adhesive disc. It is common in

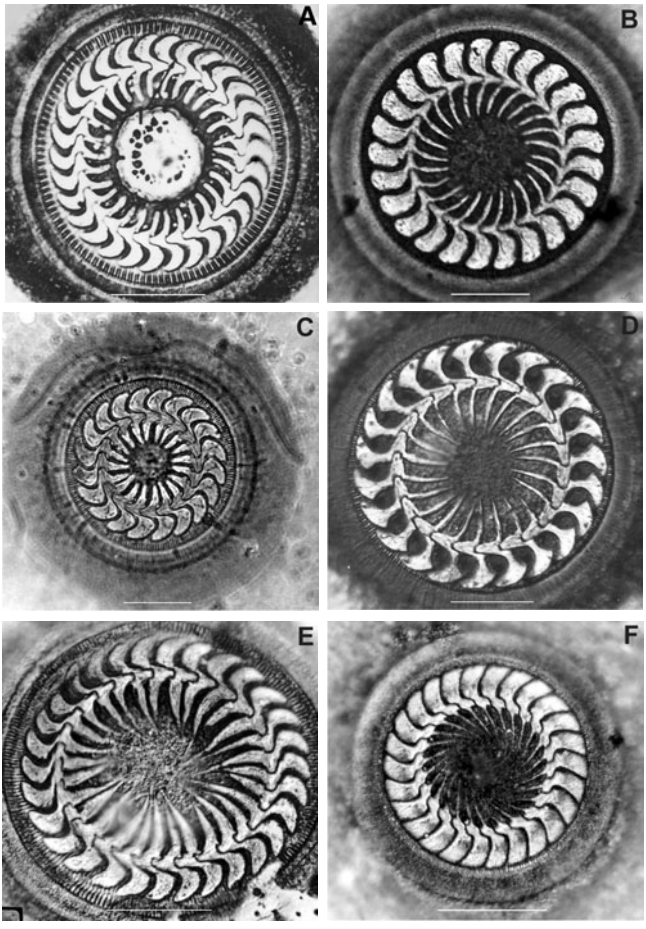


Fig. 5.4. Silver-impregnated adhesive discs of *Trichodina* species. A. *T. murmanica* (courtesy of Dr Lom), B. *T. mutabilis*, C. *T. nigra*, D. *T. nobilis*, E. *T. pediculus* (courtesy of Dr Lom), F. *T. perforata* (courtesy of Dr Lom). Scale bars = 20 μm. B–D originals.

the North Atlantic, Bering and other northern seas, where it occurs on the skin of *Gadus morhua* as well as coastal gadid and perciform fishes. The adhesive disc and denticle ring range from 40 to 70 μm and from 41 to 55 μm, respectively, and the number of denticles from 25 to 31.

The pathogenic potential of this and other marine species has not been studied in maricultures.

Trichodina mutabilis Kazubski and Migala, 1968 (Fig. 5.4B) is commonly found on the skin and gills of the common carp. It also occurs on several other fish families in Eurasia, America, the Philippines and Africa and exhibits great morphological variability (Kazubski and Migala, 1968). The adhesive disc ranges from 37 to 74 μm, the denticle ring is from 29 to 46 μm and the number of denticles ranges from 21 to 32.

Trichodina nigra Lom, 1961 (Fig. 5.4C) is a very common fish trichodinid found on the skin and gills of many freshwater fish families in Eurasia, Africa and the Philippines. It shows some preference for cyprinid hosts, but is also found on various cichlid and perciform hosts. Its morphology is very variable and some of the populations may be separate species. The diameter of the adhesive disc ranges from 32 to 65 μm

and the diameter of the denticle ring from 19 to 39 µm, with 18 to 29 denticles.

Trichodina nobilis Chen, 1963 (Fig. 5.4D) is common on the skin and rare on the gills of mostly cyprinid fish in China, Japan, Taiwan and the Philippines. It was introduced into Europe. A characteristic of this species is deep notches in the proximal anterior border of the blade. The adhesive disc ranges from 61 to 69 µm, the denticle ring ranges from 37 to 40 µm, and the number of denticles from 24 to 28.

Trichodina pediculus (O.F. Müller, 1786) Ehrenberg, 1838 (Fig. 5.4E) has a cosmopolitan freshwater distribution; it is common on the skin of adult fish and fish fry in Eurasia and America. It is disc-shaped or hourglass-shaped in lateral view. The parasite has extremely long, needle-pointed rays. Its adhesive disc ranges from 46 to 68 µm, the denticle ring from 28 to 38 µm and the number of denticles from 20 to 32.

Trichodina perforata Lom, Golemansky and Grupcheva, 1976 (Fig. 5.4F) lives on the gills of *Cyprinus carpio* and *Carassius carassius* in Eurasia. It has deep notches or even holes in the denticle blades. The adhesive disc ranges from 37 to 50 µm, the denticle ring from 23 to 29 µm and the number of denticles from 23 to 26.

Trichodina rectuncinata Raabe, 1958 (Fig. 5.5A) occurs on the gills of marine gobiid, blenniid, perciform and syngnathiid hosts. This species shows a lot of variability, suggesting the existence of more than one species.

Trichodina reticulata Hirschmann and Partsch, 1955 (Fig. 5.5B) shows specificity for *C. carassius* and *Carassius auratus* throughout its distribution in Eurasia, North America and the Philippines. The centre of the adhesive disc contains cell-like structures; the range of the adhesive disc is 31 to 57 µm, that of the denticle ring is 20 to 36 µm and the number of denticles ranges from 21 to 34. It causes disease and mortality in *C. carassius*, *C. auratus* and *C. carpio* (Ahmed, 1976, 1977).

Trichodina truttae Mueller, 1937 (Fig. 5.5C) is one of the largest species in the genus and a well-known pathogen of salmonids (usually on the skin, rarely on the gills) in hatcheries in North America and the former USSR. The adhesive disc ranges from 76 to 137 µm, the denticle ring from 45 to 72 µm and the number of denticles ranges from 27 to 34. The morphology was studied using scanning electron microscopy (Arthur and Margolis, 1984).

Endozoic trichodinids, with the exception of two species (*Trichodina fariai* da Cunha and Pinto, 1928 and *Trichodina luba* Basson, Van As and Fishelson, 1990), live in the urinary tract, where they are found rolled up to fit into the narrow lumen of the urinary bladder to the collecting ducts in the kidney. They may cause extensive desquamation of the epithelium; however, a diseased condition has not been observed and the renal damage to the fish has not been assessed. Their mode of transmission is unknown. It is assumed that they are discharged with the urine and somehow find their way to the urinary bladder of another fish.

Endozoic trichodinids as a rule tend to be more host specific than most of the ectozoic species and are not restricted to the genus *Trichodina*. Also, they have numerous and closely spaced denticles with narrow blades.

Trichodina luba Basson, Van As and Fishelson, 1990 is found in the colon of the marine fish, *Acanthurus xanthopterus* (South Africa, Hawaii and New Guinea). The adhesive disc ranges from 30 to 40 µm and the denticle ring from 18 to 29 µm, with 26 to 37 denticles.

Trichodina oviducti Poljanski, 1955 is the largest trichodinid. The adhesive disc ranges from 88 to 271 µm, and the denticle number ranges from 43 to 60. It commonly infects the urogenital system, including oviducts, copulatory sacs and seminal grooves of skates (*Raja* spp.) in the Barents Sea and northern Atlantic. It is transmitted venereally and heavy infection is associated with yellowish mucoid exudates (Khan, 1972; morphology – Khan *et al.*, 1974).

Freshwater endozoic species have marginal cilia and a well-developed velum. Many of them have structures in the centre of the adhesive disc. *Trichodina polycirra* Lom, 1960 lives in the urinary tract of *Rutilus*

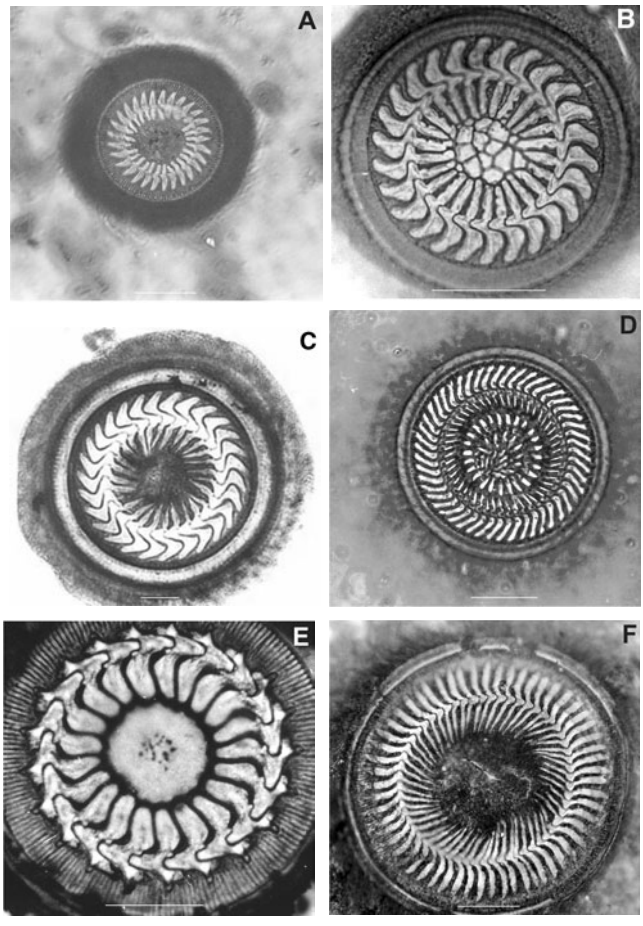


Fig. 5.5. Silver-impregnated adhesive discs of *Trichodina* species. A. *T. rectuncinata,* B. *T. reticulata*, C. *T. truttae* (courtesy of Dr Lom), D. *T. uretra*, E. *Hemitrichodina robusta*, F. *Vauchomia renicola* (courtesy of Dr Lom). Scale bars = 20 µm. A, B, D, E originals.

rutilus and *Abramis brama*. The adhesive disc ranges from 48 to 58 µm and the denticle number from 38 to 55.

Another freshwater species, *Trichodina urinaria* Dogel, 1940 has a holarctic distribution and is found in the urinary tract of *Perca fluviatilis*, *Perca flavescens*, *Stizostedion lucioperca* and *Notropis cornutus*. The adhesive disc ranges from 40 to 53 µm and the denticle number from 29 to 40. Experimental infection of *Notemigonus crysoleucas* was accomplished by keeping fish in aquaria with infected yellow perch (Li and Desser, 1983).

Trichodina uretra Basson, 1989 (Fig. 5.5D) lives in the urinary tract of the freshwater *Barbus trimaculatus* (South Africa). The adhesive disc ranges from 42 to 65 µm and the denticle ring from 27 to 48 µm, with 47 to 54 denticles.

GENUS *HEMITRICHODINA* BASSON AND VAN AS, 1989. The denticles have well-developed rays and central parts, but the blades are reduced. The adoral spiral describes an arc of more than 360°, but less than two full circles.

So far only one species has been described in this genus. It occurs on the skin and fins, occasionally also on the gills of freshwater fishes, and was reported from southern Africa.

Hemitrichodina robusta Basson and Van As, 1989 (Fig. 5.5E) has been reported from the body surface and fins of predominantly mormyrids (Mormyridae) in South Africa and Namibia. The adhesive disc ranges from 43 to 70 µm and the denticle ring from 27 to 45 µm, with 18 to 24 denticles. This species has a clear centre in the adhesive disc and is sparsely dotted, with extremely wide, well-developed rays and the blades reduced to small projections.

GENUS *VAUCHOMIA* MUELLER, 1938. These large ciliophorans have hemispherical to conical bodies. The striated border membrane consists of a ring of rays and a ring of myonemes, with these two rings fused. The denticles consist of blades, central parts and rays. The adoral spiral completes 2.5 to 3 full circles.

Two species are known, both from the USA, where they occur as endoparasites in the urinary canal of freshwater fish.

Vauchomia renicola (Mueller, 1931) (Fig. 5.5F) lives in *Esox niger* (USA) and has a flat body. The adhesive disc can be up to 97 µm in diameter, with 44 to 65 denticles, the highest number among trichodinids.

GENUS *PARATRICHODINA* LOM, 1963. These trichodinids are small and the body is shaped in a flat hemisphere. The denticles have well-developed rays and are wedged together only by the central parts. The anterior projection may be present or absent. If present, the projection is situated near the base of the blade and is not in contact with the notch in the blade of the preceding denticle. The adoral spiral makes a turn of 150–280°.

So far 11 species have been described in this genus, of which eight have been collected from the gills of freshwater and marine fishes and three from the urinary tract of fishes. Eight species occur in Eurasia, two in the USA and one, *Paratrichodina africana* Kazubski and El-Tantawy, 1986, in Africa.

Paratrichodina corlissi Lom and Haldar, 1977 (Fig. 5.6A) occurs on the gills of *Gobio* species in Eurasia and has a shallow notch in the blade. The adhesive disc diameter ranges from 19 to 25 µm, with 18 to 24 denticles.

Paratrichodina incissa (Lom, 1959) Lom, 1963 (Fig. 5.6B) is common on the gills of many fish species, with a clear preference for cyprinids in Eurasia. The denticle has a deep notch in the anterior blade margin. Its adhesive disc ranges from 16 to 31 µm, with 19 to 31 denticles.

GENUS *TRIPARTIELLA* LOM, 1959. These small trichodinids have hemispherical bodies. The delicate central part of the denticle bears a straight ray, directed backwards in most cases. The blades are slanted more or less obliquely backwards; their narrow bases, by which they join the central parts of the denticles, extend anteriorly in projections, which may be short and thin or wide and knee-like. These projections fit well into the corresponding notch in the preceding denticle. Thus the denticles are wedged together both by the central conical parts and by the projections. The adoral spiral makes a turn of 180–290°.

Representatives of this genus are all gill parasites of freshwater fishes, with 26 species described, of which 17 are considered as valid species from Eurasia, the USA, the Far East and Africa.

Tripartiella cichlidarum Basson, Van As and Paperna, 1983 (Fig. 5.6C) is found on cichlids from Israel. The adhesive disc ranges from 22 to 30 µm and the denticle ring ranges from 11 to 15 µm, with 30 to 32 denticles.

Tripartiella clavodonta Basson and Van As, 1987 (Fig. 5.6D) shows a clear preference for the gills of cichlids in South Africa and the Philippines. The adhesive disc ranges from 15 to 20 µm and the denticle ring from 9 to 12 µm, with 18 to 24 denticles.

Tripartiella copiosa Lom, 1959 is a widely distributed species in Eurasia, known from about 40 fish species, mainly cyprinids. It has rectangular backward-slanted blades, with a spike-like anterior projection. The adhesive disc diameter ranges from 14 to 31 µm, with 19 to 33 denticles.

Tripartiella orthodens Basson and Van As, 1987 (Fig. 5.6E) occurs on the gills of *Tilapia rendalli*, a cichlid in South Africa.

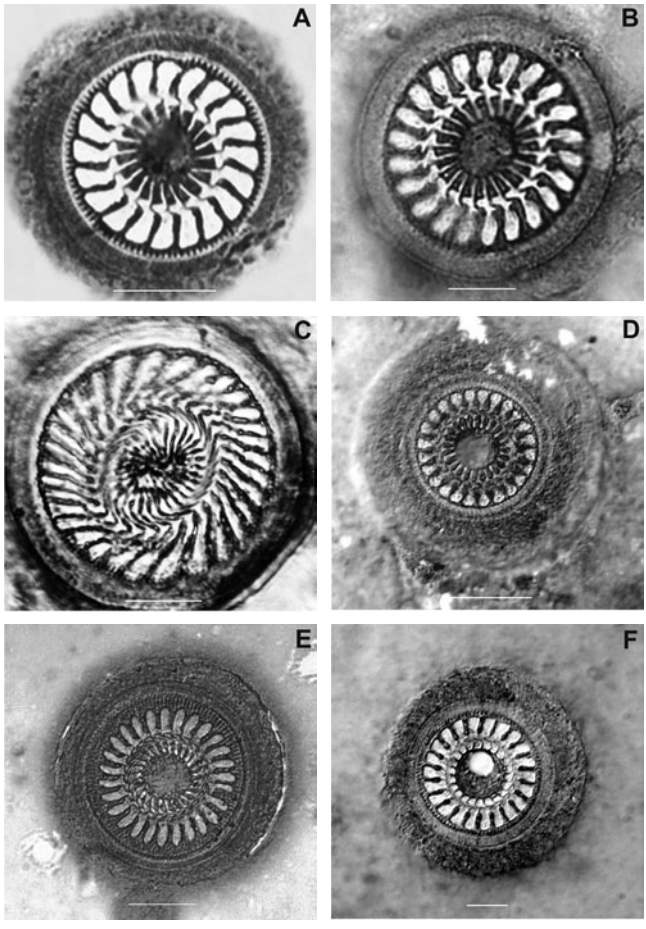


Fig. 5.6. Silver-impregnated adhesive discs of various trichodinid species. A. *Paratrichodina corlissi* (courtesy of Dr Lom), B. *Paratrichodina incissa* (courtesy of Dr Lom), C. *Tripartiella cichlidarum*, D. *Tripartiella clavodonta*, E. *Tripartiella orthodens*, F. *Trichodinella epizootica*. Scale bars = 10 μm. C–F originals.

The adhesive disc ranges from 23 to 31 μm and the denticle ring ranges from 13 to 16 μm, with 24 to 28 denticles.

GENUS *TRICHODINELLA* (RAABE, 1950) ŠRÁMEK-HUŠEK, 1953. The denticles have a delicate central part, whose anterior border extends in a projection fitting well into a notch between the central part and blade of the preceding denticle. Therefore the denticles are wedged together by both the central parts and the projections. The ray is reduced to form a short delicate hook curved along the central part. The adoral spiral performs an arc of 180–270°.

Only five species are currently recognized as valid, and as many as nine are probably synonymous with *T. epizootica*. They are mainly parasites of freshwater fishes, but sometimes marine fishes are also infested, where they occur exclusively on the gills. Representatives of this genus have been reported from Eurasia, Africa, the Middle East, Mexico, the USA and the Philippines (Lom and Dykova, 1992).

Trichodinella epizootica (Raabe, 1950) (Fig. 5.6F) is one of the most widely distributed species of freshwater trichodinids in Eurasia, but is also found in Africa and the Middle East on about 90 fishes from various

families. This parasite proliferates massively on stressed fish and becomes highly pathogenic. The adhesive disc ranges from 14 to 47 µm, with 16 to 30 denticles.

Trichodinella lawleri Lom and Haldar, 1977 is a marine species that can be pathogenic in aquarium fishes.

GENUS *DIPARTIELLA* SHTEIN, 1961. The denticle ring is composed of tightly packed denticles, consisting only of well-developed triangular blades and weakly developed central parts, which do not extend into conical protrusions. The adoral spiral describes an arc of about 270°.

This genus comprises a single species and it occurs on the gills of marine fishes from Eastern Europe and Asia.

Dipartiella simplex (Raabe, 1959) Shtein, 1961 was originally found on the gills of *Gobius niger* in the Baltic Sea. Some authors have considered this species as a mistaken identification of a *Trichodinella*, but it has been encountered recently in two cultured marine fishes from China (Xu *et al.*, 1999), as well as from Egypt (L. Basson, unpublished data).

Other Ciliophorans

OBLIGATE PARASITES

GENUS *CHILODONELLA* STRAND, 1926. The genus *Chilodonella* Strand, 1926 comprises many free-living species and two species infesting freshwater fishes. Both the latter species have cosmopolitan distributions, occur in estuarine and brackish waters (e.g. in the eastern Baltic Sea) and appear to infest most, if not all, teleost fishes. They cause the well-known chilodonellosis, a disease affecting the skin and gills, especially in fish cultures.

These ciliophorans belong to the family Chilodonellidae Deroux, 1970, order Cyrtophorida Fauré-Fremiet in Corliss, 1956 and class Kinetophragminophorea de Puytorac *et al.*, 1974.

The body of *Chilodonella* is oval and dorsoventrally flattened. The slightly convex dorsal side is without cilia, with just a short anterior ciliary row. The flat or slightly concave ventral surface has a ciliature reduced to two longitudinal belts close to the margins of the body. Three short oral kineties are found at the ventrally located buccal opening or cytostome, each comprising two rows of kinetosomes, of which only the outer row is ciliated. A conspicuous cytopharyngeal apparatus forms a circlet made up of a few large, rod-like nematodesmata surrounding the cytopharyngeal tube. The nuclear apparatus consists of a single macronucleus and a single micronucleus. An overview of the ultrastructural morphology of *Chilodonella* may be found in Soltynska (1971).

Chilodonella piscicola (Zacharias, 1894) Jankovski, 1980 (Fig. 5.7A) (synonym: *Chilodonella cyprini* (Moroff, 1902, see Shulman and Jankovski, 1984)) has an asymmetrically oval body, with a clear notch in the posterior margin, 55 µm × 43 µm (range 30–80 µm × 20–62 µm). There are mostly eight to 11 (range 7–15) kineties in the right arched ciliary band, and 12 to 13 (range 8–14) in the straight left band. The cytopharynx is curved at its inner end and reinforced by 14 to 16 nematodesmata. It may be slightly extruded for boring into and disrupting epithelial cells of the host. Two contractile vacuoles are present, one anteriorly at the right and the second posteriorly at the left. The ciliophoran survives for a period of time when removed from the host (24 h at 5°C, 1 h at 20°C). A small number may encyst and the cysts may last for a long time.

Chilodonella hexasticha (Kiernik, 1909) Kahl, 1931 (Fig. 5.7B) differs from the preceding species by the absence of a notch at the posterior body margin and in less numerous and more loosely spaced kineties (five to seven in the right, seven to nine in the left ciliary band). It is smaller (30–65 µm × 20–50 µm) and its distribution is less widespread than that of *C. piscicola*. The latter tends to infest fingerlings more than adult fish, while *C. hexasticha* prevails on older fish.

Both species may occur simultaneously on the same host. Most of the information on their biology and relationships to fish

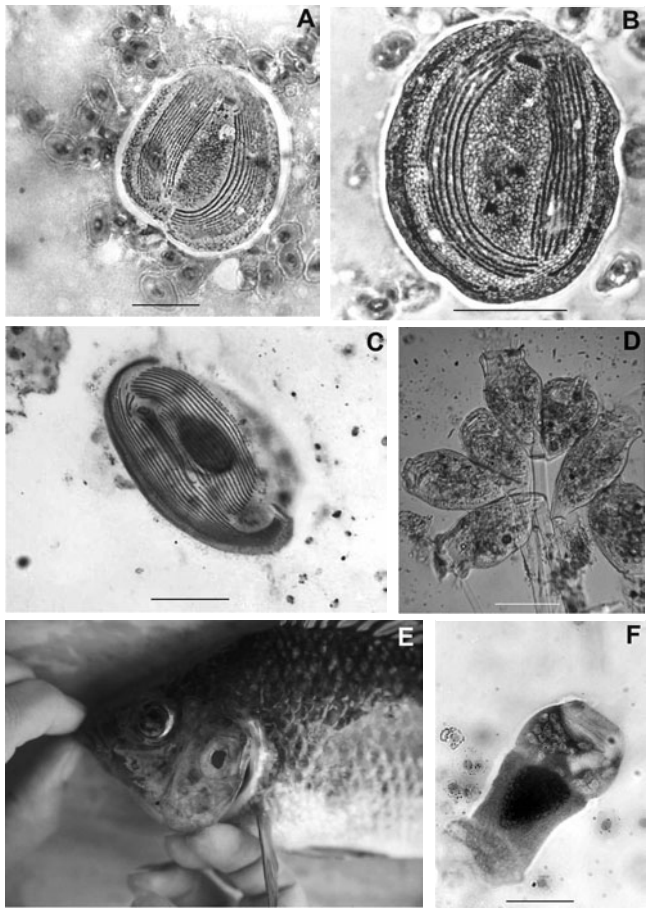


Fig. 5.7. Silver-impregnated ventral surfaces of A. *Chilodonella piscicola*, B. *Chilodonella hexasticha*, C. *Brooklynella hostilis* (C – courtesy of Prof. Lom), D. Live colony of *Epistylis transvaalensis* with feeding zooids, E. Round hole in operculum of *Oreochromis mossambicus* formed in presence of huge colonies of *E. transvaalensis*, coinciding with bacterial and fungal infections, F. *Apiosoma dermatum*, stained with haematoxylin, showing adoral cilia and nuclear apparatus. Scale bars = 20 μm. A, B, D–F originals.

was collected at a time when their specific distinction was not recognized. Prost (1952) was the first to confirm that they are indeed distinct species. The data on their distribution in Eurasia came from this period, while more recent records on the occurrence of *Chilodonella* in South Africa, Israel, the USA, Australia and Malaysia identified the species *C. hexasticha* (Hoffman *et al.*, 1979; Paperna and Van As, 1983; Shariff, 1984; Langdon *et al.*, 1985); *C. cyprini* has also been reported in Japan recently by Imai *et al.* (1985) and Urawa and Yamao (1992).

Chilodonellosis occurs throughout a wide temperature range. Mass infestations with *C. piscicola* may be found from slightly above zero (e.g. at 4°C) to slightly above 20°C, while *C. hexasticha* is known to cause morbidities even at 26 to 31°C (Shariff, 1984). Chilodonellosis accounts for the greatest economic losses due to a single disease in commercial tropical fish stores (Leibovitz, 1980). Since chilodonellosis has mostly been reported at low temperatures when fish are under stress, an assumption prevailed that chilodonellas prefer colder waters and basically only

occur in commercial set-ups. There is, however, a report on massive mortalities due to chilodonellosis (*C. hexasticha*) in free waters. This took place among native fish (bony bream) in Australia in the winter, where the temperature was 8–13°C (Langdon *et al.*, 1985). Unpublished information of the present authors also indicates that chilodonellosis does occur readily in warmer waters; we observed mortalities among cichlids on a fish farm in the Okavango River (Botswana) at water temperatures exceeding 25°C.

Under favourable conditions (e.g. in fish and especially fingerlings that are debilitated in early spring or by overcrowding), chilodonellas may virtually cover the body surface in a continuous layer. They disintegrate the body surface using their oral cytoskeletal apparatus and feed on cell debris.

Clinical signs of heavy infestations include increase of mucus, with an overall dark, slimy, patchy or mottled grey appearance. These films of mucus and cellular debris may become detached from the skin surface. Moribund fish may also show signs of hypoxia and uncoordinated swimming, are emaciated and have opaque eyes and abrased skin.

Pathogenesis in skin lesions has not been studied, but has been described in detail in gill infestations with *C. hexasticha* (Paperna and Van As, 1983; Shariff, 1984; Langdon *et al.*, 1985). *Chilodonella* initially cause localized hyperplasia of the gill epithelium, which later becomes more generalized. Proliferating epithelial cells fill the spaces between secondary lamellae, which may fuse together and coalesce into a single mass. The thin respiratory epithelium is covered by the hyperplastic epithelium and this drastically reduces the respiratory surface of the gills. The epithelium may be infiltrated with lymphocytes and eosinophil granulocytes, with an additional increased proliferation of mucus and chloride cells.

The hyperplastic epithelium may undergo changes, with dilatation of capillaries, oedema, petechia and haemorrhages. Complete destruction of the epithelium of primary and secondary lamellae may leave the fish with only cartilaginous rays. Pathological manifestations may vary, depending on the intensity of infestations. Sometimes large aggregates of melanin can also be seen along some of the primary lamellae undergoing degeneration. Disintegration of the gills and their necrosis render the gills non-functional. Fish lose osmotic balance and suffocate; this is manifested by their increased sensitivity to oxygen deficiency. Heavily infested fish die.

GENUS *BROOKLYNELLA* LOM AND NIGRELLI, 1970.

Brooklynella hostilis Lom and Nigrelli, 1970 (class Kinetophragminophorea de Puytorac *et al.*, 1974; subclass Hypostomata Schewiakoff, 1896; order Cyrtophorida Fauré-Fremiet in Corliss, 1956; family Hartmannulidae Poche, 1913) causes brooklynellosis, a severe disease of marine fishes in sea aquaria and in maricultures, where it can cause mass mortalities and repeated epizootics (e.g. in fish from Kuwait and Singapore – see Lom and Dykova, 1992). The parasite has a cosmopolitan distribution, but is more common in warmer waters. Unlike *Chilodonella*, *Brooklynella* has not been detected on feral fish. It infects most marine teleosts in aquaria and seems to be restricted to the gills of its host, unlike its freshwater counterpart *Chilodonella*.

Brooklynella hostilis is the only species in the genus and is 36–86 μm × 32–50 μm in size and kidney-shaped, with a shallow posterior notch (Fig. 5.7C). It has a flat ventral and a vaulted dorsal side. The ventral surface is uniformly ciliated, with the kineties converging anteriorly along a prebuccal suture. There are 8 to 10 postoral kineties, and a left band of 12 to 15 kineties, which do not reach the posterior margin of the body, stopping short of the glandular organelle. The right band of 8 to 11 kineties is long and arches along the posterior and anterior margin of the body. The middle postoral kinety continues as a series of non-ciliated kinetosomes in a spiral course around the posterior glandular organelle, which appears as an elevated tubercle. The organelle discharges a thin secretion, associated with attachment of the ciliophoran to the host. The preoral kinety is quite short,

while the anterior circumoral kinety is longer than the posterior one. The cytopharyngeal tube, reinforced by six to eight stout nematodesmata, capped by separate beak-like apical ends, is compressed laterally and appears as a broad slit. Descriptions of these and other ultrastructural features may be found in Lom and Corliss (1971). The oval macronucleus is about 18 μm × 12 μm. There are 13 to 22 oval micronuclei distributed around the macronucleus and several small contractile vacuoles. Cyst formation has not been recorded.

In heavy infestations the ciliophoran destroys the gill surface tissue with its cytopharyngeal armature, feeds on tissue debris and ingests blood cells. The infested fish suffer from respiratory difficulties and have haemorrhages and petechiae on the gills. A light infestation elicits a mild inflammatory reaction. The result of the proliferative reaction is fusion of the terminal lamellae. In heavy infestations desquamation, haemorrhages, cell proliferation and other tissue reactions are observed. The secondary lamellae may be completely denuded of the epithelial layer and the tissue response is manifested by macrophages and plasma cells. Such severe lesions usually cause fish mortality (Lom and Nigrelli, 1970).

OBLIGATE FISH COMMENSALS

Sessilines

The sessiline peritrichs (class Oligohymenophorea de Puytorac *et al.*, 1974; subclass Peritrichia Stein, 1859; order Sessilida Kahl, 1935) contain 12 families associated with aquatic organisms, and four of them are associated with fish. In the first family, the Epistylididae Kahl, 1935, there are six genera, of which two are commonly on fish, i.e. *Epistylis* Ehrenberg, 1830 and *Apiosoma* Blanchard, 1885 (syn. *Glossatella* Bütschli, 1889). In the case of the latter genus, all species are associated with fish, whilst species of *Epistylis* are not restricted to fish. The second family, Scyphidiidae Kahl, 1935, has six genera, and two of them are exclusively associated with fish, i.e. *Riboscyphidia* Jankovski, 1985 (syn. *Scyphidia* Dujardin, 1841 p.p.) and *Ambiphrya* Raabe, 1952. In the two remaining families, the Operculariidae Fauré-Fremiet in Corliss, 1979 and the Ellobiophryidae (Chatton and Lwoff, 1929), one genus each has representatives associated with fish, i.e. *Propyxidium* Corliss, 1979 and *Caliperia* Laird, 1953, respectively.

Sessiline peritrichs associated with fish are permanently attached to the host with a scopula, a specialized flattened thigmotactic area of the pellicle equipped with very short immobile cilia. The scopula either adheres directly to the substrate or it secretes a stalk, which may be non-contractile (e.g. in the family Epistylididae) or contractile (e.g. in the family Vorticellidae). The stalk may bear one zooid, or if branched, many zooids forming a macroscopic colony.

The vaulted apical surface inside the peristomial spiral is called the epistomial disc, which is raised above the border of the peristomial area (known as the peristomial lip) in the feeding state, but can retract inside the body. The epistomial disc is separated by a deep groove from the peristomial lip, in which the peristomial spiral is inserted.

The non-ciliated surface of the bell-shaped, conical or cylindrical zooid is finely annulated. In order to infest another host, the zooid is transformed into a mobile disc-shaped telotroch, which uses a locomotory fringe of cilia to swim towards its host after detaching itself from the stalk. Some fish sessilines also form protective cysts.

Sessiline peritrichs found on fish are essentially ectocommensals or symphorionts, which use their hosts as living substrates, from which they feed on organic debris and waterborne bacteria. Sessilines can be found on a variety of substrates, but in some cases, such as *Apiosoma* species, these are exclusively associated with freshwater fish. Sessilines never occur in large numbers on healthy, non-stressed fish; a heavy growth signifies that the fish has been predisposed by some debilitating

environmental and/or infectious factor decreasing the repellent ability of its surface. Attachment via scopula or terminal platelet of the stalk does not harm the host's epithelium, except for the increased number of microfibrils present beneath the host cell membrane (Lom and Corliss, 1968). Very often sessilines are considered to be responsible for the diseased state, but some species may, at most, merely impair the condition of the fish by irritating the surface. They are actually very seldom involved in pathology.

Family Epistylididae Kahl, 1935

The scopula produces a non-contractile stalk, either simple, bearing a solitary zooid, or dichotomously branched, bearing many zooids. The zooid is contractile, with a retractile peristomial lip, more or less divergent, encircling a wide, slightly elevated epistomial disc. A great number of species are found in this family, both free-living and epizoic. Species of two genera (*Epistylis* and *Apiosoma*) occur on fish (Lom and Dykova, 1992).

GENUS *EPISTYLIS* EHRENBERG, 1830. These are colonial ciliophorans, with bell-shaped or elongated cylindrical or conical bodies. A non-contractile stalk bears several to many zooids. The vaulted epistomial disc is slightly elevated above the peristomial lips and slanted. The macronucleus is horse-shoe-shaped or very long and sausage-shaped. Numerous species are either free-living (freshwater and marine) or epizoic, mostly on invertebrates. Five species occur on the surface of freshwater fish.

Epistylis lwoffi Fauré-Fremiet, 1943 occurs on the gills or skin of a variety of freshwater fishes in Eurasia and North America. It forms small colonies, up to eight conical zooids (40–80 μm × 20–30 μm), with a horseshoe-shaped macronucleus and a small micronucleus. The stalk is attached to the substrate by a thin terminal platelet, a ring-like termination, which fastens around the basal body end of *Apiosoma*, or a fork-like stalk end, which is wedged into the epithelium. The stalk may be extremely variable in shape, even in populations from the same host.

Heavy infestations of *Epistylis* sp. were implicated in the death of many thousands of game fish species of the family Centrarchidae in the USA. These infestations coincided with ulcers and pit-like lesions, known as 'red sore disease'. Rogers (1971) reported that, although *Epistylis* is not an obligate parasite, it would infest the surface of fish, causing proliferations and subsequent destruction of the epidermis. He also reported that bacteria would secondarily infect these lesions and that mortalities occasionally associated with these *Epistylis* infestations were probably due to the secondary bacterial infections. Lom (1966), however, found that there was no ultrastructural evidence that *E. lwoffi* inflict any serious damage by their mode of attachment. He further showed that these ciliophorans are incapable of producing histolytic enzymes that would be able to produce scale erosion, ultimately causing pit-like lesions. Esch *et al.* (1976) reported that 'red sore disease' occurred specifically in reservoirs in the USA with high levels of organic material and thermal pollution and in combination with the presence of the bacterial pathogen, *Aeromonas hydrophila*. This bacterium is most likely to be the primary cause of red sore disease, while *Epistylis*, being incapable of producing any lytic or mechanical action that would result in ulceration, was probably the secondary invader, harvesting the rich masses of bacteria in the lesions (Hazen *et al.*, 1978).

Epistylis transvaalensis Viljoen and Van As, 1983 (Fig. 5.7D) was reported from South Africa from *Pseudocrenilabrus philander*. It forms colonies with up to eight bell-shaped zooids, 27–45 μm × 20–38 μm in size. The macronucleus is ribbon-shaped.

Epistylis transvaalensis was also implicated in red sore disease in South Africa (Van As and Basson, 1988), where huge colonies were found on wild adult *Oreochromis mossambicus*. In one specimen these lesions formed a round hole in the operculum of the fish, with colonies of *Epistylis* coinciding with bacterial and fungal infections (Fig. 5.7E).

GENUS *APIOSOMA* BLANCHARD, 1885 (SYN. *GLOSSATELLA* BÜTSCHLI, 1889). These ciliophorans are solitary, with circular scopulas, either narrow or of large diameter, sometimes with long lateral projections or lobed. They secrete a short disc in the shape of a pad of fibrillar material (only seen using the electron microscope; under the light microscope, the scopula appears to be directly attached to the substrate). The macronucleus is compact, usually conical, its apex pointed towards the scopula, or ellipsoidal, situated in the aboral half of the body. Many species (more than 60) are found in this genus as epizoics on the surface of freshwater fish.

Apiosoma dermatum (Viljoen and Van As, 1983) (Fig. 5.7F) occurs on the skin of wild *P. philander* and *B. trimaculatus* (South Africa). The long cylindrical body is 40–61 μm × 25–38 μm. A triangular macronucleus is situated in the aboral part of the body, and a small oval micronucleus is situated laterally to the macronucleus.

Apiosoma micropteri (Surber, 1940) occurs on the gills and body surface of *Micropterus salmoides* and *Micropterus dolomieu*. It has an urn-shaped body, 58.6 μm × 22.2 μm, with a large, inverted, cone-shaped macronucleus and a small micronucleus. It is very similar to *Apiosoma piscicolum*, or maybe identical. It is allegedly responsible for mortalities in *M. salmoides* and *M. dolomieu* fingerlings in North America, causing suffocation.

Apiosoma piscicolum Blanchard, 1885 (Fig. 5.8A) occurs on the skin and gills of many European, North American and South African freshwater fishes. The plump to elongated body is 110 μm in length and 40 μm wide. The macronucleus is conical, with the end pointing aborally.

Family Operculariidae Fauré-Fremiet in Corliss, 1979

This family differs from Epistylididae in having a narrow, epistomial, operculum-like disc. The family contains solitary or colonial zooids, with the body ovoid and elongated or stocky and globular. The macronucleus is elongated, horseshoe-shaped or spheroid. Numerous species are known, generally freshwater, free-living or very often epizoic. A single species of a single genus (out of six) is found on freshwater fish.

GENUS *PROPYXIDIUM* CORLISS, 1979. *Propyxidium* has solitary zooids on short stalks and a horseshoe-shaped macronucleus. It contains many free-living or epizoic species.

Propyxidium tectiformis (Scheubel, 1973) occurs on the gills of *Phoxinus phoxinus*. The body is about 52 μm × 19 μm, with a short non-contractile stalk extending into a short, spoon-like shield, which protects the zooid from one side.

Family Scyphidiidae Kahl, 1935

These are solitary ciliophorans that attach directly to the substrate (mostly aquatic animals) via short immobile scopulary cilia or by an adhesive secretion of the scopula. The scopula may be very large and flattened, forming a wide, flat disc. The body is cylindrical or cylindrical-conical, with the adoral spiral making one turn around a slightly elevated epistomial disc. They are found in freshwater and marine environments and are generally epizoic, with two genera associated with fish.

GENUS *RIBOSCYPHIDIA* JANKOVSKI, 1985 (SYN. *SCYPHIDIA* DUJARDIN, 1841 P.P.). The scopula is often extremely large, with a thin flat border, with or without immobile scopulary cilia. The body is mostly cylindrical, sometimes conical, with a reversible collar and the epistomial disc is flat or slightly vaulted above the peristomial lip. A sausage-shaped macronucleus can often be sinuous in the oral half of the cell. Approximately 18 species are found exclusively on the skin and gills of freshwater and marine fish.

Riboscyphidia adunconucleata (MacKenzie, 1969) occurs on *Pleuronectes platessa* in Scottish waters. The body is almost cylindrical and up to 75 μm long, with a small micronucleus (1.5 μm).

Riboscyphidia arctica (Zhukov, 1962) occurs on the gills of marine species of the genera *Liparis*, *Melletes* and *Myoxocephalus* in the North Atlantic and Pacific Oceans. The plump conical body can be up to 80 μm

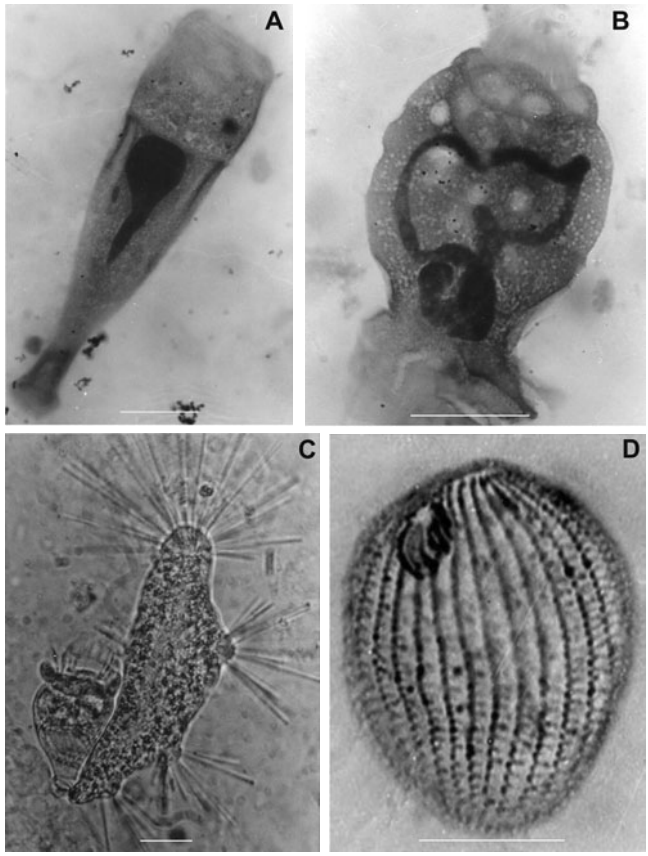


Fig. 5.8. A. *Apiosoma piscicolum*, stained with haematoxylin, showing large macronucleus and smaller micronucleus. B. *Ambiphrya neobolae* with ribbon-shaped macronucleus. C. *Erastophrya* sp., a live specimen from skin of *Barbus* attached to an apiosoma. D. *Tetrahymena corlissi*, silver-impregnated specimen (courtesy of Dr Lom). Scale bars = 20 μm. A–C originals.

long, with a sinuous macronucleus and a giant micronucleus (7 μm × 11 μm in size).

GENUS *AMBIPHRYA* RAABE, 1952. These ciliophorans are similar to *Riboscyphidia*, but have a permanent equatorial ciliary fringe, which is motionless in attached ciliophorans. The cilia are often shielded by a thin pellicular fold, called a median membrane. The macronucleus is in the shape of a long, thin and sinuous ribbon. They are known from the gills and skin of brackish and freshwater fishes, and are represented by four described species.

Ambiphrya ameiuri (Thompson, Kirkegaard and Jahn, 1947) occurs on the gills of young bullhead, *Ameiurus melas melas* (North America); it is also common on *Ictalurus punctatus* and the symbionts were found on *I. punctatus* introduced into Russia and on the fry of cyprinids (including *C. carpio* and introduced *Ctenopharyngodon idella*) in Europe. It usually has a plump conical or cylindrical body, 50–95 μm × 40–61 μm. It attaches to the gills or skin by a scopula in the form of a flat disc, often exceeding body width and often eccentric. The epistomial disc is slightly vaulted and retractile. The macronucleus is ribbon-like, 4–6 μm wide, forming an orally situated horse shoe, with the ends descending into the aboral end of the

body. The globular micronucleus is situated next to the macronucleus.

It inflicts no injury and feeds on waterborne organic particles, but may irritate the body surface and impede respiration when it occurs in very high fish densities, especially on fry.

Ambiphrya neobolae Viljoen and Van As, 1985 (Fig. 5.8B) occurs on the skin of *Neobola brevianalis* in South Africa. The cylindrical body, with constrictions at the equatorial ciliary fringe and just adoral to the scopula, is 44–84 μm × 24–45 μm. The scopula is broad, with a diameter of 17–38 μm. A ribbon-shaped macronucleus extends throughout the body, with the tips folded back.

Family Ellobiophryidae (*Chatton and Lwoff, 1929*)

These are solitary sessilines fastened to the gills of aquatic animals by two outgrowths from either side of the scopula, which encircle the gill filament and grow together to form a firm attachment ring. Of the two genera in the family, one is found associated with marine fish.

GENUS *CALIPERIA* LAIRD, 1953. The cylindrical body is slightly contractile and the attachment outgrowths have distinct central axes. Ciliophorans of the genus *Clausophrya* Naidenova and Zaika, 1969 from the skin of *Proterorhinus marmoratus* from the Black Sea are most probably synonymous with *Caliperia*, but their alleged differences (lack of central axis and contractility of the outgrowths) warrant further study. Only three species are found associated with fish.

Caliperia longipes Laird, 1953 occurs on the gills of New Zealand marine fishes *Oliverichthys melobesia* and *Ericentrus rubrus*. The body size is 51 μm × 38 μm in fixed specimens, a single rod-shaped axis runs in both the calliper-like posterior processes. The macronucleus forms a long spiral.

Suctoria

Suctorians (class Kinetophragminophorea de Puytorac *et al.*, 1974; subclass Suctoria Claparéde and Lachmann, 1858; order Suctorida Claparéde and Lachmann, 1858) are considered to be the most puzzling of all ciliophorans, with seemingly unique characteristics, i.e. the stalked, sessile adult suctorians lack cilia, they possess feeding or ingestory sucking tentacles, the swarmer (tomite) bears cilia and lastly the single mouth opening of the conventional kind is replaced by polystomy. Some species are colonial. A few species are passive floaters in aqueous habitats and some are stalkless endoparasites. Suctorians are predators for the most part, with free-living ones feeding on other ciliophorans in particular. Reproduction is mainly by a budding process, where the tomite or swarmer is formed for a very short period of time.

The most studied part of the suctorian body has been the tentacles. The number, arrangement and distribution of the tentacles are important diagnostic characters.

Suctorians are abundant in fresh and brackish waters, but the majority occur in marine environments. They typically attach to a substratum, such as algae or other vegetation, other protozoans or aquatic invertebrates of many groups. However, they also occur as symphorionts on semi-terrestrial organisms, such as turtles. They have been reported from peritrich ciliophorans and other suctorians as endosymbionts, as well as from the respiratory surfaces of invertebrates and fishes and the intestinal tract of various animals, including some mammals. Symbiotically, suctorians are the most widespread of all ciliophoran groups.

Three families of suctorians are found associated with fish. Of these families only one has representatives that attach directly to the epithelium of fish.

Family Trichophryidae Fraipont, 1878

A single genus occurs on fish, i.e. *Capriniana* Strand, 1928. Suctorians in this family have one or sometimes two or three bundles of suctorial organelles in the attachment surface. They fasten to the host cell by a special cementing layer containing spirally wound fibres. Three species are known from fishes of

which *C. piscum* is a common freshwater ectocommensal.

Capriniana piscium (Bütschli, 1889) Jankowski, 1973 (synonyms *Trichophrya piscium* Bütschli, 1889; *Trichophrya sinensis* Chen, 1955; *Trichophrya intermedia* Prost, 1952; *Trichophrya micropteri* Davis, 1947; *Trichophrya ictalurus* Davis, 1942; *Trichophrya salvelinus* Davis, 1942 and *Capriniana aurantiaca* Strand, 1926) is found as an ectocommensal on the gills of various freshwater fishes, very commonly on *P. fluviatilis* and *Esox lucius*. The body is oval or irregularly elongated and adheres to the secondary gill lamella with a flattened broad attachment surface, called the scopuloid. A prominent apical fascicle of 10 to 35 knob-tipped suctorial tentacles extends from the opposite side of attachment. Another fascicle and/or isolated tentacles can occur on the opposite side of the body. Tentacles have capitate ends bearing numerous haptocysts, which upon contact impale and attach to prey (ciliophorans), to be ingested through the tentacle. It reproduces by endogenous budding, forming a discoidal migratory larva, often settling nearby. In this way heavy infestations can build up. *Capriniana* feeds on other ciliophorans; there is no firm evidence that it feeds on the fish host cell or mucus.

Free-living ciliophorans tentatively identified as *C. piscium* were found to cause heavy infestations of gills, viscera and body muscles in marine fishes (Cheung *et al.*, 1980). These infected fish were predisposed by some environmental stress. Ciliophorans had ingested blood cells and cellular debris, and were in internal organs, specifically the kidney, urinary bladder, spinal cord and blood vessels. There was also extensive damage to body muscle, and fishes died in a relatively short time. Death was attributed to a heavy parasite load on the gills, which interfered with respiration.

The following two families of suctorians contain representatives that are indirectly associated with fish.

Family Dendrosomatidae Fraipont, 1878

They divide by endogenous budding, producing one or more daughter individuals in an internal brood pouch, out of which a free-living larva emerges through a birthpore. The adults are stalkless, without a lorica, while the larvae have transverse bands of cilia. A single genus is found on fish, i.e. *Erastophrya* Fauré-Fremiet, 1943.

The posterior end is drawn out into a semicircular projection or into two such projections that fuse to form a ring embracing the basal body part of sessiline peritrichs of the genus *Apiosoma*, ectocommensal on the surface of fishes. Two species, i.e. *Erastophrya chattoni* Fauré-Fremiet, 1943 and *Erastophrya wuchangensis* Chen, 1964 in Chen, 1973, are known.

Erastophyra chattoni lives attached to sessiline peritrichs of *Apiosoma* on the surface of various freshwater fishes, such as *Gasterosteus aculeatus*, *P. fluviatilis* and *Leuciscus cephalus* in the Palaearctic. The ovoid body (60–140 μm × 30–55 μm) has a rugged surface attached to the suprascopular narrow part of *Apiosoma*. The larvae are up to 40 μm, generally formed singly in the brood pouch. Contractile, slightly motile suctorial tentacles with knobbed tips cover the body.

Erastophyra wuchangensis was reported attached to apiosomas on several species of cyprinid fishes in China. The elongated body (60–200 μm long) possesses a single crook-like holdfast projection and three and more bundles of tentacles.

On several occasions suctorians tentatively identified as belonging to the genus *Erastophrya* have been encountered on a number of fish species in the Okavango River (Botswana). They were also found attached to species of *Apiosoma* (Fig. 5.8C).

Family Endosphaeridae Jankovski, 1978

These are small ciliophorans with neither stalks nor tentacles, and with endogenous budding. They are found solely as endoparasites of peritrich ciliophorans. One genus lives on fish.

GENUS *ENDOSPHAERA* ENGELMANN, 1876. *Endosphaera engelmani* Entz, 1896 is 9–40 μm in size and lives within the cells of various peritrichs, including *Trichodina* and *Trichodinella* species in fresh and salt water.

We have also encountered them in various species of the genus *Mantoscyphidia*, found on the gills of marine molluscs. The larval swarmers, with three equatorial ciliary bands, attach to a new host ciliophoran and enter its cytoplasm by piercing a narrow channel through the pellicle. Eventually they may kill the host ciliophoran.

FACULTATIVE FISH PARASITES

GENUS *TETRAHYMENA* FURGASON, 1940. Although these ciliophorans are mostly saprozoic, there are several records of *Tetrahymena* Furgason, 1940 (class Oligohymenophorea de Puytorac *et al.*, 1974; subclass Hymenostomata Delage and Hérouard, 1896; order Hymenostomatida Delage and Hérouard, 1896; suborder Tetrahymenina Fauré-Fremiet in Corliss, 1956; family Tetrahymenidae Corliss, 1952) infections in fish. Many species are facultative parasites in fish, but there are others that are obligate parasites (Elliot, 1973), causing 'tet' disease and mortalities of ornamental fishes. Despite the relatively common occurrence in fish, however, specific determination is mostly non-existent or uncertain.

Tetrahymena are small pyriform ciliophorans with radially symmetrical bodies, uniformly covered with 20 to 30 meridional kineties. The buccal cavity is equipped with a specialized oral ciliary apparatus consisting of the paroral membrane on the right side and of three serially arranged membranelles on the left side of the buccal cavity. The cytostome is at the bottom of the buccal cavity. One to three postoral kineties begin at the posterior margin of the buccal cavity. Comprehensive information on the morphology and ultrastructure of *Tetrahymena* may be found in Elliot (1973). Several species have been reported from fish.

Tetrahymena corlissi Thompson, 1955 (Fig. 5.8D) is found naturally on amphibians and freshwater fish with a (facultatively?) free-living stage. This ciliophoran infects various fish species and is frequently fatal to its host. It is a histophagous parasite, found pervading the skin, muscle and body cavity organs. Although it may have a cosmopolitan distribution, records on its occurrence are patchy and mostly from the USA (Hoffman *et al.*, 1975), with a recent report from Japan and other South-east Asian countries by Imai *et al.* (2000).

The body is pyriform (averaging 55 μm × 30 μm in size), with 25 to 31 kineties. It has one caudal cilium, which is more rigid and longer than other somatic cilia, an ellipsoid macronucleus, a single micronucleus and a single, posteriorly located contractile vacuole. It forms reproductive cysts, in which the ciliophorans divide to produce two to eight tomites.

As a histophagous parasite, *T. corlissi* disintegrates host tissue and feeds on cell debris. In tropical aquarium fishes the infection is manifested by whitish patches, due to masses of ciliophorans in copious amounts of mucus (Johnson, 1978). Fish scales are bristled as the outer skin is lost and the tissue swells. A mass of ciliophorans may form a rim around the eye orbit ('spectacled eye'). The entry into the body has not been well studied. Extensive necrotic changes occur in the muscles and subdermal tissue. An inflammatory reaction has only rarely been observed (I. Dyková, unpublished). The infection results in mortality. Although the ciliophorans in general are not considered a major threat to tropical fish, but more of a nuisance, they can be important in tropical fish cultures.

Infections with this and other *Tetrahymena* species in fishes may be much more prevalent than reported earlier. Ciliophorans tentatively identified as *Tetrahymena pyriformis* (Ehrenberg, 1830) were found to invade fry, especially those with injured integuments. They may destroy not only surface tissues but also internal organs (Shulman and Jankovski, 1984). These ciliophorans, including *Tetrahymena faurei* (Corliss, 1952), were recorded in the central nervous system of larvae of *C. carpio*, *A. brama* and *Ameiurus* sp. and in the spinal canal and musculature of rainbow trout (Corliss, 1960; Stolk, 1960).

Tetrahymena rostrata (Kahl, 1926) Corliss, 1952 is an edaphic histophagous organism and a facultative parasite of some invertebrates. It has 27 to 35 kineties and

forms both reproductive and resting cysts. It was probably associated with cranial ulcerations in farmed *Salmo salar* in freshwater tanks. Invasion of head tissues was associated with subacute inflammation. The clinical signs were severe fin and tail rot and depigmentation of the head (Ferguson *et al.*, 1987).

Tetrahymena are very easy to culture. Agnotobiotic cultures feed on bacteria and are easy to isolate and maintain in 2.5% proteose peptone. They can also be cultured axenically in chemically defined media (Elliot, 1973).

GENUS *BALANTIDIUM* CLAPARÉDE AND LACHMANN, 1858. Ciliophorans of the genus *Balantidium* Claparéde and Lachmann, 1858 (syn. *Stentoropsis* Dogel and Bykhovski, 1938) (class Kinetophragminophorea de Puytorac *et al.*, 1974; subclass Vestibulifera de Puytorac *et al.*, 1974; order Trichostomatida Bütschli, 1889; family Balantidiidae Reichenow, 1929) infect various homeotherm and poikilotherm vertebrates; although mostly innocuous, they have pathogenic potential. The cytostome is located at the base of an anteriorly situated vestibulum. The body is uniformly covered with longitudinal ciliary rows. Ten species are found in freshwater and marine fishes, with some species being monoxenous and some being found in different genera. These species are all endocommensals that can become histophagous parasites.

Balantidium ctenopharyngodoni Chen, 1955 lives in the intestine of grass carp, *C. idella*, in China, and also in the grass carp stocks introduced to Europe. The infection is quite common.

The body is ellipsoidal, slightly dorsoventrally flattened, about 40–80 μm × 25–65 μm. The subapical vestibular groove, almost one-third the body length, deepens towards the cytostomial opening. Its left edge is covered with densely spaced ends of adjacent somatic kineties, bearing stronger and longer coalescent cilia, while the right vestibular edge bears no cilia. The macronucleus is bean-shaped, with a single micronucleus adjacent to it. Three contractile vacuoles lie in the middle third of the body. Infection is transmitted by spherical cysts.

Balantidia are essentially harmless and are found in the middle and posterior parts of the intestine in grass carp. Little-known factors, perhaps the lack of starch in the host's food, may turn them into histophagous parasites, where they abrade the epithelium, penetrate into the subepithelial intestine layer and feed on the tissue cells. Balantidia cause extensive ulceration and elicit granuloma formation deep in the tissue. *Balantidium* enteritis is associated with hyperaemia and inflammatory changes of the intestine. Enteritis may be fatal, and losses in 1–3-year-old grass carp were recorded (Molnár and Reinhardt, 1978).

Balantidium polyvacuolum Li, 1963 commonly occurs in the rectum of *Xenocypris argentea, Xenocypris davidi* and *Plagiognathops microlepis* in China. This ciliophoran (84–142 μm × 57–115 μm in size), containing up to 22 contractile vacuoles and a broad vestibular groove, can seriously damage the intestinal tissue of its host, producing local necroses and ulcerations.

Two additional species are found in fish, *Balantidium barbi* (Dogel and Bykhovski, 1934) Jankovski, 1982 (syn. *Stentoropsis barbi* Dogel and Bykhovski, 1934) (see Shulman and Jankovski, 1984) from fish in Central Asian rivers and *Balantidium sigani* Diamant and Wilbert, 1985 from herbivorous marine fish from the Red Sea; no pathogenicity was recorded in their hosts.

URONEMA MARINUM DUJARDIN, 1841. *Uronema marinum* Dujardin, 1841 (class Oligohymenophorea de Puytorac *et al.*, 1974; subclass Hymenostomata Delage and Hérouard, 1896; order Scuticociliatida Small, 1967; family Uronematidae Thompson, 1964) is well documented as a parasite of marine fishes in aquaria. It is equipped with a thigmotactic ciliary field. It is about 30–50 μm in size. It has a long caudal cilium and a buccal area that is half the body length.

Free-living ciliophorans tentatively identified as this species were found to cause heavy infections of gills, viscera and body muscles in marine fishes (Cheung *et al.*, 1980). Infected fish were predisposed

by some environmental stress. The fish initially had white patches, which later became lesions or open wounds. Infected fish were nervous and later were listless, had breathing difficulties and had open, bleeding surface ulcers, full of ciliophorans (Bassleer, 1983). The ciliophorans had ingested blood cells and cellular debris, and were in internal organs, specifically the kidney, urinary bladder, spinal canal and blood vessels. There was also extensive damage to the body muscle and fishes died in a relatively short time. Death was attributed to the heavy parasite load on the gills, which interfered with respiration.

Others (free-living ciliophorans attacking the surface of fish)

There is a large group of ciliophorans that are mostly free-living. However, when fish are stressed by adverse environmental conditions and they are completely debilitated and moribund, these free-living ciliophorans become facultative parasites externally. These freshwater ciliophorans include species of the genus *Coleps* Nitzsch, 1827 and various histophagous ciliophorans, which feed on tissues. These fishes may also be colonized by free-living peritrichous ciliophorans (e.g. *Vorticella* Linnaeus, 1767, *Carchesium* Ehrenberg, 1830 or *Zoothamnium* Bory de St Vincent, 1826).

Fish that are seriously stressed but not moribund are also susceptible to free-living ciliophorans, such as *Chilodonella cucullulus* (O.F. Müller, 1786), *Chilodonella uncinata* (Ehrenberg, 1838), *Dexiostoma campylum* (Stokes, 1886) Jankovski, 1967, *Glaucoma scintillans* Ehrenberg, 1830, *Colpidium colpoda* (Ehrenberg, 1831) Stein, 1960, *Frontonia acuminata* Ehrenberg, 1833 and *Frontonia leucas* Ehrenberg, 1838. They may establish transient infestations of the skin and gills.

These ciliophorans live in highly polluted waters, and infect the body surface and gills of pond fish under adverse conditions, e.g. in early spring after a long, severe winter. When environmental conditions (oxygen, temperature, food, water quality) improve, they rapidly disappear (Migala and Kazubski, 1972).

Surface wounds on fish, notably on fry, predispose them to invasions by free-living ciliophorans in the marine environment. *Helicostoma buddenbrooki* Kahl, 1931, *Euplotes* Ehrenberg, 1830 sp. and *Uronema* Dujardin, 1841 sp. were recorded on O-group sole and plaice and young *Zoarces viviparus* in mariculture (Purdom and Howard, 1971). They attached to skin abrasions, enlarged the lesions and eventually caused lethal injuries.

Diagnosis

Microscopic examination (objective × 10) of wet mounts of excised gills and scrapings from skin, fins and nasal pits is the best available technique to detect ectozoic ciliophorans. Sessile suctorians are easy to find as the refractile granules in the cytoplasm make them appear very dark, in contrast to surrounding cells. Endocommensal ciliophorans (*Balantidium* etc.) can be detected by careful examination of intestinal contents and/or mucosal scrapings.

Careful examination of fresh mounts is required to detect light infestations. There are no other methods to detect these ciliophorans.

Prevention and Control

The prevention and treatment strategies are very similar to those taken with most ectozoic protozoan infestations. The outbreak of an ectoparasitic ciliophoran infestation always indicates that the defence capabilities of the fish are compromised, often due to adverse environmental conditions. Hence the host–parasite equilibrium must be shifted back in favour of the fish by changing the environment. This is a prerequisite, without which all treatment procedures are only of temporary value. Handling and transport will also have a negative impact on fish health. Treatment for most fish ciliophorans involves a chemical added

directly to the water. Before treating fish, a few things must be considered:

- Knowledge of the water in which the fish are kept (source depth, volume and chemical nature, etc.).
- Knowledge of the fish's susceptibility to chemicals (e.g. malachite green is far less toxic to catfish than it is to sunfish and bass and it might kill fry).
- Knowledge of the chemical to be used is important (e.g. copper sulphate can be used in dosages higher than 2 ppm in waters with a carbonate hardness over 200 ppm, but, in water with a carbonate hardness of 20 ppm, concentrations as low as 0.02 ppm may kill fish).
- Certain chemicals may have an indirect detrimental effect on the fish. Copper sulphate during summer months may kill enough algae to cause an eventual oxygen depletion problem. Formalin is known to also cause oxygen depletion. In both cases provide ample agitation in summer months.
- Legal considerations: certain countries have limitations for fish being produced for human consumption (e.g. malachite green is not approved for food fish by the US Food and Drug Administration (FDA), but formalin is approved).
- Advisability of treatment: in some cases where the majority of fish are moribund, it must be decided whether or not a treatment is economically feasible as the stress imposed by the treatment may add to total mortality.

The guidelines of proper health management for fish culture are summarized as follows:

- The water should be pathogen-free and it can be either from wells or sand-filtered if the supply is from a stream containing native fish. The water must also be free of pollutants, at an appropriate temperature and physiochemically balanced.
- Efforts should be made to prevent access of trash or wild fish.
- Ponds and tanks should be drained at regular intervals and their bottoms sanitized with quicklime (2.5–3 t/ha). Raceways and various tanks can be made parasite-free by flushing them with 5% formalin.
- Make sure nets, buckets and other implements used on the farm are clean.
- Ponds should be stocked with parasite-free or previously treated fish.
- New fish should never be added to existing populations in a pond, as this could introduce pathogens. Always routinely treat them for parasites before adding them to a system that has been drained and disinfected.
- If new stocks are obtained, always keep these in quarantine for a period.
- Fish should be fed a balanced diet and not over- or underfed.
- There should be a proper oxygen supply and this should be balanced in relation to temperature.
- Prevent excessive crowding and ensure minimal handling of the fish.
- Specific requirements of the fish species should be provided.
- Prevent unnecessary stress to fish, such as extremes in water quality, as well as a build-up of ammonia, nitrites, nitrates or any toxicants.
- Fish should be examined regularly for parasites.

Chemical treatment is to be used in an emergency and only after the proper management practice has failed. We recommend sodium chloride, formalin, acriflavine hydrochloride (= trypaflavin), malachite green (*p*, *p*-benzylidene bis *N*, *N*-dimethyl aniline) and/or potassium permanganate ($KMnO_4$) to treat most of the ectozoic ciliophorans. They can be used in relatively high concentrations in dip baths (D) when the fish are immersed in a net for a very short time (seconds to 1 h). These chemicals can be used in low concentrations as a flush treatment (F). Fish are exposed to the chemicals for several hours to days before the water in the tank is replaced with clear water. In a highly diluted state, the chemicals can be left in the tanks for indefinite treatment (I). Dip treatment is convenient for small fish, fry and fingerlings, while flush and indefinite treatments are suitable for

raceways, tanks, larger water bodies or aquarium systems. The choice always depends on their feasibility in a given situation. The wide range of chemical concentrations recommended by various authors can be explained by the necessity to adjust the concentration to the ambient temperature, oxygen contents and quality of water (pH, total hardness, organic load) in relation to the particular fish species and age class. Finally, the resistance of parasite populations to these chemicals should be taken into account. Thus, to assess the proper concentrations and length of exposure and to make sure that a specific species can tolerate the chemical, a trial should precede large-scale treatment.

For chilodonellas, trichodinids, sessiline peritrichs, facultative and opportunistic parasites, suctorians and other ectocommensals, the following treatments are recommended (the concentrations below are given either in percentage or ppm):

1. Sodium chloride (not for marine or euryhaline ciliophorans).
D: 1.5% to 3% for 5 min to 1 h (e.g. sessiline peritrichs: 3% for 5 to 10 min, or 1.5% for 1 h; *Chilodonella*: in common carp fry 2% for 15 min at temperatures above 15°C, 1% for 30 min at 20–25°C, in yearling carp 3% for 15–30 min at temperatures above 15°C).
F: 0.1–0.2% for 1 to 2 days.
2. Formalin (convenient also for warm-water and for marine ciliophorans).
D: 150–250 ppm for 30–60 min (e.g. in common carp and salmonid yearlings, concentration depends on temperature: below 10°C, 250 ppm; 10–15°C, 200 ppm; above 15°C, 150 ppm).
F: 250 ppm for 1 h
40–50 ppm for 24 h.
I: 15–25 ppm.
3. Acriflavin.
F: 50 ppm for one to several days.
I: 10–20 ppm.
4. Malachite green.
D: 2.5–5 ppm for 30 s
60 ppm for 10–30 s at 10–20°C.
F: 0.15–0.5 ppm for 6 days (e.g. common carp 0.5 ppm, trout fingerlings 0.15–0.2 ppm).
I: 0.1 ppm
in combination with formalin:
0.25 ppm malachite green in 125 ppm formalin for 2–6 h, depending on intensity of oxygenation.
5. Potassium permanganate.
D: 0.1% for 30–45 s
50 ppm for 10–15 min (e.g. trout fry).
F: 10 ppm for 60–90 min
5 ppm for 16 min.
I: 2–3 ppm.

For *Brooklynella*, in addition to formalin and acriflavine treatment, indefinite immersion in a copper bath is useful: 0.42 ppm cupric acetate in 5.25 ppm formalin in 4.8 ppm Tris buffer.

For *Uronema* and other marine facultative parasitic ciliophorans, use a freshwater dip for 10–15 min, followed by a 15–20 min dip in 5 ppm methylene blue or 30 ppm nitrofurazone (repeat twice daily, if necessary).

Raising the water temperature to 25°C for several days may help to restore the defence capability of the fish against *Chilodonella* infestations. This may also be useful against other ciliophorans, but not against those in the tropics.

Conclusions and Recommendations for Future Research

This review is a summary of the basic knowledge of ciliophorans that live on the surface or inside the fish body. There is an impressive diversity in genera and species, in their form and structure and many species are serious pathogens especially of cultured fish. Chemicals are readily available and are quite effective against the ciliophoran parasites.

Future studies should centre chiefly on the host–parasite relationships, although it is likely that there are many more undescribed species and genera. Basic biodiversity in large parts of the globe has not been studied; in the field of trichodinid diversity, basically nothing is known from central and northern Africa, Australia and South and Central America. To conduct studies on host–parasite relationships, we

have to have a reliable source of parasites. At the moment, there are neither *in vitro* culture methods nor dependable *in vivo* techniques to maintain ectozoic ciliophorans. Once we have a reliable source of parasites, we can then start to unravel the host–parasite relationships. The intriguing areas that we should initially pay attention to include the process of infestation (e.g. stimuli that attract the ciliophoran to the fish host), proliferation of the parasite on the fish (e.g. factors that predispose the fish to disease and allow the parasite to proliferate), nutritional requirements of the parasite and the host immune response to the parasite (e.g. protective humoral and cell-mediated responses).

The mechanism of the disease should be further studied. We know little about the pathomorphogenesis in chilodonellosis and trichodinosis and the differences in pathogenicity among various strains and races of *Chilodonella* and *Trichodina*. This basic information is necessary in order to form the basis of an efficient programme on disease prevention.

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6 Phylum Apicomplexa

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Introduction

Apicomplexans occur in a wide variety of animals (helminths to mammals). Apicomplexan parasites, primarily coccidia of warm-blooded animals, belong to the most important and harmful group of animal pathogens. The economic importance of apicomplexan infection in mammals and birds is documented every year by hundreds of reports on the development and pathogenic effect of these parasites, as well as by dozens of drugs and technologies recommended for prevention. Contrary to this fast-developing branch of coccidiology, the progress in fish apicomplexan research is rather moderate and can claim only modest results.

Since the recent knowledge on fish apicomplexans was summarized in a chapter of the first edition of this book (Molnár, 1996), only a limited number of related papers has been published. These papers concern mostly the occurrence of the known species (Cruz and Davies, 1998; Diouf *et al.*, 2000; Abollo *et al.*, 2001; Alvarez-Pellitero *et al.*, 2002; Tolonen and Karlsbakk, 2003) and the description of some new species from different continents (Diouf and Toguebaye, 1994, 1996; Lom and Dyková, 1995; Sitja-Bobadilla *et al.*, 1996; Baska, 1997; Molnár and Ogawa, 2000; Alvarez-Pellitero and Sitja-Bobadilla, 2002; Molnár *et al.*, 2003), and only a few deal with the ultrastructure (Azevedo *et al.*, 1995; Paperna, 1995; Azevedo, 2001; Diniz *et al.*, 2002), systematic position (Siddall *et al.*, 1994; Siddall, 1995; Jirku *et al.*, 2002), development (Negm-Eldin, 1998, 1999; Vilenkin and Paperna, 1997; Fournie *et al.*, 2000; Davies and Smit, 2001) and pathology (Steinhagen, 1997; Hemmer *et al.*, 1998) of fish apicomplexans. Although the first coccidium oocysts from fish had already been detected at the end of the 18th century (Thélohan, 1890), the subject has been studied by only a few specialists and there is a limited amount of data on the development and pathogenicity of both histozoic and blood-parasitic fish apicomplexans. It is likely that the number of described species represents only a small fraction of fish apicomplexans. Of these, species infecting marine fishes were summarized by Lom (1984). In another review on the coccidia proper, Dyková and Lom (1983) included 128 species, and this number has been increased up to the present by at least 100 new coccidium species (Duszynski *et al.*, 1999). A review on fish haemogregarines (coccidia *sensu lato*) was made by Davies (1995), who recorded 72 species from marine and 31 species from freshwater fishes. Coccidioses of the common, bighead and silver carp, eel and tilapia are known to cause mortality. Heavy Apicomplexa infections are also common in marine fishes, e.g. swim-bladder coccidiosis

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of the cod caused by *Goussia gadi*, liver coccidiosis of the killifish caused by *Calyptospora funduli* and testicular infection of the sardine caused by *Eimeria sardinae*. Little is known about the pathogenic effect of haemogregarinids.

Host Range of the Parasite

Apicomplexan parasites, both coccidians and adeleid blood parasites, infect fishes worldwide. They are common in both marine and freshwater fishes. While, however, haemogregarinids are mostly known from marine fishes (Davies, 1995; Davies and Johnston, 2000), coccidia proper are common in both freshwater and marine specimens (Dyková and Lom, 1981, 1983). The majority of the known apicomplexan species have been described from Europe and the Far East; there are, however, data on their occurrence in North and South America (Fatham *et al.*, 1942; Hoffman, 1965; Molnár and Fernando, 1974; Solangi and Overstreet, 1980; Békési and Molnár, 1990), in South Asia (Setna and Bana, 1935; Mandal *et al.*, 1984; Molnár *et al.*, 2003), in Australia (Molnár and Rohde, 1988; Lom and Dyková, 1995), and in Africa (Diouf and Toguebaye, 1996; Smit and Davies, 1999).

Little is known about host specificity of fish apicomplexans. The majority of known haemosporidians has been described from a single fish species. In contrast, *Haemogregarina bigemina* has been recorded in at least 85 species of fish. This may be explained by the fact that these are not based on experimental studies and that some authors described the meronts or gamonts as new species, while others identified them with the best-known species, *H. bigemina*. The majority of investigators (Khan, 1978, 1980; Becker and Overstreet, 1979; Barta and Desser, 1989; Barta, 1991) regarded only leeches as vectors, while Davies and Johnston (1976) as well as Davies (1982) suggested that *H. bigemina* could develop both in the leech *Oceanobdella blennii* and in the isopod *Gnathia maxillaris.*

Barta (1991) suggested that *Babesiosoma* species were less host specific and synonymized some of the described species. He listed 26 fish hosts for the five *Babesiosoma* spp. he considered valid. A similar conclusion was drawn by Negm-Eldin (1998, 1999), who, using the leech vector *Batracobdeloides tricarinata*, could transmit *Babesiosoma mariae* and *Cyrilia nili* to series of fishes in cross-transmission experiments. Fish coccidia seem to be less host specific than mammalian coccidia. A fish coccidium can infect several closely related host species, usually of the same genus (Belova and Krylov, 2000). However, *Goussia subepithelialis* of the common carp and *Goussia sinensis* of silver and bighead carp are relatively strictly host specific. *Eimeria anguillae,* which is found in eel species of both the Atlantic and the Pacific Oceans, and *Goussia vanasi* and *Goussia cichlidarum* of tilapia species are good examples of group specificity. Although Shulman (1984) listed more than a dozen hosts for *Goussia carpelli*, experimental data (K. Molnár, unpublished) suggest that *G. carpelli* infects only fish of the genera *Cyprinus* and *Carassius* and that coccidians in other cyprinids are morphologically similar but less well-studied species.

Cryptosporidia are considered to have an extremely broad host range. According to Levine (1984) four species, including the fish-parasitic *Cryptosporidium nasoris*, are valid, while according to Fayer *et al.* (1997) eight species should be recognized. Up to the present time, four named *Cryptosporidium* species (*C. nasoris*, *C. reichenbachklinkei*, *C. cichlidis* and *C. molnari*), as well as five *Cryptosporidium* spp., are known from fishes (Hoover *et al.*, 1981; Paperna and Vilenkin, 1996; Alvarez-Pellitero and Sitja-Bobadilla, 2002). Paperna and Vilenkin (1996) thought that fish cryptosporidia differed in morphology and in their location in the host from cryptosporidia of warm-blooded animals and created a new genus, *Piscicryptosporidium*, for them.

Systematic and Taxonomic Positions of Apicomplexans

Members of the phylum Apicomplexa Levine, 1970 have a special cell organelle, called the apical complex, which facilitates

invasion of the host cell. The majority of species form spores and/or oocysts. Fish apicomplexans are divided into two major groups. Coccidia proper are primarily intestinal parasites and produce resistant oocysts in the host. Adeleid blood parasites (Coccidia *sensu lato*) have the merogonic and some gamogonic stages in fish, while spore formation takes place in parasitic annelids or insects. Both groups are characterized by a complete apical complex, including the conoid (Conoidasida). In addition to adeleid apicomplexans, some protozoans of uncertain systematic position (*Haematractidium*, *Haemohormidium*) can also infect blood cells of the fish. These latter parasites have an incomplete apical complex and lack the conoid, and there is no spore formation in their life cycle.

The majority of adeleid apicomplexans parasitize marine fishes and only a few species occur in freshwater fishes. The majority of these parasites have not been adequately described, and in most cases only the merogonic and gamogonic stages in blood cells are known. Levine (1988) recorded 60 *Haemogregarina*, two *Cyrilia* and one *Hepatozoon* species in fish, and, in Barta's revision (1991), five *Babesiosoma* and two *Dactylosoma* have been added. Siddall (1995) created a new genus, *Desseria*, within the family Haemogregarinidae, and included 40 other species of marine and freshwater fish haemogregarines with a wide distribution.

A common characteristic of the Apicomplexa is the 'apical complex', a structure demonstrable only by electron microscopy. The organelle appears only at certain stages of development. The apical complex usually consists of a polar ring, a conoid, micronemes, rhoptries and subpellicular tubules. Apicomplexans have a complex life cycle, which involves (in most cases) merogony, gamogony and sporogony.

The following classification is based on that of Levine (1988) and is modified by Perkins *et al*. (2002). The latter authors regarded *Goussia* as a junior synonym of *Eimeria*, but molecular studies made by Jirku *et al*. (2002) showed that the genetical distance between *Eimeria* and *Goussia* is rather large; therefore *Goussia* and *Calyptospora* are tentatively included in the family Eimeriidae as independent genera.

Phylum: Apicomplexa Levine, 1970
- Class: Conoidasida Levine, 1988
  - Order: Eucoccidiorida Leger and Duboscq, 1910
    - Suborder: Adeleiorina Leger 1911
      - Family: Haemogregarinidae Neveu-Lemaire, 1901
        - Genus: *Haemogregarina* Danilewsky, 1885
        - Genus: *Cyrilia* Lainson, 1981
        - Genus: *Desseria* Siddal, 1995
      - Family: Dactylosomatidae Jakowska and Nigrelli, 1955
        - Genus: *Dactylosoma* Labbé, 1894
        - Genus: *Babesiosoma* Jakowska and Nigrelli, 1956
    - Suborder: Eimeriorina Leger, 1911
      - Family: Eimeriidae Leger, 1911
        - Genus: *Eimeria* Schneider, 1875
        - Genus: *Goussia* Labbé, 1896
        - Genus: *Crystallospora* Labbé, 1896
        - Genus: *Calyptospora* Overstreet, Hawkins and Fournie, 1984
      - Family: Cryptosporidiidae Leger, 1911
        - Genus: *Cryptosporidium* Tyzzer, 1907

Protists of uncertain taxonomic position
- Genus: *Haematractidium* Henry, 1910
- Genus: *Haemohormidium* Henry, 1910

Morphology and Development

Coccidia proper

Development

The development of fish-parasitic coccidia does not differ essentially from that of coccidia of warm-blooded animals (Fig. 6.1). The development of the Eimeriidae and the Cryptosporidiidae is divided into merogony, gamogony and sporogony. Merogony begins with invasion of the host cell by the parasite. The intruding sporozoite, enclosed in a parasitophorous vacuole in the cytoplasm or nucleus of the cell becomes rounded, begins to grow and changes into a meront. In the meronts, merozoites are formed, which, when released, invade other cells and form

second- and third-generation meronts. Gamogony begins when the last generation of merozoites forms macro- and microgamonts. In the microgamonts numerous microgametes develop. After fertilization the macrogamont develops into an oocyst. During sporulation, the zygote in the oocyst divides and forms sporocysts, in which sporozoites develop. Sporulation may take place within (endogenous sporulation) or outside (exogenous sporulation) the fish.

Morphological characteristics of the different developmental stages

MEROGONIC STAGES. Merogonic stages in naturally infected fish have been described by numerous workers. However, only Paterson and Desser (1981a), Hawkins *et al.* (1984a, b) and Steinhagen *et al.* (1989) have studied meronts and merozoites in experimentally infected fish. The merogony of *Goussia iroquoina* takes place in the intestine, while that of *C. funduli* is in the liver. According to Hawkins *et al.* (1984b), there were two merozoite generations, and Paterson and Desser (1981a) also found at least two generations. In species with epicellular development, merogony is also epicellular (Molnár and Baska, 1986; Landsberg and Paperna, 1987; Kent *et al.*, 1988; Jastrzebski and Komorowski, 1990). Usually 8–16 merozoites of 8–16 μm in length are formed in the meronts (Fig. 6.2), but in *G. cichlidarum* Landsberg and Paperna (1985) reported meronts containing large numbers of merozoites. The merozoites of fish coccidia do not differ in structure from those of warm-blooded animals: they have an easily discernible nucleus, conoid apparatus and trimembranous pellicle covering the merozoite.

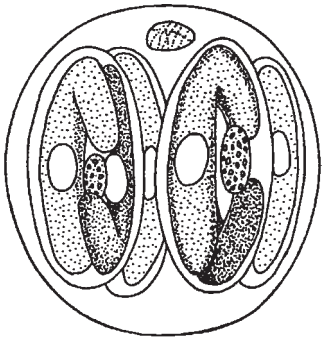


Fig. 6.1. *Goussia acipenseris*. Schematic diagram of the oocyst.

The meronts develop in the cytoplasm, or occasionally in the nucleus, within a parasitophorous vacuole. In species with epicellular development, the parasitophorous vacuole is intracellular but extracytoplasmal and is covered by a single unit membrane of the host cell. The vacuole of

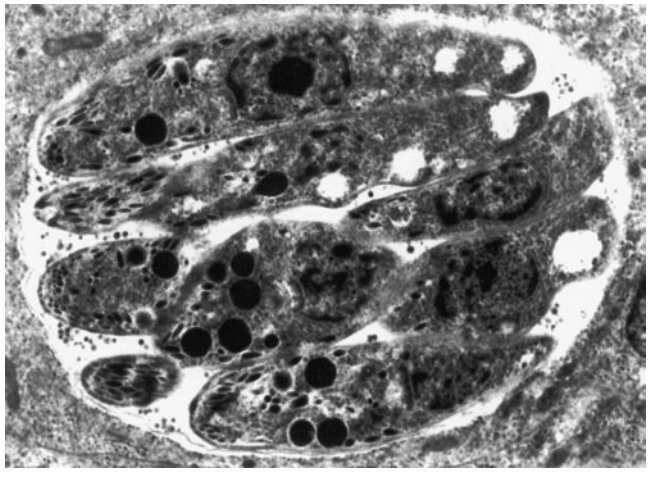


Fig. 6.2. *Goussia balatonica*. Intracellular meront with merozoites (transmission electron microscope (TEM), × 8800).

epicellular eimeriids is complete, which in Molnár and Baska's (1986) view means that its cavity completely surrounds the parasite. The wall of the vacuole is a single-layered unit membrane. Good metabolic communication between parasite cytoplasm and the host cell is ensured by parts of the vacuole protruding into the cytoplasm of the host cell.

Cryptosporidium have incomplete vacuoles. The parasitophorous vacuole formed by enterocyte microvilli surrounds the meront only on the side facing the gut lumen. Between the meront and the host cell cytoplasm there is a special adhesive zone. All stages of cryptosporidia develop epicellularly.

GAMOGONIC STAGES. Microgamogenesis begins with multiple divisions of the nucleus of the microgamont in the parasitophorous vacuole. The newly formed nuclei gradually migrate to the periphery of the microgamont, where they separate from the cytoplasm to form microgametes (see Fig. 6.4). Microgametes possess two flagella, with the exception of those of cryptosporidia, which do not have flagella. The microgamete contains a nucleus, mitochondria, microtubules and a perforatorium at the apical end. In the majority of species, macrogamogenesis takes place in the apical part of the cytoplasm of epithelial cells. However, there are species with intranuclear and epicellular development. Alvarez-Pellitero *et al.* (1997) reported that *G. sparis* might develop in both intraepithelial and supraepithelial positions. The macrogamont (Fig. 6.3), which develops from the last merozoite generation, is always surrounded by a parasitophorous vacuole. In species with epicellular development, the vacuole is extracytoplasmic and this is similar with the meront (Fig. 6.4). Electron microscopic studies of the macrogamont cytoplasm (Paterson and Desser, 1981c; Hawkins *et al.*, 1983b) indicate amylopectin, lipid granules and two distinct types of 'wall-forming body' (assumed to be involved in the formation of the thin oocyst wall).

SPOROGONIC STAGES. The oocysts of fish coccidia, except for members of the genus *Cryptosporidium*, are rather similar (see Figs 6.1, 6.6 and 6.7). The oocysts of the Eimeriidae contain four sporocysts and, occasionally, one to two polar granules. Each sporocyst contains two sporozoites and a residual body. The only important difference between genera is in the structure of the sporocyst.

The oocyst is adapted to the aquatic environment; it has a thin, sensitive oocyst wall and does not have a micropyle. The thickness of the one- or three-layered wall varies between 3 and 200 nm.

Each *Cryptosporidium* oocyst contains four naked sporozoites and a residual body.

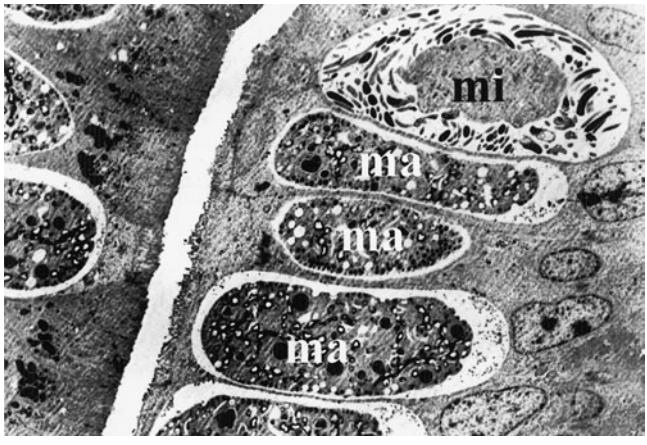


Fig. 6.3. *Goussia balatonica*. Intracellular macrogamonts (ma) and microgamont (mi) in the intestinal epithelium (TEM, × 4400).

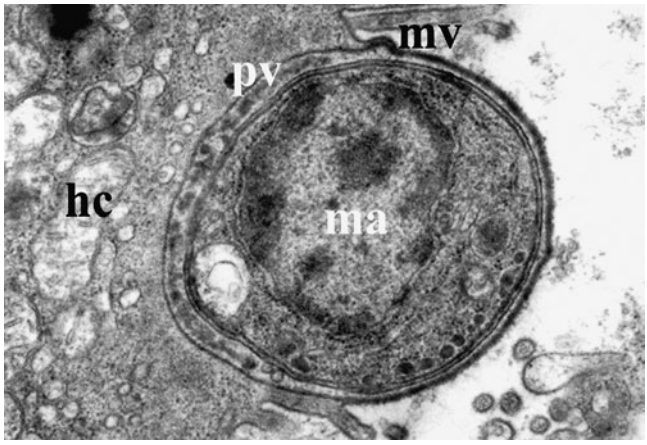


Fig. 6.4. *Eimeria anguillae*. Epicellular macrogamont (ma) in a parasitophorous vacuole (pv) surrounded by microvilli (mv) inside a host cell (hc) (TEM, × 21,800).

Fish cryptosporidia have been reported by Hoover *et al*. (1981), Pavlasek (1983), Landsberg and Paperna (1986), Paperna (1987) and Alvarez-Pellitero and Sitja-Bobadilla (2002). These authors based their diagnosis on the special adhesive zone of the developmental stages. The ultrastructure of sporulated oocysts has only been studied by Paperna (1987) and Alvarez-Pellitero and Sitja-Bobadilla (2002). Paperna (1987) found the wall to be thick and double-layered, but Alvarez-Pellitero and Sitja-Bobadilla (2002) reported the simultaneous occurrence of thick-walled and thin-walled oocysts and they observed three main layers in the wall.

Sporocysts are elliptical, oval or dodecahedral in shape. They differ from each other primarily in the mode of sporozoite release. The wall of the sporocyst is generally thin but is more resistant than that of the oocyst. However, there are species with a relatively thick sporocyst wall, e.g. *Eimeria etrumei* and *Goussia siliculiformis*.

The sporocyst wall is usually composed of two layers. However, some species, such as *G. gadi* and *Goussia lucida* have a three-layered wall (Odense and Logan, 1976; Daoudi, 1987). The thickness of the wall varies between 30 and 500 µm. Azevedo (2001) found a thick inner layer and a thinner outer layer in *Goussia clupearum*; myelin-like projections emerged from the outer layer.

Only a few fish coccidia (e.g. *Eimeria isabellae*) possess a typical *Eimeria* sporocyst (i.e. having a Stieda body). In the majority of the species there is only a thickening, plug or cap at one end of the sporocyst. This is where the sporocyst opens and the sporozoites are released in the host or intermediate host.

Goussia-type sporocysts are composed of two equal-sized, round, elliptical or coffin-shaped valves, united by a suture. This suture is hardly discernible using light microscopy but can be seen under an electron microscope. Although most fish coccidia have been described as *Eimeria*, the majority of the species undoubtedly belong to the genus *Goussia*. Sometimes the sporocyst is also surrounded by a membraneous veil, which is attached to the sporocyst by special membranes. The sporocyst veil has been reported by Lom (1971) for *Goussia degiustii* and by Baska and Molnár (1989) for *G. sinensis*.

The sporocysts of *Calyptospora* are characterized by a thickening or projection at the caudal end, by sporopodia starting from the spore surface or from the caudal projection and by a sporocyst veil supported by sporopodia and surrounding the sporocyst. The opening of the sporocyst is a longitudinal suture, which extends only to the anterior one-third of the sporocyst.

The sporocyst of *Crystallospora crystalloides* is bipyramidal and opens at a suture situated at the foot of the pyramids (Daoudi, 1987).

The sporozoites of fish coccidia are banana-, sausage- or comma-shaped. They usually lie in the sporocyst in a head-to-tail presentation. In the cryptosporidia the sporozoites are side by side and lie in the same direction. The sporozoite has a nucleus that is easily discernible by light microscopy and is situated in the middle of the body. The conoid apparatus lies at the anterior end, and the posterior end may sometimes be striated.

After the formation of sporocysts, a sporocyst residuum appears within the sporocyst. This residuum is granular in the young oocysts and may be compact in the older ones (Molnár, 1989). Certain species, e.g. *G. siliculiformis* and *G. degiustii*, are characterized by a large residuum. In coccidia parasitizing the inner organs, the residuum is used up slowly, while it is used more rapidly in intestinal coccidia.

Infection of fish

Coccidia of fish were for a long time regarded as infecting the host in a direct way. Now it has been proved that fish coccidia might develop through intermediate and paratenic hosts as well (Fig. 6.5). The possible role of intermediate hosts in the transmission of fish coccidia was first suggested by Landau *et al.* (1975), who demonstrated the sporozoites of a coccidian parasitizing a muraena (*Gymnothorax moringa*) from the intestinal tissues of a crustacean (Mysidacea). Direct infection with oocysts has been reported by Musselius *et al.* (1965), Zmerzlaya (1966), Steinhagen and Körting (1988) and Steinhagen *et al.* (1989) using *G. carpelli*, while Marincek (1973b) was successful with *G. subepithelialis* in the common carp. By feeding oocysts mixed in the feed, Paterson and Desser (1981a,b,c, 1982) produced *G. iroquoina* infection in fathead minnow (*Pimephales promelas*) and common shiner (*Notropis cornutus*), while Landsberg and Paperna (1985) produced *G. cichlidarum* infection in tilapia. In contrast, Molnár (1979) failed to produce experimental *G. carpelli* infection by feeding oocysts mixed in the diet; however, he succeeded when the oocysts were mixed with chicken liver. He consistently achieved intensive infection by feeding tubifex collected from natural habitats. Kent and Hedrick (1985) produced *G. carpelli* infection in goldfish only if they had previously fed the oocysts to tubifex or shrimps. Steinhagen and Körting (1990) produced *G. carpelli* infection both by the direct route and through vectors. On the other hand, Solangi and Overstreet (1980) and Fournie and Overstreet (1983)

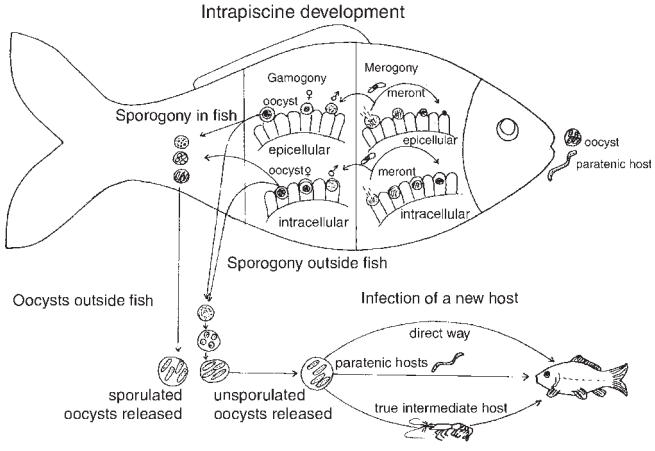


Fig. 6.5. Schematic diagram of coccidian development inside and outside fish.

reported that *C. funduli* required a true intermediate host, the grass shrimp (*Palaemonetes pugio*), for its development. On the other side, Steinhagen and Körting (1990) suggested that the invertebrate is a paratenic host in which the sporozoites do not undergo development. Fournie *et al.* (2000) have demonstrated that the sporozoites of *C. funduli* need to infect the shrimp for at least 5 days before reaching infectivity. These authors also proved that during that stage sporozoites were located in the basal epithelial cells of the shrimp's intestine. Steinhagen (1991) observed that the sporozoites of *G. carpelli* and *G. subepithelialis* invaded cells of the alimentary tract, where they were found in membrane-bound parasitophorous vacuoles and persisted for 9 weeks for *G. carpelli* and 3 weeks for *G. subepithelialis*. Steinhagen *et al.* (1998) have shown that uninfected carp can be infected by *G. carpelli* both directly and through paratenic hosts, but reinfection occurs only in the latter way. Vilenkin and Paperna (1997) infected tilapia hybrids directly with the oocysts of *Eimeria vanasi*, and 7 to 56 h post-exposure (p.e.) found sporozoites in intraepithelial lymphocyte-like or leucocyte-like cells but never in epithelial cells. Sporozoites in these cells divided by endodiogeny once or twice to form daughter sporozoites. The oocysts of fish coccidia undergo rapid sporulation and ageing in the environment. The sporozoites die soon after the residual body of the sporocyst is used up. In tubifex or shrimp vectors, on the other hand, the sporozoites are released in the gut and remain viable for a long time in epithelial cells.

Peculiarities in the development of fish coccidians

Oocysts of fish coccidians are always assumed to be sporulated when they leave the fish. Recent studies, however, have revealed that a number of species shed unsporulated oocysts (Figs 6.6 and 6.7). The majority of fish coccidians infect the intestinal epithelium, but a relatively large number of them, especially those with large oocysts, develop at non-intestinal sites, such as the kidney, spleen, liver, swim bladder and serosa. Most species produce oocysts continuously throughout the year but there are species which follow a 1 year seasonal cycle and produce oocysts only in

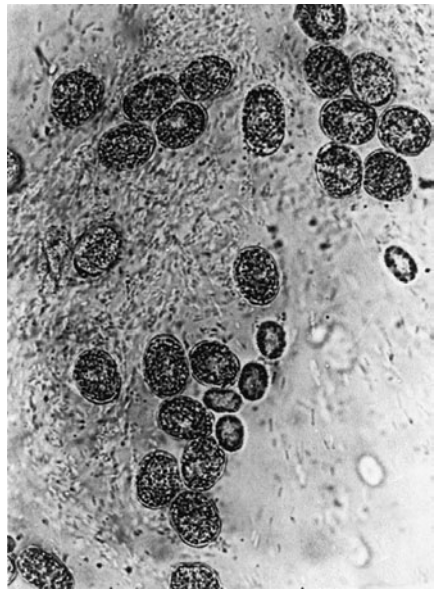


Fig. 6.6. Unsporulated oocysts from the gut of the sterlet. The larger ones are *Goussia vargai* and the smaller ones are *Goussia acipenseris* (× 320).



Fig. 6.7. Extraintestinally sporulated oocysts of *G. vargai* (× 1000).

the spring. Oocysts of warm-blooded animals have resistant oocysts, which preserve the sporocyst residuum and infectivity for years; fish coccidians have a soft, membranaceous oocyst wall. Residual bodies in warm-blooded animals are relatively steady; fish coccidia consume residual bodies very quickly and lose infectivity within a few days.

Adeleid blood parasites (coccidia *sensu lato*)

Development of adeleid blood parasites in general

Fish-parasitic coccidia *sensu lato* have heteroxenous life cycles, which involve two hosts, one being the fish and the other a parasitic leech or insect (Fig. 6.8). The development of coccidia *sensu lato* is divided into merogony, gamogony and sporogony. There is also a special association of the two gamonts prior to encystment (syzygy) and this takes place before sporogony. Although the Haemogregarinidae and the Dactylosomatidae have striking resemblances (development, morphology and vectors), there are important differences between them. At sporogony haemogregarinids develop in parasitophorous vacuoles in cells in the definitive host, while dactylosomatids are free in the cell cytoplasm. Haemogregarinids infect the fish with sporozoites while the infective stage is the merozoite in the dactylosomatids. No significant differences exist in transmission to vertebrates. Davies and Johnston (2000) think that in the case of the haemogregarinids *Haemogregarina* and *Desseria* merozoites are transmitted by the invertebrate host, while in that of *Cyrillia* sporozoites are transmitted. In the case of the dactylosomatid *Babesiosoma* merozoites are transmitted, while for *Dactylosoma* this type of transmission has not been demonstrated yet.

Development and morphology of haemogregarinids

The development of fish-parasitic haemogregarinids (Figs 6.9a,b and 6.10) is known primarily from studies of natural infections. Khan (1980) was the first to describe some developmental stages of *Cyrilia uncinata* in experimental infections. He found that sporozoites were injected into the blood by leeches, entered lymphocytes, monocytes, neutrophils or blast cells, developed into meronts and formed merozoites. The merogony of *Haemogregarina* and

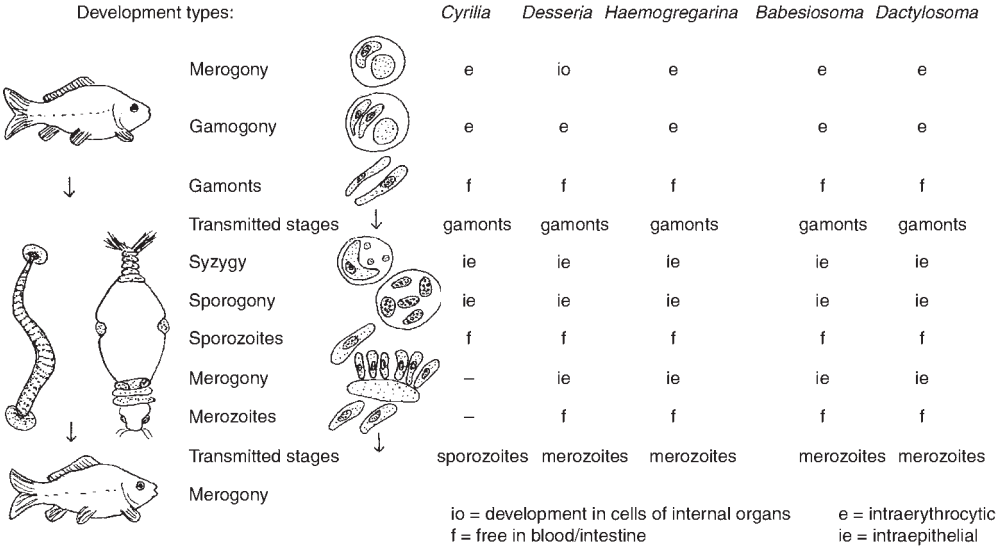


Fig. 6.8. Schematic diagram of the development of haemogregarinid and dactylosomatid apicomplexans.

Cyrilia spp. occurs in blood cells, while in *Desseria* spp. it takes place mainly in the inner organs. Negm Eldin (1999) found that *C. nili* had two successive types of merogonic stages in infected fish and the meronts of the second merogonic cycle were destined to form gamonts. The vermiform merozoites enter erythrocytes or leucocytes to form macro- and microgamonts. In some species there is some division of gamonts in the erythrocytes, while in others they directly change into micro- and macrogamonts.

Gamonts are taken in during a blood meal. In the intestine of the leech they are released from blood cells and the micro- and macrogamonts unite in syzygy. During this process they are surrounded by a thin membrane. In *Haemogregarina* and *Cyrilia* the microgamont produces four microgametes, and one of the resulting microgametes fertilizes the macrogamont. Oocyst formation takes place and, depending on the parasite species, it may occur either within an enterocyte or on the surface in a parasitophorous vacuole. The produced sporozoites migrate towards the salivary and the proboscis of the leech. Intraleucocytic meronts of *Haemogregarina* spp. usually harbour two to eight merozoites and do not significantly enlarge the size of the host cell. The length of the banana- or crescent-shaped merozoites and gamonts varies between 4 and 5 μm; intraleucocytic meronts, however, may reach 26 μm × 23 μm. *Haemogregarina bigemina* can also be transmitted to fish by gnathiid isopods. Davies and Smit (2001) observed that specimens of *Gnathia africana* taken from fish contained the following stages of *H. bigemina*: syzygy, immature and mature oocysts, sporozoites and merozoites of at least three types.

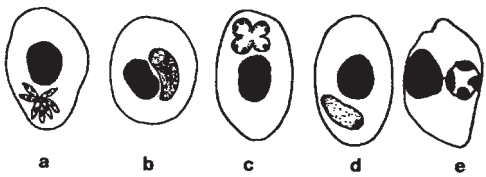


Fig. 6.9. Schematic diagrams of adeleorin apicomplexans and of *Haemohormidium beckeri* in erythrocytes. a, b. Meront with seven merozoites (a) and gamont (b) of *Haemogregarina delagei* (after Khan, 1972). c, d. Meront of tetrad form (c) and gamont (d) of *Babesiosoma hannesi* (after Paperna, 1981). e. *H. beckeri* (after Khan, 1980).

Development and morphology of dactylosomatids

For a long time the development of dactylosomatids parasitic in fishes was little studied. Only the intraerythrocytic merogonic and gamogonic stages (Fig. 6.9c,d) were known. Paperna (1981) suggested that the parasite is transmitted by leeches. Negm-Eldin (1998) was the first to experimentally infect fish with *B. mariae.* This author used the leech vector *B. tricarinata* for transmission. In the affected fish, three successive types of merogonic cycles occurred within the fish erythrocytes. The third cycle produced merozoites, destined to become gamonts. The gamonts were somewhat larger than the meronts. Parasitaemia in the fish lasted for up to 7 months.

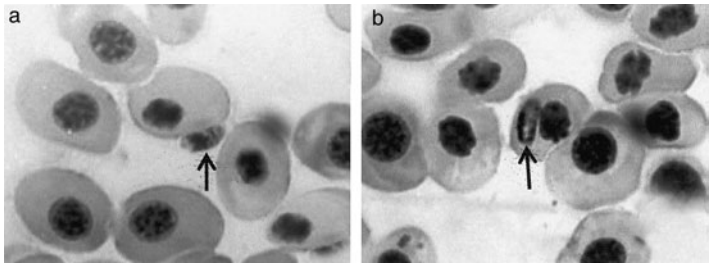


Fig. 6.10. *Haemogregarina* developmental stages in Giemsa-stained blood films. a. Free gamete of *H. acipenseris* in the cytoplasm. b. Intraerythrocytic gamont of *H. acipenseris* (× 1200). (Courtesy of Dr F. Baska.)

In the leech crop, gamonts became associated in syzygy and fused. Negm Eldin (1998) found three stages of merogony in the development of *B. mariae*, but Smit *et al.* (2003), who recovered this parasite from the African fish *Serranochromis angusticeps*, could not find these stages.

Blood protozoans with uncertain systematic position

Members of this protozoan group differ from other apicomplexans in having an apical complex without a conoid. They have no spores or sporogonic stages. They are well-known pathogens of vertebrates. Fish are also infected by *Haemohormidium* and *Haematractidium* spp. Haemohormidia are amoeboid, oval or rounded organisms in the cytoplasm of erythrocytes (Fig. 6.9e). Davies (1980), who studied *Haemohormidium cotti* using light and electron microscopy, found that the parasites (1.2 to 4.5 μm) in the red blood cells were bounded by a complex membrane like that seen in the pellicle of a typical Apicomplexa. The outer membrane was in direct contact with the host cell cytoplasm. No parasitophorous vacuole was found. Khan (1980), who studied the development of *Haemohormidium beckeri* in erythrocytes, described its division by both binary fission and merogony. He found no gamogonic stages. The leeches *Platybdella orbiti* and *Johanssonia arctica* were vectors. Dividing stages were found in their gut and proboscis; however, no oocysts were found. Natural transmission occurred when leeches fed on susceptible fish. Siddall *et al.* (1994), who studied *Haemohormidium terranovae* in American plaice, stated that there was no evidence of any ultrastructure feature characteristic of the phylum Apicomplexa. They considered *Haemohormidium* Henry as a senior synonym of *Haematractidium* Henry. Davies and Johnston (2000), who compared the ultrastructure of *H. cotti* with *Haematractidium scombri*, found that the cytoplasm of *H. cotti* was electron-lucent in comparison with that of *H. scombri*, it contained vacuoles and scattered bodies with dense centres and, crucially, it lacked the peripheral organelles characteistic of *Haematractidium*.

Pathogenicity of Apicomplexans

The majority of fish coccidians have relatively low pathogenicity. No mortality was observed even when 85–90% of the spleen or liver was infected with *Goussia* spp. or *C. funduli* (see Molnár, 1976; Solangi and Overstreet, 1980). Lethal infections occur primarily in farm ponds, but severe cases have been reported from natural waters as well. Bauer *et al.* (1981) reported fish mortality caused by *G. carpelli* and *G. sinensis*. Molnár (1976) also recorded deaths due to *G. sinensis. Eimeria anguillae* infection caused emaciation and deaths in eels in New Zealand (Hine, 1975). Fiebiger (1913), as well as Odense and Logan (1976), reported mortality in the haddock caused by *G. gadi*. MacKenzie (1978) found a species of *Eimeria* that caused 6–10% reduction in body mass in blue whiting. Pinto (1956) reported parasitic castration as a result of *E. sardinae* infection. Upton *et al.* (2000) supposed that a heavy infection of the gut with *Eimeria phyllopticis* caused significant morbidity and mortality in the aquarium-cultured sea-dragon, but the role of a joint infection with bacterial pathogens could not be excluded.

The damage to tissues depends on the intensity of infection. In *E. anguillae* infection, Hine (1975) observed partial or total destruction of the intestinal mucosa and submucosa. The epithelial cells became compressed and the submucosal connective tissue vacuolated, and mature oocysts and free sporocysts were passed out with necrotic tissue. Kent and Hedrick (1985) reported high mortality in a lethargic and emaciated 15-day-old goldfish population. They found that *G. carpelli* caused chronic enteritis, with numerous yellow bodies and inflammatory and necrotic cells in the lamina propria of the gut. The microvillous structure of the intestinal epithelium was also destroyed. Molnár (1976) studied *G. sinensis* in silver carp and also found intensive histological changes in the gut

epithelium. Most of the epithelial cells were damaged by meronts or gamonts but neither inflammation nor haemorrhagic lesions developed in the intestinal wall. He concluded that, despite intensive infection, *G. sinensis* was regarded as a moderately pathogenic parasite. In artificial infection of the common carp with *G. carpelli*, Steinhagen *et al*. (1997) reported on dilution of the blood plasma, leucocytosis, bacterial co-infection and the increase of phagocytic activity, while Hemmer *et al*. (1998) observed loss of epithelial cells and atrophy of intestinal folds, with severe damage to the infected epithelium. Heavy *G. carpelli* infection increased the number of mitotic enterocytes, and the squamous to cuboidal-shaped cells exhibited immature characteristics. The relatively mild clinical symptoms observed during *G. carpelli* infection are attributed to the high regenerative capacity of the carp intestine. Epicytoplasmic infection with *G. cichlidarum* leads to intense desquamation of the swim bladder epithelial lining in cichlids (Landsberg and Paperna, 1985).

More severe changes develop in nodular coccidiosis (Fig. 6.11). Pellérdy and Molnár (1968) reported that agglomerations of *G. subepithelialis* oocysts in common carp were frequently surrounded by inflammatory cells. Marincek (1973a) described second-generation meronts developing basal to the nucleus in the cytoplasm. These exerted a more pathogenic effect on cells than first- and third-generation meronts located in the apical region of endothelial cells. The most severe changes were seen during gamogony. The chromatin of infected cell nuclei degenerated, the nuclei became enlarged and infected cells extended above the level of the uninfected cells. The damaged cells changed their cylindrical shape and died. In severe cases the epithelium desquamated and the lamina propria was exposed. The infection was aggravated by inflammation, and lymphocytes and eosinophils appeared in the infected area. The process was characterized by increased mucus production. Large numbers of pinhead-sized yellowish-white nodules were found in the enlarged spleen of a gudgeon infected with *Goussia metchnikovi* (Pellérdy and Molnár, 1968). These nodules contained hundreds of oocysts. Young stages, mostly macrogametes, were surrounded by an inflammatory tissue reaction, while the young oocysts were surrounded by thin connective tissues. In advanced cases the capsule became thick (10 to 15 μm). A similar host reaction was reported in *C. funduli* infection in killifish (Solangi and Overstreet, 1980). Heavily infected fish had pale white to black lesions densely scattered throughout the liver parenchyma. At the early stage, inflammatory cells (mainly lymphocytes, mononuclear

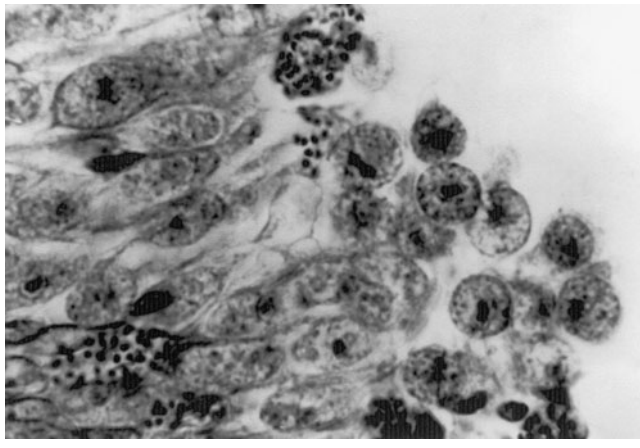


Fig. 6.11. Nodular coccidiosis in tench. Unsporulated oocysts just leaving the infected area of the intestinal epithelium (haemotoxylin and eosin (H & E) × 730).

macrophages and eosinophils) accumulated around the gamonts. Later, fibrotic capsules surrounded by inflammatory cells developed around the oocysts. As the process advanced, the inflammatory infiltration disappeared and pigmentation appeared within the fibrotic capsules. Similar pathogenesis and host reaction were observed in the *Goussia thelohani*-infected liver of a marine fish, *Symphodus tinca* (see Daoudi *et al.*, 1988). Hawkins *et al.* (1981) found few changes in the liver during merogony of *C. funduli*, which they attributed to the formation and release of merozoites. Hepatocyte degeneration, pigment formation and leucocytic infiltration occurred mostly during gamont formation; hepatocytes were replaced by parasites and leucocytes associated with them, including monocytes, heterophils and eosinophils.

The formation of a connective tissue capsule around clumps of oocysts is also common in *Goussia* spp. that infect kidneys. Pellérdy and Molnár (1968) observed a capsule around *Goussia scardinii* oocysts in the renal parenchyma, and Morrison and Poynton (1989) reported damage of the renal epithelium and granuloma formation around the affected tubules in *Goussia spraguei*-infected cod.

The infection of the swim bladder is characterized by severe changes. Both Schäperclaus (1954), who studied *G. gadi* infection in the cod, and Grabda (1983), who investigated *Eimeria jadvigae* infection in *Coryphaenoides holotrachys*, reported thickening of the swim bladder wall. In more severe cases the inner surface of the bladder was covered with a white substance, the wall had a spongy texture and the lumen was filled by a mucus exudate containing large numbers of oocysts.

There is little information on the pathogenic effect of adeleid parasites. In cultured turbot, Ferguson and Roberts (1975) reported a proliferative disease of the haematopoietic tissues; this was later discovered to be caused by a haematozoon (Kirmse and Ferguson, 1976), which Kirmse (1978) described as *Haemogregarina sachai*. Kirmse (1980) found that up to 60% of some populations of *H. sachai*-infected turbots were affected with gross tumours in the musculature and viscera. The lesions consisted of necrotic tissue with a caseous centre. Histologically, there was an accumulation of parasitized reticuloendothelial cells, cell debris and pycnotic nuclei. Parasitaemias of up to 36% of all blood cells were observed; the majority of infected cells were neutrophils and monocytes. In mackerel caught in certain areas of the Atlantic, 4% of the leucocytes were infected with meronts, and meronts were demonstrated in 100% of impression smears from the spleen. Lesions in the spleen and kidneys contained *Haemogregarina*-like organisms and were often surrounded by a connective tissue capsule (MacLean and Davies, 1990).

Coccidian infection in fish increases the propagation of immunoglobulin-positive (Ig(+)) leucocytes. In *G. carpelli* infection Steinhagen (1997) observed that during merogony and gamogony the proportion of Ig(+) cells increased and peaked during oocyst formation. Increase of Ig(+) cells was temperature-dependent but even at low temperatures local and systemic immune responses were induced in carp with enteric coccidian infection.

Clinical Signs of Infection

According to Schäperclaus (1954), Kocylowski and Myaczynski (1963) and Bauer *et al.* (1981), fish infected with *G. carpelli* do not feed properly, group in a certain area of the pond and float in the water with their heads down. In intestinal coccidiosis, the faeces are covered with a thick, sometimes reddish, mucus layer. In goldfish affected with coccidiosis, Hoffman (1965) observed a long mucus sheath containing masses of oocysts protruding from the rectum. Difficulty in swimming is indicative of swim bladder coccidiosis.

Intensive intestinal infection is always accompanied by abundant mucus production. As a result, the frequently reddened intestinal wall is lined with thick viscous mucus. This is more likely to be derived from the cytoplasm of the injured cells than from enhanced secreting activity. In nodular coccidiosis, white nodules the size of a millet seed or

lentil can be seen through the intestinal wall. Spleen and liver coccidiosis is also indicated by the presence of nodules, and swim bladder coccidiosis by a thickening of the swim bladder wall.

Host–Parasite Relationship

Fish coccidia and their hosts have a well-developed host–parasite relationship. Though Steinhager and Hespe (1997) described an enhanced phagocytic activity during the period of merogonic and gamogonic development at *G. carpelli* infection of the common carp, the host usually takes a long time before reacting to the developing parasite. Infected cells are inactive and gradually die. At this time the host response is mainly restricted to replacing the damaged cells (Molnár, 1984). In diffuse coccidiosis, e.g. in *G. carpelli* infection, the inactive epithelial cell that contains the developing macrogamont is gradually surrounded, grown over and pushed to deeper layers by neighbouring epithelial cells. The sporulated oocyst is expelled, probably as a result of macrophage activation and necrosis of the epithelium above the oocyst. The oocyst or two or three oocysts together are situated in a 'yellow body'. One component of the yellow body is the necrotic host cell, but macrophages engulfing oocysts may also become yellow bodies. According to Dyková and Lom (1981), the yellow body contains mucoid substances and ferrous ions as well as the cytoplasm of the pathologically altered host cell. Also, the yellow body contains lipofuscin and ceroid, which may be derived from degenerating cell membranes (Kent and Hedrick, 1985).

Molnár (1984) suggested a new ejection process in *G. subepithelialis* infection. The merogonic and gamogonic stages develop in the epithelial cells and, as Marincek (1973b) pointed out, only a few unsporulated oocysts are excreted from the parasitic nodule (Fig. 6.11). The remaining infected epithelial cells, which contain oocysts, are pushed downwards and grown over by proliferating intact epithelial cells. In this way large numbers of oocysts may be situated and may sporulate close to the submucosa. Another intensive host reaction is mounted against oocysts after the death of host cells. This results in secondary damage of the epithelial cells overlying the oocysts. Oocysts get into the intestinal lumen as a result of secondary damage to the epithelial layer. According to Dyková and Lom (1981), unrejected oocysts in the subepithelium became surrounded by a connective tissue capsule and gradually died. Marincek (1973a, 1978) suggested that a large portion of the infected gut lost its normal function. A similar mechanism was observed in *E. anguillae* infection. Rupture of the host cell membrane releases the majority of the young oocysts into the gut lumen and these sporulate outside the host. However, some of the macrogamonts and young oocysts are surrounded by intact epithelial cells and are driven to deep layers of the epithelium, where they sporulate. This must be why Dyková and Lom (1981) and Lacey and Williams (1983) suggested that *E. anguillae* had intracytoplasmic sporogony. A similar host reaction is supposed for *Piscicryptosporidium* spp. described by Paperna and Vilenkin (1996). Although these authors differentiated the genus *Piscicryptosporidium* from *Cryptosporidium* by the presence of intraepithelial oocysts, this author thinks that these oocysts assumed the latter position only through a secondary host reaction in a similar way to that characteristic of epicellular *Goussia* spp. Non-specific defence response parameters (leucocytosis, eosinophilia, activation of phagocytes and elevation of natural antibody titre and of ceruloplasmin) were detected in carp infected with *G. subepithelialis* (Studnicka and Siwicki, 1990).

The majority of oocysts of non-intestinal species sporulate within the fish and are released after the death of the host. These oocysts, e.g. the oocysts of *G. metchnikovi* in the spleen or those of *G. siliculiformis* on the serous membranes, have a large sporocyst residue and remain viable for a long time after their sporulation. Oocysts scattered in the parenchyma of the spleen are first localized to larger nodules, become surrounded by melanomacrophage cells (Fig. 6.12) and are later destroyed (Molnár, 1984).

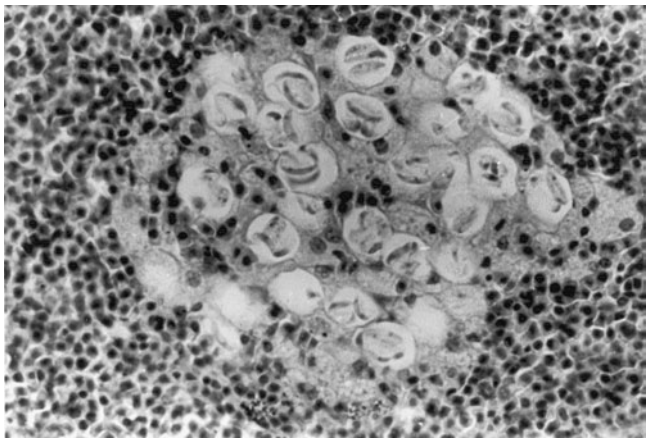


Fig. 6.12. *Goussia metchnikovi* oocysts inside a melanomacrophage, spleen of *Gobio gobio* (H & E × 730).

Certain coccidian infections persist in fish; this suggests that the mechanism observed in coccidioses of warm-blooded animals also occurs in fish. Nothing is known about the humoral immune response of the host. Hawkins *et al.* (1981) and Békési and Molnár (1990) reported a depletion of hepatocytes around *Calyptospora* macrogamonts in the liver and an increase in the number of perisinusoidal cells, which aggregated the macrogamonts into islets. Later, the macrogamonts become surrounded by granulocytes and heterophil and other leucocytes. In *G. sardinae* infection, Morrison and Hawkins (1984) observed leucocytes and phagocytes, while in *G. thelohani* infection Daoudi *et al.* (1988) saw lymphocytes and eosinophil cells around the gamonts. According to Molnár (1984), the melanomacrophage cells would gradually destroy *G. metchnikovi* oocysts congregated into islets. In *C. funduli*-infected killifish, an infection prevents reinfection (Solangi and Overstreet, 1980). On the other hand, Békési and Molnár (1990) observed three generations of *Calyptospora tucunarensis* in the liver of tucunare. The developing macrogamonts were surrounded by perisinusoidal cells and oocysts aggregated into nodules. A connective tissue capsule was formed around some oocysts from an earlier infection.

The severity of an apicomplexan infection can be increased by immunosuppression. Steinhagen *et al.* (1998), who treated common carp with corticosteroids or subjected them to temperature stress in the laboratory, could produce a higher number of oocysts during primary infection. However, resistance to reinfection was not reduced by the application of hydrocortisone, dexamethasone or X-ray irradiation, given both prior to and concurrently with reinfection. The administration of hydrocortisone or irradiation did not induce a recurrence of previous infection. Carp that had been immunosuppressed by hydrocortisone injection during a primary infection were also refractory to a secondary infection via faecal contamination, and reinfection of these fish was achieved only through tubifex paratenic hosts.

Nutrition of Fish Apicomplexans

Apicomplexans with a typical intracytoplasmic parasitophorous vacuole feed through intravacuolar blebs that contain host cell cytoplasm. According to Hawkins *et al.* (1983a,b,c), Morrison and Hawkins (1984) and Jastrzebski (1989), nutrient materials in the cisternae of the host cell endoplasmic reticulum are passed into the space of the parasitophorous vacuole, formed into blebs

and absorbed into the cytoplasm of the parasite. Epiplasmally located coccidia, such as *Cryptosporidium* and some *Goussia* and *Eimeria*, absorb nutrients only through their attachment zone or through invaginations of the parasitophorous vacuole (Fig. 6.3). In *Cryptosporidium*, the parasitophorous vacuole is not complete and there is a special adhesive zone between the cell and the parasite. This is where absorption takes place. Parasite nutrition is ensured by a special feeder organelle, which is formed by close contact of the parasitic folds and the plasma membrane at the base of the parasitophorous vacuole. In epicellular *Goussia* and *Eimeria*, the parasitophorous vacuole extends invaginations into the host cell cytoplasm (Molnár and Baska, 1986; Daoudi, 1987; Morrison and Poynton, 1989; Jastrzebski and Komorowski, 1990). Paperna and Landsberg (1987), however, reported that these invaginations are substituted by tubular formations in *G. vanasi*.

Diagnosis of Infection

The thin and fragile wall of the oocyst and sporocyst of fish coccidia does not permit the use of concentration procedures routinely used with coccidia of warm-blooded animals. Fish coccidia are demonstrated exclusively in fresh preparations or by histological methods.

Coccidia in internal organs are detected by examining small pieces of tissue under a cover slip. Low-intensity infections are detected if the organs are first digested in 0.25–0.5% trypsin solution. Intestinal coccidia are demonstrated by microscopic examination of faeces and intestinal scrapings; they are easiest to detect in the mucus from the intestinal epithelium or surface of the faeces (Molnár, 1977). If the fish is fasted for 1 or 2 days before the examination, oocysts in the yellow body are easily discernible at × 400 in the gut.

Unsporulated oocysts (small size and thin oocyst wall) (Fig. 6.6) in fresh preparations are often mistaken for granulocytes or algae, which are common in intestinal scrapings. Oocysts are often distinguished only after sporulation. Oocyst sporulation is achieved in several changes of tap-water in a watch glass or in a small Petri dish. Addition of a loopful of penicillin and streptomycin sulphate to the water to control bacterial growth increases the chances of sporulation. The oocyst wall is fragile and this does not permit prolonged storage (Molnár, 1986, 1989). Oocysts in the mucus or faeces are preserved in 1% glutaraldehyde or 4% formalin (Molnár and Hanek, 1974; Molnár, 1977) for a short period. For permanent preparations, fish coccidia are best preserved as histological slides.

Oocysts are relatively easy to detect using histological methods. Mallory's stain is particularly suitable. Mature oocysts stain yellow and stand out in sharp contrast to fish tissues.

Flotation is rarely used for fish coccidia. The only data have been published by Berland and Højgaard (1981) who used IKI stock solution (30 g I + 300 g KI + 500 ml distilled water) and 290 ml distilled water for the flotation of *Goussia clupearum* oocysts.

Davies and Stewart (2000) recommended an easy and effective method for detecting low-level infection with coccidians infecting the inner organs. These authors utilized autofluorescence of the oocysts. Using fluorescence microscopy they could detect oocysts of *Eimeria sardinae*, *Goussia clupearum* and *G. metchnikovi* in unstained squash preparations of the liver, testis and spleen, respectively, where sporocysts showed a blue autofluorescence.

Prevention and Control of Apicomplexan Infections

Little is known about the prevention of losses caused by fish coccidia. Perhaps this is because they only affect fish species (common carp, silver carp, bighead, eel and, possibly, tilapias) cultured in ponds. Only a few of the drugs developed for controlling coccidioses in warm-blooded animals have been tried on these fish parasites. Naumova and Kanaev (1962) recommended

Osarzol as a therapeutic agent against *G. carpelli* infection of the common carp, but failed to specify the dose. Furazolidone (for every 1000 fingerlings they mixed 300 mg drug with the food) is effective if fed to fish for 3 days (Musselius *et al.*, 1965). Kocylowski *et al.* (1976) reported that amprolium chloride (fed at a dose of 5 g/kg fish at 2-day intervals on three occasions) is quite efficacious against the parasite. To control *E. anguillae*, Hine (1975) successfully treated eels with Furanace (1.0–1.5 mg/kg of eel) by adding it to the food for 6 days. Reduction in oocyst count in killifish fed monensin (Solangi and Overstreet, 1980) indicated that other anticoccidials might also be efficacious. Bauer *et al.* (1981) suggested that ponds should be disinfected by freezing, drying out or the addition of lime or chlorinated lime.

Conclusions and Recommendations for the Future

Significant progress has been made since the review of Dyková and Lom (1981) and this includes the ultrastructure and intracellular development of fish coccidia. Except for a few pioneering studies, most of the literature is based on natural infections. Hence, a fundamental task in future research into fish coccidia would be to establish conditions for reproducible experimental infections. This would be necessary to better understand the pathogenicity and specificity of and immunity to fish coccidia. The possible role of vectors in transmitting these parasites would be another important area of research. There is an urgent need to apply molecular biological methods for fish coccidians. These methods are fairly advanced in the case of coccidians of warm-blooded animals, while for fish coccidians only the first such trials are in progress (Jirku *et al.*, 2002).

Further studies are needed to elucidate the life cycle and development of Haemogregarinida and Dactylosomatida coccidia. Other areas that need clarification are taxonomy and phylogenetic relationships between groups of related coccidia. For studying the host selection, specificity, antigenicity, immunity and pathogenicity of blood-dwelling apicomplexans, a laboratory model should be selected.

Coccidiosis is presumed to cause substantial losses; however, there is no chemotherapy against fish coccidia in fish farms. Hence research should also be focused on studying the pathogenicity of coccidia and selecting anticoccidials that could be used in fisheries.

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7 Phylum Microspora

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Introduction

Microsporidia are unicellular organisms living as intracellular parasites in a variety of invertebrates and in all five classes of vertebrate hosts. Many microsporidian species are widely distributed in teleosts in freshwater, estuarine and marine habitats. Some species, e.g. *Heterosporis anguillarum* (Hoshina, 1951), *Kabatana takedai* (Awakura, 1974), *Loma salmonae* (Putz *et al.*, 1965) and *Nucleospora salmonis* (Hedrick *et al.*, 1991), are serious pathogens of cultured fishes. Microsporidian infections can cause direct losses due to mortalities, which in natural conditions can be accompanied by subsequent decline of stocks, and by indirect losses due to reduction of the marketing value of infected fish (Shaw and Kent, 1999). Microsporidian infections also constitute a threat to the ornamental fish industry and to colonies of zebrafish in research facilities (Lom *et al.*, 1989, 1993; Michel *et al.*, 1989; Matthews *et al.*, 2001).

Host Range of the Parasite

Several microsporidian species (*Glugea anomala*, *Glugea stephani*, *L. salmonae*, *Ovipleistophora mirandellae*, *Pleistophora hyphessobryconis*) are not known to be host specific. Descriptions of most species are based on findings in a limited number of hosts. Until recently, it was believed that, except for a few *Pleistophora* species that infect amphibians and reptiles, the genera known from fish do not occur in hosts other than fish. Recently, a new species of *Pleistophora*, *P. ronneafiei*, has been described from the skeletal muscle of a patient who was immunocompromised (Cali and Takvorian, 2003).

The hosts, sites of infection and known geographical distribution for each species of microsporidia in fish were published recently by Lom (2002).

Systematic and Taxonomic Position

Based on analyses of various molecular markers, Microsporidia are considered a separate phylum in the kingdom Fungi (Germot *et al.*, 1997; Hirt *et al.*, 1997, 1999; Cavalier-Smith, 1998; Keeling and McFadden, 1998; Keeling *et al.*, 2000; Van de Peer *et al.*, 2000; Tanabe *et al.*, 2002; Lom and Nilsen, 2003). The structural diversity of fish microsporidia reviewed by Lom and Nilsen (2003) includes 14 genera with 156 species to date. Recently, Azevedo and Matos (2003) added a new genus and species of xenoma-forming microsporidians, *Amazonspora hassar*, from gill filaments of the Amazonian teleost *Hassar orestis*. Previous taxonomic schemes of microsporidia

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have relied exclusively on morphological features distinguishable under light and electron microscopes. Recent classification of fish-infecting microsporidia has also integrated molecular data accumulated in the last decade (Bell *et al.*, 2001). Presenting results of phylogenetic analyses, Lom and Nilsen (2003) concluded that the grouping of fish-infecting species of microsporidia based on molecular data corresponds to the morphological criteria defined by Sprague *et al.* (1992) for families Pleistophoridae Doflein, 1901 (now containing the genera *Pleistophora*, *Heterosporis* and *Ovipleistophora*) and Glugeidae Thélohan, 1892 (with the only genus *Glugea*), while the families Spragueidae Weissenberg, 1976 (with the only genus *Spraguea*) and Tetramicridae Matthews and Matthews, 1980 (with the genera *Microgemma* and *Tetramicra*) cluster together with *Kabatana* spp. and *Microsporidium seriolae*, forming a group which is morphologically heterogeneous. Also the clade formed by partial small subunit ribosomal RNA (SSU-rRNA) gene sequences of *Loma embiotocia*, *L. salmonae*, *Loma* spp. and *Microsporidium* sp. MYX1 and by SSU-rRNA gene sequences of *Pseudoloma neurophilia* and *Ichthyosporidium* sp. is morphologically heterogeneous.

Parasite Morphology and Life Cycle

Microsporidia are strictly intracellular parasites, lacking mitochondria in all stages of their life cycle, and are characterized by single-walled spores. The spores are about 2–10 μm long, mostly ellipsoidal or egg-shaped; fish-infecting species have a large posterior vacuole (Fig. 7.1). They contain a sophisticated extrusive apparatus that injects the infectious sporoplasm into the host cell via the extrudable polar tube (Fig. 7.2F). Generic and specific determination is based on spore morphology, and especially on the characters of developmental stages. These characters (fine structure of merogonial and sporogonial stages and fine structural features of interaction with host cells) can only be detected under the electron microscope.

As a rule, fish microsporidia have simple life cycles consisting of merogony and sporogony, which take place in the cytoplasm of the host cell.

Merogony serves to produce a great number of meronts (Fig. 7.2A). Meronts divide by binary or multiple fission, thus giving rise to uninucleate cells or multinucleated plasmodia. Heavy infections with, for example, *Pleistophora* or *Glugea* species may be due to the spread of merogony stages within infected tissue or to autoinfection, when mature spores discharge their infectious sporoplasm into neighbouring host cells. As a rule meronts grow into multinucleate cylindrical or ellipsoidal plasmodia (Fig. 7.2B, E). They divide by plasmotomy or by multiple fission. The surface of meronts, as well as the ultrastructure of cell organelles, varies in individual genera and helps to distinguish them (Larsson, 1986; Lom and Nilsen, 2003).

Spores are produced in the process of sporogony. The intermediate stages are sporonts, which have an electron-dense coat and either produce sporoblasts (Fig. 7.2D) by binary fission (disporoblastic sporogony) or first grow into a multinucleated plasmodial sporont (or sporogonial plasmodium). The plasmodial sporont produces sporoblasts by multiple fission, by gradual fragmentation or by producing intermediate stages, the sporoblast mother cells, e.g. in the genus *Glugea* (Fig. 7.2C). These in turn produce sporoblasts, which mature into spores.

In most genera, a special envelope is produced at the surface of the sporont and during sporogony it detaches from the parasite to demarcate a vesicle-like space around it. This is the sporophorous vesicle (SPV) (Fig. 7.1N,P). The SPV envelope may be a thick wall or a thin membrane. In muscle-infecting species of the genus *Heterosporis*, the whole development is completed within a special thick membrane, a sporophorocyst of presumably parasite origin (see Fig. 7.7B,C).

In most species mature spores are of a uniform shape and size. The development of macro- and microspores, which differ in size and number of polar tube turns, was described in *Pleistophora*, *Ovipleistophora*

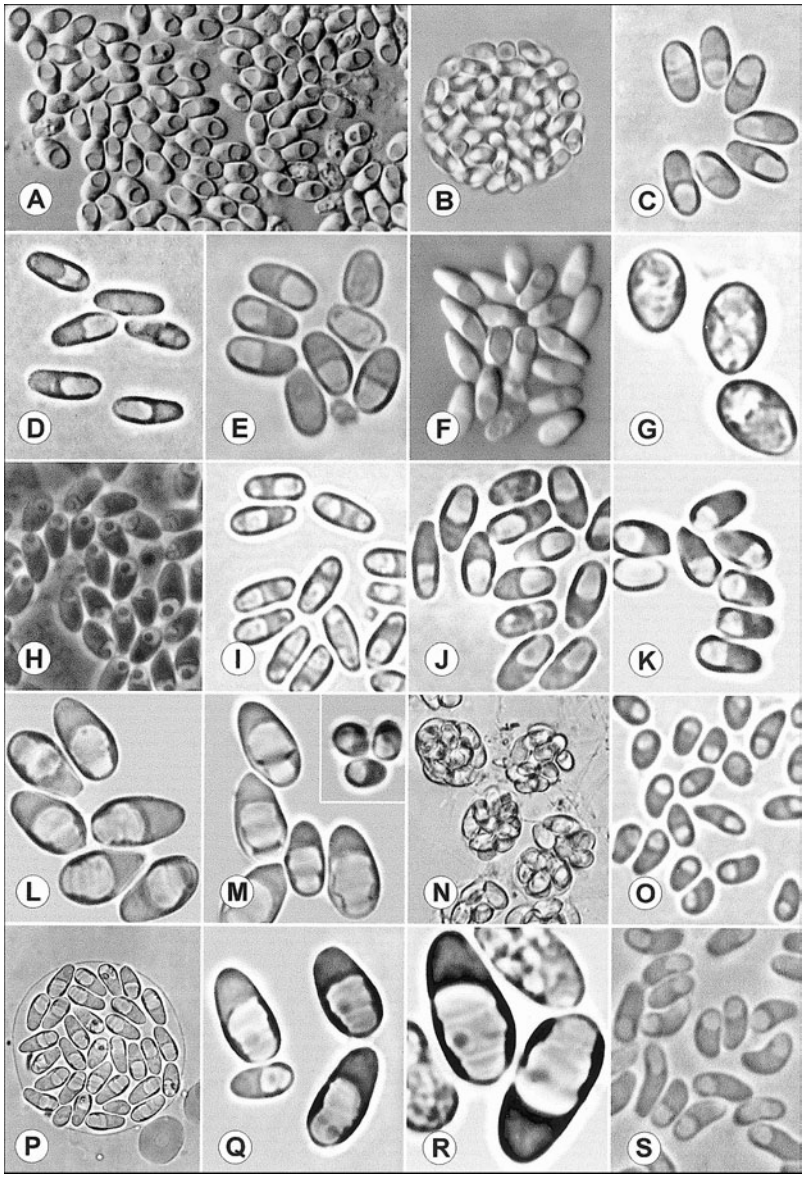


Fig. 7.1. Spores of fish-infecting microsporidia. A. *Glugea anomala* from *Nothobranchius korthausae*. B, C. *G. anomala* from *Gasterosteus aculeatus* (B. Spores within a sporophorous vesicle). D. *Glugea plecoglossi* from *Plecoglossus altivelis*. E. *Glugea stephani* from *Parophrys vetulus*. F. *Glugea atherinae* from *Atherina boyeri*. G. *Ichthyosporidium giganteum* from *Leiostomus xanthurus*. H. *Tetramicra brevifilum* from *Scophthalmus maximus*. I. *Glugea hertwigi* from *Osmerus mordax*. J. *Glugea luciopercae* from *Stizostedion lucioperca*. K. *Pleistophora hippoglossoides* from *Hippoglossoides platessoides*. L. *Pleistophora hyphessobryconis* from *Paracheirodon innesi*. M, N. *Heterosporis schuberti* from *Pseudocrenilabrus multicolor* (inset in M. Microspores; N. Spores within a sporophorous vesicle). O. *Kabatana arthuri* from *Pangasius sutchi*. P, Q. *Ovipleistophora mirandellae* from *Rutilus rutilus* (P. Spores within a sporophorous vesicle). R. *Pleistophora ovariae* from *Notemigonus crysoleucas*. S. *Spraguea lophii* from *Lophius americanus*. (The size of each species included in the plate is given in the text.)

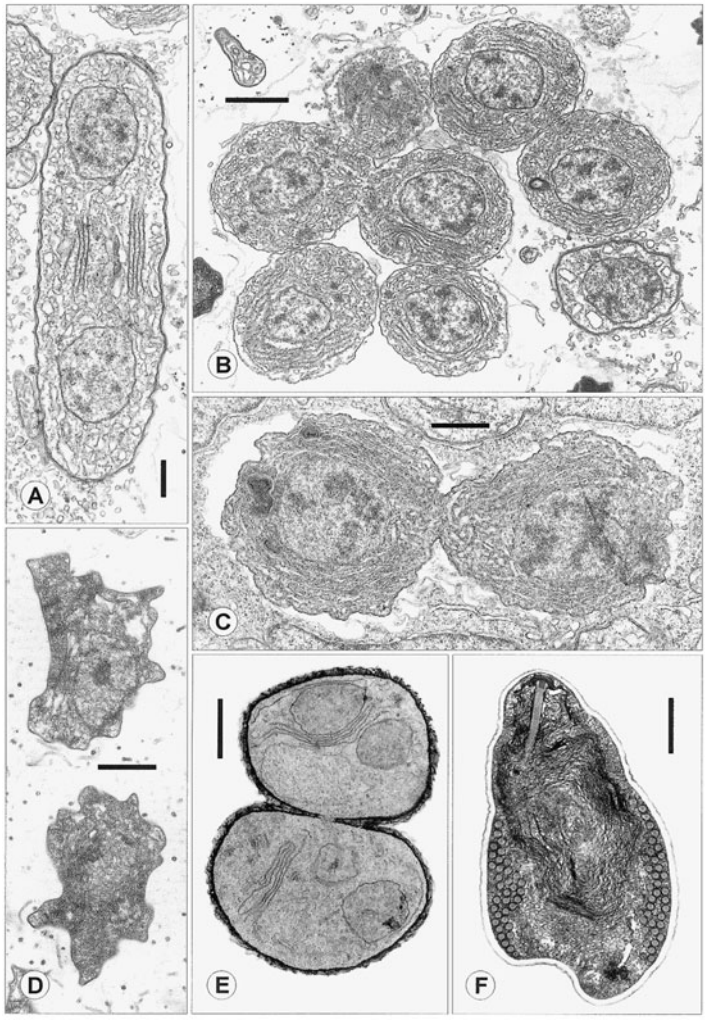


Fig. 7.2. Microsporidian life cycle stages. A. Elongated meront of *Glugea anomala*. B. Almost completely divided sporogonial plasmodium of *G. anomala*. C. Division of the sporoblast mother cell in *G. anomala*. D. Sporoblasts of *G. anomala* (common fixation shrinkage). Scale bars for A–D: 1 µm. E. *Ovipleistophora mirandellae,* sporogonial plasmodium in the process of segmentation. Scale bar: 2 µm. F. Mature spore of *O. mirandellae*. Scale bar: 1 µm.

and *Heterosporis*. In *Spraguea lophii* from the type host *Lophius piscatorius*, two developmental sequences producing two different spore forms were described and 'true spore dimorphism' was taken as an important taxonomic criterion.

Species of some genera stimulate the infected cell to enormous hypertrophy. Such hypertrophic cells with hypertrophic nuclei and surface modifications (microvilli, invaginations or thick walls) are called xenomas (Sprague and Vernick, 1968; Weissenberg, 1968). These cells and their microsporidian parasites integrate morphologically and physiologically and form a separate entity (Weissenberg, 1949) with its own development (Figs 7.3A–C, E, F and 7.7A).

Development of microsporidian infection within fish hosts depends on many factors. Among environmental factors it is the ambient temperature that is known to influence development. Thus, the growth

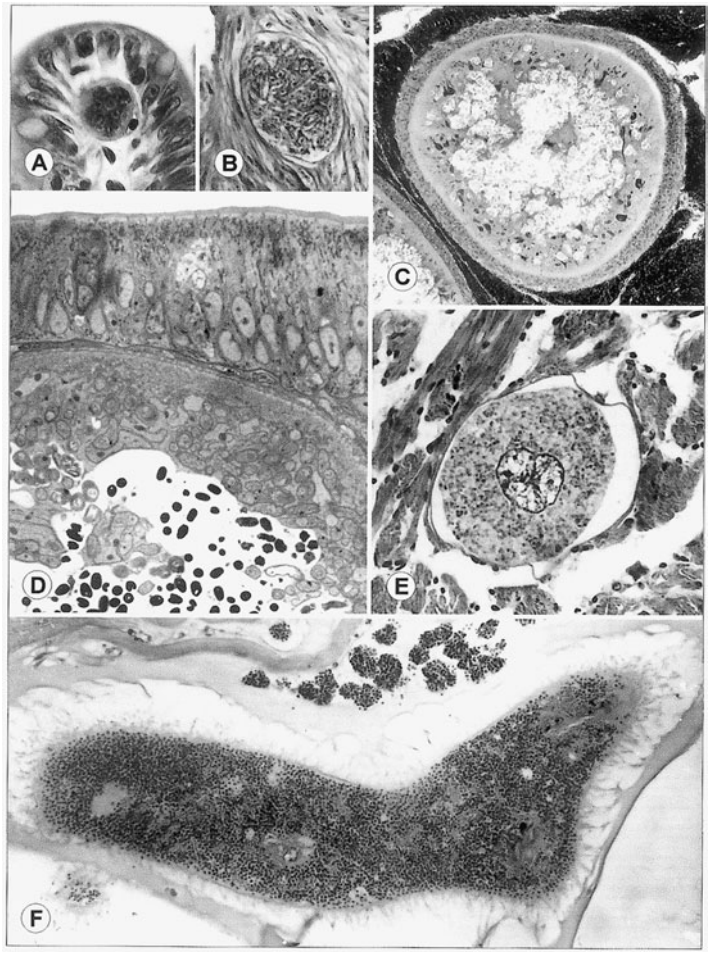


Fig. 7.3. Xenoma formations. A. Young xenoma of *Glugea anomala* in the subepithelial connective tissue of the intestine of *Nothobranchius* sp. (× 480). B. Young xenoma of *Glugea plecoglossi* with cylindrical meronts embedded in the lamina muscularis of the intestine (× 360). C. Mature xenoma of *G. plecoglossi* in the testis of *Plecoglossus altivelis* surrounded by a connective-tissue layer (× 170). D. Structure of xenoma formation of *G. anomala* with presporogonic stages on the periphery and densely stained spores accumulating inwards (semi-thin section, × 890). E. Typical small xenoma of *Glugea* sp. with hypertrophied centrally located unfragmented nucleus localized in the cardiac muscle of *Ictalurus punctatus* (× 380). F. Xenoma of *Tetramicra brevifilum* in the muscle tissue of *Scophthalmus maximus*. Note the villosities on the periphery of the xenoma and spores released from another xenoma between the remnants of muscle fibres (× 250).

of *Glugea plecoglossi* is extremely retarded at temperatures below 16°C (Takahashi and Egusa, 1977b) and experimental infections with *G. stephani* failed when the host was kept below 15°C (Olson, 1976). In *Microsporidium takedai*, low temperature (below 15°C) does not prevent infection, but the parasite only develops after the ambient temperature is raised above 15°C. Transfer of infected rainbow trout yearlings from the optimal 18°C to 8°C stops parasite development.

It is supposed that all fish-infecting microsporidia possess a direct transmission accomplished perorally, although some species described from invertebrates (*Amblyospora* spp., *Parathelohania* spp., *Dubosquina*

sp. and *Hyalinocysta chapmani*) require an intermediate host to complete the life cycle (Andreadis and Vossbrinck, 2002; Lom and Nilsen, 2003).

Experiments focused on transmission of *L. salmonae* (Shaw *et al.*, 1998) showed that exposure of *Oncorhynchus* spp. to infectious spores through cohabitation, intraperitoneal, intramuscular or intravascular injection or administration *per os* led to the development of infection, while the placement of spores on gills did not provoke infection. Transovarial (true vertical) transmission suspected in *L. salmonae* and *Pleistophora ovariae* has not been confirmed. *P. neurophilia* is spread to progeny with sexual products rather than directly within eggs (Kent and Bishop-Stewart, 2003).

Host–Parasite Relationships

The interactions of microsporidia with fish hosts have been studied mostly in natural infections (Dyková and Lom, 1980). Since the early studies of Weissenberg (1913, 1921), there have been several attempts to elucidate the host–parasite relationships in experimental infections (McVicar, 1975; Olson, 1976; Takahashi and Egusa, 1977a; Berrebi, 1978; Dyková and Lom, 1978; Matthews and Matthews, 1980; Rodríguez-Tovar *et al.*, 2002, 2003).

Site selection of microsporidians within the hosts depends on their requirements for a particular cell type within which they can develop. Some species seem to be highly cell specific (e.g. *S. lophii* develops in neurocytes, *O. mirandellae* in oocytes, *Pleistophora* species in myocytes). In some species (*Loma*) the infection was found to start in neutrophils. The cell type is difficult to recognize after it has been transformed into a xenoma. This probably explains the usage of the ambiguous term 'mesenchymal cells' for infected connective-tissue cells. Progressive (hyperbiotic) changes, manifested by extensive hypertrophy of infected cell and fragmentation of the nucleus, are set up when the xenoma starts to develop. Cells infected with non-xenoma-forming species are subject to regressive (hypobiotic) changes (see Fig. 7.5A–D). The final changes in both groups are the same. The cells are replaced with mature spores and eventually totally destroyed (Fig. 7.4D). Tissue damage elicited by xenoma-forming species is essentially the same as that caused by non-xenoma-forming species. Regressive characters (dystrophy, atrophy and necrosis) predominate in the final phase of both types of infections. The extent of regressive changes is related to the intensity of infection. When the total volume of organ or tissue is reduced, their vital functions can be seriously impaired (Ralph and Matthews, 1986).

Tissue reactions to fish-infecting microsporidia are essentially directed towards the isolation and elimination of parasites. Two patterns are recognized according to whether the parasites are localized in xenomas or disseminated in the tissue from the beginning of infection.

The first type is exemplified by *Glugea* species and comprises three successive stages: a weakly reactive stage, a productive stage with the formation of granulomas and granuloma involution (Figs 7.3A–E and 7.4A–C). The extent of the pathological changes depends on the intensity of the infection.

The weakly reactive stage is often long-lasting and overlaps with later stages. It is characteristic in infections with young, thin-walled xenomas (Fig. 7.3A, B) containing only meronts. The host tissue is damaged by pressure atrophy. This elicits proliferation of the connective tissue and results in formation of a thin, concentric layer around the xenoma (Fig. 7.3C). After they have been filled with spores, large xenomas provoke a proliferative inflammation. The host tissue reacts by granulomatous inflammation (Fig. 7.4C–E). The granulation tissue matures and the granuloma undergoes involution. It diminishes in size and the resulting fibrous connective tissue may undergo hyalinization. Gradual repair of the tissue lesions occurs quite often; however, the original function of the organ is not restored.

The second type of host tissue reaction is represented by *Pleistophora* species, which infect muscles. Host tissue reaction

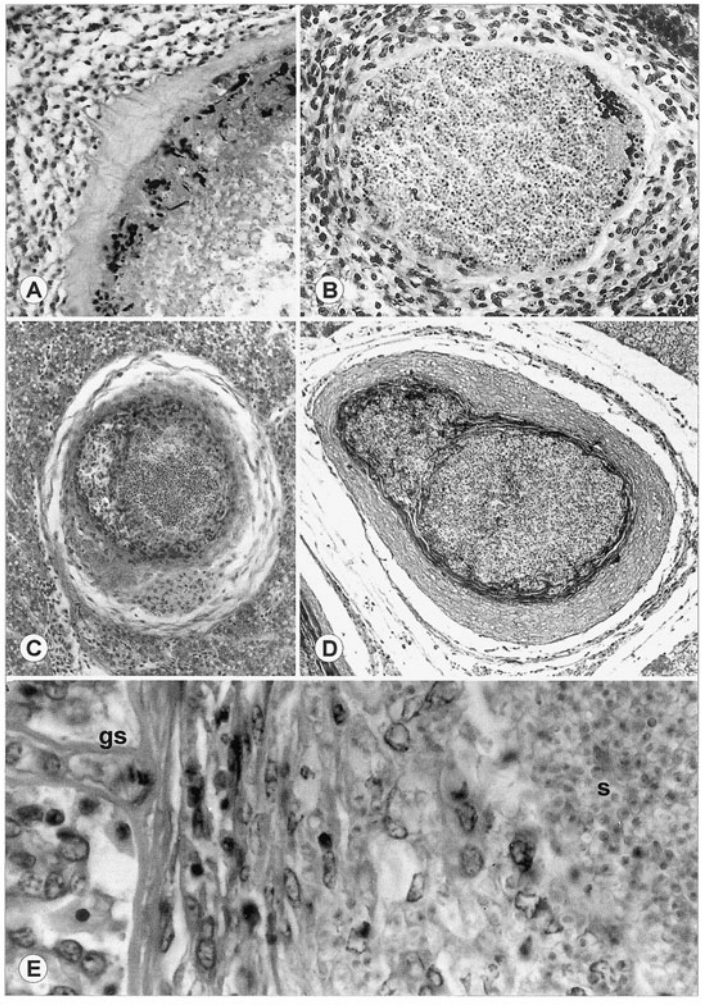


Fig. 7.4. Advanced stages of host tissue reaction to microsporidian infections. A. Inflammatory infiltration around a *Glugea plecoglossi* xenoma with dystrophically changed wall (the ovary of *Plecoglossus altivelis*, × 240). B. The remnants of *G.* cf. *anomala* xenoma surrounded by an inflammatory reaction (the liver of *Austrolebias nigripinnis*, × 320). C. Remnants of *Tetramicra brevifilum* xenoma walled off with connective tissue from the liver parenchyma of *Scophthalmus maximus* (× 220). D. *Pleistophora macrozoarcidis* spores replacing all muscle fibre of *Macrozoarces americanus* and walled off with mature connective tissue (× 150). E. Proliferation of granulation tissue triggered by spores released from xenoma of *Loma branchialis* (s, spores; gs, gill secondary lamella) (× 900).

is surprisingly slight during merogony and sporogony. There is little evidence that the invaded muscle fibres are isolated by a host response (Fig. 7.5C,E). A slight lymphocyte infiltration of the myosepta is the first indication of a tissue reaction. The productive stage of the tissue reaction occurs when mature spores completely fill the infected muscle fibre (Fig. 7.4D). A thick wall of fibroblasts may be formed to demarcate the parasite mass as soon as it undergoes necrotic changes. Phagocytic cells play a crucial role in the host defence mechanism in both types of tissue reactions. Their exact nature and origin are still not clear. In the granulation tissue, there are migratory

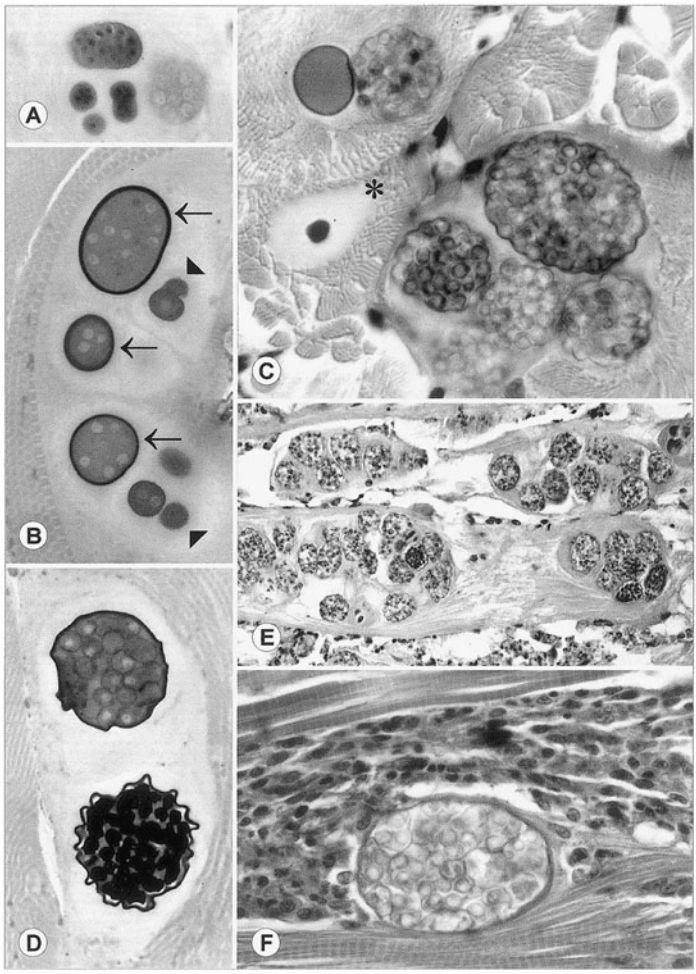


Fig. 7.5. Life cycle stages of *Pleistophora hyphessobryconis* (A–D). A, B. Early stages of the life cycle as seen in semi-thin sections: arrowheads mark meronts, arrows mark sporogonial plasmodia (× 780). C. Earlier stage of the life cycle surrounded by a halo of destroyed sarcoplasm (asterisk) and a group of sporophorous vesicles with maturing spores (× 850). D. Advanced sporogonial plasmodium (top) and sporophorous vesicle (bottom) (× 780). E. Massive infection of *P. hyphessobryconis* in the muscle tissue of *Paracheirodon innesi* (× 200). F. Sporophorocyst of *Heterosporis* sp. and inflammatory reaction in the muscle tissue of *Betta splendens* (× 730).

phagocytic cells, macrophages and fibroblasts, which ingest and digest spores (up to 30 spores were found in one cell) (Fig. 7.4E).

Many of the spore-filled macrophages in the centre of the granuloma disintegrate and other phagocytes take up the resulting spore-containing debris. Eventually, the spore content and even chitinous spore shells are completely digested.

The host reaction eventually destroys the spores produced in the course of the infection. The efficiency of the defence response depends upon the physiological condition of the host and environmental conditions. The ambient temperature affects not only the humoral response (Corbel, 1975; Roberts, 1975) but also phagocytosis. Low temperatures delay or inhibit macrophage response, fibroplasia and the activity of

fixed macrophages of the reticuloendothelial system (Finn and Nielson, 1971; Ferguson, 1976) and also impede the development of the parasite. Thus, the histopathological changes are the result of a complicated interaction of the host's response to the parasite and the environmental temperature.

The role of innate and acquired immunity against microsporidian infections in fish is far from being understood. Although phagocytosis of spores by macrophages seems to play a crucial role in the host defence (Dyková and Lom, 1980, 2000; Canning and Lom, 1986), detailed knowledge of innate resistance mechanisms is lacking. The host specificity of microsporidia provides indirect evidence of the innate immunity of fish species. Marked differences in the susceptibility to *L. salmonae* were observed between fish species (completely resistant Atlantic salmon versus susceptible chinook salmon) and even between different strains of the same fish species. Although antibody production due to microsporidian infections in fish is known, protective immunity has only been proved in rainbow trout after exposure of juveniles to the low-virulence variant of *L. salmonae* (Sanchez *et al.*, 2001a).

Microsporidia of Low Organ Specificity

Microsporidians that infect multiple organs belong to the genera *Glugea*, *Tetramicra* and *Ichthyosporidium*. Since the cells infected are connective tissue cells, xenoma formations can be found in any organ of the fish host.

G. anomala (Moniez, 1887) Gurley, 1893, the best-known species of the genus, is common in *Gasterosteus aculeatus* and *Pungitius pungitius* in Eurasia and North America. Microsporidia morphologically identical to *G. anomala* were found in several species of aquarium fish of the genera *Nothobranchius* and *Austrolebias*. The spores are elongate and slightly ovoid. The range of their highly variable size is 1.9–2.7 μm × 3–6 μm. The posterior vacuole extends to the middle of the spore (Fig. 7.1A–C). The cylindrical meronts divide by constriction into uninucleate daughter cells, which grow into sporogonial plasmodia. These divide into uninucleate sporoblast mother cells, each producing two spores.

The xenomas of *G. anomala* appear as white, usually spherical, cyst-like formations, which develop gradually to a diameter of several mm. Subcutaneously located xenomas may bulge from the organ surface and internal cysts may cause body deformities. At an early stage of infection, the host cell starts its hypertrophy (Fig. 7.3A) and later it lays down a thick xenoma wall. Hypertrophy also modifies the size and shape of the host cell nucleus. In early xenomas the meronts are distributed throughout the xenoma; in advanced xenomas they are confined to the periphery, while spores concentrate in the centre (Fig. 7.3D). In advanced xenomas fragments of host cell nuclei are observed. The ageing xenomas change into 'bags of spores', losing remnants of host cell cytoplasm and nuclei. Since the beginning of their development, xenomas cause pressure atrophy of the surrounding tissues. When xenomas are filled with mature spores, their cell membranes lose their integrity and the host inflammatory reaction starts to develop in the form of granuloma formation (Fig. 7.4B). The reparative type of inflammation that substitutes destroyed tissues with connective tissue can result in a permanent functional disorder of the infected organ.

G. plecoglossi Takahashi and Egusa, 1977 causes one of the most dangerous diseases of cultured *Plecoglossus altivelis* in Japan. *Oncorhynchus mykiss* can be infected experimentally (Takahashi and Egusa, 1977a). The sites of infection are similar to those of *G. anomala*. The development of xenomas is temperature-dependent (Takahashi and Egusa, 1977b). The spores are elongate ellipsoidal, 2.1 μm × 5.8 μm, and the posterior vacuole occupies about one-half of the spore (Fig. 7.1D). Gross lesions associated with the infection are related to the site of development of the xenomas. Large xenomas are visible through the skin and may occur as a bulge on the body surface. The sequence of tissue reaction to xenomas developing from early stages to maturity follows the pattern common in *Glugea* infections.

Glugea cepedianae (Putz, Hoffman and Dunbar, 1965) Canning and Lom, 1986 was originally described as *Pleistophora* species from *Dorosoma cepedianum* in fresh water in Ohio, USA. In the first year of life, up to 65% of young shad may be infected. Heavy infections result in high mortalities of gizzard shad, which is a valuable bait fish in some areas. The spores are slender ovoid, 2.3 μm × 4.9 μm. Single large xenomas up to 1 cm in diameter were observed to compress the visceral organs against the body wall.

Glugea atherinae Berrebi, 1978 infects the commercially important smelt *Atherina boyeri* in brackish lagoons on the French Mediterranean coast. Mathieu-Daude *et al.* (1992) performed cross infections and considered *G. atherinae* to be in fact *G. stephani*. The prevalence in separate lagoons may range from 1.2 to 21%. The infection is sex dependent. In general, females are more frequently infected than males (Berrebi, 1978). The xenomas are basically in the same organs as in other *Glugea* species. The spores are elongate ovoid, 2.9 μm × 5.7 μm in size, and the posterior vacuole occupies one-half of the spore length (Fig. 7.1F). The infection is acquired in the first and second year of life. Two types of xenomas are found in yearlings. Large xenomas are up to 14 mm in diameter, occur mainly in the body cavity and cause pressure atrophy of affected and surrounding organs. Numerous small xenomas (up to 0.2 mm in diameter) are found throughout the whole length of the post-oesophageal part of the digestive tract and may cause partial obstruction of the intestine. Experimental infections produced xenomas both in the body cavity and in the subcutaneous connective tissue. The xenomas and their effect on organs contribute to the mortality of fish.

Ichthyosporidium giganteum (Thélohan, 1895) Swarczewski, 1914 infects the marine fishes *Crenilabrus melops* along the Atlantic coast of France, *Crenilabrus ocellatus* in the Black Sea and *Leiostomus xanthurus* along the Atlantic coast of the USA. The prevalence in *L. xanthurus* may reach 25% (Schwartz, 1963). The infected cells, which transform into xenomas, may be cells of connective or fat tissue. The diplokaryotic spores are ovoid, 4 μm × 6 μm in size (Fig. 7.1G). The site of infection is subcutaneous connective tissue, fat tissue and probably also liver parenchyma (Sprague and Hussey, 1980). The infection of *L. xanthurus* is extensive and causes ventral swelling, extending from the head to the caudal fin. There are two types of xenomas. Small multilocular cystic formations are up to 0.2 mm in size and develop simultaneously in enormous numbers, while the huge, thick-walled, lobed xenomas are up to 4 mm in size. Inflammatory reactions develop around both types of xenomas. The proliferative inflammatory response is characterized by epithelioid cells in palisade-like arrangements. The early stages of *I. giganteum*, the development of xenomas and the hypothesis on the syncytial origin of 'multilocular cysts' (Sprague and Hussey, 1980), as well as the prevalence of this unique parasite, warrant further study.

Tetramicra brevifilum Matthews and Matthews, 1980 was found in feral fish populations of *Scophthalmus maximus* off the Cornish coast (UK). The prevalence in 1-year-old fish reached 10%. In a turbot farm in north-west Spain, *T. brevifilum* was diagnosed in 1990 as the agent responsible for mortalities (11.5%) and the low growth rate of surviving specimens (Figueras *et al.*, 1992). In fish with a high intensity of infection, xenomas and aggregates of spores are visible through the body wall and in organs as white nodules; muscle tissue develops a jelly-like consistency, while, in the liver, kidney, spleen and gills, globular xenomas up to 1 mm in size and xenoma-like agglomerations of spores are detected as elongated or ribbon-like xenomas, which predominate in the muscle tissue (Fig. 7.3F). These xenomas display a large mass of fragments of the host cell nuclei. The ovoid spores are uninucleate and 2 μm × 4.8 μm in size, with a large posterior vacuole (Fig. 7.1H) containing a large inclusion, which is a phenomenon very rare among fish microsporidia. A similar electron-dense body was found in the vacuole of a *Nosemoides syaciumi* spore (Faye, 1992). Except for pressure atrophy of a considerable extent due to the large size of xenomas, proliferative inflammation is elicited around the large agglomerations of

spores in parenchymatous organs. In the muscle tissue, giant xenomas displace surrounding muscle fibres in myomeres and provoke regressive changes, atrophy and liquefactive necrosis. The spores released from xenomas are surrounded by only a weak cellular response. The infection of *S. maximus* shows organ preference neither in intensity nor in prevalence.

Microsporidian Infections in the Intestine

The microsporidians that infect the intestine of marine and freshwater fishes belong to the genera *Glugea* and *Loma*.

G. stephani (Hagenmüller, 1989) Woodcock, 1904 causes infections in flatfish of the genera *Pleuronectes*, *Pseudopleuronectes*, *Parophrys* and *Rhombus* in the northern Atlantic and European seas. The prevalence may slightly exceed 50% and its development is temperature-dependent. Spores from *Pseudopleuronectes platessa* are oval and about 2.7 μm × 4.7 μm in size (Fig. 7.1E). Since xenomas average 1 mm in diameter, the intestinal lesions can be observed macroscopically. In heavy infections the masses of developing xenomas in subepithelial connective tissue cause thickening of the intestinal wall, atrophy of the epithelium and luminal occlusion. Severe morphological alterations of the intestine along with functional failure may be limiting factors that affect the growth of feral and cultured plaice and flounders.

Glugea hertwigi Weissenberg, 1911 is also highly pathogenic and occurs mainly in the intestine of the euryhaline smelt *Osmerus eperlanus eperlanus* in the Baltic Sea, and is also common in *Osmerus mordax* in Canada and the USA. In northern Russian lakes, it occurs in *Hypomesus olidus* and in fish of the genus *Coregonus*. The spores are elongate oval, 2.2 μm × 4.7 μm in size (Fig. 7.1I). *G. hertwigi* forms giant xenomas (up to 4 mm in diameter), mainly in the intestine, though in massive infections almost all organs may be affected. The maximum intensity of infection was about 250 xenomas per host (Sinderman, 1963). Severe epizootics with high mortalities were recorded in smelt in Canadian lakes, in New Hampshire (Haley, 1952, 1953, 1954) and in Russia (Annenkova-Khlopina, 1920; Bogdanova, 1957; Petrushevski and Shulman, 1958). The effect of *G. hertwigi* on the intestine of the host is basically the same as in *G. stephani* infection. In the anterior part, the most heavily infected portion of the intestine (Delisle, 1972), the intestinal wall may disintegrate completely, and dystrophic changes associated with septicaemia or intoxication may result in the death of the host. Infected fish may survive for a period of time if organ functions are not severely impaired and the host tissue reaction (i.e. granulomatous inflammatory reaction) takes place to eliminate xenomas or to repair the ulcerative losses. The influence of stress is extremely important. Mortality is high due to spawning stress in late spring and due to environmental stress in other seasons (Nepszy and Dechtiar, 1972).

Glugea luciopercae Dogiel and Bykhovski, 1939, syn. *Glugea dogieli* Gasimagomedov and Issi, 1970, occurs in *Stizostedion lucioperca* in estuarine and freshwater habitats in Europe and Asia. The spores are elongate oval 2.1 μm × 4.5 μm with a relatively narrow anterior end (Fig. 7.1J). In the intestine of pike and perch, numerous tiny xenomas fuse together to form a confluent layer in the subepithelial connective tissue.

Loma acerinae (Jírovec, 1930) Lom and Pekkarinen, 1999 is a representative of a group of species that form small xenomas where the host cell nucleus is in a central position. The developmental stages of the parasite are not limited to the periphery of the xenoma. The parasite occurs in *Gymnocephalus cernuus* in Central Europe. Spores are ellipsoid, 2.7 μm × 4 μm, with a large posterior vacuole. The xenomas are spherical and do not exceed 0.5 mm in diameter. They are located in the subepithelial connective tissue of the intestine and are sometimes also found in the lamina muscularis. The parasite causes only slight pathological changes in the digestive tract. A similar type of xenoma was observed in *Loma myrophis* infection in

the midgut of *Myrophis platyrhynchus* collected in the Amazon River (Azevedo and Matos, 2002).

Microsporidian Infections in the Liver

There are a few records of microsporidians infecting the liver parenchyma. The parasites belong to three genera, *Glugea*, *Microgemma* and *Microsporidium*, and are known from marine hosts.

Glugea machari (Jírovec, 1934) Sprague, 1977 syn. *Octosporea machari* Jírovec, 1934, has been recorded from the liver of *Dentex dentex* in the Adriatic Sea. The xenomas are up to 400 μm in diameter and are localized near blood vessels.

Microgemma hepaticus Ralph and Matthews, 1896 was described from the liver of juvenile *Chelon labrosus* off the coast of Cornwall, UK. The prevalence was about 38%. Pyriform spores, 2.4 μm × 4.2 μm in size, develop within xenomas, which are up to 0.5 mm in diameter. Meronts divide by plasmotomy and are enclosed by the host membrane. The sporogonic stages are free in the cytoplasm and divide by exogenous budding. The foci of infection are manifested as white spots in the liver. Xenomas are associated with the walls of blood vessels, with bile ducts and sometimes even with capsules of the encysted metacercariae of *Bucephalus haimeanus* (Ralph and Matthews, 1986). Degenerated xenomas elicit a typical inflammatory reaction, leading to granuloma formation. The parasite causes considerable mortalities in 0+ age-class *C. labrosus*.

Microgemma ovoidea (Thélohan, 1895) Amigo, Salvado, Gracia and Vivarès, 1996 (synonyms *Glugea ovoidea* Thélohan, 1895; *Nosema ovoideum* Labbé, 1899; *Microsporidium ovoideum* Sprague, 1977) was originally described from *Motella tricirrata*. It also occurs along the French coast in *Cepola rubescens* and *Mullus barbatus*. Spores are ovoid, 1.5 μm × 2.5 μm in size. Uninucleate meronts divide by binary fission. Sporogony starts with binucleate cells dividing in two sporonts. Each sporont divides to produce two sporoblasts.

Microsporidian Infections in Muscles

The microsporidians that infect muscle tissue are species of *Pleistophora*, *Heterosporis*, *Kabatana* and *Microsporidium*.

Pleistophora macrozoarcidis Nigrelli, 1946 is common in *Macrozoarces americanus*, in the western part of the northern Atlantic. The prevalence of infection increases with the age and size of the fish (Sheehy *et al.*, 1974). The microspores are usually ovoid, 3.5 μm × 5.5 μm, and they have a large vacuole at the wider posterior end. The rare sausage-shaped macrospores are up to 8 μm long. In early infections the parasite foci appear as small white cylinders lying along the axis of the body of the fish host. In advanced stages they appear as tumour-like masses, with large areas of body musculature replaced with cell debris and spores. In most cases a thin layer of sarcoplasm remains in each infected muscle fibre. The infected muscle fibres may be encapsulated with concentrically arranged connective tissue (Fig. 7.4D). No news on the occurrence has been available recently.

Pleistophora ehrenbaumi Reichenow, 1929 is common in *Anarhichas lupus* and *Anarhichas minor* in the North Sea. Meyer (1952) reported 10% prevalence in *A. lupus* in the waters west of Iceland. The spores are ovoid and uninucleate, with a large posterior vacuole and distinct coils of the polar tube. Spore size varies from 1.5 μm × 3 μm (microspores) to 3.5 μm × 7 μm (macrospores). In the infected muscle fibres, there are mostly small, brownish, needle-like formations, which are filled with spores. Egidius and Soleim (1986) found a giant tumour-like formation in an emaciated *A. lupus*. In the course of infection, SPVs filled with spores gradually replace muscle fibres. As long as a thin peripheral layer of sarcoplasm remains, no host tissue reaction is apparent. Enhanced phagocytosis of spores occurs when the remnants of muscle fibres disintegrate.

Pleistophora hippoglossoides Bosanquet, 1910, originally described from *Drepanopsetta hippoglossoides*, is also common in *Hippoglossoides platessoides* and *Solea solea*. The muscle tissue of heavily infected hosts is unfit for human consumption because of

the consistency and organoleptic qualities. The elongate-ovoid or pyriform spores are monomorphic, 2.7 μm × 4.7 μm in size (Fig. 7.1K). The whitish parasitic nodules are visible through the skin. They consist of a mass of sporophorous vesicles lying in a structureless matrix with the remnants of the muscle fibres. Kabata (1959) observed displacement of muscle tissue surrounding these nodules.

P. hyphessobryconis Schäperclaus, 1941 occurs commonly in freshwater aquarium fish. In addition to its two original hosts *Paracheirodon innesi* and *Hemigrammus erythrozonus*, about 16 other freshwater fishes belonging to four families have been found to be infected. Some of these are feral fish. The species was imported in fish from the upper course of the Amazon River. However, it is presently distributed all over the world. The spores are ovoid, 4 μm × 6 μm in size, and one side is slightly more vaulted. The posterior vacuole occupies more than one-half of the spore length (Fig. 7.1L). There are up to 34 coils of the polar tube. SPVs with 20 to 130 spores may reach up to 35 μm in diameter. In the neon and glowlight tetras the infected segments of muscles are clearly visible as greyish or whitish patches under the skin. In heavy infections the parasite may spread to other organs and the body cavity. Developmental stages within the individual muscle fibres may be surrounded by amorphous dystrophic sarcoplasm or by unaffected peripheral sarcoplasm (Fig. 7.5A–D). As the infection progresses, large blocks of muscles are destroyed (Fig. 7.5E). The resulting debris contains masses of SPVs and free spores, together with host phagocytes with ingested spores.

Pleistophora finisterensis Leiro, Ortega, Iglesias, Estévez and Sanmartín, 1996 was described from blue whiting, *Micromesistius poutassou*, from coastal waters in north-west Spain. The yellowish or white fusiform (3–6 mm) lesions localized in the hypaxial musculature of the host contained ovoid or slightly pear-shaped spores with a large posterior vacuole, 2 μm × 4 μm in size. SPVs containing 150–250 spores reach a diameter of up to 25 μm. The muscle fibres adjacent to the infected ones were not altered.

The genus *Heterosporis* Schubert, 1969 has three species, two of which infect ornamental fishes.

Heterosporis finki Schubert, 1969 is found in the oesophageal wall and skeletal muscles of *Pterophyllum scalare*. The spores are ovoid with a large posterior vacuole. The macrospores are 2.5 μm × 8 μm and the microspores 1.5 μm × 3 μm in size. The pathogenicity of *H. finki* in juvenile farmed *P. scalare* was studied by Michel *et al.* (1989). The parasite caused significant losses and adult fish appeared to be more resistant. In severe infections fish were emaciated, with discoloured or greyish spots on the skin. All the skeletal muscles were milky white and creamy in texture. The striated muscles contained numerous sporophorocysts.

Heterosporis schuberti Lom, Dyková, Körting and Klinger, 1989 infects the ornamental fish *Pseudocrenilabrus multicolor* and *Ancistrus cirrhosus*. Massive infection of muscle tissue has been repeatedly found in both fishes and the infection caused up to 95% mortality in *A. cirrhosus*. The spores from *P. multicolor* vary greatly in shape and size. They may be broadly ovoid microspores (3.1 μm × 4.2 μm in size), medium-size spores (about 6.5 μm in size and nine per SPV) and macrospores (4.4 μm × 7 μm) (Fig. 7.1M,N). The posterior vacuole occupies about two-thirds of the spore length. Infected specimens of *P. multicolor* were partly emaciated and showed signs of distress. Large parts of the trunk musculature were pervaded with parasites. The earliest stages identified were early meronts. They were wedged into myocytes and were without a halo of disintegrated sarcoplasm. Small or young sporophorocysts (see Fig. 7.7B) were surrounded by muscle fibrils, which extended perpendicularly from the cyst surface. In the final stages of infection, the walls of the SPVs disappeared within the cyst and a thick wall of connective tissue was built up around the sporophorocysts. Several sporophorocysts were fused together to form a large cystic structure with a common connective tissue wall. Between the sporophorocysts there was moderate cellular infiltration. Eventually, spores were released into spaces between the myocytes. The muscle was thus

converted into a disorganized mass of damaged myocytes, cystic structures, free spores and aggregates of macrophages with ingested spores (see Fig. 7.7C, D).

H. anguillarum (Hoshina, 1951) Lom, Dyková, Wang, Lo and Kou, 2000 (syn. *Pleistophora anguillarum* Hoshina, 1951) is a serious pathogen of farmed Japanese eel, *Anguilla japonica*, in Japan and Taiwan. Severe infections, localized mainly in skeletal muscles, cause growth retardation and/or considerable mortalities. Infected eels may be unsuitable for market. The spores are elongate-ovoid, with a huge posterior vacuole occupying almost two-thirds of the spore length. Microspores average 2.4 μm × 7.8 μm. The clinical signs include deformities of the body, whitish spots beneath the skin, depressed sites of the trunk musculature and its liquefaction (T'sui and Wang, 1988). Infected muscle fibres are gradually replaced with cysts, which are full of mature spores. Along with these regressive changes in the muscles, the host inflammatory reaction is initiated. In advanced infection the disappearance of muscle fibres or whole myomeres is associated with an extensive fibroblastic response.

K. takedai (Awakura, 1974) Lom, Nilsen and Urawa, 2001 (synonyms: *Glugea takedai* Awakura, 1974; *Nosema takedai* Miki and Awakura, 1977; *Microsporidium takedai* (Awakura, 1974) Canning and Lom, 1986), an important pathogen of cultured commercial fish in Japan (originally found in *O. mykiss*), infects eight salmonid species in Japan and Sakhalin. The prevalence may be 100% in *O. mykiss* and *Oncorhynchus gorbuscha*; in *Oncorhynchus masou*, *Oncorhynchus keta* and *Salvelinus leucomaenis*, it may range from 86 to 93%. The infection is temperature-dependent and is restricted to the warmer period of the year (Urawa, 1989). The parasite grows well at 18°C, whereas it stops growing at 8°C. The spores, with subapically attached polar tubes, are ovoid, 2 μm × 3.4 μm in size. *K. takedai* produces spindle-shaped cyst-like formations, up to 6 mm in size, in skeletal muscles. In the heart, they are globular, about 2 mm in diameter. Both types of 'cysts' contain proliferating microsporidia in a cytoplasmic mass, which lacks host cell nuclei, xenoma wall or any other distinct boundary. The heart muscle 'cysts' prevail in chronic infection and develop at lower temperatures. In the acute disease, there is high mortality, with enormous numbers of 'cysts' in the trunk musculature (up to 130 per gram of tissue). There is a strong negative correlation between the condition of the fish and the intensity of the infection. Mortalities were also observed in wild masu salmon (Urawa, 1989). In experimentally infected yearlings, host tissue reaction started on day 11 with inflammatory infiltration. Phagocytic cells appeared on day 17. Their number declined to a minimum on day 30. The phagocytic response was more pronounced in 2-year-old trout.

Kabatana arthuri (Lom, Dyková and Shaharom, 1990) Lom, Dyková and Tonguthai, 2000 (synonyms: *Microsporidium arthuri* Lom, Dyková and Shaharom, 1990; *Kabataia arthuri* Lom, Dyková and Tonguthai, 1999) was observed in the skeletal musles of *Pangasius sutchi* from Thailand. Sporogony proceeds without SPV formation. Multinucleate meronts transform into sporogonial plasmodia. Round, pyriform, uninucleate spores average 2.1 μm × 3.1 μm (Fig. 7.1O). In heavy infections the parasite destroyed parts of the musculature (Fig. 7.6D). Necrotic changes developed in muscle fibres around presporogonic stages as well as on the periphery of giant aggregates of mature spores (Fig. 7.6A,D). The main feature of the host defence reaction was the phagocytic activity of macrophages. A host inflammatory reaction was observed only exceptionally. Spore-laden macrophages were found in various tissues and organs (Fig. 7.6B,C). Their infiltration in the epidermis includes its outermost layers and may effectively enhance the spread of infection while the host still lives (Fig. 7.6B).

Kabatana seriolae (Egusa, 1982) Lom, Dyková and Tonguthai, 1999 (syn. *Microsporidium seriolae* Egusa, 1982) infects *Seriola quinqueradiata* in Japanese maricultures. The spores are ovoid or pyriform (2.2 μm × 3.3 μm in size) and are produced by multiple fission of multinucleate sporogonial plasmodia. As in the preceding species, SPVs and diplokarya are absent. Pathological changes are

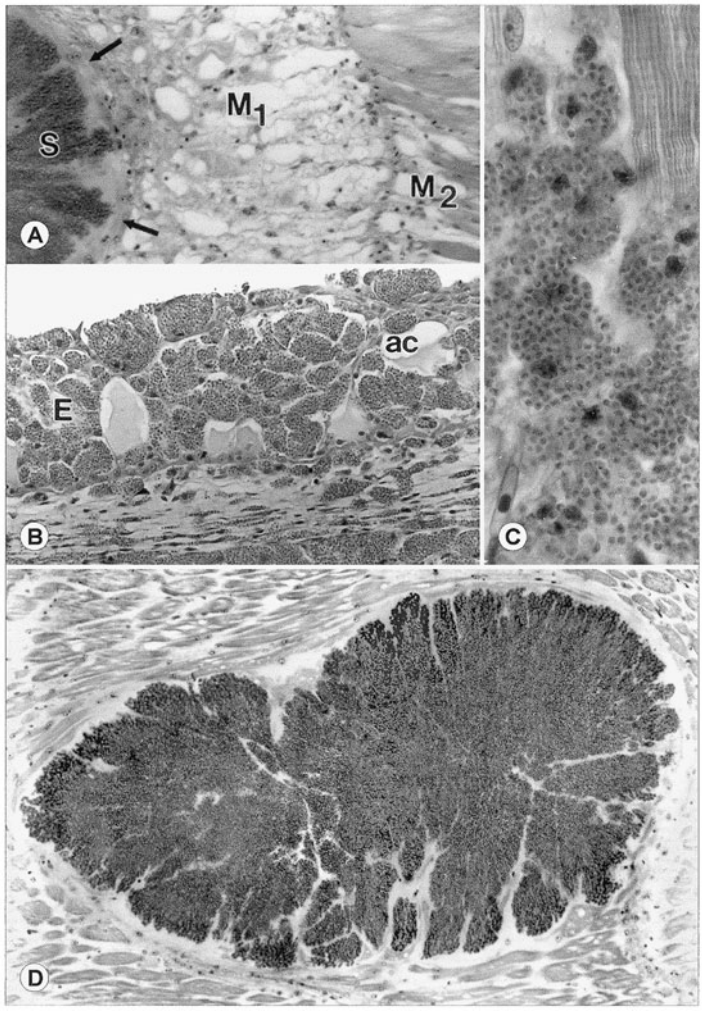


Fig. 7.6. *Kabatana arthuri* infection in *Pangasius sutchi*. A. Regressive changes of two myomeres (M_1, M_2) adjacent to a giant aggregate of spores (S). Arrows mark thin connective-tissue envelope (× 350). B. Macrophages replete with spores among still preserved muscle fibres. Macrophages in epidermis (E) and alarm cells (ac) (× 870). C. Spore containing macrophages in corium (× 340). D. Longitudinal section of the ventral muscle showing a large mass of spores (× 185).

manifested as surface depressions, which indicate disintegrated regions of muscles. The amorphous, cheese-like tissue debris contains various stages of the life cycle.

Microsporidian Infections in Gills

Species of the genus *Loma*, which are morphologically very similar, are restricted to gill filaments or lamellae. *Loma branchialis* (Nemeczek, 1911) Morrison and Sprague, 1981 (synonyms: *Nosema branchialis* Nemeczek, 1911; *Glugea branchialis* (Nemeczek, 1911) Lom and Laird, 1976; *Loma morhua* Morrison and Sprague, 1981) are found in *Gadus* (*Melanogrammus*) *aeglefinus* and six other species of gadid fish in the North Atlantic and north-western seas. The prevalence is about 10 to 60%. Spores

are ovoid, 2.3 μm × 4.8 μm. White cyst-like xenoma formations (about 0.5 mm in size) are localized in gill filaments or secondary lamellae (Fig. 7.7A). The reported infections are usually light. Distortions and atrophy or local vascular changes in gill filaments did not seem to affect the host. The tissue reaction, which eliminates xenomas and repairs altered gills, is similar to the reaction provoked by other xenoma-forming species. The overall damage to the gills correlates with the intensity of infection.

L. salmonae (Putz, Hoffman and Dunbar, 1965) Morrison and Sprague, 1981 (syn. *Pleistophora salmonae* Putz, Hoffman and Dunbar, 1965) is widespread in feral and hatchery-reared *O. mykiss*, *O. masou* and *Oncorhynchus nerka* throughout North

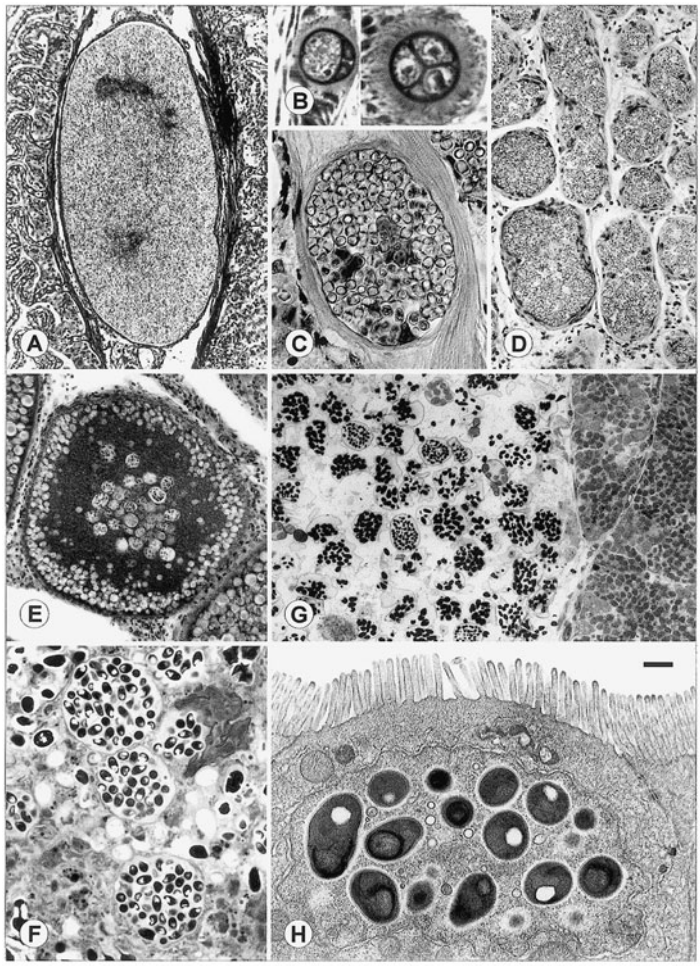


Fig. 7.7. A. Xenoma of *Loma branchialis* filled with spores in a gill filament of *Melanogramus aeglefinus* (× 150). B. Early (left) and more advanced (right) sporophorocyst with meronts of *Heterosporis schuberti* from muscles of *Ancistrus cirrhosus* (× 850). C. Sporophorocyst of *H. schuberti* in muscles of *Pseudocrenilabrus multicolor* (× 480). D. Muscle fibres replaced with masses of spores in *H. schuberti* infection in *P. multicolor* (× 150). E, F. *Ovipleistophora mirandellae* infection in oocytes of *Abramis bjoerkna* (× 140). F. Sporophorous vesicles in an oocyte (× 640). G. Sporophorous vesicles of *O. mirandellae* in the testis of *Rutilus rutilus* (× 220). H. *Nucleospora secunda,* spores developing in the nucleus of enterocyte of *Nothobranchius rubripinis*. Scale bar: 1 μm.

America and Japan. It has been imported into France with *Oncorhynchus kisutch*. Considerable mortalities were recorded in Japan and North America. In one Californian epizootic, almost all the fingerlings were lost in a hatchery (Putz, 1964). Spores are pyriform, 2.4 μm × 7.5 μm in size, two or four being produced in SPVs. Uninucleate meronts develop into elongate plasmodia, which have at least five nuclei.

The clinical signs of infection are similar to those in *L. branchialis*. In heavy infections gill filaments are distorted by xenoma formations, secondary lamellae fuse and there is a marked epithelial hyperplasia in all parts of the gills. Wales and Wolf (1955) correlated anaemia with the intensity of the *L. salmonae* infection. Using a specific digoxigenin-labelled single-stranded DNA probe, Sanchez *et al*. (2001b) detected early developmental stages of *L. salmonae* in the gut mucosal epithelium (24 h post-exposure) and in the heart (48 h post-exposure). They visualized for the first time the haematogenous distribution of this parasite and deduced that merogony takes place before *L. salmonae* reaches the gills.

Loma fontinalis Morrison and Sprague, 1983, described from *Salvelinus fontinalis* in Canada, is very similar to *L. salmonae*.

Microfilum lutjani Faye, Toguebaye and Bouix, 1991 was described from gill secondary lamellae of *Lutjanus fulgens* from coastal waters of Senegal.

Microsporidian Infections in Gonads

Several species that belong to the genera *Ovipleistophora* and *Microsporidium* were found to infect gonads of fishes. Most of them are restricted to female hosts. There is also one species assigned to the genus *Pleistophora* and two doubtful species of the genus *Thelohania* that infect oocytes (Canning and Lom, 1986).

O. mirandellae (Vaney and Conte, 1901) Pekkarinen, Lom and Nilsen, 2002 (syns *Pleistophora oolytica* Weiser, 1949; perhaps also *Pleistophora elegans* Auerbach, 1910; according to Maurand *et al*. (1988) also *Pleistophora longifilis* Schuberg, 1910 from testes of *Barbus barbus*) is common in *Abramis bjoerkna*, *Abramis brama*, *B. barbus*, *Leuciscus leuciscus*, *Rutilus rutilus* and other European cyprinids. It is sometimes also found in *Esox lucius* and *Hucho hucho*. Sporogonial plasmodia arise from oval meronts with one to four nuclei and produce sporophorous vesicles (Figs 7.1P and 7.7F). There are macrospores, with size range 7.4–12 μm × 3.9–6.4 μm (about 30 to 60 per SPV, medium-size spores (about 100 per SPV), with size range 4–6.5 μm × 2–3.5 μm, and microspores (3 μm × 1.5 μm in size) (Fig. 7.1Q). The macroscopic white lesions are obvious in the gonads of male and female fish. The infection of oocytes probably occurs before the zona radiata is formed. The parasite develops within the yolk, which is eventually replaced by the spores (Fig. 7.7E). Up to 20% of oocytes may be infected. The oocytes or their remnants are surrounded by a pronounced proliferative inflammatory reaction and contain numerous regressively changed spores. In testes the developmental stages of *O. mirandellae* are in the supporting connective tissue among the seminiferous tubules (Fig. 7.7G). *O. mirandellae* infection may reduce fecundity in both male and female fish.

P. ovariae Summerfelt, 1964 is a common parasite of *Notemigonus crysoleucas* and *Pimephales promelas* in the Midwest and southern states of the USA, with an overall prevalence of about 46% and with a much higher prevalence in bait minnow hatcheries. According to Lom (2002), this species possibly also belongs to the genus *Ovipleistophora*. The SPVs, 23 μm in diameter, contain 8 to 20 elongate ovoid spores 8.4 μm × 4.2 μm in size (Fig. 7.1R). The posterior vacuole occupies two-thirds of the spore length. Since the development of *P. ovariae* takes place in oocytes, infected oocytes become prematurely atrestic. Spores released from oocytes are removed by phagocytosis. The course of the inflammatory reaction has not been described. In the post-spawning season, a mass of proliferating connective tissue was noted in infected ovaries of fish. Mortalities were not observed but parasitic castration may reduce the fecundity of

shiners and minnows by more than 40% (Summerfelt, 1964).

Microsporidium sulci (Rašín, 1936) Canning and Lom, 1986 (syns *Cocconema sulci* Rašín, 1936; *Pleistophora sulci* (Rašín, 1936) Sprague, 1977) commonly infects oocytes of *Acipenser ruthenus* and *Acipenser gueldenstaedti* in the Danube and Volga River basins. Spherical spores, which are 2.5 μm in diameter, develop in SPVs. Sporogonial stages have diplokarya, and meronts are cylindrical.

Microsporidian Infections in the Nervous System

S. lophii (Doflein, 1898) Vávra and Sprague, 1976 (syns *Glugea lophii* Doflein, 1898; *Nosema lophii* (Doflein, 1898) Pace, 1908; *Glugea americanus* Takvorian and Cali, 1986) is a common parasite of *L. piscatorius* and of four other anglerfish species along the coast of Europe and Iceland, in the North Sea, along the US Atlantic coast and along the coast of Brazil. The prevalences vary from 32% to 100% in *L. piscatorius*, *Lophius budegassa* and *Lophius americanus*, and seem to increase with the age of the host (Priebe, 1971). Based on a single ultrastructural study by Loubès *et al.* (1979), *S. lophii* is considered to be a dimorphic microsporidian with two developmental sequences. Each produces spores of a different type. In small xenomas, there are uninucleate oval spores of the *Nosemoides* type. These are 2.5 μm × 4.5 μm in size (Fig. 7.1S). In advanced xenomas, there is a peripheral region with a different type of developmental stages and slender curved *Nosema*-type spores (1.4 μm × 3.7 μm in size) with diplokarya. Freeman *et al.* (2003) follow the claim of Takvorian and Cali (1986) that *Lophius* species other than *L. piscatorius* harbour *Spraguea* without spore dimorphism. Pathological lesions associated with the *S. lophii* infection are conspicuous when the cerebrospinal region of the host is examined. Intracellular development of *S. lophii* in the cells of cranial and spinal nerve ganglia causes hypertrophy, manifested as nodular formations resembling bunches of grapes. There is proliferation of glial cells between the xenomas, which gradually turn to granulomas. The impairment of vital functions has not been described and most xenomas in mature fish only contain a mass of phagocytosed and destroyed spores.

Pseudoloma neurophilia Matthews, Brown, Larison, Bishop-Stewart, Rogers and Kent, 2001 is a parasite of the spinal cord and brain of zebrafish, *Danio rerio*, a model organism widely used in studies of vertebrate developmental biology. The infection was detected in many laboratory colonies and pet stores (Matthews *et al.*, 2001). In addition to the central nervous system, the primary site of infection, free or phagocytosed spores are also detectable in skeletal muscles within foci of the host inflammatory reaction. Spores are ovoid to pyriform, uninucleate and 5.4 μm × 2.7 μm in size, with a large posterior vacuole. Their polar tube forms 13 to 16 coils. According to the original description (Matthews *et al.*, 2001), the SPVs containing aggregates of 8 to 16 spores are located within xenoma formations. Since the authors did not observe any meronts, a morphological re-description is warranted.

Microsporidia – Exclusive Parasites of Fish Cell Nuclei

N. salmonis Hedrick, Groff and Baxa, 1991 (syn. *Enterocytozoon salmonis* Chilmonczyk, Cox and Hedrick, 1991) infects blood leucocytes and haematopoietic cells of the lymphocyte lineage in the spleen and kidney of *Oncorhynchus tshawytscha* in the Pacific Northwest of the USA. Similar haematopoietic infections were reported in *O. mykiss* in British Columbia, Canada and France. Spores are ovoid, 1.5 μm × 2 μm in size, and have 8 to 12 turns of the polar tube. All stages develop in direct contact with the host cell nucleoplasm. In the chinook salmon *O. tshawytscha*, up to 37% of spleen haemoblasts harboured these parasites (Elston *et al.*, 1987). Non-haemopoietic cells, e.g.

epithelial cells of the urinary tubules or mesangial cells of the glomeruli, were occasionally infected, and the infection was usually associated with a high rate of infection in haematopoietic tissues. The clinical signs are anaemia, lymphoblastosis and leukaemia, but these have not been confirmed in experimental infections.

Nucleospora secunda Lom and Dyková, 2002 infects nuclei of enterocytes of ornamental fish *Nothobranchius rubripinnis* (Fig. 7.7H). Developmental stages and spores are isolated from one another in the karyoplasm. Merogony proceeds from simple uninucleate to multinucleate meronts. Transformation into sporogonial plasmodia is marked by the appearance of ellipsoidal dark bodies, evidently polar tube precursors. Primordia of the extrusion apparatus are already established in the plasmodium before it starts to cleave into separate sporoblasts. Ellipsoidal mature spores are of average size, 1.6 μm × 0.8 μm, and have four to five turns of the polar tube. Tubular inclusions appear in the karyoplasm of infected nuclei.

In Vitro Culture and Propagation of Microsporidia

In vitro culture (from meronts to infectious spores) was accomplished with *N.* (*Enterocytozoon*) *salmonis* (Wongtavatchai *et al.*, 1995). It has been propagated *in vitro* for over 17 passages in chinook salmon mononuclear leucocyte cultures. Experimental infections proved the infectivity of spores harvested from the cultures. Successful *in vitro* culture of a fish-infecting microsporidian species has also been announced by Lores *et al.* (2003). The authors reported continuous *in vitro* culture of *Glugea* sp., which was most probably *Microgemma caulleryi* (Lom, 2002). As a host cell line they used larval cells of the mosquito *Aedes albopictus* at 28°C. They failed to culture this species in a salmon cell line (CHSE-214) at 21°C. Unfortunately, only several life cycle stages were documented and the formation of xenomas was not observed.

Diagnosis of Infection

Clinical signs and gross lesions are observed in fish heavily infected with microsporidia. Multiple focal lesions due to xenoma-forming species can be observed on the body surface as well as in the body cavity, digestive tract and parenchymatous organs, depending on the number and site of the development of xenomas. They resemble plasmodial cyst-like formations due to myxosporean infections. The lesions caused by non-xenoma-forming species are of a less defined shape, manifesting themselves as whitish foci in the affected tissue. The white colour also characterizes oocytes infected with *Ovipleistophora* sp. To make a general etiological diagnosis, i.e. to recognize microsporidian infection, mature spores typical of this group of organisms have to be detected in the content of lesions.

In the light microscope, spores of microsporidia can easily be recognized in fresh tissues. The best visibility can be obtained using Nomarski differential interference contrast. The large posterior vacuole typical for fish-infecting species and the uniform size and shape of the spores differentiate them from yeasts and other objects (in most species, mature spores are of a uniform shape and size, natural variation is low and spore dimorphism is rather exceptional). When spores sampled from lesions are available, the polar tube can be expelled by, for example, 2% hydrogen peroxide. As an important diagnostic feature, the polar cap at the spore apex can be stained as a red dot using the periodic acid Schiff reaction. In tissue sections, spores stain dark blue with Giemsa but they can also be recognized in sections stained routinely with haematoxylin and eosin.

Careful ultrastructural studies following the lines drawn, for example, by Larsson (1986), Canning and Lom (1986) or Lom and Dyková (1992) constitute a basic prerequisite for generic and species determination of the agents of lesions. A practical key for the determination of microsporidian genera parasitic in fishes, together with a catalogue of described genera and species, has been given recently by Lom (2002). In total, 108 named

fish-infecting species and 59 innominate ones were included.

Serodiagnosis is not currently used in fish microsporidioses. Protein profiles of spores were characterized for several fish-infecting microsporidia with the aim of testing possible cross-reactivity with the *Trachipleistophora hominis* antiserum (Cheney *et al.*, 2001).

Several diagnostic PCR assays have been proposed for fish-infecting microsporidia (Barlough *et al.*, 1995; Docker *et al.*, 1997; Bell *et al.*, 1999; Brown and Kent, 2002), but sequence data available thus far do not permit the construction of fast and reliable tools for species identification within all genera (Nilsen, 2000; Lom and Nilsen, 2003).

Since inadequate ultrastructural observation and judgement, in the absence of molecular analysis, may result in the lumping together of several species that are not congeneric into one genus, Lom and Nilsen (2003) recommended the identification of signature sequences when describing new species (see Woese, 1987).

Prevention and Control

The direct transmission supposed thus far in all fish-infecting microsporidia, as well as the longevity of their spores, makes implementation of prevention measures impossible in natural conditions and quite difficult in rearing facilities or cultures of ornamental fishes. Strict quarantine and examination of fish collected in natural water bodies are a basic requisite for introduction into any type of aquaculture. Experimental infections of *Gasterosteus aculeatus* and natural infections of *Nothobranchius* spp. fry with *Glugea* spp. revealed the presence of mature xenoma formations as early as 21 days post-infection (Dyková and Lom, 1978, 1980; I. Dyková and Z. Pindová, unpublished). Also, in rainbow trout experimentally infected with *L. salmonae*, the first cells infected with meronts were found in gills at week 3 post-exposure (Rodríguez-Tovar *et al.*, 2002). Such time studies on phases of life cycles are extremely important, since the deficit of proven remedies for microsporidian infections gives priority to disinfection and quarantine. Although chlorine treatment of eggs at 25 ppm for 10 min is routinely used in zebrafish laboratories, the evaluation of concentrations of chlorine needed to kill microsporidian spores is still pending. When the risk of the possible presence of mature spores, liberated, for example, from the digestive tract of parental specimens, exists, the hatched fry should be separated from adult individuals before they start to feed.

For the treatment of *G. anomala* infections, Schmahl and Mehlhorn (1989) and Schmahl *et al.* (1990) recommended Toltrazuril (Bayer AG) as a short bath (5–20 μg/ml of water for 1 to 4 h) applied for 6 days at 2-day intervals in well-aerated water. The attempts to treat G. *plecoglossi* infections with fumagillin (acyclic polyene macrolide produced by the fungus *Aspergillus fumigatus*) yielded ambiguous results (Takahashi and Egusa, 1976). The same applies for amprolium treatment of *K. takedai* infections (Awakura and Kurahashi, 1967). Summarizing data from the literature, Shaw and Kent (1999) stressed that high concentrations and prolonged treatment of infections of *L. salmonae* with fumagillin might cause side effects. Lower doses (2 or 4 mg of drug/kg of fish per day) give positive results when administered in an adequate form (e.g. mixed with alcohol and sprayed on the feed). The same authors also recommend an analogue of fumagillin, TNP-470 (Takeda Chemical Industries, Ltd) for the treatment of *L. salmonae* and *N. salmonis* infections (0.1–1.0 mg of drug/kg of fish per day for 6 weeks). Nitrofurazone, a broad-spectrum bactericidal drug that possesses some antiprotozoan activity, was reported to reduce infections of *P. ovariae* in *N. crysoleucas* (Nagel and Summerfelt, 1977). Albendazole, a derivative of benzimidazole, successfully used in human infections of *Enterocytozoon bieneusi*, was not tested in fish. It was found to be teratogenic in animals in doses equivalent to doses given in humans (Gross, 2003).

Promising results (substantial lowering of the intensity of *L. salmonae* infection in rainbow trout juveniles) achieved in vaccination experiments with a live low-virulence variant of *L. salmonae* (Sanchez *et al.*, 2001a)

open a new field of studies focused on protection of fish during the grow-out period.

Summary and Conclusions

Since fish-infecting microsporidia are supposed to be transmitted directly, it is reasonable to expect that, with the development of intensive fish cultures, new microsporidians will emerge as important disease agents. The pathogenic potential of microsporidians has already been proved in feral as well as cultured fishes but newly discovered genera and species give evidence of the necessity to fill the gaps in our knowledge of the whole group of obligatory intracellular parasites. Suggestions by Lom and Nilsen (2003) have to be followed: a careful morphological study continues to be indispensable for the proper identification and subsequent recognition of existing and new species. Identifying sequences of SSU-rRNA genes should support this, especially when new species are described. On the basis of thorough knowledge of the microsporidian fauna of fish, deeper insight into the host specificity should be acquired. Also, studies of host–parasite relationships at the tissue and cellular levels have to be amplified. They have to include experimental infections focused on initial phases of life cycles to trace the route of the parasite from the digestive tract to target organs and to learn how the merogony stages are spread within the host.

Limited experience with chemoprophylaxis and chemotherapy of fish microsporidian infections has to be augmented. Selected therapeutic drugs have to comply with all regulations that ensure that the products used in disease control are safe and effective and do not leave harmful residues in food. To elaborate new methods of appropriate treatment, experimental infections as well as establishment of *in vitro* cultures in fish cell lines are of vital importance.

Of the many problems of fish microsporidian biology, especially those concerning host–parasite relationships, the foremost is that of xenoma formation. Nobody has yet successfully attempted to induce xenoma production experimentally in cells maintained *in vitro*.

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8 Phylum Myxozoa

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Introduction

Myxozoans are highly specialized metazoan parasites of aquatic hosts with a very wide host range. This diverse group of organisms is characterized by multicellular spores with polar capsules containing extrudable polar filaments. Interest in the group has intensified along with the development of aquaculture since many species cause serious disease outbreaks in farmed fish species, in both freshwater and marine environments. *Myxobolus cerebralis* (Hofer, 1903), *Tetracapsuloides bryosalmonae* (Canning, Curry, Feist, Longshaw and Okamura, 1999) and *Ceratomyxa shasta* Noble, 1950, in salmonids are examples. The economic impact of such parasites can be severe, especially where prevalence rates are high. They can also have a severe impact on wild fish stocks. Infections with multivalvulid myxozoans such as *Kudoa* spp. within the musculature of several marine fish species severely reduce the flesh quality and in some cases cause extensive myoliquefaction, rendering the product unmarketable. In freshwater environments, *M. cerebralis* has been shown to be a significant factor in population declines of wild stocks of Pacific salmonids.

Recognition of the requirement for oligochaete and bryozoan obligatory hosts in the life cycle of several freshwater species has resulted in an increased interest in the biology of the group. Many new species have been described and significant advances have been made in the understanding of the transmission biology of several species. The development of specific diagnostic methods has facilitated studies on the pathogenesis and the use of molecular phylogenetic techniques has provided fundamental advances in the taxonomy of the Myxozoa. In particular, insights into the evolution of Myxozoa have been provided by morphological and molecular studies of the class Malacosporea. Current research continues to be focused on the occurrence of disease outbreaks, pathogenesis and phylogeny of these parasites. However, for several important and well-characterized diseases, including ceratomyxosis, whirling disease and proliferative kidney disease (PKD), the emphasis is on host immunity, prevention and control. This chapter provides a summary of current knowledge of the biology of the phylum Myxozoa, with particular attention given to those parasites as agents of disease in fish. Sections covering diagnosis, control and new directions for research are also provided.

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Host Range and Distribution

Myxozoans are common parasites of teleost fish and invertebrates, with a few species infecting elasmobranchs (Arthur and Lom, 1985; Stoffregen and Anderson, 1990; Heupel and Bennett, 1996), amphibians and reptiles and a single case of infection in a digenean (Overstreet, 1976), and a *Kudoa* sp. has been reported causing myoliquefaction in octopus (Yokoyama and Masuda, 2001). However, recent studies have revealed a much wider host range amongst animal phyla than had previously been imagined. A myxozoan-like parasite in European mole, *Talpa europaea* (Friedrich *et al.*, 2000) and the presence of myxozoans in inflammatory lesions of the bile ducts and hepatic parenchyma of anatid ducks held in an enclosure at a zoological garden have been reported (Lowenstine *et al.*, 2002). In addition, there have been several reports of myxozoan spores in human stool samples. In each case, the patients were suffering from abdominal pain or diarrhoea that were related to other enteric pathogens. These are likely to have arisen through the ingestion of infected fish material (McClelland *et al.*, 1997; Boreham *et al.*, 1998; Lebbad and Wilcox, 1998; Moncada *et al.*, 2001).

There is no clear pattern regarding host specificity within the Myxozoa. Some species appear to be highly specific to their host, whilst others may be specific at the family level (e.g. *M. cerebralis* and *T. bryosalmonae* in salmonid hosts). Others possess a wide host specificity (e.g. *Myxobolus aeglefini* Auerbach, 1906, in both gadoid and pleuronectid hosts) (Nielsen *et al.*, 2002). Myxozoans appear to be distributed throughout marine, estuarine and freshwater environments worldwide. Phylogenetic studies by Kent *et al.* (2001) have shown that, at the genus level, marine taxa branch separately from genera that usually infect freshwater fish.

There are discontinuities in the prevalence of myxozoans in fish hosts with differences in prevalence being found across the range of the hosts. However, some myxozoans have become established or at least have the potential to become established in other geographical regions via the anthropogenic translocation of the hosts. *Myxobolus cerebralis* is thought to have been transferred to the USA, New Zealand, South Africa and Australia through the movement of either live fish or dead, chilled fish destined for human consumption (Bartholomew and Reno, 2002, Whipps *et al.*, 2004b). Lowers and Bartholomew (2003) demonstrated that the importation of oligochaetes into the USA carried a risk of translocating Myxozoa from Europe to the USA since actinospore stages (see later section) were found within oligochaetes destined for the pet trade. *Myxobolus artus* Akhmerov, 1960 in the musculature of carp was originally restricted to the former USSR, but through the international trade in the host species it has now been recorded in Japan, Taiwan and Indonesia (Ogawa *et al.*, 1992) and in the UK (M. Longshaw and S.W. Feist, unpublished data). Similarly, *Myxobolus koi* Kudo, 1919 has been reported outside its normal geographical range in the UK (Crawshaw and Sweeting, 1986) and a parasite morphologically identical to the European *Myxobolus pseudodispar* Gorbunova, 1936 has been reported in peamouths in the USA (Kent *et al.*, 1996). The myxozoan *Sphaeromyxa sevastopoli* Naidenova, 1970, a parasite originally recorded in gobies from the Black Sea area of the former USSR, has been reported in two recently established European goby species in Michigan, USA (Pronin *et al.*, 1997). *Sphaerospora molnari* Lom, Dyková, Pavlásková and Grupcheva, 1983 and *Sphaerospora* cf. *chinensis* (Lee and Nie, 1965) have been reported in Germany and the USA, respectively, outside their normal range (Hedrick *et al.*, 1990; Kaup *et al.*, 1995). However, the paucity of data on the diversity of myxozoans in many hosts and the geographical range for most species makes it difficult to come to firm conclusions regarding possible translocation of these parasites.

The discontinuity in parasite prevalence and distribution has been used to suggest their use as biological tags for a number of hosts. *M. aeglefini* has been used as a biological tag in studies of stock structure of *Pleuronectes platessa* and to examine

migration routes of *Gadus morhua hiemalis* (van Banning *et al.*, 1978; Timofeeva and Marasaeva, 1984). Hemmingsen *et al.* (1991) suggested that *Myxidium* spp. could be useful tags to discriminate stocks of *Gadus morhua* in northern Norway and, in particular Larsen *et al.* (1997) showed that *Myxidium oviforme* Parisi, 1912 and *Zschokkella hildae* Auerbach, 1910 were suitable tags in this case. Kabata (1963) discriminated stocks of *Trisopterus luscus* using *Ceratomyxa arcuata* Thélohan, 1892 and *Myxidium sphaericum* Thélohan, 1895 and stocks of *Melanogrammus aeglefinus* using *Myxidium* sp., *Leptotheca* sp. and *Sphaeromyxa hellandi* Auerbach, 1909.

Two species of *Myxobolus* in the central nervous system of salmonids, *Myxobolus neurobius* Schuberg and Schröder, 1905 and *Myxobolus arcticus* Pugachev and Khokhlov, 1979, have been used as biological tags to study the migration and stock structure of salmonids. Awakura *et al.* (1995) used both *M. arcticus* and *M. neurobius* as a biological tag to demonstrate that *Oncorhynchus masou* caught in Japanese coastal waters originated from a number of different river systems and that there was migration of the fish from the Sea of Okhotsk to the Sea of Japan. Furthermore, Urawa and Nagasawa (1995) and Urawa *et al.* (1998) have shown marked differences in the prevalence of *M. arcticus* both in different geographical regions and in different *Oncorhynchus* spp., which means that *M. arcticus* shows promise as a biological tag. Margolis (1993) reported a court case in Canada in which the prevalence of *M. arcticus* in sockeye salmon was used to demonstrate that the fish were caught outside the commercial fishing season.

Myxozoans can show seasonal and annual variations in prevalence and this is related to a number of factors, including availability of hosts and ambient temperature influencing parasite development. Whilst the definitive hosts (oligochaetes) can release actinospore stages throughout the year, most are released in spring and summer in temperate regions (El-Mansy *et al.*, 1998a,b; Xiao and Desser, 1998b; Özer *et al.*, 2002; Oumouna *et al.*, 2003). Similarly, *T. bryosalmonae* is present within the bryozoan definitive host throughout the year, but is released mainly in spring and summer (Gay *et al.*, 2001; Tops and Okamura, 2003). A corresponding seasonal pattern of infection in the fish host occurs, though in many cases the pattern is less clear. The confusing patterns observed may in part be due to the examination of different host age classes within a sample, as it is known that prevalence of infection alters with age (Roubal, 1994). However, in temperate climates, it appears that prevalences are generally highest in the autumn and winter (Andrews, 1979; Gonzalez-Lanza and Alvarez-Pellitero, 1985; Mitchell, 1989; Brummer-Korvenkontio *et al.*, 1991). Furthermore, inter-annual variations in prevalences within fish hosts are known to occur (Awakura *et al.*, 1995; Molnár, 1998; Molnár and Székely, 1999; Pampoulie *et al.*, 2001; M. Longshaw and S.W. Feist, unpublished data).

Extrasporogonic stages of *T. bryosalmonae* infections in fish tend to be short-lived, less than a year. Fish surviving the infection become resistant to reinfection and generally do not display disease symptoms. Myxospore infections in fish hosts, however, can be extremely long-lasting, potentially persisting for the lifetime of the host. Infections in oligochaetes can persist for at least a year under laboratory conditions and possibly for the lifetime of the oligochaete under natural conditions (El-Matbouli *et al.*, 1999a,b; Granath and Gilbert, 2002).

Systematics and Taxonomic Position

The phylum Myxozoa Grassé, 1960 currently contains two classes, the Myxosporea Buetschli, 1881 and the Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000. Following the erection of the class Myxosporea, the phylum has undergone a series of taxonomic revisions.

Despite early suggestions that myxozoans may be metazoans, they were classified as multicellular (= pluricellular) protistans for many years. Actinospores in oligochaetes

were first described by Štolc (1899) and, whilst for many years they were placed within the same phylum as myxozoans from vertebrate hosts, no attempts were made to link them biologically. As a result, the phylum Myxozoa contained two classes – the Myxosporea and the Actinosporea. Following the discovery that actinospores represented an alternate stage of the Myxosporea (Wolf and Markiw, 1984), Corliss (1985) and Kent *et al.* (1994) proposed the suppression of the class Actinosporea. In addition, they considered that all myxozoan parasites in annelid worms should be relegated to *species inquirendae* and referred to as collective groups, a suggestion supported by Lom *et al.* (1997). The actinospore genus *Tetractinomyxon* has also been reduced to the collective group level, despite some earlier reservations (El-Matbouli and Hoffmann, 1998; Kent and Lom, 1999). The proposals by Kent *et al.* (1994) and Lom *et al.* (1997) that new forms of actinospore should not be named and should be referred to as, for example, collective group sp. 1, of Smith, 1995, etc. were not universally accepted (Lester *et al.*, 1998). Various arguments were proposed by proponents of the two positions (Lester *et al.*, 1998, 1999; Kent and Lom, 1999) and currently most authors agree with Kent and Lom (1999) in publishing descriptions of new actinospore forms and referring to them as, for example, Triactinomyxon type 1 of Smith (2001); Raabeia type 7 of Smith (2001), etc. Following the discovery of myxozoan parasites in bryozoans, Canning *et al.* (2000) erected a new class and order (Malacosporea: Malacovalvulida) within the phylum Myxozoa to accommodate these parasites.

Myxozoan taxonomy is based on the structure of the spore stages, principally on the number of shell valves, spore shape and position of the polar capsules (Fig. 8.1). Over 1300 species have been described, principally on spore morphology and in some cases on tissue tropism (Bahri and Marques, 1996). The use of molecular tools has allowed further characterization of parasites to take place and in many cases confirmed the morphological criterion for species discrimination (Estzerbauer, 2002; Molnár, *et al.*, 2002). However, it has also been demonstrated in some cases that morphological criteria may not necessarily be the best method to elucidate the relationships within the group. Whipps *et al.* (2003) recently described a new species of *Kudoa* using morphological and molecular approaches. On the basis of morphological characters, the new species should have been classified within the family Septemcapsulidae as it possessed 12–13 polar capsules. Sequence information, however, clearly placed the parasite within the family Kudoidae. Subsequently, Whipps *et al.* (2004a) suppressed the multivalvulid families Pentacapsulidae, Hexacapsulidae and Septemcapsulidae on molecular evidence so that the genera *Pentacapsula, Septemcapsula* and *Hexacapsula* become junior synonyms of *Kudoa*. Phenotypic variation in actinospores with identical sequences

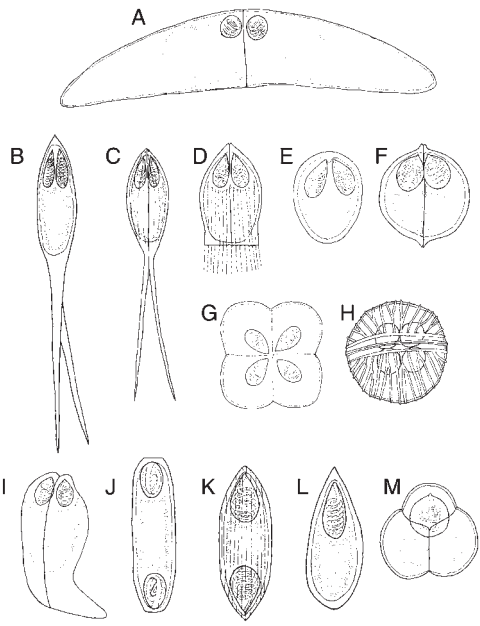


Fig. 8.1. Line drawings of representative myxospore genera showing key morphological features (not to scale). A. *Ceratomyxa*, B. *Henneguya*, C. *Myxobilatus*, D. *Hoferellus*, E. *Myxobolus*, F. *Sphaerospora*, G. *Kudoa*, H. *Chloromyxum*, I. *Parvicapsula*, J. *Sphaeromyxa*, K. *Myxidium*, L. *Thelohanellus*, M. *Unicapsula*.

are also known (Hallett *et al.*, 2002). The overlapping morphological characteristics for other genera can also be problematic, e.g. discrimination of *Zschokkella* and *Myxidium* and of *Leptotheca, Ceratomyxa* and *Sphaerospora*. A revision and re-evaluation of the morphological and molecular characteristics used to discriminate myxozoans at the species, genus and family level need to be undertaken, especially since it has been pointed out by Kent *et al.* (2001) that taxa cluster more by tissue location and mode of development than by spore morphology.

The class Myxosporea currently consists of the order Bivalvulida, composed of three suborders and 12 families, and the order Multivalvulida, containing two families. The current classification of the phylum Myxozoa is according to Grassé (1970):

Class Myxosporea Buetschli, 1881
- Order Bivalvulida Schulman, 1959
  - Suborder Platysporina Kudo, 1919
    - Family Myxobolidae Thélohan, 1892
  - Suborder Sphaeromyxina Lom & Noble, 1984
    - Family Sphaeromyxidae Lom & Noble, 1984
  - Suborder Variisporina Lom & Noble, 1984
    - Family Alatosporidae Shulman *et al.*, 1979
    - Family Auerbachiidae Evdokimova, 1973
    - Family Ceratomyxidae Doflein, 1899
    - Family Chloromyxidae Thélohan, 1892
    - Family Fabesporidae Naidenova & Zaika, 1969
    - Family Myxidiidae Thélohan, 1892
    - Family Ortholineidae Lom & Noble, 1984
    - Family Parvicapsulidae Shulman, 1953
    - Family Sinuolineidae Shulman, 1959
    - Family Sphaerosporidae Davis, 1917
- Order Multivalvulida Shulman, 1959
  - Family Kudoidae Meglitsch, 1947
  - Family Trilosporidae Shulman, 1959

Class Malacosporea Canning, Curry, Feist, Longshaw & Okamura, 2000
- Order Malacovalvulida Canning *et al.*, 2000
  - Family Saccosporidae Canning, Okamura and Curry, 1996

Seventeen collective groups of actinospores are recognized as follows: Antonactinomyxon, Aurantiactinomyxon, Echinactinomyxon, Endocapsa, Guyenotia, Hexactinomyxon, Hungactinomyxon, Neoactinomyxum, Ormieractinomyxon, Pseudotriactinomyxon, Raabeia, Siedleckiella, Sphaeractinomyxon, Synactinomyxon, Tetractinomyxon, Tetraspora and Triactinomyxon (Fig. 8.2).

Origin and Evolution

The advent of molecular techniques has led to further interest in the phylogenetic position of the Myxozoa. These parasites are allied either to the Bilateria (Smothers *et al.*, 1994; Schlegel *et al.*, 1996; Anderson *et al.*, 1998; Kim *et al.*, 1999; Zrzavý and Hypša, 2003) or to the cnidarians (Siddall *et al.*, 1995; Zrzavý *et al.*, 1998; Zrzavý, 2001) based on molecular data. The latter view has led to calls for the suppression of the phylum and its transfer to the cnidarians by Siddall *et al.* (1995). However, Anderson *et al.* (1998) showed that the parasites possessed *Hox* genes typical of bilaterians. The bilaterian nature is supported by ultrastructure (Canning *et al.*, 2002; Okamura *et al.*, 2002) and DNA sequences (Monteiro *et al.*, 2002) of the vermiform myxozoan *Buddenbrockia plumatellae* Schröder, 1910. This bryozoan parasite possesses two cell layers separated by a basal lamina and four longitudinal muscle blocks. There is no evidence of nervous tissues, gut, reproductive organs or excretory systems. The structure of the vermiform stage of *B. plumatellae* is more complex than any myxozoan stages occurring in vertebrate hosts. Myxozoans are now considered to be highly specialized bilaterian metazoans, possibly as a sister group to the Nematoda (see Canning and Okamura, 2004, and references therein). Canning *et al.* (2002) used both molecular and morphological evidence to indicate that the phylum Myxozoa should be retained within the Bilateria. However, Kelley *et al.*

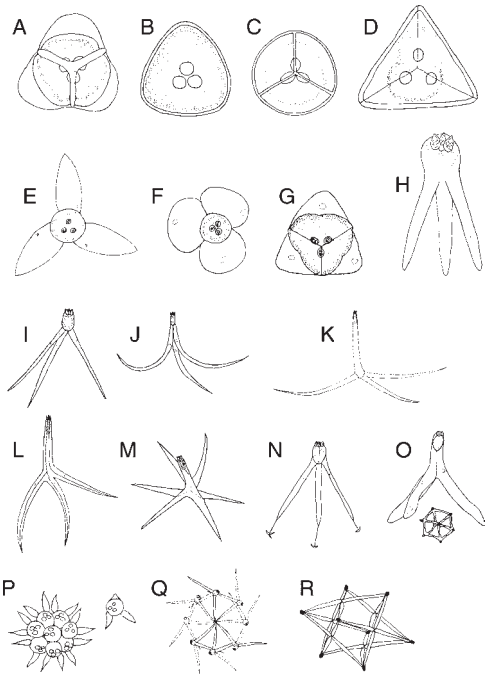


Fig. 8.2. Line drawings of the actinospore collective groups showing key morphological features (not to scale). A. Endocapsa (after Hallett *et al.*, 1999), B. Sphaeractinomyxon (after Hallett *et al.*, 1999), C. Tetraspora (after Hallett and Lester, 1999), D. Tetractinomyxon, E. Aurantiactinomyxon, F. Neoactinomyxum (after Ormières and Frézil, 1969), G. Neoactinomyxum (after El-Mansy *et al.*, 1998a), H. Guyenotia (after Naville, 1930), I. Echinactinomyxon, J. Raabeia, K. Triactinomyxon, L. Pseudotriactinomyxon (after Lowers and Bartholomew, 2003), M. Hexactinomyxon (after Janiszewska, 1957), N. Ormieractinomyxon (after Marques, 1984), O. Siedleckiella (after Janiszewska, 1955), inset, collection of eight spores forming characteristic net, P. Synactinomyxon (after McGeorge *et al.*, 1997), inset, individual spore shown, Q. Synactinomyxon, R. Antonactinomyxon (after Janiszewska, 1955).

(2004) utilized actin cDNA sequence data to suggest that the myxozoans may branch outside of the metazoans.

Before the recognition of invertebrates as obligatory hosts for Myxozoa, discussion on the evolution of these parasites was based only on evidence from forms infecting fish hosts. Schulman (1966) considered that primitive forms were probably initially coelozoic parasites of the gall bladder and urinary bladder of marine teleosts of the subclass Actinopterygii ('ray fins') from the Cretaceous period (approximately 100 million years ago). Radiation of the Myxosporea followed, as they became histozoic, infecting a variety of tissues and new hosts as teleost fish radiated into the freshwater environment (particularly the Platysporina) and then returned to the marine environment. However, members of the Actinopterygii and sharks and rays (Chondrichthyes), which are hosts to modern Myxosporea, evolved much earlier, during the lower Devonian period, about 400 million years ago. Extinct groups of ancient fish, such as the cephalaspids ('armoured fish'), were amongst the first vertebrates and evolved even earlier, during the upper Ordovician period. It is possible that myxozoans or their ancestors were able to utilize these hosts, either as parasites or as symbionts. However, fossil evidence of myxozoan infections is lacking. Reinvestigation of well-preserved fossil fish assemblages, such as those from the Solnhofen limestone (Jurassic period) or the more recent Miocene deposits at Monte Bolca in Italy and others, may reveal evidence of myxosporean cysts and even myxospores, particularly in specimens where some soft tissue preservation has occurred. Unfortunately, direct evidence of infections in ancient cephalapsids and chondrichthyans is highly unlikely since preservation of muscle and other soft tissues is extremely rare.

The origin of myxozoan invertebrate hosts goes back even further in time. Bryozoa first emerged during the early Ordovician prior to the cephalapsids and rapidly diversified into approximately 30 families, which are well represented in the fossil record (Taylor, 1985; Paul, 1989). Oligochaete annelid and nematode fossils are known from the Carboniferous period (approximately 300 million years ago) onwards (Conway Morris, 1981). They must have originated much earlier from primitive annelids, whose remains have been found in Precambrian deposits, such as the Ediacaran fauna from Australia (McMenamin, 1989). Similarly, the Burgess shale fauna from

Canada, deposited during the 'Cambrian explosion' 570 million years ago, when there was a huge diversification of life forms, contains several annelid forms (Gould, 1990). If one accepts the bilaterian origin of the Myxozoa, it is interesting to speculate that their evolution into parasitic forms could have occurred shortly after the evolution of their primary annelid hosts, with parasitism of bryozoan and possibly fish hosts occurring later during the Ordovician period 450 to 500 million years ago and thereafter.

Parasite Morphology and Life Cycle

Morphology

Guidelines for the description of myxospore and actinospore stages have been proposed by Lom and Arthur (1989) and Lom *et al.* (1997), respectively. Caution should be exercised when examining hosts for myxozoan spores as it is recognized that some spores can show morphological plasticity and malformed spores have been reported (Meglitsch, 1960; Arthur and Lom, 1985; Feist *et al.*, 1991; McGeorge *et al.*, 1997; Morris, D.C. *et al.*, 2002; see Fig. 8.5).

Myxozoans are classed as either histozoic (within tissues) or coelozoic (inhabiting cavities) and most organs in the fish hosts can be infected. Myxospore size in fish hosts is usually between 10–20 µm in length or width. Spores comprise between two and 12 shell valves, between one and 13 polar capsules and the infective sporoplasm, which normally consists of one binucleate or two uninucleate cells. *Polysporoplasma* can contain up to 12 sporoplasm cells. Myxospore shell valves are held together along a sutural line that splits to release the infective sporoplasm within the oligochaete definitive host. The shell valves can be smooth or ridged and in some genera there may be protrusions from the shell valve (Fig. 8.3). Additionally, some spores may be surrounded, either completely or partially, by a mucus envelope. The polar capsules contain a coiled polar filament and the orientation and type of coiling can be species-specific. The polar filament is twisted longitudinally on itself as well as coiled within the polar capsule. The number of polar filament coils is a useful diagnostic feature for species identification. Myxospores develop within plasmodia or pseudoplasmodia and can have extrasporogonic proliferative stages within the fish host (Fig. 8.4). Additionally, hyperparasitic microsporidia may be noted within the plasmodia (Diamant and Paperna, 1985, 1989; Dyková and Lom, 1999; Tin Tun *et al.*, 2000).

Within oligochaetes, actinospore stages develop generally within the intestinal epithelium. The actinospore stage released from the oligochaete host is always triradiate, with either three or six spore valves (caudal processes) (Fig. 8.5). The exception to this is the actinospore collective group Tetractinomyxon, which has four spore valves. The spore valves are considered to act as floats to assist in maintaining the actinospore position in the water column. Each caudal process contains a single nucleus and the length can range from 10 µm for members of the collective group Neoactinomyxum to several hundred µm for Triactinomyxon and Raabeia types. All actinospores described so far contain three polar capsules, regardless of the number of caudal processes and contain fewer polar filament coils compared with their myxospore counterparts.

Malacospores in bryozoan hosts develop within hollow rounded sacs or larger vermiform forms, in the case of *B. plumatellae*, measuring up to 2 mm in length. Spores, approximately 19 µm in diameter, comprise eight cells forming the valvular cells, four spherical to subspherical polar capsules contained within pyriform capsular cells at the apical end of the spore and two sporoplasms, each of which may contain a secondary cell (Fig. 8.6). The spore shell valve cells contain haplosporosomes, elongate nuclei, mitochondria and lipid vacuoles and are joined together by septate junctions. The spores of *B. plumatellae* and *T. bryosalmonae* do not contain extraneous ornamentation. However, an unnamed species from the bryozoan *Plumatella repens* possesses subspherical spores with small

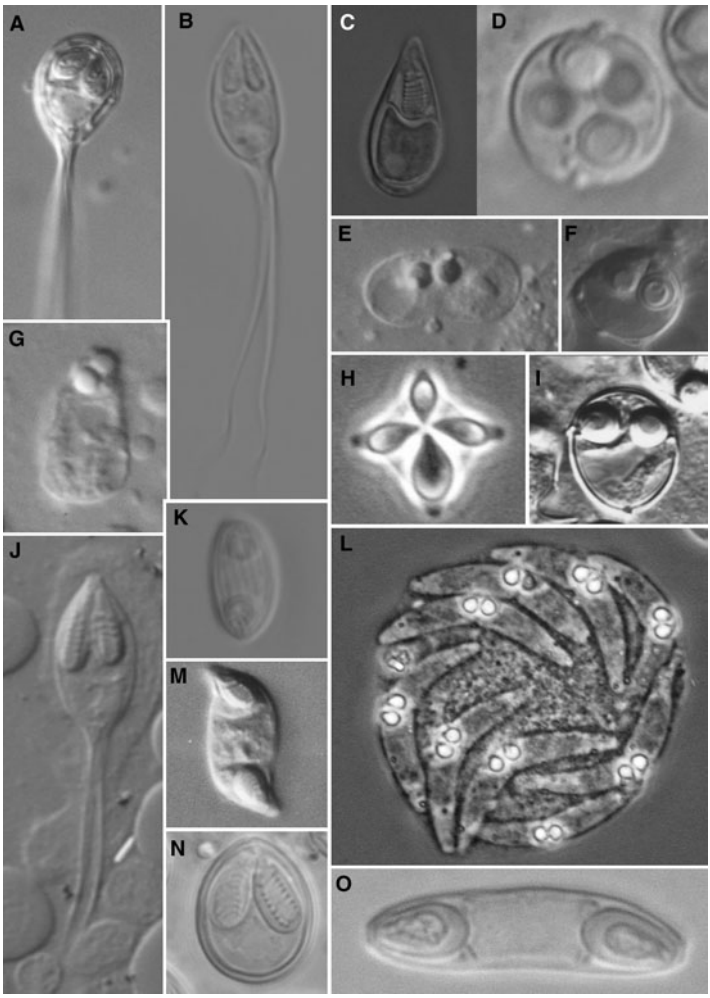


Fig. 8.3. Photomicrographs of representative myxospore genera. A. *Henneguya zschokkei* from coho salmon (*Oncorhynchus kisutch*), B. *Henneguya psorospermica* from pike (*Esox lucius*) gills, C. *Thelohanellus pyriformis* from gill of tench (*Tinca tinca*), D. *Chloromyxum* sp. from *P. phoxinus* gall bladder, E. *Leptotheca* sp. from gall bladder of tadpole fish (*Raniceps raninus*), F. *Sphaerospora elegans* from stickleback (*Gasterosteus aculeatus*) kidney, G. *Parvicapsula assymetrica* from *Cyclopterus lumpus* urinary bladder, H. *Kudoa thyrsites* from scabbardfish (*Lepidopus caudatus*) muscle, I. *Myxoproteus ambiguus* from the urinary bladder of anglerfish (*Lophius piscatorius*), J. *Myxobilatus gasterostei* from the kidney of stickleback *Gasterosteus aculeatus*, K. *Myxidium* sp. from the gall bladder of rudd (*Scardinius erythrophthalmus*), L. *Ceratomyxa* sp. from common goby (*Pomatoschistus microps*), M. *Myxidium gadi* from the gall bladder of whiting (*Merlangius merlangus*), N. *Myxobolus* sp. from dace (*Leuciscus leuciscus*) buccal cavity cyst, O. *Sphaeromyxa* sp. from two-spot goby (*Gobiosculus flavescens*) gall bladder.

extensions to the shell valves (Morris, D.J. *et al.*, 2002). Discrimination of species is currently based on differences in the development of spores and DNA sequence, as there are a limited number of features of taxonomic value (Canning and Okamura, 2004). To date, only two morphologically identical malacospore forms have been reported in fish, one in salmonid hosts (causing PKD) and one in common carp (*Cyprinus carpio*) (Voronin, 1993; Voronin and Chernysheva, 1993). In carp, sporogonic forms have not

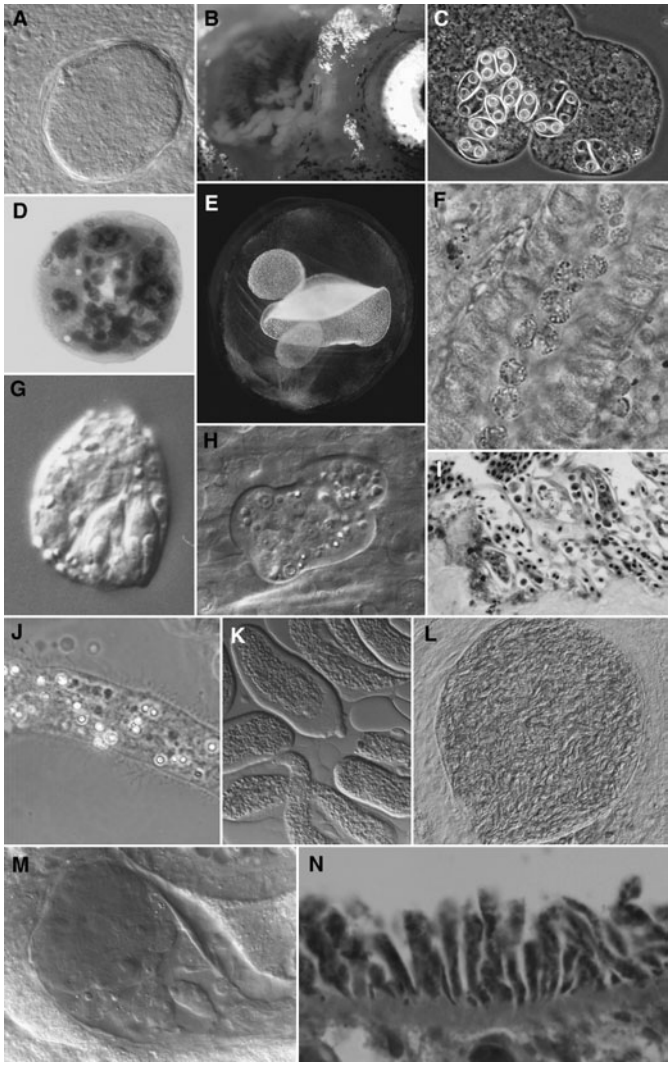


Fig. 8.4. Myxosporean extrasporogonic and plasmodial stages. Fresh preparations unless otherwise stated. A. *Myxobolus artus* plasmodium encysted in the renal tissue of koi carp (*Cyprinus carpio*). B. Gill cysts of *Myxobolus macrocapsularis* in juvenile chub, *Leuciscus cephalus*. C. Sporogonic plasmodium of *Zschokkella* sp. from the gall bladder of three-bearded rockling (*Gaidropsaurus vulgaris*). D. May–Grünwald Giemsa-stained smear of a *Myxidium incurvatum* plasmodium from the gall bladder of flounder (*Platichthys flesus*). E. *Sphaeromyxa* sp. plasmodia contained within the gall bladder of two-spot goby (*Gobiusculus flavescens*). F. *Sphaerospora truttae* pseudoplasmodia within renal tubule of brown trout (*Salmo trutta*). G. Plasmodium of *Myxobilatus gasterostei* containing two mature spores from the kidney of the three-spined stickleback (*Gasterosteus aculeatus*). H. Extrasporogonic stage from the rete mirabile in the eye of *G. aculeatus*. I. Giemsa-stained section of the rete mirabile with the parasites located within the capillaries. J. Phase-contrast image of a plasmodium of *Myxidium lieberkuehni* from the urinary bladder of pike (*Esox lucius*), showing the characteristic villous projections on the surface of the plasmodium. K. Interference-contrast image of *M. lieberkuehni* plasmodia showing the clear ectoplasmic layer. L. Cyst of *Myxidium rhodei* from the kidney of roach (*Rutilus rutilus*). M. Presporogonic stages of *Sphaerospora elegans* in Bowman's space of the glomerulus in the kidney of *G. aculeatus*. N. Numerous plasmodia attached to the epithelium of the urinary bladder of a juvenile dace (*Leuciscus leuciscus*).

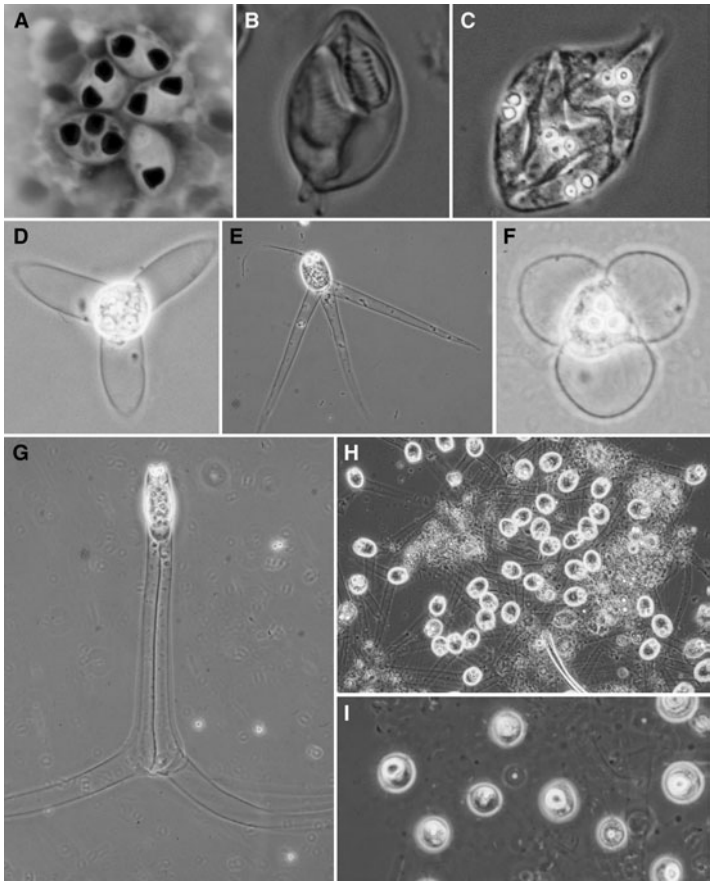


Fig. 8.5. A–C. Deformed myxospores; D–I. Actinospores released from oligochaetes. A. Giemsa-stained smear of *Myxidium giardi* spores from eel *Anguilla anguilla,* one of which contains three polar capsules. B. Deformed *Thelohanellus pyriformis* spore from gill of tench (*Tinca tinca*). C. Triradiate spores of *Ceratomyxa* sp. from the gall bladder of common goby (*Pomatoschistus microps*). D. Aurantiactinomyxon-type actinospore. E. Echinactinomyxon-type actinospore. F. Neoactinomyxum-type actinospore. G. Triactinomyxon-type actinospore. Note presence of large style and sporoplasm and polar capsules at apex of spore. H. Collection of Synactinomyxon-type actinospores. I. Secondary cells released from the sporoplasm of a Triactinomyxon-type actinospore.

been observed, whilst in salmonids a limited form of sporogony can occur (Kent *et al.*, 2000).

Life cycle

Until the pioneering studies by Wolf and Markiw (1984), it was thought that myxospores were transmitted directly from fish to fish and that the spores needed to be 'aged' in mud to be infective. However, Wolf and Markiw (1984) demonstrated that an intermediate or alternate oligochaete host was an essential requirement in the development of *M. cerebralis* (the causative agent of whirling disease in salmonids). The oligochaete stage, known as an actinospore, is the infective stage to fish. Subsequently, a number of other studies confirmed that some myxozoans undergo a two-host life cycle (Table 8.1). These include representatives of the genera *Myxidium*, *Sphaerospora*, *Hoferellus*, *Myxobolus*, *Thelohanellus* and *Zschokkella*. In general, *Myxobolus* spp. appear to produce

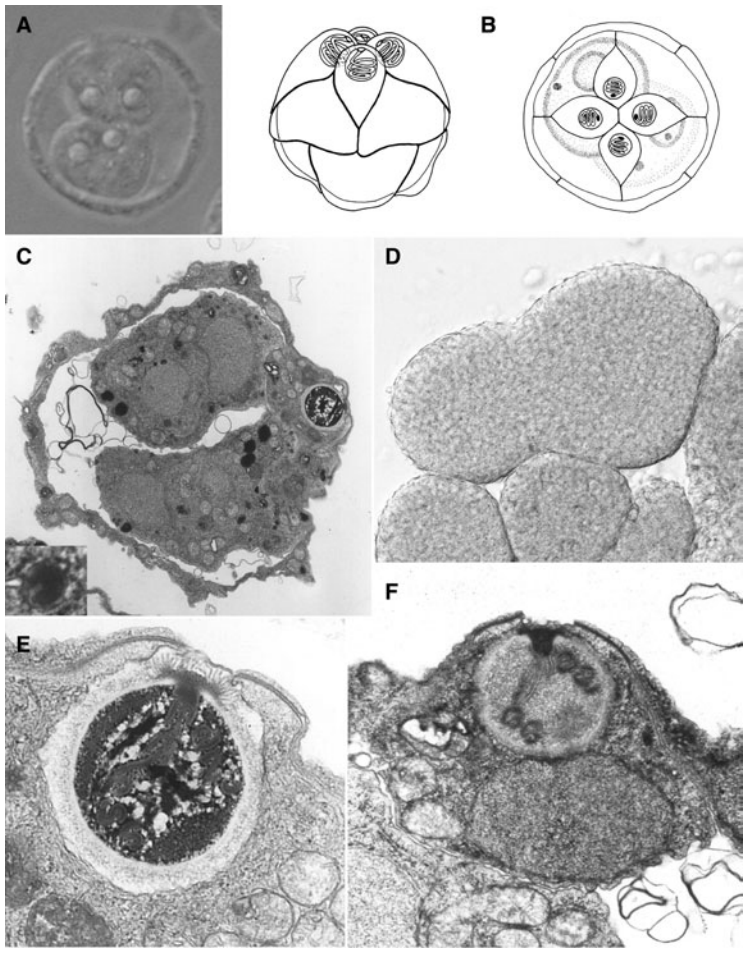


Fig. 8.6. Sporogonic stages of *Tetracapsuloides bryosalmonae*. A. Fresh spore of *T. bryosalmonae* showing the four polar capsules and two sporoplasm cells surrounded by valvogenic cells. B. Diagrams of *T. bryosalmonae* spores in three-dimensional view and apical view. Note presence of four capsular cells and eight valvular cells. C. Section through a complete spore showing the sporoplasm cells with each containing a secondary cell. A single polar capsule can also be seen. Inset: Characteristic sporoplasmosome within the cytoplasm of the sporoplasm cell, showing the typical bar-like invagination, also seen in the histozoic fish stage of the parasite. D. Sacs of *T. bryosalmonae* released from the bryozoan host. E. Section through the polar capsule showing the exit pore for the filament and characteristic reticulated cap. Note the gap between the valvogenic cells at the exit pore of the polar filament. F. As E, showing the nucleus of the capsulogenic cell and sections through the coiled polar filament.

Triactinomyxon-type actinospores, with the exceptions of *Myxobolus cultus* Yokoyama, Ogawa and Wakabayashi, 1995, which produces *Raabeia*-type actinospores (Yokoyama *et al.*, 1995b), and *Myxobolus pavlovskii* Akhmerov, 1954, which produces Hexactinomyxon-type actinospores (Ruidisch *et al.*, 1991). Aurantiactinomyxon-type actinospores have been demonstrated for *Myxidium giardi* Cépede, 1906 (Benajiba and Marques, 1993), *Hoferellus carassii* Akhmerov, 1960 and *Hoferellus cyprini* (Doflein, 1898) (El-Matbouli *et al.*, 1992b; Grossheider and Körting, 1992) and *Thelohanellus hovorkai* Akhmerov, 1960 and *Thelohanellus nikolskii* Akhmerov, 1960 (Yokoyama, 1997; Székely *et al.*, 1998). Other actinospore types that have been shown to

Table 8.1. List of known myxozoan life cycles.

Myxozoan species	Fish host	Actinospore type	Invertebrate host	Reference
Ceratomyxa shasta	*Oncorhynchus mykiss*	Tetractinomyxon	*Manayunkia speciosa*	Bartholomew *et al.* (1997)
Ellipsomyxa gobii	*Pomatoschistus microps*	Tetractinomyxon	*Nereis* spp.	Køie *et al.* (2004)
Enteromyxum fugu	*Takifugu rubripes*	None – direct transmission	None	Yasuda *et al.* (2002)
Enteromyxum leei	*Sparus aurata*	None – direct transmission	None	Diamant (1997)
Enteromyxum scophthalmi	*Scophthalmus maximus*	None – direct transmission	None	Redondo *et al.* (2002)
Henneguya exilis[1]	*Ictalurus punctatus*	Aurantiactinomyxon	*Dero digitata*	Lin *et al.* (1999)
Henneguya ictaluri	*Ictalurus punctatus*	Aurantiactinomyxon	*D. digitata*	Pote *et al.* (2000)
Henneguya nuesslini	*Salmo trutta, Salvelinus fontinalis*	Triactinomyxon	*Tubifex tubifex*	Kallert *et al.* (2005a)
Hoferellus carassii	*Carassius auratus*	Aurantiactinomyxon	*Nais* cf. *elingius*	Troullier *et al.* (1996)
Hoferellus carassii	*Carassius auratus*	Neoactinomyxum	*Branchiura sowerbyi*	Yokoyama *et al.* (1993b)
Hoferellus cyprini	*Cyprinus carpio*	Aurantiactinomyxon	*Nais* sp.	Grossheider and Körting (1992)
Kudoa ovivora[2]	*Thalassoma bifasciatum*	None – direct transmission?	Via infected host eggs?	Swearer and Robertson (1999)
Myxidium giardi	*Anguilla anguilla*	Aurantiactinomyxon	*T. tubifex*	Benajiba and Marques (1993)
Myxobolus arcticus	*Oncorhynchus nerka*	Triactinomyxon	*Stylodrilus heringianus*	Kent *et al.* (1993)
Myxobolus arcticus	*Oncorhynchus masu*	Triactinomyxon	*Lumbriculus variegatus*	Urawa (1994)
Myxobolus bramae	*Abramis brama*	Triactinomyxon	*T. tubifex*	Eszterbauer *et al.* (2000)
Myxobolus carassii	*Leuciscus idus*	Triactinomyxon	*T. tubifex*	El-Matbouli and Hoffmann (1993)
Myxobolus cerebralis	*Oncorhynchus mykiss*	Triactinomyxon	*T. tubifex*	Wolf and Markiw (1984); El-Matbouli *et al.* (1999b)
Myxobolus cotti	*Cottus gobio*	Triactinomyxon	'tubifex'	El-Matbouli and Hoffmann(1989)
Myxobolus cultus	*Carassius auratus*	Raabeia	*B. sowerbyi*	Yokoyama *et al.* (1995b)
Myxobolus dispar	*Cyprinus carpio*	Raabeia	*T. tubifex*	Molnár *et al.* (1999b)
Myxobolus drjagini	*Hypophthalmichthys molitrix*	Triactinomyxon	*T. tubifex* *Limnodrilus hoffmeisteri*	El-Mansy and Molnár (1997a)
Myxobolus hungaricus	*Abramis abramis*	Triactinomyxon	*T. tubifex,* *L. hoffmeisteri*	El-Mansy and Molnár (1997b)

Continued

Table 8.1. *Continued*. List of known myxozoan life cycles.

Myxozoan species	Fish host	Actinospore type	Invertebrate host	Reference
Myxobolus intimus	*Rutilus rutilus*	Triactinomyxon	*T. tubifex*	Rácz *et al.* (2004)
Myxobolus macrocapsularis	*Abramis brama*	Triactinomyxon	*T. tubifex*	Székely *et al.* (2002)
Myxobolus parviformis	*Abramis brama*	Triactinomyxon	*L. hoffmeisteri*	Kallert *et al.* (2005b)
Myxobolus pavlovskii	*Hypophthalmichthys molitrix*	Hexactinomyxon	*T. tubifex*	El-Matbouli and Hoffmann (1991a)
Myxobolus portucalensis	*Anguilla anguilla*	Triactinomyxon	*T. tubifex*	El-Mansy and Székely (1998)
Myxobolus pseudodispar	*Rutilus rutilus*	Triactinomyxon	*T. tubifex*, *L. hoffmeisteri*	Székely *et al.* (1999)
Sphaerospora renicola	*Cyprinus carpio*	Neoactinomyxum	*B. sowerbyi*, *T. tubifex*	Molnár *et al.* (1999a)
Sphaerospora truttae	*Salmo trutta*	Echinactinomyxon	*L. variegatus*, *T. tubifex*	Özer and Wootten (2000)
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	None	*Fredericella sultana*, *Plumatella repens*	Feist *et al.* (2001)
Thelohanellus hovorkai	*Cyprinus carpio*	Aurantiactinomyxon	*B. sowerbyi*	Yokoyama (1997); Székely *et al.* (1998)
Thelohanellus nikolskii	*Cyprinus carpio*	Aurantiactinomyxon	*T. tubifex*	Székely *et al.* (1998)
Zschokkella nova	*Carassius carassius*	Siedleckiella	*T. tubifex*	Uspenskaya (1995)
Zschokkella sp.	*Carassius auratus*	Echinactinomyxon	*B. sowerbyi*	Yokoyama *et al.* (1991)

[1]Lin *et al.* (1999) used sequence information to link the actinospore stage with the myxospore stage – no experimental transmission trials have confirmed this finding.
[2]This report needs confirming as Swearer and Robertson (1999) used fish collected from the field for both control and challenge fish.

be the infective stage of myxospores include Siedleckiella (*Zschokkella nova* Klokacheva, 1914) and Neoactinomyxum (*Sphaerospora renicola* Dyková and Lom, 1982).

However, only two life cycles have been replicated consistently in laboratory studies, namely *M. cerebralis* (El-Matbouli *et al.*, 1999b) and *M. pseudodispar* (Székely *et al.*, 2001). Studies on other species have provided conflicting results. El-Matbouli *et al.* (1992b) reported that the actinospore stage of *H. carassii* was an Aurantiactinomyxon type, whereas Yokoyama *et al.* (1993b) considered it a Neoactinomyxum type. Whilst the myxospore forms used in these studies were morphologically identical, it is possible that they represented two different species. However, no DNA sequence analysis of the forms has been carried out to establish relatedness. In addition, the actinospores were released from different oligochaete hosts, suggesting the possibility that the oligochaete influences the type of actinospore produced.

Xiao and Desser (2000b) employed a cladistic approach to examine the relatedness of the two myxozoan life stages and found that conventional classification of the actinospore stage at the supraspecific level was not supported. There was a lack of congruence between the two stages, which is supported from studies of myxozoan life cycles carried out to date. Different life stages are subject to various environmental selection

pressures, which may account for the morphological differences (Xiao and Desser, 2000b). Understanding of the morphological adaptations and the ecology of the actinospore stages will assist in elucidating the reasons for the different phenotypes observed.

Although actinospores have been recorded from a number of different oligochaete genera of Tubificidae and Nadidae, only representatives of *Stylodrilus*, *Tubifex*, *Limnodrilus*, *Lumbriculus*, *Branchiura*, *Dero* and *Nais* have been demonstrated to be hosts. In addition, the polychaete *Manayunkia speciosa* has been shown to act as the invertebrate host for *C. shasta* (Bartholomew *et al.*, 1997), the marine polychaetes *Nereis* spp. as hosts for *Ellipsomyxa gobii* (Køie *et al.*, 2004) and freshwater bryozoans as hosts for *B. plumatellae* and *Tetracapsuloides* species, including the agent for salmonid PKD (Anderson *et al.*, 1999a,b; Longshaw *et al.*, 1999; Canning *et al.*, 2000, 2002; Feist *et al.*, 2001). However, life cycles for most myxozoans have not been elucidated and, although it is likely that a two-host life cycle will apply to most species, it is recognized that some can be transmitted directly between fish. Diamant (1997), using cohabitation experiments, demonstrated that *Enteromyxum* (= *Myxidium*) *leei* (Diamant, Lom and Dyková, 1994) could be transmitted to uninfected fish and Redondo *et al.* (2002) showed direct transmission with *Enteromyxum scophthalmi* Palenzuela, Redondo and Álvarez-Pellitero, 2002. Yasuda *et al.* (2002) demonstrated that *Enteromyxum* (= *Myxidium*) *fugu* (Tin Tun, Yokoyama, Ogawa and Wakabayashi, 2000) and *E. leei* (= *Myxidium* sp. TP of Tin Tun, Yokoyama, Ogawa and Wakabayashi, 2000), parasitic in cultured tiger pufferfish, were both capable of fish-to-fish transmission. They suggested that, although an alternate host may be available in the natural environment, the parasites were able to transmit from fish to fish via trophozoites and spores passed out with the faeces.

El-Matbouli *et al.* (1995) and El-Matbouli and Hoffmann (1998), described the development of *M. cerebralis* in the fish and oligochaete hosts, respectively. Their findings confirmed that sexual reproduction occurred in the oligochaete host and thus that host should be considered as the final or definitive host. However, descriptions of some myxozoan life cycles continue to refer to oligochaetes as alternate hosts. Similarly, Bryozoa are the definitive hosts of malacosporeans described to date. Fish appear to be accidental or dead-end hosts (Hedrick *et al.*, 2004; Henderson and Okamura, 2004; Tops *et al.*, 2004).

Intra-oligochaete development

Intra-oligochaete development of myxozoan life cycles undergoes three phases – schizogony, or proliferative stage, gametogamy and sporogony (Fig. 8.7). In myxozoans with a two-host life cycle, the oligochaete phase is initiated by infection with myxospores from the fish host, either over the lifetime of the host or on death with subsequent decomposition of the host. This decomposition may occur either naturally or, as in the case of some *Kudoa* infections, more rapidly due to the release of proteolytic enzymes (Moran *et al.*, 1999b).

Myxospores are ingested by oligochaetes and, on contact with the oligochaete epithelium, polar filaments are released, which anchor the parasite to the host. The spore valves separate along the sutural line and the amoeboid sporoplasms are released and penetrate between the epithelial cells of the gut wall. In *M. cerebralis*, the binucleate sporoplasm undergoes several nuclear divisions to produce a multinucleate cell. It is presumed that a similar pattern of development occurs in myxospores with two uninucleate sporoplasms, each producing a multinucleate cell, rather than fusion to form a binucleate cell. The multinucleate cell undergoes plasmotomy to produce numerous uninucleate cells, which invade other intercellular spaces. Some of these cells may then undergo another schizogonic phase, giving rise to more multinucleate cells and subsequently more uninucleate cells. Fusion by plasmogamy of uninucleate cells produces a binucleate stage.

The next phase in the development is gametogony and is initiated by the

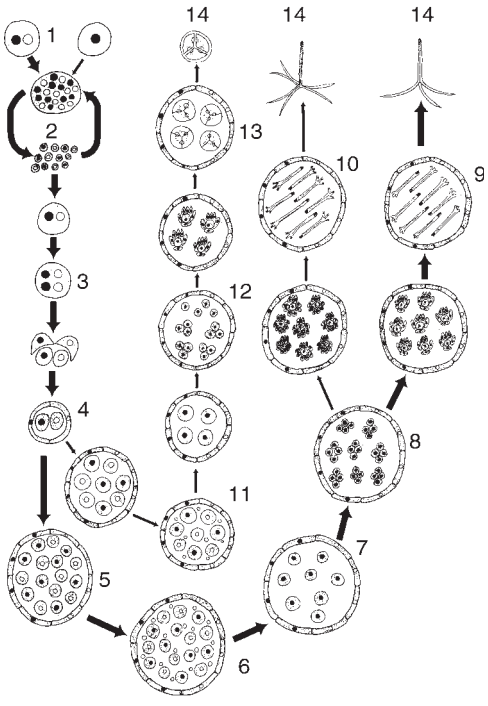


Fig. 8.7. Intra-oligochaete myxozoan life cycle (actinospore phase), where heavy arrow = life cycle of *M. cerebralis* demonstrated experimentally by El-Matbouli and Hoffmann (1998) and light arrow = life cycle for other actinospores.

A binucleate or uninucleate sporoplasm (1) released from a myxospore following ingestion by the oligochaete undergoes a series of nuclear divisions to produce a multinucleate cell. Following plasmotomy, the cells either undergo further nuclear divisions to produce more multinucleate stages (2) or produce a two- and then four-nuclei cell (3). Plasmotomy follows to produce four cells, two of which envelop the other two cells (4). For all actinospore types, the outer cells undergo two mitotic divisions to produce a pansporocyst wall containing eight cells. For *M. cerebralis* and other actinospores producing eight actinospores per pansporocyst, division of the inner cells produces 16 diploid cells via three mitotic divisions (5). Following one meiotic division, 16 haploid cells are produced (6). Fusion of the α and β cells, the sexual phase, producing an early pansporocyst containing eight cells (7), which each undergo two mitotic divisions to produce eight four-cell stages (8). For *M. cerebralis*, three of these cells will undergo one mitotic division to produce the capsulogenic and valvogenic cells. Subsequent development will produce a pansporocyst containing eight actinospores (9). For actinospores with six caudal processes (e.g. Hexactinomyxon type), following production of eight four-cell stages (8), three of the cells undergo one mitotic division. Three of these new cells will undergo a further mitotic division, giving rise to six valvogenic, three capsulogenic and one sporoplasmogenic cell, and develop into eight actinospores (10). For *Tetraspora*, following the division of the early developmental stage into a two-cell early pansporocyst (4), the inner cells undergo two mitotic divisions and one meiotic division to produce eight haploid cells (11). Fusion of the α and β cells produces an early pansporocyst containing four cells. Two mitotic divisions produce four four-cell stages within the pansporocyst (12). Within each of these, a mitotic division of three of the cells leads to the production of capsulogenic and valvogenic primordia. Subsequent development leads to the production of a pansporocyst containing four actinospores (13). Release of actinospore stages into the water allows the life cycle to continue (14).

binucleate stage producing a four-nuclei stage by karyogamy. This stage produces four uninucleate cells by plasmotomy, two of which become enveloping somatic cells and the other two becoming generative cells, designated as α- and β-cells. In this early pansporocyst, the two enveloping cells undergo mitosis twice to produce eight somatic cells. In all actinospore types with pansporocysts that ultimately contain eight actinospores, the α- and β-cells undergo three mitotic divisions to produce 16 diploid gametocytes, composed of eight α-cells and eight β-cells. One meiotic division produces 16 haploid gametocytes and 16 polar bodies. Each α-cell fuses with a β-cell to produce a pansporocyst consisting of eight zygotes surrounded by eight somatic cells. For Tetraspora, which only contains four actinospores in the pansporocyst, the generative α- and β-cells in the early pansporocyst presumably undergo two mitotic divisions to produce eight diploid gametocytes consisting of four α-cells and four β-cells. One meiotic division leads to the production of eight haploid gametocytes and eight polar bodies. The fusion of each α-cell with a corresponding β-cell produces a pansporocyst containing four zygotes surrounded by eight somatic cells in Tetraspora.

The fusion of the α- and β-cells is the sexual phase of development, demonstrating that oligochaetes are the definitive or final hosts, i.e. hosts in which fusion of gametes takes place. Sporogony is the final phase of development. This begins with two mitotic divisions of the zygotes, leading to a four-cell stage, so that sporocysts contain 32 cells in eight groups of four cells. The exception to this is the Tetraspora pansporocyst, which contains a total of 16 cells in four groups of four cells. For actinospores containing three valves, such as *M. cerebralis* and Tetraspora, three of these cells surround the fourth and divide by mitosis, resulting in six cells. Three of these become capsulogenic cells and the other three valvogenic cells. Valvogenic cell primordia of actinospores containing six valve cells, such as Pseudotriactinomyxon and Hexactinomyxon, undergo a further mitotic division to produce six valvogenic cells. The fourth cell in the four-cell stage becomes the sporoplasm cell, initially dividing by internal cleavage, leading to a generative cell surrounded by a somatic cell. The generative cell divides by mitosis to produce infective germ cells in various multiples, normally 2, 4, 8, 16, 32, 64, 128 and so on. This number is species-dependent. Further development of the capsulogenic and valvogenic cells leads to the formation of polar capsules and valve cells, respectively. Polar capsule ontogeny in actinospores follows a similar pattern to that in myxospores, with the polar filament being formed by the inversion of an external tube. The exit pore for the polar capsule is covered by a 'plug' of moderately dense material, topped by a cone, which is then covered by the cell membrane of the capsulogenic cell (Lom and Dyková, 1997; Alvarez-Pellitero *et al.*, 2002). At maturity, each pansporocyst contains eight (or four in Tetraspora) actinospores. Within the pansporocyst, the shell valves are usually folded up or concertinaed, expanding on contact with water. Exceptions are members of the collective group Endocapsa, whose caudal processes do not expand on contact with water. The mature actinospores are either released into the lumen of the oligochaete, passed into the environment by defecation or released on death of the host.

Intra-piscine development

The actinospore stage is infective to a fish host. On contact with a suitable host, the polar filaments are extruded from the actinospore to anchor it to the host. The actinospore sporoplasm penetrates the epidermis of the host. In some cases a favoured entry point is via mucus cells (Yokoyama and Urawa, 1997; El-Matbouli *et al.*, 1999b). Malacospores of *T. bryosalmonae* released from the bryozoan phase also enter the fish host via the mucus cells (Morris *et al.*, 2000b; Longshaw *et al.*, 2002). In the initial intercellular stage, the cell wall surrounding the actinospore sporoplasm disintegrates and the sporoplasm cells (infective germ cells) invade surrounding tissues (Fig. 8.8). Endogenous budding gives rise to an enveloping primary cell and an internal secondary cell. At this point, development can continue in several ways. These range from the production of small uninucleate pseudoplasmodia to large complex extrasporogonic proliferative stages prior to spore formation. The type of intra-piscine development appears to be species-dependent and varies between genera. It is probable that most myxozoans will undergo at least one proliferative stage prior to spore formation in order to maximize potential spore production. The formation of pseudoplasmodia directly from individual actinospore sporoplasm cells appears unlikely since only limited numbers could be produced.

Extrasporogonic cycles, which occur in sites other than those in which sporogony takes place, have been reported from several genera, including *Sphaerospora*, *Hoferellus*, *Myxidium*, *Kudoa* and *Myxobolus*. In the extrasporogonic phase, the secondary cell within the primary cell undergoes a series of mitotic divisions to produce numerous secondary cells. Endogenous budding of the secondary cells gives rise to tertiary cell(s) within each secondary cell. In *M. cerebralis*, this first extrasporogonic phase takes place intraepithelially in the dermis, and within the blood for *S. renicola*. Rupture of the

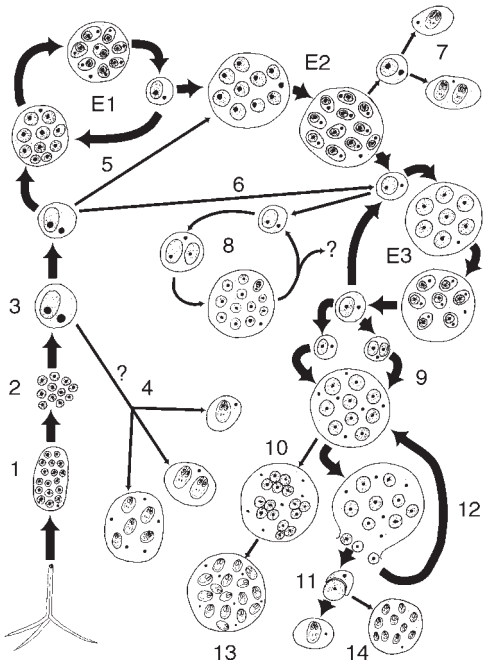


Fig. 8.8. Intra-piscine myxozoan life cycle (myxospore phase). This applies to those myxozoans undergoing a two-host life cycle. Heavy arrow = life cycle of *M. cerebralis*, demonstrated experimentally by El-Matbouli *et al.* (1995); light arrow = postulated life cycle for other myxospores.

On contact with a suitable host, the sporoplasm is released from an actinospore and invades the epidermis (1). The cell wall of the sporoplasm disintegrates and the inner secondary cells invade surrounding host cells (2). Endogenous budding produces a secondary cell surrounded by a primary cell (3). Several routes of development are possible for the myxospore following this stage. Production of mono-, di- or slightly polysporic pseudoplasmodia without a proliferative stage may be possible (4). The primary cell containing a secondary cell can undergo one (E3), two (E2 and E3) or three (E1, E2 and E3) extrasporogonic proliferative stages in which endogenous budding gives rise to numerous secondary cells containing tertiary cells. (5) Development route with two proliferative stages (E2 and E3); (6) Development route in which only one proliferative stage occurs (E3). Production of mono- or disporic pseudoplasmodia following one or two proliferative stages (7). In the proliferative stage of *Sphaerospora renicola* occurring in the swim bladder, secondary and tertiary cells become trapped within the swim-bladder wall (8). At the site of sporogony, the primary cell releases the secondary cells into the host cytoplasm or into the blood system. *M. cerebralis* undergoes a further two proliferative stages, one intercellularly in the subcutis and a second in the nervous tissues. For *S. renicola,* one of the proliferative stages occurs within the swim bladder wall. Molnár (1994) considered this stage equivalent to the blood stage, as proliferation occurs within the capillaries of the swim bladder. Extrasporogonic stages have also been reported in the rete mirabile of sticklebacks co-infected with *Sphaerospora elegans* Thélohan, 1892 and *Myxobilatus gasterostei* (Parisi, 1912), and in the renal tubule or collecting duct epithelium and glomeruli in carp infected with *S. renicola*. At the site in which sporogony occurs, the parasite develops into a plasmodium (trophozoite) or a pseudoplasmodium, in which sporogony takes place. Pseudoplasmodia can be monosporic, disporic or slightly polysporic (Lom and Dyková, 1992), but in all cases the cytoplasm contains one vegetative nucleus. Conversely, plasmodia contain multiple nuclei. Spores in pseudoplasmodia and in plasmodia are either formed within a pansporoblast, which can be monosporic, disporic or polysporic, or by the aggregation of generative cells (Feist, 1995). Pansporoblast formation is initiated by the envelopment of a generative cell by another cell (the pericyte). Division of the enveloped cell (sporogonic cell) produces the required number of capsulogenic, valvogenic and sporoplasmogenic cells required for the formation of the spore.

production of a plasmodium containing numerous nuclei and secondary cells (9). Sporogony is initiated either by the aggregation of generative cells (10) or by the production of a pansporoblast derived from a pericyte enclosing a sporogonic cell (11). In plasmodia containing pansporoblasts, it is possible for a final proliferative stage to occur (12). Sporogony is completed by formation of spores after aggregation of valvogenic, capsulogenic and sporoplasmogenic cells in a plasmodium (13) or pansporoblast (14). Release of the spores into the water over the lifetime or on the death and decay of the host allows the life cycle to continue in the invertebrate host.

Alternatively, in spore formation occurring by aggregation of generative cells, each differentiates into the requisite cell types. Polar filaments within the polar capsules are formed by the inversion of an external tube that is attached to the capsula primordium. On completion of polar filament development, the exit pore of the polar capsules is sealed with a plug-like structure, usually lacking the cap typically reported in actinospores.

Myxospores within the fish host possess one to several uni- or binucleate sporoplasms, which initiate the new infection in the invertebrate host. Sporoplasms often contain electron-dense sporoplasmosomes within the cytoplasm and spores may contain an iodinophorous vacuole. Myxospores are released into the environment via a number of different routes, either rupture of cysts on body surfaces with excretory products or upon the death and decay of the host. Myxospore stages are highly resilient, as demonstrated for *M. cerebralis* spores, which retain infectivity after 4 months in mud, freezing at –20°C for 2 months and passage through the alimentary canal of predatory birds and fish (El-Matbouli and Hoffmann, 1991b). Ingestion of myxospores by oligochaetes initiates the developmental cycle.

Malacospore development

The development of *T. bryosalmonae* does not follow the patterns observed in the myxospore–actinospore life cycle. The route of entry into and the initial early development of the parasite within the bryozoan host have not been elucidated. In *T. bryosalmonae*, the wall of the sac in which spores develop consists of a single layer of flattened (mural) cells. Proliferation of these cells leads to the formation of a sac wall and production of sporogonic cells within the sac. These sporogonic cells take one of two forms – either sporoplasmogenic (pale) cells or denser, stellate cells. The sporoplasmogenic cells, containing characteristic cytoplasmic sporoplasmosomes (Fig. 8.6), undergo a series of meiotic divisions, which have not been observed in stellate cells. Division of the sporoplasmogenic cells leads to the production of two uninucleate sporoplasms, both of which may contain a secondary cell derived by endogenous budding. The sporoplasms are enveloped by a group of stellate cells, which differentiate to form at least four valve cells and four capsulogenic cells. Synchronous maturation of the spores occurs within the sac, which contains up to several hundred spores. Unlike myxospores, the valve cells do not degenerate at maturity and they remain ‘soft’. The ontogeny of polar capsules in malacosporeans is still unclear. The formation of an external tube prior to inversion into the capsule has not been conclusively observed and may be an extremely transitory event (Canning *et al.*, 1996, 1999, 2002). However, unlike actinospores and myxospores, the exit pore of the polar filament is not covered by a cap or stopper-like structure, nor is it covered by the valve walls. Malacospores are short-lived, surviving for less than 12 h (De Kinkelin *et al.*, 2002).

Feist *et al.* (2001) and Longshaw *et al.* (2002) experimentally induced PKD in naïve rainbow trout fish exposed to the bryozoan stages of the parasite. They demonstrated that the parasite entered the fish via the mucus cells of the skin epithelium and not via the gut epithelium. Morris *et al.* (2000b) showed that the parasite was present in the gill epithelium after 3 days’ exposure at an enzootic field site. Thus it is likely that all external epithelia exposed to the environment are suitable sites for infection. Entry of the parasite into the fish is apparently rapid, with *T. bryosalmonae* cells present in the skin of rainbow trout at 1 min post-exposure (Longshaw *et al.*, 2002). Parasites are transported around the body via the blood, during which time they may undergo a series of multiplications (Kent and Hedrick, 1985). These extrasporogonic stages undergo further development, primarily within the kidney and spleen but they can also be localized within many other tissues and organs of the host. The earliest identifiable stage of *T. bryosalmonae* (PKX cells) in the fish host tissues

is a mononucleate primary cell containing prominent cytoplasmic granules. Following endogenous cleavage, secondary cells are produced within the primary cell. Secondary cells may also themselves divide by binary fission and form tertiary cells by endogeny. In rainbow trout cultured within the UK this is most often the final stage observed in fish. *Tetracapsuloides bryosalmonae* undergoes sporogony within the lumen of renal tubules and collecting ducts in some hosts, particularly in salmonids from the USA (Kent and Hedrick, 1985). The spores are characterized as being ovoid, with indistinct valves and two polar capsules (Kent *et al.*, 2000). The complete life cycle of *T. bryosalmonae* has not been elucidated, since infections of bryozoan hosts have not been achieved and the possible role of other hosts cannot be discounted (Tops *et al.* 2004). The possibility that propagation of malacosporeans can occur within bryozoan hosts has been suggested by Taticchi *et al.* (2004). These authors described the presence of malacosporean-like vermiform stages, similar to those of *B. plumatellae*, hatching from *Plumatella fungosa* flotablasts. This finding suggests the possibility of parasite survival and dispersal in the statoblast stages and that other hosts may not be required, at least for some species.

Ecological factors affecting transmission

Actinospore release follows a circadian pattern in oligochaetes, with most spores being released during late evening and early morning (Yokoyama *et al.*, 1993a; Özer and Wootten, 2001). The maximum number of spores released daily by an individual worm can be as many as 80,000 for Echinactinomyxon types (Özer and Wootten, 2001). Actinospores are released from the oligochaete host throughout the year; however, most are released during the summer (El-Mansy *et al.*, 1998a,b; Özer *et al.*, 2002). There have been limited studies on the longevity of actinospores following release from the oligochaete, with estimates ranging from 11 to 25 days (Yokoyama *et al.*, 1993a; Xiao and Desser, 2000a). Actinospores appear to release their sporoplasms in response to mucus from the specific fish host (Yokoyama *et al.*, 1993a, 1995a).

The general paucity of data on actinospore biology for most species contrasts markedly with those gained on the actinospore of *M. cerebralis*. The susceptibility of different genetic strains of *Tubifex tubifex* to *M. cerebralis* myxospores and the factors that influence the distribution of actinospore infections in oligochaetes have been determined (Allen and Bergersen, 2002; Beauchamp *et al.*, 2002). The *M. cerebralis* actinospore develops optimally at 15°C, and developmental stages degenerate at temperatures above 25°C (El-Matbouli *et al.*, 1999a). The effect of different chemicals and salinity on the viability of the Triactinomyxon stages has also been investigated (Wagner *et al.*, 2003). Whilst it is recognized that sediment type affects the distribution of oligochaete species (Xiao and Desser, 1998b), Blazer *et al.* (2003) showed that the substrate type affected the number and duration of release of *M. cerebralis* actinospores. Actinospore release was greatest in oligochaetes maintained in mud and sand, and least in a leaf litter substrate (Blazer *et al.*, 2003). Stevens *et al.* (2001) found that the initial myxospore dose affected the number of actinospores produced and that the parasite reduced the biomass, abundance and individual weights of oligochaetes. There is a need to conduct more wide-ranging studies on actinospore biology and ecology in order to ascertain whether the patterns that are apparent in *M. cerebralis* actinospores apply to other actinosporean infections.

Vector specificity and factors affecting vector

Most actinospores have been recorded in oligochaetes, predominantly in the families Tubificidae and Nadidae, although there are a few records in polychaetes (Ikeda, 1912; Bartholomew *et al.*, 1997; Hallett *et al.*, 1998; Køie, 2000, 2002; Køie *et al.*, 2004). The life cycle for *C. shasta* is the only one that has been completed in which a freshwater

polychaete has been implicated. Whilst Bartholomew *et al.* (1997) also reported the presence of an Aurantiactinomyxon type in the same host, thus far all other records of actinospores in marine polychaetes are of the Tetractinomyxon type (Ikeda, 1912; Køie, 2000, 2002). Despite a number of publications on the actinospore fauna of marine oligochaetes and polychaetes, no links have been made between the stages in this host and in a vertebrate counterpart (Caullery and Mesnil, 1905; Hallett *et al.*, 1995, 1997, 1998, 1999, 2001; Hallett and Lester, 1999).

Parasite specificity in the invertebrate host is unclear. Actinospores of *M. cerebralis* appear to be specific to genetic lineages of *T. tubifex*, whilst other actinospores may have a wide variety of oligochaete types as hosts (Xiao and Desser 1998a,b; Koprivnikar and Desser, 2002; Székely *et al.*, 2003). Whilst the malacosporean *T. bryosalmonae* appears to be relatively specific to the salmonid host (with the exception of pike, *Esox lucius*), it has been reported from a wide range of bryozoan hosts in both Europe and the USA (Longshaw *et al.*, 1999; Okamura *et al.*, 2001; Okamura and Wood, 2002).

As with any free-living organism, polychaetes, oligochaetes and bryozoans are affected by the environment in which they are found. *Tubifex tubifex* distributions are determined by the composition and organic content in the substratum and the presence of other oligochaetes (Granath and Gilbert, 2002) and, whilst they are often associated with sites of poor water quality, they can survive in many different habitat types. In general, poor water quality, low oxygen content and low flow rates are detrimental to the definitive hosts of myxozoans.

Diseases According to Host Organ

Gills

Infections of the gill can compromise the respiratory capability if present in sufficient numbers, and disruption of the gill epithelium by the release of myxospores can provide a route of entry for secondary infections. Pathology associated with myxozoan infections in the gills includes fusion of lamellae, inflammation, hyperplasia, pressure atrophy and cellular necrosis. Molnár (2002b) proposed a system to describe the specific site locations of myxozoans in gills. This distinguishes between interlamellar epithelial and intralamellar vascular types for plasmodia in the gill lamellae, with either chondroidal, vascular or epithelial intrafilamental types developing in the gill filaments.

Gill sphaerosporosis of carp, caused by *S. molnari*, has been reported in *C. carpio* and *Carassius carassius* in Europe (Dyková and Lom, 1988a; Fig. 8.9). Additionally, Hedrick *et al.* (1990) reported the presence of *S.* cf. *chinensis* in the gills of *Carassius auratus.* In both infections, moderate to severe gill hyperplasia results and a large proportion of the respiratory epithelium can be replaced by sporogonic stages. In mild *S. molnari* infections, local circulatory disorders and dystrophic changes also occur. *S. molnari* spores measure 10.5 μm × 10.3 μm and those of *S. chinensis* measure 7.4 μm × 7 μm. Both the parasites form monosporic pseudoplasmodia.

Proliferative gill disease (PGD, hamburger gill disease), caused by the extrasporogonic stage of *Henneguya ictaluri* Pote, Hanson and Shivaji, 2000, is a major disease of channel catfish (*Ictalurus punctatus*) and can result in high mortalities amongst juvenile farmed catfish (Pote *et al.*, 2000). The parasite elicits a strong granulomatous inflammatory response in the gills. Styer *et al.* (1991) demonstrated experimentally that the life cycle alternated between the fish host and the oligochaete *Dero digitata*. Both stages in the life cycle have been confirmed using molecular techniques (Pote *et al.*, 2000; Hanson *et al.*, 2001). The spore body of the myxospore measures 24 μm × 6 μm and the total spore length is 48–80 μm.

Another pathogenic *Henneguya* sp. in the gills of *I. punctatus* is *Henneguya exilis* Kudo, 1929. The presence of plasmodia at the base of the secondary lamellae (epithelial filamental type of Molnár, 2002b) elicits

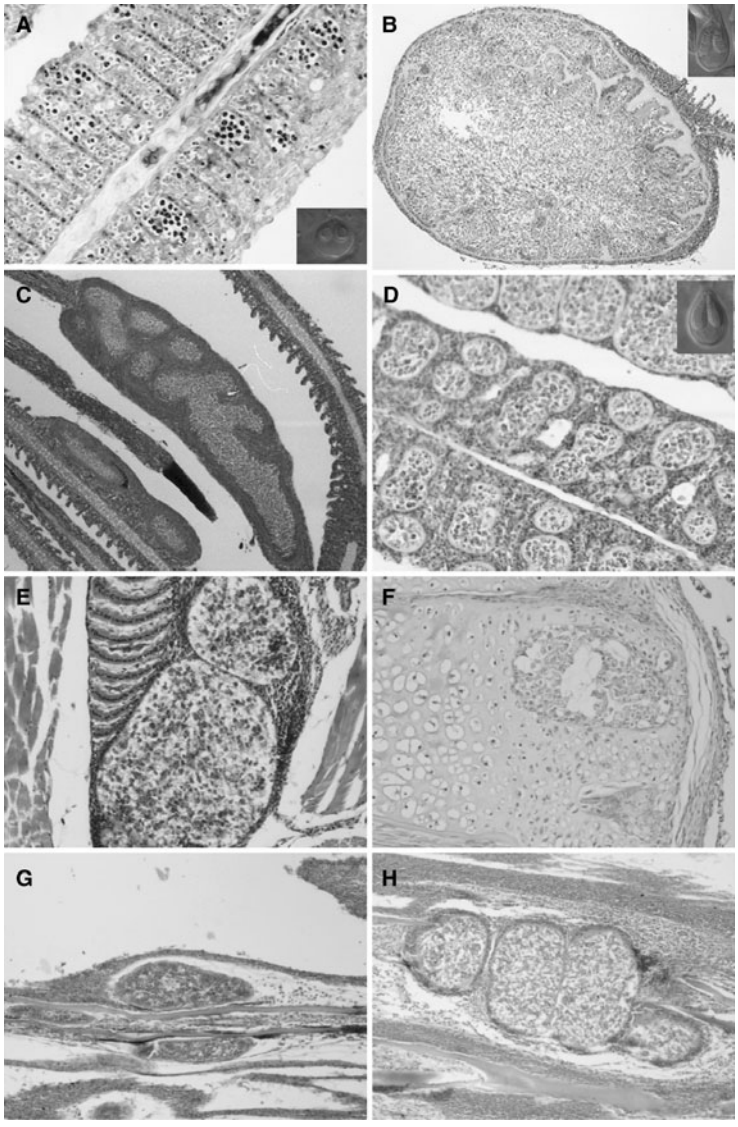


Fig. 8.9. Histological sections of gill, pseudobranch and fin infections. A. Giemsa-stained section of carp (*Cyprinus carpio*) gill infected with *Sphaerospora molnari* (inset: mature spore in sutural view). B. Large cyst of *Myxobolus koi* in gill of koi carp (*C. carpio*) (inset: mature spore in valvular view). C. Multiple cysts of *Henneguya psorospermica* in gill of pike (*Esox lucius*). D. Multiple cysts of *Myxobolus macrocapsularis* in gill of chub (*L. cephalus*) (inset: mature spore in valvular view). E. Cysts of *M. macrocapsularis* in pseudobranch of dace (*L. leuciscus*). F. Sporogonic plasmodium of an unidentified *Myxobolus* sp. in cartilage of caudal peduncle of *L. cephalus*. G. Longitudinal section through fin of a juvenile roach (*R. rutilus*) showing *Myxobolus* sp. cysts within the epithelium. H. *Myxobolus* sp. cysts in connective tissue of fin of barbel (*Barbus barbus*).

a severe tissue proliferation in this atypical site. Known as 'lamellar disease', it can lead to high mortalities of fingerlings. The formation of cysts in other sites is not pathogenic.

The alternate stage is an Aurantiactinomyxon type in the oligochaete *D. digitata* (Lin *et al.*, 1999). Spores measure 17.6 µm × 4.9 µm, and total length is 52.3 µm.

The pathology associated with *Henneguya creplini* (Gurley, 1894) on the gills of *Stizostedion lucioperca* in Lake Balaton was described by Molnár (1998). Plasmodia on the gills are large, reaching 900 μm × 750 μm in size, with an average of 30–60 plasmodia per fish. During cyst development, host responses are minimal. Following sporogenesis and rupture of plasmodia, an intense host response, consisting of epithelial proliferation, overgrowth of the damaged plasmodium and cell necrosis, becomes apparent. Spores are 14.3 μm × 5.5 μm, with a total length of 43 μm including caudal appendages. Infections in pike (*E. lucius*) and perch (*Perca fluviatilis*) caused by *Henneguya psorospermica* Thélohan, 1895 also give rise to multiple large plasmodia displacing the gill tissue (Fig. 8.9).

Henneguya piaractus Martins and de Souza, 1997 in the gills of captive *Piaractus mesopotamicus* can cause mortalities (Martins and de Souza, 1997; Martins *et al.*, 1997). Clinical signs of infection include decreased feeding, lethargy, erratic swimming and loss of equilibrium. Histologically, haemorrhaging and severe inflammation occur in the gill epithelium and larger cysts cause pressure atrophy on adjacent lamellae. Spores measure 11.3 μm × 3.2 μm with a total length of 48.4 μm including caudal process.

Infections with *Myxobolus basilamellaris* Lom and Molnár, 1983 cause a localized pathological response to the parasite cysts within the gills of its host (Kovács-Gayer and Molnár, 1983). The cysts develop in a basifilamental position on the gill. Growth of the cyst lifts the basal part of the gill filament and deforms adjoining lamellae, reducing the respiratory surface. Those cysts developing in the gill arch restrict nerves and blood vessels passing through the gill arch, leading to local blockages.

M. koi forms large and small-type cysts in the gills of *C. carpio* (Fig. 8.9). The smaller cysts develop in an interlamellar position within the gills and host responses are minimal. In contrast, large cysts produce extensive pathological changes in the gill, including hypertrophy of the branchial epithelium and clubbing of the gill filaments (Yokoyama *et al.*, 1997). Spores measure 12–15 μm × 5–9 μm and contain two equal-sized polar capsules measuring 6–7.4 μm × 1.6–2.7 μm.

M. pavlovskii infects the gills of bighead (*Aristichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) and in young cultured fish can cause pathological damage to the gill structure due to the development of plasmodia affecting respiratory function (El-Matbouli *et al.*, 1992a). Parasite plasmodia develop in an interlamellar and basifilamental position on the gills (Molnár, 2002b). The parasite has been shown to undergo a two-host life cycle, producing Hexactinomyxon-type actinospores in the oligochaete *T. tubifex* (El-Matbouli and Hoffmann, 1991a). Myxospores measure 7–9.5 μm × 5–8 μm.

Serious infections of the pseudobranch are uncommon, although infections of this organ have been recorded (Molnár, 2002b). Mortality of farmed *Salmo salar* associated with *Parvicapsula pseudobranchicola* Karlsbakk, Sæther, Høstlund, Fjellsøy and Nylund, 2002 has been reported. The *Parvicapsula* species reported by Sterud *et al.* (2003) is likely to be *P. pseudobranchicola*, based on tissue location, pathogenesis and geographical location, although there are some minor differences in the spore dimensions. Clinical signs include increased surface swimming, lethargy, lack of feeding, exophthalmia and mortality. Petechial haemorrhaging of infected pseudobranchs is observed. It has been suggested that the infection ultimately leads to blindness of the host due to loss of pseudobranch function which supplies blood to the eye (Karlsbakk *et al.*, 2002; Sterud *et al.*, 2003). The spores measure 12 μm in length and 6.2 μm in thickness and possess two slightly unequal polar capsules measuring 2.4 and 2.3 μm in diameter (Karlsbakk *et al.*, 2002).

Skin, scales, fins and subcutaneous tissues

Infections of the skin, scales or subcutaneous tissues are generally not considered serious (Fig. 8.9). However, in fish destined for the aquarium trade or for human consumption, they can be considered problematic

as they reduce the aesthetic appeal of the fish and their value. Typical of this group is *Myxobolus diversus* Nie and Li, 1973 in the fins of goldfish. Whilst serious pathological changes are generally absent, the obvious presence of myxozoan cysts on the fins reduces the value of the fish (Molnár and Székely, 2003).

Molnár (2002a) described the characteristic types and development of myxozoan plasmodia in fins. Plasmodia developing within the skin between the fin-rays and inside the lumen of the fin-rays produced limited host responses. However, plasmodia developing on the outer surfaces of the fin-rays, as typified by *T. nikolskii*, markedly deform the cartilaginous elements of the fin-rays of carp (Molnár, 1982; Moshu and Molnár, 1997). *T. nikolskii* has also been reported on the scales of *C. carpio carpio* (common carp), where plasmodial development is associated with calcified collagen but with low pathogenicity (Moshu and Molnár, 1997). *C. c. carpio*, *C. auratus* (goldfish) and *C. carpio haematopterus* (koi carp) possess differential susceptibility to infections by *T. nikolskii* with *C. c. haematopterus* being less susceptible compared with *C. c. carpio*, and *C. auratus* being resistant (Molnár, 2002c). Székely *et al*. (1998) demonstrated that *T. nikolskii* alternates through *T. tubifex*, producing an Aurantiactinomyxon type with a spore body measuring 21 µm in diameter and possessing caudal processes measuring 13 µm × 9 µm. The myxospore measures 16.5 µm × 10 µm and contains a single polar capsule measuring 6.5 µm × 5.6 µm.

T. hovorkai, a parasite of the connective tissue, causes haemorrhagic thelohanellosis in koi carp. The disease is manifested by haemorrhaging and oedema in skin lesions, with destruction of the capillary network caused by the release of mature spores into the surrounding tissues, and chronic mortalities (Yokoyama *et al.*, 1998). The parasite alternates through the oligochaete *Branchiura sowerbyi*, producing an Aurantiactinomyxon-type actinospore with a spore body 19 µm in diameter and possessing caudal processes measuring 29 µm × 9 µm (Székely *et al.*, 1998). Actinospores enter the fish host via the gills (Yokoyama and Urawa, 1997). Myxospores measure 20 µm × 10 µm and possess a single polar capsule measuring 9 µm × 8 µm.

Thelohanellus wuhanensis Xiao and Chen, 1993 is a serious disease of *C. auratus gibelio* that causes large swellings on the skin of affected fish and leads to mortality of infected fry (Wang *et al*., 2001). Myxospores measure 23 µm × 14 µm and contain a single polar capsule measuring 10.5 µm × 8 µm.

Several *Myxobolus* species in the skin and subcutaneous tissues have been reported as pathogens. *Myxobolus drjagini* (Akhmerov, 1954) forms small plasmodia in the skin of the operculum, head and buccal cavity of *H. molitrix* and causes twist disease in China (Wu *et al*., 1975). El-Mansy and Molnár (1997a) did not consider that it caused losses in Hungary. The parasite alternates through *T. tubifex* and produces a triactinomyxon-type actinospore. Myxospores measure 13 µm × 7 µm and contain two unequal-sized polar capsules measuring 5.8 µm × 3.7 µm and 3.4 µm × 2.5 µm. The market value of *C. auratus auratus* is reduced as a result of infections with *Myxobolus rotundus* (Nemezek, 1911) that cause large, visible cysts on the body surface (Lu *et al.*, 2003).

Myxobolus episquamalis Egusa, Maeno and Sorimachi, 1990 infects the scales of *Mugil cephalus*. Small, pale, raised lesions are present on infected fish and can lead to erosion of the scales and attenuation of the dermis. Whilst the infection is of little pathological consequence, Rothwell *et al*. (1997) considered that up to 6% of the catch were unsaleable due to the loss of aesthetic quality of the fish.

Muscle

There are many reports of myxozoan infections in muscle of both marine and freshwater fishes worldwide. They range from innocuous infections with minimal host response to intense infections leading to mortality or spoilage of the musculature through enzymatic degradation on the death of the host. Typically, host responses

to the parasite are usually not initiated until sporogenesis of the myxospores is complete. Whilst the majority of infections are reported from somatic muscle, any organ containing muscle tissue, including the heart, intestine and gills, can be affected. In addition, the presence of myxozoan development stages in muscle, which normally occur in other host tissues, can lead to pathological changes in the musculature (Fernández-de-Luco *et al*., 1997).

The synonymy of *Henneguya zschokkei* Doflein, 1901 from whitefishes (*Coregonus* spp.) and *Henneguya salminicola* Ward, 1919 from salmonids (*Oncorhynchus* and *Salmo* spp.) is problematic. Whilst some authors consider *H. salminicola* as a junior synonym of *H. zschokkei*, Eiras (2002) included them as separate species in a synopsis of the genus. Recent molecular data have provided further evidence that they are distinct species (Kent *et al*., 2001). For both, heavy infections within the somatic muscle can render the flesh unmarketable as the production of fillets can cut through these cysts to produce a milky exudate (Awakura and Kimura, 1977). The spore body of *H. salminicola* measures 12 μm × 8 μm and total spore length is 43–52 μm. The spore body of *H. zschokkei* measures 10 μm × 7 μm and total spore length is 55 μm (Fig. 8.3).

In heavy infections with *M. artus* in *C. carpio*, the body surface of the host is 'irregular and uneven' due to the presence of numerous cysts in the muscle (Ogawa *et al*., 1992). Two types of cysts are found – a larger, interfibrillar type and a smaller intrafibrillar type. Following sporogenesis, spores are subjected to a host response and the rupture of pseudocysts leads to the lysis of adjacent muscle fibres. Spores are phagocytosed and transported to other organs in the body via the blood (Fig. 8.10). Yokoyama *et al*. (1996) showed that carp release up to 3×10^5 *M. artus* spores per fish per day throughout their lifetime, possibly via the intestine and skin. Spores measure 8 μm × 11 μm in size.

Cyprinid fishes are parasitized by a number of *Myxobolus* spp. in the musculature (Fig. 8.10). Some workers have considered that *M. pseudodispar* from the musculature of roach is a junior synonym of *Myxobolus cyprini* Doflein, 1898 due to the pleomorphic nature of the spore. Molnár *et al*. (2002) used DNA sequence data to demonstrate that *M. pseudodispar* Gorbunova, 1936 from a number of hosts, *Myxobolus musculi* Keysselitz, 1908 from barbel (*Barbus barbus*) and *M. cyprini* from *C. carpio* were distinct species. In addition, barbel are hosts to *Myxobolus pfeifferi* Thélohan, 1895 in the muscle, which has caused epizootics of 'barbel boil disease'. Longshaw *et al*. (2005) found *M. pfeifferi* in the musculature of juvenile *Leuciscus cephalus*, *Leuciscus leuciscus* and *Rutilus rutilus* without significant host response. For all four *Myxobolus* spp. the pathogenesis of the disease is similar to that reported for *M. artus*, whereby spores develop within cysts in the myofibrils. Growth of cysts results in marked enlargement of infected muscle fibres and pressure atrophy of adjacent myofibrils. On completion of sporogenesis, spores are subjected to a vigorous granulomatous response involving epitheloid-type cells and phagocytic cells. Infected myofibrils are destroyed and localized blockage of vessels and necrosis of the surrounding tissue can occur (Fig. 8.10). Spores are transported around the body via the blood and lymphatic system to various sites, including the thymus, liver, kidney, spleen, gills and intestine. The majority of infections in carp by *M. cyprini* are considered to cause only subclinical damage to the muscle and local necroses in other organs but can, in some cases, cause hydropic degeneration and dropsy (Molnár and Kovács-Gayer, 1985). In roach infected with *M. pseudodispar*, similar effects have not been reported (Baska, 1987; Athanassopoulou and Sommerville, 1993b). Spore measurements are as follows: *M. cyprini* 10–16 μm × 8–12 μm (polar capsules equal in size, measuring 5–7 μm in length); *M. musculi* 9–13 μm × 8–11 μm (polar capsules unequal in size, measuring 4.5–7 μm × 3–4 μm and 4–6 μm × 2–3.5 μm); *M. pseudodispar* 10–12 μm × 7–9.5 μm (polar capsules unequal in size, measuring 4.5–5.5 μm × 3 μm and 4 μm × 3 μm); *M. pfeifferi* 10–13 μm × 9–12 μm

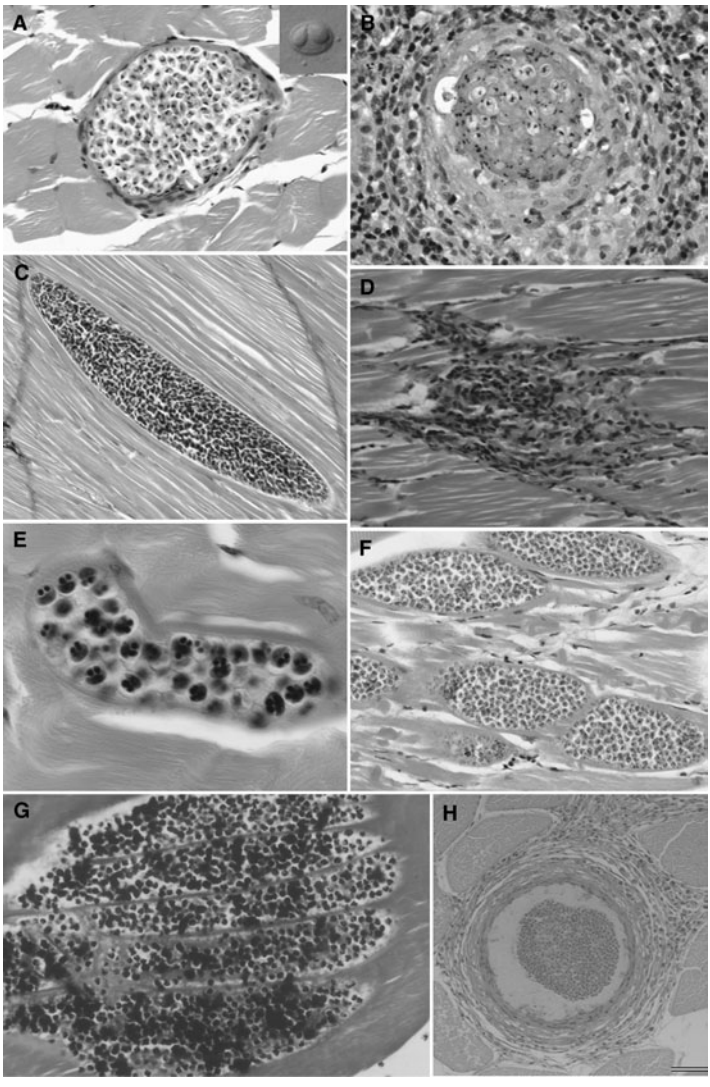


Fig. 8.10. Histological features of muscle-invading species. A. Sporogonic plasmodia of *Myxobolus artus* in the skeletal muscle of carp (*C. carpio*) (inset: mature spore in valvular view). B. Granulomatous host response to a plasmodium of *M. artus* within the renal tissue (same fish as in Fig. 8.10A). C. A plasmodium of *Myxobolus pseudodispar* within the muscle of a juvenile roach (*Rutilus rutilus*). D. Focal inflammatory response to *M. pseudodispar* spores and sporogonic stages from a ruptured plasmodium. E. Intramyofibrillar *Kudoa* infection in cow-nosed ray (*Rhinoptera bonasus*). F. Multiple plasmodia of *Kudoa* sp. in the myofibrils of common goby (*Pomatoschistus microps*). G. Giemsa-stained section of *Kudoa thyrsites* infection in cod (*Gadus morhua*). H. Fibrous encapsulation of a *Kudoa* sp. plasmodium in viviparous blenny (*Zoarces viviparus*).

(polar capsules equal in size, measuring 5–6 μm × 2.5–3.5 μm).

Myxobolus lentisuturalis Dyková, Fiala and Nie, 2002 from *Carassius gibelio* induces severe regressive changes in the dorsal somatic musculature. Externally, the disease can manifest itself as a large swelling anterior to the dorsal fin, caused by the developing parasite cyst. In heavily infected individuals, necrotic changes occur in

infected myofibrils. Spores measure 11.8 μm × 7.6 μm, with equal-sized polar capsules measuring 4.2 μm × 2.5 μm.

Intense infections with a variant of *Myxobolus procercus* (Kudo, 1934) were reported in the musculature of *Percopsis omiscomaycus* by Cone *et al.* (1997). Host responses to sporogonic forms of the parasites were in approximately 5% of the fish examined; otherwise fish appeared healthy. Cone *et al.* (1997) considered that the extremely large numbers of myxozoan cysts in the muscle may be due to an over abundance of oligochaetes and the depressed immune system of the fish host. Spores measure 14 μm × 6 μm, and polar capsules are often dissimilar in size measuring approximately 7.2 μm × 2.2 μm.

Most muscle infections in freshwater fishes are caused by bivalvulid myxospores and the host response is usually towards mature spores. In marine fish, however, myxozoan infections in the muscle are frequently caused by multivalvulid myxospores, which in a few cases result in the enzymatic destruction of the muscle tissue following the death of the host. Typical of this group are members of the genus *Kudoa*, reviewed by Moran *et al.* (1999b) (Fig. 8.10). Fish can be unmarketable due either to the presence of large, macroscopic, melanized cysts within the musculature or to post-mortem myoliquefaction. At least 50 species of *Kudoa* have been described from marine fishes worldwide. *Kudoa thyrsites* is known to infect more than 20 species of fish from a number of different families worldwide, including wild and farmed fish (Kent, 2000). Myoliquefaction has also been reported in *Seriola holandi* infected with *Unicapsula seriolae* Lester, 1982 and *Kudoa* (= *Hexacapsula*) *neothunni* (Arai and Matsumoto, 1953).

Ulcerative lesions in young-of-the-year *Brevoortia tyrannus* have been attributed to a number of aetiologies, including bacteria, fungi and harmful algal blooms. Reimschuessel *et al.* (2003) demonstrated that ulcers and chronic inflammatory infiltrates in this host were associated with invasive extrasporogonic plasmodia of *Kudoa clupeidae* (Hahn, 1917). Spores and plasmodia were present within the musculature of these fish and, whilst the authors were unable to unequivocally demonstrate that the plasmodia causing ulceration were early stages of *K. clupeidae*, strong circumstantial evidence was provided to suggest that these invasive extrasporogonic stages were responsible for the ulcerations.

Cartilage and ossified elements

Infections of cartilage and bone are relatively rare, and parasites infecting the host prior to ossification often become trapped within the ossifying or fully ossified elements. Infections of the cartilage can result in hypertrophy of cartilage and inflammation of surrounding tissues.

M. cerebralis is the causative agent of salmonid whirling disease. Clinical signs of the disease can include melanization of the tail region and skeletal deformities. The characteristic 'whirling' motion and tail chasing of infected fish are thought to be a consequence of constriction and pressure on the spinal cord (Rose *et al.*, 2000). *M. cerebralis* was first recorded infecting brown trout from Central Europe and northeast Asia, which were transferred to a number of sites worldwide through the movement of live fish and fish products (Bartholomew and Reno, 2002; Bartholomew and Wilson, 2002). Evidence from the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) confirmed that *M. cerebralis* was introduced to several countries from a limited geographical source (Andree *et al.*, 1999a; Whipps *et al.*, 2004b). It now occurs in Europe, the USA, South Africa and New Zealand. Movements of live infected fish from hatcheries and fish farms possibly increased the geographical spread of the pathogen within individual countries. Whilst there is strong evidence from other studies in some regions of the USA that *M. cerebralis* is responsible for declines of wild rainbow trout (Hedrick *et al.*, 1998), Modin (1998) considered that 'there is little clear evidence of significant impacts on wild salmonids in California'.

Wolf and Markiw (1984) demonstrated that the parasite required an oligochaete host in its life cycle and subsequent studies have shown that the parasite is extremely specific to *T. tubifex* (Granath and Gilbert, 2002) and possibly even to distinct genetic lineages of *T. tubifex* (Beauchamp *et al.*, 2001). Like numerous other myxozoans, it has been presumed that myxospores from fish were only available to the oligochaete host upon the death and decay of the fish host. However, Nehring *et al.* (2002) have demonstrated experimentally that live brown trout expel viable *M. cerebralis* myxospores, which are infective to *T. tubifex*. Sporoplasms from waterborne triactinomyxon actinospores enter the host via a number of entry points, including the mucus cells of the epithelium, gills and buccal cavity (Markiw, 1989; El-Matbouli *et al.*, 1995, 1999b). Entry and establishment of the disease in fish is determined by the degree of ossification and, whilst adult, ossified fish can be infected, they are asymptomatic for the disease (Markiw, 1992). Following penetration of the epithelium by the triactinomyxon, sporoplasms reach the peripheral nerves, then the central nervous system and eventually the cartilaginous elements. During migration through the nervous tissues, the parasite undergoes a series of proliferative stages and appears to be unrecognized during this stage by the host immune system. Within the cartilaginous elements, phagocytosis of chondrocytes by plasmodia has been reported and the lysis of cartilage by the parasite can induce a vigorous host response (Fig. 8.11). Inflammatory cells involved in the host response include mononuclear leucocytes, and the combination of cartilage lysis with a vigorous host response can lead to severe disruption of the cartilage and result in skeletal deformities. *M. cerebralis* spores are highly variable in morphology, but in general are oval to circular in valvular view and measure 7–10 μm × 7–10 μm in size. Polar capsules are equal in size, measuring 4–6 μm × 3–3.5 μm, and contain five to six turns of the polar filament. The advent of molecular tools such as PCR and *in situ* hybridization has assisted in the identification and phylogeny of the parasite in the oligochaete and fish hosts and provided further insights into its biology (Andree *et al.*, 1997, 1999b; Antonio *et al.*, 1998).

Infections with *Myxobolus buckei* Longshaw, Frear and Feist, 2003, originally reported by Bucke and Andrews (1985) as *Myxobolus ellipsoides* Thélohan, 1892, cause serious disease in juvenile cyprinids in the UK, resulting in marked spinal compression in infected fish. Normal and burst swimming of infected fish is compromised and few juvenile fish survive to adulthood (Longshaw *et al.*, 2003). Histological lesions in infected fish range from mild hypertrophy of the zygapophyseal elements to complete fusion of the vertebrae (Fig. 8.11). Hypertrophy of the vertebral elements and expansion of the intervertebral membrane to accommodate the developing plasmodia cause pressure on the spinal cord and adjacent blood vessels. Fish are infected prior to ossification and the parasite develops initially within the remnants of the embryonic notochord. A vigorous inflammatory response to the developing plasmodia and spores leads to the radial expansion and fusion of the vertebral centra during the cartilaginous phase of host development. Following ossification, parasites remain trapped within bony elements. Spores measure 14 μm × 11.5 μm in size.

M. aeglefini has been reported from a number of marine fishes in the North Atlantic, North Sea and Baltic Sea (Lom and Dyková, 1992). It has a wide host specificity, having been reported in flatfish and gadoids (Nielsen *et al.*, 2002). The report by Yokoyama and Wakabayashi (2000) of *M. aeglefini* in cartilaginous tissues of the skeletal musculature of Japanese porous-head eelpout may represent a morphologically similar but genetically different species of *Myxobolus*. Whilst *M. aeglefini* is normally considered as a parasite with a specificity for head cartilage, it can be found in the eyes of gadoid hosts (Fig. 8.11). As with other cartilage-infecting myxozoans, damage to the host is caused by a combination of the host response and lysis of the cartilage by the action of the parasite. Spores measure 11–13 μm × 10–12 μm in size.

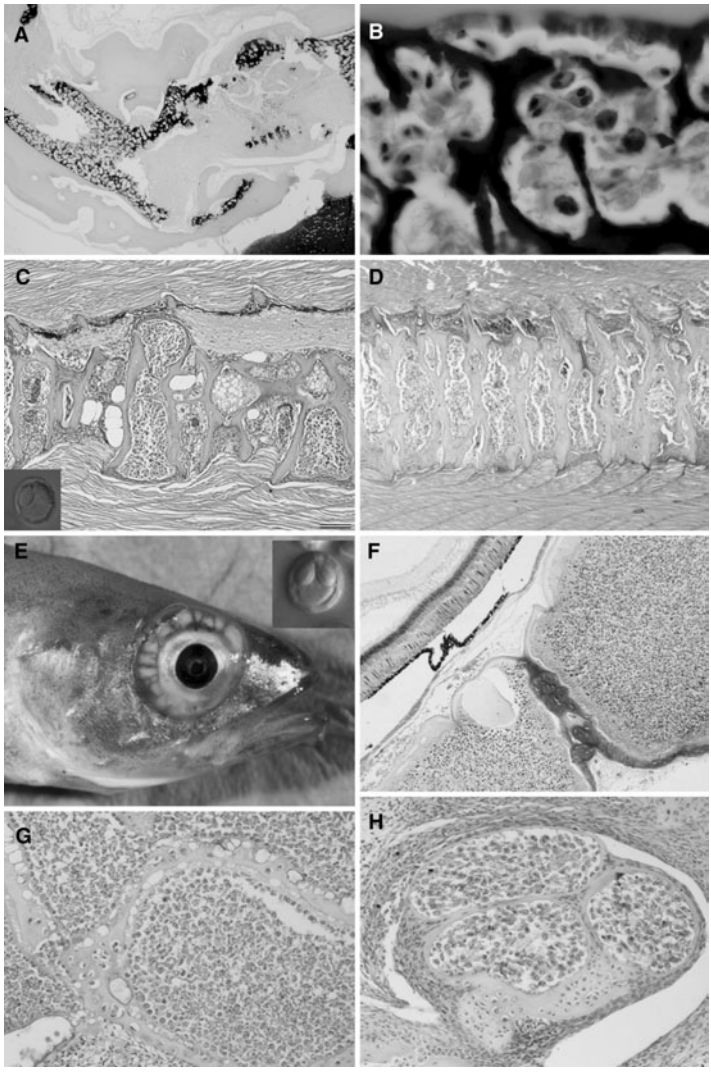


Fig. 8.11. Pathological features of cartilage infections. A. Low-power view of head cartilage of rainbow trout (*Oncorhynchus mykiss*) infected with *Myxobolus cerebralis,* showing disintegration of cartilaginous elements. B. High-power view showing spores and developmental stages of *M. cerebralis* amongst destroyed cartilage (A and B are from Giemsa-stained sections). C and D. Vertebral deformation and complete fusion of vertebrae in chub (*L. cephalus*) caused by *Myxobolus buckei* (C. inset: mature spore of *M. buckei* in valvular view). E. Cysts of *Myxobolus aeglifini* in the scleral cartilage of the eye of poor cod (*Trisopterus minutus*) (inset: mature spore of *M. aeglifini* in valvular view). F. Section through two large cysts of *M. aeglifini* in the scleral cartilage of whiting (*Merlangius merlangus*). G. *Myxobolus* sp. in the cranial cartilage of common goby (*P. microps*). H. Cysts of *Myxobolus* sp. in the cranial cartilage of roach (*R. rutilus*).

Reproductive tissues

Myxozoan infections of gonadal tissue are relatively rare and appear to be sex specific. Infections of the ovaries occur either within oocytes or within the interstitial or connective tissues, with those species occurring within the oocytes being more serious.

In both male and female fish, infections can lead to reductions in fecundity by castration or gonadal regression. Negative impacts at the host population level may be possible but have not been reported to date.

Sphaerospora testicularis Sitja-Bobadilla and Alvarez-Pellitero, 1990 infects the seminiferous tubules in the testis of *Dicentrarchus labrax*. Infections lead to testicular hyalinization and hypertrophy, occlusion of the tubular lumens, ascites in the abdominal cavity and damage to the germ and Sertoli cells. Disporic pseudoplasmodia contain spores measuring 12 μm × 15 μm in size. *Agarella gracilis* Dunkerley, 1915 has been reported in the testis of *Lepidosiren paradoxa* in Brazil, with no data on the impact of the parasite. Spores measure 28–35 μm × 13–16.5 μm in size. Testicular tissue of *Girardinus januarius* is destroyed by spores of *Myxobolus lutzi* Aragão, 1919. Spores measure 10 μm × 7 μm in size.

Henneguya oviperda (Cohn, 1895) Labbé, 1899 has been reported from the ovaries of pike *E. lucius* in Russia and other European countries. In heavy infections, it causes complete degeneration of ovarian tissue and oocytes. Parasite cysts are found in the connective tissue and follicle epithelium of the ovary, whilst spores are found in the oocytes. Spores measure 27–42 μm × 5–10 μm, including caudal appendages.

Sphaerospora ovophila Xiao and Desser, 1997 spores in the ovary of *Lepomis gibbosus* produce visible cysts measuring up to 500 μm in diameter. Spores measure 8.2 μm × 6.2 μm in size. *Myxobolus algonquinensis* Xiao and Desser 1997 from *Notemigonus crysoleucas* produces cysts in the connective tissue of ovaries or interstitial tissue among oocytes measuring up to 800 μm in diameter. Spores measure 14.7 μm × 10.9 μm in size. No pathology was reported with these two forms (Xiao and Desser, 1997).

Kudoa ovivora Swearer and Robertson, 1999 occurs in the ovaries of *Thalassoma bifasciatum* and other labroid fishes. White-coloured plasmodia are restricted to the inner wall of the oocyte membrane and spores are only found in late developmental stage oocytes undergoing or completing vitellogenesis. Eggs are non-viable and infected females show reduced growth, fecundity and/or spawning activity. Spores measure 6.5 μm × 7.7 μm in size.

Myxobolus dahmeyensis (Siau, 1977) was reported in the ovaries of *Tilapia zilli* and *Sarotherodon melanotheron melanotheron* by Gbankoto *et al.* (2001). No parasite cysts were produced and spores were only present in mature oocytes, which were destroyed by the parasite, leading to castration of the host.

Henneguya amazonica Rocha, Matos and Azevedo, 1992, originally reported from the gills of *Crenicichla lepidota*, is found mainly within the ovarian stroma but also within the follicles of *Hoplosternum littorale*. Whilst Torres *et al.* (1994) reported that the morphology of infected oocytes was not significantly affected, they could not discard the possibility that ovaries may be destroyed by the parasite. Spore body measures 13.9 μm × 5.7 μm; total spore length, including caudal appendages, is 59.3 μm.

Central nervous system

Over 20 myxosporean species have been reported infecting the brain and spinal cord of teleosts (Hoffmannn *et al.*, 1991; Frasca *et al.*, 1999; Cho and Kim, 2003). In addition, unidentified myxozoan-like parasites have been described infecting the spinal cord of the toad (*Bufo bufo*) (Stensaas *et al.*, 1967) and more recently from the brain of the mole (*Talpa europea*) (Friedrich *et al.*, 2000). In these cases, only plasmodial stages containing secondary (occasionally tertiary cells in the mole parasite) were observed and no significant host response was recorded.

In general, myxosporeans infecting nervous tissue do not elicit a significant host response. Examples include *Myxobolus galaxii* Szidat, 1953 from the spinal cord of *Galaxias olidus*, a salmoniform freshwater fish from Australia (Langdon, 1990), *Myxobolus neurophilus* (Guilford, 1963) in yellow perch (*Perca flavescens*) (Dzulinksy *et al.*, 1994) and *Myxobolus inaequus* Kent and

Hoffmann, 1984 in South American knife fish (*Eigemannia virescens*) (Kent and Hoffmann, 1984). However, encephalitis associated with presporogonic and sporogonic stages occurs with some species (Dyková and Lom, 1988a; Frasca *et al.*, 1998). In others, a significant proportion of the nervous tissue can be replaced with parasitic cysts. Infections with *Myxobolus hendricksoni* Mitchell, Seymour and Gamble, 1985 in fathead minnows (*Pimephales promelas*) result in macroscopic cysts up to 1.5 mm in diameter throughout the brain, in particular the optic lobes and corpus cerebrelli (Mitchell *et al.*, 1985). Even with extensive infections, behavioural abnormalities have rarely been reported. Fish infected with *M. arcticus* have reduced mean swimming speed compared with uninfected fish (Moles and Heifetz, 1998), and infections with *Myxobolus encephalicus* (Mulsow, 1911) in carp fry can result in loss of equilibrium and circling motion. Plasmodia of *M. encephalicus* grow within the blood vessels of the brain and cause congestion of the vessels and focal oedema. Sporogonic stages invoke a severe granulomatous response (Dyková and Lom, 1988a). Neurological symptoms, including uncoordinated darting and rolling movements, are associated with infections of *Myxobolus balantiocheirli* Levsen, Alvik and Grotmol, 2004 in tricolour sharkminnow (*Balantiocheilos melanopterius*). In this case, parasite-induced inflammation is largely absent (Levsen *et al.*, 2004).

There have been several reports of skeletal deformities associated with myxosporidiosis of neural tissues. *Triangula percae* Langdon, 1987 was described from redfin perch (*P. fluviatilis*) from Victoria, Australia, where juveniles appear to be more susceptible to the infection and develop marked curvature (lordodis) of the spine. The parasites are located in the white matter of the mesencephalon, the diencephalon and the medulla oblongata (Langdon, 1987). In Scotland, *Myxobolus sandrae* Reuss, 1906 also causes lordosis and vertebral compression of the spine in the same host. Atrophy and demyelinization of the white matter of the spinal cord is the principal host response to the presence of sporogonic plasmodia (Lom *et al.*, 1991). Egusa (1985) described infections with *Myxobolus buri* Egusa, 1985 causing severe scoliosis in cultured *Seriola quinqueradiata* in Japan. Several regions of the brain were affected, including the olfactory and optic lobes, cerebellum and medulla oblongata. Parasite stages are surrounded by a fibrous capsule and pericystic inflammation. Spinal curvature in mullet (*M. cephalus*) from Japan is caused by *Myxobolus spinacurvatura* Maeno, Sorimachi, Ogawa and Egusa, 1990. Cysts approximately 1 mm in diameter occupy several regions in the brain, including the olfactory and optic lobes, and also infect mesenteric tissue and the spleen (Maeno *et al.*, 1990).

Bullheads (*Cottus gobio*) from the Czech Republic, southern England and Germany are hosts to *Myxobolus cotti* El-Matbouli, Fischer-Scherl and Hoffmannn, 1990, where plasmodia infect several regions of the brain and are also found within axons (Lom *et al.*, 1989b; El-Matbouli *et al.*, 1990; see Fig. 8.15). Intra-axonal myxosporean plasmodia have also been described from the spinal cord and brainstem of the common shiner (*Notropis cornutus*) (Ferguson *et al.*, 1985).

Salmonids are hosts to several brain-infecting myxosporeans. *M. neurobius* infects brown trout (*Salmo trutta*), Atlantic salmon (*S. salar*) and grayling (*Thymallus thymallus*) in Europe and Asia and Pacific salmonids in North America (Gonzales-Lanza and Alvarez-Pellitero, 1984; Maloney *et al.*, 1991). Plasmodia are up to 400 μm in diameter. Spores are ovoid, 10–14 μm × 8–9.2 μm in size. Although generally regarded as non-pathogenic, intense infections can induce pressure atrophy of surrounding tissues. A similar *Myxobolus* sp. causes 'sleeping disease' in cultured masu and amago salmon in western Japan (Murakami, 1979, cited by Urawa and Awakura, 1994). In Pacific salmonids *M. arcticus* can be found at prevalences approaching 100% (Kent *et al.*, 1993; Awakura *et al.*, 1995). Spores are pyriform, measuring approximately 15 μm × 7.5 μm. *M. arcticus* is currently the only myxosporean parasite with a tropism for neural tissue for which the life cycle has been elucidated. Transformation of

M. arcticus myxospores occurs in the oligochaete *Styrodrilus heringianus*, with triactinomyxon spores released from that host being infective to sockeye salmon. Mature myxospores develop in the neural tissue 3–4 months after exposure (Kent *et al.*, 1993).

Neurotropic presporogonic stages of *M. cerebralis*, identified using small subunit rDNA sequence data and *in situ* hybridization, have been linked with neurological disease and mass mortality of Atlantic salmon smolts at a marine culture facility in Ireland (Frasca *et al.*, 1998, 1999). Clinical signs are absent. Plasmodia are present between axons, most frequently in the optic tectum of the mesencephalon. Pathology ranged from mild to severe focal encephalitis, involving glial cells, macrophages and lymphocytes. It was hypothesized that infective stages originated from nearby riverine inputs to the farm location (Frasca *et al.*, 1999).

Multivalvulid myxosporeans of the genus *Kudoa* have been described in the brain of cultured and wild marine fish. *Kudoa paralichthys* Cho and Kim, 2003 infects olive flounder in Korea (Cho and Kim, 2003), whilst *Kudoa tetraspora* Narasimhamurti and Kalavati, 1979 and *Kudoa cerebralis* Paperna and Zwerna, 1974 infect mullet (*M. cephalus*) and striped bass (*Morone saxatilis*), respectively (Paperna and Zwerna, 1974 Narasimhamurti and Kalavati, 1979). In all of these infections, the host response is minimal. Whereas all neurotropic myxosporeans appear to sporulate in the central nervous system (CNS), a *Kudoa* sp. infecting *Thunnus maccoyii* from Western Australia usually sporulates in peripheral nerves (Langdon, 1990). *Kudoa* (= *Pentacapsula*) *neurophila* (Grossel, Dykova, Handlinger and Munday, 2003) causes severe granulomatous meningoencephalomyelitis amongst cultured juvenile striped trumpeter (*Latris lineata*) in Tasmania (Grossel *et al.*, 2003) and *Kudoa* (= *Septemcapsula*) *yasunagai* (Hsieh and Chen, 1984) forms large parasitic cysts in cultured *Lateolabrax japonicus* and *Oplegnathus fasciatus* in China and Japan (Hsieh and Chen, 1984).

Infections of the kidney and urinary tract

The kidney is the main target organ for a large number of myxosporean species. The various metabolic functions of the organ related to excretion, osmoregulation and haematopoiesis, as well as the presence of endocrine tissue, such as the corpus of Stannius, and inter-renal tissue, are reflected in its complex structure. Coelozoic species reside in all cavities, from Bowman's capsule of the glomerulus and the various compartments of the nephron to the renal collecting ducts and the urinary bladder. Histozoic species (e.g. *Myxobolus* spp.) are commonly found as small accumulations of loose or phagocytosed spores or contained in larger groups within macrophage aggregates, where they may have been transported from other organs for elimination from the host. In addition, they can occur as encysted plasmodia within the haematopoietic interstitial tissues (e.g. *Myxidium rhodei* Léger, 1905). Histozoic and intracellular proliferative extrasporogonic stages of some myxosporean and malacosporean species induce a significant pathological host response, resulting in diseases such as PKD of salmonids, caused by *T. bryosalmonae*, and sphaerosporosis in carp, caused by *S. renicola*. Renal infections of endocrine tissues have not been reported thus far. The principal histopathological features of renal myxosporidiosis range from acute to chronic inflammation to extensive tissue necrosis and fibrosis and are dependent on a variety of factors, including the immune status of the host, the pathogenicity of the parasite and the stage of development of the infection. Renal damage can be extensive, resulting in functional impairment. However, the host is often able to survive unless placed under additional environmental stress and/or secondary challenge from other pathogens.

Infections with the malacosporean *T. bryosalmonae* (formerly PKX) are responsible for serious economic loss in salmonid culture throughout Europe and North America, although the impacts on wild fish populations are less certain (Feist *et al.*, 2002; Wahli *et al.*, 2002). Infection of fish

hosts is initiated when infective spores released from bryozoans enter the fish through the skin and gill epithelium, either between epithelial cells or intracellularly via mucus cells (Morris *et al.*, 2000b; Longshaw *et al.*, 2002). The spore has characteristic soft shell valves, comprising eight cells surrounding four small subspherical polar capsules and two sporoplasm cells, which may each contain a single secondary cell. The sporoplasm cells contain characteristic sporoplasmosomes, identical to those seen in the extrasporogonic histozoic fish stage (Fig. 8.12).

Most reports of PKD involve rainbow or steelhead trout (*Oncorhynchus mykiss*),

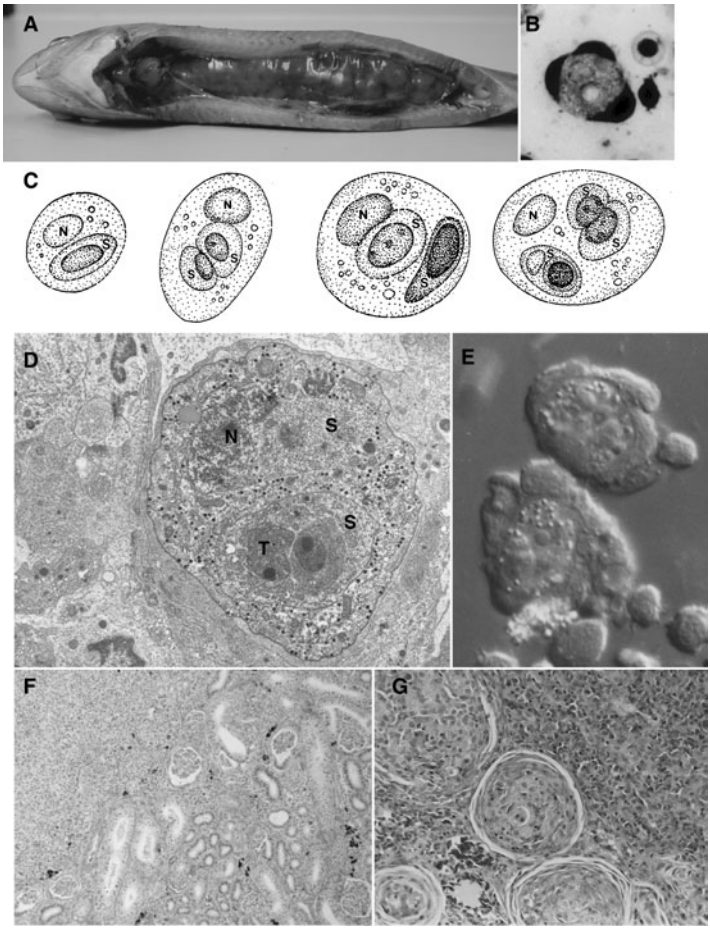


Fig. 8.12. Proliferative kidney disease, pathology and morphology of the causative agent *Tetracapsuloides bryosalmonae*. A. Rainbow trout (*Oncorhynchus mykiss*) fingerling exhibiting renal hypertrophy. B. Giemsa stained renal impression smear showing extrasporogonic stage of *T. bryosalmonae* surrounded by host phagocytes. C. Diagrammatic representations of stages in the development of histozoic *T. bryosalmonae* from rainbow trout kidney (primary cell nucleus, N; secondary cell, S; tertiary cell, T). D. Electron micrograph of a renal interstitial stage from rainbow trout kidney. Primary cell contains a prominent nucleus (N) with two secondary cells (S), one of which contains two tertiary cells (T). E. Fresh preparation of *T. bryosalmonae* from trout kidney with characteristic cytoplasmic granules and secondary cells within the primary cell, which is itself surrounded by phagocytic cells. F. Low-power view of a section of infected renal tissue showing loss of excretory elements and proliferation of interstitial haemopoietic tissue (upper left). G. Chronic granulomatous response in pike (*E. lucius*) spleen in response to *T. bryosalmonae* stages.

although outbreaks in brown trout (*S. trutta*) and Atlantic salmon (*S. salar*) in Europe and in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) in North America are common (Hedrick *et al.*, 1993). In Europe, Arctic charr (*Salvelinus alpinus*) and grayling (*T. thymallus*) are also known to be hosts for the parasite, and may exhibit signs of the disease (Feist and Bucke, 1993). Pike (*E. lucius*) is the only non-salmonid fish known to be susceptible (Seagrave *et al.*, 1981; Morris *et al.*, 2000a). Brook trout (*Salmo fontinalis*) can become infected but do not exhibit clinical signs of PKD (Feist and Bucke, 1993). Juvenile fish are most at risk from the disease but naïve fish of any age are susceptible. Frequently 100% of susceptible stocks become infected. Outbreaks of PKD are seasonal in nature, occurring in late spring, with clinical signs being seen typically between June and September when temperatures reach 15°C and above (Clifton-Hadley *et al.*, 1986). Parasites are usually seen within renal tissue about 4 weeks post-exposure, with lesions developing shortly thereafter. The histozoic stage consists of a primary cell containing one or more secondary cells and occasional tertiary cells (Seagrave *et al.*, 1980a,b). The cytoplasm of the primary cell contains numerous characteristic sporoplasmosomes, morphologically identical to those in the sporoplasm cell of the bryozoan stage (Feist, 1997; Kent *et al.*, 1998). Pericyte formation has been observed (Feist and Bucke, 1987). The main presenting features of the disease are abdominal distension, bilateral exophthalmia and pale gills. Internally, marked renal and splenic hypertrophy is typical and the kidney may have a mottled appearance.

The extrasporogonic stage of the parasite proliferates rapidly within the host, reaching almost every organ via the bloodstream and eliciting focal or multifocal inflammation within the tissues. In the skeletal musculature, severe granulomatous myositis can result (Fernández-de-Luco *et al.*, 1997). In the kidney and spleen, both the proliferation of the parasite and the host response are more vigorous. Interstitial haematopoietic cell hyperplasia, with degenerative changes in the excretory elements, and vascular pathology, including haemoglobin crystallization, occur (Clifton-Hadley *et al.*, 1987; Fig. 8.12). In chronic infections, granulomatous interstitial lesions, with phagocytic cells surrounding the parasites, are commonly seen (MacConnell *et al.*, 1989). Eventually the parasite is eliminated and the lesions resolve. Some parasites traverse the renal tubule epithelium, becoming coelozoic within the tubules, where sporogony is initiated. This generally occurs at a low level, with only a few, apparently immature, ovoid spores being produced. An exception is in Arctic charr, where large numbers of spores are formed without an extensive host reaction to the extrasporogonic stages (Kent *et al.*, 2000). The spores are formed within monosporous pseudoplasmodia and measure 12 µm in length and 7 µm in width. They contain two subspherical polar capsules and are surrounded by indistinct valvogenic cells. Hedrick *et al.* (2004) described more advanced stages in the urine of fish recovering from PKD. These were subspherical with a width of 16 µm and a height of 14 µm, with two spherical polar capsules. Transmission studies have so far failed to produce infections of bryozoans by these stages (Tops *et al.*, 2004).

Management strategies aimed at reducing exposure to the infective stage are the most effective means to control the disease. Reduced water temperature suppresses the effects of the disease (Clifton-Hadley *et al.*, 1986) and fish exposed late in the season, before ambient temperatures fall, survive the infection and appear less susceptible to the disease the following year. The infective stage is present throughout the year (Gay *et al.*, 2001; Tops and Okamura, 2003). Practical measures to reduce the numbers and distribution of the bryozoan hosts in and around aquaculture facilities have yet to be evaluated. Treatment regimes are covered in the section on 'Prevention and Control'.

Two species of *Parvicapsula* infecting salmonid renal tissue have been reported. *Parvicapsula minibicornis*, Kent, Whitaker and Dawe, 1997 is known from several Pacific salmonids from Canada

(Jones *et al.*, 2003, 2004). Affected fish exhibit slight renal swelling. Trophozoites reside within Bowman's space and capillaries of the glomeruli and occasionally within the renal tubule epithelium. Sporogonic stages occur within the lumen of renal tubules. Pyriform spores contain two equal-sized pyriform polar capsules and measure 11 μm in length and 7.5 μm in width (Kent *et al.*, 1997). The parasite has been associated with prespawn mortality among Fraser River stocks of sockeye salmon (*Oncorhynchus nerka*) in British Columbia (St-Hilaire *et al.*, 2002). Prevalence rates amongst Pacific salmon species range from 47 to 100% (Jones *et al.*, 2003). A second unidentified *Parvicapsula* species infects marine-cultured coho salmon in North America (Hoffmann, 1984). Other Pacific salmonids and Atlantic salmon are also susceptible. The disease has not been reported from Europe. Infection occurs 8 to 10 weeks post-introduction to an infected site, with up to 50% losses being reported (Johnstone, 1984). The kidney is the primary site of infection and appears hypertrophied and haemorrhagic. Renal tubules become occluded with trophozoites and developing spores (Fig. 8.13). Tubule epithelium may also harbour sporulating trophozoites. Histologically, the tubule epithelium becomes atrophied. The release of parasite stages to

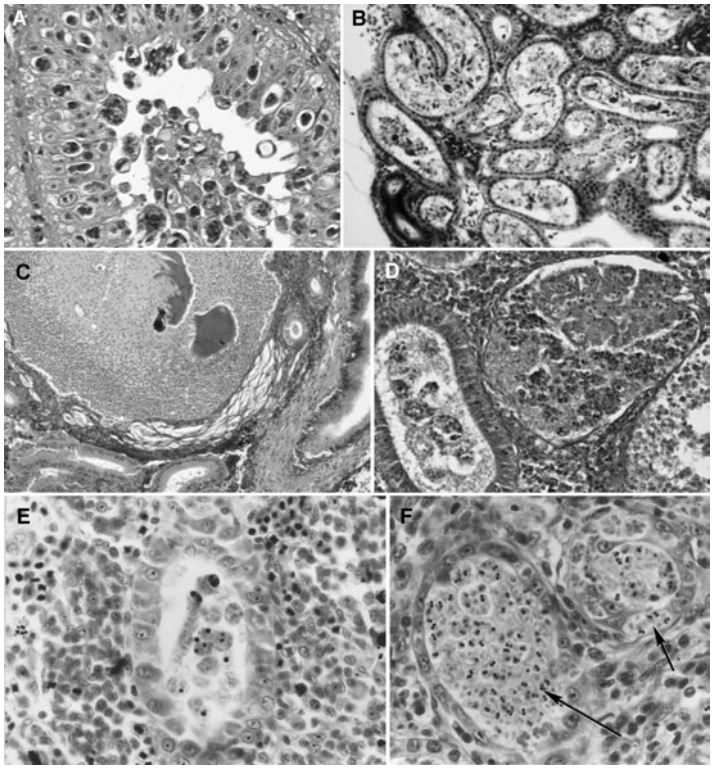


Fig. 8.13. Pathology of renal infections. A. Intracellular and coelozoic stages of *Hoferellus carassii* in the renal tubule of goldfish (*Carassius auratus*). B. Three-spined stickleback (*G. aculeatus*) kidney showing distension of renal tubules caused by numerous sporogonic stages of *Myxobilatus gasterostei*. C. Large xenoma of extrasporogonic stages of *Myxidium lieberkuehni*, which has replaced the glomerular tissue in the kidney of pike (*E. lucius*). D. Glomerular and tubule infections of *Myxobilatus platessae* in the kidney of European flounder (*Platichthys flesus*). E. Sporogonic stages of *Myxidium minteri* in the renal tubule lumen of chinook salmon (*Oncorhynchus tshawytscha*). F. *Parvicapsula* sp. sporogonic stages within the renal tubule epithelium and lumen of coho salmon (*O. kisutch*).

the renal interstitium following rupture of affected tubules elicits only a mild host response. Glomerular infections have not been reported with this species. Pseudobranch infections, similar to those occurring in cultured Atlantic salmon infected with *Parvicapsula pseudobranchiola* have also been reported (Yasutake and Elliott, 2003). Relationships between *Parvicapsula* sp., *P. pseudobranchiola* and *P. minibicornis* require further analysis.

Marked renal hypertrophy associated with *Parvicapsula renalis* Landsberg, 1993 infections have been reported in red drum (*Sciaenops ocellatus*) from the Atlantic Ocean off Florida (Landsberg, 1993). The parasite infects proximal tubules of the posterior kidney with both extrasporogonic and sporogonic stages in the lumen of the tubules. Epithelial changes include vacuolation and the presence of hyaline droplets, sometimes with the presence of rodlet cells. Interstitial renal tissue is not affected.

M. rhodei is a common parasite of the roach (*Rutilus rutilus*) and other cyprinids in Europe (Dyková *et al.*, 1987; Alvarez-Pellitero, 1989; Athanassapoulou and Sommerville, 1993a). Plasmodia are frequently found in Bowman's space within the glomerulus, resulting in atrophy of the glomerular tuft (Fig. 8.14). Development within the haemopoietic interstitial tissues results in an intense granulomatous inflammatory reaction, which is often effective in destroying the parasite. Mortalities have not been associated with *M. rhodei* infections (Dyková *et al.*, 1987). Other species of *Myxidium* have been associated with significant disease. *Myxidium minteri* Yasutake and Wood, 1957 is restricted to wild and hatchery stocks of salmonids of the Pacific north-west of the USA. The parasite is coelozoic in renal tubules (Fig. 8.13), with occasional spores present in interstitial tissues in heavy infections (Yasutake and Wood, 1957). Spores are fusiform, 9.3 to 12.6 μm in length and 6 to 7 μm in width, with equal-sized pyriform polar capsules. Coho salmon are most susceptible to infection (up to 44%), with prevalence rates in chinook salmon and rainbow trout generally less than 10% (Sanders and Fryer, 1970). However, in these cases, the parasite was located in the gall bladder, with rare

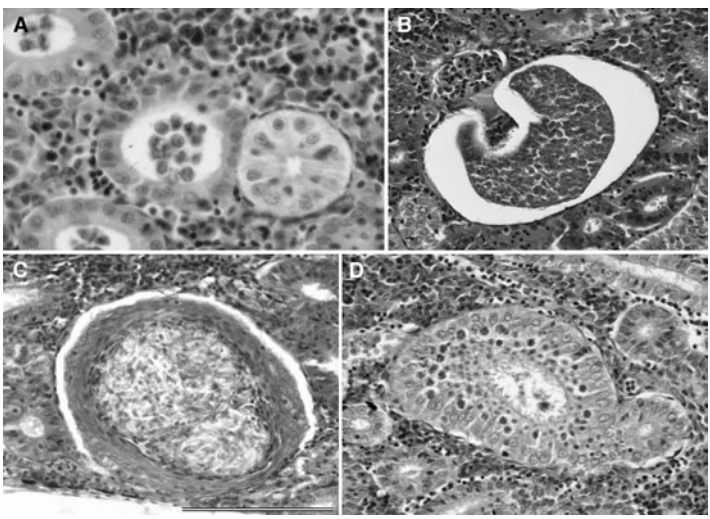


Fig. 8.14. Pathology of renal infections. A. *Sphaerospora* sp. in the kidney of dace (*Leuciscus leuciscus*) causing dilatation of renal tubule with reduction in tubule epithelial height. B. Atrophy of the glomerular tuft surrounded by a coelozoic plasmodium of *Myxidium rhodei* in the kidney of dace (*L. leuciscus*). C. Fibrous encapsulation of sporogonic plasmodia of *M. rhodei* in the renal interstitial tissue of *R. rutilus*. D. Intracellular extrasporogonic stage of an unidentified myxosporean in the renal tubule epithelium of dace (*L. leuciscus*).

cysts found in the liver. Myxidiosis caused by *M. giardi* in eels, *Anguilla anguilla*, affects many tissues, including the kidney, where significant damage to the excretory elements occurs. Granulomatous inflammation and mortality among elvers can result.

In pike (*E. lucius*), extrasporogonic stages of *Myxidium lieberkuehni* Buetschli, 1882 invade the endothelial cells of the glomerular tuft, resulting in grossly enlarged cells containing numerous parasites and a hugely hypertrophied nucleus, the whole structure constituting a parasitic xenoma. The cells contained within the xenoma frequently contain secondary and tertiary cells, which, on release from the xenoma, pass down the proximal tubule and develop into small plasmodia (Lom *et al.*, 1989a; Feist, 1997). Many infected glomeruli become infiltrated by host phagocytes and granulation tissue and are destroyed by the host. Large polysporic plasmodia are commonly found attached to the epithelium of the renal collecting ducts, and in the urinary bladder they impart an orange tinge due to the pigment granules contained within the plasmodia (Fig. 8.13). A second extrasporogonic stage is located intracellularly within the epithelial cells of the collecting ducts. Infected cells become hypertrophied and show degenerative changes without xenoma formation. It is presumed that these stages are also able to become sporogonic if they enter the lumen of the ducts. Affected epithelium becomes hyperplastic, forming papillomatous folds into the lumen of the duct, which itself becomes extremely dilated. Gross pathology in juvenile fish resulting from these changes is reminiscent of PKD, with renal hypertrophy and a greyish mottled appearance being typical features (Fig. 8.13). Mature spores of *M. lieberkuehni* are fusiform with longitudinal striations typical of the genus and measure approximately 20 µm in length and 6 µm in width.

Chloromyxum species also infect renal tissues, although most do not cause significant pathology. *Chloromyxum majori* Yasutake and Wood, 1957 in salmonids can result in the destruction of glomerular capillaries, and plasmodia of *Chloromyxum inexpectatum* Baska, 1990 in sterlet (*Acipenser ruthenus*) from the River Danube also infect glomeruli, displacing the glomerular tuft. Stages within the lumen of the tubules and urinary bladder have no discernible pathogenic effect (Baska, 1990).

Hoferellosis in goldfish (*C. auratus*) caused by *H. carassii* gives rise to the condition known as 'kidney enlargement disease' (KED) (Molnár *et al.*, 1989). The disease has been reported from Europe, Asia and North America. Clinical signs include abdominal distension, protrusion of scales and loss of balance. The disease is seasonal in nature, with fish becoming infected during the late summer and autumn, developing clinical signs the following spring, when mortalities may result (Yokoyama *et al.*, 1990a). Internally, marked renal hypertrophy is caused by a cystic papillomatous hyperplasia of the tubule epithelium (Fig. 8.13). Extrasporogonic stages of *H. carassii* invade tubule epithelial cells. The stages consist of primary cells up to 17 µm in diameter, with secondary, tertiary and even quaternary cells contained within them. Affected cells become elongate and protrude into the lumen of the tubule. Division of the epithelial cells, presumably in response to the parasite, offers further opportunities for intracellular development and attachment of coelozoic plasmodial stages. These are approximately 100 µm in diameter and contain spores measuring 13 µm in length and 8.4 µm in diameter. The oligochaete *Nais* cf. *elinguis* has been identified as the host for the actinospore stage (Aurantiactinomyxon) of *H. carassii*. However, transmission experiments have not been successful in inducing typical disease symptoms in susceptible fish (Trouillier *et al.*, 1996).

Cultured gilthead sea bream (*Sparus aurata*) from the Mediterranean and Atlantic coast of Spain are susceptible to glomerular disease caused by *Polysporoplasma sparis* Sitjà-Bobadilla and Alvarez-Pellitero, 1995. Only semi-intensive culture systems are affected and the infection is not seasonal. Prevalence rates increase with increasing fish size, reaching over 50% in fish greater than 100 g (Palenzuela *et al.*, 1999). Infected fish do not exhibit external clinical signs,

but internally glomeruli of the mesonephros harbour numerous sporogonic stages of the parasite within the capillaries, replacing blood cells and reducing the filtration capacity of affected glomeruli. Hypertrophy of the corpuscle and thickening of the capsule are typically seen and, eventually, necrosis and fibrosis result. Parasites can also infect the renal tubule epithelium and occupy the lumen. In heavy infections, atrophy of renal interstitial tissue, with inflammation and haemorrhage, is present (Palenzuela *et al.*, 1999). Spores are subspherical, 19.8 µm in length, 21.3 µm in thickness and 18.1 µm in width, with large capsulogenic cells. The characteristic feature of the spores, which differentiates the species from the genus *Sphaerospora*, is the presence of between 4 and 12 sporoplasm cells (Sitjà-Bobadilla and Alvarez-Pellitero, 1995). Gilthead sea bream and common dentex (*Dentex dentex*) are hosts to *Leptotheca sparidarum* Sitjà-Bobadilla and Alvarez-Pellitero, 2001. Large numbers of sporogonic stages within renal tubules cause blockage and pressure atrophy of the tubule epithelium (Sitjà-Bobadilla and Alvarez-Pellitero, 1995).

Several members of the genus *Sphaerospora* are pathogenic within the kidney of fish, although most frequently the changes produced are mild and induce only localized host reactions, especially where only the renal tubule lumens are infected (Fig. 8.14). Extrasporogonic stages appear to be typical for the genus and have been reported for many *Sphaerospora* species (Lom *et al.*, 1985; Hedrick *et al.*, 1988a, 1990; Feist *et al.*, 1991; Supamattaya *et al.*, 1993; McGeorge *et al.*, 1994; Jones *et al.*, 2004).

Sphaerosporosis in carp caused by *S. renicola* occurs amongst cultured common carp throughout Europe and in Israel. Prevalence may reach 100%. Sporogonic stages of the parasite are found in the lumen of renal tubules, comprising disporic pseudoplasmodia and subspherical spores measuring approximately 7.3 µm in diameter. These stages cause tubule dilatation and attenuation of the epithelium. In severe infections, the epithelium becomes atrophied and necrotic, with an associated inflammatory reaction in the interstitial tissues. Spores are infective to the oligochaete *B. sowerbyi*, with Neoactinomyxum actinospores being released after 98 days of development within the oligochaete host (Molnár *et al.*, 1999a). However, experimental proof that these stages are infective to naïve carp is lacking. *S. renicola* has two proliferative extrasporogonic cycles. Blood stages, comprising primary cells containing a number of secondary and tertiary cells, invade the swim bladder, where they continue to proliferate, increase in size and induce swim bladder inflammation (see above) (Dyková *et al.*, 1990). These swim bladder stages can be transmitted experimentally but have not been shown to give rise to the stages occurring within the renal tubule epithelium (Molnár and Kovács-Gayer, 1986). The marked cellular hypertrophy and stenosis of affected tubules are thought to be due to extrasporogonic stages of *H. cyprini* rather that *S. renicola*. Molnár (1988) reported that similar intracellular tubule epithelium stages occur in cyprinid fishes infected with *Myxobilatus legeri* (Cépede, 1905).

Mass mortality among cultured cobia (*Rachycentron canadum*) from Taiwan has been caused by infections with an unidentified *Sphaerospora*-like species (Chen *et al.*, 2001). Cumulative mortality may reach 90% within 30 days of introduction to marine cages. Affected fish exhibit pale livers, ascites, gill pallor and marked renal hypertrophy, with the surface having a knobbly appearance and pale or haemorrhagic patches. Parasitic stages infect glomeruli, renal tubules and the interstitial tissues. Tubules become occluded with parasites and cellular debris, with the epithelium showing hypertrophy and hyperplastic changes. Ruptured tubules invoke a vigorous host response to the parasites, with granuloma formation being typical. Mature spores have not been described.

Wild and cultured groupers (*Epinephelus malabaricus*) from Thailand infected with *Sphaerospora epinepheli* Supamattaya, Fischer-Scherl, Hoffmann and Boonyaratpalin, 1991 exhibit highly vacuolated tubule epithelial cells with pycnotic nuclei. Other changes include

dilation of glomerular capillaries, with degeneration of endothelial cells, thickening of the basement membrane and adhesion of the capillary membrane to the parietal layer of Bowman's capsule. Severely affected corpuscles become necrotic and surrounded by a fibrous layer (Supamattaya *et al.*, 1993). Interstitial haematopoietic tissues are unaffected.

Serious outbreaks of histozoic sphaerosporosis involving significant losses among cultured juvenile tench (*Tinca tinca*) from Germany have been reported. Prevalence is generally low but can reach 100%. The causative agent, *Sphaerospora tincae* Plehn, 1925, produces large numbers of disporic pseudoplasmodia, replacing tissues of the pronephros and resulting in hypertrophy of the organ. Inflammation is usually absent. Concurrent infections with the coelozoic *Sphaerospora galinae* Evlanov, 1981 exert no discernible pathological effect (Lom *et al.*, 1985).

Sphaerospora truttae Fischer-Scherl, El-Matbouli and Hoffmann, 1986 infects brown trout (*S. trutta*), grayling (*T. thymallus*) and Atlantic salmon (*S. salar*). Hatchery-reared fish become infected during June, when extrasporogonic stages may be found in the blood and renal interstitial tissues. Primary cells contain up to 100 secondary cells, which in turn may have tertiary cells within them. These stages are transmissible to naïve fish by intraperitoneal injection. Coelozoic disporic pseudoplasmodia develop within renal tubules from August onwards, with mature spores present from September. These may be retained within the kidney for up to 18 months when held in fresh water but are lost within 4 months following transfer to sea water (McGeorge *et al.*, 1996a,b).

Gall bladder and liver

A large number of coelozoic myxosporean species inhabit the gall bladder, especially in marine fish, and most infections are rather innocuous, without a significant host response. Infection proceeds via the hepatic vascular system into the biliary ductules and finally the gall bladder (Dyková and Lom, 1988b). Many myxosporeans cause changes to the colour and consistency of the bile, depending on the intensity of infection, and cellular necrosis of bladder epithelium is occasionally reported (Morrison *et al.*, 1996). Plasmodia of pathogenic species frequently become lodged in the biliary collecting ducts in the liver, where they can be associated with proliferation of bile ducts, inflammation of the surrounding tissues (pericholangitis) and occasionally hepatocellular necrosis (Walliker, 1968; Bucher *et al.*, 1992; Fig. 8.15). Attenuation of bile ductule epithelium in the liver is a common finding amongst gall-bladder-infecting species. It is likely that many myxosporeans inhabiting the biliary system are capable of inducing pathological changes similar to those described above. However, the majority of species have been described without histological examination of the bladder, bile duct or liver, which would reveal these.

Several *Zschokkella* species are known to be pathogenic. *Zschokkella russeli* Tripathi, 1948 in the five-bearded rockling (*Ciliata mustela*) is found at prevalences up to 89% at intertidal localities in Wales (Davies, 1985) and also occurs in many other fish species. Plasmodia are found folded within the bile ducts in the liver, floating freely in the bile and loosely attached to the gall bladder epithelium. Pathological changes, including cholangiofibrosis, pericholangitis and flattened epithelium with reduced microvilli, have been reported in the hepatic bile ducts but the parasite induces no pathology in the gall bladder itself.

Zschokkella icterica Diamant and Paperna, 1992 infecting rabbitfish (*Signanus luridus*) from the Red Sea produces large plasmodia up to 400 μm in diameter in the hepatic bile ducts, whilst smaller plasmodia reside within the gall bladder, where they are associated with desquamation of the epithelial lining (Diamant and Paperna, 1992). Congestion of the ducts leads to cholestasis, breakdown of the ducts and, in severe infections, hepatocellular necrosis, and cholangitis results, with clinical signs of ascites and jaundice in affected fish. Mullets of the genera *Mugil* and *Liza* from the

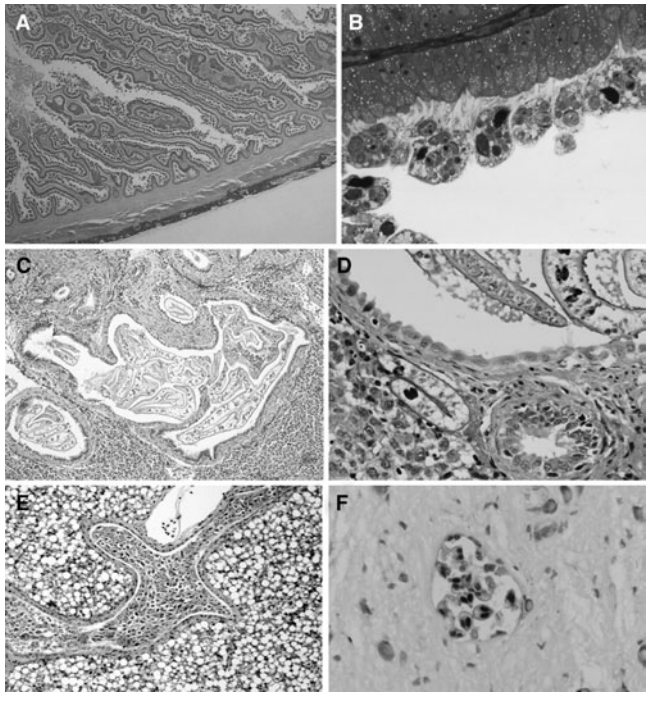


Fig. 8.15. Infections of the gall bladder, liver and neural tissues. A. Low-power view of papillomatous ingrowths of the gall-bladder epithelium of saithe (*Pollachius virens*) infected with *Myxidium gadi*. B. Semi-thin resin section from the previous specimen showing attachment of sporogonic *M. gadi* plasmodia. C. Hepatobiliary fibrosis associated with invasion of the bile ductules with elongate plasmodia of *Myxidium truttae* infecting brown trout (*Salmo trutta*). D. High-power view showing attenuation of gall-bladder epithelium and the presence of a small plasmodium of *M. truttae* within the hepatic parenchyma. E. Infection of bile ducts in *Callionymus lyra* with a *Myxidium* sp. F. Spores of *Myxobolus cotti* within the brain of bullhead (*Cottus gobio*).

Mediterranean are hosts to *Zschokkella mugilis* Sitjà-Bobadilla and Alvarez-Pellitero, 1993. Sitjà-Bobadilla and Alvarez-Pellitero (1993) recorded prevalences of up to 70%. Plasmodia measuring up to 50 µm in diameter induce degenerative changes in the bladder epithelium.

Chloromyxum truttae Léger, 1906 is a common parasite of brown trout (*S. trutta*) and other salmonids (Alvarez-Pellitero *et al*., 1982; Lom and Dyková, 1992). Infections in wild fish do not usually exhibit clinical signs of disease, but heavy infections in cultured stocks may result in emaciation and yellowish discoloration of the skin and fins. A discoloured liver, swollen gall bladder and enteritis are the main presenting features, and mortalities due to severe infections have been reported (Schulman, 1966). Up to 100% prevalence of infections in cultured sea trout (*S. trutta*) (anadromous) and 48% in Atlantic salmon (*S. salar*) from Finland have been recorded without the presence of histopathological changes or mortalities (Feist and Rintamäki, 1994). Sporogonic plasmodia of *Chloromyxum cristatum* Léger, 1906 infecting common carp (*C. carpio*) and grass carp (*Ctenopharyngodon idella*) induce hepatocellular necrosis (Lom and Dyková, 1981). Similar pathology occurs in carp infected with *Chloromyxum cyprini*, where plasmodia invade the liver parenchyma. Regeneration of necrotic liver tissue in such cases is limited and the prognosis for infected fish poor (Dyková and Lom, 1988b). Necrosis of the gall bladder epithelium resulting from infections of *Chloromyxum*

trijugum Kudo, 1919 has also been reported (Mitchell *et al.*, 1980).

Myxidium gadi Georgèvitch, 1916 is a well-known parasite of gadoid fish such as saithe (*Pollachius virens*) and pollack (*Pollachius pollachius*), occurring at high prevalences in fish from the North Sea. Infected gall bladders are atrophied and have a pale coloration. Depending on the intensity of infection, the bile may be slightly discoloured and viscous, leading to complete occlusion of the gall bladder and bile duct with parasitic stages. Plasmodia attach themselves to the bile duct epithelium, resulting in extensive papillomatous ingrowths of the epithelium and necrosis (Feist and Bucke, 1992; Fig. 8.15). Intracellular epithelial and intrahepatic stages have not been reported.

Ceratomyxosis caused by *Ceratomyxa sparusaurati* Sitjà-Bobadilla, Palenzuela and Alvarez-Pellitero, 1995 is a significant pathogen of cultured gilthead sea bream (*S. aurata*) in the Mediterranean (Sitjà-Bobadilla *et al.*, 1995). Prevalence of infection can reach 60% in infected stocks and low-level mortalities have been reported. Severe infections induce abdominal distension with inflammation and ascites. Cytological damage to the bladder epithelium includes hypertrophy and vacuolization. Sloughing of the epithelium and inflammation of the underlying tissues can occur. The parasite is regarded as a threat to sea bream culture (Palenzuela *et al.*, 1997).

Infections of the intestine

Members of several genera of myxosporeans infect intestinal tissues, although relatively few are exclusive pathogens of the intestine. Most infections are not regarded as pathogenic, typically forming discrete sporogonic cysts with minimal localized host reaction to the parasites. Examples of pathogenic species are given below.

Ceratomyxosis of hatchery-reared and wild salmonids from the Pacific north-west of North America is caused by *C. shasta* (Ratliff, 1983; Bartholomew *et al.*, 1989a; Hendrickson *et al.*, 1989). The parasite is responsible for losses among hatchery stocks, juvenile wild salmonids and pre-spawning adults. Differences in susceptibility between salmonid species and strains have been noted (Ibarra *et al.*, 1991, 1992), and fish surviving the infection are typically undersized and emaciated (Tipping, 1988). Susceptible fish acquire the infection from actinospores released from the freshwater polychaete *M. speciosa* (Bartholomew *et al.*, 1997). External clinical signs vary according to the salmonid species and include anorexia, lethargy, abdominal distension, darkened coloration and exophthalmia. Internally, there is widespread enteritis and haemorrhaging, with the formation of mucoid deposits and caseous material within the intestine and pyloric caecae, resulting from destruction of the intestinal mucosa. As the infection proceeds, proliferative stages spread via the bloodstream and infect the visceral tissues and skeletal muscle by causing widespread haemorrhaging and necrosis (Bartholomew *et al.*, 1989b).

The histozoic nature of *C. shasta* is unique amongst the usually benign coelozoic members of the genus inhabiting the urinary bladder and gall bladder of marine fish. The actinospore stage released from *M. speciosa* invades the epithelium of the posterior intestine, with small plasmodia initiating local lymphocytic infiltration by 18 days post-exposure at 12°C. Between 30 and 50 days post-exposure to the infective stage, all intestinal layers become invaded with the presporogonic stages of the parasite, necrosis is evident and mortalities ensue (Fig. 8.16). At 21°C the pathogenesis of the disease is more rapid. Sporogenesis is complete by day 18 and mortalities occur by day 20 (Bartholomew *et al.*, 1989b). Gross lesions may also affect the spleen and renal and muscle tissue. Myxospores are arcuate, with a diameter of 14–23 μm, containing two uninucleate sporoplasm cells with a subspherical polar capsule positioned each side of the sutural line.

A few members of the genus *Myxobolus* infect intestinal as well as other tissues. Most species are located in the tissues of the lamina propria underlying the mucosal epithelium, suggesting that plasmodial

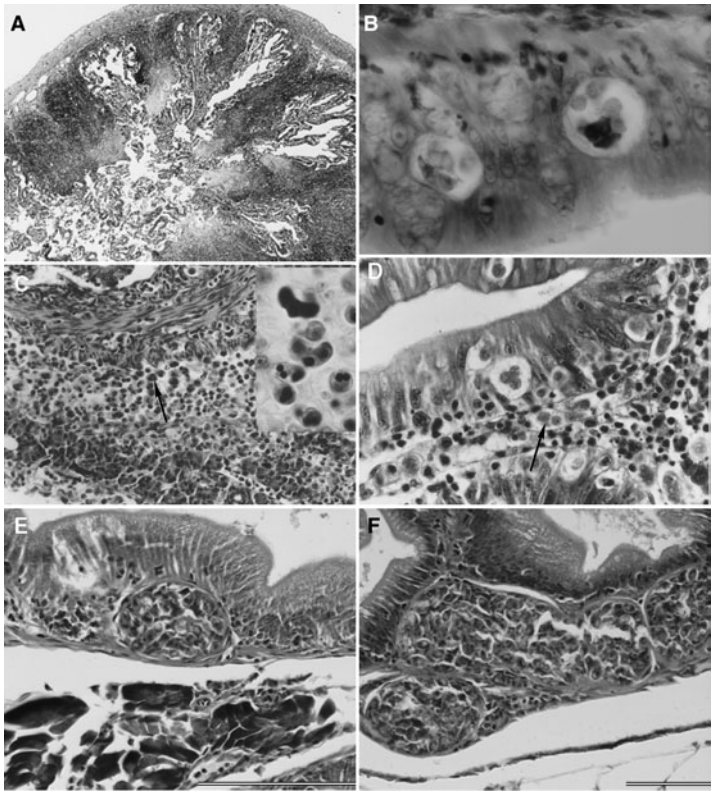


Fig. 8.16. Infections of the intestine and associated tissues. A. Destruction of the intestinal mucosa of gilthead seabream (*Sparus aurata*) caused by *Enteromyxum leei*. B. Intraepithelial stages of *E. leei* in the intestinal mucosa of *S. aurata*. C. Section of pyloric caecae with large numbers of plasmodia and spores of *Ceratomyxa shasta* (arrows) in the underlying connective tissue. Inset: developing spores of *C. shasta*. D. Intraepithelial stages of *C. shasta* in the mucosal epithelium and submucosal tissues (arrow). E and F. Cysts of *Myxobolus* sp. in the thin connective-tissue layer underlying the intestinal epithelium of minnow (*Phoxinus phoxinus*) and roach (*R. rutilus*), respectively.

development is initiated within capillaries (Molnár, 2002d). Rupture of the cysts is likely to result in the death of the host. Plasmodia vary greatly in size, depending on the species, from microscopic to over 5 mm in diameter. *Myxobolus nodulo-intestinalis* Masoumian, Baska and Molnár, 1996 produces large cysts in the intestinal wall of *Barbus sharpeyi* from south-west Iran, constricting the gut lumen. *Thelohanellus kitauei* (Egusa and Nakajima, 1981) causes serious disease in Israel carp (*C. carpio nudus*) in Korea. The parasite forms large cysts on the intestinal mucosa, surrounded by a thin connective tissue capsule. Development of the cysts is rapid during July, with maturation leading to cyst rupture and release of spores occurring in August and September (Rhee *et al.*, 1993). Infections of catfish (*Clarias gariepinus*) from the River Nile, Egypt, with *Henneguya supra-branchiae* Landsberg, 1987 give rise to large intestinal cysts measuring up to 4.5 mm in diameter (El-Mansy, 2002). Prevalence was 21.2%. Plasmodia invade the outer layer of the intestine and cause pressure atrophy to the smooth muscle layer, which becomes discontinuous around the intestine.

Although *Kudoa* species are well-known parasites of striated muscle (see above), some species invade other tissues, including the smooth muscle of the intestinal wall.

Kudoa dianae Dyková, Fajer Avila and Fiala, 2002, a parasite of the bullseye puffer, *Sphoeroides annulatus*, was found in 20% of adults collected off the coast of Mexico. Spores measure 4.5–5.5 µm in width and 5.5–6.5 µm in thickness. Cysts were also located in mesenteric tissues. Although there is no significant inflammatory reaction to intact cysts, spores from ruptured plasmodia are phagocytosed by macrophages and transported to the mucosal epithelium, where they can totally replace the epithelial tissue (Dyková *et al*., 2002). *Kudoa intestinalis* Maeno, Nagasawa and Sorimachi, 1993 infecting striped mullet, *M. cephalus*, from Japan also infects the smooth muscle of the intestine but is apparently non-pathogenic.

E. scophthalmi is also a highly pathogenic histozoic parasite which causes the disease enteromyxosis, characterized by severe enteritis and mortalites in cultured turbot (*Scophthalmus maximus*) (Branson *et al*., 1999; Palenzuela *et al*., 2002). Transmission is direct, via cohabitation with infected fish, exposure to water contaminated with spores and oral administration of infected intestinal tissue. Early developmental stages are intracellular within epithelial cells and can be detected in the anterior intestine as early as day 8 following oral intubation. Proliferative stages become abundant intraepithelially and within the lumen of the intestine (Redondo *et al*., 2002, 2003). Pathogenesis of the disease is dependent on temperature and mode of infection. Typical pathology includes vacuolation and sloughing of the epithelial cells and oedema of the subepithelial tissues. Mortalities occur within 3 weeks of exposure to infected material and reach 100% in most experimental trials. Spores (22.2 µm in length and 11.7 µm in width) are scarce in infected animals, are not formed until late in the infections and do not appear to be responsible for transmission to susceptible hosts (Redondo *et al*., 2002).

E. leei was first noted as a pathogen of cultured gilthead sea bream (*S. aurata*) by Diamant (1992) and subsequently in several other cultured marine species in the Mediterranean area (Diamant *et al*., 1994; Le Breton and Marques, 1995; Diamant, 1998). Direct fish-to-fish transmission occurs, presumably by ingestion of exfoliated intestinal tissues infected with spores or presporogonic stages of the parasite, or by coprophagy. Experimental infections in *S. aurata* resulted in prevalence levels of up to 33% and the onset of mortalities within 2 weeks of exposure by cohabitation or exposure to effluent from a tank containing infected fish (Diamant, 1997). Pathological changes are similar to those in enteromyxosis caused by *E. scophthalmi* (Fig. 8.16). Both species are serious threats to the mariculture of several fish species in the Mediterranean region.

Emaciation disease of tiger puffer (*Takifugu rubripes*) in the Kyushu district of Japan has been noted since 1996 (Tin Tun *et al*., 2000). Clinical signs are principally general emaciation of the body, including enophthalmia and the appearance of bony ridges on the head. The disease can result in mortalities of up to 60%. Three myxozoans have been detected in the intestine of infected fish (namely *E. fugu, E. leei* and *Leptotheca fugu* Tin Tun, Yokoyama, Ogawa and Wakabayashi, 2000) (Ogawa and Yokoyama, 2001). Of these, *E. leei* and *E. fugu* can be transmitted by feeding on infected tissues, cohabitation with infected fish and exposure to effluent from tanks containing infected fish (Yasuda *et al*., 2002). Histopathological changes include infiltration with inflammatory cells into the lamina propria and epithelium, with eventual detachment of the mucosal layer as infection with *E. leei* and *E. fugu* progresses. *Enteromyxum fugu* does not appear to be pathogenic (Tin Tun *et al*., 2002). The tissue tropism, morphological characteristics and pathogenicity of *E. fugu* are similar to those of *Enteromyxum* infections.

Infections of the swim bladder

There are several cyst-forming myxosporean parasites that infect the wall of the swim bladder. In most cases, there is little significant host reaction to the parasite since sporogonic stages are not exposed to the

host immune system. However, as is the case for such infections in other locations within the host, rupture of cysts results in a vigorous inflammatory response. In the case of swim bladder infections, the likely result is organ dysfunction, impaired swimming performance and possibly death. However, such an outcome has rarely been reported. In chub (*L. cephalus*) infected with *Myxobolus cycloides* Gurley, 1893, infiltration of trophozoites with fibroblasts results in the isolation of small clusters of spores by fibrous tissue and macrophages. Eventually there is complete encapsulation of the trophozoite and necrosis of the trapped spores. *M. cycloides* is not regarded as a serious pathogen (Holzer and Schachner, 2002).

The swim bladder is one site for the extrasporogonic proliferative stage of *S. renicola* in common carp fingerlings, which causes swim bladder inflammation (SBI). The stages comprise primary cells containing up to approximately 50 secondary cells, each containing one or two tertiary cells. Rupture of the primary cell releases the secondary cells, which may then grow, with internal division of the enclosed cells to repeat the cycle. The presence of large numbers of parasites in the swim bladder wall elicits a strong inflammatory response. Hyperplasia of the connective and epithelial tissues results in extreme thickening of the swim bladder wall, and haemorrhaging is frequently present. Affected fish display reduced swimming capability and poor growth. In the acute phase, peritonitis and renal hypertrophy can occur. Prognosis for fish with SBI is poor and mortalities may occur (Körting, 1982; Csaba *et al.*, 1984; Dyková and Lom, 1988a; Molnár, 1988).

In Vitro Culture and Propagation of the Parasite

There is a paucity of data on *in vitro* culture methods for myxozoans. For a number of freshwater myxozoans, transmission via the invertebrate hosts is possible, although reliable challenge methods have thus far only been achieved for *M. cerebralis* and *M. pseudodispar* (El-Matbouli *et al.*, 1999b; Székely *et al.*, 2001). Myxozoans may be transmitted to naïve hosts via exposure of fish to waters enzootic for the parasite in question and, for myxozoans with direct life cycles, cohabitation with infected fish is also possible (Diamant, 1997; Redondo *et al.*, 2002; Yasuda *et al.*, 2002). Intraperitoneal injection of extrasporogonic stages into naïve fish has been demonstrated for *T. bryosalmonae* (Kent and Hedrick, 1985), *C. shasta* (Ibarra *et al.*, 1991), *S. renicola* (Molnár and Kovács-Gayer, 1986), *S. truttae* (McGeorge *et al.*, 1996b) and *K. thyrsites* (Moran *et al.*, 1999a). Wolf and Markiw (1976) induced sporulation of *M. cerebralis* trophozoites in media and Siau (1977) was able to culture *Myxobolus exiguus* Thélohan, 1895 sporoplasms in trout tissue culture cells. Attempts by Redondo *et al.* (2003) to culture and maintain *E. scophthalmi* in a variety of media were unsuccessful.

Diagnosis of Infection

Diagnosis of myxozoans is still mainly reliant on morphological characteristics of mature spore stages, using established criteria (Lom and Arthur, 1989; Lom *et al.*, 1997). Detection of developmental stages from fresh material, smear preparations and histological sections is dependent on the experience of the investigator and the intensity of the infection. However, specific identification is generally not possible. Ultrastructural investigations using sectioned material are rarely able to identify species, but scanning electron microscopy provides valuable diagnostic data on the surface morphology of spore stages (Lom and Dyková, 1993). Fresh or fixed spores are best visualized using phase contrast or differential interference contrast microscopy, whilst smears or imprints can be stained with Giemsa, May–Grünwald–Giemsa or silver nitrate (Wolf and Markiw, 1979; Clifton-Hadley *et al.*, 1983; Baska and Molnár, 1988). Biotinylated lectins have been used to characterize the carbohydrate types within myxozoans and have some diagnostic value

(Castagnaro *et al.*, 1991; Marín de Mateo *et al.*, 1996; Muñoz *et al.*, 1999a, 2000), and the fluorescent dye 5(6)-carboxyfluorescein diacetate succinimidyl-ester (CFSE) has been used to determine routes of entry by actinospores into the fish host (Yokoyama and Urawa, 1997).

Monoclonal and polyclonal antibodies have been developed for a number of myxozoans that are important fish pathogens and used to detect both extrasporogonic and spore stages and to study parasite antigens. Antibodies have been raised against *T. bryosalmonae* (Adams *et al.*, 1992; Marín de Mateo *et al.*, 1996; Saulnier and de Kinkelin, 1996; Morris *et al.*, 1997, 2000a), *C. shasta* (Bartholomew *et al.*, 1989c), *K. thyrsites* (Chase *et al.*, 2001), *H. salminicola* (Clouthier *et al.*, 1997), *M. cerebralis* (Griffin and Davis, 1978; Markiw and Wolf, 1978; Hamilton and Canning, 1988), *Sphaerospora dicentrarchi* Sitja-Bobadilla and Alvarez-Pellitero, 1992 (Muñoz *et al.*, 1998, 1999b) and *M. rotundus* (Lu *et al.*, 2003).

The advent of molecular biology-based tools has led to the development of sensitive methods for the detection of myxozoan parasites of economic concern by targeting specific rDNA sequences, in particular, the use of PCR to link the alternate stages of myxozoan life cycles (Andree *et al.*, 1997; Bartholomew *et al.*, 1997; Lin *et al.*, 1999; Pote *et al.*, 2000; Feist *et al.*, 2001; Hanson *et al.*, 2001). Primers have been developed that target both the 18S and the ITS regions of the rDNA (Andree *et al.*, 1999a) and include generic myxozoan primers (Kent *et al.*, 2001), genus-specific (Andree *et al.*, 1999b) and species-specific primers (Hervio *et al.*, 1997; Saulnier and de Kinkelin, 1997; Andree *et al.*, 1998; Kent *et al.*, 1998; Morris, D.C. *et al.*, 2002). DNA sequence data from the PCR product provide confirmation on species identity and in addition are routinely used for phylogenetic analysis. In addition, restriction fragment length polymorphism (RFLP) on PCR products can be used to discriminate species and has been used to demonstrate phylogenetic links between species (Xiao and Desser, 2000c; Eszterbauer *et al.*, 2001; Eszterbauer, 2002).

One of the major constraints of the PCR technique is that, whilst it demonstrates the presence of parasite DNA, it does not provide information on the viability or pathological effect of the parasite. Therefore, a further refinement of the PCR method has been in the development of the *in situ* hybridization technique to demonstrate the presence of myxozoans in tissue sections (Antonio *et al.*, 1998; Frasca *et al.*, 1999; Morris *et al.*, 1999, 2000b; Longshaw *et al.*, 2002).

Prevention and Control

A number of chemical and biological methods currently exist to negate or reduce the impact of myxozoan infections in fish. Many of the treatments proposed were developed prior to the discovery of myxozoan life cycles and it may prove useful to examine the effectiveness of the various treatments on actinospore stages as these may well be more susceptible to treatment. However, conflicting data are provided for chemical treatments in the literature. The efficacy of drugs against myxozoans shows clear differences within both the myxozoan species and the genera tested. The reasons for these differences are rarely discussed but may be related to pharmacodynamics, metabolism and excretion altering the mode of action of the drug, as well as host susceptibility to the disease and parasite loading. The use of reliable, controlled, laboratory challenge experiments, coupled with fish of a known provenance, provides a real possibility of carrying out comparable drug trials to determine the mode of action and efficacy of chemical treatments against important pathogenic myxozoans.

Clifton-Hadley and Alderman (1987) and Alderman and Clifton-Hadley (1988) demonstrated that repeat treatment with malachite green of subclinical fish affected by PKD controlled the development of the disease and the presence of the extrasporogonic parasites in susceptible rainbow trout. Malachite green is ineffective against ceratomyxosis caused by *C. shasta* in rainbow trout (Ibarra *et al.*, 1990). It is

recognized that repeated doses of malachite green can lead to pathological alterations of livers and gills in exposed fish (Gerundo *et al.*, 1991). The use of malachite green in food fish is banned due to the accumulation of the chemical or its metabolites in fish tissues and its potentially carcinogenic properties.

The oral administration of fumagillin or its analogue TNP-470 has been shown to be efficacious in the treatment of *T. bryosalmonae* in chinook salmon and sockeye salmon (Hedrick *et al.*, 1988b; Higgins and Kent, 1998), *H. carassii* in *C. auratus* (Yokoyama *et al.*, 1990b), renal sporogonic stages of *S. renicola* in *C. carpio* (Molnár *et al.*, 1987), *Myxobolus* sp. in *O. masou* and *Oncorhynchus rhodurus* (Murakami, 1980), *T. wuhanensis* in *C. a. gibelio* (Wang *et al.*, 2001) and *M. giardi* in *A. anguilla* (Székely *et al.*, 1988). However, as with malachite green, toxic effects have been reported in fish exposed to higher doses of fumagillin, including reduced growth rates, direct mortality and reductions in the size of kidney and spleen (Wishkovsky *et al.*, 1990). These results were further confirmed by Le Gouvello *et al.* (1999), who showed that whilst a single treatment of fumagillin administered at 3 mg kg/body weight/day was sufficient to reduce the impact of PKD on rainbow trout farms, it led to a loss of appetite in treated fish. Whilst they did not find evidence of pathological changes associated with a toxic effect due to fumagillin, they did report the possibility that fumagillin, at least in the early post-medication phase, may in fact depress the immune system, leading to an increase in stress and bacterial infections. In contrast, Morris *et al.* (2003) demonstrated that in rainbow trout TNP-470 had a similar pathological mechanism to fumagillin. They suggested that, whilst a treatment regime of 1.0 mg TNP-470/kg fish/day for 6 days was successful at removing parasites from the host, there was conflicting evidence regarding the mortality effects of the drug and further work would be required to test the effects of the drug on different host species. Additionally, Staton *et al.* (2002) demonstrated that neither fumagillin nor TNP-470 significantly reduced the number of *M. cerebralis* spores, although they did report some spore deformation, in contrast to El-Matbouli and Hoffmann (1991c), who were able to prevent the clinical outbreak of whirling disease in *O. mykiss* fed fumagillin DCH. Molnár *et al.* (1987) showed that, while fumagillin DCH worked against renal sphaerosporosis, it was ineffective against early stages of *S. renicola* and against *M. cyprini* and *T. nikolskii* in the same hosts. Similarly, fumagillin is ineffective against *S. testicularis* and *C. shasta* (Ibarra *et al.*, 1990; Sitja-Bobadilla and Alvarez-Pellitero, 1992).

The anticoccidial drug toltrazuril affects developmental stages of *Myxobolus* sp. in the gills of *Abramis brama* (Schmahl *et al.*, 1989) but is ineffective against *H. carassii* and *S. renicola* (Yokoyama *et al.*, 1990b; Molnár, 1993). Taylor *et al.* (1973) showed that oral administration of furazolidone reduced the numbers of *M. cerebralis* spores in fish and Alderman (1986) suggested that a combined dose of proguanil HCl and clamoxyquin could also reduce *M. cerebralis* spore production and associated lesions.

As with many diseases, good husbandry practice in farm situations can help to alleviate the symptoms of disease and reduce the impact of co-infections with other diseases. To reduce the spread of any infections, the movement of potentially infected fish should be avoided. Avoidance of the actinospore stages, again in fish farms, can reduce the transmission of myxozoans to fish, either by rearing fish in borehole, filtered or ultraviolet (UV)- or ozone-treated water (Tipping, 1988; Nehring *et al.*, 2003). Rearing fish outside the critical period of transmission for infective stages may also be useful. De Kinkelin and Loriot (2001) exposed naïve fish to infective stages of *T. bryosalmonae* and held them at between 10 and 11°C for up to 4 months prior to an increase in temperature permissive for full development of PKD. An effective immune response was instigated that provided protection against PKD. Due to the inherent nature of fish farming, in many farms it is impossible to control the temperature of the

inflowing water and thus this approach has limited value. However, many farmers are able to manage the disease by a controlled exposure programme involving the exposure of salmonids to infective stages in the late autumn and overwintering the fish. By the following summer most fish are immune to PKD (Longshaw *et al.*, 2002). El-Matbouli *et al.* (1999a) showed that high temperatures were successful at stopping actinospore production in *T. tubifex* and killing all developmental stages in the host.

In both wild and farmed fish, the removal of dead and moribund fish from a system is critical to limit the release of spores from these animals. A possibility for reducing the availability of myxospores in wild situations may be either to remove clinically affected fish and fish from areas with high levels of infection or to remove the older age classes, which would release large numbers of myxospores to the environment following death. The draining, drying out and liming of fish ponds reduces the numbers of both myxospores and oligochaetes. Wagner (2002) reviewed the current state of knowledge regarding the prevention, control and management of whirling disease.

The alteration of the habitat may have an impact on myxozoan infections. Reducing the availability of suitable habitats for oligochaetes and/or bryozoans, such as by rearing fish in concrete raceways, reduces the numbers of alternate hosts available to transmit the parasite to fish, thus reducing the overall prevalence. There is evidence to suggest that this may be a possibility as it has been shown that *T. tubifex* maintained in mud and sand released more actinospores than counterparts held in leaf litter (Blazer *et al.*, 2003). Conversely, habitat degradation may increase the numbers and types of oligochaetes and reduce host defence mechanisms to allow an increase in severity and prevalence of certain Myxozoa. Stevens *et al.* (2001) found that the initial *M. cerebralis* myxospore dose affected the number of actinospores produced in *T. tubifex* and that the parasite reduced the biomass, abundance and individual weights of oligochaetes.

Two methods that may show promise as a control mechanism are interfering with the actinospore's ability to penetrate the host by manipulation of the triggers that the actinospore responds to, such as mucus and its constituents, and disrupting the biochemical pathways involved in the pathogenesis of the parasites (Longshaw *et al.*, 2003).

The susceptibility of different fish species to myxozoan infections is known, particularly for *C. shasta*, *M. cerebralis* and *T. bryosalmonae*, which may have some value in reducing the impact of these diseases at certain sites by selecting resistant species for culture, thus reducing the availability of hosts for the parasite and potentially breaking the life cycle. Rainbow trout (*O. mykiss*), steelhead trout (*O. mykiss*) and chinook salmon (*O. tshawytscha*) are more susceptible to *M. cerebralis* infections compared with coho salmon (*O. kisutch*) and brown trout (*S. trutta*) (Hedrick *et al.*, 1999, 2001b). Similarly, *O. mykiss* and *Oncorhynchus clarki* are susceptible to *C. shasta* infections, whereas *S. fontinalis*, *S. salar, S. trutta, O. nerka, O. tshawytscha, O. kisutch* and *Oncorhynchus keta* are relatively resistant to the disease (Bartholomew, 1998) and a similar differential susceptibility is known for *T. bryosalmonae* (Hedrick *et al.*, 1993). However, the factors that confer resistance for one disease are not necessarily the same for all myxozoan diseases, since it is known that *C. shasta*-resistant rainbow trout are susceptible to infections with *M. cerebralis* (Hedrick *et al.*, 2001a).

The host strain also has an impact on susceptibility, which has led some authors to suggest that there may be development of resistance through natural selection, which is passed on to the progeny. Evidence of a genetic basis to disease resistance is only now becoming available and has been demonstrated through selective breeding and species hybridization, coupled with molecular markers to identify the genetic loci involved (Ibarra *et al.*, 1994; Bosworth *et al.*, 2003; Nichols *et al.*, 2003). Manipulation and integration of the resistance 'genes' into strains of fish may provide a useful means of controlling these important diseases.

Fish can respond to myxozoan infections through a number of specific and non-specific mechanisms. Long-term acquired immune responses are known to occur in fish with a historical exposure to *T. bryosalmonae*, *E. scophthalmi* and *C. shasta* (Foott and Hedrick, 1987; Bartholomew, 1998; Sitjà-Bobadilla *et al.*, 2004). Detailed understanding of these responses will assist in the development of strategies towards negating the impact of diseases on the host. Studies investigating the molecular basis of the immune response via expression of immune-regulatory genes during the pathogenesis of myxozoan diseases will identify specific dynamic changes occurring during the disease process (Holland *et al.*, 2003). Ultimately, techniques to boost the immune system to combat important myxozoan infections and the development of vaccines against these diseases will provide a successful means of reducing the impact of these parasites. Another approach for the possible control of whirling disease by inhibiting parasite protease activity has been suggested (Kelley *et al.*, 2004).

Summary and Conclusions

During the past decade there have been several significant advances in the knowledge of myxozoan parasites and the diseases they cause. Many new species have been identified and the increasing use of rDNA sequencing has and will continue to play a pivotal role in the confident assignment of these, particularly since many 'species' exhibit spore pleomorphism, both within individual plasmodia and in those occurring in multiple sites within hosts. Although most species cause little harm, a few have become recognized as serious pathogens, especially in aquaculture situations. Detailed studies on host–parasite interactions have provided valuable information on the pathogenesis of these, and the cellular aspects of the host response to myxozoan infections is reasonably well understood. However, this is true only for a small percentage of known species. For most, the mechanisms of migration and replication within the host remain a mystery. Host immunology during myxozoan infections remains little studied, apart from recent investigations into diseases such as PKD and ceratomyxosis in salmonids. Increasing knowledge in this area will offer insights into the molecular aspects of host–parasite responses, which may offer potential for the development of DNA-based vaccines. However, it currently seems unlikely that these will be developed in the near future. The application of genomic, proteomic and metabolomic methodology offers powerful tools to characterize the changes occurring within the hosts during the disease process and may reveal molecular targets for diagnosis and treatment. The elucidation of the life cycle for several myxosporeans and the malacosporean *T. bryosalmonae* offers the most immediate opportunity for amelioration of the diseases caused by these organisms. With this knowledge, management strategies aimed at avoidance of the infectious stage or deliberate exposure aimed at eliciting an effective host response without the manifestation of clinical disease will continue to be refined according to local situations until proprietary treatments become available.

Significant advances regarding the phylogeny of the Myxozoa have been made in recent years, in particular, associated with the suppression of the class Actinosporea and the erection of the class Malacosporea following the recognition of bryozoans as hosts. The recognition of the enigmatic parasite *B. plumatellae* as a myxozoan and subsequent morphological and molecular studies have provided compelling evidence that myxozoans are highly specialized triploblast animals, possibly related to the Nematoda. There are likely to be several interesting developments in this area as new invertebrate hosts for similar parasites become recognized. Fundamental understanding of the evolutionary aspects of myxozoan biology will also be refined. Interest in the life cycles of marine species and their anticipated invertebrate hosts will surely provide many fascinating discoveries. Practical applications for infections

in mariculture species can be anticipated. In addition, the discovery of myxozoan parasites in mammals and birds indicates the possibility that many other hosts are yet to be identified.

Most investigations consider myxozoan pathogenicity at the individual level, with comment on the extent of mortalities in captive populations. However, with the notable exception of whirling disease, there have been few investigations on the effect of myxozoan parasites on wild populations. That the pathogenicity of myxozoan parasites and other pathogens is dependent on a wide range of factors, both internal and external to the host, is accepted. Usually, descriptions of the 'diseases' caused indicate the more severe manifestations and may comment on individual survivability in the short term. However, most myxozoans, even if they cause acute disease, do not kill their host unless secondary stressors and/or reduced immunocompetence has a significant effect. Consequently, myxozoan parasitism tends to be chronic, with the host able to survive for several years, depending on the species. There are very few data on the effects of such chronic parasitism in wild fish populations and only in the case of whirling disease have population declines been associated with the infection. An important area of research will be to understand the long-term impacts of these infections at the population level. Knowledge of the population biology of target species and of invertebrate hosts will be essential in this regard.

With the continuing increase in aquaculture and the introduction of new species, it is likely that myxozoan parasites will also continue to pose health challenges to the industry, both from previously undescribed species and from others able to exploit a new niche in high-density host populations. Parasites, such as *E. leei*, with direct transmission capabilities will pose the most serious threats. Epidemiological approaches and the application of risk analysis for assessment of potential disease spread have begun to be used for fish diseases, particularly those listed by the Office International des Epizooties (OIE). Similar approaches applied to the study of myxozoan infections in wild populations will be useful for understanding temporal and spatial changes in infection rates, especially where environmental data are also considered.

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9 Monogenea (Phylum Platyhelminthes)

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GENERAL

Introduction

Monogeneans are flatworms (Platyhelminthes) with representatives in freshwater, brackish and marine habitats. The vast majority of species are ectoparasitic and they all have a direct life cycle, i.e. without intermediate hosts. Although a number of species parasitize cephalopods, amphibians, reptiles and mammals, most of these platyhelminths are fish parasites with a relatively high host specificity. Thus, it is generally assumed that many fish hosts (agnathans, cartilaginous and bony fish) harbour at least one unique monogenean species and this presumption can be used to estimate the total number of species present. Since there are more than 25,000 known teleost species, it is tempting to suggest that the total number of monogenean species exceeds this number, although fewer than 3000 species have been described (Whittington, 1998). Furthermore, it was recently suggested (Bakke *et al.*, 2002) that there are about 20,000 species in the genus *Gyrodactylus* alone. The potential for expansion of our knowledge about this group of platyhelminths was further stressed by Lim (1998), who stated that only 8% of the South-east Asian monogenean species are known.

The majority of the monogeneans are on external surfaces of fish (skin, fins, gills, mouth cavity, nostrils) but a few species have adopted an endoparasitic life. Thus, the bladder and urinary ducts (*Acolpenteron ureteroecetes* in labrids), the foregut and stomach (e.g. *Enterogyrus* sp. in *Pomacanthus paru* (Cone *et al.*, 1987)) and even the cloaca (*Calicotyle kroyeri* in skates and rays) may serve as a microhabitat for a few species. In addition, the gill-parasitic amphibdellids have juvenile stages in the blood of electric rays (Llewellyn, 1960). However, even relatively homogeneous organs such as the gills, the fins or the skin comprise subcompartments which serve as microhabitats. Monogeneans tend to select these rather specific microhabitats on their fish hosts (e.g. Rohde, 1977; Euzet and Combes, 1998).

The main character of the group is the opisthaptor (main adhesive apparatus in the hind part of the worm) (Figs 9.1, 9.2 and 9.3). This organ of attachment is normally equipped with sclerotinized structures (hooks, clamps and suckers). Also the fore part of the worm has attaching capacities (adhesive pads, cephalic openings) and is referred to as the prohaptor.

Monogeneans are an evolutionarily old animal group that is believed to have evolved from ancestral ecto-commensal turbellarians several hundred millions of years

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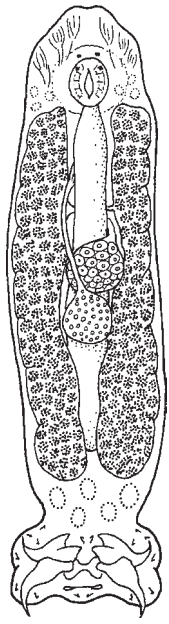


Fig. 9.1. Oviparous monopisthocotylean monogenean (*Tetraonchus* sp.). Drawing by Beth Beyerholm.

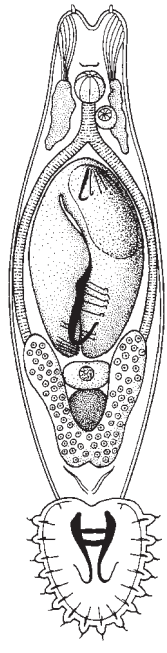


Fig. 9.2. Viviparous gyrodactylid monogenean (*Gyrodactylus* sp.). Drawing by Beth Beyerholm.

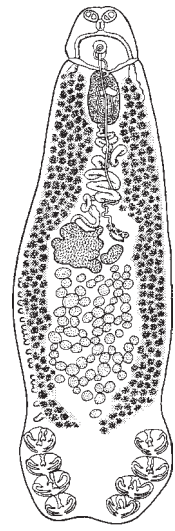


Fig. 9.3. Oviparous polyopisthocotylean (*Discocotyle sagittata*). Drawing by Beth Beyerholm.

ago (Llewellyn, 1982; Kearn, 1994). These platyhelminths have demonstrated an impressive adaptability to changing environmental and host conditions. Thus, the high number of species is probably not only the result of co-evolution between host and parasite but may also result from host switching (Bakke *et al.*, 2002; Zietara and Lumme, 2002). This is especially true of the gyrodactylids, a group of parasites that spread directly from fish to fish or via a transient residence on the substrate before infecting a range of hosts that reside in a specific habitat. Thus, a wide range of fish species may be exposed to infection and some of these exhibit some degree of susceptibility, which provides the basis for a new parasite–host system.

Two quite different dominant lineages have been suggested for the monogeneans, and they are the monopisthocotyleans and the polyopisthocotyleans. An alternative nomenclature based on an analysis of anatomical and ultrastructural features defines the subclass Polyonchoinea (equivalent to Monopisthocotylea, with 18 families) and a clade corresponding to Polyopisthocotylea comprising the subclasses Polystomatoinea (two families) and Oligonchoinea (30 families) (Boeger and Kritsky, 1997). These two

main groups differ markedly in morphology, anatomy, nutrition, physiology and reproduction. Molecular and ultrastructural studies have even suggested that Monogenea comprising these two groups is not a monophyletic group (Justine, 1998). The differences in anatomy, physiology and particular feeding activity between these two groups have important implications for their pathogenicity. Polyopisthocotyleans are blood feeders, whereas monopisthocotyleans are epithelial feeders browsing the host surface and ingesting epithelial cells, mucus and only occasionally a limited amount of blood leaking from haemorrhages. This is the basis for a clear difference in gut morphology, nutrition and physiology between the two groups (Smyth and Halton, 1983). Likewise, the direct ingestion of host blood (with its high content of immune factors) in polyopisthocotyleans probably demands a quite special immune evasion strategy compared with monopisthocotyleans ingesting mucus, epithelial cells and occasionally some blood cells leaking from haemorrhages.

Epizootics in Wild Fish Stocks

Despite the fact that many host–monogenean systems appear well adapted, several reports have described high pathogenicity of monogeneans to certain host species, not only in aquaculture systems but also in natural lakes, rivers and seas. Thus, dramatic decreases of wild fish stocks due to heavy and uncontrolled infections by monogeneans are known from the Aral Sea, where the local population of the spiny sturgeon (*Acipenser nudiventris*) experienced mass mortality and became severely decimated after introduction of the capsalid monogenean *Nitzschia sturionis* into this marine habitat. This parasite, occupying the gills and buccal cavity of the host, has a body length of up to 20 mm and was probably introduced into the Aral Sea with infected stellate sturgeon (*Acipenser stellatus*) from the Caspian Sea in the 1930s. Ninety spawners of the stellate sturgeon, which had not been examined for infections, were introduced into the Aral Sea in 1934, with 350,000 and 7,000,000 larvae in 1933 and 1934, respectively. Following its introduction in the 1930s, the parasite propagated in the highly susceptible host, leading to heavy parasite burdens and high mortality. The intensities of infection reached 600 worms per fish and the prevalence was 100% in most areas. The devastating effect of the worm on the gills impaired gas exchange and thereby survival, which left the local fish stock in a very bad state (Lutta, 1941; Petrushevski and Shulman, 1961).

Similarly, wild populations of Atlantic salmon (*Salmo salar*) in more than 44 Norwegian rivers have been infected by *Gyrodactylus salaris* since 1970 and this has resulted in heavy losses of wild salmon fry in affected rivers (Johnsen and Jensen, 1986; Mo, 1994). The parasite originated from an isolated stock of Baltic salmon in Sweden and was probably imported on several occasions into Norwegian rivers and fish production systems in the 1970s (Hansen *et al.*, 2003). The Baltic salmon seems to be relatively resistant compared with the Atlantic salmon in Norway and Scotland (Bakke *et al.*, 1990; Bakke and MacKenzie, 1993; Dalgaard *et al.*, 2003). The resistance is probably because the Baltic salmon had coexisted with the parasite for a few thousand years. In contrast, the Norwegian salmon had no history of coexistence with the parasite before 1970, whereby no selection of resistance had occurred. It appears that the introduction of certain species of monogeneans to previously unexposed host species may lead to uncontrolled and epidemic outbreaks of monogenean infections. A number of other epidemics with fatal outbreaks of monogenean infections have been reported. Other reports of mass mortality include the death of 87,000 Japanese anchovy (*Engraulis japonica*) infected with *Pseudanthocotyloides* sp. in the semi-enclosed Sea of Iyo, Japan (Yamamoto *et al.*, 1984). These mazocraeid monogeneans are sanguinivorous gill parasites and intensities of up to 60 worms per fish were found. Considering that the hosts had body lengths of 5.8–6.8 cm, it was suggested that anaemia was the cause of the mortality. Correspondingly, in Japanese

marine waters, the monogenean *Neoheterobothrium hirame* has been found to be associated with a decline of Japanese flounders, *Paralichthys olivaceus* (Ogawa, 2002). Recent studies indicated that the pathogenicity of *N. hirame* is increased by concomitant infections with marine strains of the viral haemorrhagic septicaema (VHS) virus (Shirakashi *et al.*, 2003).

Epizootics in Cultured Fish

In many aquaculture systems (both freshwater and marine) where fish are at a high stocking density and in confined environments, monogeneans are often important pathogens because they propagate rapidly and are transferred readily between fish (Thoney and Hargis, 1991). In fish culture systems where monogenean infections are allowed to develop due to appropriate abiotic and biotic conditions, uncontrolled high morbidity and mortality occur. If left untreated, this will lead to serious economic loss for fish farmers. The mass infections may be based partly on the innate susceptibility of the fish, which can be influenced by stressful environmental conditions. Thus, cortisol released in response to stress or artificially administered glucocorticoids decrease the resistance of fish to monogenean infections (Harris *et al.*, 2000).

Freshwater facilities

Common carp, *Cyprinus carpio*, suffers from infections with several species of *Dactylogyrus* and *Gyrodactylus* (Wunder, 1929; Wilde, 1935; Bauer *et al.*, 1973). Recently, mass mortality of koi carp occurred in connection with heavy infections by mainly *Dactylogyrus formosus* and five other congeneric species (Kritsky and Heckmann, 2002). Similar problems exist in production facilities for other cyprinids, such as grass carp (*Ctenopharyngodon idella*), bighead carp (*Aristichthys nobilis*), tench (*Tinca tinca*) and silver carp (*Hypophthalmichthys molitrix*) (Bauer *et al.*, 1973). Production of eels, such as the European eel, *Anguilla anguilla*, and the Japanese eel, *Anguilla japonica*, has experienced severe problems caused by the gill parasites *Pseudodactylogyrus bini*, *Pseudodactylogyrus anguillae* and *Gyrodactylus anguillae* (Ogawa and Egusa, 1976, 1980; Buchmann, 1997). Rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, cultivated in Europe suffer from skin and fin infections with *Gyrodactylus derjavini* (Buchmann and Uldal, 1997). Also gill infections with the blood-feeding polyopisthocotylean *Discocotyle sagittata* are a health problem, eliciting serious anaemia in trout (Rubio-Godoy *et al.*, 2003b). Likewise, salmonids kept in freshwater rearing facilities in North America have infections with *Gyrodactylus salmonis* and *Gyrodactylus colemanensis* (Cone and Cusack, 1988). Atlantic salmon, *S. salar*, can harbour lethal infections with *G. salaris*; these are primarily on the fins and skin and only occasionally on the gills (Mo, 1994). In Japan, other salmonids such as Masou salmon, *Oncorhynchus masou*, are hosts for *Tetraonchus awakurai* and *Tetraonchus oncorhynchi*, which can cause severe mortality (Ogawa and Egusa, 1985). Various catfish species are hosts for monogeneans. The European catfish *Silurus glanis* can have high populations of *Ancylodiscoides vistulensis* (Molnar, 1968; Szekely and Molnar, 1990). Severe morbidity due to *Gyrodactylus groschafti* infections on African catfish *Clarias gariepinus* was reported by Obiekezie and Taege (1991). The guppy *Poecilia reticulata*, which is an important ornamental fish, is host for the congeners *Gyrodactylus bullatarudis* and *Gyrodactylus turnbulli* (Scott, 1985).

As a specialized variant of aquaculture enterprises, public freshwater aquaria and exhibition facilities may have serious problems due to monogenean infections on their fish. Thus, the pirarucu, *Arapaima gigas*, can experience severe morbidity and heavy mortality due to *Dawestrema cycloancistrium* (Buchmann *et al.*, 1994). This is not confined to monogeneans parasitizing the external surfaces of the host but also those colonizing the internal organs. For example, *Acolpenteron ureteroecetes* in the ureters of largemouth bass, *Micropterus salmoides*, may reach intensities in

hatching facilities which elicit mortalities (Petrie-Hanson, 2001).

Mariculture

Mariculture enterprises may also be hampered by severe monogenean infections. Sea bass, *Dicentrarchus labrax*, production has experienced severe problems with the gill parasites *Diplectanum aequans* and *Diplectanum laubieri* (Oliver, 1977; Gonzales-Lanza *et al.*, 1991). Related diplectanid monogeneans have produced problems in grouper culture in South-east Asia (Leong, 1997). Likewise representatives of the related genus *Pseudorhabdosynochus* are expected to pose problems in Brazilian culture of *Epinephelus niveatus* (Santos *et al.*, 2000). Gilthead sea bream, *Sparus aurata*, cultured in the Mediterranean, is host for problematic monogeneans, such as *Furnestia echeneis* (Paperna *et al.*, 1977) and *Microcotyle chrysophrii* (Oliver, 1984; Faisal and Imam, 1990). Monogenean problems in Japanese mariculture have been intensely studied for many years. Thus, Japanese yellowtail, *Seriola quinqueradiata*, cultures have suffered from heavy infections by *Benedenia seriolae* and *Heteraxine heterocerca* (Egusa, 1983). Red Sea bream, *Pagrus major*, cultured in Japanese waters, suffer from *Anoplodiscus tai* infections (Ogawa, 1994) and Japanese flounder, *P. olivaceus*, both wild and in culture, can be severely affected by *N. hirame* (Hayward *et al.*, 2001; Ogawa, 2002). Similarly, tiger puffer, *Takifugu rubripes*, suffers from infections with *Heterobothrium okamotoi* (Ogawa, 2002).

Apart from food-fish production, other aquaculture production types face similar monogenean problems. Marine exhibition aquaria are excellent habitats for monogenean propagation. The capsalid *Neobenedenia melleni* was reported to have caused morbidity and mortality of a range of marine teleosts in the New York aquarium well before 1930 (Jahn and Kuhn, 1932; Nigrelli and Breder, 1934). This parasite showed an unusually broad host spectrum and infected several species of fishes. Also sharks and rays may be severely affected by various microbothriids, such as *Dermophthirius* (Cheung *et al.*, 1982; Rand *et al.*, 1986), and monocotylids, such as *Dendromonocotyle* (Chisholm and Whittington, 2003). Mariculture of tilapia experienced problematic infections with *N. melleni* (Kaneko *et al.*, 1988) and the emerging fin fish culture in Australian marine waters has also suffered devastating infections with this parasite (Deveney *et al.*, 2001). Deveney *et al.* (2001) described an outbreak of *N. melleni* in cultured barramundi (*Lates calcarifer*), resulting in the death of 200,000 fish. Yellowtail cultured in this region also suffers from infections with *B. seriolae* and *Zeuxapta seriolae* (Ernst *et al.*, 2003). Related parasites, such as *Benedenia monticelli*, are considered pathogenic in mullets (Paperna *et al.*, 1984) and, as mentioned above, *B. seriolae* affects yellowtail culture. Common sole, *Solea solea*, which is normally only lightly infected under natural conditions, can have dense populations of *Entobdella soleae* in captivity (Anderson and Conroy, 1968; Kearn, 2002). Generally it seems that many monogenean parasites can propagate in an uncontrolled way if suitable conditions (e.g. temperature, salinity, pH, confined environments) for reproduction are available (Thoney and Hargis, 1991).

Morphology of Monogeneans

Monogeneans comprise two very distinct groups, the Monopisthocotylea (Figs 9.1 and 9.2) and Polyopisthocotylea (Fig. 9.3), which differ considerably, with important implications for pathogenicity, treatment and host response.

General adult monogenean parasite

Opisthaptor

The most important adhesion apparatus is the opisthaptor, which is located posteriorly (Fig. 9.4). It is often equipped with characteristic sclerotinized structures used for attachment to the host. The attachment sclerites can be formed as large hooks or anchors (hamuli) (Fig. 9.5). Some may be able to penetrate

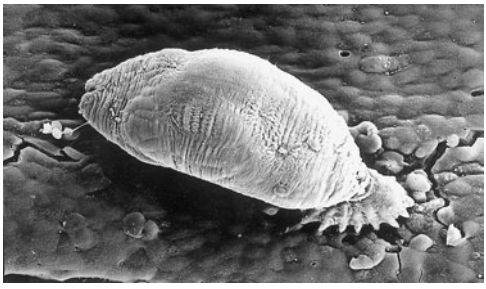


Fig. 9.4. *Gyrodactylus derjavini,* dorsal view showing opisthaptor firmly attached to fin of rainbow trout.

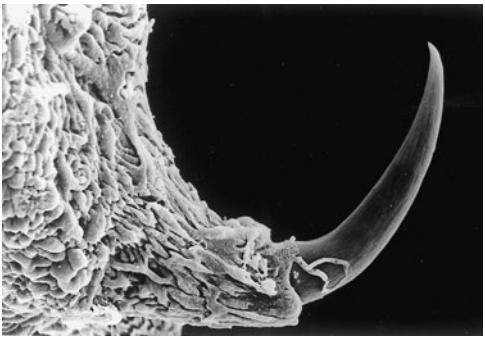


Fig. 9.6. *Pseudodactylogyrus anguillae,* distal part of one hamulus able to penetrate gill tissue.

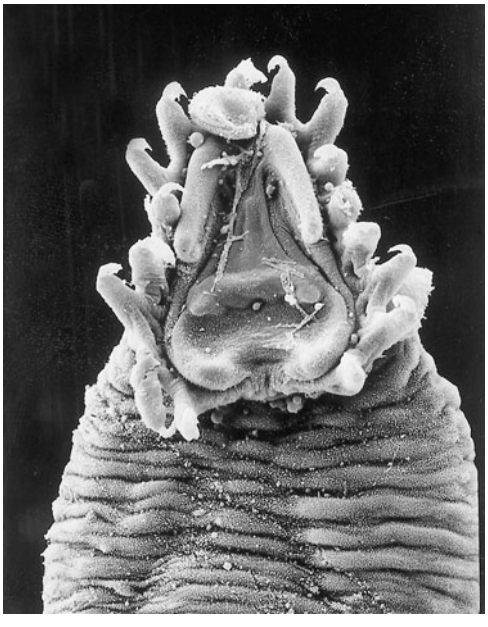


Fig. 9.5. *Gyrodactylus derjavini,* ventral view of opisthaptor showing two large hamuli in sheaths and 16 marginal hooklets.



Fig. 9.7. *Gyrodactylus derjavini,* marginal hooklets on opisthaptor able to penetrate epithelial cells of fins.

host tissue (Fig. 9.6), whereas others may serve a non-penetrating spring function, securing tension of attachment sites. These are often connected by transverse bars and plates. In addition, smaller marginal hooklets (Fig. 9.7) assist the attachment. They are able to penetrate epithelial cells and generate a firm contact with the host. Polyopisthocotyleans can possess sclerotized clamps, often in large numbers, which are extremely important for pinching the secondary lamellae of the host gill (Fig. 9.8 and 9.9). Several other specialized structures, such as suckers, septae or squamodiscs, may be found in association with the opisthaptor.

Tegument

The outer lining of the monogenean is a syncytial tegument composed of an outer anucleated layer and a basal nucleated layer in the parenchyma (Lyons, 1970; El-Naggar and Kearn, 1983). The outer tegumental plasma membrane is covered by a glycocalyx showing positive reactions for various carbohydrates. Although the tegument of many species is relatively smooth, it can be equipped with microvilli, especially in the adhesive regions of the parasite, such as cephalic openings in *Gyrodactylus* (Buchmann and Bresciani, 1999). Spines also occur. The tegument seems to be quite active and may serve osmoregulatory and excretory functions. It is well equipped with

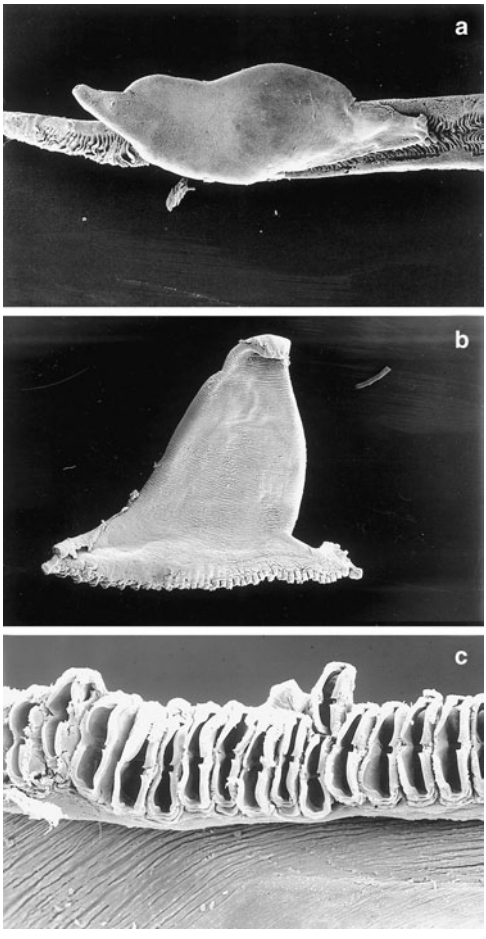


Fig. 9.8. The polyopisthocotylean gill monogenean *Axine bellone* (length 2.5 mm), a. attached to gill lamellae of garfish, b. ventral view showing numerous clamps on opisthaptor, c. clamps on opisthaptor (width of clamp 20 μm). Parasites kindly provided by Eric Buchmann.

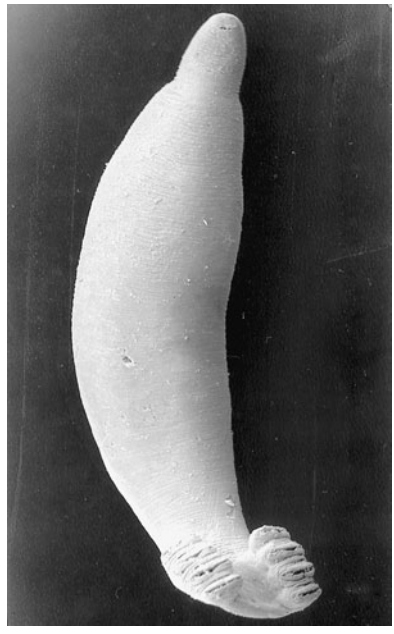


Fig. 9.9. The opisthocotylean *Discocotyle sagittata,* adult worm (length 4 mm) with clamps on opisthaptor. Parasite kindly provided by Miguel Rubio-Godoy.

mitochondria and secretory bodies. It has been suggested that some trans-tegumental uptake of nutrients may occur (Halton, 1978).

Anterior adhesive pads

The fore part of monogeneans plays a crucial role in attachment, movement, feeding and reproduction (Whittington *et al.*, 2000). It has been suggested that at least three types of gland cells secrete products involved in attachment of the parasite to the substrate (host or external items) (Kritsky, 1978; El-Naggar and Kearn, 1980; Kearn and Evans-Gowing, 1998). Sticky secretions may allow the worm to attach to the substrate (fish or other materials) and additional secretions released can subsequently eliminate the adhesive and allow the worm to detach. Preliminary studies suggested that the material is proteinaceous in nature (Whittington *et al.*, 2000). Although only a limited number of studies have been conducted on gland cells in the cephalic region, it is generally assumed that these play a crucial role in attachment and communication with the host (El-Naggar and Kearn, 1980; Whittington *et al.*, 2000).

Digestive gland cells

Different gland cells serving various purposes, such as adhesion or digestion, are found in monogeneans (Smyth and Halton, 1983). Enzymes produced in association with the gut of gyrodactylids include alkaline and acid phosphatases, esterases and proteases (Buchmann, 1998b). The

purposes of these enzymes have been only partly explored but may have importance for pathogenicity of the parasite when enzymes are released into host tissue, initiating extracorporeal digestion. Likewise, enzymes released from the worms may act as important antigens in the host immune response.

Sensory system

Various types of sense organs exist in monogeneans (Lyons, 1969; Watson and Rohde, 1994). From *in vitro* studies with these platyhelminths, it is evident that they respond actively to both mechanical and chemical stimuli. Chemotaxis in host finding has been described (Kearn, 1967). Likewise, reactions to light (photopositive or photonegative behaviour) have been studied in larval monogeneans (e.g. Bychowsky, 1957). It is therefore believed that chemoreceptors, tangoreceptors, rheoreceptors and photoreceptors function in monogeneans. Many larval monopisthocotyleans (oncomiracidia) have two pairs of pigmented eyes, which may be equipped with lenses. Polyopisthocotylean larvae have one pair of eyes, which normally are without lenses (see Smyth and Halton, 1983). The eye may be equipped with melanin in the supporting cells, but ciliary eyes consisting of a single cell may be found. Isolated, single, unicellulated receptors are embedded in the tegument and can be seen projecting from the general worm surface and may register touch or flow in the water. Groups of unicellulated receptors are called compound receptors. They are mostly distributed in the anterior part of the worm and it is speculated that they function as chemo- and tangosensors. Also, compound multiciliated receptors made of a number of nerve endings, each bearing modified cilia, can be found. Monogeneans may have papillae equipped with and penetrated by nerves, which may serve a function in attachment to the host.

Nervous system

Most monogeneans are mobile and move actively in their microhabitat. Likewise, feeding on host material is active and requires a developed and coordinated neuromuscular system. The worms generally possess an orthogonal central nervous system, composed of three pairs of longitudinal nerve trunks (dorsal, ventral and lateral) interconnected with transverse commissures (Halton *et al.*, 1993, 1998). There is a rudimentary brain, composed of cerebral ganglionic collections of nerve cells, which are connected by commissures, from where the longitudinal nerve cords extend anteriorly or posteriorly (Fig. 9.10a). In the opisthaptor, the nerve roots (Fig. 9.10b) extend to the periphery for innervation of attachment musculature connected to hooks or clamps. All major anatomical elements (cephalic openings, pharynx, buccal suckers, mouth, gonopore, cirrus, subtegumental musculature) are innervated by the peripheral nervous system. Neurotransmitters in the nervous system and the neuromuscular system have been studied extensively (Halton *et al.*, 1993, 1998). Histochemically, esterases such as acetylcholinesterase can be found both in the central and peripheral system (Fig. 9.10a,b). In addition to these cholinergic nerves, a number of aminergic nerves are found, both histochemically and by use of immunocytochemistry. Serotonin is generally less abundant but may be found in the major compartments. Further, a range of peptides can be localized immunocytochemically in the worms. Monogeneans do not possess a circulatory system through which hormones may fulfil their function. The presence of hormones and peptides in the monogenean nerves indicates that this system may have a role to play for hormonal function in platyhelminths. In this context it could be suggested that various host substances (steroids, peptides, proteins) could affect the behaviour of the parasite. Correspondingly, the signal molecules in the parasites could interfere with epidermal functions in the host.

Muscular system

Many monogeneans are able to move rapidly. They can be seen moving in a leech-like manner if they are isolated or moving on

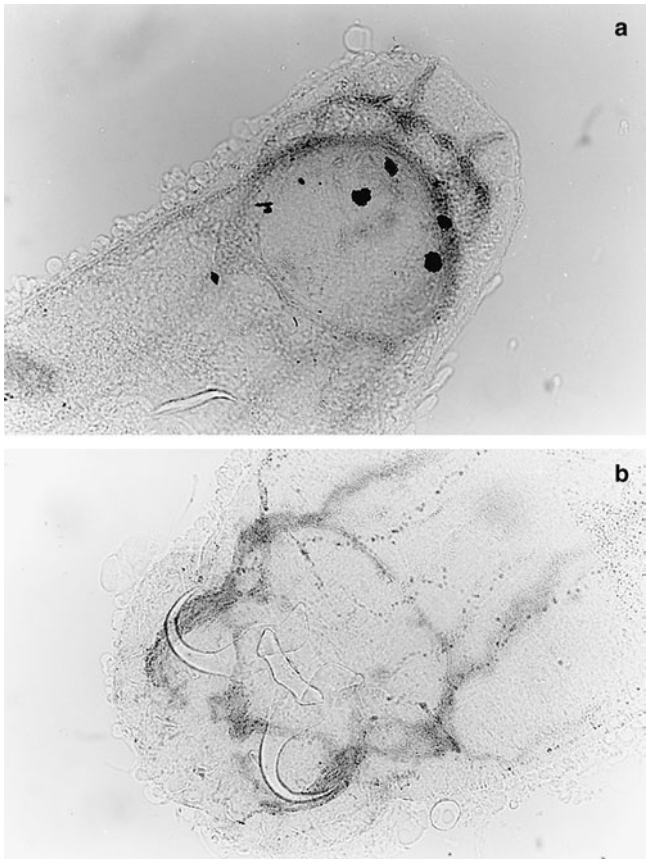


Fig. 9.10. Histochemical demonstration of the nervous system of *Pseudodactylogyrus bini* visualized due to activity of cholinesterases, a. anterior part of worm with four eye spots and cerebral ganglia, b. posterior body and opisthaptor with hamuli.

fins, skin or gills. Typically, a sequence of movements consists of stretching the body, attaching the anteriormost part of the worm, detaching and lifting the opisthaptor, bending the body and attaching the haptor next to the fore part, which will then detach. Likewise, feeding, including attachment of the feeding organ to the host surface and active function of the mouth and pharynx, is based on coordinated muscular actions. Reproduction involves movement of male and female copulatory organs and reproductive tracts, with sphincters coordinating neuromuscular functions (see Halton *et al.*, 1998). Muscles in monogeneans are generally arranged below the worm surface as an inner layer of longitudinal and outer layer of transverse fibres. Additional oblique, diagonal, dorsoventral or radial fibres can be found. Thus, the opisthaptor, with its hooks, suckers and clamps, the pharynx and the male copulatory organs are richly supplied with muscles. They are all non-striated and contain many mitochondria, allowing the typical coordinated movements composed of stretching, shortening and bending.

Alimentary canal

The digestive system in monogeneans is comprised of the mouth, pharynx and intestinal caeca. These latter are normally paired and can be simple and unbranched

or can branch or anastomose. A range of enzymes are released by gland cells associated with the mouth, pharynx or caeca. The gastrodermis serves for absorption of nutrients and is developed according to the food type. Polyopisthocotyleans, which are primarily sanguinivorous, are adapted for the absorption of haemoglobin (Halton, 1974), whereas the monopisthocotylean surface browsers are adapted to digestion of epithelial cells and mucus. Most investigated monogeneans possess cellular gastodermis. However, the intestinal lining of gyrodactylids is a syncytium (Kritsky *et al.*, 1994).

Excretory system

Worm excretory products are released though two anterolateral openings leading to the collecting ducts, which again are connected to a protonephridial system, consisting of flame cells, capillaries and ducts. The ultrastructure of the flame bulbs and capillaries (presence of septate junctions and lamellae) differs among groups and reflects the phylogeny of the worms (Rohde, 1993). It is likely that the system serves an osmoregulatory role, in addition to elimination of waste material. The distribution of flame cells and ducts is an important taxonomic character (e.g. Malmberg, 1970).

Reproductive system

Monogeneans are hermaphroditic flatworms and are equipped with an ovary and testes. In addition, vitellaria, an ootype and a seminal receptacle may be present. Sclerotinized structures, such as a cirrus (penis), an accessory cirrus and a vagina, may be found. These hard structures, together with the opisthaptoral elements, represent important diagnostic characters. The main differences concerning the anatomy of the reproductive system between monopisthocotyleans and polyopisthocotyleans are the presence of the genito-intestinal canal in the latter group (Kearn, 1994) and a different organization of spermatozoan microtubules (Justine, 1993).

Parasite Identification

Classical morphological method

Sclerotinized elements – hard parts

Classical descriptions of monogeneans have been based on drawings and measurements from slide preparations, i.e. helminths embedded in Canada balsam or other lipophilic mounting media or hydrophilic media such as glycerine gelatin. Gyrodactylids are especially well suited for embedding in ammonium picrate glycerine (Malmberg, 1970). Studies on live worms are used for description of the excretory systems and distribution of flame cells. The primary characters are the shape and size of hard sclerotinized parts, such as hamuli, clamps and marginal hooklets, and their arrangement on the opisthaptor. Additionally, hard reproductive organs, such as the vagina, cirrus and accessory cirrus, are important characters. However, body length and width, arrangements of testes, ovary, vitellaria, pharynx and intestinal caeca and gland cell distribution may also supplement the description. It should be noted that the water temperature at sampling sites may influence the size of sclerotized structures (Mo, 1991, 1993). Following treatment with silver nitrate and ultraviolet (UV) exposure, various sensillae on the parasites may be visible. The arrangement and distribution of these (chaetotaxy) represent important taxonomic characters (Shinn *et al.*, 1997).

Molecular methods

Nuclear DNA encoding ribosomal RNA from both large and small subunits of monogenean ribosomes has been the subject of extensive studies (Mollaret *et al.*, 2000; Olson and Littlewood, 2002). These genes have been shown to provide excellent tools for phylogenetic studies and for diagnostic purposes. Further, mitochondrial DNA has recently been shown to provide a high degree of sensitivity in phylogenetic studies of gyrodactylids (Zietara

and Lumme, 2002; Hansen *et al.*, 2003). Sequencing of entire PCR products will, of course, elucidate differences and variations between species. The genes for 18S, 5.8S and 28S ribosomal RNA and the spacers between them have shown themselves to be particularly interesting because the intraspecific variation is extremely low. Fast and yet reliable methods to differentiate between species have been developed. Based on slight variations between species in the internal transcribed spacer (ITS) regions 1 and 2, comprising also the 5.8S gene, a reliable diagnostic tool the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), has been developed by Cunningham (1997) to distinguish different gyrodactylid species. Thus, conducting PCR with specific primers for this region in the *Gyrodactylus* species infecting salmonids in Europe, *G. truttae*, *G. derjavini*, *G. salaris* and *G. teuchis*, a 1300 bp fragment is obtained. Various restriction enzymes can cut the ITS sequence but, due to differences between the four species, different fragments will result. By running an agarose gel with ethidium bromide of the restriction products from the different species and subsequent UV illumination, specific band profiles will be visualized (Cunningham, 1997; Cunningham *et al.*, 2001). The repetitive ribosomal RNA genes are separated by intergenic spacer (IGS) regions. A very sensitive discrimination between populations of gyrodactylids may be obtained from studying the IGS regions of ribosomal DNA (rDNA) (Collins and Cunningham, 2000). By using random amplified polymorphic DNA (RAPD) methods, it has also been possible to differentiate between populations of *G. salaris* (Cunningham and Mo, 1997). Knowledge of specific differences between species in certain genes may allow the use of fast methods for species differentiation. Thus, it is possible to conduct hybridization, in which DNA from a worm is immobilized on a membrane and subsequently hybridized with specific probes conjugated with a marker, allowing visualization of positive reactions (Cunningham, 2002).

Reproduction and Life Cycle

These platyhelminths are hermaphroditic, with both male and female copulatory organs. Some monogeneans are oviparous with a simple life cycle (Fig. 9.11). The worms produce and release eggs into the aquatic habitat (Fig. 9.12a,b,c). Egg morphology differs considerably. *Dactylogyrus* and *Pseudodactylogyrus* produce small oval eggs equipped with a sticky stalk, allowing eggs to glue to the substrate (Fig. 9.12a), while other eggs have short (Fig. 9.12b) or long extensions (e.g. Bychowsky, 1957; Ogawa, 2002). Dependent on temperature, the egg will in most species embryonate and hatch by releasing a ciliated larva, the oncomiracidium (Fig. 9.13), although some species may hatch by releasing a non-ciliated larva, e.g. *Acanthocotyle greeni* (MacDonald and Llewellyn, 1980). Following a crawling or free-swimming phase of short duration (normally less than 24 h, dependent on temperature), the

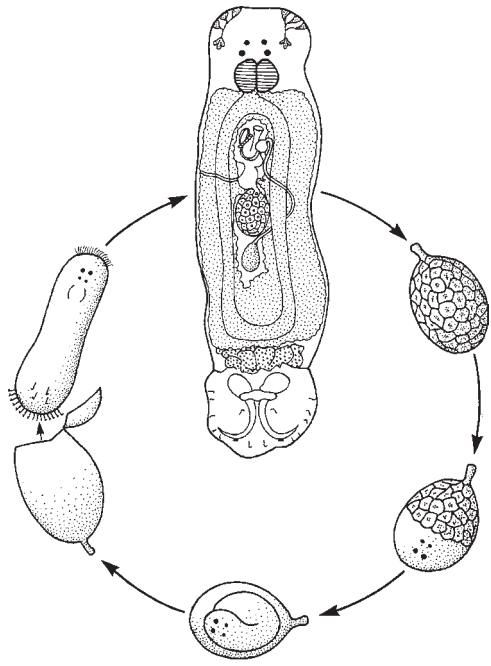


Fig. 9.11. Life cycle of the oviparous monogenean *Pseudodactylogyrus anguillae*. Drawing by Beth Beyerholm.

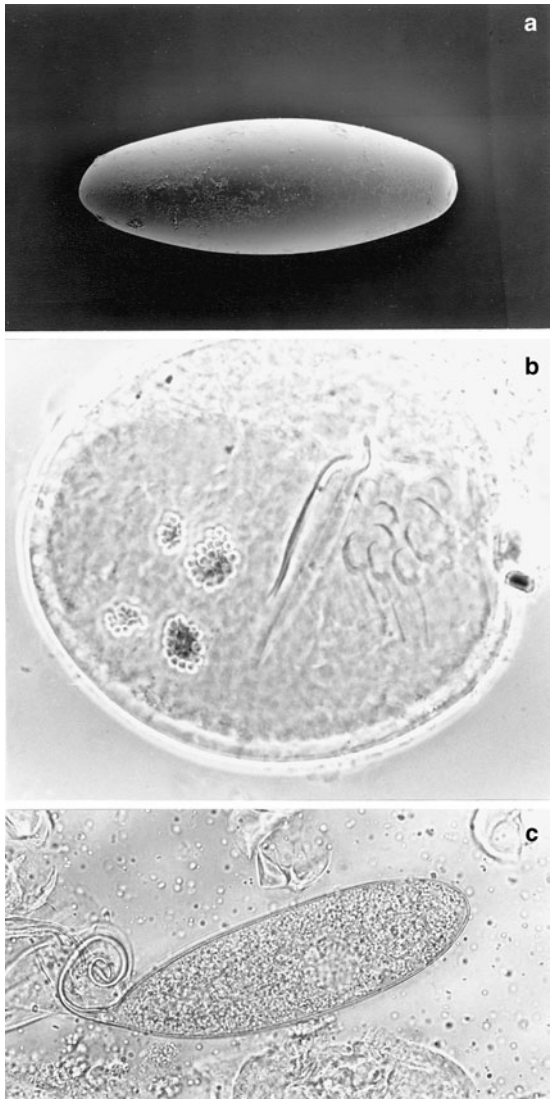


Fig. 9.12. Eggs of monogeneans. a. *Discocotyle sagittata* egg (length 300 µm), kindly provided by Miguel Rubio-Godoy, b. *Pseudodactylogyrus anguillae,* embryonated egg with oncomiracidium ready to hatch, c. *Dawestrema cycloancistrium* egg with extended filament.

oncomiracidium attaches to the host and sheds the ciliated cells, whereupon this postlarva develops into the adult stage. Oncomiracidia of gill monogeneans may attach directly to the host gills or settle on the body and subsequently migrate as postlarvae towards the gills (Cone and Burt, 1981).

The oviposition rate, the embryonation and hatching time, the free-living phase, the postlarval development and the adult lifespan are clearly influenced by temperature (Bauer *et al.*, 1973; Buchmann, 1997; Gannicott and Tinsley, 1997). A number of species are ovoviviparous and a large group of monogeneans, represented by most gyrodactylids, are viviparous, i.e. they give rise to live offspring. In the genus *Gyrodactylus*, most representatives are viviparous and have a well-developed uterus in which the

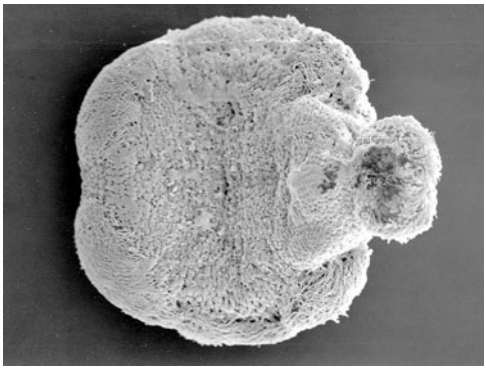


Fig. 9.13. *Discocotyle sagittata,* oncomiracidium (length 250 µm). Specimen kindly provided by Miguel Rubio-Godoy.

offspring is located. Remarkably, this also bears a maturing embryo (Fig. 9.2), whereby three generations can be discerned in one mother worm (Cable and Harris, 2002; Cable *et al.*, 2002). The reproductive rate of gyrodactylid monogeneans is also temperature dependent (Malmberg, 1970; Gelnar, 1987; Jansen and Bakke, 1991; Andersen and Buchmann, 1998). Although the variations in temperature preference of these worms are extremely high because their natural habitat spans from deep marine waters to tropical freshwater lakes, reproduction is positively correlated with temperature within certain limits. However, because gyrodactylids reproduce and deliver offspring directly on the fish, some indirect temperature effects may counteract this. Thus, the host response to monogeneans is highly influenced by temperature and will increase with temperature, whereby the high reproductive rate at higher temperatures will be counteracted by the elevated host immune responses (Andersen and Buchmann, 1998).

Host–Parasite Relationships

Egg hatching

Both abiotic factors and host factors can affect even the earliest stage of an oviparous monogenean parasite, the egg. It has been described that changes of light intensities may initiate hatching in certain species. Thus, *E. soleae* egg hatching is stimulated by illumination after a period of darkness (Kearn, 1963). In contrast, *D. sagittata* egg hatching is induced by darkness (Gannicott and Tinsley, 1997). These reactions are interpreted as adaptations to the host behaviour. Thus, sole burrow into the sand in the morning and become easily available for oncomiracidial invasion. However, host substances can also influence the egg-hatching process. Thus, mucus extract from a fish host has in several systems been observed to act as an efficient hatching stimuli. This has been observed in as different systems as *E. soleae* from sole (Kearn, 1974), *Acanthocotyle lobianchi* from *Raja* spp. (MacDonald, 1974), *Pseudodactylogyrus* from eel (Chan and Wu, 1984) and *Ancyrocephalus mogurndae* from mandarin fish (Xia *et al.*, 1996). When the fully embryonated egg is subjected to mucus extracts, the oncomiracidium will initiate active movements and subsequently escape the egg through the opercular opening. The hatching stimulus in mucus was suggested to be urea, although this could not be confirmed for *E. soleae* (Kearn, 2002).

Host finding

The selection of the correct fish host in a highly complex ecosystem is impressive. The basic mechanisms behind this choice are still insufficiently studied. However, due to the high host specificity in monogenean–fish systems, it has been speculated that host factors can be recognized by and attract the monogenean transmitted stage. Controlled studies were conducted by Kearn (1967) using *E. soleae* on *S. solea*. It appeared that there was some chemoattraction of the infective oncomiracidia. Isolated epithelium-covered scales from various fish species were exposed to larvae and the larvae clearly selected the sole scales and showed low attraction to dab, plaice, solenette and thickback sole. Dissected epithelia from corneae in fish were presented to the parasite as well but appeared to be without the

same attracting force. As corneae have no mucus cells, they did not attract the larvae, which suggested that a host mucus factor(s) is involved in host finding. In fact, some oncomiracidia selected agar plugs with sole mucus substances, although subsequent studies were less convincing (Kearn, 2002).

Similar studies have been conducted in gyrodactylid systems. Investigations of host selection by *Gyrodactylus salaris* and *G. derjavini* support the view of host preference. *G. salaris* infects salmon, *S. salar*, when it has the choice between rainbow trout, carp and salmon. Likewise, *G. derjavini* preferentially infect rainbow trout when offered the same three host possibilities (Buchmann *et al.*, 2004). This ability to select the correct host was also found in *in vitro* studies using fish scales covered with live host cells. Involvement of lectins in trout skin and carbohydrates in *G. derjavini* was suggested by Buchmann (2001) to be part of the communication between host and parasite. However, preliminary *in vitro* investigations on migration of isolated gyrodactylids offered mucus or in agar plugs in Petri dishes did not indicate specific selection of host material. Therefore, other factors, e.g. volatile host substances from intact cells only, could be involved in host selection. It has also been suggested that the pH of mucus plays an important role for host identification (Hirazawa *et al.*, 2003). These researchers found that oncomiracidia of *H. okamotoi* are sensitive to the pH of mucus from tiger puffer. Thus, a pH of 6.4, which occurs naturally in this natural host, was preferred by the parasite. Further studies on attachment induction in *Neobenedenia girellae* oncomiracidia indicated that lyophilized extracts of fish skin epithelia induce infection by parasite settlement. Lectin studies indicated that sugar/lectin associations are responsible for this communication (Yoshinaga *et al.*, 2000). Similar associations between fish epithelia and *Benedenia seriolae* were described by Yoshinaga *et al.* (2002). The studies showed that the attachment induction was also obtained by using inappropriate host material. This indicates that the settling of this parasite species is not specific.

Host factors affecting nutrition and reproduction

Correct host attachment is only a minor part of the life cycle. Stimuli to induce feeding, maturation, mating and finally production of offspring are important elements of the parasite's life. Therefore host factors may induce proper feeding and absorption of nutrients, maturation and reproduction. For example, hormonal treatment of unsuitable hosts elicits gyrodactylid reproduction. Thus, brown trout treated with hydrocortisone allows *G. salaris* to reproduce on this otherwise innately resistant host (Harris *et al.*, 2000). Salmon treated with dexamethasone allows the reproduction of *G. derjavini* on this unsuitable host (Olafsdottir *et al.*, 2003). The mechanism is not known and further studies should be conducted to elucidate these effects.

Host responses and acquired immunity

In many monogenean–fish systems, the parasite infrapopulation under normal circumstances will not increase to a lethal level for the host. Several physiological and anatomical factors may be responsible and the host immune response is one of these. The first reports indicating immunity in fish against monogenean infections came from the New York Aquarium, where *N.* (*Epibdella*) *melleni* had high numbers in naïve fish but only showed limited infection following subsequent exposures (Jahn and Kuhn, 1932; Nigrelli and Breder, 1934). The related capsalid *N. girellae* infecting Japanese flounder induced acquired immunity in the flatfish against reinfection (Bondad-Reantaso *et al.*, 1995b). Paperna (1963, 1964) found that common carp infected with *Dactylogyrus vastator* and *Dactylogyrus extensus* responded well and eliminated part of the dactylogyrid population. This was confirmed by Vladimirov (1971), working with a similar cyprinid–dactylogyrid system.

Further, this author actively immunized carp with *Dactylogyrus* antigens and subsequently detected a host response. In the same period, grass carp (*C. idella*) was described to react against its gill parasite *Dactylogyrus lamellatus* (Molnar, 1972). Viviparous gyrodactylids are also known to induce a host response in some hosts. Thus, Lester (1972) and Lester and Adams (1974) indicated significant elimination of parasites in later stages of infection. They detected a limited refractory period in the host following parasite elimination. Corresponding reactions were further described by Scott and Robinson (1984), Scott (1985) and Richards and Chubb (1996) using the guppy *P. reticulata* and *G. bullatarudis* and *G. turnbulli* association. Gyrodactylids infecting salmonids were studied by Cone and Cusack (1988). *G. salmonis* and *G. colemanensis* both propagated rapidly on the hosts but in later stages of infection a population decrease was noted. A number of challenge experiments elucidating the different ability of various salmonid hosts to respond to *G. salaris* were described by Bakke *et al.* (2002). Likewise, host reactions in salmonid hosts to *G. derjavini* were noted by Buchmann and Uldal (1997), Lindenstrøm and Buchmann (2000) and Lindenstrøm *et al.* (2003a,b). Anguillid eels show some reaction to the congeners *P. bini* and *P. anguillae* (Slotved and Buchmann, 1993). These authors infected European eel *A. anguilla* with both species, eliminated the infection after an infection period of 1 month and challenged the fish after 14 or 33 days post-clearance. It was seen that primed eels in a number of cases had a significantly lower infection compared with naïve eels.

The sanguinivorous monogenean *Microcotyle sebastis* infecting the rockfish *Sebastes schlegeli* in Korea induces host responses, as judged from vaccination studies conducted by Kim *et al.* (2000). Likewise, the blood feeder *D. sagittata* infects both brown trout and rainbow trout but survives and establishes better on brown trout (Rubio-Godoy and Tinsley, 2003). The parasite elicits a weak anti-parasitic response (Rubio-Godoy and Tinsley, 2002) in the host, including production of detectable antibodies (Rubio-Godoy *et al.*, 2003b). This was further indicated in vaccination experiments (Rubio-Godoy *et al.*, 2003a). In fact, immunity factors, such as the alternative pathway of complement activation, were found to be associated with differences in host susceptibility of brown and rainbow trout (Rubio-Godoy *et al.*, 2004). The blood-feeding diclidophorid *H. okamotoi* elicits antibody production in naturally infected tiger puffer (Wang *et al.*, 1997) and some hosts develop resistance to infection (see Ogawa, 2002). Although the number of studies on host responses with reduction or elimination of infrapopulations of monogeneans are limited, it should not be concluded that host responses are uncommon. Most histopathological studies of monogenean infections have demonstrated pronounced cellular reactions at the infection site. Hyperplasia, haemorrhages and infiltration with macrophages, neutrophils and other reactive cells are commonly discerned in histological sections of fish tissue infected with monogeneans (e.g. Paperna, 1963; Molnar, 1972; Oliver, 1977).

The mechanisms of host response to monogeneans have been studied. The reaction pattern varies from system to system but it seems that both innate and acquired elements are involved (Buchmann, 1999). Both these responses involve humoral elements, such as lectins, complement factors, prostaglandins, leukotrienes and acute-phase proteins, in the non-specific path and antibodies in specific reactions. Cytokines in host epithelia may take part in the orchestration of the reactions (Lindenstrøm *et al.*, 2003a,b). Cellular elements, such as neutrophils and macrophages, are involved (Buchmann, 1999). *In vitro* studies have also provided evidence that macrophages may recognize epitopes on monogeneans (Buchmann and Bresciani, 1999). These cells are able to produce reactive oxygen and nitrogen species with adverse effects on monogeneans.

Adaptive responses comprise antibodies (immunoglobulins). However, it is interesting that the few recorded cases of antibody production in infected fish all involved gill parasites (see above). Some gill

monogeneans (e.g. *Heteraxinoides xanthophilis* on the gills of *Leiostomus xanthurus*) are not known to induce antibody production in the host (Thoney and Burreson, 1988). As monogenean–fish host systems are extremely varied and diverse, the mechanisms involved in the host reactions may also vary considerably. However, recent studies on *G. derjavini* have indicated that some of these are influenced by a cytokine network in rainbow trout skin (Lindenstrøm *et al.*, 2003a). Within a few days post-infection, expression of genes encoding interleukin 1-beta and tumour necrosis factor alpha can be detected. These genes are involved in the inflammatory response in fish skin, leading to the initial anti-parasitic reactions. Further, expression of inducible nitrogen oxide synthetase (iNOS), leading to production of reactive N species, is subsequently seen. Also cyclo-oxygenase, responsible for prostaglandin production with regulating effects on the inflammation process, is expressed. Expression of genes for transforming growth factor (TGF) was found in later stages of the infection. This suggests an immune regulating and skin-repairing role of the cytokine (Lindenstrøm *et al.*, 2003b). There was no evidence of T-cell receptor (TCR) and major histocompatibility complex (MHC) up-regulation due to infection. This suggests that the responses are primarily autonomous innate reactions, at least in rainbow trout skin infected with *G. derjavini* (Lindenstrøm *et al.*, 2003a,b).

Although some gill monogeneans are able to induce an antibody response in their host, the importance of this for protection has not been confirmed. Various other components of cellular or humoral components may be more important. These include mucus and peptide secretion from epithelial mucus cells, epidermal mast-cell degranulation, complement and lectin production (Wells and Cone, 1990; Buchmann, 1998a, 1999; Harris *et al.*, 1998; Sterud *et al.*, 1998; Bakke *et al.*, 2000) and release of reactive substances from epidermal cells and macrophages (Buchmann and Bresciani, 1999; Lindenstrøm *et al.*, 2003a,b). The roles of prostaglandins, leukotrienes and other acute-phase reactants are still to be elucidated.

In Vitro Culture and Propagation of Monogeneans

Limited studies have been concerned with *in vitro* culture of monogeneans. Isolated *G. derjavini* showed temperature-dependent survival in fresh water, with the longest survival being 4 days at 3–12°C. Parasites bearing embryos were able to deliver their developed offspring present in the uterus when isolated from the fish but with no further reproduction if maintained in water only (Buchmann and Bresciani, 1999). *Diclidophora merlangi* survived for 28 days in sea water and increased its lifespan to 33 days if the medium was supplemented by amino acids and glucose (Halton, 1978). With the use of an artificial epithelial cell layer, EPC cells, Buchmann *et al.* (2000) kept attached specimens of *G. derjavini* alive for almost 6 days at 12°C, although reproduction was not induced. *In vitro* cultivation of monogeneans seems to be an area with opportunities for future development and improvements.

Pathogenicity

The anatomy of attachment organs, physiology, gland secretions and feeding strategy vary considerably among monogeneans, even within the Monopisthocotylea and Polyopisthocotylea.

Feeding

Direct blood feeding by polyopisthocotyleans can result in anaemia in the host. The higher the infection intensity, the higher the loss of blood. Thus, infection with a high number of blood-feeding worms firmly anchored in host tissue leads to severe anaemia. This was described by Eto *et al.* (1976) studying *Heteraxine heterocerca* infections of yellowtail, *S. quinqueradiata*. Erythrocyte

counts, haemotocrit values, haemoglobin content and mean corpuscular measures of blood from parasitized fish were lower than in normal fish. The anaemia was characterized as a microcytic, hypochromic type. The condition was associated with lethargy, anorexia, dark skin colour and paleness of the muscle, gills, kidney and liver of the infected fish. The proteins in the serum were lowered. Enzyme activities of lactate dehydrogenase (LDH), alkaline phosphatase (AlP) and glutamic pyruvic transaminase were also low. Urea and creatinine levels were higher in infected fish. Likewise, Paperna *et al*. (1984), studying *Allobivagina* sp. infection in *Siganus luridus*, showed a clear negative correlation between the infection intensity and haematocrit values. Fish without infection had a 25–50% haematocrit, while fish with 70–100 worms had values of 15–30% and fish with more than 150 worms had values below 10%. The lowest value was in a fish with 985 worms. Blood smears from infected fish showed mainly immature erythrocytes. The pathogenicity of the sanguinivorous *N. hirame* infecting olive flounder, *P. olivaceus*, is associated with severe anaemia (Ogawa, 2002). However, recent work has demonstrated that the mortality of hosts is further increased by concomitant infection with a marine form of the VHS virus (Shirakashi *et al*., 2003). The viral infection alone did not produce noteworthy mortality but the effect of the monogenean infection made the host increasingly susceptible to this viral pathogen.

Browsing of epithelia by monopisthocotyleans elicits another type of pathological disturbance, and this is dependent on the size and number of monogeneans. Thus, high densities of *G. salaris* or *G. derjavini* on fins of salmonids severely injure the epithelia (Fig. 9.14). However, large capsalids, such as *B. seriolae*, may produce large feeding wounds with heavy erosion of epidermal layers on yellowtail, amberjack and kingfish (Ernst *et al*., 2003). Extensive hyperplasia and tissue reactions at the site of attachment are also associated with even smaller monogeneans. Such host reactions will, of course, impair the normal physiological function of the organ. Gill tissue developed into club-shaped filaments with fused lamellae or extensive outgrowths, partly embedding the invading parasite. This decreases the respiratory surface of gills and will lead to mortality due to hypoxia (Fig. 9.15). The pathological reaction is evidently produced by the host as a response to the invasion (Roubal, 1995). However, this reaction is highly disadvantagous to the host respiration. Carp infected with *D. vastator* are more sensitive to low oxygen content compared with uninfected fish (Molnar, 1994). In addition, several observations indicate that some gill-dwelling parasites benefit from these pathological reactions. Thus, worms with a weak attachment capacity will obtain a safer and more secure hold of the host tissue if it is partly surrounded with host tissue. This is seen in *Linguadactyla molvae* living on *Molva dipterygia elongata* (Bychowsky, 1957), *D. aequans* on gills of sea bass (Oliver, 1977), *Callorhynchicola multitesticulatus* on the holocephalan fish *Callorhynchus millii* (Llewellyn and Simmons, 1984) and *P. bini* on eel gills (Buchmann, 1997). The tissue ingested by the monopisthocotyleans comprises epithelial cells, mucus and blood (if haemorrhages occur). Apart from the pathological reactions this activity produces, the injuries on the host may be severe. The large capsalids, such as *Benedenia*, *Entobdella* and *Neobenedenia*, occurring in high numbers elicit severe damage to the skin during feeding.

Mechanical effects of opisthaptoral hooks, suckers and clamps

The hamuli, marginal hooklets, suckers and clamps of monogeneans all have direct contact with host tissue and may afflict mechanical damage directly. This has been extensively reported for cyprinids (e.g. Bychowsky, 1957; Paperna, 1963; Prost, 1963; Molnar, 1972; Bauer *et al*., 1973), anguillids (Chan and Wu, 1984; Buchmann, 1997) and salmonids (Cone and Odense, 1984). Even light infections with *Bothitrema bothi* on *Scophthalmus aquosus* may result in depressions of host tissue caused by the hard structures on the opisthaptor (Hendrix, 2004).



Fig. 9.14. Gyrodactylid effects on fins of salmonids. a. *Gyrodactylus salaris* on Atlantic salmon fin, b. *Gyrodactylus derjavini* on rainbow trout fin, c. marginal hooklets of *G. derjavini* penetrating fin epithelial cells.

In addition, the mechanical effect can lead to a host reaction of epithelia, including hyperplasia. However, the impact varies. Hamuli in gyrodactylids do not penetrate the fish skin, whereas the marginal hooklets may each penetrate an epithelial cell. These mobile and active worms can easily move to another position on the skin if there is

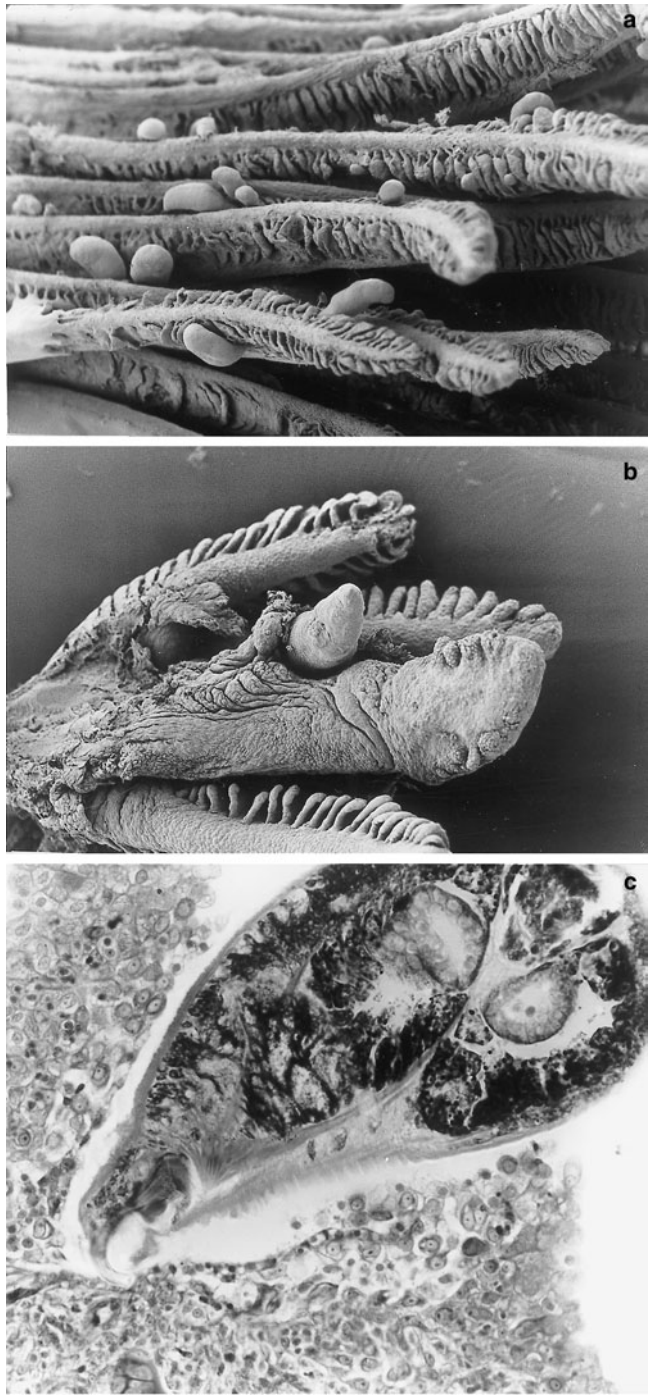


Fig. 9.15. Pathological reactions of eel gills, a. Numerous specimens of *Pseudodactylogyrus bini* on eels gills, b. hyperplasia and fusion of gill filaments and lamellae due to infection with *P. bini*, c. partly embedded *P. bini* in tissue reaction of eel gills.

significant reaction at the site of attachment. In contrast, clamps need intact secondary lamellae in order to attach to the gills and the strategy of these worms is to focus on a minimum of damage to the host structure, although prolonged exposure may produce gill tissue changes. A combination of infection intensity and damage produced by the monogenean is well illustrated in *G. salaris* infecting susceptible Norwegian salmon. Fry of this vulnerable host may have several thousand parasites. Each worm produces with its marginal hooklets 16 minute holes in epithelial cells at each attachment site. Further, part of the infrapopulation moves around on the fins and skin of the fish. All these will probably have a devastating impact on osmoregulation in the fish. Studies on the effect of *G. derjavini* on rainbow trout showed a positive correlation between mortality of hosts and intensity of infection (Busch *et al.*, 2003).

Effect of gland secretions

The enzymes, such as proteases, phosphatases and others in the pharynx, oesophagus and intestinal caeca, associated with digestion (Smyth and Halton, 1983) may affect the host epithelia and provoke reactions. Gland secretions from cephalic glands involved in attachment of the prohaptor and gland secretions from opisthaptoral glands may all have effects on host tissue. Although monogeneans inflict extensive pathogenic effects on the fish, the host reactions to eliminate them are only partly expressed. This indicates that the parasites are able to produce immune-evading or immunosuppressive substances. The nature of such molecules has to be investigated.

Monogeneans as Vectors for other Pathogens

It has been speculated by several authors (see Cusack and Cone, 1986) that monogeneans could act as vectors for other pathogens, such as bacteria and viruses. Bacteria and *Mycoplasma*-like organisms have been detected in *D. merlangi* from whiting gills (Morris and Halton, 1975) and Cusack and Cone (1985) found the tegument of *Gyrodactylus avalonia* on stickleback in brackish water colonized by rod-shaped bacteria. Correspondingly, bacteria have been observed on the tegument of *G. salmonis* on rainbow trout (Cone and Odense, 1984) and on the gill parasite *P. bini* from eel gills (Buchmann, 1997), and Justine and Bonami (1993) detected virus-like particles in *Microcotyle* sp. from marine fish. Thus, it can be suggested that the injuries caused by monogenean hooks or worm-feeding activities could act as a portal of entry for various pathogens. In a controlled experiment, Busch *et al.* (2003) investigated whether *G. derjavini* infections on rainbow trout augmented infections by *Flavobacterium psychrophilum*. The fish, either infected or uninfected with monogeneans, were exposed to a bacterial solution. Only one fish with *G. derjavini* became infected – which is only weak evidence that the gyrodactylids could be involved in bacterial invasion.

Prevention and Control

Worm control in wild fish populations

Devastating monogenean infections in wild fish populations have been treated with chemicals. Thus, aiming at eradicating *G. salaris* infections from Norwegian rivers, extensive rotenone treatments have been conducted for a number of years (Mo, 1994). This practice differs from normal treatments because both fish and parasites are killed by rotenone. Subsequently, uninfected salmon from pathogen-free gene bank rearing systems are released for re-establishment of the fish stock in the treated river. These drastic measures have been replaced in certain rivers by the addition of aluminium products because this metal is quite toxic to gyrodactylids and is tolerated by fish in the concentrations applied (Soleng *et al.*, 1999).

Worm control in cultured populations

It is possible to interfere with and control propagation of monogeneans in most

aquaculture systems. Thus chemical and medical treatment of infections in fish-rearing facilities is widely used. In these cases, chemicals and drugs with a high antiparasitic effect and low toxicity to the fish are selected. Some salts will merely interfere with osmoregulation, whereas others, such as oxidizers (hydrogen peroxide, sodium percarbonate), destroy the worm surface. Other drugs interfere directly with specific molecules in the worms. Thus, organophosphates inhibit the acetylcholinesterases in the nervous system and the neuromusculature of the worms, resulting in paralysis. Benzimidazoles bind to tubulin monomers and prevent microtubule assembly in worm cells, interfering with a number of functions in the monogenean (Buchmann, 1997). However, it should be stressed that the sensitivity of host fishes to the toxic effects of drugs vary considerably and drug legislation differs between countries. Specific approval of a specific drug for a specific disease is required in many countries.

Eggs and oncomiracidia in some species are large enough to make filtering a possible way to remove them. Filtering eggs and oncomiracidia by nylon mesh with pore sizes less than 80 μm have been used in recirculated systems to control pseudodactylogyrosis. Several monogeneans have eggs with long appendages or polar filaments, which allow them to cluster and entangle various objects in the aquatic environment. This is the basis for a suggested parasite control method by removal of eggs. Thus submersion of ropes and nets into net pens with tiger puffer, *T. rubripes*, infected by *H. okamotoi* is a practical way to remove large numbers of eggs. At intervals, ropes or culture nets entangled with eggs are replaced by new ropes (Ogawa, 2002). The eggs form long strings and chains on ropes up to more than 2 m long and these must be replaced before hatching of parasite eggs occurs. At summer temperatures (25°C), this means every 4 days. Such labour-intensive measures may put a limit on implementation of this mechanical control method.

Biological control

Some fish species are not particularly selective in their food choice and will readily ingest both adult and larval parasites. It is well established that labrids can be used for keeping salmon and cod lice, *Lepeophtheirus salmonis* and *Caligus elongatus*, respectively, at an acceptable level in salmon net pens. Some monogeneans attached to the surface of a fish host may also be easily discernible by cleaner fish. Several studies have been conducted indicating that cleaner fish can ingest considerable numbers of monogeneans. Control of *Macrogyrodactylus polypteri* by tilapia ingesting ectoparasites from the body surface of African lungfish was reported by Khalil (1964). Likewise, Kearn (1978) observed injuries in large monogeneans on rays probably produced by smaller cleaner fish. Further, *N. melleni* on red tilapia was ingested by cleaner fish (Cowell *et al.*, 1993), and controlled experiments conducted by Grutter *et al.* (2002) demonstrated that another capsalid monogenean, *Benedenia lolo*, parasitizing *Hemigymnus melapterus* was subjected to predation by the cleaner fish *Labroides dimidiatus*. Thus, future control measures may involve the use of cleaner fish.

Studying a recirculated eel farming system with fish tanks covered with extensive biofilm layers, it was found that turbellarians of the genus *Stenostomum* were able to ingest newly produced eggs of *Pseudodactylogyrus* spp. attached to the biofilm. Copepods and other crustaceans are frequently found in fish tank systems and they may have an impact on monogenean populations by feeding on oncomiracidia. Cyclopoid copepods were found to ingest oncomiracidia of *Pseudodactylogyrus* spp. (Buchmann, 1988).

Chemical control

Numerous chemicals have been used with varying success to control monogenean infections. The most widely used are copper sulphate, formaldehyde, sodium chloride

and hydrogen peroxide and, to a lesser extent, potassium permanganate. However, the toxicity of these substances to hosts and parasites varies considerably and is dependent on species and biotic and abiotic conditions, and each parasite–host system should be tested specifically before treatment is used on a large scale. Formaldehyde addition to fish tank water is a widely applied non-specific and crude way to remove monogeneans from fish skin, gills and tanks. Typically, concentrations applied vary between 30 and 100 ppm formaldehyde for short periods. Addition of sodium chloride may compromise the physiology of monogeneans infecting freshwater fish and freshwater dips may on the other hand have effects on monogeneans infecting marine fish. Normally this practice is used for short time treatments in order to minimize the adverse effects on the host physiology. Ammonia is toxic both to fish and parasites but, due to slight differences in toxicity of ammonia to host and parasite, it has been used in water-bath control of monogeneans (Chan and Wu, 1984). Hydrogen peroxide will oxidize the organic constituents of monogeneans, which is the basis for the extensive use of this compound. Due to the high surface/volume ratio of ectoparasitic worms compared with the host, this substance has often shown a satisfactorily high parasiticidal effect compared with lower host toxicity. Hydrogen peroxide can also be administered in the form of sodium percarbonate, which is sodium carbonate with hydrogen peroxide bound in the molecule instead of water (Buchmann and Kristensson, 2003). This will allow a slower release and prolonged action of hydrogen peroxide in the water bath. The finding that various metals may have adverse effects on worms was also the basis for studies showing that *P. anguillae* could be partly controlled by addition of aluminium and zinc chloride to fish tank water with infected eels (Larsen and Buchmann, 2003).

The advent of effective anthelmintics in human and veterinary medicine inspired fish parasitologists to investigate the effects of various drugs on monogeneans. The main groups are the organophosphates, such as metrifonate and dichlorvos, and benzimidazoles (levamisole and praziquantel). All these are efficacious not only in laboratory trials but also under farm conditions. However, the toxicity of these chemicals for different fish hosts differs. Thus, while European eel, *A. anguilla,* tolerates mebendazole bath exposure well, the effect is very severe in goldfish, *Carassius auratus*. Likewise, where organophosphates are effective on monogenean infections when applied in flow-through systems or in large outdoor fish ponds, they are less suitable in modern recirculated systems, where the drugs accumulate and elicit mortalities in fish. Therefore, use of anthelmintics in fish cultures should not be conducted before substantial toxicological and tolerance tests have been carried out for the specific purpose. The tolerance of the specific host to the drug, temperature conditions, salinity, water content of organic material, retention time of the drug in the rearing facility and possible accumulation should be taken into consideration.

Benzimidazoles bind to tubulin monomers in the parasite to destroy microtubules in cytoskeletons and cell transport functions. A number of members of this group have been tested, including thiabendazole, oxibendazole, albendazole, oxfendazole, mebendazole, flubendazole, parbendazole, fenbendazole, triclabendazole and luxabendazole (Szekely and Molnar, 1987; Buchmann and Bjerregaard, 1990; Tojo *et al.*, 1992; Buchmann, 1997; Tojo and Santamarina, 1998). Mebendazole baths were first used by Goven and Amend (1982) against gyrodactylids parasitizing goldfish. Later it was found that bath exposure also eradicated *P. anguillae* and *P. bini* from European eel (Szekely and Molnar, 1987; Buchmann and Bjerregaard, 1990), and *D. extensus* and *Dactylogyrus minutus* (Buchmann *et al.*, 1993) and *Anacanthorus penilabiatus* from pacu, *Piaractus mesopotamicus* (Martins *et al.*, 2001).

Praziquantel interferes with integumental integrity (Schmahl and Mehlhorn, 1985) and may possibly expose hidden parasitic antigens to the host immune system.

The drug has wide application against monogeneans in both marine and freshwater fish culture. It has high efficacy against *Dactylogyrus* (Schmahl and Mehlhorn, 1985) and *P. bini* and *P. anguillae* (Buchmann, 1997) in freshwater aquaculture. Also, under marine conditions, praziquantel in a bath treatment or in feed is effective. Thus, monogenicidal action was shown against *Benedeniella posterocolpa* (Thoney, 1990). Effective oral treatment with praziquantel against *M. sebastis* was reported by Kim and Cho (2000). *B. seriolae* on kingfish (Ernst *et al.*, 2003) and *Clemacotyle australis* on white-spotted eagle rays (*Aetobatus narinari*) (Janse and Borgsteede, 2003) can also be controlled by the drug.

Organophosphates paralyse the parasite by inhibiting cholinesterases in the nervous system and neuromuscular transmission. The widely used representative of this group is metrifonate, which is used in low concentrations (0.25–0.5 ppm) for bath exposure of fish infected with monogeneans (e.g. Sarig *et al.*, 1965; Chan and Wu, 1984). Dichlorvos is chemically related to metrifonate and has a similar mode of action (Buchmann, 1997).

The anthelmintic niclosamide has a profound effect on monogeneans (Buchmann, 1997), but the effect on fish is even stronger, which makes this compound unsuitable.

Vaccination against monogenean infections

It is generally agreed that fish mount various host responses against monogenean infection. A few studies have indicated that vaccination will confer some protection to fish. It appears that innate mechanisms in the skin are activated during monogenean infections (Lindenstrøm *et al.*, 2003a,b) and several examples of cross-protection between different species of monogeneans have been described (Richards and Chubb, 1996; Buchmann *et al.*, 1999; Larsen *et al.*, 2002).

A few studies have been conducted in the field of anti-monogenean vaccines. The common carp, *C. carpio*, suffers from heavy infections with the gill parasite *D. vastator* and early Russian researchers attempted to produce a protective vaccine against this parasitosis (Vladimirov, 1971). Homogenates of the collected worms were injected intraperitoneally into carp and subsequent challenge infections showed some protection. Korean parasitologists working with the rockfish *S. schlegeli* and the polyopisthocotylean gill parasite *M. sebastis* demonstrated that injection of host fish with homogenates of the worms conferred some protection against reinfections. Furthermore, the adjuvant in the vaccine also induced some protection, which indicates that some innate and non-specific mechanisms were involved in protection (Kim *et al.*, 2000). Rainbow trout, *O. mykiss*, is host for the blood-feeding *D. sagittata* and an experimental vaccine without adjuvant was developed and tested by Rubio-Godoy *et al.* (2003a). Various formulations of the parasite homogenate injected intraperitoneally into the host induced some protection in experimental fish. Injection of a homogenate of *H. okamotoi* into tiger puffer did not result in protection against juvenile parasites on the gill filaments but decreased the number of adult parasites infecting the branchial gill wall during the later stages of infection (see Ogawa, 2002). Although no absolute protection was conferred in these cases, it was clearly shown that some immunity was induced. In all three studies, the vaccinated hosts mounted specific antibody responses, although it was suggested that other immune effector mechanisms (complement, leukocytes) might be involved as well. The experiments were all performed with gill monogeneans, where the barrier between the parasite and the host is extremely delicate, which may suggest that host protective factors can easily reach the worm. Similar positive results were not obtained by Bondad-Reantaso *et al.* (1995b) working with a skin parasite. Although these researchers recorded acquired immunity in Japanese flounder infected with *N. girellae*, they were not able to induce protection by injecting homogenate of the parasite into flounders.

Zoosanitary measures

Due to lack of technology to control infections at an acceptable level, it is often necessary to use more drastic measures. Thus, some producers use excessive amounts of anti-parasitic drugs or chemicals to achieve a profitable production of fish. This is, of course, problematic for the environment. Further, the possible use of vaccination or of immunostimulants is still not developed for use in commercial fish farming. Thus, it becomes necessary to implement eradication measures to obtain satisfactory control of monogenean infections. This has been applied both in natural systems and in aquacultural enterprises. The pathogenic *G. salaris* has been cleared from more than 16 Norwegian rivers by using rotenone on the entire water system (Mo, 1994). In this way, both fish and parasites were killed and uninfected fish were subsequently stocked in the rivers. Further, recirculated aquaculture plants have been cleared for infection by emptying, drying and subsequent disinfection. Following these measures, uninfected sterilized fish eggs have been introduced for hatching and restocking to obtain parasite-free facilities. Cage culture of fishes suffering from monogenean infections may benefit from fallowing and movements of facilities to new uninfected sites at regular intervals. Likewise, strategic placement of net pens and farms may take into consideration the fact that infective stages of parasites are spread to neighbouring plants by water currents. Thus, spread of *B. seriolae* and *Z. seriolae* parasites from one kingfish facility to another is dependent on water currents (Chambers and Ernst, 2003).

Legislation, approved zones and import restrictions

Strict enforcement of legislation is often a prerequisite for keeping certain areas free from infection. The basis for this should be monitoring programmes and restriction of fish transportation between approved zones.

The spread of *N. sturionis*, *G. salaris*, *P. bini* and *P. anguillae*, *N. hirame* and other monogeneans to vulnerable and susceptible host populations has produced unambiguous evidence for the devastating effects of uncontrolled transfer of pathogens. Although the transportation of fish for aquaculture purposes, the ornamental fish trade and consumption has confused the original zoogeographical distribution of monogeneans, it is still possible to conduct a strict record of parasite distribution. In Europe, certain areas (England, Scotland) are free from *G. salaris* infection (Shinn *et al.*, 1995). Monitoring programmes are conducted and the importation of fish products from infected areas is restricted.

Integrated control

Prevention of the introduction of monogeneans to a particular facility is the preferred approach. If this is not possible, a range of methods may be used to alleviate the parasite problems. This will include primarily a strict monitoring of infection levels concomitant with implementation of control measures aiming at reducing the infection pressure. These actions include: (i) fallowing of infected systems; (ii) regular movements of facilities to new uninfected sites; (iii) placement of new production entities in areas with low spreading potential and away from existing facilities; (iv) mechanical removal of eggs and larvae by filtration or by using replaceable ropes or nets; (v) reducing entrapment of eggs and larvae in a stationary production system (e.g. net cages); (vi) use of cleaner fish, if available; and (vii) strategic treatments using chemicals or anthelmintics.

SHORT DESCRIPTION OF SELECTED MONOGENEANS CAUSING DISEASE IN AQUACULTURAL ENTERPRISES

Although it is expected that most monogeneans have the ability to produce disease in host populations under appropriate biotic and abiotic conditions, the number of reports on this issue is limited. Below are monogeneans that are known to cause

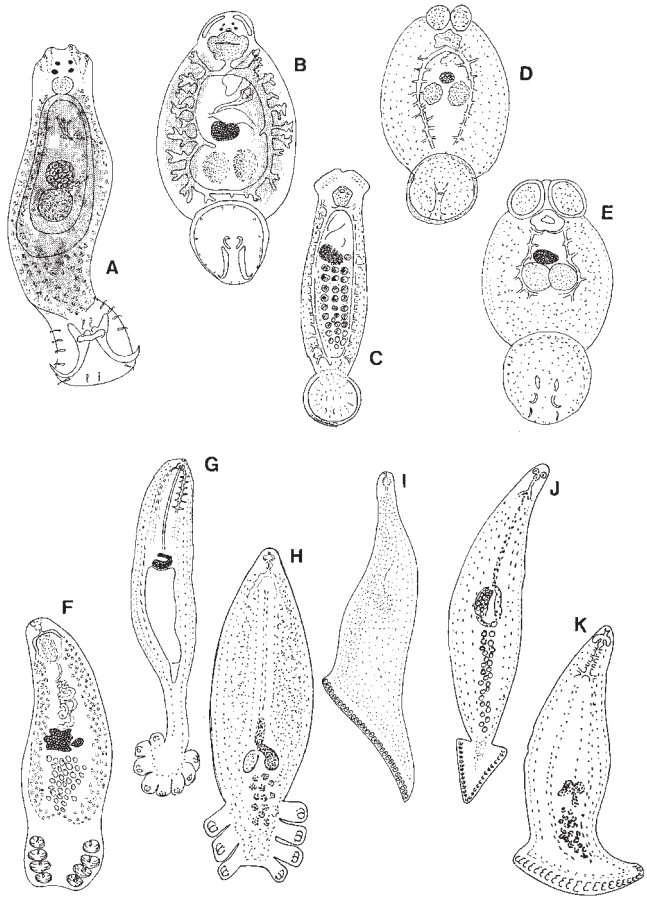


Fig. 9.16. Different monogenean types, A. *Dactylogyrus*, B. *Entobdella*, C. *Nitzschia*, D. *Neobenedenia*, E. *Benedenia*, F. *Discocotyle*, G. *Neoheterobothrium*, H. *Diclidophora*, I. *Axine*, J. *Microcotyle*, K. *Heteraxine*.

disease, and the morphology of some of the monogenean types is outlined in Fig. 9.16.

Monopisthocotyleans

Family Dactylogyridae

Dactylogyrus vastator

- Host. Common carp, *Cyprinus carpio*, and goldfish, *Carassius auratus.*
- Macrohabitat. Fresh water.
- Geographical distribution. Originally endemic in Asia but transferred with hosts to Europe and North America.
- Morphology. Body length 0.328–0.388 mm, width 0.081 mm. Hamulus length 35–41 µm with 14 marginal hooklets.
- Microhabitat. Gills.
- Life cycle. Eggs embryonate and hatch within 2–3 days. The oncomiracidial phase is shorter than 24 h and the postlarval development takes 5 days at 24–28°C. The lifespan of the adult worm at this temperature is 5 days (Bauer *et al.*, 1973).
- Pathogenicity. Attachment and/or feeding of the worm induces extensive reaction on gill tissue, with hyperplasia of

epithelia and mucus cells (Paperna, 1963).
- Control. A sodium chloride bath has varying effectiveness on the parasite, with adult parasites being most resistant. The organophosphorus compound metrifonate used as a bath treatment is effective (Sarig *et al.*, 1965) and so is praziquantel (Schmahl and Mehlhorn, 1985). Management as a preventive tool was suggested by Prost (1963). Draining and drying of ponds to kill overwintering parasite eggs and the separation of water to prevent contamination from infected ponds to nursery ponds are helpful. There are indications that immune mechanisms are involved in the regulation of parasite loads.

Dactylogyrus extensus

- Host. Common carp, *Cyprinus carpio*, and goldfish, *Carassius auratus*.
- Macrohabitat. Fresh water.
- Geographical distribution. Endemic in Asia, Europe, Japan, North America.
- Morphology. Length is 0.99–1.584 mm and width 0.158 mm. Four eye spots. Cirrus and accessory cirrus are species characteristic. Dorsally directed hamuli. Hamulus length 75–88 µm with 14 marginal hooklets (Bauer *et al.*, 1973).
- Microhabitat. Gill filaments and lamellae.
- Life cycle. At 24–25°C postlarvae mature and initiate egg production after 6–7 days (Prost, 1963).
- Pathogenicity. Considered to be less pathogenic than *Dactylogyrus vastator*. Light infections of carp fry co-occurred with *Dactylogyrus minutus* (Buchmann *et al.*, 1993). Attachment and/or feeding of the worm induces hyperplasia and epithelial reactions, which can partly embed the worm (Buchmann *et al.*, 1993). Increased mucus production may result (Prost, 1963).
- Control. A sodium chloride bath has varying effectiveness on the parasite, with adult parasites being most resistant. Organophosphates like metrifonate and praziquantel may be effective for bath treatments. Mebendazole is an effective anthelmintic but may have adverse effects on the hosts (Buchmann *et al.*, 1993). Schmahl and Mehlhorn (1985) found praziquantel to affect worm integrity and structure. Management as a preventive tool, as suggested by Prost (1963) for other dactylogyrids, may be applicable. Draining and drying of ponds to kill overwintering parasite eggs and separation of water to prevent contamination from infected ponds to nursery water can be applicable. There are indications that the host immune system is involved in regulation of parasite levels.

Dactylogyrus minutus

- Host. Common carp, *Cyprinus carpio*.
- Macrohabitat. Fresh water.
- Geographical distribution. Originally endemic in Asia.
- Microhabitat. Gill filaments and lamellae.
- Pathogenicity. Heavy infections of carp fry are associated with mortalities (Buchmann *et al.*, 1993). Attachment and/or feeding of the worm induces hyperplasia of epithelia. Thus clubbing of filaments and fusion of lamellae is a common reaction.
- Control. Sodium chloride has varying effects on the parasite, with adult parasites being most resistant. Organophosphorus compounds like metrifonate and praziquantel may be effective for bath treatments. Mebendazole is an effective anthelmintic but may have adverse effects on the host (Buchmann *et al.*, 1993). Management as a preventive tool as suggested by Prost (1963) for other dactylogyrids may be applicable. Draining and drying of ponds to kill overwintering parasite eggs and separation of water to prevent contamination from infected ponds to nursery ponds can be applicable. There are indications that the host immune system is involved in regulation of parasite levels.

Dactylogyrus lamellatus

- Host. Grass carp (*Ctenopharyngodon idella*).

- Macrohabitat. Fresh water.
- Geographical distribution. Far East but transplanted with export of infected fish to Europe.
- Morphology. Length 0.48 mm, width 0.11 mm. Four eye spots. Hamuli, connecting bar, marginal hooklets, vagina, cirrus and accessory cirrus are species specific (Bauer *et al.*, 1973).
- Microhabitat. Gill filaments.
- Life cycle. Each adult worm may produce two to four eggs per hour. The egg has a sticky stalk and adheres to the substrate. Embryonation and hatching are temperature dependent and take 6–7 days at 15°C. The oncomiracidium swims freely for 2–3 h and attaches to the host surface before it migrates to the gills. The postlarval development lasts 5–8 days.
- Pathogenicity. Heavy infections elicit lethargy, morbidity and mortality. Severe hyperplasia of gill filaments, including clubbing, occurs (Molnar, 1972).
- Control. Treatments with formaldehyde and organophosphorus agents such as metrifonate have been used. Other anthelmintics may have effects on the parasite.

Dactylogyrus ctenopharyngodonis

- Host. Grass carp, *Ctenopharyngodon idella*.
- Macrohabitat. Fresh water.
- Geographical distribution. Originally endemic in Asia but transplanted with export of fishes to Europe and other parts of the world.
- Morphology. Length 0.5 mm, width 0.08 mm. Four eye spots. Hamuli, marginal hooklets, cirrus and accessory cirrus are specific for species.
- Microhabitat. Gill filaments and lamellae.
- Life cycle. The life cycle is temperature dependent and the parasite occurs abundantly in the warm season (Bauer *et al.*, 1973).
- Pathogenicity. Heavy infections of grass carp fry (several thousands per fish) were associated with mortalities. Attachment and/or feeding of the worm induces injuries with haemorrhages. A tissue reaction, with hyperplasia of epithelia, clubbing of filaments and fusion of lamellae, is common (Bauer *et al.*, 1973).
- Control. Sodium chloride bath treatments affect the worm stages to different degrees, with adult parasites being the most resistant. The organophosphorus compound metrifonate used as a bath treatment like praziquantel may be effective. Mebendazole may be effective, although toxicity of the drug to grass carp has not been studied. Management as a preventive tool against dactylogyrids as suggested by Prost (1963) may be applicable. Draining and drying of ponds in order to kill overwintering parasite eggs and separation of water to prevent contamination from infected ponds to nursery water can be applicable. Some host response has been detected, indicating that immune mechanisms may be involved in regulation of parasite levels.

Dactylogyrus hypophthalmichthys

- Host. Silver carp (*Hypophthalmichthys molitrix*).
- Macrohabitat. Fresh water.
- Geographical distribution. Asia, from where it has been transplanted with export of infected hosts to other parts of the world.
- Morphology. Length 0.42 mm, width 0.11 mm. Hamuli, marginal hooklets, cirrus and accessory cirrus are specifically shaped (Bauer *et al.*, 1973).
- Microhabitat. Gill filaments.
- Life cycle. Similar to those of other dactylogyrids. Its development is temperature dependent.
- Pathogenicity. Heavy infections can occur in older fish, with intensities up to thousands of parasites per host.
- Control. As for other dactylogyrids.

Dactylogyrus aristichthys

- Host. Bighead carp (*Aristichthys nobilis*).
- Macrohabitat. Fresh water.

- Geographical distribution. Asia, from where it has been transplanted with export of infected hosts to other parts of the world.
- Morphology. Length 0.48 mm, width 0.1 mm. Hamuli, marginal hooklets, cirrus and accessory cirrus are specifically shaped. Larva elongate with seven pairs of hooklets at primordial opisthaptor (Bauer *et al.*, 1973).
- Microhabitat. Gill filaments.
- Life cycle. Temperature dependent development between 14 and 30°C. Fastest embryonation and hatching at 30°C (2 days). This process takes 10 days at 12°C. Oncomiracidium swims freely 8–11 h at 17–23°C. It attaches initially to the oral cavity of the host, whereupon it migrates to the gill filaments.
- Pathogenicity. Heavy infections can occur in older fish, with intensities up to thousands of parasites per host. Thus counts of more than 6000 parasites per host (2-year-old bighead carp).
- Control. As for other dactylogyrids.

Acolpenteron ureteroecetes

- Host. Largemouth bass, *Micropterus salmoides* (Petrie-Hanson, 2001). Representatives of the genus in Perciformes, Cypriniformes and Centrarchidae.
- Macrohabitat. Fresh water.
- Microhabitat. Urinary system, urinary bladder and ureters of teleosts.
- Geographcal distribution. North America. Representatives of the genus also in Eurasia and Africa.
- Morphology. Body elongate, length 1.17–1.25 mm, width 0.11–0.18 mm. Head organs poorly developed. Four eye spots. Haptors without hamuli and bars but with 14 marginal hooklets (0.028 mm). Cirrus and vagina present. Eggs measure 0.06 mm × 0.04 mm, with a blunt process at one end (attaching stalk).
- Life cycle. Oviparous hermaphroditic worm produces eggs, which hatch, with the appearance of oncomiracidia.
- Pathogenicity. The adverse effect is correlated to the intensity of infection. Worm loads (*Acolpenteron* sp.) may vary from 1 to 52 per fish (Fischthal and Allison, 1941). Estimates of at least 560 *A. ureteroecetes* worms per host have been presented (Petrie-Hanson, 2001). The presence of a high number of parasites in the lumen of ureters and bladders elicits necrosis, inflammation and fibrosis. Urinary obstruction can occur. The kidney may appear enlarged and pale. Morbidity and mortality may result. Clinical signs are anorexia, discoloration, disorientation, balance disturbances and spasms.
- Control. No controlled treatment experiments have been conducted but anthelmintics such as mebendazole, praziquantel or others may have an effect.

Family Pseudodactylogyridae

Pseudodactylogyrus anguillae *and* Pseudodactylogyrus bini

- Host. Anguillid eels. Registered from European eel, *Anguilla anguilla*, Japanese eel, *Anguilla japonica*, *Anguilla reinhardtii* and *Anguilla bicolor* (Ogawa and Egusa, 1976; Buchmann, 1997).
- Macrohabitat. Fresh water or brackish water.
- Geographical distribution. It has been suggested that the two species originated in the Pacific area, from where they were introduced to Europe through transportation of live eels. Known localities are Japan, China, Taiwan, Australia, Indonesia, Russia, most European countries and North America.
- Morphology. Length is generally around 1–1.5 mm. The opisthaptor is equipped with two ventrally pointed large hamuli and 14 marginal hooklets. Sclerotinized cirrus, accessory cirrus and vagina. Four eye spots. The two species are differentiated based on hamulus size (Ogawa and Egusa, 1976).
- Microhabitat. The two species may select different microhabitats in the gill apparatus (Buchmann, 1997).
- Life cycle. The life cycle is temperature dependent and finds its optimum at 25–30°C, whereas development below

10°C is extremely slow. These monogeneans are oviparous and each worm may produce up to 24 eggs per day. The eggs embyonate and hatch within 1–2 days and the postlarval development is 1 week at 25°C. The worm may survive more than 60 days at 25°C (Buchmann, 1997).

- Pathogenicity. Infected eels develop hyperplasia of gill structures, including fusion of gill lamellae. Direct mechanical action by hamuli, marginal hooklets and feeding activity may elicit the reactions.
- Control. Bath treatment, using various drugs such as mebendazole (1 ppm) (or other benzimidazoles) or praziquantel (10 ppm), is feasible (Buchmann, 1997). Formaldehyde (50–100 ppm) is commonly used by farmers. Trials have shown that aluminium chloride (1–10 ppm) may have an adverse effect on the infections (Larsen and Buchmann, 2003). Predation of parasite eggs and oncomiracidia by turbellaria and copepods, respectively, has been observed, which suggests alternative control strategies (Buchmann, 1988).

Family Ancyrocephalidae

Ancylodiscoides vistulensis

- Host. European catfish, *Silurus glanis.*
- Macrohabitat. Fresh water.
- Geographical distribution. Europe.
- Microhabitat. Gills.
- Pathogenicity. Heavy infections obtained in confined environments may cause mortalities (Molnar, 1968).
- Control. Bath treatments with ammonium hydroxide have been found to be more effective than treatments with praziquantel and mebendazole (Szekely and Molnar, 1990).

Dawestrema cycloancistrium

- Host. Pirarucu (*Arapaima gigas*).
- Macrohabitat. Fresh water.
- Geographical distribution. Northern part of South America, such as the geographic region Amazonas, transplanted to public aquaria around the world, e.g. Denmark (Buchmann *et al.*, 1994).
- Morphology. Length up to 2.552 mm, width up to 0.138 mm, cirrus coiled with five to seven rings, proximally coiled tubular vagina present, four eye spots, gut connects behind testis, ventral anchor 0.035 mm long, dorsal anchor 0.051 mm long, 14 hooklets, eggs elongate-ovate with proximal filament (Kritsky *et al.*, 1985).
- Microhabitat. Gills.
- Life cycle. Oviparous reproduction.
- Pathogenicity. Serious morbidity and mortality due to heavy infections with several hundreds of parasites per host in confined environments. Gross pathological changes include necrotic areas and inflammation of infected tissue (Buchmann *et al.*, 1994).
- Control. No published information available. However, anthelmintics such as praziquantel or benzimidazoles may be effective.

Family Tetraonchidae

Tetraonchus awakurai *and* Tetraonchus oncorhynchi

- Host. Masou salmon (*Oncorhynchus masou*).
- Macrohabitat. Fresh water.
- Geographical distribution. Japan.
- Morphology. Most parasites with a body length of 1.5 mm, with maximum size up to 2.5 mm.
- Microhabitat. *T. awakurai* preferentially occupies the lower or middle part of the gill filament whereas *T. oncorhynchi* tends to colonize the distal part of the filament (Ogawa and Egusa, 1985).
- Life cycle. Oncomiracidia hatch from embryonated eggs and transmission occurs when the temperature is above 10°C (Ogawa and Egusa, 1985).
- Pathogenicity. Mass mortality has been reported associated with heavy infections in which intensities exceed 1000 parasites per fish.

- Control. Prevention of access of wild infected fish into farms. Probably formaldehyde or anthelmintics such as praziquantel or mebendazole may be effective.

Family Diplectanidae

Diplectanum aequans *and* Diplectanum laubieri (*Paperna and Laurencin, 1979*)

- Host. Sea bass, *Dicentrarchus labrax*.
- Macrohabitat. Marine waters.
- Geographical distribution. Mediterranean, Atlantic Ocean.
- Morphology. *D. laubieri*: length 0.53–1.45 mm, width 0.13–0.27 mm. Four eye spots, spherical pharynx, haptor with two squamodiscs (each with 11–16 rows of sclerites) placed ventrally and dorsally. Dorsal hamuli length 0.042–0.064 mm, ventral hamuli length 0.042–0.062 mm. Seven pairs of symmetrical marginal hooklets 11–12 μm in length. Cirrus straight with a length of 0.101–0.132 mm (Gonzales-Lanza *et al.*, 1991).
- Microhabitat. Gills.
- Pathogenicity. Both species can easily be found in densities of several hundred parasites per host. Haemorrhages in gills may be caused by heavy infections. Hyperproduction of mucus is seen. Inflammation and hyperplasia of gill epithelium, with inclusion of leukocytes, appear (Gonzales-Lanza *et al.*, 1991). Fusion of lamellae with decreased respiratory surface may impair gas exchange. Localized cellular reactions can partly encapsulate *D. aequans* (Oliver, 1977).
- Control. Various anthelmintics may be effective against these parasites.

Furnestia echeneis

- Host. Gilthead sea bream, *Sparus aurata.*
- Macrohabitat. Marine waters.
- Geographical distribution. Mediterranean, Atlantic Ocean and Red Sea.
- Microhabitat. Gills and inner operculum (in heavy infections).
- Pathogenicity. Heavy infections with up to 239 parasites per fish (8–13 cm body length) were found associated with gill rot (Paperna *et al.*, 1977).
- Control. Formaldehyde bath treatment (Paperna *et al.*, 1977).

Family Gyrodactylidae

Gyrodactylus anguillae

- Host. Anguillid eels such as European eel, *Anguilla anguilla*, American eel, *Anguilla rostrata,* and Japanese eel, *Anguilla japonica* (Ogawa and Egusa, 1980; Ogawa and Hioki, 1986).
- Macrohabitat. Fresh water and brackish water.
- Geographical distribution. Europe, North America, Japan.
- Morphology. Hamulus length 0.036 mm. Marginal hooklet sickle length 5.6–6.1 μm. No anterolateral processes of ventral bar.
- Microhabitat. Parasites are mostly found on gills but in heavy infections may be found on fins and skin and in nostrils and pharynx.
- Life cycle. Viviparous reproduction.
- Pathogenicity. Morbidity and mortality of heavily infected fish are probably associated with injuries of gills in connection with attachment and feeding activities of worms. Intensities of up to 20,000 parasites per fish were recorded by Ogawa and Egusa (1980).
- Control. No investigations on effective methods have been conducted. However, addition of sodium chloride to tanks with infected eels kept in fresh water stimulated population increase.

Gyrodactylus salaris

- Host. Salmonids and particularly Atlantic salmon, *Salmo salar*. However, Arctic charr and rainbow trout may serve as susceptible reservoir hosts (Bakke *et al.*, 2002).
- Macrohabitat. Freshwater rivers and lakes. The parasite may survive salinities

up to 15 ppt in fjords and brackish water areas for some time but full-strength sea water kills the worm within minutes.
- Geographical distribution. Norway, Sweden, Finland, Denmark, Russia and Germany.
- Morphology. General anatomy as other gyrodactylids. The shape and size of marginal hooklets, ventral bar and hamuli are species-characteristic. The size of these varies dependent on temperature during development (Mo, 1991).
- Microhabitat. The parasite occurs preferentially on fins, to some extent on the body skin, cornea and nostrils but may also be found rarely in the gill apparatus.
- Life cycle. The parasite is viviparous and three generations (parent individual with embryo-bearing offspring in the uterus) may be seen in one parasite.
- Pathogenicity. Susceptible salmonids allow an extensive population increase of parasites on the fish skin, eventually leading to the death of the host. This probably occurs due to osmoregulatory impairment and secondary infections.
- Control. In Norway, stamping-out procedures of infected hatcheries are conducted. Preferred treatment in hatcheries is formaldehyde addition to tank water. Anthelmintics may be used but are not in general use. In natural waters, such as Norwegian rivers, eradication programmes have been performed for more than a decade to clear infected rivers. Rotenone treatment of rivers kills both hosts and parasites and subsequent restocking with the same fish strain has been performed with some success, but a number of treated rivers have been reinfected. Addition of aluminium compounds to rivers is now in progress due to the known effects of aluminium on gyrodactylids (Soleng *et al.*, 1999).

Gyrodactylus derjavini

- Host. Salmonids. Brown trout and rainbow trout are particularly susceptible, whereas Atlantic salmon is relatively resistant but may harbour a few parasites in infected areas (Buchmann and Uldal, 1997).
- Macrohabitat. Freshwater rivers, lakes and trout farms.
- Geographical distribution. Present in Norway, Sweden, Denmark, Scotland, France, Poland and Germany.
- Morphology. General gyrodactylid shape and anatomy. May be discerned from the shape and size of marginal hooklets, ventral bar and hamuli. The size of these vary dependent on temperature during development (Mo, 1993).
- Microhabitat. Selects preferentially fins of the host and the body skin to a lesser extent. However, the site selection is dynamic and may change from primary preference for pectoral and pelvic fins to a higher affinity for corneal surfaces when the host reacts (Buchmann and Uldal, 1997).
- Life cycle. The parasite is viviparous and three generations may be seen in one individual with pregnant offspring in uterus.
- Pathogenicity. Parasite populations may reach high levels, approximately 1500 specimens in one fingerling. Mortality is correlated to infection level in fry (Busch *et al.*, 2003).
- Control. Preferred treatments in ponds are formaldehyde, although more environmentally friendly oxidizing agents such as sodium percarbonate have been introduced (Buchmann and Kristensson, 2003).

Gyrodactylus colemanensis

- Host. Salmonids such as *Oncorhynchus mykiss*, *Salmo trutta*, *Salvelinus fontinalis* and *Salvelinus namaycush* and hybrids between these (Cone *et al.*, 1983).
- Macrohabitat. Fresh water.
- Geographical distribution. North America.
- Morphology. General gyrodactylid outline with an approximate length of 0.45 mm and width of 0.11 mm. Two large hamuli (length 43–51 μm) with ventral bar equipped with well-developed anterolateral processes.

Marginal hooklet with sickle length of 5–6 µm (Cone *et al.*, 1983).

- Microhabitat. Skin and particular caudal, pectoral and pelvic fins, of which the margins are preferred by the frequently relocating worm (Cone and Cusack, 1989).
- Life cycle. Typical gyrodactylid viviparous reproduction.
- Pathogenicity. Although heavy parasite burdens may develop on susceptible hosts, clinical signs are not obvious. This may be related to the lack of inflammation and ulceration at the attachment site (Cone and Wiles, 1989). Whether this is related to the active translocation of parasites is not known.
- Control. Host responses result in marked decreases of the parasite population within 85 days at 10°C (Cusack, 1986). Formaldehyde bathing or exposure to sodium chloride solutions may adversely affect parasite populations without eliminating these totally.

Gyrodactylus salmonis

- Host. Salmonids such as *Oncorhynchus mykiss*, *Oncorhynchus kisutch*, *Salmo clarki*, *Salmo salar*, *Salmo trutta*, *Salmo aquabonita* and *Salvelinus fontinalis*.
- Macrohabitat. Fresh water.
- Geographical distribution. North America.
- Morphology. Typical gyrodactylid outline with a body length of approximately 0.5 mm and a width of 0.15 mm. Hamuli with a length of 53–65 µm and ventral bar with small anterolateral processes. Marginal hooklets with sickle length of 7–9 µm.
- Microhabitat. Body skin, fins, buccal cavity and nostrils.
- Life cycle. Viviparous gyrodactylid reproduction. When reproducing on *S. fontinalis*, populations may reach intensities of 376 parasites per fish 38 to 55 days post-infection.
- Pathogenicity. Mortality and morbidity of *S. fontinalis* fry are associated with infection. Clinical signs of infections in fry are cachexia, lethargy and discoloration. These may be elicited by the adverse effects of marginal hooklets penetrating host epithelia (Cone and Odense, 1984). Heavy infections may cause osmoregulatory disturbances.
- Control. Formaldehyde bath will deparasitize hosts (Cone and Cusack, 1988).

Gyrodactylus katharineri

- Host. Main host is common carp, *Cyprinus carpio*, but occurs occasionally on other cyprinids.
- Macrohabitat. Fresh water.
- Geographical distribution. Originally the parasite was in Asia and has been introduced to Europe and North America with the introduction of carp.
- Morphology. Body length 0.6–1.1 mm. Hamuli are 70–112 µm long. Ventral bar with very long anterolateral processes (14–34 µm). The marginal hooklet sickle length is 8–10 µm.
- Microhabitat. The parasite selects body skin and fins but may occur on the gills and in the buccal cavity.
- Life cycle. Viviparous reproduction as in other gyrodactylids.
- Pathogenicity. Adverse effects on hosts are dependent on intensity of infection and size of host. More than a million parasites were found on an adult carp (Solomatova and Luzin, 1988). Fry and young fish are particularly vulnerable to infection, with the parasite causing morbidity and mortality (Ergens, 1983). Probably the damage caused by the attachment hooks and the feeding activities is responsible for the epithelial damage.
- Control. Bath treatments with formaldehyde and ammonia are partly effective (Solomatova and Luzin, 1988). Mirror carp seems to be relatively resistant to infection compared with the scaled form of carp (Ergens, 1983).

Gyrodactylus turnbulli *and* Gyrodactylus bullatarudis

- Host. Guppies (*Poecilia reticulata*).
- Macrohabitat. Fresh water, both natural waters and aquaria.

- Geographical distribution. Asia, Europe, North and South America.
- Morphology. Hamuli with a length of 57 µm, marginal hooklet sickle length 8.5 µm. Anterolateral processes of the ventral bar are small.
- Microhabitat. Fins and body skin. Preferentially the caudal fin region is parasitized, although other fins are parasitized as well (Harris, 1988).
- Life cycle. Viviparous reproduction and it is dependent on temperature. At 25°C, parasite may give birth four times at 1- to 2-day intervals.
- Pathogenicity. Clinical signs of heavily infected fish are cachexia and discoloration. Frayed fins can be due to mechanical injuries caused by attachment sclerites and feeding activities. The parasite is considered a problem for aquarists.
- Control. Generally infections are treated with formaldehyde, hydrogen peroxide or sodium chloride. Studies by Richards and Chubb (1996) showed that the host mounts a response, leading to decrease of parasite populations. This was also shown by Scott (1985). In addition, a genetic factor has been suggested to play a role in susceptibility, which may lead to breeding for resistance (Madhavi and Anderson, 1985).

Gyrodactyloides bychowskii

- Host. Atlantic salmon, *Salmo salar.*
- Geographical distribution. White Sea, Norwegian Sea (Mo and MacKenzie, 1991).
- Macrohabitat. Marine waters.
- Morphology. Resembles *Gyrodactylus* but the shape of the opisthaptor is different. Thus, the opisthaptor is more oval and the 16 marginal hooklets are arranged in three groups. Two anterolateral groups each composed of four hooklets and one posterior group of eight hooklets are found. In addition, a median sucker is found on the ventral side of the haptor. This is reinforced by sclerotized structures. The marginal hooklets consist of very long and two-segmented handles (Mo and MacKenzie, 1991).
- Microhabitat. Gills.
- Life cycle. Viviparous reproduction.
- Pathogenicity. Many hundreds may be found on larger fish. Infested fish are in poor condition. Histological sections of gill tissue show hyperplasia and hypertrophy of epithelia concomitantly infected with ectoparasitic flagellates and ciliates (Mo and MacKenzie, 1991).

Family Anoplodiscidae

Anoplodiscus tai

- Host. Red sea bream, *Pagrus major.*
- Macrohabitat. Marine waters.
- Geographical distribution. Japanese waters.
- Morphology. Body elongate, slightly tapering at both ends, with a length of 3.2–4.3 mm and a width of 1.35–1.6 mm. Opisthaptor sucker-like without hamuli, bars or marginal hooklets. Four eye spots dorsal to pharynx. The pharynx leads to a single intestine with short irregular side branches. The testis is single and rounded and located in the anterior part of the body. Cirrus tubular. The ovary is anterior to the testis and a vagina is present (Ogawa, 1994).
- Microhabitat. Fins, occasionally on body skin (Ogawa, 1994).
- Pathogenicity. Fins become emaciated, probably due to feeding of the parasite on host epithelia. Haemorrhages occur. Epithelium erodes at the site of haptor attachment. The muscular haptor produces sucking and pinching, eliciting haemorrhages. Inflammatory reactions occur and epithelial hyperplasia around the parasite results. There is massive infiltration of lymphocytes, macrophages and eosinophilic granular cells to the area of parasite attachment.
- Control. No information available but anthelmintics may be effective.

Family Microbothriidae

Dermophthirius

- Host. (*D. melanopteri* on the Pacific blacktip reef shark *Carcharhinus melanopterus*, *D. carcharhini* on the Galapagos shark *Carcharhinus galapagensis*, *D. nigrellii* on the lemon shark *Negaprion brevirostris*)
- Macrohabitat. Marine waters.
- Geographical distribution. Atlantic and Pacific Oceans.
- Morphology. Flat-leaf like worms. Two adhesive organs in the prohaptor. Buccal cavity present. The opisthaptor is small and cup-shaped.
- Microhabitat. Adult parasites attach to the placoid scales by the use of secretory adhesive substances. The haptor may have a sucker-like mode of attachment (Rand *et al.*, 1986).
- Pathogenicity. Clinical signs are erratic swimming with the rubbing of the body surface against objects and the bottom of the fish tank. Parasites feed on the epidermal tissue and ulcerated lesions occur, with loss of scales.
- Control. Chemical treatment with metrifonate or formaldehyde has been shown to be effective (Cheung *et al.*, 1982).

Family Capsalidae

Neobenedenia melleni

- Host. Not host specific, with more than 100 marine teleost species infected (Jahn and Kuhn, 1932; Nigrelli and Breder, 1934; Whittington, 2004).
- Macrohabitat. Marine waters.
- Geographical distribution. North America, South America, Australia.
- Morphology. Length 2–5 mm, width 1.5–3 mm. Two anterior adhesive discs and a posterior ventrally directed haptor with sclerites. The anterior sclerites are 21.6 µm long, anterior hamuli are 29.7 µm, the posterior hamuli are 11.8 µm and the marginal hooklets are 9.8 µm. A vagina is absent.
- Microhabitat. The skin and eyes of the host are preferentially infected but the worm may occur in gills and nostrils.
- Life cycle. Eggs are filamented, tanned, operculate and tetrahedral. They hatch within 5–8 days at room temperature.
- Pathogenicity. Attachment and feeding on the host produce considerable injuries, which can lead to mortality (Deveney *et al.*, 2001). Heavy uncontrolled infections are associated with blindness, scale loss and erosion of epidermis and underlying tissue. Hyperproduction of mucus, discoloration, hyperaemia and haemorrhages can be seen (Kaneko *et al.*, 1988).
- Control. A short freshwater bath may limit the infection. Praziquantel has been reported to be effective against capsalids (Ernst *et al.*, 2003). Cleaner fish have been demonstrated to eliminate parasites from infected hosts (Cowell *et al.*, 1993).

Neobenedenia girellae

- Host. Many species of marine fishes but amberjack (*Seriola dumerili*) and Japanese flounder (*Paralichthys olivaceus*) are particular good hosts.
- Macrohabitat. Marine waters.
- Geographical distribution. Tropical seas, e.g. Mexico, California, Japan.
- Morphology. Body length 3.9–5.2 mm. Oncomiracidium 0.187–0.212 mm.
- Microhabitat. Body skin and fins. Young stages appear on the fins and the adult parasites on the body. Older parasites on the skin, particularly the mouth and eye region, occur on Japanese flounder (Bondad-Reantaso *et al.*, 1995a).
- Life cycle. Eggs with egg filaments are released from the adult worm. Eighteen to 49 eggs may be produced per hour. Eggs hatch within 4 days at 25°C but development is inhibited at 15°C. Oncomiracidia first invade fins and perform their postlarval stages on fins before migration to the skin. First oviposition was observed after 10 days at 25°C (Bondad-Reantaso *et al.*, 1995a).

- Pathogenicity. The parasite feeds on epithelia and may cause similar injuries and reactions to those described for *Neobenedenia melleni*.
- Control. Freshwater bathing may clear infections. Sodium chloride-supplemented sea water or anthelmintics such as praziquantel may be effective. Some acquired protection against challenge infections was found by Bondad-Reantaso *et al*. (1995b) in naturally infected fish. This would suggest that immunoprophylactic strategies can be applied. However, hosts injected with worm homogenate were not significantly protected.

Benedenia monticelli

- Host. Mugilid fish, including *Liza carinata*, *Crenimugil crenilabris*, *Mugil auratus*, *Mugil capito*, *Mugil subviridis* and *Valamugil seheli* (Paperna and Overstreet, 1981; Paperna *et al*., 1984).
- Macrohabitat. Marine waters.
- Geographical distribution. Mediterranean, Red Sea, Gulf of Elat and Suez.
- Morphology. Body length up to 5 mm, width 1.5 mm. Presence of vagina.
- Microhabitat. Mouth of the host is preferentially infected but skin and gills may serve as substrate.
- Pathogenicity. Attachment and feeding on the host produce considerable injuries, which can lead to mortality. Haemorrhages are elicited in the oral submucosa. Intensities of 50–300 worms were found associated with moribund fish.
- Control. No studies are available but presumably formaldehyde or anthelmintic treatments may be effective.

Benedenia seriolae

- Host. Yellowtail, *Seriola quinqueradiata* (Hoshina, 1968; Egusa, 1983).
- Macrohabitat. Marine waters.
- Geographical distribution. Pacific Ocean.
- Morphology. The body is oval and dorsoventrally flattened. The length is up to 9 mm, the width 3–4 mm. The haptor has three pairs of central hooks. Eggs are triangular with a long filament, up to 2.7 mm in length. Egg colour is yellow-brown. The larva is 0.5 mm in length and 0.2 mm in width. Four eye spots are present, which disappear during postlarval development.
- Microhabitat. Fish skin and fins.
- Life cycle. Egg production is temperature dependent. One egg per 50 s is the maximum production rate and a total of 60–200 eggs are delivered at a time. Eggs do not develop below 9°C or above 30°C and 18–24°C is optimum. Larvae are phototactic and survive only 1 day. Development to the adult stage takes at least 2 weeks during the summer season and more than 1.5 months at 17–20°C. The total life cycle takes 20 days during the warm season (Egusa, 1983).
- Pathogenicity. Fish rub against the net of enclosures in order to remove parasites. This will result in injuries and haemorrhages of the skin and secondary infections may occur. Infections are associated with anorexia and growth retardation (Hoshina, 1968).
- Control. Fresh water will kill the parasite (Hoshina, 1968). Sea water supplemented with disodium phosphate peroxyhydrate or pyrophosphate peroxyhydrate is effective (Egusa, 1983).

Entobdella soleae

- Host. Common sole, *Solea solea*.
- Macrohabitat. Marine waters.
- Geographical distribution. North Sea.
- Morphology. The adult worm has a length of approximately 2–5 mm and a width of 1–2.5 mm. The worm is dorsoventrally flattened and equipped with four tiny eyes. The anterior adhesive area is important for attachment and movement. From the pharynx the intestine divides into two lateral branches. A cirrus with an ejaculatory duct is present, as well as a vagina. The ovary is placed centrally in the body, anterior to the two testes. The opisthaptor has both anterior and posterior hamuli, as well as accessory sclerites and marginal hooklets.

The oncomiracidium is equipped with four well-developed eye spots with lenses. The opisthaptor posseses 14 marginal hooklets and both anterior and posterior hamuli with accessory sclerites are present.

- Microhabitat. Skin. The upper part of the body is used for initial settling and later the postlarva migrates to the lower side of the body for reproduction.
- Life cycle. Eggs of *E. soleae* are tetrahedral and possess a stalk with adhesive material for attachment to sand grains or other materials in the environment. Egg development takes 27 days at 13–17°C. The oncomiracidium escapes through an operculum and the hatching process can be induced by host mucus or light (Kearn, 1963, 1974).
- Pathogenicity. A heavy build-up of *E. soleae* populations on the skin of sole kept in captivity was reported by Anderson and Conroy (1968). Several hundred worms may be found on hosts, which can be lethal if left untreated (Kearn, 2002).
- Control. Formaldehyde bath treatments at intervals can cure the infection (Anderson and Conroy, 1968). Other anthelmintics may show effects.

Polyopisthocotyleans

Family Diclidophoridae

Heterobothrium okamotoi (*see Ogawa, 2002*)

- Host. Tiger puffer, *Takifugu rubripes.*
- Macrohabitat. Marine and brackish waters.
- Geographical distribution. Japan.
- Morphology. Length 12–23 mm, eggs with polar filaments.
- Microhabitat. Adult parasites on branchial cavity wall, juveniles on gill filaments.
- Life cycle. Eggs hatch at temperatures between 15 and 25°C and at salinities between 6.7 and 33.4 ppt. The oncomiracidium has a lifespan of 5–9 days and this is dependent on temperature. Self-fertilization can occur.
- Pathogenicity. Lethargy, discoloration and severe anaemia. Adult parasites penetrate to muscle layers of the branchial wall, inducing epithelial hyperplasia and inflammatory reactions in the dermis and subcutaneous tissue. There is necrosis of tissues surrounding the parasite.
- Control. Regular mechanical collection of egg clusters on rope or nets in net pens may reduce the population of parasites.
- Chemical treatment. Hydrogen peroxide bath treatments are effective only against juvenile gill stages. Oral application of caprylic acid may have an effect.

Neoheterobothrium hirame
(*see Ogawa, 1999, 2002*)

- Host. Japanese flounder, *Paralichthys olivaceus.*
- Macrohabitat. Marine waters.
- Geographical distribution. Japanese waters (Ogawa, 1999).
- Morphology. Elongate and fusiform body with a total length of 14–32.9 mm, width 2.4–3.6 mm. The haptor is separated from the body proper by a slender isthmus. The haptor is equipped with eight asymmetrical clamps. The mouth opening is subterminal, with a pair of oral suckers on both sides of the oral cavity. The pharynx leads to a bifurcating intestine with branches. There are numerous testes in the posterior half of the body. The copulatory organ is muscular and equipped with hooks. The ovary is in front of the testes and C-shaped. There is no vagina but there is a genital pore in front of the male organ. The uterus is narrow with only a few eggs. Eggs have a total length of 0.44–0.57 mm with straight appendages at both ends (Ogawa, 1999). The oncomiracidium is 0.25–0.320 mm with five pairs of marginal hooks and one pair of hamuli. No eye spots have been seen (Ogawa, 2000).
- Microhabitat. The parasite first attaches to the gill filaments and then migrates to the gill rakers and finally to

the buccal cavity wall, where oviposition starts (Anshary and Ogawa, 2001).

- Life cycle. Oviposition exceeds 500 per worm above 15°C (Ogawa, 2002). Eggs hatch in filtered sea water at 20°C within 24 h (Ogawa, 2000). Egg production occurs at the buccal cavity wall microhabitat. To reach the adult stage at this site takes 59 days at 15°C but only 31 days at 25°C.
- Pathogenicity. The parasite feeds exclusively on host blood, eliciting anaemia, and this may elicit mortality (Ogawa, 2002). The strong inflammatory reaction is associated with necrosis (Anshary and Ogawa, 2001).
- Control. A sodium chloride-supplemented seawater (30 g/l sea water) bath for 1 h is effective against immature parasites on the gills.

Family Discocotylidae

Discocotyle sagittata

- Host. Salmonids, e.g. brown trout, *Salmo trutta,* and rainbow trout, *Oncorhynchus mykiss* (see Rubio-Godoy and Tinsley, 2003).
- Macrohabitat. Fresh water. The parasite will survive in sea water for a limited period.
- Geographical distribution. Extensive distribution in Northern Hemisphere, Europe, North America and Asia.
- Morphology. Length up to 12 mm, width up to 3–4 mm. The rectangular opisthaptor is equipped with four pairs of clamps for attachment to gill lamellae. Freshly hatched oncomiracidia have one pair of clamps. The additional three pairs of clamps develop in succession until the adult stage has been reached. The adult has numerous follicular testes and a double V-shaped vagina.
- Microhabitat. Gills, particularly secondary gill lamellae.
- Life cycle. Oviposition and development are temperature dependent. The optimum temperature range is 13–18°C, where the developmental rate is highest. Eggs released from the adults embryonate and hatch within 28 days at 13°C. Most embryonated worm eggs hatch within 1 h from onset of darkness (Gannicott and Tinsley, 1997). The oncomiracidia have a lifespan of less than 24 h at 13°C. They attach to the gills, shed their ciliary plates and develop into adults. However, no transmission occurs during cold winter months and accumulated eggs hatch during spring when temperatures rise above 10°C (Gannicott and Tinsley, 1998).
- Pathogenicity. The worm is blood-feeding and anaemia may develop in heavily infected hosts. Intensities of more than 1000 parasites per host occur in trout farms, although wild hosts normally bear only a few parasites (Rubio-Godoy and Tinsley, 2002).
- Control. Anthelmintics such as praziquantel and benzimidazoles may have some effect. It has been shown that hosts produce specific antibodies against the worm and immunization with worm material confers some slight protection against challenge infections (Rubio-Godoy *et al.*, 2003a,b).

Family Microcotylidae

Microcotyle sebastis

- Host. Rockfish, *Sebastes schlegeli*.
- Macrohabitat. Marine waters.
- Morphology. Adult worms about 4 mm in length, 0.5–1.0 mm in width.
- Microhabitat. Gills.
- Morphology. Opisthaptor is well developed, arrow-shaped with about 30 clamps for attachment to gill lamellae.
- Pathogenicity. Morbidity and mortality of infected fish have been reported (Kim *et al.*, 2000).
- Control. Anthelmintics such as praziquantel and mebendazole have effects (Kim and Cho, 2000). Immunization of hosts with worm homogenate confers some protection against challenge infections (Kim *et al.*, 2000).

Allobivagina *sp.*

- Host. Siganid fishes, such as *Siganus luridus*, *Siganus rivulatus* and *Siganus argenteus* (see Paperna *et al.*, 1984).
- Macrohabitat. Marine waters.
- Geographical distribution. Red Sea, Mediterranean.
- Microhabitat. Gills.
- Pathogenicity. Mass mortality due to heavy infections has been reported. Studies on these blood-feeding worms on siganids, showed a significant negative correlation between intensity of infection and host haematocrit values (Paperna *et al.*, 1984). Intensities varied between 50 and 1133 worms per fish and the lower haematocrit values (less than 10%) were found in fish with more than 150 worms. The lowest value (2.5%) was found in a fish infected with 985 parasites.
- Control. No trials have been conducted but anthelmintics such as praziquantel and mebendazole may be effective. Evidence for development of acquired immunity has been noted (Paperna *et al.*, 1984).

Heteraxine heterocerca

- Host. Japanese yellowtail, *Seriola quinqueradiata.*
- Macrohabitat. Marine waters.
- Geographical distribution. Japanese marine waters.
- Morphology. Length up to 17 mm but mostly 5–10 mm.
- Microhabitat. Gills.
- Life cycle. Eggs with long filaments entangle material in the environment, e.g. net meshing in cage culture. Oviposition occurs between 10 and 28°C, with an optimum at 18–25°C. Often 300–800 eggs are delivered at a time (Egusa, 1983).
- Pathogenicity. Infected fish appear lethargic and anorexic. Discoloration of skin, muscle, gills, liver and kidney is found. Anaemia is hypochromic and microcytic and it is often associated with the infection as the adults are blood feeders. Condition factors, lipid and glycogen stores in muscle and liver were lowered. Serum protein picture was disturbed (Eto *et al.*, 1976).
- Control. Sea water supplemented with sodium chloride may affect adult and juvenile parasites. Likewise, supplementation of sea water with oxidizing agents, such as pyrophosphate peroxyhydrate or disodium phosphate peroxyhydrate, is effective for eradication of worms. Anti-fouling agents containing organic tin suppress the infestation (Egusa, 1983). Anthelmintics, such as praziquantel or others, may be effective.

Summary and Conclusions

Monogeneans comprise a group of parasitic flatworms with a relatively high host specificity and high species richness, occupying both marine and freshwater ecosystems. Despite a well-adapted relationship between host and parasite, severe morbidity and mortality may develop in association with epizootic outbreaks in confined environments with excessive infection pressure. This is particularly evident in aquacultural enterprises. Thus, a large number of monogenean species are now known to cause severe problems in both marine and freshwater rearing facilities and it is expected that numerous additional monogeneans will show their pathogenicity whenever their preferred hosts are domesticated with their corresponding monogeneans. In addition, serious outbreaks, leading to mass mortalities of hosts, have been recorded when anthropogenic transfer of parasites has resulted in the spread of the parasite in wild host populations with a low innate resistance to the parasite. The direct life cycle of monogeneans, which are oviparous (most of the described species), ovoviviparous or viviparous (e.g. most gyrodactylids), is a prerequisite for the fast development of outbreaks whenever suitable abiotic and biotic conditions are present.

The pathogenicity of the worms is dependent on their reproductive potential,

mode of attachment and feeding mechanisms. Thus the monopisthocotyleans are epithelial feeders, whereas polyopisthocotyleans are blood feeders. Generally the number of worms in relation to host size is important for the development of disease. Severe hyperplasia of affected tissue due to parasite activities will impair normal physiological activities of the host. Thus, destruction and fusion of gill filaments and lamellae in gill parasite infections (both monopisthocotyleans and polyopisthocotyleans) may lead to asphyxiation. Direct blood feeding of polyopisthocotyleans can cause severe anaemia.

Classical diagnoses of worms are mainly based on morphological characters of sclerotized structures, such as hamuli, hooklets, connecting bars, cirrus, accessory cirrus or vagina, if present. Modern molecular techniques using DNA sequences encoding genes for ribosomal RNA are now widely applied. Likewise, variations of gene sequences of mitochondrial DNA in monogeneans have found wide application for differentiation of strains and lineages. Additional gene sequences may be used in the future.

Control (e.g. mechanical, biological, chemical) methods are widely applied. In addition, immunological studies have suggested that immunoprophylactic measures may have some effect. Hygienic measures may reduce or even prevent introduction of monogeneans into new production systems. If parasites are present, mechanical systems may be used to collect and remove monogenean eggs in order to reduce infection pressure. In addition, filtration of production water can eliminate eggs and larvae. Studies have suggested that biological control may be applicable in culture. Thus, cleaner fish take monogeneans from the body surface of infected fish and microfaunistic elements such as turbellarians and copepods may ingest monogenean eggs and larvae, respectively. Traditional chemical control efforts have been based on the use of various salts and formaldehyde. Hydrogen peroxide formulations are now widely used as environmentally safe control-bath treatments. Also metal salts are now being tested, both in culture systems and in natural waters. Several anthelmintics have been tested for their efficacy and have proved their effect. Thus, organophosphates (metrifonate, dichlorvos), benzimidazoles (mebendazole, flubendazole) and isoquinoline pyrazines (praziquantel) have been used succesfully. However, due to drug legislation, many of these drugs are not allowed in many countries. Immunoprophylactic measures may add to antiparasitic control programmes in the future. A number of studies have indicated that fish respond to monogenean infections by using a range of immunological elements. Most studies suggest that primarily non-specific elements of both cellular and humoral origins contribute to the anti-parasitic response. However, specific immunoglobulins have also been detected and vaccination efforts have been found protective especially against gill parasite infections. Due to the serious impact of monogeneans in both cultured and wild host populations, these helminths should be effectively surveyed and actions should be taken to prevent introduction of monogeneans into new host populations. Basic studies on the biology, ecology and physiology of the parasite and host–parasite interactions may provide new ways to control diseases caused by monogeneans.

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10 Digenea (Phylum Platyhelminthes)

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Introduction

The Digenea (previously termed digenetic trematodes) are one of the three major taxa of parasitic Platyhelminthes, the other two being the Cestoda and the Monogenea. Digeneans are heteroxenous (i.e. they require more than one host to complete their life cycle), and their adult stage is parasitic in vertebrates. All major groups of vertebrates serve as hosts for adult digeneans. Apart from being hosts to adult digeneans, fish may also be infected by the metacercarial larval stage. With one exception, digeneans undergo part or all of their larval development in molluscs. Members of the genus *Aporocotyle* complete their larval development in polychaete annelids (Koie, 1982).

Very few adult-stage digeneans are known to cause significant harm to the fish host. Notable exceptions are the extra-intestinal parasites, such as sanguinicoliid blood flukes, the cyst-forming didymozoids and the skin-inhabiting *Transversotrema* spp.

Metacercarial infection in fish is the main source of disease, with subsequent economic loss. Metacercariae may affect growth and survival, or disfigure fish so that they lose their market value as a food or ornamental product (Paperna, 1991, 1996). Some metacercariae in fisheries and aquaculture products (fish and shellfish) are a source of infections in humans and domestic animals (Ito, 1964; Deardorff and Overstreet, 1991). Metacercariae of *Nanophyetus salmonis* also transmit rickettsial infection ('salmon poisoning') to dogs (Philip, 1955).

In the first edition we introduced the reader to many aspects of fish trematode biology and the wide range of interactions between digeneans and their piscine hosts. The focus of the current review is on parasites that cause disease in fish and the circumstances that promote the deterioration of the steady-state balance between the fish and its associated parasites. We shall also update the reader with the recent practical aspects of the relationships between digeneans and their fish hosts. The development in molecular methodologies did not bypass research on digeneans. We shall have a special section on molecular methodologies for the study and diagnosis of fish trematode infections. These methodologies have also been applied succesfully to explore digenean phylogenesis and, more importantly, to unveil life histories and consequently to provide a precise tool for specific identification (Cribb *et al.*, 1998; Levy *et al.*, 2002).

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Distribution and Prevalence

Host range and geographical distribution

The most important limiting factor for digenean dispersal is the molluscan hosts. Molluscs that serve as the first intermediate host belong to the Gastropoda and Pelecypoda (bivalves), but a few digeneans also develop in Scaphopoda (e.g. an elasmobranch parasite, *Ptychogonimus megastoma*, in *Dentalium* spp. (Palombi, 1941, Wright, 1971)). Marine and freshwater prosobranch snails are the most common intermediate hosts of fish digeneans. All known freshwater and marine Bucephalidae develop in bivalve molluscs. Bivalves are also the first intermediate hosts of Fellodistomatidae (*Fellodistomum*, *Bacciger*), Gorgoderidae (*Phyllodistomum* and *Gorgodera*) and Allocreadiidae (*Allocreadium, Bunodera*, *Crepidostomum*) (Yamaguti, 1958; Hoffman, 1967; Schell, 1970). Except Cyathocotylidae and *Apatemon* spp., which develop in freshwater prosobranch operculids, all other strigeids, as well as Clinostomidae and Plagiorchidae, are associated primarily with pulmonate snails (Yamaguti, 1958; Hoffman, 1960, 1967; Lo *et al.*, 1982; Finkelman, 1988). However, Sanguinicolidae of marine and freshwater fishes diverge in their choice of intermediate hosts, and they include both pulmonates and operculids. The marine members can also be found in bivalves and in polychaetes (Smith, 1972). Specificity of individual digenean species to the molluscan host seems to be very restricted, usually to species, or even to subspecies. However, this has only been demonstrated in non-piscine digeneans, notably in human schistosomes (Paperna, 1968a; Wright, 1971). Some digeneans infecting widely distributed fish (such as the grey mullet, *Mugil cephalus*) seem to be less specific in their choice of molluscan host (Paperna and Overstreet, 1981).

Many digeneans of land vertebrates use fish for their metacercarial stages. Metacercariae are frequently the most common infections in fishes in the marine, estuarine and lacustrine littoral zones. These nutrient-rich waters are equally attractive to fish, molluscs and piscivorous birds; hence infections by skin, gill and visceral metacercariae are high. The high natural infections of young-of-the-year plaice with *Cryptocotyle lingua* and *Stephanostomum baccatum* in the north-east Atlantic littoral zones are well documented (MacKenzie, 1968; MacKenzie and Liversidge, 1975). There are also heavy heterophyid muscle infections in grey mullets and in juveniles of other fishes in lagoons and inshore marshes and estuaries in the eastern Mediterranean Sea. These areas are inhabited by high populations of the intermediate host *Pirenella conica* (Fig. 10.1A; Paperna, 1975; Paperna and Overstreet, 1981; Taraschewski and Paperna, 1981; Taraschewski, 1984). The Syrian–African rift is a major migratory route for birds between Europe and Africa. Water bodies from the Jordan to the East African Great Lakes have common fishes (cichlids, *Clarias* and *Barbus*), snails (*Melanoides tuberculata* (Fig. 10.2), *Bulinus* (Fig. 10.1B) and *Lymnaea* spp.) and metacercariae whose definitive hosts are herons (Ardeidae), cormorants (*Phalacrocorax* spp.) and pelicans (*Pelecanus onochrotalus*). Gill infection

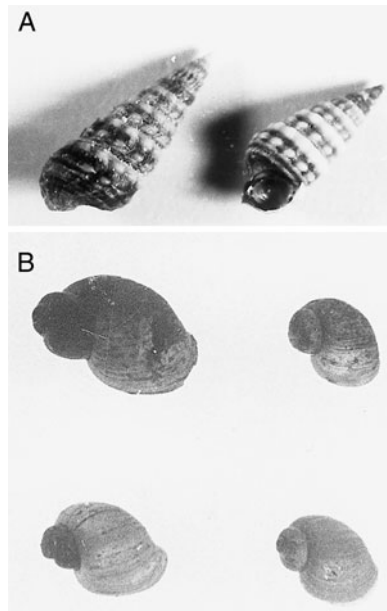


Fig. 10.1. A. *Pirenella conica* (actual size 15 mm). B. *Bulinus truncatus* (actual size 6–11 mm).

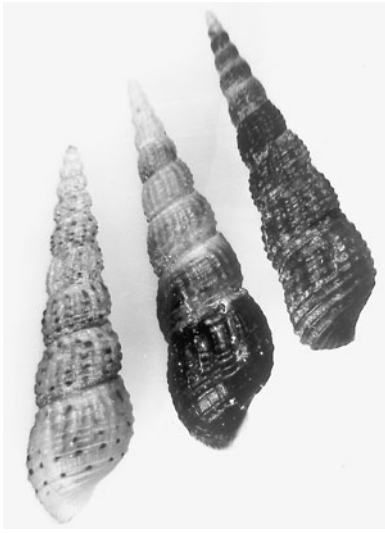


Fig. 10.2. *Melanoides tuberculata* (actual size 20 mm).

with the heterophyids *Centrocestus* spp. and *Haplorchis* spp., transmitted by *M. tuberculata*, occur in all young-of-the-year cichlids that inhabit shallow waters (Farstey, 1986; Paperna, 1996). *Bulinus* and *Lymnaea* are more fastidious in their habitat demands, and demonstrate, particularly in the seasonal Mediterranean zone, greater seasonal and annual variations in abundance (Paperna, 1968b, c), and likewise the metacercariae they transmit (*Neascus* (black spot) and flesh and brain diplostomatids and clinostomids) (Khalil, 1963, 1969; Paperna, 1964a, 1996; Britz *et al.*, 1985; Yekutiel, 1985; Finkelman, 1988; Dzikowski *et al.*, 2003a). In the temperate inland waters of the northern hemisphere, infections by metacercariae of strigeoid digeneans are particularly common. The common infections in North America are 'white grub' (*Posthodiplostomum minimum*), encysting in the viscera (Hoffman, 1960; Grizzle and Goldsby, 1996), species of *Bolbophorus*, encysting in muscles (Overstreet *et al.*, 2002), 'black grub' (pigmented skin metacercariae – *Uvulifer ambloplitis* and *Crassiphiala bulboglossa*), muscle metacercaria of *Hysteromorpha triloba* (*Neascus musculicola* (Hoffman, 1960; Huggins, 1972)) and 'yellow grub' (*Clinostomum marginatum* (Hunter and Hunter, 1934)). *C. marginatum*, together with 'diplostomulum' and 'neascus', has been reported to infect fish (*Poecilia gillii*) in dry-season pools fringing a stream in Costa Rica (Chandler *et al.*, 1995). The diplostomatid eye infections are important and common in European and North American freshwater fishes. Dubois (1953) considered the American *Diplostomum flexicaudum* to be the European *Diplostomum spathaceum.* Both parasites develop to adult stage in gulls (Hoffman, 1960). *D. spathaceum* is in more than 125 fish species (Hoglund, 1991). Parasitic cataract, leading to blindness, has been reported in rainbow trout farmed in Patagonia, Argentina, where the local snail *Chilina dombeiana* is the molluscan host (Semenas, 1998). From five sites in the St Lawrence River, 12 fish species have been found infected with metacercariae of *Diplostomum* spp., with benthic fish (brown bullhead, *Ictalurus nebulosus*, and white sucker, *Catastomus commersoni*) having the heaviest infection. *Diplostomum* spp. metacercariae are in the vitreous humour of brown bullheads, and this is the same for the vast majority of diplostomatids in the other fishes (Marcogliese and Compagna, 1999). Wooten (1974) and Lester and Huizinga (1977) reported *Diplostomum adamsi*, *Diplostomum gasterostei* and *Tylodelphis clavata* from the humour or the retina of the eyes. Ocular diplostomiasis has been reported to occur in juvenile cichlids and *Barbus* spp. from East and South Africa (Thurston, 1965; Mashego, 1982; Paperna, 1996), while in Lake Kariba eye infection in *Tilapia rendalli* and *Oreochromis mortimeri* has been about 85% (Douellou, 1992).

The host range and geographical distribution of trematodes have also been altered through human intervention. Snails (carrying larval trematodes) have been translocated with fish culture stock or the ornamental fish trade from one geographical region to another (Scholtz and Salgado, 2000; Scholtz *et al.*, 2001). This allowed the expansion of piscivorous bird trematodes, with metacercariae that are not piscine host specific. Some of these resulting infections, most notoriously *Centrocestus formosanus*

and *Haplorchis pumilio*, dispersed through the introduction of the Afro-Asian snail *M. tuberculata*, became health risks for both wild and farmed fish in many water bodies in the USA and Mexico (Mitchell *et al.*, 2000; Brandt southwest.fws.gov/fishery/trematode.pdf; Scholtz *et al.*, 2001). Four aquacultured fish species (*Ictalurus punctatus*, hybrid *Morone*, *Notemigonus crysoleucas* and *Pimephales promelas*) became readily infected when exposed to *C. formosanus* cercariae (Mitchell *et al.*, 2002); in Mexico, farmed carp were also infected. The only definitive host identified was the heron *Butorides striatus* (Scholtz *et al.*, 2001).

Seasonal distribution of adult stages

The seasonal occurrences of digeneans in freshwater fishes have been extensively reviewed (Chubb, 1979). However, a similar review on marine fish is not available. In some digenean infections, fish acquire their parasites by direct cercariae penetration (Sanguinicolidae and Transversotrematidae). *Sanguinicola inermis*, which infects the common carp (*Cyprinus carpio*) in Europe, is transmitted during the summer (Lucky, 1964). *Sanguinicola* sp. infecting cichlid fishes in Lake Kinnereth and the nearby fish ponds in Israel appears by spring and later by autumn (Paperna, 1996). Cercariae of *Aporocotyle simplex* have been found in December, and flatfishes are infected in 6°C water (Koie, 1982). The tube-dwelling polychaete *Lancides vayssieri*, host of larval-stage aporocotyles, occurs in Antarctic waters with temperatures not exceeding 1.6°C (Martin, 1952). The ratio of juvenile to adult digeneans in a fish enables a determination of time of infection. In general, transmission usually takes place during the summer or early autumn (Chubb, 1979).

Infection transmitted via the predation of metacercariae-infected intermediate hosts can be only partially correlated with climatic fluctuations (Scott, 1969; Shotter, 1973). In the North Atlantic, *Lecithobothrys bothryophorum* infections in juvenile *Argentina silus* peak in May (Scott, 1969). The same has been reported for whiting (*Odontagatus merlangus*) infected with *Derogenes varicus*, *Hemiurus communis*, *Stephanostomum pristis* and *Lecithaster gibbosus* (Shotter, 1973). In the North Atlantic, infection by *Derogenes* and *Hemiurus* may be linked to peak abundance of zooplankton in the summer (Shotter, 1973). It becomes more complicated when there are several optional or consecutive intermediate hosts (e.g. *D. varicus*; see Koie, 1979a, 1985). European sea bass (*Dicentrarchus labrax*) become infected with *Bucephalus haimeanus* upon entry into estuarine habitats, where both molluscan hosts (*Cardium edule*) and metacercariae-infected gobies occur (Matthews, 1973b). Feeding on plankton has been correlated to *Bunodera luciopercae* infection of *Perca fluviatilis* in fresh water (Chubb, 1979). There is seasonal transmission of *Allocreadium fasciatus* in tropical waters in India and this has been assumed to be related to blooms of copepod populations following the monsoon season (Madhavi, 1979).

Seasonal distribution of metacercariae infections

It is difficult to extrapolate seasonal variations from direct counts of metacercariae on fish, since such infections normally persist for over 12 months (Donges, 1969). Under these circumstances, infection accumulates with fish age (presumably with size) (Karlsbakk, 2001). Marcogliese and Compagna (1999) suggested that, in the St Lawrence River, fish become infected with metacercariae in their first year of life, and that benthic fish are more prone to infections. In northern Finland, where eye diplostomiasis is widespread in numerous fish species (habitats ranging from freshwater lakes to brackish marine), infestation is stable despite the extremely narrow transmission window between the snail and the fish. Extended longevity and continuous accumulation precluded detection of any seasonal pattern (Valtonen and Gibson, 1997). This is similar in brain infections of *Phoxinus phoxinus* by *Diplostomum phoxini* in two habitats in

Scotland (Barber and Crompton, 1997). In wild *Rutilus rutilus*, in the UK, the abundance of eye diplostomiasis peaks in late June and mid-September each year, while in farmed fish abundance is continuous. This has been explained by mortality among infected wild fish (McKeown and Irwin, 1997). In the Jiangkou reservoir, China, metacercariae of the bucephalid *Dolfustrema vaneyi* infect *Pseudobagrus fulvidraco* in late spring and summer, and the metacercaria has a predilection for the eyes (Wang *et al.*, 2001). Presence of young metacercariae may indicate active transmission; however, differentiation of metacercariae by age is feasible only when they transform with time (e.g. *Bolbophorus levantinus*: Paperna and Lengy, 1963; *Centrocestus* sp.: Farstey, 1986). Seasonal fluctuations in infection can be detected among the young-of-the-year fish or in transitory fish after they enter a new habitat (Lemly and Esch, 1984a). Active shedding of cercariae and infection of fish commonly take place during the warmer times of the year in fresh water (Chubb, 1979; Lemly and Esch, 1984a; Stables and Chappell, 1986) and in marine and estuarine habitats (Matthews, 1973b; Koie, 1975; Cottrell, 1977). In some habitats, infection in snails only peaks in the autumn (McDaniel and Coggins, 1972). Transmission during the winter is rare since snails do not shed their cercariae below 10°C (Stables and Chappell, 1986). There is, however, a report on invasion of *Oncorhynchus mykiss* by *Diplostomum opataceum* at low winter temperatures. This infection had apparently been acquired by predation on *Lymnaea* containing precocious (progenetic) metacercariae (Becker and Brunson, 1966). Another report involves *Oncorhynchus kisutch* in Oregon, which hatch in March and become infected with *Nanophyetus salmincola* by mid-April (Millemann and Knapp, 1970).

D. phoxini infections in *P. phoxinus* in a Swiss lowland alpine lake and in a Scottish highland lake peak by summer (Meuller, 1995; Barber and Crompton, 1997). The favourable ambient temperatures in boreal or alpine habitats are between 10 and 17°C (Bauer, 1959; Wooten, 1974). This represents the low winter temperatures at which activity of pulmonate snails, such as *Bulinus truncatus* and *Lymnaea* (= *Stagnicola*) *palustris*, is interrupted in the south-eastern Mediterranean (Yekutiel, 1985; Farstey, 1986). The latter snails live in aquatic habitats fringing Lake Kinneret. *M. tuberculata*, which inhabits the lake proper, may also be found in deeper waters in the winter. During winter, some snails retain sporocysts that contain xiphidiocercariae, and some rediae may have pleurolophocercous cercariae (Farstey, 1986). Shedding of *Bolbophorus damnificus* cercariae by its snail, *Planorbella trivolis*, is also temperature dependent (Terhune *et al.*, 2002). The pulmonates inhabiting the flood pools fringing the lake are more susceptible to annual flood–drought transitions in their habitat. Successive years of drought and flooding resulted in the elimination or reduction of pulmonate snails and the disappearance of metacercariae of *Neascus*, *Bolbophorus levantinus*, *Clinostomum tilapiae* and *Euclinostomum heterostomum* from the lake-dwelling cichlids for several years (Dzikowski *et al.*, 2003a). However, infections transmitted by lake-inhabiting snails (*M. tuberculata* transmitting *Centrocestus* and *Haplorchis* spp., *Melanopsis costata* transmitting *Pygidiopsis genata* and *Lymnasa* (= *Radix*) *auricularia* transmitting *Clinostomum complanatum*) are not affected (Paperna, 1964a; Yekutiel, 1985; Farstey, 1986; Finkelman, 1988; Dzikowski *et al.*, 2003a). In north-east Thailand, the seasonal pattern of *Opistorchis viverini* occurrence in cyprinid fish fluctuates between high abundance during the rainy season and a low during the dry season ('winter'). The number of metacercariae in fish is often positively associated with infection levels in humans (Sithithaworn *et al.*, 1997). Recruitment of *Haplorchis taichui* in north Thailand is highest during the dry season (Sukontason *et al.*, 1999).

P. conica in marine lagoons fringing the south-eastern Mediterranean and the northern gulfs of the Red Sea continues to shed cercariae (of *Heterophyes* and others) throughout the winter months, when water temperatures of fringing and landlocked sites may drop to below 10°C (Taraschewski

and Paperna, 1981, 1982). Year-round infection by larval digeneans has been reported in *Cerithidea californica* from mudflats in southern California (Martin, 1955). In the perennial habitats of the East African lakes, the *M. tuberculata*-transmitted metacercariae (*Centrocestus* and *Haplorchis*) and a variety of pigmented skin metacercariae (*Bulinus*-transmitted) accumulate uninterruptedly until the young cichlids migrate to deeper waters (Paperna, 1996). A similar year-round recruitment occurs in India of *C. formosanus* and of *Postdiplostomum grayii* in *Apocheilus panchax* (Madhavi and Rukmini, 1991, 1992) and in China of *C. formosanus* in grass carp (Zeng and Liao, 2000).

Infections in farmed fishes

Extensive fish ponds and dam reservoirs, with usually a low fish biomass and established hydrophilic vegetation, provide a stable habitat for freshwater snails, which include vectors of *Sanguinicola* (blood flukes) and metacercariae harmful to fish, notably eye flukes. These habitats are also attractive to piscivorous birds. In warm latitudes, as in Israel, intensive-culture earth ponds, cultivating mainly carp, with their heavy organic and nitrogenous load and muddy (eutrophic) bottom are considered unfavourable habitats for all snails. Omnivorous fish, such as carp and siluroid catfishes, eat thin-shelled snails and snail eggs. Metacercarial infections are episodal only when ponds become obstructed from their routine intensive cultivation (Paperna, 1996). Diversification of the fish culture routine – introduction of new species with growing emphasis on cichlid fish ('tilapia') culture – will result in gradual adjustments of the water-holding facilities. Smaller fish ponds with a firmer substrate (earth or gravel), often plastic-sheltered during winter, will provide an excellent environment for snails (predominantly *M. tuberculata*) to proliferate. These snails are good intermediate hosts for a whole range of heterophyid parasites, in particular *Centrocestus* spp. The new structures do not prevent entry of piscivorous birds that are definitive hosts of these trematodes (egrets, night herons and cormorants). Further intensification has led to the employment of circulation systems – earth and concrete raceways and circular ponds – which additionally favour proliferation of *M. tuberculata*, *B. truncatus* and sometimes species of *Lymnaea* (Paperna, 1996).

In the USA, circulation systems such as raceways and hatcheries similarly become heavily populated with snails (Stables and Chappell, 1986), but transmission is often limited to sanguinicolids (Wales, 1958; Hoffman *et al.*, 1985). Metacercarial infections are usually prevented when piscivorous birds are excluded because of an efficient net system.

Blood flukes (*Sanguinicola* spp.) were implicated in massive mortalities of hatchery rainbow, cut-throat (*Salmo clarki*) and brook trout (*Salvelinus fontinalis*) after their snail hosts *Oxytrema* spp., *Flumincola* spp. and *Leptoxis* (*Mudalia*) spp. became established in the culture system (Davis *et al.*, 1961; Evans, 1974b; Hoffman *et al.*, 1985). *S. inermis* (transmitted by *Lymnaea* spp.) has been reported in pond-reared common carp in Eastern Europe (Lucky, 1964). Anderson and Shaharom-Harrison (1986) reported the introduction of *Sanguinicola armata* with infected bighead carp (*Aristichthys nobilis*) and grass carp (*Ctenopharyngodon idellus*) into fish farms in tropical Malaysia. An unidentified blood fluke became established in cichlids reared in circulation systems in Israel (Paperna, 1996).

Massive metacercarial infections of the gills by *Centrocestus* sp. and of subcutaneous tissues by *Haplorchis* are transmitted by *M. tuberculata* (Sommerville, 1982; Paperna, 1996). Gill infections by *C. formosanus* have resulted in mass mortality in farmed Japanese eels (*Anguilla japonica*) in Japan (Yanohara and Kagei, 1983). Common carp fry are also affected by *Centrocestus* sp. in Indian fish farms (Mohan *et al.*, 1999). Infections have been reported from grass carp (*Ctenopharyngodon idella*) in China (Zeng and Liao, 2000) and from *Puntius* spp. and other cyprinids in north Thailand, where the infection coincided with *H. pumilio*, *H. taichui* and the human

bile fluke *O. viverini* (Sukontason *et al.*, 1999). Another troublesome heterophyid, *Phagicola longa*, accumulates in the truncus arteriosus in farmed cichlids in Israel. Its snail intermediate host is unknown. Skin *Neascus* ('black spot'), muscle infection with *B. levantinus* (Paperna, 1996), and visceral infections of *C. tilapiae* and *E. heterostomum* (Lombard, 1968; Britz *et al.*, 1985; Paperna, 1996), transmitted by *B. truncatus* (Donges, 1974; Finkelman, 1988), interfere with growth and compromise survival of farmed cichlid fishes in Israel and in tropical and southern Africa. Visceral infections by *Euclinostomum clarias* are abundant in farmed and wild *Clarias* spp. in Nigeria (Ezenwaji and Inyang, 1998).

Lymnaea (*Radix*)-transmitted *C. complanatum* are responsible for heavy infection in farmed loach (*Misgurnus anguillicaudatus*) and ayu (*Plecoglossus altivelis*) in Taiwan, and these contribute to growth retardation and reduce survival (Liu, 1979; Lo *et al.*, 1981). *Procerovum cheni* metacercariae cause severe muscle and connective-tissue infections in farmed eels in Taiwan (Ooi *et al.*, 1999). In the USA, Mitchell *et al.* (1982) reported heavy mortality of commercially farmed fathead minnows (*P. promelas*), in Missouri from intensive visceral infection by *P. minimum minimum.* The snail involved is *Physa.*

Trout in farms in Europe are often troubled by eye infections with *D. spathaceum*, which cause blindness (Shariff *et al.*, 1980; Stables and Chappell, 1986). The snails, *Lymnaea* spp., thrive in both earth ponds and raceways. Ocular diplostomiasis also affects farmed channel catfish (*I. punctatus*) in the southern USA (Rogers, 1972). Diplostomatid eye infections have also been reported from pond-reared largemouth bass (*Micropterus salmoides*) and rainbow trout in South Africa (Lombard, 1968). Ocular and brain infections of diplostomatid metacercariae in introduced cichlids (*Oreochromis* spp.) have been reported from Mexico (Garcia Luis *et al.*, 1993).

The snail *Planorbella*, vector of *Bolbophorus* spp. and *C. marginatum* (Lorio, 1989), often occur in channel catfish (*I. punctatus*) ponds (Overstreet *et al.*, 2002; Terhune *et al.*, 2002). Metacercariae of two *Bolbophorus* spp. cause severe losses among pond-reared young channel catfish in the southern USA and infected fish are often rejected by commercial processors (Terhune *et al.*, 2002). *B. damnificus* is vectored by the snail *P. trivolis*, and its definitive host is the American white pelican (*Pelecanus erythrorhynchos*). The life history of the second species, in pelicans and causing protodiplostomulum-stage infections in catfish, awaits further studies (Overstreet *et al.*, 2002). Metacercariae of *C. marginatum* in the flesh of farmed channel catfish in the southern USA pose a potentially similar marketing problem for fish farmers (Lorio, 1989). Infection is also found in other farmed fish, such as hybrid bass (Mitchell, 1995). There is also a report of clinostomiasis (reported as *C. complanatum*, apparently *C. marginatum* (Dzikowski *et al.*, 2004a) in introduced *Oreochromis* spp. from Mexico (Garcia Luis *et al.*, 1993).

Metacercarial exotic infections, most notoriously *C. formosanus* and *H. pumilio*, have been dispersed through the introduction of the Afro-Asian snail *M. tuberculata* and have become a health risk for farmed fish in many water bodies in the USA and Mexico (Mitchell *et al.*, 2000; Scholtz *et al.*, 2001; Brandt southwest.fws.gov/fishery/trematode.pdf). Four aquacultured fish species (*I. punctatus*, hybrid *Morone*, *N. crysoleucas* and *P. promelas*) are readily infected when exposed to *C. formosanus* cercariae (Mitchell *et al.*, 2002). In Mexico infections have spread to the common carp (Velez Hernandez *et al.*, 1998).

In the marine environment, in the eastern Mediterranean, extensively used seawater ponds often support dense populations of *P. conica* (Taraschewski and Paperna, 1981) and in the western Mediterranean, *Hydrobia* spp. (Maillard *et al.*, 1980). *P. conica* is first intermediate host of a number of heterophyids, including *Heterophyes heterophyes*, which primarily infect grey mullets (Mugilidae), but also cichlids and *D. labrax* (Paperna and Overstreet, 1981). *Sparus auratus* postlarvae, in a culture system in

southern France, succumbed to massive infection with *Acanthostomum imbutiforme* metacercariae transmitted via *Hydrobia acuta* (Maillard *et al*., 1980; Euzet and Raibaut, 1985). Less information is available on metacercarial infection in cage-cultured fish: Lysne *et al*. (1994) report *Cryptocotyle* spp. infections in caged Atlantic cod.

Parasite Morphology

Adult-stage morphology

Adult digeneans (Fig. 10.3) usually have a dorsoventrally flattened oval body with a smooth, spiny or corrugated surface (Fig. 10.4), a sucker around the anteroventral mouth and an additional ventral sucker, or acetabulum. Both suckers are used for attachment and locomotion. The digestive system consists of a pharynx connected to the mouth opening, a short oesophagus and two blind intestinal caeca. The excretory system includes flame cells connected to a duct network, which is connected to a posteriorly opening bladder. Most trematodes are hermaphroditic (with cross-insemination). The male system consists of testes, vasa efferentia and vas deferens, and a cirrus pouch containing a seminal vesicle, ductus ejaculatorius and prostate glands. Some also contain a specialized copulatory organ (gonotyl in *Heterophyes* spp.). The female system is comprised of an ovary and paired vitellaria, an oviduct, vitelline ducts, Mehlis' glands and the receptaculum seminis, which open into an ootype. The latter is also connected with a rudimetary vagina – the ductus laureri – and the uterus, through which eggs are evacuated to the genital opening (after Dawes, 1946; Yamaguti, 1958). Eggs are usually oval and operculated (Fig. 10.5).

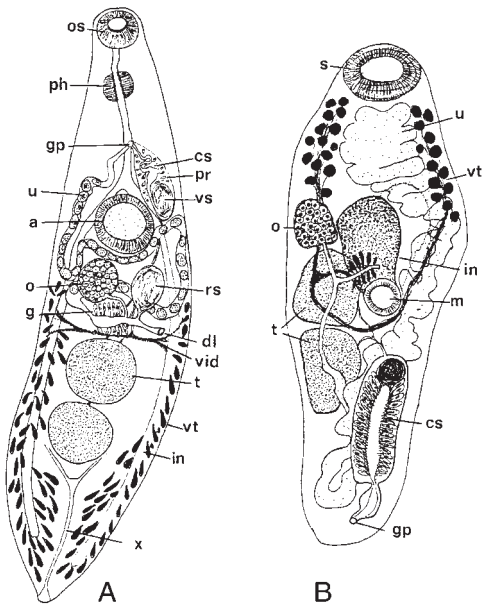


Fig. 10.3. Examples of a 'distome' digenean (A) *Allocreadium isoporum* (drawn with reference to Dawes, 1946, actual size 3–5 mm) and a 'monostome' digenean (B) *Bucephalopsis gracilescens* (Bucephalidae, drawn with reference to Dawes, 1946 and Bychowskaya-Pavlovskaya *et al*., 1962, actual size 6 mm). Abbreviations: a = acetabulum; cs = cirrus pouch (sac); dl = ductus laureri; g = Mehlis glands; gp = genital opening; in = intestine; o = ovary; od = oviduct; os = oral sucker; ot = ootype; ph = pharynx; pr = prostate; s = sucker; t = testis; vd = vas deferens; vid = vitelline ducts; vs = seminal vesicle; vt = vitellaria; x = excretory ducts/bladder.

Extra-intestinal digeneans, which include most adult trematodes pathogenic to fish, are also morphologically and developmentally notable exceptions; these are the sanguinicoliid blood flukes, the cyst-forming didymozoids and the skin inhabiting *Transversotrema* spp. Sanguinicolidae (= Aporocotylidae) are parasites of the blood system. They are slender and spiny and do not have anterior and ventral suckers and pharynx. The intestinal caeca are short and are either X- or H-shaped (Figs 10.6 and 10.7). Eggs are thin-shelled and do not have an operculum; a metacercarial stage is lacking (Smith, 1972).

Didymozoidae are thread-like tissue parasites (*Atalostrophion*, *Metanematobothrium*, for example), with or without an expanded posterior region. They occur either as solitary individuals or in groups or pairs in cysts or cyst-like cavities (Fig. 10.8). Many are hermaphroditic (*Didymozoon*, *Didymocystis*). Equal-sized hermaphroditic individuals of some didymozoids

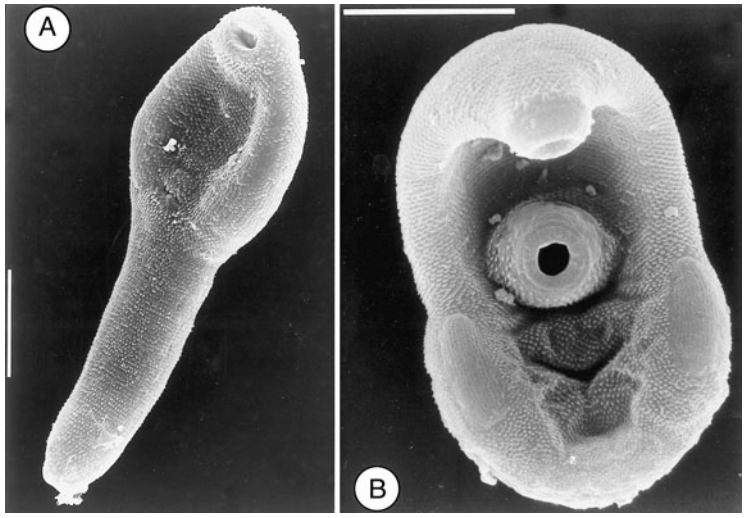


Fig. 10.4. Scanning electron micrographs of adult-stage *Haplorchis pumilio* (A) and *Pygidiopsis geneta* (B) from the gut of a cormorant (*Phalacrocorax carbo*), scales 50 µm.

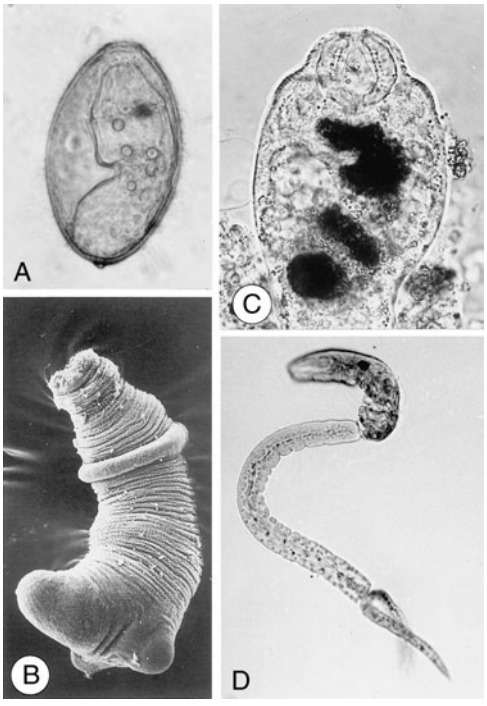


Fig. 10.5. A. *Clinostomum tilapiae* eggs containing ready-to-hatch miracidium (actual size 125 µm × 83 µm). B. Redia of *Mesorchis denticulatum* (scanning electron micrograph, scale 10 µm, courtesy of M. Køie). C. Redia of *C. tilapiae* (live, actual size). D. Furcocercaria of *C. tilapiae* (live, actual size).

share the same cyst, with fused posterior parts. They include *Colocyntotrema* spp., *Phacelotrema* spp. and *Neodiplotrema pelamydis* (Dawes, 1946; Yamaguti, 1958; Fig. 10.9). Others show variable degrees of separation into sexes, with intermediate rudimentary hermaphrodites (Gonapodasmiinae and *Nematobothrium* spp.). Their opposite sex systems are reduced, rudimentary or lacking, and only the eggs of the larger 'female' develop (see Yamaguti, 1958).

The known genera of Transversotrematidae – *Transversotrema* and *Prototransversotrema* – are ectoparasitic under the scales of fish. They have leaf-like, transversely elongated bodies, with or without an oral sucker and with pigmented eye spots (Fig. 10.10; Witenberg, 1944a; Velasquez, 1958; Angel, 1969). The oral sucker is used for attachment to the host skin (Mills, 1979).

Larval stages

Free-swimming miracidia are pyriform in shape and are covered with cilia-bearing epithelial plates. Sporocysts in the intermediate host are tegumental sacs, which enclose germinal cells and developing daughter sporocysts or rediae. The redia has a muscular

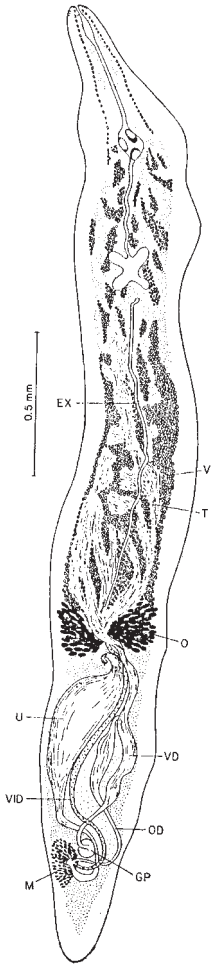


Fig. 10.6. *Sanguinicola dentata* (Paperna, 1964) from *Clarias gariepinus* circulatory system (eggs are released into the kidneys). Actual size 3.4 mm, Abbreviations: EX = excretory duct; GP = genital pore; M = Mehlis glands; O = ovary; OD = oviduct; T = testis; U = uterus; V = vitellaria; VD = vas deferens; VID = vitelline duct.

pharynx connected to a sac-like intestine, a birth pore located near the pharynx, germinal cells and developing daughter larval stages (Fig. 10.11). Cercariae are formed in either sporocysts or rediae (Fig. 10.11). They have a mouth, pharynx, branched intestine, ventral sucker, excretory vesicles and primordia of the genital organs. Cercariae have a pair of eyes and may also have a tail and fins, which are used as locomotive devices. The latter structures are all lost when cercariae transform into metacercariae. The structural diversity of the cercariae (Dawes, 1946; Schell, 1970) reflects the numerous strategies employed to reach the subsequent hosts. Furcocercariae with forked tails are characteristic of Strigeoidea, Sanguinicolidae and Clinostomidae (Fig. 10.5). The bucephalids have typical gasterostome organization and a bifid tail with a very short and broad tail base, and pleurolophocercariae common to Heterophyidae have non-divided tails. Some sanguinicolid and clinostomatid cercariae carry a dorsomedian fin fold. All these cercariae actively penetrate the integument of their hosts. They have anteriorly opening penetration glands, which are absent in cercariae encysting on a substrate (Fares and Maillard, 1974) and in those that infect their hosts via ingestion (of Bivesiculidae (Pearson, 1968) and Fellodistomidae (Koie, 1979b)). For detailed descriptions of the morphological evolution and diversity of digeneans through their various stages of life history readers are referred to the first edition of this volume (Paperna, 1995).

Ultrastructural studies

For a general review of the structure and ultrastructure of adult digeneans readers are referred to earlier reviews (Erasmus, 1977 Smyth and Halton, 1983; Threadgold, 1984). Koie (1971a, b, c, 1985) published detailed observations using scanning and transmission electron microscopy on the intramolluscan stage of fish digeneans. There are numerous recent scanning electron microscopic studies of digeneans (e.g. Hughes *et al.*, 1999; Armitage, 2000; Chai *et al.*, 2000) and a very noteworthy electron microscopic description of the ectoparasitic digenean *Transversotrema licinum* (Abdul-Salam and Sreelatha, 1992).

The digenean integument (e.g. see Cohen *et al.*, 1996, 2001; Halton, 1997; Hughes *et al.*, 1999) consists of an external layer of cytoplasm joined by cytoplasmic bridges, with nucleated masses of cytoplasm lying internal to the body musculature (Fig. 10.12).

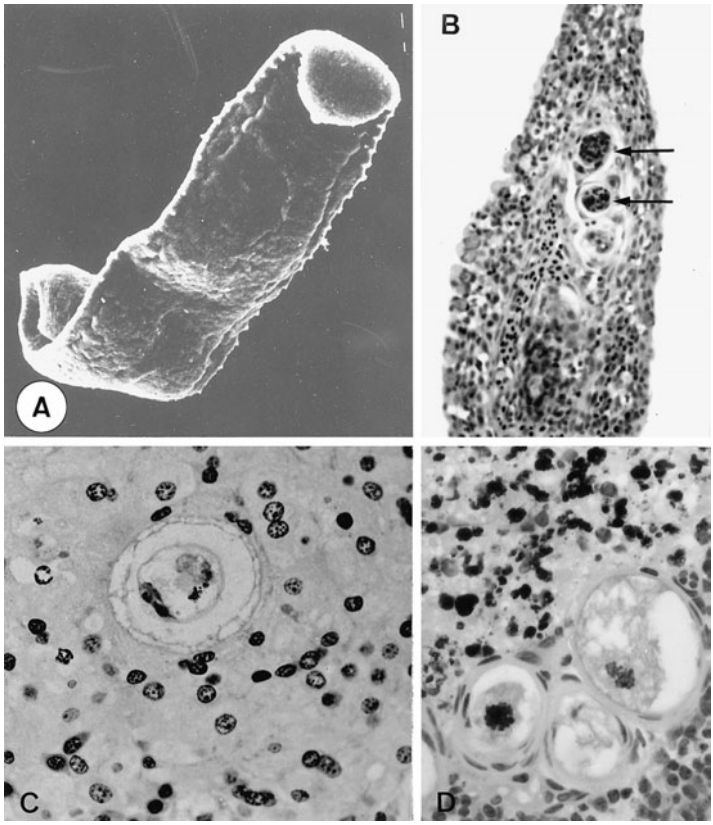


Fig. 10.7. Blood flukes. A. Scanning electron micrograph of *Sanguinicola fontianalis* (from Hoffman *et al.*, 1985, courtesy of the author; bar = 100 μm). B. Eggs of *Sanguinicola* sp. in gills of *Oreochromis aurea*, Lake Kinnereth. C. Egg in gill tissue of *Baryanchistus* sp. (Plecostomidae), Amazon, Brazil. D. Eggs in spleen of *O. aurea*, Lake Kinnereth.

Many digeneans (notably Heterophyidae) have spines protruding through the tegument (Figs 10.4 and 10.13). Regional specializations of the surface are related to functions such as secretion and absorption (for example, the lamellate and highly infolded tegument on the inner face of the adhesive organ of *Apatemon gracilis* (see Erasmus, 1977)). Ultrastructural and biochemical studies have implicated the tegument as a major participant in nutrition: it is replete with organelles of metabolic function and its surface architecture typifies an absorptive epithelium, with folds, microvilli or similar features that amplify the free surface area. Cytochemical uptake and membrane-transport studies have further clarified the dynamic aspects of the tegument (Lumsden, 1975; Pappas and Read, 1975). Lumsden (1975) discussed the possible role of the external layer, particularly the outer glycocalyx, in permeability control, ionic regulation, protection of the parasites against host enzymes and involvement in antibody/antigen reactions.

The syncytial tegument confers a number of paramount advantages on parasitic organisms. First, the absence of cell boundaries means it is less vulnerable to attack and breakdown by host agents, such as digestive enzymes, detergent bile acids and components of the immune system. Secondly, it means that movement of substrates and ions is possible without restriction. The sunken nucleated region is distant from any adverse influence of the host.

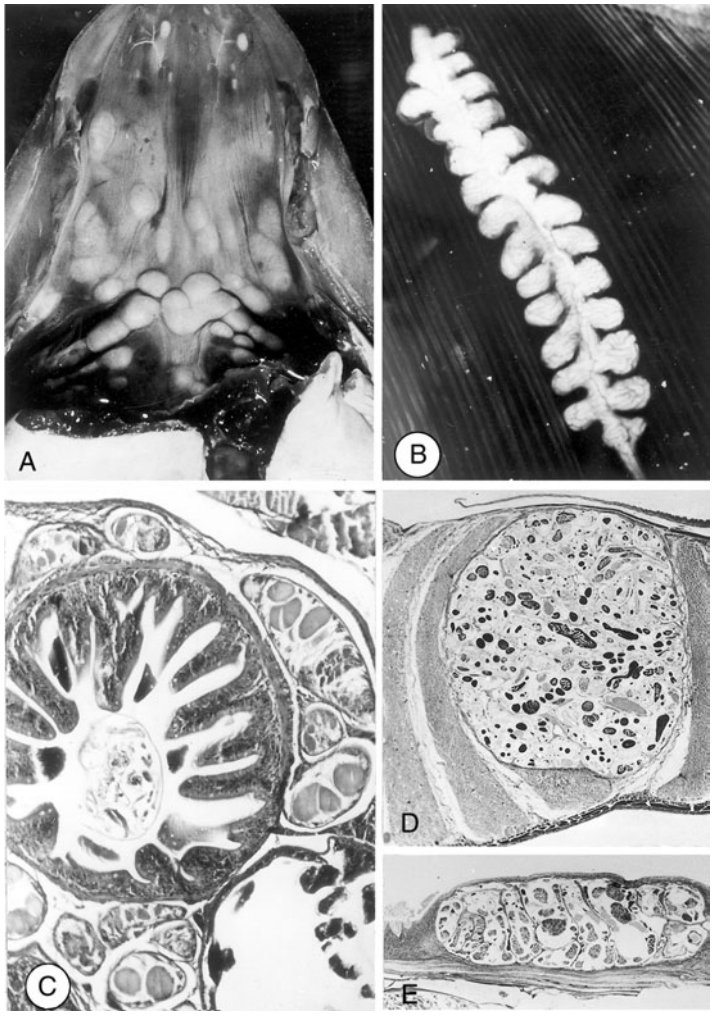


Fig. 10.8. Didymozoidae. A. Palate of *Platycephalus fuscus* infected with *Neometadidymozoon helicis* (in life, bright yellow). From Lester, 1980, courtesy of the author. B. *Lobatozoum multidacculatum* on gills of *Katsumonus pelamis*, New Zealand. C. Larval stages of didymozoids encysted on the surface of the intestine of *Favonigobius exquisitus*. D. Section of *Nematobothrium spinneri* in the body wall muscle of *Acanthocybium solndri*, Queensland. E. *N. helicis* capsule in the body wall of *P. fuscus*. (B–E courtesy of B. Lester.)

The separation of the nucleated regions, although attached to the same cytoplasmic mass, allows regional differentiation and specialization (Halton, 1997).

The addition of scanning electron microscopic studies has provided detailed knowledge of the outer surface (Figs 10.4, 10.7A and 10.13). Tegument surfaces of sporocysts and rediae (Fig. 10.5) show different surface topographies, which probably reflect different nutritive demands (Koie, 1985): *Podocotyle reflexa* sporocysts are covered by long, thin microvilli (Koie, 1971b); the *Neophasis lageniformis* redia has numerous folds, whereas that of *Zoogonoides viviparus* has slightly increased surface area (Koie, 1971a, 1985). Rediae of *Aporocotyle simplex* (Sanguinicolidae) have a nearly smooth surface with numerous canal openings on the tegument (Koie, 1982).

The surface of the cercariae contains oral or cephalic spines and structures possibly connected with sensory function (Koie, 1971c, 1975, 1976, 1977, 1985, 1987). Presumed endocytotic vesicles opening to the surface of *N. lageniformis* cercariae suggest active nutrient absorption by the tegument. These tailless cercariae remain in their rediae until the snail host is eaten by the definitive host (Koie, 1971a, 1973).

All encysting metacercariae are first enclosed in a self-produced, non-cellular granular matrix wall, before becoming enclosed by a cyst wall, which is of host origin (Fig. 10.12; Halton and Johnston, 1982; Faliex, 1991; Walker and Wittrock, 1992). The parasite wall consists of either acid or neutral mucopolysaccharides and is sometimes two to three layers with different textures (in *U. ambloplitis* and *Neascus pyriformis* (Wittrock *et al.*, 1991)). The tegumental surface of encysting metacercariae changes and seems to become specialized for absorption of nutrients by forming short microvillus-like projections (Koie, 1981) or anastomosing folds (Koie, 1977).

Cercaria-type spines (such as are used for penetration) disappear or are transformed into the spines that are characteristic of the adult stage (Koie, 1985, 1987). In *Cryptogonimus chyli*, the parasite cyst stained intensely for acid mucopolysaccharides and moderately for neutral polysaccharides and proteins. The host capsule stained strongly for connective tissue and protein and moderately for lipids, nucleic acids, non-specific esterase activity and acid and alkaline phosphatase activities (Walker and Wittrock, 1999). The gut epithelium plasma membrane is complex. In some species, microvilli with supporting filaments are apparent and the plasma membrane is covered by a granular or filamentous glycocalyx. The ultrastructure of the gastrodermal cytoplasm reflects its

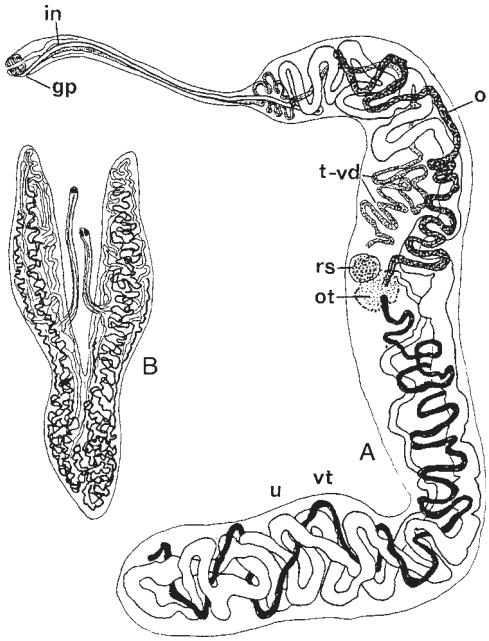


Fig. 10.9. Didymozoidae. A. *Didymozoon faciale* (actual size 16.3 mm). B. *Neodiplotrema pelamydis* – fused pair (drawn with reference to Dawes, 1946 (A) and Yamaguti, 1958 (B); for abbreviations see legend to Fig. 10.3).

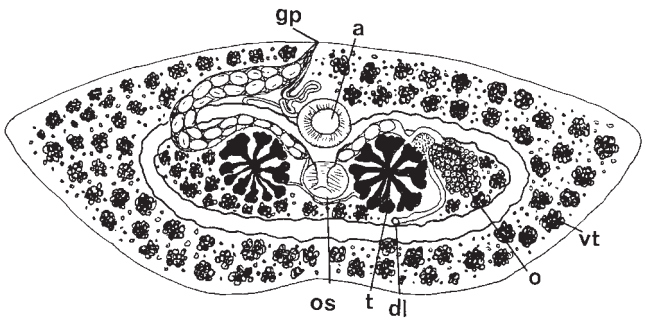


Fig. 10.10. *Transversotrema haasi* (drawn with reference to Witenberg, 1944a; for abbreviations see legend to Fig. 10.3).

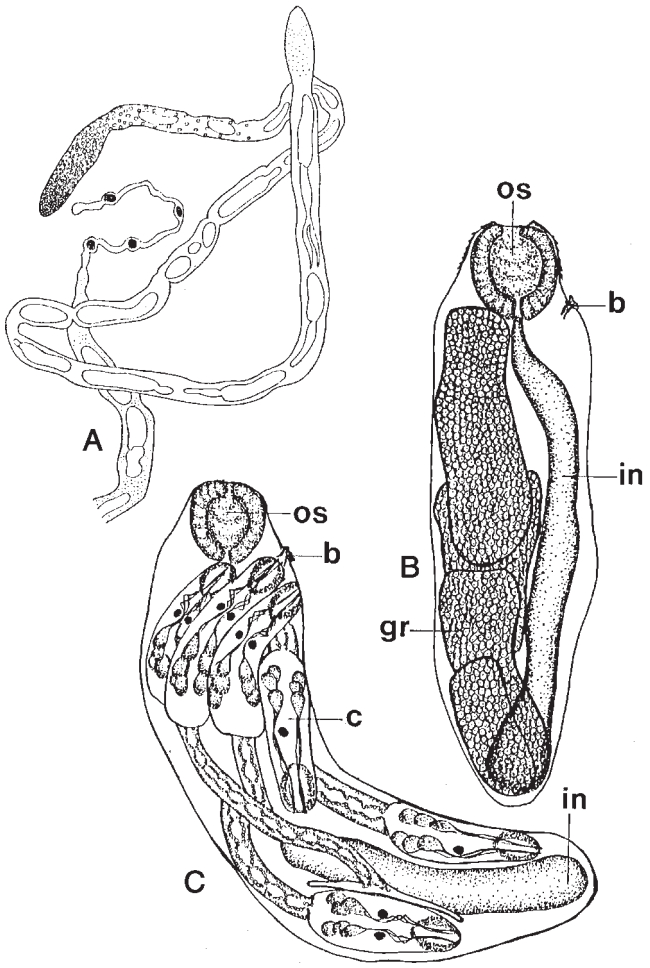


Fig. 10.11. A. *Bolbophorus levantinum* daughter sporocyst with cercariae, characteristic of strigeoid and schistosomatid digeneans (redrawn from Paperna and Lengy, 1963). B.Young and C, mature, daughter redia containing cercariae of *Clinostomum tilapiae*. Abbreviations: b = birth pore; c = cercariae; gr = germinating stages; in = intestine; os = oral sucker.

metabolic activity: it is rich in granular endoplasmic reticulum, mitochondria, Golgi complexes and various inclusions (Erasmus, 1977).

In the redia, the surface of the oral sucker and the oesophagus are continuations of the external integument and are surrounded by secretory glands. The gut epithelial cell plasma membrane forms large (up to 2 μm) projections, folds or microvilli. Gut cells are linked to each other by long septate desmosomes, and contain granular endoplasmic reticulum, free ribosomes, Golgi complexes and particles of alpha-glycogen. The basal membrane forms tubular invaginations, which project into the cell cytoplasm. Beneath the basal membrane is a thin layer of muscle (Koie, 1971a,b; Erasmus, 1977). The cytological features of the cercarial digestive tract resemble those in the redia (Koie, 1971a, 1973). The energy required for cercarial activity is apparently derived from endogenous sources. Gut cells, however, have the ultrastructural characteristics of a metabolically active epithelium and in some instances also

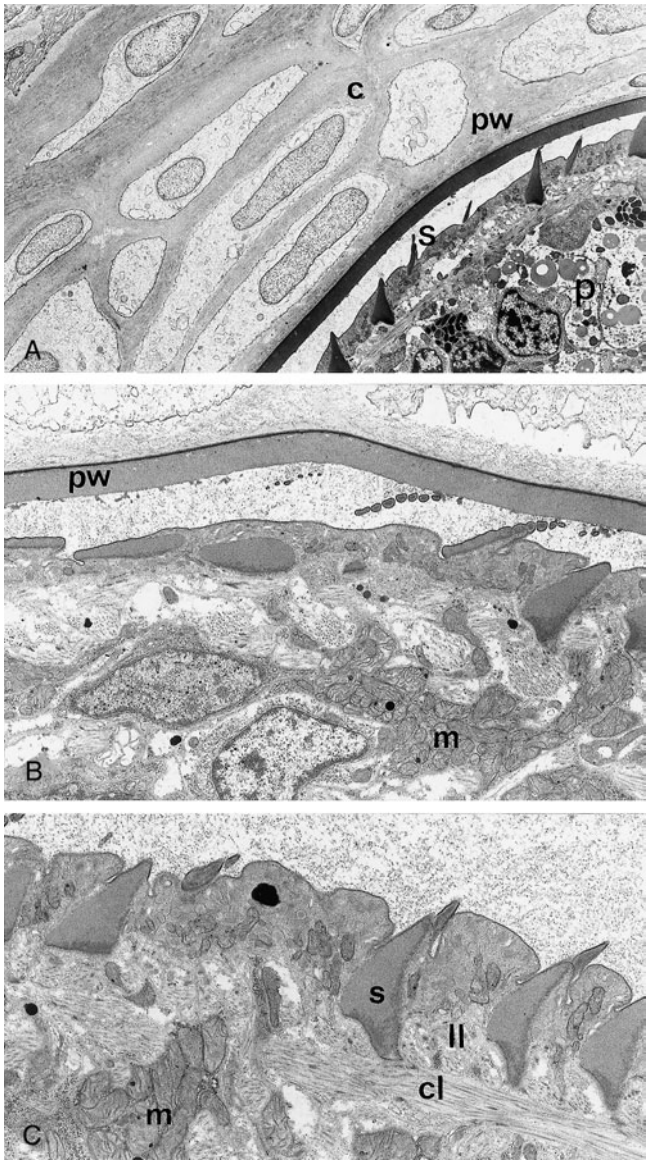


Fig. 10.12. Transmission electron micrographs of *Centrocestus* sp. metacercariae from gills of *Oreochromis aurea*. A. View of the host-produced cartilaginous cyst (c), the parasite-produced wall (pw) enclosing the parasite (p) with the spiny tegument (s). B. Parasite's wall (pw) and the syncytial tegument. C. Enlarged view of the tegument with its spine-carrying border. Abbreviations: cl = circular muscles; ll = longitudinal muscles; m = mitochondrion; n = nuclei of the tegument; s = spines.

demonstrate enzyme activity (see Erasmus, 1977). The surface area of the gut is usually amplified by increasing the size of the absorptive caeca. In Glyauchenidae, parasitic in herbivorous fish (Siganidae), the caeca are relatively small while the foregut is greatly expanded (Jones-Malcolm *et al.*, 2000). The lining of the digestive tube displays a peculiar form of surface amplifications, interpreted to be an adaptation to the predominantly

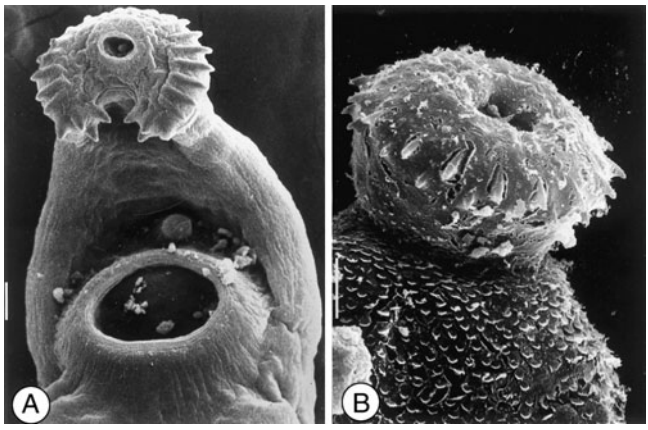


Fig. 10.13. Scanning electron micrographs of adult-stage trematodes from cormorant's gut: A. *Paryphostomum radiatum* (Echinostomatidae) with perioral spines and smooth tegument, scale bar 100 μm. B. *Centrocestus* sp. (Heterophyiidae) with perioral and tegumental spines, scale bar 10 μm.

herbivorous diets of the definitive host. The intestine (as well as the excretory vesicle) of old encysted metacercariae becomes specialized to store excretory products, which are visible as highly refractile bodies (Koie, 1981).

Insight into the penetration mechanism of the miracidium has been provided by an ultrastructural study by McMichael-Phillips *et al.* (1992). In the miracidium of *S. inermis*, a stylet and a rodlet complex are located within the single apical gland, which lies between a pair of lateral glands. The miracidial stylet consists of an arrangement of hollow tubes, composed of microtubules, connected at their base to large secretory granules and opening anteriorly through the apical papilla. A single rodlet lies adjacent to the stylet base and at least eight rodlets are arranged at the apical tip of the stylet. Two sensory endings are associated with the apical papilla. Niewiadomska and Czubaj (2000) provide a detailed fine structural description of the proto- and paranephridial excretory systems in the metacercaria of *Diplostomum peudospataceum*. All protonephridial canals have nuclei and septate desmosomes between neighbouring cells, as well as desmosomes enclosing the canals. Contrary to the protonephridial system, the paranephridial one has a syncytial structure with few cell nuclei in the wall.

Evolution and Taxonomy

The diversity of forms, structures and developmental strategies and a fairly uniform larval development in the molluscan host have led to the suggestion that the protodigenean was a molluscan parasite (Pearson, 1972). The phenomenon of progenesis, i.e. larval stages attaining sexual maturity and yielding eggs while still in the invertebrate host, has been interpreted as a remnant of past life histories (Llewellyn, 1986). In this context an evolutionary relationship has been suggested between Mesozoa and digeneans (see Wright, 1971). Manter (1957) writes of the difficulties in differential diagnosis of Digenea. The life histories of only a fraction of digeneans are known. Many of the cercariae from molluscan hosts or metacercariae on fishes cannot be correlated with established species or genera, and many cannot be affiliated with certainty, if at all, with a particular parasite family. Six undescribed species of *Didymozoon* (recognized by morphological criteria) and *Neometadidymozoon helicis* were confirmed to be distinct species using ribosomal DNA (rDNA) (Anderson and

Barker, 1993). These alternative methods of molecular taxonomy are now gathering momentum as the new methodology for both taxonomy and resolving life history of trematodes.

Molecular diagnosis

The molecular knowledge of Digenea is accumulating rapidly and has great potential in resolving life histories and obtaining new perspectives on their phylogeny (Cribb *et al.*, 2001; Olson *et al.*, 2003). DNA data provide a useful tool to define limits of taxons and for unveiling cryptic species (Overstreet *et al.*, 2002). Specific determination of digenean larval stages, cercariae and metacercariae, by morphological traits is difficult and ambiguous, because of the lack of morphological traits related to the reproductive system. Experimental demonstration of the life history is often unachievable due to the lack of knowledge of the specific intermediate or definitive host. Although hosts and morphology change throughout the life history of these parasites, the genetic composition remains constant.

The use of these methodologies has proved their great potential in creating the genetic linkage between all stages in digenean life history by either comparing ribosomal gene sequences or creating specific DNA probes. Using PCR methodologies enables the targeting of genes even from a single cerceria without DNA extraction (Grevelding *et al.*, 1997). Various ribosomal genes, along with the interspecific polymorphic regions, contain highly conserved regions for which 'universal primers' can be designed in order to amplify this gene from a newly studied species (Hillis and Dixon, 1991; Littlewood and Olson, 2001).

There are already several achievements in resolving life histories of marine fish Digenea, where experimental studies are particularly difficult. The metacercaria of *Indodidymozoon pearsoni* was identified using the DNA sequence of the internal transcribed spacer 2 (ITS2) rDNA (Anderson, 1999). Cribb *et al.* (1998) have demonstrated the three-host life cycle of *Bivesicula claviformis*, showing the genetic identity in the ITS regions of mesocercariae collected from the intestine of the herbivorous fish *Thalassoma lunare* (Labridae) and the adult collected from the intestine of the carnivore *Epinephelus fasciatus* (Serranidae). Using the sequence of this region, together with a PCR-linked restriction fragment length polymorphism (RFLP) approach, Jousson *et al.* (1998) have linked three cerceria species of the Mesometridae family to their adult forms and found that three snail species, *Rissoa similes*, *Rissoa auriscalpium* and *Rissoa ventricosa*, serve as the first intermediate hosts of *Centroderma spinosissima*. Using this methodology, Bartoli *et al.* (2000) have demonstrated the life cycle of *Monorchis parvus*. However, intraspecific variation in the rDNA ITS loci of some echinostomes and of *Paragonimus westhermani* (Sorensen *et al.*, 1998; Van Herwerden *et al.*, 1999) has raised questions regarding the utility of the ITS regions as a diagnostic tool. The small subunit of the ribosomal DNA (SSU-rDNA) due to its systematic versatility was found to be suitable for many phylogenetic studies because of its interspecific polymorphic regions (Hillis and Dixon, 1991; Littlewood and Olson, 2001). The ITS regions may be used as diagnostic confirmation and further be used to investigate intra- and interspecific phylogenetic variations among isolates of poorly differentiated groups.

Sequencing and aligning the 18S rDNA genes allows the development of species-specific DNA probes to distinguish adult *B. daminificus* and an undescribed *Bolbophorus* sp. ('Type 2') collected from pelicans (Levy *et al.*, 2002). Levy *et al.* (2002) have developed and applied DNA probes, designed for polymorphic regions in the 18S rDNA genes, to link adults to their cercariae and metacercariae. Moreover, these probes have been used as an early diagnostic tool to detect infections of young rediae in the planorbid snail *P. trivolis* even before the snail starts shedding cercariae. Similar methodologies have allowed scientists to develop differential assays between *B. levantinus* and *Bolbophorus confuses*, as well as between *C. complanatum* and

C. marginatum (Dzikowski *et al.*, 2003b, 2004a). Species-specific probes for six heterophyid species were developed and applied to demonstrate the genetic linkage between cercariae shed by snails, metacercariae infecting cichlid fish and adult stages found in cormorants (R. Dzikowski *et al.*, 2004b).

We should emphasize that the location of the polymorphic sites of the 18S sequences may not be valid for all species. Additional species with identical sequences in this location and polymorphism will have to be confirmed. Therefore, until more sequence data on poorly investigated groups are available, it is recommended to verify the gene sequence when positive results of the developed species-specific assays are achieved.

Parasite Life History

Host and site specificity

According to Manter (1957), piscine digeneans are host specific, since the majority have been from a single host species or from hosts of the same or related genera. Adult-stage digeneans are primarily parasites of the digestive tract. However, several digenean families have become specialized to extra-intestinal habitats. These digeneans have special structural adaptations as well as functional specializations. Sanguinicolidae (e.g. *Aporocotyle*, *Sanguinicola* and *Cardicola*) are in the blood vessels (Smith, 1972). Transversotrematidae are ectoparasitic, attached under the scales of marine (Witenberg, 1944a), brackish-water (Velasquez, 1958; Angel, 1969) and freshwater fishes (Mills, 1979). Didymozoidae are in cavities and in tissues. Worms occur in the body cavity in *M. cephalus* (Skinner, 1975), in the orbits of *Labeo* spp. (Khalil, 1969) and encysted in the tissue near the body cavity, the mouth, the branchial cavity or the fins (see Yamaguti, 1958). *Nemarobibothrioides histoldi* lives in the skeletal muscles of the ocean sunfish (*Mola mola*) (Thulin, 1980), and *Nemotobothrium texomensis* in the ovaries of *Ictobius bubalis* (Hoffman, 1967). *Didymocystis palali* and *Didymocystis superpalati* are in cysts in the palate of *Neothunnus macropterus.* Their late larval stages and adults are in the same fish host (Yamaguti, 1970). Species of *Phyllodistomum* (Phyllodistominae; Gorgoderiidae) are parasitic in the urinary bladder of fishes.

Other extra-intestinal digeneans belong to predominantly gut-inhabiting taxa. The bucephalid *Paurorhynchus hiodontis* occurs in the body cavity of *Hiodon tergisus* (Manter, 1957). The rhynchi, which are characteristic in other bucephalids, are degenerate in this species. One hemiurid, *Gonocerca macroformis* (Manter, 1957), infects cod (*Gadus morhua*) ovaries. *Acetodextra ameiuri* (Cryptogonimidae) infects the swim bladder, body cavity and ovaries of *Ictalurus* spp. (Hoffman, 1967).

Many digenean metacercariae in fishes have the ability to infect a wide range of hosts. *D. spathaceum* has been reported from more than 125 fish species (Hoglund, 1991). Only a few metacercariae have some degree of predilection for one or a few closely related piscine hosts. Metacercariae of *C. tilapiae, Clinostomum cutaneum* and *B. levantinus* occur only in cichlids, which mostly belong to *Oreochromis* and *Sarotherodon* (Paperna, 1964b; Ukoli, 1966; Britz *et al.*, 1985; Yekutiel, 1985; Finkelman, 1988) Also, some metacercariae which have a wide range of hosts nevertheless have preferences for particular fishes. *Heterophyes* spp. in the eastern Mediterranean prefer grey mullet (Mugilidae) over other fishes and, among the grey mullets, *Liza ramada* (= *Mugil capito*) is the most preferred species (Paperna and Overstreet, 1981).

Lifespan of digeneans

Information on the lifespan of adult digeneans is scanty and has been mostly extrapolated from field data. The lifespan of an adult digenean is highly variable, even between members within the same family. Some adults apparently live for one or two seasons (spring to autumn – *Rhipidocotyle septapapillata* (Krull, 1934), *Asymphylodora kubanica* (Evans, 1978)), whereas others,

mainly in colder habitats, survive for more than a year and are replaced annually (Awachie, 1968; see also Margolis and Boyce (1969) data for *Tubulovesicula lindbergi* and *L. gibbosus* studied in captive Pacific salmon). Survival is temperature-dependent, and overwintering usually prolongs the lifespan. Laboratory studies on *Transversotrema patialense* suggest that, in addition to its dependence on ambient temperatures, its lifespan is also density-dependent (Mills *et al.*, 1979).

Egg release and development into miracidium

Eggs either are undeveloped when laid and begin embryonic development only after evacuation from the host (in Clinostomidae – Donges, 1974; Finkelman, 1988; Fig. 10.5A) or (as in many piscine digeneans) contain fully developed miracidia before being laid and these hatch immediately or soon after evacuation from the definitive host (in *Asymphylodora tincae* – Van den Broek and de Jong, 1979; in *Paucivitellosus fragilis* – Pearson, 1968; in all Haploporidae and Haplosplanchnidae – Manter, 1957; Fares and Maillard, 1974). Hatching in Haploporidae is stimulated by light (Fares and Maillard, 1974). Fully embryonated eggs of Heterophyidae and Azigiidae do not hatch in the aquatic environment, but infect snail hosts after being ingested (Khalil, 1937; Sillman, 1962; Schell, 1970). Eggs produced by digeneans in the kidney or gonads are evacuated from their host with products of the organs. Didymozoid eggs seem to remain viable in tissues long after the worm is dead (Lester, 1980; Gibson *et al.*, 1981), and are liberated only after the death of the host. Host death is a necessary condition also for the transmission of *Aphalloides ceolomicola* parasitic on the gobiid *Pomatoschistus microps*: the eggs accumulate in a fine membrane of parasitic origin in the abdominal cavity of the fish host (Pampoulie *et al.*, 2000). Eggs of blood flukes (Sanguinicolidae), which contain fully developed miracidia, accumulate in the terminal (distal) blood capillaries. Those that reach blood vessels of the gill filaments release their miracidia, which then actively break through the gill tissue into the water (Davis *et al.*, 1961; Smith, 1972; Figs 10.7B, C and 10.14).

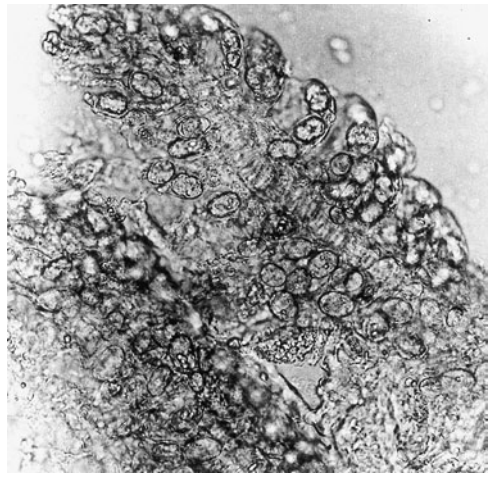


Fig. 10.14. Eggs of a sanguinicolid in gills of juvenile *Liza* sp., Kowie estuary, south-east Cape, South Africa.

Yield of cercariae

The intramolluscan infection with rediae-forming digeneans is usually considerably longer than that with sporocyst-forming digeneans; the sporocyst elapses after yielding rediae or cercaricae, while rediae remain to yield daughter rediae or cercariae. Heterophyid infections in *P. conica* (H. Taraschewski and I. Paperna, unpublished) and in *M. tuberculata* (Farstey, 1986) last over a year, and up to 5 years in *C. lingua*-infected *Littorina littorea* (Meyerhof and Rothschild, 1940). *C. lingua*-infected *L. littorea* shed about 3330 cercariae per day at the early stage of infection, and 830 towards the end of the 5-year period. Daily or periodic cercarial output in pulmonate snails is often similar (Wright, 1971; I. Paperna, unpublished) or even higher (Paperna and Lengy, 1963), but the overall production time is shorter for digeneans in pulmonates, which have only sporocyst stages (Strigeata, Sanguinicolidae, Plagiorchidae). Cercarial production has daily

and periodic fluctuations (Wright, 1971). The periods of low cercarial production correspond to renewals of the germinal cell stocks in the sporocysts or in the rediae (Theron, 1986). Shedding may be stimulated by salinity (tide periods) and temperature, as well as light (day–night cycles), all of which interact with intrinsic periodicity (Wright, 1971). In schistosomes, the timing of the daily emission correlates with periods of activities by the definitive host (Theron, 1986). An adaptive correlation between cercarial shedding and piscine host behaviour has not been established. *T. patialense* are shed at night (Rao and Ganapati, 1967), as are cercariae of *A. gracilis* (Wright, 1971), whereas peak emission of *Heterophyes* spp. occurs by the middle of the day. In *B. levantinus*, the observed daily cycle, however, was not affected by continuous illumination or darkness (Paperna and Lengy, 1963).

Lifespan of metacercariae

Development of *D. spathaceum* is completed at 12°C in 28 days in *P. phoxinus* and *Gobio gobio*, while it takes 35–40 days in *R. rutilus*, 120 days in *P. fluviatilis* (Sweeting, 1974) and 65–85 days in *Salmo trutta* (Chubb, 1979). The lifespan of *D. spathaceum* varies in different hosts and becomes shorter in less compatible ones (Shigin, 1964; Donges, 1969). Sommerville (1981) reported differences in tissue responses among plaice (*Plathichthys platessa*), turbot (*Scophthalmus maximum*), Dover sole (*Solea solea*) and the common dab (*Limanda limanda*) exposed to *Stephanochasmus* (= *Stephanostomus*) *baccatum* infection. In the turbot, which is the least compatible host, the tissue response was the most intense and there was mortality among the encysted metacercariae.

Metacercarial stages in fish

Most digeneans that encyst as metacercariae in fish (Heterophyidae, Diplostomatidae, Clinostomidae) subsequently reach maturity in piscivorous birds. Members of the Diplostomatidae form free (*Diplostomulum*) or encysted (*Neascus*) metacercariae in the eyes of fish (Hoffman, 1960; Donges, 1969). Cercariae of *D. spathaceum* penetrate mainly through the gills and migration via the blood system to the eyes and this is completed within 24 h (Hoglund, 1991), although some migrating diplostomulae may also be found in the subcutis and the muscles of the trunk (Ratanart-Brockelman, 1974). Bucephalidae are the best example of a major group of digeneans with fish as both intermediate and definitive hosts. Definitive hosts are usually predatory fishes (*Esox lucius*, *P. fluviatilis*, *Lophius piscatorius*, *Conger conger* (Dawes, 1946), *Morone saxatilis* (Matthews, 1973a,b), *Micropterus dolomeiu* and *Lepidosteus* spp. (Hoffman, 1967)). However, adult bucephalids have been found in grey mullets, which are not usually predators (Paperna and Overstreet, 1981). The intermediate piscine hosts of digeneans establishing as adults in fishes are small-sized and/or juvenile fishes (Ginetsinskaya, 1958; Schell, 1970; Koie, 1978, 1985).

Metacercarial stages in molluscs and other aquatic organisms

Cercariae may encyst in the tissue of their own molluscan host or emerge and encyst in another individual of the same species (Ginetsinskaya, 1958; Stunkard, 1959; Van den Broek and de Jong, 1979) or in molluscs of different species and taxa (Hopkins, 1937; Palombi, 1937; DeMartini and Pratt, 1964). When encysting in their own host, cercariae may form metacercariae in the rediae, or in the sporocysts in digeneans that do not have a redia stage. An example is *D. spathaceum* in sporocysts in *Stagnicola emarginata* (Dawes, 1946; Lester and Lee, 1976).

The majority of digeneans reach their definitive piscine host via planktonic or benthic organisms. In marine habitats, the second intermediate hosts of *Fellodiplostomum felis* are brittle stars (Koie, 1980); the second intermediate hosts of *Podocotyle atomon* (Opecoelidae) are crustaceans (Amphipoda, Isopoda and Mysidacea) (Ginetsinskaya, 1958; Koie, 1985). Metacercariae of marine lepocreadiids *Opechona bacillaris* and

Lepidapedon rachion occur in Coelenterata, Ctenophora, Chaetognatha and Polychaeta (Koie, 1985) and *Neopechona cablei* occurs in hydrozoan and scyphozoan coelenterates and in ctenophores (Stunkard, 1980), whereas *L. elongatum* occurs in Polychaeta and Mollusca (Koie, 1985). Metacercariae of the zoogoniid *Z. viviparus* have been found in brittle stars, *Ophiura albida,* Polychaeta, lamellibranchs and gastropods (Koie, 1975, 1985). Hemiurid transmission in the marine environment involves planktonic organisms such as *Sagitta*, several medusae, benthic crustaceans, amphipods, copepods and shrimps (Chabaud and Biguet, 1954). The greatest versatility is in *D. varicus*, which has been found in more than 100 fish species, in temperate and subpolar waters and in deep waters of warm seas. The molluscan hosts are *Notica cateria* and *Notica pallida.* Copepods (calanoid and harpacticoid) are the common second intermediate hosts, but infections occur in other crustaceans as well, including hermit crabs. *Sagitta* may become infected after consuming copepods.

Premature adults in fish may be passed via predation to mature in a predator fish (e.g. cod). Mature *D. varicus* may also reestablish themselves following predation in a new host fish (Koie, 1979a). Didymozoidae apparently have similar life histories via planktonic prey–predator chains from copepods via coelenterates, ctenophores, chaetognathes and polychaetes to juvenile fish (Nikolaeva, 1965; Koie and Lester, 1985). Larval forms have also been found in *Lepas* (Cable and Nahhas, 1962). Common second intermediate hosts of Allocreadiidae (*Crepidostomum* and *Allocreadium* spp.) of freshwater fishes are larvae of aquatic insects: mayflies (Ephemeroptera), caddis flies (Trichoptera), dragonflies (Odonata) and Chironomidae. Other intermediate hosts include copepods, cladocerans and annelids (Dawes, 1946; Ginetsinskaya, 1958; Hoffman, 1967; Schell, 1970).

Infection of definitive host

Cercariae develop in fish either to adult stages (Sanguinicolidae, Transversotrematidae and *Azygia* spp.) or to encysted or unencysted metacercariae. Cercariae can penetrate skin or gills (Hoglund, 1991; Sommerville and Iqbal, 1991) or are ingested by the fish host (cercariae of Bivesiculidae, Azygiidae and Fellodistomidae (Hoffman, 1967; Pearson, 1968; Koie, 1979b)). These develop to maturity if ingested by a suitable host, or remain a waiting larval stage, which will develop to maturity only if the paratenic host is predated upon by the definitive host (Van Cleave and Mueller, 1934; Ginetsinskaya, 1958; Koie, 1979a,b). Another option is for the cercariae to enter invertebrate hosts, which are subsequently eaten by fish. Cercariae may also attach to plant material or to some other substrate (encysting into metacercariae) to be later browsed by potential host fishes (e.g. Haplosplanchiidae and Haploporidae) (Cable, 1954; Fares and Maillard, 1974). Although *S. inermis* cercariae can penetrate common carp over its entire body surface, the majority (68%) penetrate the fins (Sommerville and Iqbal, 1991). *Cardicola* (= *Sanguinicola*) *alseae* is only capable of penetrating the soft, ventral surface, and *Cardicola klamathensis* can only enter through the less heavily scaled areas in salmonids (Meade and Pratt, 1965; Meade, 1967). *A. simplex* easily penetrates through the entire body surface of dabs (*L. limanda*), flounders (*Plathychthys flesus*) and plaice (Koie, 1982). *S. inermis* remains in fins of carp for over 3 months following entry and all the worms are in the circulatory system after 5 months (Sommerville and Iqbal, 1991).

A. simplex remains in the lymphatic system and among the muscles for over 94 days while its entry into blood vessels is completed only 180 days after exposure to cercariae (Koie, 1982).

Progenetic generations

Sexually mature stages containing fully developed eggs are sometimes found in the first intermediate molluscan host and in other aquatic invertebrates that are hosts of metacercariae. Eggs are released only after disintegration of the host tissue following

death or predation. In *Asymphylodora* spp., progenetic generation in the molluscan host and also in an annelid second intermediate host (*Nereis diversicolor*, Polychaeta) of the marine species (Reimer, 1973) has been shown to alternate or coexist with adult-stage generation in the definitive host (Stunkard, 1959; Van den Brock and de Jong, 1979). Progenetic stages have also been found in other non-molluscan second intermediate hosts, in amphipods (Hunninen and Cable, 1943; De Giusti, 1962) and in crustaceans and chaetognaths (Koie, l979b). Progenetic metacercariae of *D. varicus* have been found in the parasitic copepod *Lernaeocera lusci*, which infects *Gadus luscus* (Dollfus, 1954).

Feeding and nutrition

As envisaged by Llewellyn (1965), the earliest of flatworm parasites most probably arose from free-swimming rhabdocoel-like ancestors that fed as opportunistic micropredators. The external surface of all parasitic flatworms departs from the turbellarian character and is composed of a multifunctional syncytial tegument. This ultimate boundary between the parasite and the host is a living plasma membrane and is associated with polyanionic coating or a glycocalyx. This boundary is permeable to a variety of small organic solutes, some crossing by passive diffusion and others via facilitated or actively mediated transport (Halton, 1997).

The trematodes inhabit a wide variety of environments within their host, each distinct in terms of the concentration and physicochemical properties of nutrients. In general, most of them reside within a physiological fluid, whether it is blood, extracellular fluid or bile, and thereby, it is assumed, have the opportunity to indulge in tegumental uptake of substrates from the host. If the parasite surface is alive, it can respond dynamically to changes in environmental conditions. It also opens up the possibility, not only of selective transport of materials into the parasite, but also of a regulated two-way flux of molecules and information between host and parasite (Halton, 1997).

The relative roles of the tegument and gut in trematode nutrition are not easy to assess, with variation between species that is often dependent on environmental and other conditions (Pappas, 1988). In the two best-studied trematodes, schistosomes and the liver fluke (*Fasciola hepatica*), the tegument appears to be the route of absorption of the low-molecular-weight solutes glucose and amino acids (Isseroff and Read, 1974; Mercer and Chappel, 1985), but the alimentary tract *in vivo* plays the major role in nutrition and functions primarily in macromolecular digestion and subsequent absorption of soluble digestive products (Halton, 1997).

Little is known about the nutrition and metabolic function of fish digeneans. The blood-dwelling flukes (Sanguinicolidae) feed on blood, while the encysted didymozoids obtain plasma from the capillaries around the cyst and also ingest blood cells (Yamaguti, 1970; Lester, 1980). In the ectoparasitic Transversotrematidae, feeding has been suggested to be similar to the skin-inhabiting capsaloid monogeneans (*Benedenia*), i.e. digestion of the host epidermal cells by proteolytic enzymes excreted from the pharynx (Mills, 1979).

The elaborate tegumental surface seen in ultrastructural studies of sporocysts, rediae and encysted metacercariae of piscine digeneans strongly suggests active nutrient absorption (Koie, 1971a,b, 1985). During migration, the metacercariae of *D. spathaceum* have been shown to utilize their glycogen reserves (Ratanart-Brockelman, 1974). In metacercarial cysts of *Bucephaloides gracilescens*, uptake of labelled substances across the cyst wall and subsequent incorporation by the metacercaria indicates that the encysted larva retains communication with the external environment. The degree of encapsulation varies with encystment site and increases with age; substrate incorporation has been found to be lowered in thicker-walled cysts (Halton and Johnston, 1982). The cyst wall of the large clinostomids is vascularized, which facilitates efficient glucose transport to the

encysted worm. It also lacks the acellular, parasite-induced inner wall. Larson *et al.* (1988) showed that the rate of diffusion through the cyst wall exceeded the rate of transport into the worm. Glucose uptake by encysted *C. marginatum* is not affected by glucose transport inhibitors (phlorizin and phloretin) (Uglem and Larson, 1987). Apparently, the cyst wall is selectively permeable, permitting rapid diffusion of glucose to the worm surface while restricting the movement of the inhibitors.

Host–Parasite Relationships

Pathology

Adult trematodes

Adult intestinal trematodes are normally considered not to cause disease even when their numbers are high. Extra-intestinal parasites are, however, potentially pathogenic. Blood flukes (sanguinicolids and aporocotyles) cause considerable damage to the gills and impair respiration. Adult worms and eggs can physically obstruct the passage of blood and cause thrombosis and subsequent tissue necrosis (Hoffman *et al.*, 1985; Ogawa *et al.*, 1989). Extensive rupture of the gill lining by emerging miracidia and tissue response around trapped eggs in capillaries and internal organs appeared to be the direct cause of mortality of bloodfluke-infected fish (Figs 10.7B–D, 10.14 and 10.15). This is the case in *Sanguinicola fontinalis*-infected brook trout (*S. fontinalis*) in Pennsylvania, USA (Hoffman *et al.*, 1985). In flatfishes (*Hippoglossoides platessoides*, *L. limanda* and *Pleuronectes platessa*) infected with *A. simplex*, fragments of disintegrated worms in the efferent gill filament arteries cause the gill filament to become stunted and necrotic (Koie, 1982). The blood fluke *Paradeontacylix* sp. causes mass mortalities of cage-cultured *Seriola purpurascens.* Infections with up to 1000 worms per single gill filament have been found and they invoke gill hyperplasia. Eggs in the gills and the ventriculum are encapsulated and they induce papillate proliferation of the endothelium of afferent branchial arteries. However, there is necrosis in the gills or elsewhere (Ogawa *et al.*, 1989). Proliferation of the arterial endothelium has also been reported in common carp infected with *S. inermis* (Prost, M., quoted in Lucky, 1964). Heavy blood loss occurs when miracidia escape from the gills and apparently this is the main cause of mortality in trout fingerlings infected with *Sanguinicola davisi* and *Sanguinicola klamathensis* (Wales, 1958; Davis *et al.*, 1961). Loss of blood is evidenced by the pale colour of the gills and the decline in

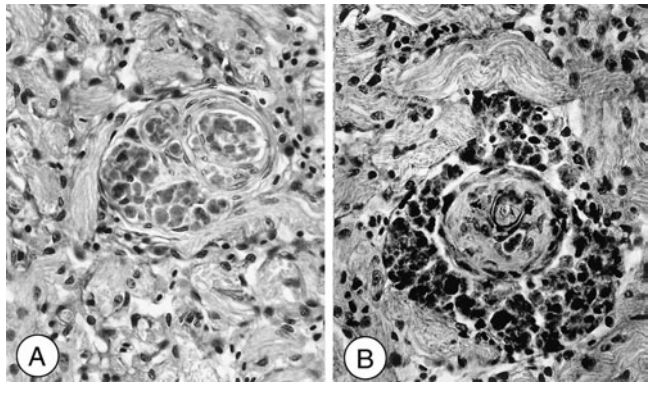


Fig. 10.15. Degenerate eggs of the sanguinicolis *Pearsonellum corventum* in the heart of the fish *Plectropomus leopardus*. A. Encapsulated egg mass enclosed by infiltrated macrophages. B. Granuloma-encased egg residue within a melanomacrophage. (From Overstreet and Thulin, 1989, courtesy of R. Overstreet.)

packed cell volumes and oxyhaemoglobin levels (Evans, 1974b). Heavily infected cultured carp apparently die of suffocation during transportation (Lucky, 1964; Smith, 1972). In chronic infections, adult worms disperse and become stranded in the heart, kidneys and caudal vessels. Some eggs become encapsulated, and may also become surrounded with focal granuloma. Nodular foci occur in the heart, head kidney and spleen of *Oreochromis* spp. (Fig. 10.7D), in the gills, heart and kidneys of carp (Scheuring, 1922) and in the liver of *Paracardicola hawaiensis*-infected *Tetraodon hispidus* (Martin, 1969). Eggs of *S. armata* are widespread in the viscera of grass carp (*C. idella*) and bighead (*A. nobilis*), but the tissue response is negligible (Anderson and Shaharom-Harrison, 1986). Eggs in the kidneys of *S. klamathensis*-infected cutthroat trout (*S. clarki*) cause hypertrophy or necrosis of the renal epithelium and renal calcifications (Evans, 1974a). In sanguinicolosis of cichlid fishes and in *Pearsonellum corventum*-infected serranids (*Plectropomus leopardus*), granulomas form around eggs in the viscera and these are accompanied by melanomacrophage aggregates (Fig. 10.15; Overstreet and Thulin, 1989). Dead *A. simplex* are encapsulated in the superficial parts of the liver (Koie, 1982). General effects on growth and condition are not always evident however, and they are correlated with the duration and severity of the infection. Encapsulated lesions around residues of *Sanguinicola* eggs in the heart have been found in a dying native population of *Sarotherodon galilaeus* in Lake Kinnereth, Israel (Paperna, 1996). Smith and Willams (1967) did not find any apparent effect on weight in hake (*Merluccius merluccius*) infected with *Aporocotyle spinosicanalis.*

Encapsulation of didymozoid worms induces no more than limited local reactions (Fig. 10.8; Lester, 1980; Perera, 1992a, b), although at times there is also haemorrhaging of peripheral capillaries in the buccal dermis of *Platycephalus fuscus* (Lester, 1980). *Nematobothrium spinneri* in the muscles of *Acanthocybium solandri* is enclosed in a thick-walled capsule. Connective tissue and blood capillaries are interdigitated with worm coils, which facilitates worm feeding on blood (Lester, 1980). Only natural infections have been recorded, and these are likely to be moderate. It is not known whether these infections are harmful to maricultured fish. *T. patialense*, an ectoparasite on the *Brachydanio rerio* integument, leaves pressure and feeding indentations on the body surface. Tissue regeneration occurs soon after the worm changes position (Mills, 1979). Kidney damage induced by heavy infection of the urethra with *Phyllodistomum umblae* (= *conostomum*) adversely affects survival of charr migrating from fresh water to sea water (Berland, 1987). *A. kubanica* are pathogenic to *R. rutilus heckeli* in the Sea of Azov when they infect the kidneys rather than the intestine (Bauer, 1958).

Metacercariae

Clinical effects of infection are often not obvious. Metacercariae in supposedly sensitive organs, such as the brain, cranial nerves or spinal cord, e.g. *B. gracilescens* in cod, *G. morhua* (Matthews, 1974), *Diplostomum mashonense* and *Diplostomum tregenna* in *Clarias* spp. (Beverly-Burton, 1963; Khalil, 1963) and *Ornithodiplostomum ptychocheilus* in *P. promelas* (Hoffman, 1958; So and Wittrock, 1982), do not necessarily have obvious debilitating effects on fish even when the number of parasites is relatively high and despite visible structural damage to the organs.

A sudden massive outbreak of infection is often fatal. Exposure to massive numbers of cercariae will kill fry within a few hours (Sommerville, 1982), but such exposures do not normally occur in nature. Cercariae penetrate and encyst deeper in the tissues of small fishes and the relatively larger cysts may interfere with organ function (Fig. 10.16). The effects of cercarial infestation are most severe in 0-year-class plaice and minimal in the 1+-year class (Sommerville, 1981). Hoffmann *et al.* (1990) reported a massive *Bucephalus polymorphus* infestation and mortality of cyprinid fishes after the water temperature was suddenly increased from 12–14 to 20°C.

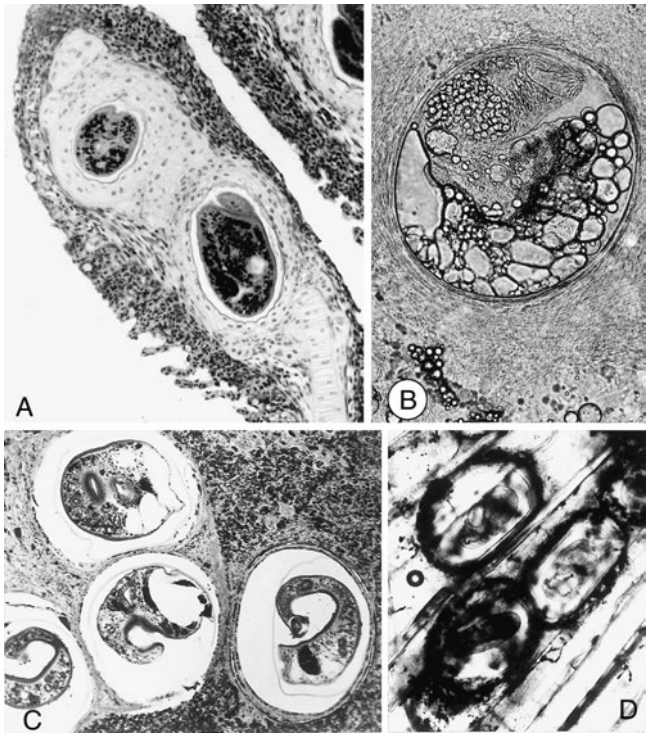


Fig. 10.16. Metacercariae in fish tissues. A. Cartilaginous cyst around *Centrocestus* sp. metacercariae in gills of *Oreochromis aurea,* Lake Kinnereth. B. *Ascocotyle coelostoma* (live) in the truncus arteriosus of *Liza ramada*. C. Encysted *Phagicola nana* in the gut wall of *Micropterus salmoides*. D. Pigmented *Neascus* ('black spot') encysted on fins of young *Oreochromis* hybrids.

Massive metacercariae infections in juvenile (0-class) fish have been incriminated as an important cause of natural mortalities, e.g. *C. lingua* infections of plaice, *P. platessa* (MacKenzie, 1968), *Bolbophorus levantinus* in cichlid fishes (Fig. 10.17B; Yekutiel, 1985; Paperna, 1991). Population studies and field observations suggest that fish heavily infected with metacercariae are lost from the host population (Chubb, 1979; Lemly and Esch 1984a,b). Lemly and Esch (1984b) confirmed that heavily *U. ambloplitis*-infected young-of-the-year bluegill sunfish (50 per fish) did not survive the winter because of depleted reserves.

Entry into tissue and encystment

A pronounced inflammatory response often accompanies penetration and early migration. This is particularly obvious following heavy exposure. Fish may even die from the penetration wounds caused by *D. spathaceum* (Ferguson and Hayford, 1941) and from tissue damage (focal haemorrhages) caused during *H. pumilio* migration in muscles (Sommerville, 1982) and in the brain caused by *D. adamsi* (Lester and Huizinga, 1977). Cercariae of *C. lingua* and *S. baccatus* induce temporary epidermal lesions. They may also cause some disruption of connective tissues, inflammatory cell proliferation, sometimes myofibrillar necrosis (associated with bacteria) and reactive swelling of the intermuscular septa (McQueen *et al.*, 1973; Sommerville, 1981). The inflammatory reaction is particularly intense around non-encysting metacercariae (e.g. *Rhipidocotyle johnstonei* and *Prosorhynchus crucibulum* in plaice and turbot) (Matthews, 1973a, 1974) and precedes the eventual enclosure of the parasite in a fibrous capsule

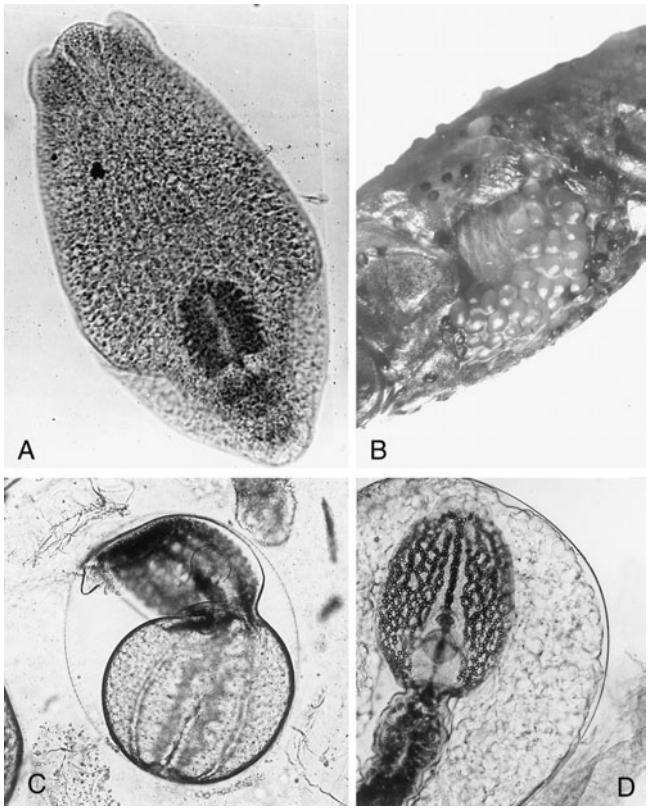


Fig. 10.17. Diplostomatid metacercariae: A. *Diplostomum* sp. removed from a visceral cyst in *Clarias gariepinus,* Uganda. B. Heavy *Bolbophorus levantinum* metacercaria in muscles of young *Oreochromis aurea* from Lake Kinnereth. C. Early-stage *B. levantinum* metacercaria with expanded posterior half. D. Later-stage *B. levantinum* metacercaria with emptied posterior end (actual size 0.6–0.8 mm).

(Yekutiel, 1985; Fig. 10.18A). The fibrous capsule produced by the host is superimposed on the acellular wall secreted by the encysting cercaria (Fig. 10.18B). The peripheral inflammatory cellular infiltrate that precedes fibrous capsule formation consists mainly of macrophages (Sommerville, 1981). Inflammatory cells become stratified into an epitheloid (Fig. 10.18B), while the innermost layer gradually degenerates. This early capsule, which has the form of a chronic granuloma (and also contains giant cells) is gradually replaced by the fibrous capsule (Fig. 10.16C; Sommerville, 1981; Yekutiel, 1985). At 5°C, cyst formation is much slower than at 15°C (McQueen *et al.*, 1973). *Centrocestus* metacercariae on the gills become encysted in cartilaginous capsules (Fig. 10.16A). The 'sand-grain' grub of yellow perch is caused by metacercarial cysts of *Apophallus brevis.* It is composed of bone, which becomes clustered around the parasite within the peripheral blood vessels. Such cysts have also been identified in ancient fossil perch. The bony structure has two opposite escape canals for the parasites and has lines interpreted as the growth rings of the bone (Sinclair, 1972). Metacercariae of *B. levantinus* migrate in tissues before encysting in the muscles (Paperna and Lengy, 1963). The inflammatory response and macrophage proliferation are limited to host tissues damaged in the earlier stages of the encystment (Yekutiel, 1985). Cellular damage around *Heterophyes* spp. infecting grey

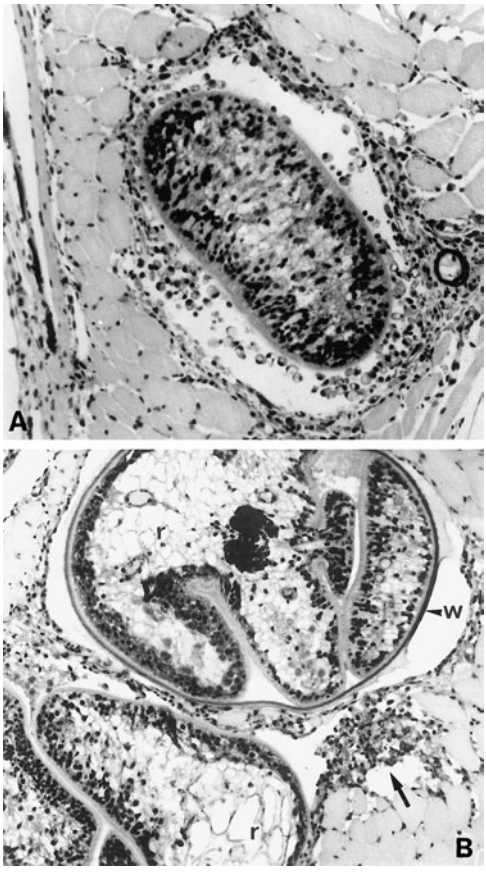


Fig. 10.18. Histopathology of *Bolbophorus levantinum* infection in young *Oreochromis aurea*. A. Macrophage infiltration around young unencysted metacercaria. B. Maturing metacercaria with 'reserve cells' (r) enclosed in a parasite-derived wall (w) and foci of macrophage proliferations in the surrounding muscles (arrows).

mullet is minimal even in heavy infections (6000 metacercariae per gram muscle) (Paperna, 1975). Under certain circumstances, however, the same metacercariae can induce more extensive cellular changes, particularly during the late stages of infection when damaged tissues and dead parasites have accumulated (Font *et al.*, 1984). In muscles of grey mullet infected with *Stellantchasmus falcatus*, myofibrils degenerating near the parasitic cysts are replaced by large fat cells (Lee and Cheng, 1970). Cellular and deposited ground substances may vary with species, but may also be affected by the host tissues. Collagen may be deposited in the liver (Mitchell, 1974) and in the spleen (Font *et al.*, 1984), but it is usually absent. When crowded on the serosal surface of the pyloric caeca (Fig. 10.16C), metacercariae of *Phagicola nana* evoke intense proliferation of the connective tissues, with a considerable amount of collagen. When the parasite is in the submocosa, the proliferation includes smooth muscle (Font *et al.*, 1984).

Gill infection due to Centrocestus *spp.*

Natural infections by *Centrocestus* sp. are common in cichlids (as well as in introduced *Gambussia affinis*) throughout the distribution range of its snail host *M. tuberculata* along the Syrio-African rift valley and nearby fish farms (Farstey, 1986; Paperna, 1996). *Centrocestus* is also in carp fry in India (Mohan *et al.*, 1999). In the Far East, a different species, *C. formosanus*, infects farmed eel (Yanohara and Kagei, 1983) and grass carp (*C. idella*) (Zeng and Liao, 2000). Introduction of *M. tuberculata* to Mexico and to the USA extended *C. formosanus* infection to farmed (carp) and native fishes (Mitchell *et al.*, 2000; Scholtz *et al.*, 2001). Metacercariae encysted on the gill filament cartilage are enclosed by a cartilagenous cell capsule (Fig. 10.12A). Cercariae penetrate the gill filaments and encyst in the connective tissue adjacent to the cartilage layer. Chondroblasts proliferate from the ray perichondrium and, following deposition of ground substance, the metacercaria becomes encapsulated (by day 7 post-infection) within a cartilaginous extension of the filament ray (Fig. 10.16A; Farstey, 1986; Paperna, 1996). Infection induces epithelial hyperplasia; infected gill filaments are thickened and often shortened and distorted when infection is heavy, and sometimes haemorrhagic. The proliferated cartilage causes filaments to expand several times the normal diameter, and this leads to fusion of filaments and disruption of the normal gill morphology. Heavy gill infection appears to lower respiratory efficiency. Heavily infected fish seem to be selectively dying out from the natural and farmed populations,

particularly under stressful circumstances (e.g. overwintering and extreme oxygen conditions). Heavily infected fish do not survive well during transportation. This has been demonstrated under simulated experimental conditions: all young cichlids (*S. galilaeus*) heavily infected with *Centrocestus* (116 ± 48 per fish) succumbed, while all same-size lightly infected fish (with 15 ± 15 per fish) survived during a 3 h journey at ambient temperature of 24°C (Farstey, 1986). Infections in common carp fry in India similarly cause both acute and chronic mortalities (Mohan *et al.*, 1999).

Dermal encystment

Cysts formed around certain dermal metacercariae may contain cells heavily loaded with melanin (and, exceptionally, other pigments). These are commonly called 'black spot', and are formed by *Neascus* metacercariae from the genera *Crassophiala*, *Ornithodiplostomum* and *Uvulifer* (Hoffman, 1960; Wittrock *et al.*, 1991) (Figs 10.16D and 10.19A,B) and heterophyids of the genera *Cryptocotyle* and *Haplorchis.* Cutaneous melanosis and cloudy eye have been reported in flounders heavily infected with pigmented *C. lingua* (Mawdesley-Thomas and Young, 1967). Metacercariae of a species of *Clinostomum* ('*C. cutaneum*'; Paperna, 1964a,b) encyst, sometimes in large numbers, beneath the scales of cichlid fish in Lake Kinnereth, Israel (Fig. 10.19C).

Heart infections

Metacercariae of some *Ascocotyle* and *Phagicola* have a predilection for the heart or truncus arteriosus (Sogandares-Bernal and Lumsden, 1964), but in heavy infections they may spread to other tissues. Cardiac pathology, resulting from thickening due to fibrogranulomatosis of the epicardium, is associated with infection of the pericardium with the strigeid *A. gracilis* (Watson *et al.*, 1992). Tort *et al.* (1987) demonstrated that the *in vitro* pumping performance of hearts in infected fish was reduced by as much as 50%. Cardiac infections are also commonly caused by heterophyids of the

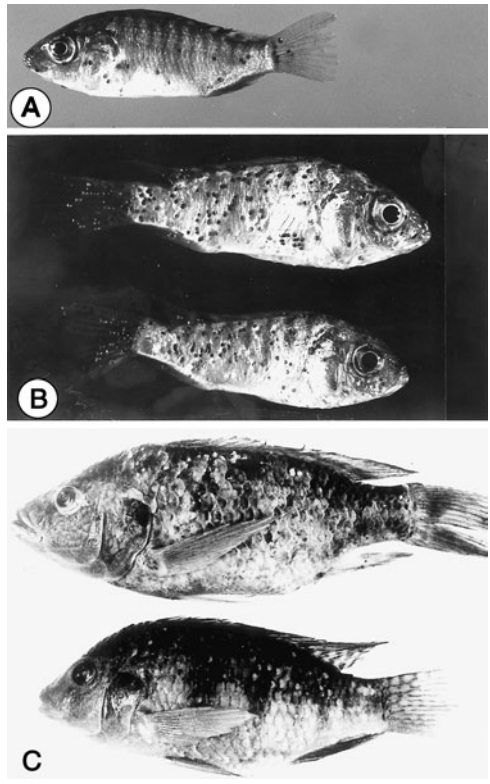


Fig. 10.19. Metacercarial skin infection in Lake Kinnereth cichlids. A, B. Low and heavy 'black spot' (*Neascus*) infection in young pond-reared *Oreochromis aurea* × *nilotica*. C. *Clinostomum 'cutaneus'*-infected *Tristramella simonis* (top) and *Tilapia zilli* (bottom).

genera *Phagicola* and *Ascocotyle* (Fig. 10.16B; Sogandares-Bernal and Lumsden, 1964; Stein and Lumsden, 1971). *Ascocotyle pachycystis* encysts in the lumen of the bulbus arteriosus, infection accumulates with age (up to 6800 per fish) and heavy infections reduce growth and overwintering survival (Coleman and Travis, 1998).

Visceral infections

Metacercariae of *Haplorchis* are found throughout the integumental connective tissues (skin, fins and gills) (Paperna, 1964a; Sommerville, 1982; Yekutiel, 1985). Heavy visceral infection of *P. minimum* ('white grub'; 500–2300 metacercariae in 50–65 mm long fish) in fathead minnow causes

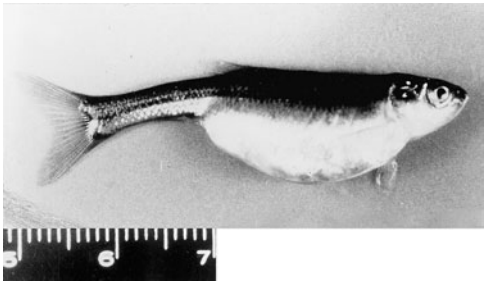


Fig. 10.20. Abdominal dropsy in *Postdiplostomum minimum*-infected *Pimephales promelas* (43 mm long) (after Mitchell *et al.,* 1982, courtesy of G.L. Hoffman).

abdominal dropsy (Fig. 10.20) with milky ascitic fluid that contains up to 92% leucocytes and 8% erythrocytes. Granulomas develop around degenerating parasites and parasitic debris (Mitchell *et al.*, 1982). Milder or no clinical impacts have been reported in *P. minimum centrarchi* (white grub) infections of largemouth bass (*M. salmoides*) (Grizzle and Goldsby, 1996). Massive invasion by the heterophyid *P. cheni* in Taiwan of Japanese eels caused severe morbidity. Their eyes were cloudy white and swollen with oedema and haemorrhages. Metacercariae, encysted in the muscle tissue, were numerous around the eyeball. Once transmission was interrupted, recovery occurred within 2 weeks (Ooi *et al.*, 1999). *Stephanostomum tenue* in the pericardial cavity of elvers of *Anguilla rostrata* impedes swimming (Oliveira and Campbell, 1998). *Bolbophorus* sp. invades as a protodiplostomum and then encapsulates in the muscles (Figs 10.17C, D and 10.18). Heavy infections of *B. levantinus* in Israel caused mortalities among young-of-the-year cichlids in Lake Kinneret and in farmed stock. Parasites invaded the major part of the somatic muscles and disfigured the fish (Fig. 10.17B); Yekutiel, 1985; Paperna, 1996). In the USA, *B. damnificus* and another, yet to be identified, *Bolbophorus* similarly invade and cause muscle damage to channel catfish farmed in southern states (Overstreet *et al.*, 2002; Terhune *et al.*, 2002).

Several species of clinostomiids occur in fish. *C. tilapiae* encysts in the connective tissues of the ventral articulation of gill arches, and sometimes also on the surface of the kidneys. *C. complanatum* becomes established in the muscles, with a preference in *Tor* (*Barbus*) *canis* for red muscles (92%) and a predilection for the branchial and mandibular muscles (62%) (Finkelman, 1988). *C. cutaneum* settles beneath the scales (Paperna, 1964a,b; Finkelman, 1988). Natural infections are sometimes exceptionally heavy; none the less, a full-sized fish can tolerate a remarkably high number of worms. A *Tristramella sacra* in Lake Kinneret, Israel, was found virtually covered with *C. cutaneum*; 130 *Nephrocephalus* sp. were recovered from a *Heterotis niloticus*; 70 *Clinostomum* sp. from *Synodontis membranaceus* (Ukoli, 1966); and the same number of *C. complanatum* from *T.* (*B.*) *canis* (Finkelman, 1988). Farmed juvenile cichlids, *Oreochromis* crossbreeds in Israel and *Oreochromis mossambicus* in Africa succumbed to infections as low as fewer than ten, or even three to five, of either *C. tilapiae* or *Euclinostomum heterostomum*; worms invaded the kidneys and the branchial cavity. Mortalities have also been reported among *Clarias* spp. due to *E. clarias* (Ezenwaji and Llozumba, 1992). Experimentally infected *O. mossambicus* fingerlings (30–35 mm long) died 62 days after being experimentally infected by 75–81 *E. heterostomus* (Donges, 1974). *C. complanatum* infections are regarded as a serious problem in Taiwan, causing death among farmed *P. altivelis* (ayu). The body wall of infected fish is profusely encysted with metacercariae, leading to its perforation (Lo *et al.*, 1981). Heavy loads of *Clinostomum van der horsti* metacercariae often congest the body cavity of their fish host, the mormyrid *Gnathonemus macrolepidotus* in southern Africa (Ortlepp, 1935). In the USA, metacercariae of *C. marginatum* (yellow grub) encyst in the flesh of farm-raised channel catfish. Apart from direct pathological damage, infected fish may not be marketable (Lorio, 1989). There are two reports of *Clinostomum* infections in humans, causing acute laryngitis, one in Israel and the other in Japan (Witenberg, 1944b; Kamo *et al.*, 1962). The worms were adults and

the infections were probably due to consumption of metacercaria-infected fish.

Eye damage in metacercarial infections

Damage to eyes may either be caused by metacercariae with a predilection for the organ (e.g. *D. spathaceum*) or be a non-specific side effect (e.g. corneal infection by integument-encysting metacercariae). Impairment of vision is aggravated when metacercarial cysts are accompanied by melanin pigment (black spot). Severe infection leads to exophthalmos, cataracts and even complete collapse of the eye. Blindness can be uni- or bilateral (Sindermann and Resenfield, 1954; Mawdesley-Thomas and Young, 1967). *D. spathaceum* is a specific pathogen of the eye. The non-encysted metacercariae invade the lens, the vitreous humour and the retina. Detailed pathological and histopathological descriptions of diplostomiasis in catfish (*Ictalurus melas*) are in Larson (1965) (Fig. 10.21) and in rainbow trout in Shariff *et al.* (1980). Farmed rainbow trout yearlings with infection limited to a cataract (small white opacity in the lens with parasites visible beneath the lens) feed normally and remain in good condition. However, in 2-year-old trout, the cataract is more prominent, with the lens dislocated into the anterior chamber or absent. Fish at this stage are dark, apparently blind and emaciated and feed only from the bottom of the tank. Parasites are most frequently in the cortical region of the lens, within a cavity with fine debris, which results from liquefaction of the cortical fibres. Degeneration of the lens is evident. In the chronic condition, the lens becomes irregular and wrinkled from adherences with the inner components of the eye and is often ruptured. Thickening of the lens capsule leads to exfoliation, with proliferation of the epithelial layer. The iris is greatly distorted and is in contact with the lens. In catfishes (*I. melas* and *I. nebulosus*), the lens herniates near the point of attachment to the dorsal ligament, with metacercariae spilling into the protrusion and leaving the remainder of the lens clear for visual function (Fig. 10.21; Larson, 1965). Entry of

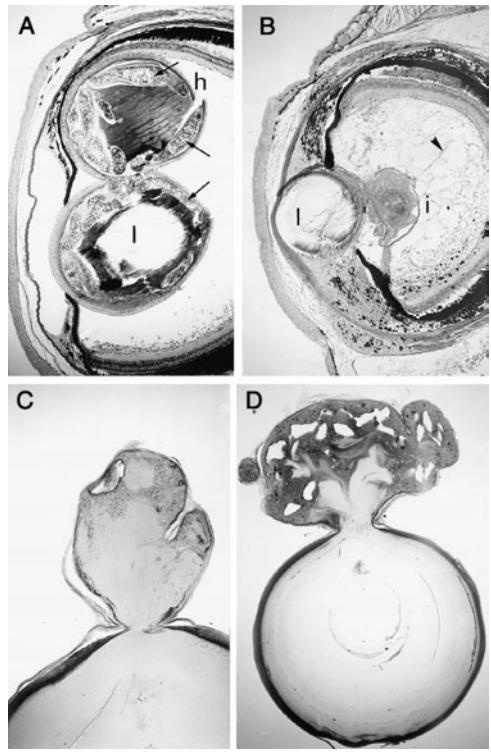


Fig. 10.21. Eye diplostomiasis in *Ictalurus melas*. A. Cross-section of the eye showing herniation (h) of the lens (l) and the metacercariae inside (arrows). B. Damaged lens (l) with proliferated epithelium around a necrotic core (i); note vascularization of the vitreous humour (arrow). C, D. Cross-sections of herniated lenses; empty spaces contained worms before processing. (After Larson, 1965, courtesy of the author.)

parasites to the subretinal level results in retinal detachment. A cellular response usually becomes evident only in later-stage infections. Macrophages aggregate around the debris from ruptured lenses, in the damaged periphery of the iris, in the choroid and around necrotic foci in the optic nerve. The combined effects of cataracts and retinal detachment cause blindness (Shariff *et al.*, 1980).

Crowden and Broom (1980) described loss of efficiency and increase in time devoted to feeding. As the infection progresses, fish spend more time in the surface layer of the water. This would increase their risk of predation by gulls. When *D. adamsi*

cercariae enter (in groups of 20 or more) the retina of *Perca flavescens* and remain unencysted, they form a cavity between the photoreceptor layer and the pigment epithelium. *Diplostomum schudderi* becomes established in a similar manner in the retina of *Gasterosteus aculeatus.* In both fish hosts, cytological damage and alterations occur in both layers of the retina. This suggests loss of vision in the foci of infection in the retina, but an overall minimal visual loss due to the peripheral location of the parasites (Lester and Huizinga, 1977). Metacercariae of *Diplostomum compactum* infected *Oreochromis aureus* and *O. mossambicus* introduced into Lake Amela, Mexico. The metacercariae enter the aqueous and vitreous humours of the eye, causing corneal oedema, conjunctivitis, neuritis of the optical nerve, eosinophilic iridoclitis and uveitis. They also invaded the brain, causing multifocal gliosis, eosinophilic meningitis, brain oedema and spongiosis (Garcia-Luis *et al.*, 1993). Metacercariae of *Posthodiplostomum brevicaudatum* encysted only in the vitreous humour and in the retina and caused less obvious eye damage (Donges, 1969).

Estimating the extent of mortality in affected fish populations

Estimation of metacercaria-induced host mortality in natural fish populations has been attempted by extrapolating quantitative data on frequency distribution of infection. The decline in variance was considered to result from the loss of heavily parasitized fish. Hence a measure of overdispersion, in comparison with negative binomial distribution or by calculating the ratio of variance to mean, was advocated as an indirect method of estimating fish mortality (Lester, 1977, 1984; Anderson and Gordon, 1982; Gordon and Rau, 1982; Kennedy, 1984). Seasonal changes in the degree of overdispersion were related to suggested parasite-caused mortality in bluegill during the winter (Lemly and Esch, 1984b). However, extrapolations from field data have met with a variable degree of success and at times have produced ambiguous results (Kennedy, 1984; Lester, 1984).

Marketing food fish aspects

Fish containing conspicuously visible (large or coloured) free or encysted worms are rejected by consumers (Kabunda and Sommerville, 1984; Lorio, 1989; Paperna, 1996; Overstreet *et al.*, 2002). Fish with badly damaged eyes (trout affected by eye diplostomiasis) are likely to be sold at depreciated prices. The mere suspicion of infection affects demand and depreciates market prices of the entire consignment of fish. Examples are the large yellow clinostomids, which also tend to encyst and crawl around after the death of the fish (Fig. 10.19C), metacercariae of diplostomatids in muscles (Fig. 10.17B); (Paperna, 1996), 'black spot' (Fig. 10.19A, B) and fish containing large didymozoids in skin and gills or in the muscles (Lester, 1980; Fig. 10.8). (For risk to public health, see Chapter 16 in this book.)

Immune responses

In contrast to the wealth of information that has accumulated on the immunology of digeneans in higher vertebrates (Butterworth, 1984), data on fish-infecting digeneans are more preliminary in nature. The main difference between immune responses of fish and those of mammals and birds is that the ambient temperature affects antibody production in fish (Avtalion *et al.*, 1973; van Muiswinkel, 1995). Eosinophilic granulocytes, which play an important role in protective immunity in mammalian digenean (schistosome) infections (Butterworth, 1984), do not seem to be as important in piscine infections (Hoglund and Thuvander, 1990). However, it is not certain whether piscine eosinophils are homologous with those in mammals (Ellis, 1977). Aaltonen *et al.* (1997) demonstrated humoral immune response of *Rhipidocotyle fennica*, it was detected in wild *R. rutilus* in lakes where fish are infected by the parasite.

Infection by *R. fennica* increases towards late summer: antibody levels are higher in sera collected in September than in those collected in June. Antibodies were produced against homogenized cercariae in experimentally immunized *R. rutilus*; however, the response was more pronounced after exposure to live cercariae. Western blots showed that responses in fish injected with homogenized cercariae were different from those exposed to live cercariae. Fish injected with the homogenate were protected on challenge.

Trout repeatedly exposed to cercariae of *Diplostomum* over a 12-week period developed protective immunity, but specific antibodies could not be detected in the blood (Hoglund and Thuvander, 1990). The protection seemed to be due to cell-mediated immunity or to a non-specific mechanism of protection against the migrating diplostomulae. In mammals, induction of cellular immunity is required for protection against helminth infections (Butterworth, 1984). Migrating diplostomulae induce non-specific cellular responses, which involve infiltration of neutrophils and monocytes (Ratanart-Brockelmann, 1974). Speed and Pauly (1985) showed that the survival time of immunized, challenged fish increased by 4 months over that of non-immunized controls. An immunocytochemical study demonstrated putative neurotransmitters in metacercariae of *Diplostomum* sp. and *Cotylurus erraticus* from rainbow trout. Cholinergic and serotoninergic staining was found primarily in the central nervous system (CNS) and in cell bodies associated with the ventral and dorsal nerve cords. Peptidergic immunoreactivity was localized in the CNS and the peripheral nervous system, revealing an extensive innervation within the holdfast organ and around the oral and ventral suckers (Barton *et al*., 1993).

Precipitating antibodies against antigen from the intestinal digenean *Telogaster opisthorchis* were demonstrated in the sera and gut mucus of eels, *Anguilla australis schmidtii* and *Anguilla dieffenbachii* (McArthur, 1978). Wood and Matthews (1987) reported induction of humoral antibodies, sensitized pronephric leucocytes and cytotoxic serum factors in *Chelon labrosus* exposed to *C. lingua* cercariae. Precipitating antibodies were detected, using the Ouchterlony technique, in plaice (*P. platessa*) infected with *C. lingua* and *R. johnstonei*. The immune sera had an immobilizing effect on the cercariae. Antibodies reacted with the tegument and with secretory glands in cercariae of both parasite species. Antibody production was temperature-dependent and did not occur at 5°C (Cottrell, 1977). The antibodies were macroglobulins, and resembled mammalian immunoglobulin M (IgM). Bortz *et al*. (1984) detected humoral antibodies in trout with natural infections of *D. spathaceum* and trout injected with sonicated metacercariae using an enzyme-linked immunosorbent assay (ELISA). In trout immunized with antigens prepared from cercariae and diplostomulae, anti-cercarial and anti-diplostomulae circulating antibodies were demonstrated using ELISA and immunofluorescence. The two cross-reacted, although the reaction between anticercarial sera and diplostomatid antigen was weaker. Fluorescence studies indicated the tail region of the cercariae to be strongly antigenic (Whyte *et al*., 1987).

In Vitro Culture

Prior to the era of molecular biology (see above), metacercariae could not provide morphological cues for specific identifications. *In vitro* studies of piscine digeneans have been concentrated on the induction of sexual maturation of metacercariae, rather than on the long-term maintenance of larval or adult stages. Stunkard (1930) was the first to attempt cultivation of metacercariae to adult stages and this has now been accomplished for a number of species (Kannangara and Smyth, 1974). Metacercariae that already possess genital primordia are more readily cultured to the adult stage, with the production of eggs capable of hatching (Basch *et al*., 1973; Mitchell *et al*., 1978). Moreover, some metacercariae (*Gynaecotyle adunca*, *Pleurogenoides sitapurri*, *Microphallus papillorobustus*) produce eggs after only minimal

stimulation, e.g. release from the cyst, exposure to light or changes in temperature (Kannangara and Smyth, 1974). Metacercariae that do not have genital rudiments take longer to develop and are more fastidious in their *in vitro* maintenance needs. *Diplostomum phoxini* was first cultivated in a medium that contained egg yolk (Bell and Smyth, 1958), but subsequent studies demonstrated that the egg yolk had little or no beneficial effect. *D. phoxini* and *D. spathaceum* reached the egg production stage, but eggs of the latter were not viable (Kannangara and Smyth, 1974). In a more recent study, Leno and Holloway (1986) reported cultivation of *D. spathaceum* on chick chorioallantois. Whyte *et al.* (1988) transformed *D. spathaceum* cercariae into metacercariae *in vitro.* These were then further maintained for 72 h. Cercariae were allowed to penetrate via trout skin using a modified version of the Clegg and Smithers (1972) mouse-skin technique for schistosomes. The best results for metacercarial maintenance *in vitro* were obtained with L-15 (Gibco) medium supplemented with added fetal calf serum (Whyte *et al.*, 1988).

Prevention and Control

Transmission control

There is no practical way to prevent contamination of pond waters by droppings of presumably infected piscivorous birds, the definitive hosts of most metacercariae infecting fish. The best preventive strategy for controlling digenean infections in farmed fish is the elimination of the intermediate host snail. This includes the use of chemical molluscicides, environmental manipulation and the use of molluscophagous fish. There is extensive literature on the control of snails that are intermediate hosts of schistosomes and *Fasciola* (McCullough and Mott, 1983; Madsen, 1990). Of all the many molluscicides developed to control these snails, only copper sulphate is of practical use in fish ponds and circulation systems. Molluscicidal concentrations of niclosamide (= Bayluscide, Bayer 73) and *N*-tritylmorpholine (= Frescon, WL 8008, Shell) recommended for schistosomiasis snail vector control are toxic to fish (Cowper, 1971). Copper sulphate (5-hydrate) is tolerated by most fish (although some species and younger fish may be more susceptible). It is inexpensive, widely used in fish ponds as an algicide (Sarig, 1971) and can be safely applied at a dose of 3.5 ppm to sea water and brackish water ponds and to neutral and hard freshwater ponds. However, in acid and soft freshwater ponds (pH 6.8, calcium ions > 12 ppm), the same or lower concentrations become toxic to fish. Copper salt may be applied continuously at a lower concentration (1 ppm) or as a low-soluble formulation (as copper carbonate or copper oxide) to produce long-term effects (Hoffman, 1970). Treatment of drained ponds or raceways with copper sulphate prior to stocking delayed, but did not prevent, repopulation by snails (Stables and Chappell, 1986; I. Paperna, personal communication of current experience of fish farmers in Israel). The environmental limits imposed on snail survival in fish-farm systems were discussed earlier and regular weed control, performed manually or with herbicides (Paperna, 1991), can decimate snail populations. Of the many recommended molluscophagous fishes (De Bont and De Bont Hers, 1952; Carothers and Allison, 1968; Taraschewski and Paperna, 1981; Paperna, 1996), only black carp (*Mylopharyngodon piceus*) in fresh water and sea bream (*Sparus* spp.) in seawater ponds are compatible with fish-rearing practices and economies.

Parasite control

Praziquantel (Droncit, Biltricide®, Bayer AG, Germany) is safe and effective against digeneans and cestodes of man and animals (Andrews *et al.*, 1983). It demonstrated high efficacy over a wide range of metacercariae: Szekely and Molnar (1991) reported on the elimination of all *D. spathaceum* metacercariae from herbivorous carp that were fed a single dose of the drug (300 mg/kg body mass). Three sequential lower doses of

35–100 mg/kg yielded 88–100% efficacy, and bath treatments of 1 mg/l for 9 h or 10 mg/l for 1 h showed 100% and 93–94% efficiency, respectively. Short (30 min–4 h) and long (1–8 days) bath treatments were also effective against *Diplostomum pusillum* and *A. gracilis* (Zhatkanbayeva and Heckmann, 1990). Fish growers in Israel used effectively veterinary formulations (Droncit) to eliminate metacercarial infections of *Centrocestus*, *Haplorchis* and *B. levantinus* in juvenile tilapia (> 70 mm in length); available formulations, however, are too costly for this farming system. Lorio (1989) compared the efficacy of Droncit (praziquantel), by bath and injection, with Ivermectin (by injection) and Masoten (by bath treatment) in *C. marginatum*-infected channel catfish. Praziquantel significantly reduced infection, Ivermectin was not as effective and Masoten was ineffective. One noteworthy limitation is potential post-treatment side effects: since dead metacercariae remain in the tissue, they may release toxins harmful to the fish (Mitchell, 1995).

Conclusions

Digeneans that parasitize fish are numerous and diverse in their morphologies and life histories. Studies on adult digeneans and metacercariae require different approaches. The importance of digeneans to fish culture has long been underestimated; also their risk to public health has not received adequate recognition. With the rapid development of warmwater aquaculture, as well as mariculture, and the spread of exotic culinary practices to Western societies risks to cultured fish and to consumers of infected fish are likely to become significant (Ko, 1995). Intra-intestinal adult digeneans are potentially pathogenic to maricultured fish: however, piscine digeneans are receiving less attention now than in the past. In the last decade, the burden due to massive infections by metacercariae has increasingly alarmed freshwater fish farmers. Some of the most troubling infections are of the gills by *Centrocestus* spp. and of the muscles and connective tissue by *Bolbophorus* spp. and by clinostomids (yellow grub), causing continuous losses or marketing problems in warmwater fish farms, notably of catfish in the USA and cichlids in Afro-Asia. To make matters worse, during the last decade, the snail vector, *M. tuberculata*, of *C. formosanus* and subsequently the parasite have been introduced into Mexico and the southern USA.

Future Studies

In the last decade the transition in parasitology from an essentially zoological to a biochemical, immunological and molecular science has had only a marginal impact on fish digenean research. Future studies should include nutritional physiology, immunology and taxonomy that make use of DNA analysis. Temperature dependence of the immune response, combined with the peculiarities of the defence mechanisms against helminthic infections, offers an attractive and challenging research model. The emerging methodology of DNA taxonomy is potentially the best option for resolving taxonomic affinities and revealing life histories of digeneans by specific recognition of larval stages.

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11 Cestoidea (Phylum Platyhelminthes)

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Introduction

Tapeworms have been observed in fish since antiquity, with the best known being the broad fish tapeworm (*Diphyllobothrium*) of humans. Most conspicuous are the cysts of tapeworms that harbour the infective stage in fish flesh and viscera. These include cysts of *Diphyllobothrium* spp. (Ando *et al.*, 2001; Dick *et al.*, 2001), cysts of *Triaenophorus crassus* in the flesh of coregonids and some salmonids (Fig. 11.1) and massive infections of *Diphyllobothrium* spp. along the viscera in salmonids (Fig. 11.2). Very heavy infections are commonly reported in the flesh or along the viscera, but remarkably few have been shown to cause mortality of fish. The human–fish tapeworm *Diphyllobothrium* has received the most attention, followed by the large larval tapeworms *Ligula* and *Schistocephalus* of birds and those of economic importance, such as *Triaenophorus* of fish. On the other hand, entire orders, such as the Tetraphyllidea and Tetrarhynchidea, have received relatively little attention until recently. With the exception of the Cyclophyllidea and Aporidea, every order of cestodes has members that infect fish. While much has been made of the human–fish tapeworm *Diphyllobothrium* in textbooks and anecdotal reports, it is *Triaenophorus* that has been most extensively studied, not because it is an important human parasite or due to its pathology in fish (Rosen and Dick, 1983) but as a contaminant in the flesh of economically important coregonids. More recently, fish tapeworms have been used to address questions of host specificity and co-evolutionary relationships between host and parasite (Caira and Jensen, 2001; Skerikova *et al.*, 2001). Both *Schistocephalus* and *Ligula* provide new insights into host–parasite associations (Reimchen, 1997; Heins *et al.*, 1999; Loot *et al.*, 2001; Overli *et al.*, 2001; Brown *et al.*, 2002; Bagamian *et al.*, 2004).

Recently several major groups of fish cestodes have received much needed reviews of their taxonomy and systematics (Beveridge *et al.*, 1999; Zehnder and Mariaux, 1999; Skerikova *et al.*, 2001; Logan *et al.*, 2004). The cestode genus *Proteocephalus* occurs worldwide; however, very little is known about the mechanisms of species distribution within the host range. The current debate is whether the distribution of species is the result of a long-lasting association or through a series of co-speciation events or whether other factors are involved (Zehnder and Mariaux, 1999; Carney and Dick, 2000; Skerikova *et al.*, 2001). Using both morphological (Carney and Dick, 2000) and molecular analyses (Skerikova *et al.*, 2001), it was concluded that the European species

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of *Proteocephalus* reflect a series of host switches rather than host–parasite co-speciations. On the other hand, studying different fish groups, Choudhury and Dick (1998) and Nelson and Dick (2002) showed that there is a strong correlation between some parasite species and host groups.

The term host specificity has been used rather loosely and with something of a sliding scale for the host–parasite association. Carney and Dick (1999), using fish sister taxa from two continents, sparked off some interesting responses on the use of the term host specificity. Poulin and Mouillot (2003) computed a 'new index of host specificity' from a phylogenetic viewpoint and questioned the index of specificity proposed by Caira *et al.* (2003), which also incorporated phylogenetic information. *Proteocephalus* species are considered to be relatively narrow in their host preference; however, several species have been shown to infect a range of fish hosts (Freze, 1965; Willemse, 1968, 1969; Chubb *et al.*, 1987; Dubinina, 1987; Hanzelova *et al.*, 1996; Scholz and Hanzelova, 1998; Scholz, 1999a).

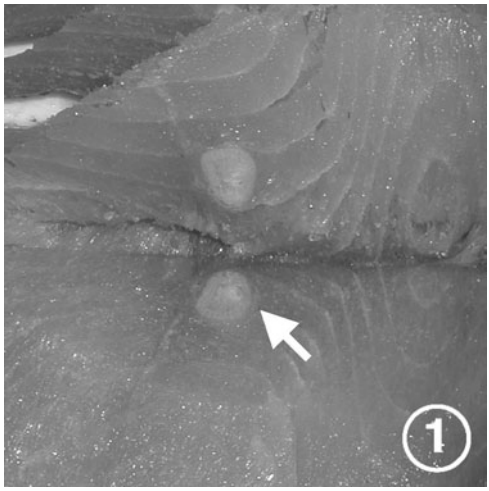


Fig. 11.1. Lake trout fillet with section through a *Triaenophorus crassus* cyst (white arrow).

Historically, most studies on fish cestodes have focused on taxonomy, life cycles, surveys, epizootiology and population dynamics. Recently, the expansion of the aquaculture industry, as well as basic curiosity about the defence mechanisms of lower vertebrates, has made the study of immunopathological responses in fish relevant. An increasing worldwide awareness of potential fish-transmitted human pathogens (Rim, 1998; Dick *et al.*, 2001) has also raised the profile of fish cestodes.

Economic importance

Numerous tapeworms cause disease in fish (Table 11.1) and the problems of tapeworms

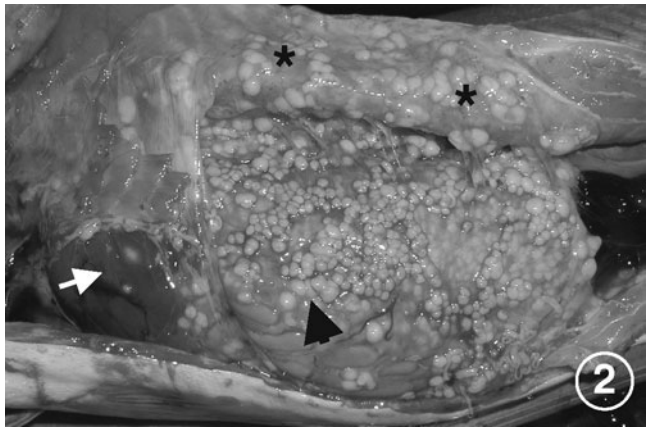


Fig. 11.2. *Diphyllobothrium ditremum* and *Diphyllobothrium dendriticum* encysted in the viscera and along the body wall of Arctic charr. Cysts in the liver (white arrow), large numbers of cysts on the caecae and stomach wall (black arrow) and asterisks show cysts along the hypaxial muscles.

Table 11.1. Pathogenic fish tapeworms, their distribution and hosts.

Order and species	Distribution	Fish host	Location in host
Amphilinidea			
Amphilina foliacea	Eurasia	Sturgeon	Liver, body cavity
Amphilina bipunctata	N. America	White sturgeon	Body cavity
Caryophyllidea			
Caryophyllaeus spp.	Eurasia, N. America	Cypriniforms	Intestine
Khawia sp.	Eurasia	Cypriniforms and siluriforms	Intestine
Pseudophyllidea			
Adults			
Bothriocephalus acheilognathi	Eurasia, N. America	Cypriniforms, centrachids, antherinids, goodeids	Intestine
Cyathocephalus truncatus	Europe	Trout	Intestine
Eubothrium salvelini	N. America	Arctic charr	Intestine
Triaenophorus crassus	Eurasia, N. America	Pike	Intestine
Triaenophorus nodulosus	Eurasia, N. America	Pike	Intestine
Larvae			
Diphyllobothrium dendriticum	Europe, N. America	Salmonids	Viscera
Diphyllobothrium latum	Worldwide	Percids	Muscle
Diphyllobothrium sebago	N. America	Trout, salmon	Viscera
Ligula intestinalis	Eurasia, N. America	Cypriniforms	Viscera
Schistocephalus sp.	Eurasia, N. America	Sticklebacks	Viscera, muscle
T. crassus	Eurasia, N. America	Whitefish, trout	Muscle
T. nodulosus	Eurasia, N. America	Trout	Viscera, liver
Proteocephalidae			
Adults			
Corallobothrium sp.	N. America	Catfish	Intestine
Proteocephalus exiguus	Europe	*Thymallus*	Intestine
Larvae			
Proteocephalus ambloplitis	N. America	Small-mouth bass	Viscera, ovaries

Pathology caused by larval marine tapeworms has been reviewed by Sindermann (1970) and Williams(1967).

in aquaculture and fisheries (marine and freshwater) have been reviewed (Sindermann, 1970; Hoffman, 1975, 1999). The most important tapeworm in warmwater aquaculture is the Asian pseudophyllidean tapeworm *Bothriocephalus acheilognathi* (Paperna, 1991; Luo *et al.*, 2003). It is an excellent example of translocation of tapeworms from their original location (in this case, Siberia) with the introduction of their hosts (ex. cyprinids) into other parts of the world (Bauer and Hoffman, 1976). Similarly, *Proteocephalus ambloplitis* was introduced and established in a warmwater reservoir on the Canadian prairies and had potential for further dissemination through stocking programmes (Szalai and Dick, 1991; T. Dick, unpublished). *Caryophyllaeus* spp. and *Khawia* spp. are also of concern in Europe and North America, and the effects of *Triaenophorus* on fisheries are well documented (Lawler, 1970). *Triaenophorus nodulosus* is considered to be the most pathogenic of the *Triaenophorus* species in Eurasia (Kuperman, 1973). It causes epizootic outbreaks with heavy mortalities in juvenile rainbow trout (*Oncorhynchus mykiss*) held in rearing ponds and under

hatchery conditions (Scheuring, 1923; Bauer, 1959; Kuperman, 1973) although natural infections of trout are relatively rare. In contrast, the most important North American freshwater parasite is *T. crassus*, since it causes unsightly infestation in the muscles of whitefish (*Coregonus clupeaformis*). High *T. crassus* infections can affect the value of exported whitefish. The culture of salmonids and coregonids in fresh water and the increasing use of fish stocking as a management strategy can be affected by *T. crassus*. *T. crassus* was found in juvenile brown trout (*Salmo trutta*) in 1991 in a hatchery in Manitoba, Canada (T. Dick, unpublished). Similarly, *T. crassus* is a problem in European coregonids as it has an impact on the growth of *Coregonus laveratus*, which is age/size-related, but does not induce host mortality (Pulkkinen *et al.*, 1999).

Experimental infections of *Eubothrium* sp. were reported to affect the growth and survival of Atlantic salmon (*Salmo salar*) (Saksvik *et al.*, 2001). Saksvik *et al.* (2001) found that infected fish had a slower growth rate than uninfected fish and the difference became greater over time.

Proteocephalids are common parasites of fishes in hatcheries; however, infection levels remain relatively low in most cases and they are generally not considered to be economically important. For example, Buchmann *et al.* (1995) found that, in Danish trout hatcheries, *Proteocephalus* sp. occurred only in farms that received water from surrounding lakes and, in all instances, infections remained low and pathology in the host was not detected. A similar result was shown for farm-raised trout (*O. mykiss*) in Michigan (Muzzall, 2000). The highest infections of *Proteocephalus* appear in Arctic populations of charr (*Salvelinus alpinus*) (Knudsen *et al.*, 1997); however, even in these populations, infection levels can be low (Kolasa and Curtis, 1995). Because all but one species do not mature in homeothermic vertebrates and are therefore unable to infect humans, the medical importance of this genus is also considered negligible (Muller, 2002).

Although *Proteocephalus* has shown little economic importance and few impacts on the health of host populations, along with several other helminths it has been used in toxicological studies as an indicator of community health. For example, pollutants may increase parasitism by increasing host susceptibility or by increasing the abundance of intermediate hosts and vectors (Turcekova *et al.*, 2002). Pollutants may also decrease parasitism as infected hosts may suffer higher mortality or pollutants may negatively affect intermediate hosts and vectors (Turcekova *et al.*, 2002). The concentrations of heavy metals such as As, Cd, Cu, Pb and Zn in *Proteocephalus* have been found to be six to 280 times higher than those in the tissues of its definitive host and this result may be used as a criterion for the assessment of fish and ecosystem health. More studies in this area are required in order to make community-based decisions with respect to ecosystem health as a whole.

Host Range/Geographical Distribution

Most of the economically important fish tapeworms are in temperate, north temperate and Arctic regions of the world, with some species (*Diphyllobothrium*, *Triaenophorus* and *Ligula*) having a circumpolar distribution. The Asian tapeworm has a remarkable ability to infect new host groups and its geographical range is expanding as new infections are discovered (Scholz, 1999a). This chapter will focus on selected species (Table 11.1) because there are adequate catalogues of most tapeworms and their hosts in other reviews (Wardle and McLeod, 1952; Hoffman, 1967, 1999; Margolis and Arthur, 1979; MacDonald and Margolis, 1995). Other reviews (e.g. deChambrier and Vaucher, 1997; Scholz, 1997; Beveridge *et al.*, 1999; Rego *et al.*, 1999; Zehnder and Mariaux, 1999; Caira and Jensen, 2001; Palm, 2002; Blend and Dronen, 2003; deChambrier *et al.*, 2004) of major groups of fish parasites are recommended for further reading.

Species diversity of fish tapeworms appears to be greatest in subtropical and temperate regions but this is also the region where most survey work has been done. As one moves north, species diversity declines but the numbers of parasites are higher, to the point where intestines may be distended with masses of adult tapeworms. For example, 1000

encysted larval *Diphyllobothrium* (mean intensity of over 400) and about 700 adult tapeworms of *Proteocephalus* and *Eubothrium* per Arctic charr are frequently found (Dick and Belosevic, 1981). Also the prevalences can be up to 97%. One must be careful in drawing conclusions on intensities and prevalence since most fish parasites have seasonal peaks and many potential hosts have not been adequately sampled. Seasonal cycles are more pronounced in temperate, north temperate and Arctic regions and are most evident in the contracted growing season of the Arctic. This seasonality is less pronounced for larval tapeworms than for adult tapeworms. Larval *Triaenophorus* tend to accumulate with the age of the fish (Watson and Dick, 1979), as do *Diphyllobothrium* larvae in whitefish and cisco (T. Dick, unpublished).

Systematics and Taxonomic Position

The majority of cestodes that produce diseases fall within three orders: Caryophyllidea (*Caryophyllaeus* and *Khawia*), Pseudophyllidea (*Ligula*, *Schistocephalus*, *Diphyllobothrium* and *Triaenophorus*) and Proteocephalidea (*Proteocephalus* spp.). The systematic relationships among various groups of tapeworms have been reviewed. Accordingly, the two lines of descent that derive the Pseudophyllidea (via the Tetrarhynchidea) and the Cyclophyllidea (via the Proteocephalidea) from a common Tetraphyllidean-like ancestor are supported by life cycle and developmental studies (Stunkard, 1983). The position of the Caryophyllidea has been the subject of considerable controversy but their historical status as 'primitive' cestodes with affinities to the cestodarians or as monozoic pseudophyllideans has been replaced by a more independent one (Mackiewicz, 1981). Phylogenetic analysis of the Platyhelminthes (Brooks, 1989) derives the Amphilinidea (*Amphilina* sp.) and Gyrocotylidea independently from an ancestor common to these two and the remaining tapeworm orders (i.e. the Eucestoda, including the Caryophyllidea).

The systematic relationships of Proteocephalidae have been studied in detail by La Rue (1911, 1914), Wardle and McLeod (1952), Freze (1965), Schmidt (1986), Rego (1994), Rego *et al.* (1999), Scholz (1999a), Zehnder and Mariaux (1999) and Kodedova *et al.* (2000). Over the past decade considerable effort has focused on the Proteocephalids, including the clarifying of some of the taxonomy by Scholz and Hanzelova (1994, 1998, 1999), Snabel *et al.* (1994, 1996), Hanzelova *et al.* (1995a,b), Scholz *et al.* (1995, 1997, 1998a,b), Kral'ova-Hromadova (1996), Kral'ova-Hromadova and Spakulova (1996), Kral'ova-Hromadova *et al.* (1997), Carney and Dick (2000), Skerikova *et al.* (2001), and Scholz and deChambrier (2003).

Rego *et al.* (1999) provide a good description of the characters for the genus *Proteocephalus.* In the past, species identification was based on a small number of morphological characteristics, most of which contain a large degree of intraspecific variability within different host species (Chubb *et al.* 1987; Dubinina, 1987). However, Scholz *et al.* (1997), Scholz (1999a) and Skerikova *et al.* (2001) caution that the systematics of this genus has not been sufficiently studied and that the identification of individual taxa is still problematic. Several species are polymorphic (Chubb *et al.* 1987; Dubinina, 1987; Scholz, 1989; Anikieva, 1992a, b, 1993, 1995; Snabel, *et al.*, 1994; Hanzelova *et al.*, 1995a,b; Scholz *et al.*, 1998a; Zehnder and Mariaux, 1999) and as such contain few morphological or biometrical features for use in species identification (Scholz, 1999a).

Recent multidisciplinary approaches have not only confirmed the validity of many *Proteocephalus* species (Gil de Pertierra, 2002; Scholz *et al.*, 2003a), but reduced the number of species listed in the genus as well. For example, upon re-evaluation of the genus throughout its European distribution, Scholz and Hanzelova (1998) recognized only 11 individual taxa rather than the multiple species listed previously.

Parasite Morphology and Life Cycles

The amphilinids and gyrocotylideans have been reviewed by Dubinina (1974), Skrjabina (1974) and Bandoni and Brooks (1987a,b).

The morphology and biology of the Caryophyllidea have been reviewed by Mackiewicz (1972). The only common feature between the caryophyllids and cestodarians is their monozoic body plan. Differences include the arrangement of the reproductive apparatus (separation of male and female gonopores and the presence of the uterine pore at the opposite end in the amphilinids) and the hosts they parasitize (amphilinids and gyrocotylids are parasites of more 'ancient' fish, such as acipenserids and chimaerids, while caryophyllids are typically parasites of cyprinid and catostomid fishes). Plerocercoids of pseudophyllideans often occupy similar sites in the fish host, and correct diagnoses require a thorough knowledge of the morphology of the larvae. The morphology of plerocercoids of *Schistocephalus* and *Ligula* is well known (Hoffman, 1967; Smyth and McManus, 1989) and the parasites are distinguished by lack of segmentation in *Ligula* (conspicuous in *Schistocephalus*). The plerocercoids of *Triaenophorus* are unique in that they bear tricuspid hooks, which vary in size, depending on the species (see review by Kuperman, 1973). The lack of conspicuous morphological differences makes identification of *Diphyllobothrium* plerocercoids difficult. Some researchers use the external tegumental morphology, i.e. wrinkled versus smooth tegument, and the form/shape of the bothrial grooves and a key has been developed using these characteristics (Andersen *et al*., 1987; Andersen and Gibson, 1989). The tetrarhynchideans are readily separated from tetraphyllideans by the presence of spiny proboscides in the former. For information on marine fish cestodes causing disease (particularly Tetraphyllidea, Tetrarhynchidea and Diphyllidea), readers are referred to Sindermann (1970) and Grabda and Bier (1988).

Ultrastructural studies of plerocercoids and adults of fish cestodes are scarce. Studies show that plerocercoids of *Schistocephalus* and *Ligula* (Charles, 1971) develop thicker outer tegumental layers, surface ridge systems and microtriches characteristic of each species. With age, the number of 'spine precursor' vesicles declines with concomitant increases in specialized vesicles with possible endolysosome-like activity (Charles, 1971). In contrast to the adult tegument, the tegument of *P. ambloplitis* plerocercoids possesses short conoid microtriches and lacks vacuolization as well as lacking unicellular tegumental gland cells (Coggins, 1980b). Coggins (1980b) also described the end organ in plerocercoids and demonstrated the presence of proteolytic enzymes (Coggins, 1980a), a structure not present in adult worms and probably used by the plerocercoid during visceral migration. In contrast, the teguments of plerocercoid and adult stages of *Haplobothrium globuliforme* were found to be 'remarkably similar' to each other, although differences in the neck region were noted. This was interpreted as a pre-adaptation of the plerocercoid to the adult existence (MacKinnon and Burt, 1984). Comparative studies on pseudophyllidean plerocercoids have revealed gland cells in the anterior end of the body (head and bothria) in *Eubothrium* and *Triaenophorus*, which release their contents via ducts (Kuperman and Davydov, 1982). In contrast, plerocercoids of *Diphyllobothrium latum* have an extensive syncytial gland complex throughout the parenchyma, while the Ligulidae possess frontal and lateral glands (Kuperman and Davydov, 1982). Few studies have documented ultrastructural interactions at the host–parasite interface in adult fish tapeworms. McVicar (1972) has shown that unique structures on the tegument of the holdfasts of some adult tetraphyllideans (*Echineibothrium*, *Phyllobothrium* and *Acanthobothrium*) may be specialized microtriches with osmiophilic ends and presents evidence that the myzorhynchus apical pad of *Echineibothrium* is involved in direct nutrient uptake from the host submucosa. Additional information on tegumental ultrastructure comes from physiological studies, often *in vitro* (see Smyth and McManus, 1989), and more recently from immunopathological interactions (Hoole and Arme, 1986, 1988; see section on pathology).

Figure 11.3 illustrates a generalized life cycle of fish tapeworms. Fish tapeworms do not exhibit modifications such as resistant eggs or cysts to withstand abiotic factors

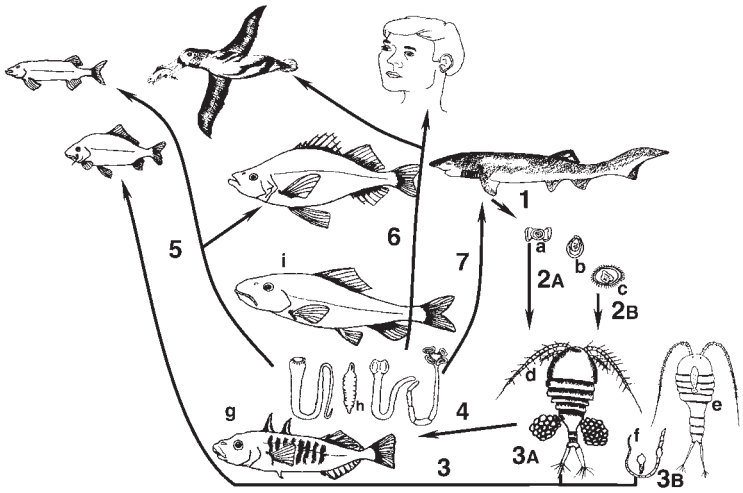


Fig. 11.3. Diagram represents life cycles of important freshwater fish tapeworms. 1. Eggs from definitive host enter water. 2A. Embryonated egg of Proteocephalidea infective to invertebrates. 2B. Hatched coracidium of Pseudophyllidea, Tetraphyllidea and Tetrarhynchidea infective to invertebrate intermediate hosts. 3. Infection of definitive host via infected invertebrates. 3A. Pseudophyllidea and Proteocephalidea. 3B. Caryophyllidea. 4. Infection of fish intermediate hosts via infected invertebrates (*Diphyllobothrium* spp., *Triaenophorus* spp., *Proteocephalus* spp., Tetraphyllidea, Tetrarhynchidea). 5. Infection of piscivorous fish definitive hosts via infected prey fish (*Proteocephalus* spp., *Triaenophorus* spp.). 6. Infection of homeotherm definitive host via infected prey fish (*Ligula, Schistocephalus, Diphyllobothrium* spp. (*D. latum* of humans and *D. dendriticum* and *D. ditremum* of gulls also utilize paratenic fish hosts)). 7. Infection of definitive cartilaginous fish hosts (sharks, rays, etc.) by larvae of Tetrarhynchidea and Tetraphyllidea via infected prey fish. a: Embryonated egg of Proteocephalidea, often with modifications to facilitate floating and dispersal; b: embryonated operculate eggs of Pseudophyllidea, Tetraphyllidea, Tetrarhynchidea; c: ciliated motile coracidia released from b; d and e: procercoids in invertebrate hosts; f: larva of Caryophyllidea in tubificids; g: fish intermediate hosts; h: larval stages (plerocercoids and plerocerci) infective to definitive hosts; i: paratenic fish host.

and are transferred passively. These tapeworms usually release eggs that complete embryonation in water and commonly hatch into ciliated, motile and short-lived larvae called coracidia. Non-motile embryos possess egg envelopes modified to maintain motion and position or attract invertebrate hosts in the water column, thereby enhancing the chance of being ingested by invertebrates (Jarecka, 1961). In the haemocoel (body cavity) of the invertebrate host, the coracidium differentiates and develops into a procercoid stage. Although not much is known about the factors that affect development, we know that space and nutrients are important (Shostak *et al.*, 1984). The migration of the procercoid and its differentiation into a plerocercoid in fish muscle is best illustrated by *T. crassus* in coregonids (Rosen and Dick, 1983). The magnitude of size increase is spectacular in larval *Ligula* and *Schistocephalus*, where, in the body cavity of the fish, the biomass of the parasite may reach 40% of the body weight of the host.

The use of paratenic hosts is well documented in fish parasites (Fig. 11.3). In the genus *Diphyllobothrium*, a piscivorous fish feeds on a smaller infected fish of the same or different species and the plerocercoid stage re-encysts in the new host. *D. dendriticum* and *D. ditremum* are good examples of this phenomenon as high numbers of larval stages accumulate in large Arctic charr (*S. alpinus*) in Arctic lakes. Numerous studies have indicated that certain larval tapeworms (*Ligula* and *Schistocephalus*) influence the behaviour of their fish hosts and enhance transmission, and parasitized intermediate hosts may be more susceptible to predation by natural

definitive hosts (Mackiewicz, 1988). Transmission to the definitive host (fish, bird or mammal) is through ingestion of the infected intermediate host and can involve consumption of carrion (natural mortality) or offal left by sports and commercial fishermen.

Host specificity of fish parasites is difficult to determine as few studies have adequately evaluated all possible routes of transmission. For example, new hosts are constantly being found for larval tapeworms (Hoffman, 1967; Margolis and Arthur, 1979). Some parasites, e.g. Caryophyllidea, are more specific in their invertebrate hosts than others, e.g. Pseudophyllidea. There can be narrow host specificity even in species that have a wide geographical distribution, but the distribution is usually closely tied to the distribution of the definitive hosts. For example, *Triaenophorus* has a worldwide distribution that is restricted and closely correlated to its host, *Esox* (pike). *T. nodulosus* is the most widely distributed species, with 78 species of intermediate fish hosts. However, *T. crassus*, with 39 reported intermediate hosts (Kuperman, 1973; Margolis and Arthur, 1979), has two preferred hosts, whitefish and cisco, in North America. Plerocercoids of *Ligula* occur in a wide range of hosts (e.g. catastomids, centrarchids, cyprinids, percids, salmonids), but the definitive host is *Larus canus* (common gull). Host specificity is also evident in *Diphyllobothrium* plerocercoids in fish from Quigly Lake, Canada, where *D. dendriticum* occurs in whitefish and cisco (*Coregonus artedii*) while *D. latum* occurs in pike and walleye (*Sander vitreus*).

As fish are poikilotherms, the effects of abiotic factors are pronounced on their tapeworms, where growth and maturation of these parasites are tied to temperature. Most infections of fish occur during the summer and early autumn and adult tapeworms grow and mature during late spring and early summer. In spring and early summer, most tapeworms reach sexual maturity and release eggs; this is the time when populations of the invertebrate hosts reach their peak. Differentiation into procercoids is also rapid, due to high water temperatures, and there is sufficient time for infections of fish to occur prior to winter. Adult tapeworms may be shed during the summer, with new recruitment in the late summer and early autumn, e.g. *T. crassus* in pike and most proteocephalid species. The mechanism of synchronization of tapeworm maturation and the physiological state of the pike is not well understood but we know that loss of *T. crassus* in pike is closely synchronized with the spring spawning of pike. The entire tapeworm population in a fish is shed at spawning and this may occur in about a week. This has the added advantage that tapeworm eggs are concentrated in shallow, warm water, the same areas that young whitefish and cisco frequent during their early stages of development.

The acquisition of larval tapeworms is similar to that of adults. If there is encystment, it usually occurs over a 2-month period in the first summer and with some associated pathology (see pathology section). Most studies report an accumulation of larval stages of the other tapeworms over time but *D. latum* in the flesh of pike and walleye shows an annual increase in the summer and a decline during the winter months. Studies on *Ligula* suggest an annual recruitment with some intraspecific interaction, apparently tied to space and nutrients in the fish host. Studies using *Ligula* and *Schistocephalus* are providing some fundamental insights into parasitism. Some of the interactions are expressed through reduced growth of the parasite through a crowding effect or, alternatively, differences in size of parasites may be the result of new parasite recruitment (Szalai *et al.*, 1989). Bagamian *et al.* (2004) reports that *Schistocephalus solidus* significantly lowered body condition in three-spined stickleback (*Gasterosteus aculeatus*) during the latter part of the breeding season, and Overli *et al.* (2001) concluded that sticklebacks infected with *S. solidus* experience chronic stress, as evidenced through monoaminergic activity by elevated 5-hydroxyindoleacetic acid (5-HIAA) : 5-hydroxytryptamine (5-HT). Heins *et al.* (1999) found that substantial numbers of female three-spined stickleback produced eggs even though they harboured infections of *S. solidus*, but they did not evaluate clutch size and mass or egg size.

Reimchen (1997) concluded that infection of stickleback with *S. solidus*, *Cyathocephalus truncatus* and *Eustrongyloides* sp. was correlated with pelvic asymmetry and concluded that it was a phenotypic signal of parasitism. Under field conditions, Loot *et al.* (2001) found that the behaviour in roach infected with *Ligula intestinalis* is modified and parasitized fish occur close to the bank. They concluded that this behaviour enhances transmission to piscivorous birds.

The typical proteocephalid life cycle includes an intermediate, definitive and paratenic (or reservoir) host (Wagner, 1954). Few studies have focused on the life cycles of individual *Proteocephalus* species and, to date, they remain one of the least studied groups of cestodes. More information on life cycles, growth and development can be found in Scholz (1999a).

Copepods of the orders Calanoida and rarely Cyclopoda serve as intermediate hosts (Zaika, 1965; Evseeva, 1996; Scholz, 1999a,b) and peak abundances of the larval stages often follow closely the seasonal abundances of the host (Evseeva, 1996; Rusinek *et al.* 1996; Hanzelova and Gerdeaux, 2003). Oncospheres ingested by the host migrate to the body cavity and quickly grow into developing 'metacestodes' (Scholz, 1999a). This stage metamorphoses into the procercoid stage, which is infective to the paratenic or other intermediate hosts. The procercoid possesses a well-developed anterior scolex with four muscular suckers and, although it is much smaller than that of the adult tapeworm, its morphology remains the same (Scholz, 1999a,b). Although it is difficult to distinguish between species of *Proteocephalus* using procercoid morphology, Sysoev *et al.* (1994) found differences between the procercoids of four species of *Proteocephalus* using body and scolex shape, body size and relative position of suckers. Other methods used to identify procercoids are excretory system morphology, embryonic hook size and the motion of larvae once liberated from the host (Doby and Jarecka, 1966; Scholz, 1999a).

Paratenic hosts of *Proteocephalus* species have not been reported extensively in the literature; however, Rusinek *et al.* (1996) found that the gobiid *Cottocomephorus grewingki* serves as a paratenic host for *Proteocephalus exiguus* in Lake Baikal. Similarly, the common sculpin is a natural paratenic host to *Proteocephalus longicollis* in Europe (Moravec, 2001). Vojtkova and Koubkova (1990), Kennedy *et al.* (1992) and Scholz and Moravec (1993) found *Proteocephalus* larvae in the alder-fly larvae (Megaloptera); however, its role in the transmission of this parasite has not been investigated.

Infection of the definitive host occurs by the ingestion of infected intermediate hosts. A low establishment rate within the definitive host gut has been documented by several authors (Meggitt, 1914; Jarecka and Doby, 1965; Doby and Jarecka, 1966; Willemse, 1968, 1969; Malakhova and Anikieva, 1976; Rintamaki and Valtonen, 1988; Morandi and Ponton, 1989; Hanzelova *et al.*, 1990; Scholz, 1991, 1993; Kennedy *et al.*, 1992), but reasons for this have not been sufficiently studied.

Recent morphological studies have revealed differences between *Proteocephalus* species that have not been previously described. These include spermiogenesis (Brunanska *et al.*, 2003a,b, 2004), ultrastructural studies on the male genital system (Korneva and Davydov, 2001; Brunanska *et al.*, 2003a,b; Iqbal, 2003) and the female genital system (Swiderski and Conn, 1999; Korneva, 2001), embryonic envelope (Brunanska, 1999; Iqbal and Wooten, 2002), scolex morphology and sensory receptor ultrastructure (Stoitsova *et al.*, 1995; Brunanska *et al.*, 1998, 2000; Scholz *et al.*, 1998b; Rego, 1999; Zd'arska and Nebesarova, 1999).

Host–Parasite Relationships

Fish have a well-developed immune system (Chapter 18), which is often temperature-dependent. Most insults to the fish host by a helminth produce a strong cellular response, with non-specific inflammation, while the main immunoglobulin (antibody) is of the IgM class. Acute inflammation

from heavy metazoan parasite infections commonly produces lesions manifested by cell death and necrosis and distortion of tissues. Generally, migrating parasites (larval forms) produce the most serious reactions: leucocytosis and fibrosis and, more rarely, haemorrhage, hyperaemia and necrosis (Arme *et al.*, 1983).

Cellular responses and tissue pathology

The host cellular response to tapeworm infections may result in total (as in *Diphyllobothrium*) or partial (as in *Ligula*) encapsulation of the parasites. The cellular exudates that initially accompany visceral reactions consist primarily of fibroblasts. Infiltration in tissues or in the body cavity by leucocytes is dominated by macrophages, neutrophils and lymphocytes (Hoole and Arme, 1983). Sharp *et al.* (1992) found that a cellular response to *D. dendriticum* plerocercoids occurred during the first 2 weeks post-infection, followed by an increase in neutrophils and a large influx of macrophages, which eventually transformed into epithelioid cells. A blood vascular network eventually developed, followed by fibroplasia and the deposition of a collagenous tissue matrix (Sharp *et al.*, 1992). The lymphoid organs of fish such as roach (*Rutilus rutilus*) infected with *Ligula* undergo changes, such as an increase in melano-macrophage centres in the spleen and an increase in 'vacuolated granulocytes' in the pronephros (Taylor and Hoole, 1989). With time, cellular activity decreases as the parasite is encapsulated and isolated. Encapsulation is a general phenomenon and may occur in the viscera (with *Diphyllobothrium* spp., *T. nodulosus*, *Proteocephalus*) or in the muscle (*T. crassus*). Although the parasites are encapsulated, they often live as long as their hosts.

The pathophysiology caused by the plerocercoids of *Ligula* and *Schistocephalus* has been reviewed in detail (see Arme *et al.*, 1983). This includes growth retardation and abdominal distension, as well as displacement and morphological/physiological alteration of internal organs. Metabolic changes, such as inhibition of gametogenesis and reduced liver glycogen, muscle carbohydrates, proteins, blood amino acids and lipids, are frequently associated with infections. Other effects are reduction in oxygen consumption by the host and an increase in serum proteins and alkaline phosphatase. The parasite may increase its own phospholipids at the expense of the host muscle (Arme *et al.*, 1983; Kurovskaya and Kititsyna, 1986; Rzeczkowska and Honowska, 1988). An increase of enzymes was also correlated with *T. nodulosus* infection of the liver and lesion production (Scheinert and Hoffman, 1986).

Reduced fecundity in small-mouth bass has been associated with *P. ambloplitis* plerocercoids, which cause gonad tissue destruction (McCormick and Stokes, 1982). Outright mortalities can be caused by larval tapeworms such as *Diphyllobothrium* and *Triaenophorus*, especially in aquaculture situations (Rodger, 1991), and mortality may be dependent on the intensity of infections (Rosen and Dick, 1983; Halvorsen and Andersen, 1984). The causes of mortality include destruction of the tissue–water interface, extensive haemorrhaging and secondary microbial infection. The liver is a favourite site of infection and the pathology may include haemorrhaging, necrosis, fibrosis, oedema and discoloration (Arme *et al.*, 1983). Mortality is often attributable to liver dysfunction and blood loss (in salmonids infected with *D. ditremum* (Weiland and Meyers, 1989; Rodger, 1991), in acipenserids infected with *Amphilina* spp. (Popova and Davydov, 1988), in trout infected with *T. nodulosus* (Scheuring, 1923; Bauer, 1959; Kuperman, 1973) and in bluegills and bass infected with larvae of *Proteocephalus* (Mitchell *et al.*, 1983; Joy and Madan, 1989)).

The early lesions in whitefish muscle infected with *T. crassus* include haemorrhaging and discoloration. The extent of the pathology due to differentiating and migrating plerocercoids (Fig. 11.4) is usually underestimated. Rosen and Dick (1983) showed by timed and serial sections that damage is quite extensive (Fig. 11.4). It has been suggested that the pathogenicity of *D. dendriticum* depends on the host's defence mechanism. Fish with weak (in salmonids)

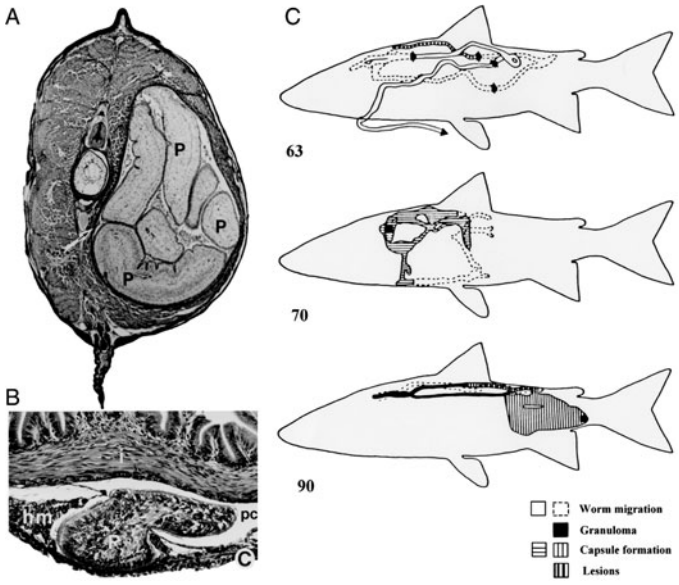


Fig. 11.4. Pathology caused by *T. crassus* in experimentally infected whitefish. A. Cross-section through posterior region showing encapsulated plerocercoid (arrow) at 90 days post-infection. B. Section through region of small intestine showing the migrating plerocercoid at 5 days post-infection. C. Reconstruction of the extensive migration of the plerocercoid and associated tissue changes at 63, 70 and 90 days post-infection. I, intestine, hm, hypaxial muscle, P, plerocercoid, pc, peritoneal cavity. From Rosen and Dick (1983), courtesy of the *Canadian Journal of Zoology*.

or no cellular response (in *Coregonus albula*) have more lesions, which lead to higher mortality; fish (such as *C. laveratus*) that produce a strong cellular encapsulation response show lower mortality (Bylund, 1972). This was also shown with *T. crassus* infections in whitefish (Rosen and Dick, 1983) and rainbow trout (Rosen and Dick, 1984).

Although some species of *Proteocephalus* are found in several host species, their prevalence, mean intensity of infection, ratio of gravid to mature and juvenile worms and worm size indicate that they are more successful in only a few hosts (Hanzelova *et al.*, 1996). Hanzelova *et al.* (1996) found that prevalence and mean intensity increase with the body size of the host; however, infections often remain low. In addition to interspecific differences in parasite loads between host species, some species of the genus exhibit differences with respect to infection level between males and females of the definitive host. Balling and Pfeiffer (1997) found that female perch (*Perca fluviatilis*) maintained higher infections of *Proteocephalus percae* during the spawning period than males. The authors speculated that female perch have higher energy demands during spawning and consume a larger amount of food infected with the parasite during this time period.

Although generally considered to exert negligible pathogenic influences on their hosts (Kassai, 1999), species such as *P. longicollis* may negatively affect growth and the general health of cage-cultured fish species, as in rainbow trout in Europe and North America (Scholz and deChambrier, 2003). Plerocercoids of *P. ambloplitis* have been reported to cause host castration due to the mechanical atrophy of gonads in the fish intermediate host (Freze, 1965; Williams and Jones, 1994).

Pathology in the gut

The pathology caused by tapeworms in the gut may cause tissue alteration or

destruction, mechanical blockage and reduced nutrient absorption. With cestodes that penetrate the mucosal layer (such as *Triaenophorus* and many tetrarhynchideans), the establishment of gut infections usually begins with an initial phase of acute inflammation in the region of contact. The inflammation is characterized by subepithelial oedema, leucocyte infiltration and, in heavy infections, epithelial erosion and necrosis. As infections become more chronic, a relatively low level inflammation is maintained. Also vascularization is decreased to the infected areas and this is accompanied by epithelial hyperplasia (Pronina and Pronin, 1982; Shostak and Dick, 1986). High numbers of parasites (200 worms) did not cause mortality, although the diameter of the lumen was reduced by more than 50% and this probably affects movement of food through the intestine (Shostak and Dick, 1986).

The pathology in the gut is often related to the morphology of the holdfast organs of the parasite. Species of Caryophyllidea that possess well-developed/specialized but non-invasive attachment organs elicit little or no pathology, whereas those with developed holdfasts or possessing an introverted terminal on the holdfast are associated with ulcers and nodules (Mackiewicz *et al.*, 1972). Also, the size of the holdfast and hooks determines the extent of pathology, as has been shown for *Triaenophorus* spp. (see Kuperman, 1973; Shostak and Dick, 1986).

Heavy gut infections may cause intestinal distension, mechanical obstruction and perforation, as in *B. acheilognathi*. The clinical signs include emaciation and anaemia in *Bothriocephalus* (Caryophyllidea) (Scott and Grizzle, 1979). Nutritional demands made by gut tapeworms may adversely affect growth, condition and fitness; *Eubothrium salvelini* in juvenile coho salmon (*Oncorhynchus kisutch*) (Boyce and Yamada, 1977; Boyce, 1979), may cause a change in blood composition or may increase susceptibility to environmental stress, such as zinc. Shostak and Dick (1986) did not find evidence for interspecific or intraspecific competition for nutrients among helminths in the intestine of pike. This suggests that nutrients were not a limiting factor.

Immunity to cestodes

Although the antibody response in fish to microbial pathogens is well documented, knowledge on specific immunity in helminth infections is sparse (Evans and Gratzek, 1989; Sharp *et al.*, 1992). The demonstration of precipitating antibodies in *Abramis brama* (bream) to *Ligula* (see Molnar and Berczi, 1965) must be viewed with some caution. Precipitation reactions may be confused with C-reactive protein activity, which has been demonstrated in turbot (*Reinhardtius hippoglossoides*) with phosphorylcholine-rich extracts from its tapeworm *Bothriocephalus scorpii* (see Fletcher *et al.*, 1980). A complement factor and C-reactive protein have also been implicated in leucocyte adherence to the plerocercoid of *Ligula* (Hoole and Arme, 1986, 1988). Precipitins to phosphorylcholine epitopes, which are common to a variety of organisms, including microbial pathogens and metazoans, have been observed in lake sturgeon, *Acipenser fulvescens* (Choudhury and Dick, 1993). Other studies have only inferred the presence of antibodies to tapeworms (Kennedy and Walker, 1969; McVicar and Fletcher, 1970; Sweeting, 1977). However, recent studies using ELISA have demonstrated unequivocally the antibody response of rainbow trout to *Diphyllobothrium* spp. (Sharp *et al.*, 1989, 1992).

No detectable cellular response was demonstrated in the gudgeon (*Gobio gobio*) infected with *Ligula* (Arme *et al.*, 1983) and pike and walleye infected with *D. latum* (Dick and Poole, l985). However, recent information (Taylor and Hoole, 1989) indicated an increase of melano-macrophages in the spleen of infected gudgeon, although cell counts in the pronephros remained unchanged. There was also evidence to suggest that plerocercoids of *Ligula* and *Schistocephalus* adsorbed host proteins, which helped them evade host immune responses (Hoole and Arme, 1986). Parasite diversity appears to be influenced by major histocompatibility complex (MHC) class IIB variation and this suggests a self-reactive T-cell elimination (Wegner *et al.*, 2003).

Reduced pathogenicity is often taken to indicate a long evolutionary period of host–parasite association. *T. nodulosus* causes little pathology in its obligatory hosts, smelt (*Osmerus*) and perch (*Perca*), but severe pathology in trout. Similarly, *Proteocephalus* evokes a weak host response in its natural hosts (*C. laveratus* and *C. laveratus migratorius*) but causes persistent inflammation in *Thymallus* (grayling). Experimental infections of rainbow trout with *T. crassus* showed extensive worm migrations. The clinical signs included pop-eye, extensive haemorrhaging in the hypaxial muscles and death (T.A. Dick, unpublished). This may account, in part, for the failure of rainbow trout stocking in pike/whitefish/*Triaenophorus* systems. It is commonly held that host-specific helminths are not pathogenic to their hosts. This generalization should be modified as *Amphilina* spp. cause extensive liver pathology in their acipenserid hosts (Popova and Davydov, 1988), for which the genus is specific.

In Vitro Culture

The absence of a digestive tract in cestodes has made them an interesting challenge for culture studies (Taylor and Baker, 1987; Smyth and McManus, 1989). Culture of cestodes may include the hatching processes (mostly due to physical factors), short-term culture or maintenance (which may or may not include the following: nutrient requirements, physicochemical aspects of the media and long-term culture involving differentiation of the parasite). The present discussion will focus on the culture to mature adult stages only. The criteria for development and maturation usually accepted are segmentation, organogeny, gametogenesis and eggshell formation (Smyth and McManus, 1989). Cestodes also pose more of a challenge because of their long flat shape and tendency to 'knot up' in culture. Consequently, in early studies, considerable effort was expended on the physical set-up of the system, i.e. roller tubes vs. continuous flow (Smyth, 1946, 1947, 1982; Berntzen, 1961, 1962; Berntzen and Voge, 1965).

With the exception of *Schistocephalus* and *Ligula*, little work has been done on fish parasites, but these two genera are unusual in that the plerocercoid stage is large with extensive energy reserves in the form of glycogen. Since external nutrients were not required for the culture of *Schistocephalus* and *Ligula*, researchers focused on the physicochemical requirements of the culture system (Smyth, 1946, 1947, 1954). Smyth (1982) developed a special culture apparatus, with ports to add and replace the culture medium, and used cellulose dialysis tubing to compress the worms. Additional culture conditions included highly buffered media to neutralize acid metabolic by-products, 100% horse serum or other well-buffered culture media, anaerobic or very low O_2 pressure (pO_2) to prevent premature oxidation of the phenolic precursors in the eggshells and gentle agitation of the culture medium to ensure diffusion (Smyth and McManus, 1989). Similar conditions were applied successfully to *Ligula*, but due to its larger size the culture system was scaled up accordingly. Smyth and McManus (1989) also pointed out that segments of *Ligula* cut from the median region of the plerocercoid would mature *in vitro*. Culturing the less differentiated plerocercoids of *D. latum* to adults remains a major challenge. In general, much more work needs to be done on cestode culture. Taylor and Baker (1987) summarize the problems and lack of knowledge of *in vitro* culture for cestodes, which include insufficient details on culture procedures, lack of precise criteria for assessing the quality of the media and culture technique, in addition to morphogenesis, lack of defined media, incomplete culture of the entire life cycle and the absence of cell lines.

Nutrition and Physiology

The biochemical composition of cestodes has been compared by Smyth (1976). For most of the fish tapeworms, values are as follows: dry weight as a percentage of fresh wet weight

varies from 27.0% to 31.8%; glycogen as a percentage of dry weight varied from 13.8% in *Triaenophorus* to 38–50% in *Ligula*; lipid as a percentage of dry weight is about 16%; protein varies from about 35% in *Ligula* to 60% in *D. latum.* These values fall within the range reported for other tapeworms. However, in the case of inorganic compounds, the few reports indicate that fish tapeworms have lower values than most other tapeworms (Smyth and McManus, 1989). Smyth and McManus (1989) summarize the general lipid composition of cestodes as being similar to that of other organisms. As cestodes have lipid compositions similar to their hosts, it is not surprising that fish tapeworms have higher levels (70–80%) of unsaturated fatty acids and this is higher than in most other cestodes. The beta-oxidation enzymes (acyl-coenzyme A (CoA) synthetase (short and long chains), acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and acetyl-CoA acyltransferase) are present in *Ligula* and *Schistocephalus* (Smyth and McManus, 1989).

The role of carbohydrates as an energy source in cestodes has been extensively studied, with the end products of carbohydrate breakdown (aerobic and anaerobic) being acetate and propionate in *Schistocephalus*, succinate and lactate in *Diphyllobothrium* and lactate, succinate, acetate and propionate in *Ligula*. In the case of the glycolytic enzymes, fish cestodes are similar to other cestodes. Key glycolytic enzymes, such as hexokinase (in *Bothriocephalus*), phosphofructokinase (in *Schistocephalus*), pyruvate kinase (in *Ligula* and *Khawia*) and lactate dehydrogenase (in *Schistocephalus*), have been demonstrated (Smyth and McManus, 1989). It appears that *Schistocephalus* is capable of utilizing carbohydrates by a functional tricarboxylic acid (TCA) cycle (Koerting and Barret, 1977). Oxidative phosphorylation may occur in *D. latum*, since its mitochondria can oxidize nicotinamide adenine dinucleotide (NADH) and succinate, and there is some fragmentary evidence for the pentose-phosphate pathway in *Ligula* and *Schistocephalus*. *B. scorpii*, in contrast to other cestodes, excretes methyl butyrate as an end product of respiration (Smyth and McManus, 1989).

The amino acid composition of all cestodes is similar to that of other organisms. Collagen is the most common structural protein in tapeworms and has been characterized from *Bothriocephalus* and *Nybelinia* (Smyth and McManus, 1989). Proteases have been reported from *Schistocephalus* and *Ligula*, but their functions remain obscure as amino acids do not appear to have an important role in cestode energetics. However, a much more plausible role for proteases is for tissue penetration by larval cestodes.

There are extensive reviews on the transport of nutrients across the cestode tegument (Arme and Pappas, 1983; Threadgold, 1984). Although the presence of a sodium pump has been demonstrated in cestodes, enzymes such as sodium or potassium adenosine triphosphatase (ATPase) have not been detected in most cestodes (Threadgold, 1984; Smyth and McManus, 1989). Pinocytosis as a means of nutrient uptake has been demonstrated in *Schistocephalus* (Hopkins *et al.*, 1981). Functional differences along the tegument of *Bothriocephalus gregarius* suggest different physiological functions (Berrada-Rkhami *et al.*, 1990). Glycoconjugates of the neck appear to be involved in recognition, adherence, fixation and protection. Those glycoconjugates on the strobila are primarily nutritional, but may also protect against digestive enzymes of the host.

Identification of Parasites

Identification of adult fish tapeworms is generally done with the aid of morphological keys, and it is the larval tapeworms (especially the pseudophyllideans) that present the most problems. Morphological keys are available for *Diphyllobothrium* (Andersen and Gibson, 1989) and general references (such as Hoffman, 1967) are useful. Isozymes were used to distinguish between *D. latum* and *D. dendriticum*

(deVos and Dick, 1989) and between *D. latum* and *Diphyllobothrium nihonkaiense* (Fukumoto *et al.*, 1987). Populations of *Bothriocephalus* have also been examined using enzyme electrophoresis. *Bothriocephalus barbatus* was found to be homogeneous, but *B. gregarius* could be divided into two groups based on geographical distribution (Renaud *et al.*, 1986). McManus (1985) showed that natural populations of *L. intestinalis* were polymorphic for the enzyme phosphoglucomutase (PGM) and populations of *L. intestinalis* were not correlated to geography or host species, while other enzymes such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glucophosphate isomerase (GPI) were not.

Antigenic affinities were demonstrated between *D. dendriticum* and *D. ditremum* and between *Diphyllobothrium vogeli* and *D. ditremum* whereas *D. latum* showed no cross-reactivity with the other three species (Freze *et al.*, 1983). Molecular techniques utilizing ribosomal DNA (rDNA) probes hybridized against endonuclease-restricted genomic DNA l (deVos *et al.*, 1990) were used to identify *Diphyllobothrium* plerocercoids. Oligonucleotide primers from sequenced intergenic spacer regions of rDNA from *D. latum* and *D. dendriticum* have been used to identify plerocercoid and adult *D. latum* and *D. dendriticum* (T.A. Dick, unpublished). Numerous recent papers have been on the use of molecular techniques to study members of the order Proteocephalidea (Zehnder and Mariaux, 1999) and the phylogenetic position of the Caryophyllidea, Pseudophyllidea and Proteocephalidea (Kodedova *et al.*, 2000), *D. nihonkaiense* (Ando *et al.*, 2001), *Proteocephalus* sp. (Scholz *et al.*, 1997, 2003b) and diphyllobothriid tapeworms (Logan *et al.*, 2004). A number of nucleic acid sequences are currently being used and they include internal transcribed spacer (ITS) rDNA (Kral'ova-Hromadova *et al.*, 2003; Logan *et al.*, 2004), ITS-1 and ITS-2 and 5.8 ribosomal gene (Kral'ova-Hromadova *et al.*, 2001) microsatellite markers (Luo *et al.*, 2003) and 28 rDNA (Scholz *et al.*, 2003a,b).

Prevention and Control of Tapeworms in Fish

Numerous studies, including some field applications, have evaluated the role of drugs for fish tapeworms and some are effective, especially those against *Bothriocephalus* and *Proteocephalus* (Table 11.2). Niclosamide has proven effective against fish tapeworms when given orally but is highly toxic to fish when added to water (Schmahl, 1991). Short exposure and low concentrations are satisfactory for sticklebacks (*G. aculeatus*).

Use of vaccines to prevent tapeworm infections in fish is not well developed, but new suggested approaches include conjugating a therapeutic drug to a monoclonal antibody (Scholz, 1999b).

Aquaculture facilities frequently use nearby natural water bodies as their source of water. These natural systems often have hosts (invertebrate and vertebrate) infected with tapeworms such as *Diphyllobothrium*, *Proteocephalus* and *Triaenophorus*. This may lead to the appearance of *Triaenophorus* (larvae) and *Proteocephalus* (adults) in cultured fish (usually trout) with the introduction of infected copepods into the water. Water filtration systems (sand, screens) or use of groundwater will help to prevent the spread of these parasites to aquaculture facilities.

Numerous environmental manipulations of natural systems have been attempted and these include: (i) extirpation of fish-eating birds in the vicinity of an aquatic system; (ii) extirpation of pike in the case of *T. crassus* (Lawler, 1970); and (iii) the removal of cisco to reduce larval cysts in the commercially valuable whitefish (Miller, 1952; T.A. Dick, unpublished). An intensive programme to remove pike from Hemming Lake, Manitoba, succeeded in the extirpation of *T. crassus*. However, cisco were inadvertently extirpated, with a substantial decrease in the number of whitefish, and, while pike size decreased, the total number of pike actually increased. The intensities of *T. crassus* plerocercoids in whitefish were reduced by 42% in whitefish in Quigly Lake through the selective removal of cisco. This was conducted prior to spawning in the autumn, but at the

Table 11.2. Chemotherapy in the control of fish tapeworms.

Parasite	Target host (location)	Chemical	Result	Reference
Bothriocephalus acheilognathi	Carp (China)	Cucurbita, areca[1] (ground up in feed)	Effective	Nie and Pan, 1985
	Carp (E. Europe)	Taenifugin carp[2] (minimum 2% of fish wt)	Successful in feed, two times over 3 weeks	Zitnan *et al.*, 1984
	Carp (Europe)	Zestocarp[2]	80–100% reduction	Weiroski, 1984
Bothriocephalus gowkongensis	Carp	Mansonil, Yomesan[2]	1 g/kg fish, 100% effective	Par *et al.*, 1977
	Carp	Taenifugin carp[2]	100% effective	Kral *et al.*, 1980
	Carp, grass carp	Niclosamide	Successful, incorporated into fish pellets	Schmahl and Taraschewski, 1987
Bothriocephalus opsariichthydis	Carp	Phenasal	100% effective	Albertova and Michurin, 1984
Bothriocephalus sp.	Carp (S. Africa)	Lintex[3] (50 mg/kg fish wt)	100% eradication	Brandt *et al.*, 1981
Caryophyllaeus sp.	Carp	Kamala[1]	Infection reduced	Bauer, 1959
	Carp	Rottlera[1]	Infection reduced	Bauer, 1959
Caryophyllaeus and *Khawia*	Carp	Zetocarp[2] (Cestocarp)	(> 12°C, day 1: 20 g/kg fish, day 2: 10 g/kg fish) 93–100% effective	Schaperclaus, 1992
Proteocephalus ambloplitis	Bass (N. America)	Mebendazole (100 mg/kg fish wt per day in feed)	90% reduction	Boonyaratpalin and Rogers, 1984

[1]Herbal extracts.
[2]Active ingredient niclosamide.
[3]Active ingredient 2′, 5-dichloro-4′-nitrosalicylanilide. See Schaperclaus (1992) for details.

same time maintaining a reproductively viable cisco population (T.A. Dick, unpublished). The removal of invertebrate hosts (copepods) of *T. crassus* was considered but was unacceptable as they are important components of the food chain of many aquatic animals (Miller, 1952).

Conclusions

Significant contributions have been made to our understanding of the proteocephalids as a group and the genus *Proteocephalus*. More work is still needed on this group of unrelated taxa. While it does not appear to have much of a negative impact on fish, it may be useful as a model to study phylogenetic and co-evolutionary associations, or lack thereof, between fish and their parasites. Studies are needed to relate larval stages of tetraphyllideans and tetrarhynchideans to their adult stages through life-cycle studies, and the use of the name *Scolex pleuronectis* should be discontinued. Also, there is very little known about the intraspecific variability (i.e. morphological, biological and genetic) in the genus *Diphyllobothrium*. The importance of *B. acheilognathi* as a worldwide pathogen of fish, with over 40 recorded fish hosts to date, indicates that the biology, infectivity and genetics of this tapeworm need further study. Significant challenges include its transfer across watersheds through engineered changes in water movements and its potential impacts on endangered and threatened fish species worldwide.

Morphological identification of larval tapeworms still relies on the local 'expert', but increased application of nucleic acid sequences is changing this approach. The perception of narrow host specificity in fish tapeworms should be viewed with caution. More needs to be known about potential hosts in a given system and the significance of these hosts in transmission. Basic information is lacking for most fish parasite systems, especially the significance of biological and genetic variability. Manipulations of portions of the aquatic ecosystem to control the spread of tapeworms in fish need more research. Parasite numbers can be reduced without drastically altering the fish species composition in a system, but an understanding of fish population dynamics, as well as parasite transmission, is essential. Knowledge of predator–prey interactions, including sports and commercial fishing, must be integrated into the concept of parasite transmission. Knowledge of food chains may help to reduce *D. latum*, *D. dendriticum* and *D. ditremum* from Arctic and north temperate systems. Little is known of the effects on the indigenous fish parasite populations following stocking of an exotic piscivorous fish species. Increased efforts to use fish tapeworms and other fish parasites as natural indicators to gain new insights into trophic strucuture and food webs in marine and freshwater ecosystems would give greater resolving power to current tools, such as stable isotopes.

As freshwater aquaculture operations expand, particularly cage culture, and the use of surface water by hatcheries becomes more common, we can anticipate additional problems with parasitic tapeworms. These include mortality, decreased growth and unsaleable products for human consumption. This is due to the difficulty of excluding copepods from the water supply and increased susceptibility of cultured species (cyprinids, coregonids, percids, salmonids) to indigenous tapeworms. Careful selection of aquaculture sites and monitoring of the water supply will have to be done to minimize some of these problems.

Only a few tapeworm diseases of fish have been closely monitored and carefully documented. In general, cell-mediated immunity appears to be the primary host response. The pathology is progressive, with the formation of large pustules and eventually host encapsulation. It may result in the destruction of the parasite, formation of a granuloma and finally its resorption. The humoral antibody response to tapeworm infections is not well understood. *T. crassus* is a useful model system to study the interaction of cellular and humoral responses since the parasite produces extensive pathology in

the intestine of pike and in the muscle of cisco and whitefish. Chemotherapy and/or vaccines to control tapeworm infections are unlikely options, although they may have a role in intensive cage-culture operations. However, much more will have to be known about the efficacy of the drugs and their persistence in fish tissues. Similarly, considerable basic research is required to understand the fish immune system and its relation to tapeworms.

Finally, there are only a few examples where a migrating and differentiating larval helminth does not seem to elicit a cellular response in the host. In this respect, the *D. latum*/pike/walleye system and *Schistocephalus*/*Ligula* may be useful models.

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12 Phylum Nematoda

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Introduction

Parasitic nematodes constitute one of the earliest known groups of helminths in fishes. They infect freshwater, marine and brackish-water fish species and sometimes cause substantial damage to the host. Although parasitic nematodes can infect almost all organs in a fish, the majority of the currently known species have been described from the intestine. Most nematodes infect fish as adults, but a large proportion of them occur as larval stages. These are usually parasites of piscivorous birds, mammals or reptiles, or less frequently of predatory fishes.

The majority of nematodes reach sexual maturity through a complicated developmental cycle involving an intermediate or possibly paratenic hosts. Species living in the temperate zone are usually characterized by seasonal occurrence, and annual life cycles are common. Because of the complicated, multi-host life cycle, the development of fish nematodes is successful in non-disrupted ecosystems. In fish taken from their natural surroundings, nematode infections are less likely to develop. For these reasons, nematodes cause less damage in cultured fishes than do other helminths. At the same time, certain nematodes can give rise to massive infections with high fish mortality in natural waters.

Although nematodes are important pathogens, their direct pathogenic effect on fishes is much less important than the role played by them as causative agents of zoonoses. Nematode infections in marine fishes cause a range of problems. Some of these are associated with pathogenicity of the parasite to the fish host, while others are health hazards connected to human ingestion of live nematodes in fresh or undercooked fish. Furthermore, consumer attitudes to the presence of nematodes in foods also have a great impact on the market value of fish products and may stress the need for strict monitoring and diagnosis of nematode infections in marine fish stocks.

The prevalence of parasitic nematodes on the different continents is not equally well known. The majority of species have been described from Europe. Detailed data on nematodes of freshwater fishes (Avdeev *et al.*, 1987; Moravec, 1994) and those in North American fishes (Hoffman, 1999) are readily available. Recently major progress has been made in the study of fish-parasitic nematodes in the Neotropical region (Moravec, 1998). Of the nematodes parasitizing marine fishes, anisakid species causing human infections are relatively well documented (Hauck and May, 1977; Smith and Wootten, 1978; Berland, 1981; Sindermann, 1990; Koie *et al.*, 1995). Relevant data on the

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occurrence and pathological role of fish-parasitic nematodes are found in the first edition of this publication (Dick and Choudhury, 1995). Since the publication of the work, relatively little new information has been added to our existing knowledge of fish-parasitic nematodes. The new data concern primarily the development, pathological effect and human health implications of a few selected groups (*Anguillicola*, *Philometra*, *Skrjabillanus*, *Anisakis*).

Economic Importance

In some cases, parasitic nematodes can produce very spectacular and massive infections in fishes. The masses of *Philometra ovata* (Fig. 12.1) in the abdominal cavity of gudgeon or the white-coloured *Hysterothylacium bidentatum* (Fig. 12.2) in the stomach of sterlet (*Acipenser ruthenus*) definitely indicate that nematodes are important pathogens. In spite of this, Williams (1967) rightly stated that among the helminth parasites of fishes the pathological effect of nematodes had been studied the least. The situation has not changed. Although the number of known species has gradually increased, there are still few new data on the pathological effects of nematodes. In freshwater fish species, the new data mostly concern *Anguillicola crassus* (Fig. 12.3) infection in eel (*Anguilla anguilla*). As regards marine fish parasites, the anisakid species (*Anisakis*, *Contracaecum*, *Hysterothylacium*, *Pseudoterranova*) have come into prominence because of infections in humans.

Fish nematodes might harm their host in a variety of ways. They can cause mechanical injuries, atrophy of tissues, occlusion of the alimentary canal, blood vessels and other ducts and toxication from their metabolic products, and they can deprive the host of food, enzymes and vitamins.



Fig. 12.2. *Hysterothylacium bidentatum* specimens in the stomach of the sterlet (*Acipenser ruthenus*) (× 1.2). Photo by György Csaba.



Fig. 12.1. *Philometra ovata* filling the abdominal cavity of a gudgeon (*Gobio gobio*) (× 0.6).



Fig. 12.3. Heavy and moderate infections with *Anguillicola crassus* in opened swim bladders of European eels (× 0.6).

Host Range

Nematode infection occurs in practically all fish species and in all locations. The prevalence, intensity and economic importance of nematode infections, however, vary by region and by fish species.

The host range of these parasites is primarily influenced by the host specificity of the nematode species for the final host and for the intermediate host. Species having a narrow host range are usually in a fish host that is prevalent in a given habitat, while species with a broad host range are in fish species all over the world. These nematodes can often cause mild disease in all members of a fish genus and, after colonizing a new host species, they can give rise to much more severe disease. Such a species is *A. crassus*, a nematode of Japanese eel (*Anguilla japonica*), which causes a mild disease in its original hosts. However, this nematode is much more pathogenic in European and American eel species (*Anguilla rostrata*).

The typical examples of highly specific nematodes are the members of different skrjabillanid genera (*Skrjabillanus tincae*, *Skrjabillanus cyprini*, *Molnaria intestinalis*, *Sinoichthyonema amuri*), which in most cases infect a single fish species.

Nematodes with a broad host range are represented by *Capillaria* species, which can colonize numerous fish species of different taxonomic positions. Fish parasites characterized by a global range include the larval stages of nematodes of seabirds and marine mammals in their adult stage (*Anisakis*, *Pseudoterranova*). The distribution of the majority of freshwater nematode species is mostly restricted to a single continent or specific zoogeographical zones thereof.

Systematic and Taxonomic Position

The phylum Nematoda consists of about 40,000 species of free-living and parasitic nematodes (Anderson, 2000). Nematodes occur also in fish, though the number of species infecting fishes is relatively few if they are compared with the terrestrial hosts, which are highly diverse and rich in taxa (Anderson, 1996). Phylum Nematoda is divided both by cladistic and molecular methods into two major classes, Adenophorea and Secernentea. Adenophorea consist mostly of free-living marine and freshwater species, as well as terrestrial soil nematodes with only a small number of parasitic organisms. These include three families: Dioctophymatidae, Capillariidae and Cystiopsidae. Secernentea also have free-living taxa, but the vast majority of this class are parasitic organisms.

Classification of fish nematodes in this chapter is based on Anderson's (2000) system, but some modifications have been made in lower taxons, as suggested by Moravec (1994, 1998).

Parasite Morphology and Life Cycle

Morphology

Nematodes in marine and freshwater fishes are highly variable in size, from microscopic to 10–20 cm in length, as with *H. bidentatum*, *Philometra obturans* and *Philometroides cyprini* (Fig. 12.4), and there are specimens as small as *Lucionema balatonense*. Adult nematodes are sexually dimorphic and they have five stages (L1, L2, L3, L4 and adult stage) with four moults. Most species have a fusiform shape, being widest in the middle and tapering at the anterior and posterior ends. Capillariids and skrjabillanids are typically fusiform with a uniform diameter and an extremely thin, elongated body (Fig. 12.5). Female *Cystoopsis acipenseris* have filiform anterior and globular posterior body regions. Nematodes have a cuticle and are without cilia. They do not have protonephridia, respiratory organs or blood systems.

Female nematodes are usually larger than males. While the size of female ascaridoids, camallanoids and capillariids are only rarely double the size of the males, philometrid females can be 100 times the length and up to 100,000 times the body weight of males. Most nematodes have a yellow or whitish colour but those that live in the blood vessels (*P. obturans*, *Philometroides sanguinea*) or feed on blood (*A. crassus*) may be a red or dark brown colour. Some intestinal nematodes may have a transparent body but with a distinct yellow buccal capsule (*Camallanus*).

The cuticle of nematodes is elastic, and it is thick in gut-dwelling species (*Hysterothylacium*, *Eustrongylides*) and relatively delicate in histozoic specimens (*Philometra rischta*, *Daniconema anguillae*). The surface of the cuticle may be smooth but it can bear longitudinal striations or transverse rows. The cuticle of some nematodes is covered entirely or partly with spines (*Spinitectus*, *Gnathosoma*), and distinct bosses might also cover the cuticular surface (*Philometroides nodulosa*, *P. cyprini*). The cuticle may also have papillae with a tactile function and which serve as chemoreceptors. All papillae are connected by nerve endings.



Fig. 12.5. Female specimen of *Capillaria pterophylli* in the gut of a discus fish (*Symphysodon discus*). (× 66) Photo by Ferenc Baska.



Fig. 12.4. *Philometroides cyprini* female freed from the scale pocket of common carp (*Cyprinus carpio*) (× 0.3).

Most papillae are located at the cephalic and caudal region. According to their location and function, they are called labial, cephalic, cervical and genital papillae.

The structure of the mouth shows great variations. It may be a simple slit-like opening at the anterior end surrounded by distinct or indistinct papillae (*Capillaria*, *Philometra*), but it can form large labia or cuticular outgrowths (Fig. 12.6), called interlabia (*Hysterothylacium*, *Anisakis*, *Raphidascaris*). The mouth leads into the buccal capsule (Figs 12.7 and 12.8), which can be sclerotized and furnished with large denticles, ridges, plates or tridents (*Camallanus*, *Cucullanus*, *Skrjabillanus*, *Anguillicola*). The buccal cavity is followed by the oesophagus. In some species a pharynx is located between the buccal capsule and the oesophagus. The structure of the oesophagus varies; in some groups (ascaridoids, camallanoids, etc.) it is entirely muscular, while in others (e.g. capillariids, philometrids) it has a muscular and a glandular part. The oesophagus joins the intestine directly or through a subglobular or elongated ventriculus. This ventriculus forms an elongated, posteriorly directed tube in ascaridids (*Raphidascaris*). Similar but anteriorly extending elongation of the intestine (intestinal caecum) exists in other ascaridids (*Pseudoterranova*, *Porrocaecum*) and in several genera of this group (*Contracaecum*, *Hysterothylacium*) both ventricular and intestinal appendices exist. The intestine is usually a straight tube finishing in the rectum. The rectum opens on the ventral side near the posterior end. In the males the rectum and the ejaculatory duct form a joint cloaca.

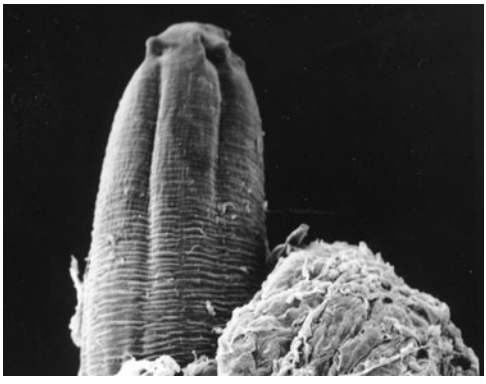


Fig. 12.6. Anterior end of *Anisakis simplex* with interlabia around mouth opening. Scanning electron microscope (SEM) (× 115). Photo by José Bresciani

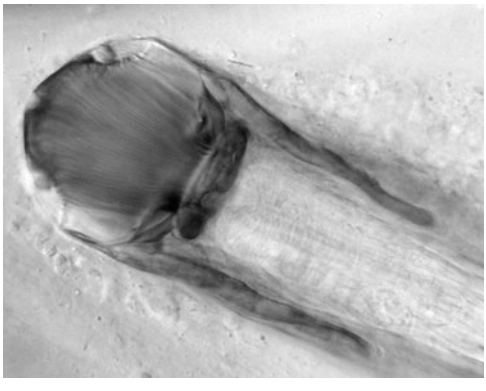


Fig. 12.7. Buccal capsule of *Camallanus truncatus* from the gut of pike perch (*Sander lucioperca*) (× 115).

An oesophageal nerve ring represents the central nervous system, and special structures (papillae and dierids), located mostly in the anterior and posterior ends of body, serve a sensory function.

In some fish nematodes, there are no great differences in the general appearance of the two sexes and without microscopic examination males and females, especially juvenile forms, are hard to distinguish (*Anguillicola*, *Raphidascaris*). Other fish nematodes have striking differences between male and female, both in size and the structure of the tail (*Philometra*, *Skrjabillanus*, *Capillospirura*).

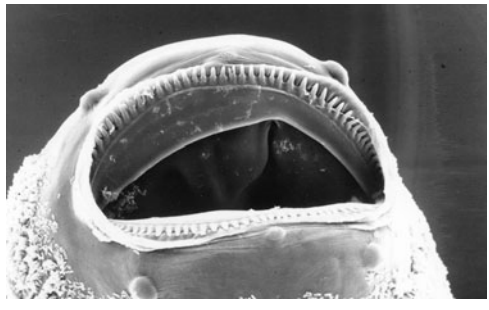


Fig. 12.8. The anterior part of *Cucullanus heterochrous* showing mouth opening. SEM (× 340). Photo by José Bresciani

The male reproductive organ usually consists of testis, vas deferens, seminal vesicle and ductus ejaculatorius. The ejaculary duct, opening into the cloaca, has some accessory copulatory organs. The most common accessory organs are the sclerotized spicules. Most fish nematodes have two spicules. They can be equal or different in size. The number of spicules in Capillariidae is one. No spicules exist in Anguillicolidae. The spicules are often supported by another sclerotized organ, the gubernaculum, which directs the movement of the spicules. In some skrjabillanids, a sclerotized copulatory plate substitutes for the spicules. A typical bursa copulatrix with membranous lateral alae forms the copulatory apparatus of skrjabillanids and a similar structure is found in male *Capillospirura*.

The female reproductive organs are composed of ovaries, oviducts, uteri, vagina and vulva. In most cases there are two sets of tubular ovaries locating anteriorly and posteriorly from the vulva opening. Ovaries continue to oviducts and uteri, from which eggs or larvae pass into a muscular common vagina. The vulva may be located in different parts of the fish body, characterizing families and genera of the nematodes. In *Capillaria*, *Skrjabillanus* and *Raphidascaris* spp., the vulva is found in the first part of the body length, but in *Camallanus* and *Rhabdochona* spp. it is situated postequatorially. The *Capillospirura* vulva is located approximately at mid-length. In adult *Philometra* specimens, the vulva and vagina are absent.

Development

The majority of fish nematodes have rather complicated development. Most are heteroxenous and have final, intermediate and paratenic (transport) hosts. In the life cycle of fish nematodes, there is always a single intermediate host. In the majority of cases, food animals such as aquatic crustaceans (Copepoda, Amphipoda, Mysidacea, Decapoda), annelids, coelenterates, molluscs and small fishes are intermediate hosts. Also parasitic organisms infecting fish, such as cyclostomes and crustaceans of the class Branchiura (fish lice), can transmit developmental stages to fish. Both fish and invertebrates may serve as transport (paratenic) hosts for some nematodes. A transport host is an organism in which no development necessary for the progress of the life cycle takes place. A high number of larvae can accumulate in such hosts. Although direct development without an intermediate host may be a possibility for some groups (e.g. for Capillariidae), this has not been unequivocally proven experimentally. Recently it was shown by Køie (2001c) that the marine *Capillaria gracilis*, which invades the rectum of Atlantic cod, needs intermediate hosts. Thus, chironomids and oligochaetes ingest the larvated eggs, where hatching and some growth of the larvae occurs. Further, the obligate fish intermediate host allows considerable growth of the worm before infection of the final host. In the case of direct development, host finding is often promoted by paratenic hosts, e.g. by oligochaetes carrying the larvated eggs (Bell and Beverley-Burton, 1980; Lomakin and Trofimenko, 1982; Moravec, 1983). Percutaneous transmission by migrating larvae, which is common in terrestrial animals, does not exist in fishes.

Some worms lay unsegmented eggs, and these worms are oviparous. Ovoviviparous worms lay eggs containing the first- or second-stage larva (Fig. 12.9a), and viviparous worms discharge free first-stage larva (Fig. 12.9b).

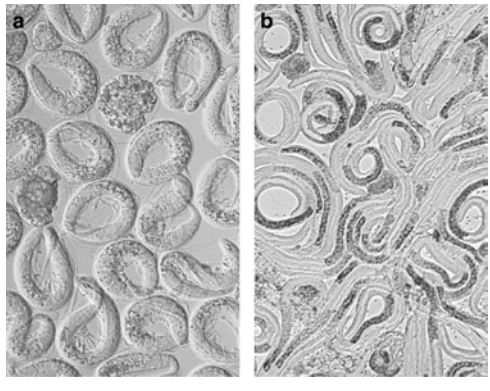


Fig. 12.9. (a) Ovoviviparous eggs of *Anguillicola crassus*. Second-stage larvae inside the extremely thin egg shells, (b) Viviparous larvae of *Philometra ovata* released from a ruptured female (× 85).

Thus the egg development, embryonation of the egg and/or subsequent survival of the larva are highly dependent on the environmental conditions. Hatching of eggs can occur in the environment after release with host faeces or after ingestion by intermediate hosts (oviparity), just after oviposition (ovoviviparity) or in the female worm (viviparity).

Final hosts of fish nematodes are both homeothermic and poikilothermic animals. In the case of some nematodes with a worldwide distribution, marine mammals and fish-eating birds serve as final hosts. Whales are the final hosts of the ascaridoid nematode *Anisakis simplex*, while for another marine ascaridoid, the seal worm *Pseudoterranova decipiens*, seals play the role of the final hosts. *Contracaecum* and *Porrocaecum* spp. have a wide host range and both birds and mammals can be final hosts.

The majority of the known fish nematodes develop to the adult stage in fish. Marine nematodes of this type include *Hysterothylacium aduncum*, *Cucullanus cirratus*, *Cucullanus heterochrous* and *Dichelyne* (*Cucullanellus*) *minutus*, while the genera *Capillaria*, *Camallanus*, *Rhabdochona*, *Philometra*, *Philometroides*, *Anguillicola* and *Raphidascaris* are in freshwater fishes and are regarded as the best studied and economically most important nematodes.

The most common route for infection of the intermediate host is when the eggs or free larvae are consumed by these animals. The first-stage larvae attract the attention of their intermediate hosts by their movement before being eaten by the intermediate host. Nematode larvae of the *Philometra*, *Anguillicola* or *Camallanus* genera penetrate into the haemocoel of the copepod intermediate host (Fig. 12.10) within a very short time and, depending on the water temperature, third-stage infective larvae are formed (Molnár, 1966b; Moravec, 1971; Hirose *et al.*, 1976). Non-motile eggs of *Rhabdochona*, *Cystidicoloides* or *Cystidicola* species are ingested by their mayfly or gammarid intermediate hosts. A special way of development in the intermediate host is when the vector is also a parasite. To date, only the branchiurid *Argulus* spp. (Fig. 12.11) and cyclostomes are known to transmit infection from one fish to another as intermediate hosts.

According to Moravec (1994), there is always a single intermediate host in fish nematodes, and host finding of nematodes is promoted by one or more paratenic hosts. The developmental cycle of capillariid nematodes seems to be monoxenous. Eggs, however, are often taken up by oligochaete paratenic hosts, in which the larvated eggs preserve their viability for a long time and serve as sources of infection. In the development of

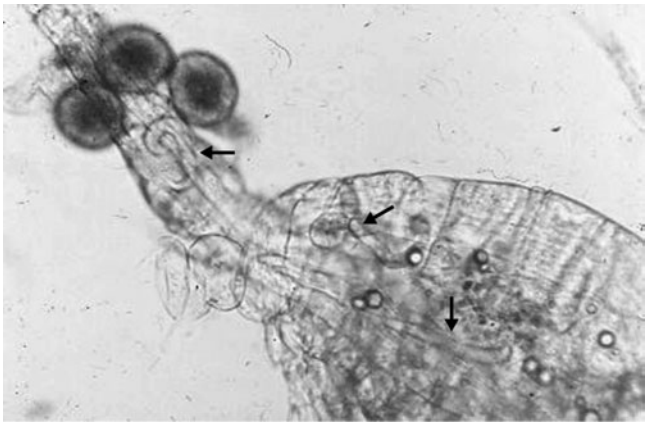


Fig. 12.10. Third-stage larvae of *Anguillicola crassus* (arrows) in the haemocoel of the intermediate host cyclops (× 80).

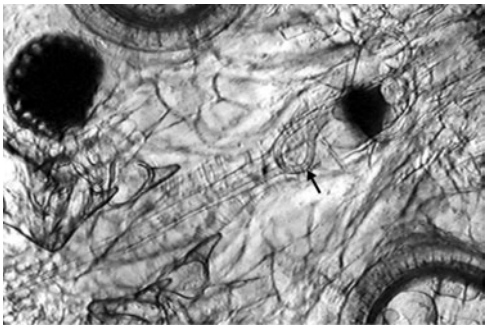


Fig. 12.11. Third-stage larva of a *Skrjabillanus* sp. (arrow) entering the stylet of the intermediate host carp lice (*Argulus foliaceus*) (× 40).

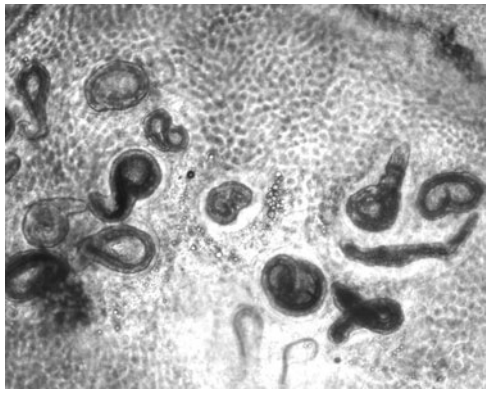


Fig. 12.12. Encapsulated dead and live third-stage larvae of *Anguillicola crassus* in the gut wall of the paratenic host ruffe (*Gymnocephalus cernuus*) (× 25).

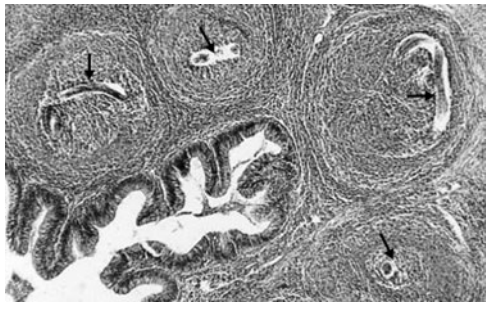


Fig. 12.13. Histological section of the gut of a fingerling of the paratenic host sheatfish (*Silurus glanis*). Third-stage larvae (arrows) of *A. crassus* encapsulated by epithelioid cells and connective tissue. Haematoxylin and eosin (H & E) (× 80).

the ascaridoid *Raphidascaris acus* oligochaetes, snails and crustaceans may serve as paratenic hosts before non-developing larvae get into the obligatory small fishes in which they reach the infective third stage. In this species the final host can also serve as intermediate host. For *A. simplex* the euphasiaceans, for *Contracaecum osculatum* and *P. decipiens* copepods and for *H. aduncum* copepods, amphipods, isopods and mysids can be the intermediate hosts (Smith and Wootten, 1978; Køie, 1993, 2001b; Køie *et al.*, 1995). It is also small fish which act as the true intermediate host for *C. cirratus* (Køie, 2000a), while polychaetes are intermediate hosts for *Cucullanus heterochrous* and *Dichelyne minutus* (Køie, 2000b, 2001a).

In most cases, paratenic hosts (crustaceans, snails, oligochaetes, small fishes and tadpoles) are food organisms of the final host. The role of paratenic hosts in the development of various nematode families is different. In the family Philometridae, the larvae of *P. obturans* from the copepod intermediate hosts are transmitted to the predacious pike through the prey fishes. The role of paratenic hosts is more important with the anisakids. Hosts (in the majority large predacious fishes, marine mammals and fish-eating birds) get infected with these parasites by several paratenic hosts. When a paratenic host is consumed, the infective third-stage larvae can survive to infect the new organism until they get to the final host. The process was studied in detail in *A. crassus* infection in European eel. The paratenic host fishes of this nematode may have massive infections (Figs 12.12 and 12.13) (Thomas and Ollevier, 1992; Székely, 1994, 1995).

Homeothermic animals as final hosts

A. simplex is an ascaridoid nematode with a worldwide distribution. The final hosts are whales, intermediate hosts are euphausiaceans and transport hosts can be copepods, fishes and cephalopods (Smith and Wootten, 1978; Køie, 2001b). In the final host, the nematodes reside in the stomach, while they are in the haemocoel in

the intermediate crustacean host. In the fish transport hosts, the worms are in organs such as gonads, liver, spleen and the musculature. (Smith and Wootten, 1978). Adult worms copulate in the final host. Eggs are delivered to the sea with host faeces. The eggs embryonate and two moults occur in the egg before hatching. The larva is third-stage (Køie *et al.*, 1995) and, before getting into the obligatory euphausiacean intermediate host, the free-living, ensheathed larvae are ingested by transport hosts such as copepods. The intermediate host can also be ingested by various types of transport hosts (fishes, cephalopods) (see Køie, 2001b).

Another marine ascaridoid with a worldwide distribution is the seal worm *P. decipiens*. Final hosts are seals, intermediate hosts are copepods and benthic crustaceans. Fish may act as paratenic hosts. The site in fish is the musculature. Adult worms live in the stomach of the seal. Eggs pass from the intestine to the marine environment, where embryonation and two moults occur inside the egg. The third-stage larva emerges on hatching (Køie *et al.*, 1995). Copepods ingest the larvae. These can be eaten by benthic crustaceans. Invertebrates are eaten by fish, in which the muscle becomes infected. Seals ingest the fish and final moults to the adult stage take place in the seal.

C. osculatum also has a worldwide distribution and uses warm-blooded animals (seals) as final hosts. Intermediate hosts are crustaceans and fish carry the third-stage larva (Køie and Fagerholm, 1995). A number of fish species, such as cod, salmon, sculpin, herring and others, can be infected (Fagerholm, 1982; Hemmingsen *et al.*, 1993). In gadoids the liver is the preferred site (Fagerholm, 1988). Adult female worms deliver eggs to the environment. Two moults occur inside the egg and the third-stage larva emerges from the egg. Before infecting marine crustaceans, copepods can act as transport hosts. Fish acquire infection by predation on crustaceans. In gadoids the liver carries the majority of larvae. Final development takes place after ingestion of transport hosts by warm-blooded animals.

Poikilothermic animals as final hosts

One widely distributed nematode in fishes is *H. aduncum*. Eel pout, *Zoarches viviparous*, is one of the best final hosts. However, the worm may be found in numerous other species, such as burbot, cod, flounder, four-horned sculpin and whitefish (Fagerholm, 1982). The digestive tract (stomach and anterior intestine) is the site of infection for the adult worm. Eggs from the adult female are passed with host faeces to the sea water, where embryonation takes place, with two moults in the egg. The third-stage larva develops in copepods, amphipods, isopods and mysids (Køie, 1993). Other organisms, such as ctenophores, chaetognaths, polychaetes and ophiurids, which obtain infection by ingesting infected crustaceans, can serve as transport hosts. Often prey fishes serve as transport hosts as well. Larvae may be found in the body cavity and encysted on various organs in the fish (Fagerholm, 1982). Larvae longer than 3 mm moult into the fourth stage within the intestinal lumen of the host.

The cucullanids have another variation: Atlantic cod, *Gadus morhua*, and other gadoids are the final hosts of *C. cirratus* in the North Atlantic and adjacent waters. The preferred microhabitats are the pyloric caeca and the intestine. Eggs pass with faecal matter to the sea water, where embryonation occurs. The third-stage larvae hatch from the eggs. Intermediate hosts are small fishes, such as sand gobies, which become infected by eating the transport host, copepods. When the final host, the cod, ingests the infected intermediate fish hosts, the third-stage larva invades the stomach mucosa, where moulting to the fourth stage occurs. Subsequently, this larva passes to the pyloric caeca and the anterior intestine, where the final moult takes place and the worm develops into the adult stage (Køie, 2000a).

A similar development is seen in *C. heterochrous*, a common worm of flatfishes in the Baltic, Atlantic and North Sea. Final hosts include flounder, dab, plaice, sole, halibut and long rough dab (Berland, 1970). The site is the intestine, primarily the

posterior half of the intestine of the flounder (Buchmann, 1991). Eggs pass with host faeces to the sea water, where embryonation occurs. Probably the third-stage larva emerges from the egg following ingestion by a polychaete, which serves as intermediate host (Køie, 2000b). Larvae from polychaetes are infective to fish, where they moult to the fourth stage in the submucosa. After the final moult, the larva develops into the adult stage in the intestine (Køie, 2000b).

The related nematode *D.* (*Cucullanellus*) *minutus* is in flounder, plaice and goby in the Baltic Sea, North Sea, Mediterranean Sea and Black Sea (Køie, 2001a). It is in the anterior part of the intestine (Buchmann, 1991). Eggs from the female worm pass with faeces to the sea bottom, where embryonation takes place. Probably two moults occur in the egg, and the larva (440 μm long) that hatches from the egg is considered a third-stage larva (Køie, 2001a). The polychaete *Nereis diversicolor* is the obligate intermediate host. Following growth, the larva is infective to fish such as flounders. The fourth moult occurs in the gut wall of the final host and the adult worms reside in the anterior intestine.

Host–Parasite Relationship

Immune reactions

Nematodes elicit specific antibody production in the host. Migration of the larval stages in host cavities and host tissue may expose both structural and metabolic antigens to the host immune system. It has been demonstrated by immunoblotting that the European eel produces specific antibodies against a number of antigens of the swim bladder nematode *A. crassus* (Buchmann *et al.*, 1991; Höglund and Pilström, 1994; Békési *et al.*, 1997; Nielsen and Buchmann, 1997; Knopf *et al.*, 2000). Third-stage larvae of *A. simplex* in the fish host provoke the production of specific antibodies in naturally infected saithe (Priebe *et al.*, 1991). In addition, a range of Antarctic teleosts has been shown to possess reactive antibodies against molecules from *C. osculatum* (Coscia and Oreste, 1998). Similar positive plasma and bile antibody reactivities in fish against *P. decipiens* larvae were recorded by Coscia and Oreste (2000). Two main types of antigens are generally recognized in nematodes. These are the soluble excretory and secretory (E/S) antigens and the somatic antigens associated with surfaces on the outer or inner part of the worm. These may be partly protective. However, it is generally agreed that cellular reactions play a major role in protection of the host against nematode infections. Both humoral and cellular host reactions have been detected in various hosts against invading nematodes and these immune factors may function in host elimination and killing of infective stages. However, the roundworms seem to have some evasion mechanisms. Thus, the cuticle of nematodes consists in most cases of a protective proteinaceous layer, which protects the inner vital organs of the worm against aggressive immune effectors. Even encapsulated *Anisakis* and *Anguillicola* worms recovered from infected fish are fully capable of moving vividly upon removal of the host encapsulation (Santamarina *et al.*, 1994; Székely, 1994; Larsen *et al.*, 2002). These mechanisms have not been adequately investigated.

Pathogenicity

Pathological effect

The pathological effect of nematode infection in fish is little studied, and most information is based on field observations. There are only a few reported cases of mortality due to nematode infections. Most authors (Bauer *et al.*, 1977; Moravec, 1994; Dick and Choudhury, 1995) agree that fish nematodes damage the hosts by depriving the fish of digested food; by feeding on host tissues, sera or blood; and by direct mechanical damage through fixing to host tissues and developing or migrating in them (Fig. 12.14). Nematodes generally possess a range of enzymes, such as proteases, which may have tissue-degrading functions (Newton and Munn, 1999). Large-sized parasites compress organs (Platzer and Adams, 1967),

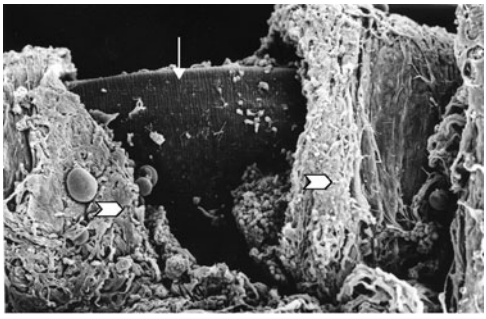


Fig. 12.14. *Anisakis simplex* (arrow) penetrating pyloric caecum of rainbow trout and causing mechanical damage of the wall (arrowheads) (experimental infection) (SEM × 50). Photo by José Bresciani.



Fig. 12.15. Damaged swim bladders of the European eels that died during the eel mortality in 1991 in Lake Balaton. One of the swim bladders contains numerous *A. crassus* specimens, while others have thickened fibrous walls as a consequence of past infection (× 0.5).

deform the shape of the body (Molnár, 1966a), increase or reduce the size of organs (Paperna, 1974) and cause haemorrhages (Jilek and Crites, 1982; Dunn *et al.*, 1983), inflammation (Measures, 1988; Molnár *et al*, 1993), granulomas (Hauck and May, 1977; Sindermann, 1990), ascites (Bauer *et al.*, 1977) and mesenteric and visceral adhesions (Sindermann, 1990). The pathogenic effect depends on the species and the size and the number of parasites (Fig. 12.3), and survival of the fish also depends on the site of infection.

Mortality

Mortality caused by nematodes was described by Bauer and Zmerzlaya (1972), who indicated that *R. acus* larvae caused heavy mortalities in bream (*Abramis brama*). According to Bauer *et al.* (1977), the heavily infected bream lost their balance and swam on their side, their body was covered by a thick layer of slime, there was local destruction of their sexual glands and bloody exudates accumulated in their abdominal cavity. Eiras and Reichenbach-Klinke (1982) also described heavy infection and deformation of the rainbow trout's intestine due to large parasitic nodules caused by *R. acus*. Moravec and Gut (1982) and Moravec *et al.* (1984) reported on the mortality of ornamental fishes due to massive infection with *Pseudocapillaria brevispicula* and *Capillaria pterophylli* (Fig. 12.5).

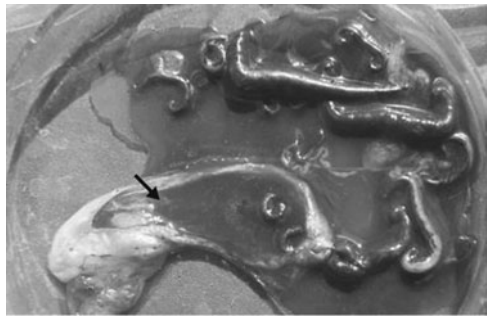


Fig. 12.16. With heavy infections with *A. crassus*, the worms die and decay in the lumen of the thickened swim bladder (arrow) (× 0.7).

Schäperclaus (1992) also found that *Pseudocapillaria tomentosa* can severely damage tench (*Tinca tinca*). A devastating effect of nematode infection was observed by Molnár *et al.* (1991, 1993), who reported on massive eel mortality in Lake Balaton, Hungary, due to *A. crassus* (Figs 12.15 and 12.16) infection. In this lake an estimated 400 t of eels died in 1991, 1992 and 1995. Similar heavy mortalities caused by this nematode were observed in the Czech Republic by Barus (1995). *Goezia* spp. infecting the stomach of the fish seem to have a relatively high pathogenic effect. These worms bore their anterior ends deep into mucosa up to the muscularis layer. Gaines and Rogers (1972) observed that

they form deep nodules in the stomach wall. These authors also reported mortalities in striped bass (*Morone saxatilis*) and tilapia in Florida. Mortalities were observed by Freitas and Lent (1946) in *Arapaima gigas* in Brazil caused by *Goezia spinulosa*.

Trichuroid nematodes are generally considered pathogenic. These nematodes damage epithelial cells by feeding and penetrating deeply into the intestinal mucosa. The necrotic changes they cause often lead to morbidity of the hosts. Dubinin (1952) in Russia reported on disease through gut inflammation of acipenserids (*A. ruthenus*, *Acipenser nudiventris*) caused by *Pseudocapillaria tuberculata*. Severe debilitating conditions also developed on some North American centrarchid fishes. *Capillaria catostomi* caused enteritis in the caeca and intestine (Hoffman, 1982). *Eustrongylides* spp. are also pathogenic. The pathogenicity of *Eustrongylides* spp. in sturgeons (*A. nudiventris*) was studied by Dubinin (1952) and Dogiel and Bykhovskiy (1939) in Russia, who considered *Eustrongylides* larvae to be highly pathogenic, causing heavy infections leading to complete destruction of gonads and to parasitic castration of infected fishes. It was also Dubinin (1952) who found that, in heavy infections with *H. bidentatum*, inflammatory processes were found in the intestinal walls of sterlets (*A. ruthenus*) and sometimes perforation of the swim bladder by migrating worms was recorded. Kall *et al.* (2004), who examined *P. obturans* infection of pike (*Esox lucius*), reported that the pikes infected by this large worm inhabiting gill arteries were less active, showed lethargy and died in aquaria shortly after transportation to the laboratory. Sprengel and Lüchtenberg (1991) experimentally proved that *A. crassus* infection in eels reduced swimming performance, and in another experiment Molnár (1993) found that heavily infected eels were more susceptible to decreased oxygen content in the water.

Intestinal tract

Most Nematoda species infect the intestinal tract. Some of them (e.g. *Hysterothylacium*, *Goezia*, *Cystidicola* spp.) prefer the anterior part of the alimentary tract (oesophagus, stomach, pyloric region), while others are in the intestine. The major damage caused by these worms is associated with their consumption of intestinal contents, thereby depriving the host of nutrients. A less important effect comes from direct mechanical blockage by the worms.

H. bidentatum (Fig. 12.2), a common parasite of the acipenserids, may cause heavy infections in sterlet. These large-sized nematodes can completely occlude the stomach and thus reduce digestion and block the passage of food. After the death of heavily infected fish, these nematodes frequently migrate out through the mouth or the gill slit.

In less intensive infections, nematodes may evoke pathological changes, mostly around their attachment sites. Damage to the mucosa or deeper tissues is usually caused by lips, the buccal capsule, teeth or spines.

Rhabdochona species, when in high numbers, can cause perforation of the intestinal wall at attachment points (Moravec, 1975). Similar damage occurs with *Camallanus*, *Procamallanus* and *Paracamallanus* species as they 'grab' the intestinal wall with their buccal capsules while feeding on blood. Usually there is a local inflammatory reaction at the attachment site. Thatcher (1991), as well as Sinha and Sinha (1988), suggested that nematodes could cause primary anaemia by feeding on blood. In intensive infections, especially in small fishes, these camallanids can reduce growth rates and also cause intestinal blockage. More severe changes were recorded in ornamental fishes. Several authors (Petter *et al.*, 1974; Stumpp, 1975; Campana-Rouget *et al.*, 1976; Schäperclaus, 1992) reported on complete destruction of the intestinal mucosa and death of the fish in the presence of large numbers of *Camallanus fotedari* or *Camallanus moraveci*. Heavy infection with *Camallanus cotti* caused a reduced sexual display rate in *Poecilia reticulata* (McMinn, 1990).

Cucullanus truttae has similar effects on rainbow trout. According to Dunn *et al.* (1983), there is loss of epithelium and mucosal hyperplasia, as well as haemorrhage and

fibrosis in the laminar propria at the point of attachment. Growth rate, food consumption and swimming activity are reduced in infected fish. The spiruroid nematodes *Camallanus oxycephalus* and *Spinitectus carolini* of the green sunfish penetrate to the mucosal layer of the gut and cause damage to the columnar epithelium. At the site of penetration, ulcers developed in the mucosal and submucosal layers and there was growth of granulomatous tissue with extensive fibrosis (Meguid and Eure, 1996). Local changes in the intestine can also be provoked by seemingly less pathogenic nematodes.

In the case of *Echinocephalus daileyi*, where there is a special cephalic inflation and rows of hooks for attachment of the worm to the intestinal mucosa, Thatcher (1991) observed inflammation and formation of a fibrous capsule around the head bulb. Formation of capsules filled by tissue debris, oedematous fluid, fibrous exudate and leucocytes at the attachment point of the nematodes was also observed by Ko *et al.* (1975) with *Echinocephalus sinensis* in the ray *Aetabulus flagellum.*

Deardorff and Overstreet (1980) remarked that *Goezia pelagia* apparently feeds on both the host elements and partially digested food. The parasite forms a deep nodule in the intestinal wall, with the development of ulcers. A connective-tissue capsule surrounds the head part of the parasite, and primary exudates, inflammatory cells, red blood cells and necrotic tissue are in the nodules. *Aeromonas* bacteria could be cultured from some of the nodules. It is often larval penetration and migration that cause severe reactions. Thus, invasion of host tissue by marine nematode larvae has been described to cause pathological reactions. Haemorrhages, tissue compression and necrosis are often found in tissue with invading *P. decipiens* larvae (Ramakrishna *et al.*, 1993). The host reaction may be expressed in tissue proliferation, degeneration and inflammation.

Molnár (1994) found hundreds of *A. crassus* larvae in nodules in the intestinal wall; some of the larvae were alive while others were dead and calcified. Similar observations were reported by Janiszewska (1939) with *D. minutus* in flatfishes. Jilek and Crites (1982), who studied the pathogenicity of the habronematoid *S. carolini* in centrarchid fishes, described the third-stage larvae penetrating the intestinal wall, causing traumatic enteritis, the growth of epithelioid fibroblasts around worms and accumulation of granule cells, leucocytes and macrophages. An expanding fibrocytic layer formed a capsule around the larvae; the innermost layer became necrotic but encapsulated worms were able to develop into adults. *A. simplex* larvae have been seen to induce severe inflammatory reactions in the wall of the stomach of cod. Thus clusters of larvae gathered in local inflammatory foci in the stomach wall of the fish host (Berland, 1981). Arai (1969) found large ulcers caused by *Anisakis* larvae in the stomach wall of *Ophiodon elongatus*, and Williams and Richards (1968) observed prominent host reactions in *Raja radiata* against *Pseudanisakis rotunda*, especially the head, which was in the lamina submucosa granulation tissue.

Body cavity

The body cavity is the frequent location of *Philometra* and *Philonema*; however, little is known about their pathogenic effect. *Philonema* spp. are known to be responsible for multiple mesenteric and visceral adhesions in salmonid fishes (Nagasawa, 1985; Garnick and Margolis, 1990; Sindermann, 1990). Both *Philonema* and *Philometra* infection can cause atrophy or destruction of gonads, ascites and extension of the abdomen (Molnár, 1966a, 1967; Platzer and Adams, 1967; Williams, 1967; Hoffman, 1975; Moravec *et al.*, 2003). While studying *Eustrongylides* sp. larvae infecting mesenteries and inner organs of African fishes of the genera *Haplochromis*, *Bagrus* and *Clarias*, Paperna (1974) found that the most heavily infected fish were emaciated. In these fish an extensive lysis was observed around the worms, which penetrated the somatic muscles. Inner organs were infiltrated by lymphocytes and macrophages and inflammatory necrosis was observed.

Eiras and Rego (1988) reported on similar changes in South American fishes (*Pygocentrus nattereri*), while Measures (1988) in North America found granulomatous inflammation and exudates containing erythrocytes and macrophages. However, Kennedy and Lie (1976) reported that heavy infections of encapsulated *Eustrongylides* did not cause weight loss or changes in the outward appearance and condition factor of fish. Experimental infections of rainbow trout with *A. simplex* third-stage larvae are known to cause a pronounced cellular reaction in the body cavity and in tissue penetrated, especially in the pyloric caeca (Santamarina *et al.*, 1994; Larsen *et al.,* 2002). Larvae of the genera *Hysterothylacium*, *Anisakis*, *Pseudoterranova* and *Contracaecum* and of *Spiroxis contortus* are frequently found on the serosa of the gut, where they are coiled and covered only by a thin serosa layer; in other cases they are encapsulated by a row of connective-tissue layers (Smith and Wootten, 1978).

Liver

Of the nematodes damaging the liver, *Schulmanela* (*Hepaticola*) *petruschewskii* is the best-known species. Kutzer and Otte (1966) studied the pathological effects of *H. petruschewskii* in salmonid, percid and cyprinid fishes. Macroscopic changes of the severely infected fish included greyish discoloration of the liver, with the appearance of nodules the size of a pinhead or larger, which was sometimes accompanied by hyperaemia, petechial haemorrhages and icterus. Histologically, in addition to the presence of helminths in numerous convoluted passages, haemorrhage and hyperaemia of the liver capillaries, aggravated by the appearance of fibrinous–serous exudate, were found. Leucocytic infiltration, epithelioid cell proliferation and even the appearance of giant cells could also be observed.

Devastating effects on host liver tissue were described by Petrushevski and Shulman (1961). Nematode larvae, probably *C. osculatum*, which normally reside in the liver of Baltic cod (*G. morhua*), were suggested to affect the size and function of the liver. Thus, heavy parasite burdens were seen in small livers and only a few nematode larvae were in large livers. Although tissue-penetrating worms may cause adverse reactions, further studies should be conducted to elucidate this question. In this context it should be recalled that liver size in gadoids is highly influenced by food composition and energy content (Buchmann and Børresen, 1988). Thus, feeding on low-energy diets such as crustaceans (serving as an intermediate host for *C. osculatum*) instead of high-energy feed (clupeids) may lead to high parasite burdens and, secondarily, low liver weight.

Of the nematodes causing heavy pathological changes of the liver in the larval stage, *R. acus* is the best-studied species. In the adult stage this parasite infects the gut of piscivorous fishes, primarily pike. In the latter fish, only local inflammations were registered in the gut. In intermediate host fishes, however, heavy infections and mass mortalities were often registered in rainbow trout, common bream, yellow perch and experimentally infected loach (*Barbatula barbatula*) (Moravec, 1970; Bauer *et al.*, 1977; Poole and Dick, 1984). Third- and fourth-stage larvae in their true intermediate hosts invaded various internal organs, destroyed blood vessels during larval migration and caused numerous nodules in the intestinal wall, liver, peritoneum and mesentery. Valtonen *et al.* (1994), who followed *R. acus* migration in roach (*Rutilus rutilus*), recorded a chronic granulomatous inflammatory reaction. They remarked that larvae occurred more often in pancreatic tissue than in the liver. These authors also remarked that the infection rate in polluted eutrophic lakes was much higher than in oligotrophic ones. Dezfuli *et al.* (2000), who studied the effect of larval raphidascariosis in the liver of *Phoxinus phoxinus*, reported that the inflammatory process was characterized by an increase of rodlet cells, besides granulocytes and epithelioid granulomata.

Authors who examined the effect of *Anisakis* larvae on host tissues (Mikailova *et al.*, 1964; Prusevich, 1964; Hauck and May, 1977; Smith, 1984) reported that

Anisakis larvae invading tissues caused destruction of the liver parenchyma, ruptures of the wall of the blood vessels, small haemorrhages and thrombus formation. The larvae were covered with fibrin infiltrated by leucocytes. At a later stage, a connective-tissue capsule was formed around the larvae.

Swim bladder

The swim bladder of fishes is damaged mostly by *Cystidicola* and *Anguillicola* species. Van Banning and Haenen (1990) and Molnár *et al.* (1993) reported that the acute process of *A. crassus* larval migration was characterized by epithelial hyperplasia and hyperaemia of the swim bladder wall. In cases of chronic swim bladder inflammation, oedema and hyperplasia of tissues of the tunica propria, submucosa and serosa were observed, as well as granulomatoid infiltration by mononuclear cells and fibrinoid degeneration around larvae. Molnár (1994) remarked that inside the oedematous connective tissue of the swim bladder larvae migrate without causing an observable host reaction in the swim-bladder wall or the gas glands (Figs 12.17 and 12.18), but in more advanced cases granulation tissue containing mononuclear cells stops larvae (Fig. 12.19.) The granulation tissue, built up from epithelioid macrophages, forms a nodule around the larvae, which become surrounded by a capsule. Larvae die and, together with the necrotized epithelioid cells, give rise to amorphous tissue debris. Secondary bacterial infections may change nodules into pustules filled with degenerate cells, inflammatory cells and serum. Kamstra (1990), Van Banning and Haenen (1990) and Molnár *et al.* (1993) reported that adult *A. crassus* specimens filling the whole lumen of the swim bladder feed on blood, causing anaemia, epithelial hyperaemia and dilatation of the ductus pneumaticus. Due to the damage caused by larvae and adult worms, the wall of the swim bladder thickens and becomes fibrotic (Fig. 12.15). Worms in the lumen either leave the bladder and migrate to the gut through the ductus pneumaticus or they die. Dead worms and blood form a hard brown-black mass in the lumen of the swim bladder (Fig. 12.16), which will eventually contain facultative pathogenic bacteria.

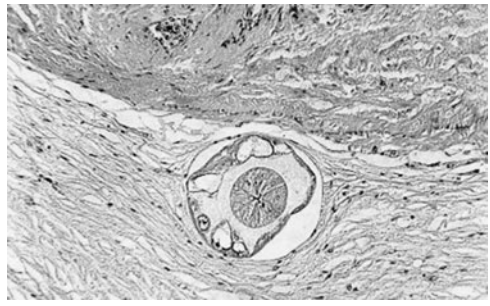


Fig. 12.17. Cross-section of a third-stage *A. crassus* larva in the oedematous tissue of the submucosa of the eel's swim bladder (H & E × 145).



Fig. 12.18. Fourth-stage larvae of *A. crassus* accumulating in the gas gland, before entering the lumen of the swim bladder (× 12).

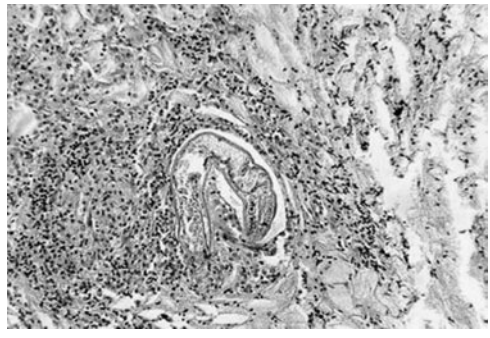


Fig. 12.19. Cross-section of a third-stage *A. crassus* larva inside the swim-bladder wall surrounded by granulation tissue and mononuclear cell infiltration (H & E × 85).

Skin

The best-known parasites of the skin come from the genera *Philometra* and *Philometroides*. By the time they reach full maturity, the large females of *Philometra rischta* destroy the subepithelial layer of the gill cover of bleak (*Alburnus alburnus*), and the worms are separated from both the gill and the aquatic environment by an epithelium bordered by basement membrane. Worms cause ulceration of the gill cover, which becomes completely disintegrated in some cases (Molnár, 1966a; K. Molnár, unpublished). Similar injuries may arise after colonization of the skin of sucker (*Catostomus commersonii*) by *P. nodulosa* (Dailey, 1966; Hoffman, 1975, 1999). The very tiny *S. cyprini* is not pathogenic, while the large females (up to 20 cm in length) of *Philometroides cyprini* (Fig. 12.4), a parasite of the Amur wild carp, can cause swellings of the scale sacs, accumulation of serous infiltration and haemorrhages under the scales. After the expulsion of worms, the areas without scales and with ulcers may have secondary microbial infections (Vasilkov, 1967, 1975; Sekretaryuk, 1983). Similar changes in the fins are caused by *P. sanguinea* infection in *Carassius* species (Fig. 12.20) and by *Philometra huronensis* infection in sucker. These changes lead to fraying of the fin rays, which may break and come off in fragments (Molnár, 1966a; Uhazy, 1978). In sturgeon fishes, especially in the sterlet, *C. acipenseris* forms large nodules in the connective tissue on the ventral surface of the skin (Fig. 12.21), causing capsule formation and oedema (Bauer *et al.*, 1977). There is usually a female and a male worm in each nodule. Rockfish infected with adult trichuroid nematodes in the skin have inflammatory reactions with intraepithelial deposition of eggs (Conboy and Speare, 2002).



Fig. 12.20. Females of *Philometroides sanguinea* in the blood vessels of the caudal fin of a gibel carp (*Carassius gibelio*) (× 0.85).

Gills

Nematode infection of the gills is relatively rare. The best-known nematode is *P. obturans*, a parasite of the gill arteries of the European pike. This large-sized nematode, measuring

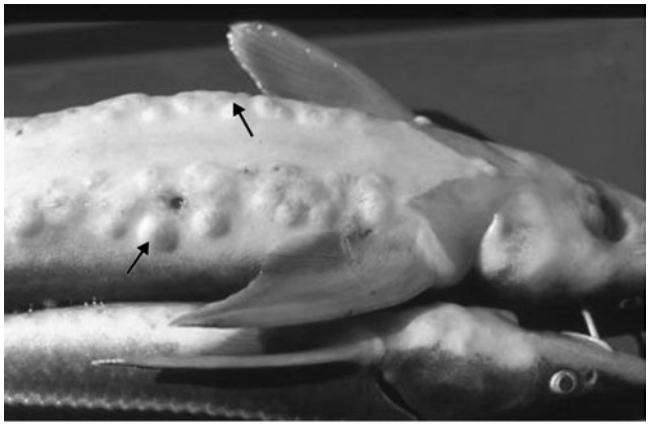


Fig. 12.21. Parasitic nodules (arrows) in the skin caused by *Cystoopsis acipenseris* in the abdominal side of sterlet (*Acipenser ruthenus*) (× 0.4). Photo by Ferenc Baska.

20 cm in length, is known to obstruct blood circulation, to feed on blood and to perforate gill arteries when releasing larvae. Moravec and Dyková (1978) and Kall *et al.* (2004) found parietal trombi in the bulbus arteriosus and endothelial hypertrophy in the ventral aorta. The worms clearly obstructed the arteries; the elastic wall of the vessel was stretched around the parasite and appeared thinner. In cases when the female worm was damaged and only the gonads filled the arteries, the arterial wall became irregular, showing signs of hypertrophy and hyperplasia. In contrast to this large Nematoda species, Ribu and Lester (2004) found tiny histozoic nematodes in gill filaments and described them as *Moravecia australiensis.*

Eyes

The eyes of fishes may frequently have worms. For example, Parukhin (1975) found the females of *Philometra oveni* in the orbits of a percid fish (*Serranelus hepatus*) and Moravec and Dyková (1978) found larval stages of *P. obturans* in the vitreous humour.

Zoonotic aspects of fish nematodes

The presence of nematode larvae in fish may present a risk for humans. Third-stage larvae of *Anisakis* spp., *Pseudoterranova* spp., *Phocascaris* spp. and *Contracaecum* spp. occur in fish musculature and their adult stage is in homeothermic animals such as whales and seals. Thus, the stimuli for further development of the larva to the final moult are high temperature, acidic pH and pepsinogen, which occur in the stomach of homeothermic animals. Following ingestion by humans of live larvae in fish products, the larvae get activated and penetrate the gastric or intestinal mucosa, eliciting abdominal symptoms. Extra-intestinal migration of worms also occurs. This is anisakiasis (anisakiosis) or pseudoterranoviasis (pseudoterranovosis) (Smith and Wootten, 1978; Möller and Anders, 1986; McClelland, 2002).

Freezing (–20°C for 24 h), heating above 60°C or salting (250 g NaCl/l) will kill *Anisakis* larvae. Likewise, storage of *Pseudoterranova* larvae in fish meat at –30°C for 15 h or at –20°C for 7 days is lethal for the worms (McClelland, 2002). Even dead worms (killed by freezing, heating or salting) still contain immunogenic molecules, which can trigger severe allergic reactions following ingestion of fish products. In fact, the disease provoked by the marine larvae is to a high degree associated with the host reaction, such as immunoglobulin E (IgE) production, mast-cell degranulation, eosinophilia, oedema and urticaria. It has been speculated that nematodes living in the adult stage in fish, such as *Hysterothylacium*, could elicit disease in humans following ingestion of live larvae (Norris and Overstreet, 1976). It has been reported that adult nematodes of *Philometra* from fish tissue can infect humans through open wounds in skin and elicit disease (Deardorff *et al.*, 1986). Considering the high prevalence of philometrids in fishes (Moravec *et al.*, 2003), this suggestion should be further studied.

Spoilage of fish products by marine nematode larvae

Nematode larvae in fish fillets may lead to rejection by consumers due to the unattractive appearance of the product. Therefore considerable efforts are being made to detect and remove larval nematodes from fish products (Möller and Anders, 1986). It has been estimated that up to half of the production costs in certain processing plants consist in the detection and removal of *Pseudoterranova* from cod fillets; the problem is worldwide due to the extensive distribution of these anisakid larvae (McClelland, 2002). These concerns are mainly with wild fishes because several studies have shown that aquacultured salmon are free from infection, since in this case fish conduct their entire life in captivity and are fed with non-infected feed (Lunestad, 2003).

In Vitro Culture of the Parasite

Studies on the *in vitro* culture of marine nematodes have concentrated on marine anisakids. Thus, incubation of third-stage larvae of *A. simplex* (Carrajal *et al.*, 1981), *P. decipiens* (Likely and Burt, 1989) and *C. osculatum* (Likely and Burt, 1992) in appropriate physiological media containing serum, haemin and amino acids resulted in moulting and in some cases in development of adult egg-producing worms. Similar successful results were obtained with *in vitro* culture of *H. aduncum* from third-stage larvae to adult worms by Iglesias *et al.* (2002). The process was developed by Adroher *et al.* (2004), who managed to follow the development process of *H. aduncum in vitro* from the third-stage larvae to the hatching of the new generation third-stage larvae. Further studies could also focus on the culture of all stages for a repeatable, complete *in vitro* life cycle.

Identification and Diagnosis of Infection

General methods for identifying nematodes are described in textbooks by Bykhovskaya-Pavlovskaya (1969), Fernando *et al.* (1972), Bauer *et al.* (1977), Bauer (1987), Sindermann (1990) and Moravec (1994).

Methods for diagnosis of nematodes much depend on the size and location of the parasites. In live fish, only large parasites on or close to the surface of the body are recognized. Red-coloured *Philometra* species in the opercula and the fins or parasite nodules in the skin around *C. acipenseris* can easily be observed. Large nematodes inhabiting the gut and inner organs are easily detected on dissection of freshly killed, frozen or formalin-fixed animals; in the case of small nematodes, a dissecting microscope is necessary. Infection with histozoic species, such as the small skrjabillanid nematodes, can be diagnosed on live material under a microscope. Scrapings of intestinal serosa can be examined under a dissecting microscope for the delicate capillariid nematodes and larval stages. Larval nematodes in the internal organs are usually found in squash preparations between two glass plates. Physiological 0.6% fish saline is necessary to keep nematodes alive. Before fixing, nematodes are rinsed in saline. For fixation, a hot mixture of 70% ethanol and glycerine (9 : 1 part) or a hot mixture of saline and 40% formalin can be used. For diagnosis of *Anguillicola* infection in the lumen of the swim bladder, Beregi *et al.* (1998) suggested X-ray (Fig. 12.22) and Székely *et al.* (2004) used computer tomographic methods.

Male genital organs can be studied by placing the worms in glycerine or lactophenol under a cover slip for clearing. For staining permanent mounts, carmine staining

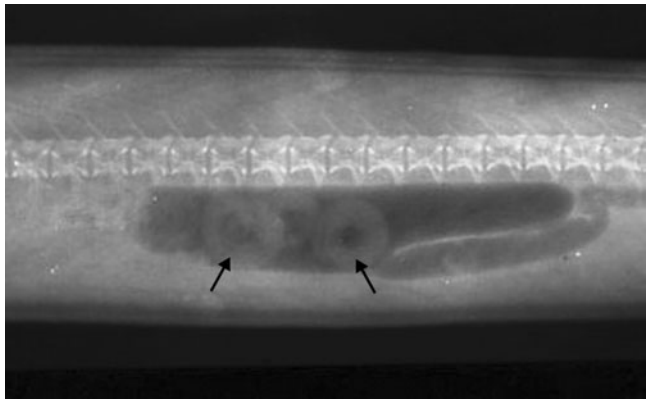


Fig. 12.22. X-ray as a tool for diagnosis of *A. crassus* infection of eel swim bladder. Note the large convoluted worms (arrows) in the swim bladder and the ductus pneumaticus (× 1).

or Thatcher's method (Petter and Thatcher, 1988) is suggested. Oral papillae can be studied by *en face* preparations (e.g. as suggested by Anderson, 1958).

Identification is based on morphological characters. Thus, relative proportion of length and width is used. An important feature is the overall shape of the worm, specifically the presence of papillae, alae, boring tooth, striations, oral opening, excretory pore opening location, caeca and appendages on oesophagus, ventriculus and intestine. As an example, it can be mentioned that the identification of a number of species (*P. decipiens*, *Pseudoterranova krabbei*, *Pseudoterranova bulbosa* and *Pseudoterranova azarasi*), comprising a species complex previously referred to as *P. decipiens* (Paggi *et al.*, 2000), has traditionally been based on such structures. Morphologically, the worm is characterized by an anteriorly directed intestinal caecum, no ventricular caecum and an excretory pore opening at the nerve ring. Most ascaridoids are clearly differentiated from *Anisakis* spp. *Anisakis* larvae are easily identified by the lack of caeca on the intestine and ventriculus. Likewise a characteristic tail spine is found. The excretory pore is located anterior to the nerve ring (Smith and Wootten, 1978). The differential diagnosis of *C. osculatum* is also feasible using morphology. Both ventricular and intestinal caeca are found and the excretory pore opens anterior to the nerve ring. Species of *Porrocaecum* and *Phocascaris* are difficult to distinguish morphologically, but the former genus is presumed to be in birds while the latter genus is in seals (Paggi *et al.*, 2000). Some similarity exists between *Contracaecum* spp. and *Hysterothylacium* spp. larvae. However, several differences are found, among which the location of the opening of the excretory pore is important. The third-stage larva of *Hysterothylacium* has no lips and carries characteristic caeca on both the intestine and ventriculus, but the excretory pore opens below the nerve ring (Fagerholm, 1982). Likewise, morphological differences are obviously used to diffentiate adult nematodes in fish. The identification of cucullanids is based on the shape and length of the worms, the location of the vulva and the shape of the spicule (Berland, 1970).

Molecular tools

The recent development of molecular techniques has provided alternative and, in some cases, more accurate diagnostic tools. Thus, PCR-based methods include amplification of genes encoding ribosomal RNA. Direct alignment and comparison of DNA sequences may reveal differences between species. By using the PCR-restriction fragment length polymorphism (RFLP) method, various closely related nematodes can be differentiated on their banding pattern in an agarose gel (Kijewska *et al.*, 2002; Szostakowska *et al.*, 2002). Further, mitochondrial DNA sequences have been found to be valuable for differentiation between sibling species within *Contracaecum ogmorhini* (Mattiucci *et al.*, 2003). In addition, electrophoresis of enzymes from various nematodes and subsequent comparison of gel migration distances allow separation of such species. These techniques were also used for confirmation of mitochondrial DNA results from *C. ogmorhini* studies (Mattiucci *et al.*, 2003). These have been found to be feasible for differentiation between the genera *Anisakis, Contracaecum* and *Pseudoterranova* (Mattiucci *et al.*, 1998). *P. decipiens* was formerly considered to be a single, well-defined species, but recent allozyme work has shown that this entity comprises a species complex like *P. decipiens* (*sensu stricto*), *P. krabbei, P. bulbosa* and *P. azarasi* (Paggi *et al.*, 2000). Similar techniques were used by Mattiucci *et al.* (2002) for differentiation between *Anisakis typica* and other species within the genus.

Prevention and Control

Parasitic nematodes primarily affect fish in natural waters. Only a smaller proportion of them cause problems in fish ponds and aquaria.

Lo (1988) summarized the methods for treating the valuable discus fish (*Symphysodon*) in an aquarium. Anthelmintics, such as garlic oil, piperazine, trichlorphon and levamisole, have varying effectiveness when administered in food. He suggested trichlorphon in a bath (concentration of 4 ppm or 7 ppm), but several side effects were recorded. Pena *et al.* (1988) found that fresh minced garlic (200 mg/l water) and its hexane extract were 100% effective in treating *Capillaria* in carp, while ammonium potassium tartrate (1.5 mg/l, twice daily) was 86% effective.

Avdosev (1978) suggested that *Philometroides lusiana* could be controlled during the larval shedding period by killing the intermediate host cyclops in the pond with 0.325 g/m^3 trichlorphon administered three times at 10-day intervals. Vasilkov *et al.* (1974) found that ditrazine citrate, administered in an oral dose of 0.4 g/kg of fish, was effective against *P. cyprini* (*P. lusiana*) developing in common carp, and the same drug was effective even in a dose of 0.3 g/kg when inoculated into the abdominal cavity.

The effects were examined of a few anthelmintics against *A. crassus* infection of the eels. Taraschewski *et al.* (1988) tested five drugs but only levamisole-HCl proved to be effective. Hartmann (1989) found that levamisole in bath treatment was effective in 2 and 5 mg/l doses.

Controlled studies by Tojo *et al.* (1994) showed that experimental infections of rainbow trout with *A. simplex* third-stage larvae were only weakly influenced 6 days post-treatment by anthelmintics such as mebendazole, flubendazole, parbendazole, triclabendazole, piperazine, netobimin, trichlorphon and nitroscanate. However, previous *in vitro* studies have shown effects of mebendazole, flubendazole, triclabendazole, nitroscanate and various types of Chinese herbal medicine on *Anisakis* spp. larvae (Tojo *et al.*, 1994). Other *in vitro* studies showed clear effects of ivermectin on larvae and adults of *P. decipiens* (Manley and Embil, 1989). Therefore, it is likely that some anthelmintics used to treat nematode infections in humans and animals may be effective against nematodes of marine animals if dosage and exposure time are appropriate. Benzimidazoles, pyrantel, morantel, avermectins and levamisole are all drugs with high nematicidal potential.

Prophylaxis

Bauer and Hoffman (1976) as well as Molnár (1987) emphasized the importance of the control of intercontinental transfer of fishes. These authors, as well as Bauer *et al.* (1981) and Schäperclaus (1992), recommended that all wild fish should be examined for internal and external parasites before stocking. Parasites spread by fish-eating birds can be the most effectively controlled if the definitive host birds are kept away from ponds or the water supply containing intermediate hosts. Bauer *et al.* (1981) suggested that, at the time of larval release of *P. cyprini*, brood fishes should be isolated from susceptible young fish.

Heat inactivation (60°C) or freezing (–20°C for 24 h) will kill infective third-stage larvae of *Anisakis* spp. (Smith and Wootten, 1978). Likewise, *Pseudoterranova* larvae are killed in fish products by storage at –30°C for a minimum of 15 h or at –20°C for at least 7 days (McClelland, 2002). Immunoprophylaxis in fish against nematode infections has not been achieved, although it is known from veterinary immunoparasitology that immune reactions in farm animals can eliminate infections. We suggest that it is time to initiate studies on developing vaccines against some of the more important pathogenic nematodes in fishes.

Summary and Conclusions

Knowledge of fish nematodes varies greatly. The nematode fauna of fish in Europe and the northern parts of Asia and America is relatively well studied. Little is known, however, about fish nematodes of South Asia, Africa, Australia and Latin America. All nematodes in marine fishes with zoonotic relevance have been studied and, of these, large-sized ascaridoid and philometrid nematodes are the best known.

Small-sized nematodes, such as the skrjabillanids, are known mostly from Europe, although recent studies on fishes of Central America show that their distribution may also be high in other continents. In most cases, only the intestinal tract is examined and histozoic nematodes infecting inner organs, serous membranes and muscles may not be found by inexperienced examiners.

There is an urgent need for the introduction of molecular methods in the research of fish nematodes. This method can be useful in synonymizing or separating morphologically similar species and finding latent infections with small histozoic nematodes.

Pathogenicity of fish parasites is mostly based on field observations, except studies done on *A. crassus* infections. *R. acus* and *A. simplex* also seem to be experimental tools for studying pathogenic effect of nematodes.

There is no effective drug against fish nematodes. Although nematodes cause relatively few problems in propagated fishes in fish farms or cage cultures, they are relatively important in aquarium fishes.

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13 Phylum Acanthocephala

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Introduction

Acanthocephalans are endoparasitic worms comprising approximately 1100 species (Golvan, 1994), nearly one-half of which are found as adults in the intestine of fishes. Juvenile worms of many other species occur in the viscera, especially the mesentery and liver, of fishes that act as paratenic hosts. Although these parasites are not usually regarded as having significant economic importance, epizootics are known from hatcheries (Bullock, 1963) and they have been linked to local extinction of natural populations (Schmidt *et al*., 1974). There is little knowledge of the more subtle effects the parasite has on host populations, but mortality might be significant when hosts are stressed (Jilek, 1979; Connors and Nickol, 1991).

Host and Geographical Distribution

Freshwater and marine fishes from the Arctic (Van Cleave, 1920) to the Antarctic (Zdzitowiecki, 2001) and from surface waters to the depths of the ocean (Noble, 1973; Wayland *et al*., 1999) harbour acanthocephalans. Fishes of most systematic groups are parasitized. Although these parasites are rare in agnathans and elasmobranchs, a few species are known to occur in lampreys (Hoffman, 1999), rays (Brooks and Amato, 1992; Sanmartin *et al*., 2000; Knoff *et al*., 2001) and sharks (Yamaguti, 1961). Sturgeons, primitive ray-fin fishes (Chondrostei), have one acanthocephalan species specific to them (Choudhury and Dick, 2001) and are reported to host another 14 species incidentally (Choudhury and Dick, 1998). Bowfins and gars, intermediate ray-fin fishes, harbour another few species (Golvan and de Buron, 1988; Hoffman, 1999). Most of the acanthocephalans of piscine hosts are found in the bony fishes (Teleostei). More species of Acanthocephala are found in the cypriniform families Cyprinidae and Catostomidae than in any other piscine family.

Seasonal Fluctuations

A review of seasonal occurrence of acanthocephalan species in piscine hosts (Chubb, 1982) reveals many differences among species and geographical localities. Kennedy (1972) found that feeding response was an important factor in controlling the intensity of *Pomphorhynchus laevis* in dace (*Leuciscus leuciscus*) and that water temperature influenced this response. Unless negated by another factor, such as temperature-dependent rejection (Kennedy, 1972), prevalence and

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intensity should be greatest during times when intermediate hosts with infective larvae constitute an appropriate portion of the diet of definitive hosts. When this occurs throughout the year, there may be no seasonal periodicity in the occurrence of acanthocephalans. For example, *P. laevis* shows no seasonal periodicity in prevalence, intensity or development and maturation in chub (*Leuciscus cephalus*) in the Danube River basin of the Czech and Slovak republics where the amphipod intermediate host is available to fish throughout the year (Moravec and Scholz, 1991).

Absence of seasonal periodicity does not imply that rates of recruitment and mortality are constant throughout the year. High water temperatures (under laboratory conditions) reduce the success with which *P. laevis* establishes in goldfish (*Carassius auratus*); hence Kennedy (1972) suggested that increased feeding by fish in the summer offsets a lower rate of parasite establishment. Thus, seasonal differences in rates of recruitment and mortality (from failure to establish in fish) could occur even if parasite occurrence shows no periodicity.

There are instances in which appropriate invertebrate intermediate hosts are consumed by definitive hosts throughout the year, but there are definite seasonal cycles in infections. Awachie (1965) demonstrated that, even though infective larvae of *Echinorhynchus truttae* occur in *Gammarus pulex* and the amphipods are an important part of the food of brown trout (*Salmo trutta*) all year, seasonal differences in densities of infective larvae lead to fluctuating occurrence in fish. Variations in habits of hosts during the year, for instance, changes in diet, also cause seasonal differences in prevalence and intensity of infections (O'Neill and Whelan, 2002).

Kennedy (1970) suggested that, when maturation of helminths from freshwater fishes shows seasonal cycles, peak egg production almost always occurs late in spring or early in summer. This generalization applies to many, but not all, species of Acanthocephala. Annual cycles in fishes frequently begin with recruitment in spring, and most transmission between fish and invertebrate hosts occurs during summer and early autumn. Development in hosts infected late in the season is retarded by low temperatures (Olson and Nickol, 1995) until spring.

Some acanthocephalans show adaptations directly related to seasonal events in the life histories of fishes. Peak egg production by *Fessisentis friedi* apparently occurs as pickerel (*Esox americanus*) move into shallow water to spawn (Muzzall, 1978). Maximum intensity and egg production of *Pomphorhynchus bulbocolli* in white suckers (*Catostomus commersoni*) have also been related to migration and spawning (Muzzall, 1980). In Lake Michigan, *Echinorhynchus salmonis* reaches peak sexual maturity in chinook salmon (*Oncorhynchus tshawytscha*) during spawning (Amin, 1978). These seasonal patterns result in peak egg production by the acanthocephalans when fishes are in shallow waters, where appropriate invertebrate intermediate hosts are most abundant.

A common type of cycle consists of spring recruitment by fishes after the environment has warmed and fish and invertebrate hosts have become active, summer transmission between fishes and invertebrates, slow development in invertebrates during the winter and completion of larval development with rising temperatures in the spring.

Deviations from the spring 'recruitment cycle' are often related to deviations in fish–invertebrate relationships and environmental disturbances. Prevalence of *E. salmonis* in yellow perch (*Perca flavescens*) in Lake Ontario rises in the autumn, after which it declines until the parasite is absent from fish during the summer (Tedla and Fernando, 1970). Watson and Dick (1979) also found that *E. salmonis* was most common in white fish (*Coregonus clupeaformis*) from Southern Indian Lake, Manitoba, in late autumn when the amphipod intermediate host was a prominent white fish food item.

In instances where invertebrates are rare or absent during winter, the cycle may be altered. This results in recruitment to the definitive host population in the autumn. Maturation then is slow, with egg production occurring in the spring. Eure (1976) found this for *Neoechinorhynchus*

cylindratus in a heated reservoir in South Carolina. Recruitment into largemouth bass (*Micropterus salmoides*) occurs in the autumn, while ostracod numbers decrease. By November, ostracods are scarce and most acanthocephalans are in bass. Egg production is delayed until spring, when ostracods are again abundant.

Environmental disturbances might not affect host relationships of all parasites similarly. Contrary to Eure's (1976) findings, Boxrucker (1979) detected no seasonal difference in prevalence or intensity of *P. bulbocolli* in a thermally heated area in Lake Monona, Wisconsin. Instead, he found that, in an unheated area, this species displayed the familiar pattern of increasing prevalence and intensity during the spring followed by declining numbers in autumn. Elevated winter temperatures may have resulted in more rapid parasite development in intermediate hosts, and in fish feeding more extensively on them, than during winter in unheated environments.

Origin and Systematics

Phylogenetic affinities of the phylum Acanthocephala have been debated among biologists for more than a century. Some have regarded the invaginable proboscis, the general body form and the pseudocoelom as evidence of affinity to the nematodes, priapulids and kinorhynchs (Meyer, 1933; Morris and Crompton, 1982). Van Cleave (1941) emphasized that the life cycle with no free-living stage points to an ancestral group that was already parasitic. He considered embryology, the types of intermediate hosts and anatomical features to indicate a close relationship with cestodes.

More recently, molecular techniques for genetic analysis suggest that Acanthocephala and Rotifera are sister taxa (Garcia-Varela *et al.*, 2000; Herlyn *et al.*, 2003) or even that Acanthocephala is a clade within Rotifera (Garey *et al.*, 1998; Welch, 2000). Although molecular data indicating close affinity with rotifers appear convincing, conclusions have been based on nucleotide sequences from only two genes (18S ribosomal RNA (rRNA) and 16S rRNA) and a relatively small number of species. The 'unequal rate effect', or 'long branch attraction', is a common source of error in phylogenetic analyses of molecular data that has often been ignored (Garey and Schmidt-Rhaesa, 1998). Genetic differentiation found among populations of *P. laevis* in European fishes (Kral'ova-Hromadova *et al.*, 2003) suggests that at least some acanthocephalan taxa are evolving rapidly and thus are likely candidates for inducing the long branch attraction error. The variety of conclusions reached from analysis of molecular data (Zrzavy, 2001) suggests that phylogenetic relationships of the group likely will remain controversial until analysis of other suitable genes is completed and more species are studied.

As presently constituted, the phylum Acanthocephala comprises four classes, totalling nine orders (Table 13.1). Members of the class Archiacanthocephala do not occur in fish. The entire class Polyacanthocephala contains only four species, most commonly parasites of South American caimans. However, at least two of the species occur as larvae in the liver of fishes that apparently act as paratenic hosts (Amin *et al.*, 1996; Aloo and Dezfuli, 1997). Nearly all of the species found in fishes belong to the classes Eoacanthocephala and Palaeacanthocephala. With the exception of nine species found in North American turtles (Barger and Nickol, 2004), all eoacanthocephalans are parasites of fishes. Among the palaeacanthocephalans, all of the order Echinorhynchida are parasites of fishes except for a few species that occur as adults in amphibians. Acanthocephalans in the other palaeacanthocephalan order, Polymorphida, occur as adults in birds and mammals, but larvae of many species are found in viscera of fishes that act as paratenic hosts.

Morphology

Acanthocephalans are bilaterally symmetrical, dioecious, pseudocoelomate worms that lack an alimentary canal. They are characterized by a spined proboscis that is invaginable and retractable into a saccular

Table 13.1. Characterization of the classes and orders of the phylum Acanthocephala.

Taxon	Key recognition features[1]	Principal definitive hosts	Principal intermediate hosts[2]
Class Polyacanthocephala	Main lacunar canals dorsal and ventral; cement glands 8, elongate with multiple large nuclei; proboscis receptacle closed sac with single muscle layer; tegumental nuclei numerous, amitotic fragments	Crocodilians	Unknown
Order Polyacanthorhynchida	Only order of the class		
Class Archiacanthocephala	Main lacunar canals dorsal and ventral; cement glands 8, spherical with single nucleus; proboscis receptacle single-walled, often with ventral cleft; tegumental nuclei few, elongate or branched, if fragmented, fragments close together	Birds and mammals	Terrestrial insects
Order Apororhynchida	Proboscis globular, not retractable, spineless or with rootless spines usually not reaching surface	Birds	Unknown
Order Gigantorhynchida	Proboscis conical, retractable, with distinct regions of large anterior hooks and small posterior spines	Birds and mammals	Orthoptera, Coleoptera
Order Moniliformida	Proboscis cylindrical, retractable, with strongly recurved hooks diminishing in size anterior to posterior and arranged in longitudinal rows	Mammals	Cockroaches
Order Oligacanthorhynchida	Proboscis spherical, retractable, with few large hooks arranged in spirals	Birds and mammals	Orthoptera, Coleoptera
Class Palaeacanthocephala	Main lacunar canals lateral; cement glands 2–8, with fragmented nuclei; proboscis receptacle closed sac with 2 muscle layers; tegumental nuclei numerous, amitotic fragments or few highly branched fragments	Fishes, amphibians, birds and mammals	Crustacea
Order Echinorhynchida	Palaeacanthocephala of lower vertebrates	Fishes and amphibians	Amphipods, isopods
Order Polymorphida	Palaeacanthocephala of higher vertebrates	Birds and mammals	Amphipods, isopods, decapods
Class Eoacanthocephala	Main lacunar canals dorsal and ventral; cement gland single syncytium with large nuclei; proboscis receptacle closed sac with single muscle layer; tegumental nuclei few, large	Fishes	Crustacea
Order Gyracanthocephala	Eoacanthocephalans with trunk spines	Fishes	Copepods
Order Neoechinorhynchida	Eoacanthocephala without trunk spines	Fishes	Ostracods

[1]For a complete key to classes, orders, families and subfamilies, see Amin (1987).
[2]For a complete list of known intermediate hosts, see Schmidt (1985).

proboscis receptacle (Fig. 13.1). Body length of adults varies greatly among species, ranging from less than 2 mm to greater than 700 mm. Acanthocephalans of most species are about 10 mm long.

Attachment to the host intestine is by means of a proboscis (Fig. 13.2). Hooks and spines on the proboscis are arranged in a variety of patterns, which serve as important taxonomic characters. The neck (Fig. 13.1) is an unarmed region immediately behind the proboscis, bounded anteriorly by the most proximal proboscis hooks and posteriorly by an invagination of body wall (Van Cleave, 1947). The neck is usually short and inconspicuous, but in some genera it is extremely elongated and may be inflated to form a bulb. The remainder of the body is a trunk, which, in some species, bears tegumental spines, which may enhance attachment to the host intestine (Aznar *et al.*, 2002). As all ontogenetic stages lack alimentary canals, a genital pore is the only opening visible by light microscopy. The genital pore occurs posteriorly on the trunk, and in males it is surrounded by an invaginable bursa (Fig. 13.1).

Apart from the anterior lemnisci and proboscis receptacle, the only conspicuous internal organs are those of the reproductive system. In males there are two testes,

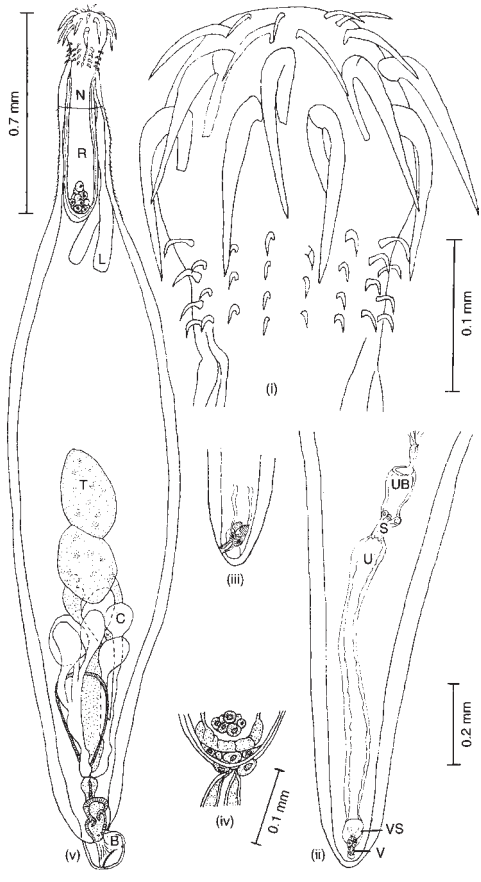


Fig. 13.1. Camera lucida drawings of *Arhythmacanthus paraplagusiarum* from the tonguefish *Paraplagusia guttata*. (i) Proboscis. (ii) Ventral view of posterior extremity of female. (iii) Lateral view of posterior extremity of female. (iv) Posterior tip of proboscis receptacle. (v) Male, entire. B, bursa; C, cement gland; L, lemniscus; N, neck; R, proboscis receptacle; S, selector apparatus; T, anterior testis; U, uterus; UB, uterine bell; V, vagina; VS, vaginal sphincter. (From Nickol, 1972.)



Fig. 13.2. Scanning electron micrograph of the proboscis and neck of *Acanthocephalus lucii*, a parasite of several species of European fishes. Scale bar = 0.2 mm. (Unpublished micrograph, courtesy of D.W.T. Crompton and O.L. Lassiere.)

followed posteriorly by one to eight cement glands (Fig. 13.1). In members of the class Eoacanthocephala, there is a separate cement reservoir, whereas in those of other classes ducts from cement glands may be dilated to store secretions of the cement glands. Efferent ducts from the testes and cement glands, or cement reservoir, join posteriorly and discharge through a small copulatory organ.

Acanthocephalan ovaries fragment early in development to form ovarian balls, which float free in the pseudocoelom. As development proceeds, the pseudocoelom fills with eggs, which have four membranes surrounding the developing larva (acanthor). Eggs are passed through a funnel-shaped uterine bell to a selector apparatus, which allows only fully formed eggs into the uterus (Fig. 13.1). Other eggs are directed back to the pseudocoelom through a series of canals and pores. The female system terminates in a vagina, regulated by a vaginal sphincter, which discharges through a genital pore.

The body is covered by a thin glycocalyx (Beermann *et al.*, 1974), beneath which is a bilayered plasma membrane. Numerous foldings of the plasma membrane form canals (about 15 nm in diameter) in the outer layer of the tegument (Stranack *et al.*, 1966). The fibrous syncytial tegument, sometimes called hypodermis, comprises three layers: an outer striped layer, pierced by numerous crypts formed by the infolded plasma membrane (Fig. 13.3), a middle felt layer, which contains large concentrations of glycogen (von Brand, 1939), and an inner radial layer, which contains the tegumental nuclei and a network of unlined channels that form the lacunar system. In the neck, the radial layer extends into the pseudocoelom as a pair of projections called lemnisci (Fig. 13.1).

Beneath the tegument, the body wall consists of an outer layer of circular and an inner layer of longitudinal muscles, a series of tubular canals that may have a circulatory function, and the rete system, which is associated with the body wall musculature (Miller and Dunagan, 1985).

The nervous system consists of a cerebral ganglion located in the proboscis receptacle, nerves branching from the cerebral ganglion and, in males, a pair of genital ganglia and a bursal ganglion. Sensory receptors are simple and inconspicuous. An apical sense organ, varying in form from a papilla to a depression surrounded by a small elevated rim, occurs at the tip of the proboscis (Dunagan and Bozzola, 1992). In the class Eoacanthocephala, this structure appears to lack a sensory function (Herlyn, 2001) and it might not be homologous to apical organs of other acanthocephalans. Paired lateral sense organs appear as depressions on the neck, but not all species possess apical or lateral sense organs. Males possess sense organs on the bursa.

Most acanthocephalans, including all of those found in fishes, do not possess an excretory system. When an excretory system is present, the protonephridial organs discharge through ducts that for males enter the vas deferens and for females enter the uterus.

Life Cycle and Transmission

Life history

Acanthocephalans require vertebrate animals as definitive hosts and arthropods as intermediate hosts. Those with terrestrial definitive hosts usually have insect intermediate hosts, whereas those with aquatic definitive hosts usually require microcrustaceans (amphipods, copepods, isopods or ostracods). Schmidt (1985) provided a complete list of acanthocephalan intermediate hosts known at the time.

Intermediate hosts are infected by ingestion of eggs voided with the faeces of definitive hosts. Eggs hatch in the alimentary tract of the intermediate host. The acanthor, armed with an aclid organ, penetrates the gut and continues to develop through several ontogenetic stages (known as acanthellae) in the serosa. During these stages, primordia of adult organs are laid down. Late in acanthella development, the proboscis invaginates and all structures of adult worms are present. This juvenile, or cystacanth, is the infective stage for a definitive host. Details of larval development were described by Ward (1940) for

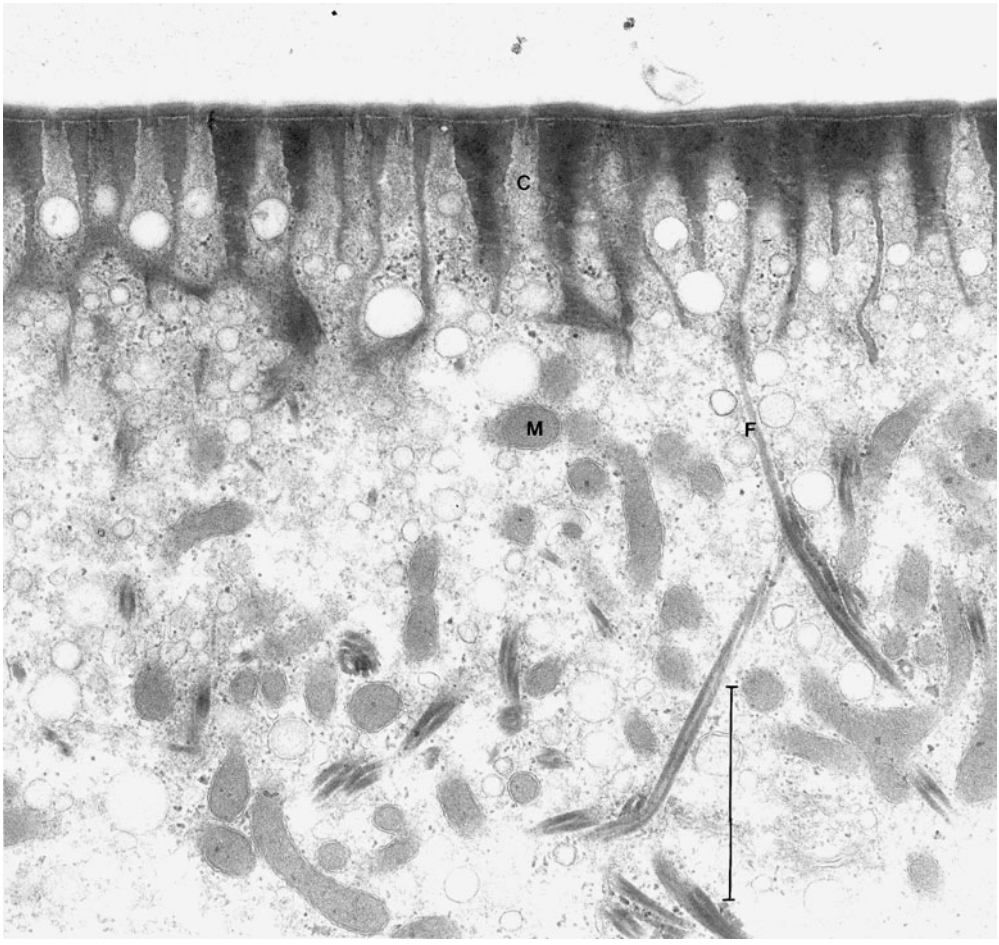


Fig. 13.3. Transmission electron micrograph of the tegument of *Leptorhynchoides thecatus* from a green sunfish (*Lepomis cyanellus*) showing crypts (C), fibres (F) of the felt layer and mitochondria (M). Scale bar = 0.005 mm. (Unpublished micrograph, courtesy of J.A. Ewald.)

N. cylindratus, a common acanthocephalan of bass, and by DeGiusti (1949) for *Leptorhynchoides thecatus*, a species reported from at least 122 piscine species. Ingestion of a cystacanth-containing intermediate host results in infection of a definitive host.

Intercalated hosts

In all documented cases, fully formed cystacanths are infective to definitive hosts directly from their invertebrate intermediate host. When cystacanths of some species are ingested by vertebrates that are unsuitable as definitive hosts, they penetrate the intestine and localize in extra-intestinal sites, where they remain as cystacanths in these paratenic hosts. Predatory definitive hosts, therefore, frequently acquire acanthocephalans from prey animals that serve as paratenic hosts. Fishes are important paratenic hosts for many species, especially those of the genus *Corynosoma* that are found as adults in marine birds and mammals (Valtonen *et al.*, 2001).

Transfer of adult worms from one definitive host to another is often assumed not to occur. When ingested as adults within their definitive hosts, however, some species of

parasites survive and parasitize the predator. Transmission of this sort, known as post-cyclic transmission, has been reported for 11 species of Acanthocephala, eight of which are transferred from fish to fish in this manner (Nickol, 2003).

Enhancement of transmission

Eggs of several acanthocephalan species possess filaments that entangle in aquatic vegetation and apparently anchor them in the habitat occupied by their intermediate hosts (Fig. 13.4). Habitat segregation resulting from differences in structure among eggs increases the likelihood of transmission to the correct host (Barger and Nickol, 1998) and helps avoid deleterious effects of interspecific infections (Barger and Nickol, 1999).

Transmission is enhanced for many species by parasite-induced changes that alter the behaviour of intermediate hosts (Bakker *et al.*, 1997; Moore, 2002) or render them more conspicuous to predators (Mazzi and Bakker, 2003). Although the degree to which behavioural modifications actually promote transmission is largely untested experimentally, three-spined sticklebacks (*Gasterosteus aculeatus*) preferentially consume *P. laevis*-infected prey when given a choice between equal numbers of infected and uninfected amphipods (Bakker and Mundwiler, 1999).

Host–Parasite Relationships

Site selection

After ingestion by the definitive host, cystacanths are activated and attach to the wall of the small intestine. Many species apparently remain at the activation site and develop to maturity. Others migrate and localize in specific regions of the intestine (Crompton 1975; Kennedy, 1985). Several factors, including physiological differences along the alimentary tract (Richardson and Nickol, 1999), maximization of sexual congress (Richardson and Nickol, 2000) and ageing (Taraschewski, 2000), have been implicated in site specificity. In most cases,

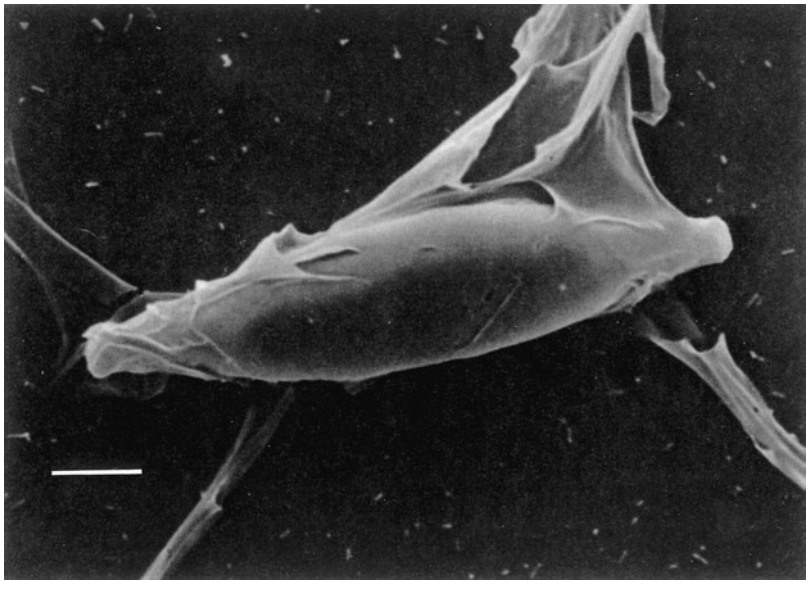


Fig. 13.4. Scanning electron micrograph of an egg from *Leptorhynchoides thecatus* showing unwrapping of embryonic membranes. Unwrapped membranes entangle aquatic vegetation and anchor eggs in habitats of intermediate host feeding. Scale bar = 0.01 mm. (Unpublished micrograph courtesy of M.A. Barger.)

however, it is unknown whether differences in sites occupied result from differences in sites of activation, differential mortality or emigration by the parasites.

Course of infection

Shortly after infection, the proboscis is surrounded by necrotic tissue, which becomes haemorrhagic and inflamed after a few days. During the second week after infection by species that penetrate deeply (e.g. in fish, *Acanthocephalus anguillae*, *P. bulbocolli*, *P. laevis*), inflamed tissue around the anterior portion of the worm is dominated by monocytes and macrophages maturing into epithelioid cells, and an outer belt of connective tissue appears (Taraschewski, 2000). This chronic stage often results in a fibrous nodule visible on the outer surface of the intestine. Species that do not penetrate deeply (e.g. in fish, *Acanthocephalus lucii*, *E. truttae*, *N. cylindratus*) move about in the intestine and change their point of attachment before the connective-tissue response results in nodules (Adel-Meguid *et al.*, 1995).

Copulation in the definitive host may occur within 24 h of infection (Muzzall and Rabalais, 1975). In laboratory infections of green sunfish (*Lepomis cyanellus*), copulation within a group of *L. thecatus* continued at least 12 weeks after infection (Richardson *et al.*, 1997). For most species, egg production starts between 4 and 8 weeks after infection and continues for approximately 2 months. The number of eggs produced daily by each female acanthocephalan is unknown for most species, but it appears to be related to the size of the worm. At peak production, a female *Macracanthorhynchus hirudinaceus* (large worms found in pigs) may produce about 260,000 eggs per day (Kates, 1944), a female *Moniliformis moniliformis* (intermediate-sized worms in rats) about 4800 (Reyda and Nickol, 2001) and a female *Polymorphus minutus* (small worms found in waterfowl) about 1700 (Crompton and Whitfield, 1968).

Male worms have shorter lifespans than do females; death of males and subsequent loss from the host may begin shortly after copulation. Although females of some species, especially those parasitic in mammals, may live longer than a year, most in poikilothermic hosts live about one season. There may be more than one generation each year.

Clinical signs and gross pathology

Acanthocephalans in moribund or dead animals are frequently assumed to indicate deleterious effects; worms have been observed extending from the rectum (Schmidt *et al.*, 1974; Fig. 13.5) or protruding through the trunk (Taraschewski, 2000) of infected fishes. There are, however, numerous instances of exceedingly heavy infections in animals that do not show any obvious disease.

Bullock (1963) described trout of several species infected with *Acanthocephalus dirus*

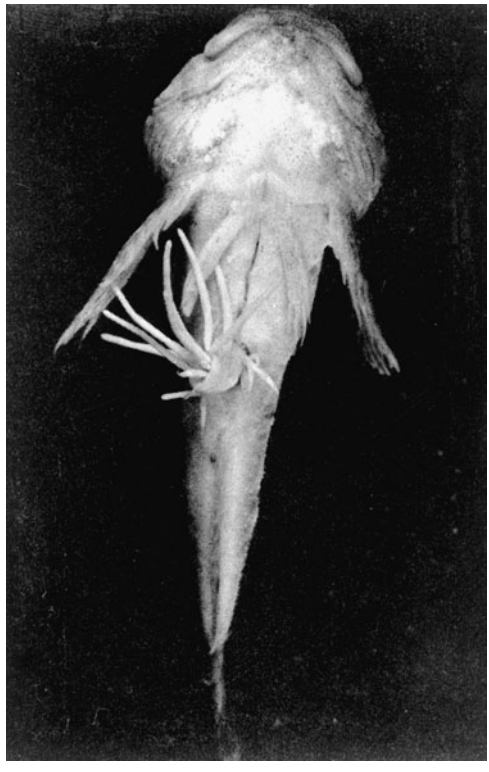


Fig. 13.5. Photograph of *Acanthocephalus dirus* attached to the prolapsed rectum of a mottled sculpin (*Cottus bairdi*). Photograph = actual size. (From Schmidt *et al.*, 1974).

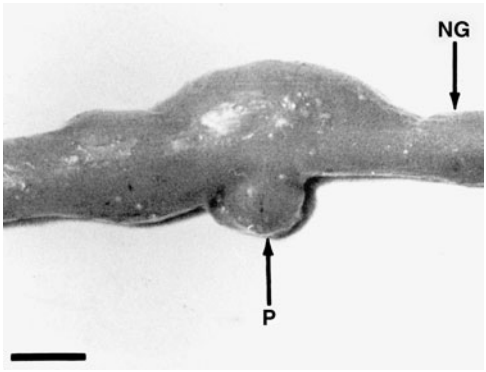


Fig. 13.6. External view of intestine from a quillback (*Carpiodes cyprinus*) showing a parasite-induced nodule (P) and expansion of the intestine compared with the normal gut (NG) from a fish infected with *Neoechinorhynchus carpiodi*. Scale bar = 5 mm. (From Szalai and Dick, 1987.)

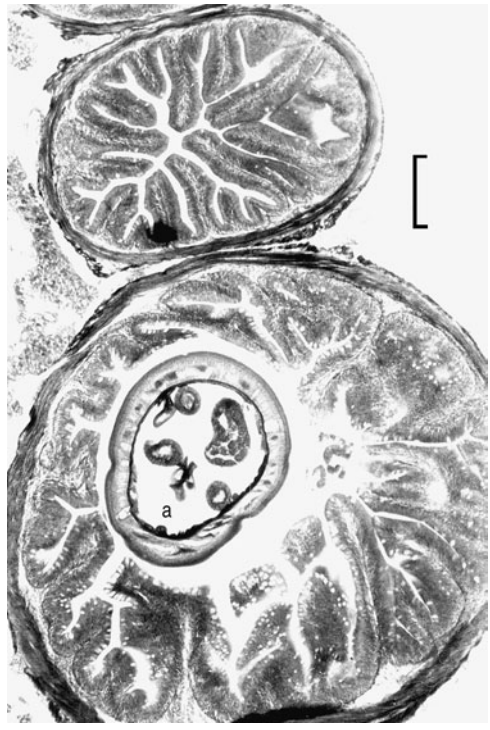


Fig. 13.7. Transverse section through the region of the pyloric caeca of a green sunfish (*Lepomis cyanellus*) infected with *Leptorhynchoides thecatus,* showing unparasitized caecum (top) and enlarged parasitized caecum (bottom). Note the compressed and eroded villi along the trunk of the parasite (a) compared with normal villi in the unparasitized caecum. Scale bar = 0.1 mm. (Unpublished micrograph, courtesy of I. de Buron.)

as having underdeveloped musculature, heads that were disproportionately large and a slightly concave dorsolateral surface. He interpreted these as signs of malnutrition.

Fibrotic nodules induced on the intestinal surface (Fig. 13.6) by deeply penetrating worms are the most frequent signs of infection. At times the viscera may be discoloured or the infected intestine enlarged and inflamed. Pyloric caeca of green sunfish (*L. cyanellus*) infected with *L. thecatus* are nearly twice the diameter of those in uninfected fish of the same size (de Buron and Nickol, 1994; Fig. 13.7). Acanthocephalans occasionally perforate the intestinal wall and protrude into the coelom or attach their proboscides to another organ.

Histopathology

Acanthocephalans embed their spiny proboscis into the mucosal epithelium. Attachment is frequently between villi. At the site of attachment, cells are destroyed and fibroblasts, lymphocytes and macrophages are mobilized below the lamina propria (Dezfuli *et al.*, 1990), where chronic fibrinous inflammation, resulting in an increased amount of connective tissue, causes thickening (Bullock, 1963). In some species, fibroplasia extends to layers of the muscularis mucosa (de Buron and Nickol, 1994).

Worms of the deeply penetrating species often possess very long necks and bulbous proboscides, which anchor them deep within the gut wall of their piscine hosts. Dezfuli *et al.* (2002a) described the cellular structure of a fibrotic tunnel that forms around the neck and proboscis bulb of *P. laevis*. The tunnel terminates in a capsule, covered by serosa and mesentery, which protrudes several millimetres into the coelomic cavity (Fig. 13.8). Sometimes these capsules persist as conspicuous fibrinous nodules on the external surface of the

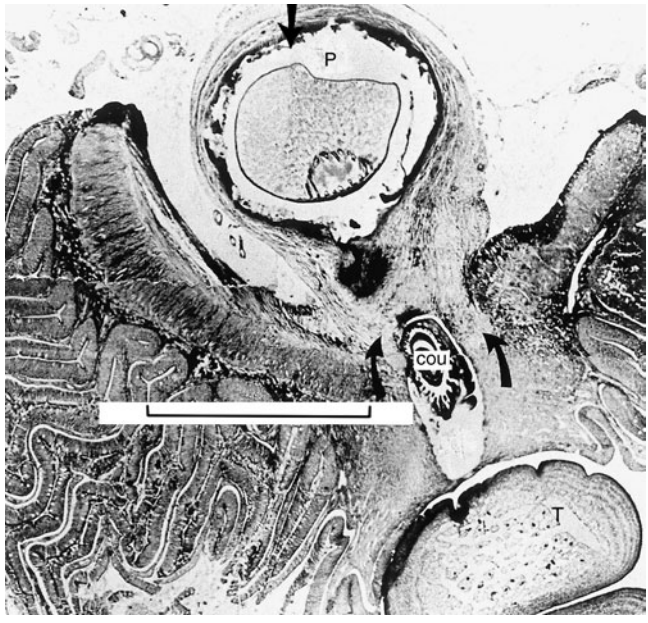


Fig. 13.8. Section of chub (*Leuciscus cephalus*) intestine showing penetration of *Pomphorhynchus laevis*. Note hyperplasia of the lamina propria (arrows) around the neck (cou) and bulbous proboscis (P) of the worm, forming a nodule in the coelom. An anterior portion of the trunk (T) of the worm shows in the intestinal lumen. Scale bar = 1 mm. (Unpublished micrograph, courtesy of I. de Buron.)

alimentary canal, or the proboscis perforates the capsule to emerge free in the coelom or to penetrate the liver or another visceral organ. Similar findings were reported for *P. bulbocolli* in rainbow darters (*Etheostoma caeruleum*) (McDonough and Gleason, 1981) and two species of catostomids (Chaicharn and Bullock, 1967). *A. anguillae* is also known to perforate the intestine of its host and attach to the liver. In goldfish (*C. auratus*), large portions of this organ are replaced by proliferative tissue, often with patches of pancreas, surrounding the embedded proboscis (Taraschewski, 1989a).

Most studies of acanthocephalan-induced lesions reveal areas along the trunk of the worm where the mucosal surface is compressed or desquamated (Fig. 13.7). In these cases, mucosal folds and tips of villi may be absent and the paramucosal lumen contains large amounts of mucoid material originating from goblet-cell hyperplasia. In green sunfish infected with *L. thecatus*, there is a significantly greater number of goblet cells in parasitized pyloric caeca than in unparasitized caeca in the same fish (de Buron and Nickol, 1994). This suggests a parasite-induced response that might lessen damage from the erosive nature of the worms. Taraschewski (2000) concluded that mucins from goblet cells also contribute to the expulsion of acanthocephalans from immunized hosts.

Pathophysiology

Chronic fibrosis, destruction of intestinal villi and necrotic and degenerative changes in mucosal epithelium adversely affect motility and the absorptive efficiency of the fish intestine. This might affect the general health and growth of the host. According to Bristol *et al.* (1984), there is a negative correlation between the number of acanthocephalans and the amount of body lipid in trout, and Buchmann (1986) demonstrated a negative correlation between the number of *Echinorhynchus gadi* present and energy

stores in Baltic cod (*Gadus morhua*). Trout infected with *P. laevis* have lower muscle protein than uninfected fish (Wanstall *et al.*, 1982). Seasonal factors, such as host spawning or reduced winter feeding, exacerbate the protein depletion. Wanstall *et al.* (1982) suggested that *P. laevis* induced mobilization of endogenous protein for energy in preference to endogenous carbohydrates or lipids. This phenomenon is not uncommon in fishes under stress. Further, the acanthocephalan *Plagiorhynchus cylindraceus*, considered of little pathological consequence, has a significant detrimental effect on the flow of food energy through the host and alters its basal metabolism (Connors and Nickol, 1991).

There are few field studies that relate acanthocephalan infections to the condition factor or survivorship of fishes. Probably this is because less fit animals are sampled less frequently as a result of more efficient predation on sick and weak hosts. Nevertheless, Bakker and Mundwiler (1999) and Sasal *et al.* (2001) have shown acanthocephalans to have an adverse effect on the fitness of their fish hosts.

Mechanism of disease

Several biologists have suggested that acanthocephalans secrete toxic substances that paralyse or kill their hosts or that promote other patent pathological changes, such as emaciation, discoloured viscera and prolapse of the rectum (Holloway, 1966; Fig. 13.5). No such substance has been isolated, however, and Schmidt *et al.* (1974) attributed such damage to localized toxaemia and chronic fibrinous inflammation, which result from laceration of cells at the site of attachment.

Action of the spined proboscis results in destruction of mucosal cells and penetration of the intestinal wall, with an accompanying loss of absorptive capability, impaired gut motility and sometimes perforation of the intestinal wall. Apparently this damage is exacerbated by biochemical reactions. Miller and Dunagan (1971) described a pore-like opening and groove on acanthocephalan hooks, and they postulated delivery of a secretion via the proboscis hooks. Taraschewski (1989b) demonstrated proteolytic enzymes incorporated within osmiophilic material associated with the proboscis of *Paratenuisentis ambiguus* in eels (*Anguilla anguilla*) and postulated that they are discharged from the worms through pores in the proboscis hooks. Such enzymes with trypsin-like activity secreted by *P. laevis* in chub (*L. cephalus*) degrade collagen (Polzer and Taraschewski, 1994) and thus are capable of degrading one or more of the major components of the host's gastrointestinal tissue.

In addition to aggravating damage done to cells and tissues by the proboscis, biochemical reactions might also increase loss of gut motility caused by parasite-induced fibrosis in the intestinal wall. Dezfuli *et al.* (2002b) found that, when infected with *P. laevis*, brown trout (*S. trutta*) have altered distribution and concentrations of calcitonin gene-related peptide, beta-endorphin, met-enkephalin, neuropeptide Y, substance P, vasoactive intestinal peptide and bombesin-, cholecistokinin-8- leu-enkephalin-, and serotonin-(5-hydroxytryptamine (5-HT))-like immunoreactive cells. Most of these neuromodulators are known to control gut motility and digestive and absorptive processes. Changes in these functions probably occur in infected fishes.

Host Immune Response

Cellular

The range of cellular and humoral immune mechanisms described in recent years for teleosts makes it clear that the piscine immune system is relatively well developed (Buchmann *et al.*, 2001). Although there are few studies to demonstrate effective immunological protection against acanthocephalans, there is evidence to suggest that some degree of immunity exists.

Goblet-cell hyperplasia occurs widely in acanthocephalan infections. The covering created by copious secretion of mucus and the presumed presence of antibodies

within probably reduce the number of parasites that succeed in establishing (Thomas, 2002).

Apart from a proliferation of goblet cells, increased numbers of eosinophils, neutrophils and monocytes at the attachment site characterize histopathological effects of acanthocephalans. Mobilization of leucocytes occurs regardless of whether the fish species is suitable for development of the parasite, and Hamers *et al.* (1992) found interspecific differences in the response of leucocytes in fishes parasitized by *P. ambiguus.* In eels (*A. anguilla*), a suitable definitive host, the response was much less intense than in carp (*Cyprinus carpio*) or rainbow trout (*Oncorhynchus mykiss*), both unsuitable hosts that expel the acanthocephalans within a few days. The leucocytes damage acanthocephalan tegument extensively in carp; hence Hamers *et al.* (1992) concluded that cellular defence is a factor in determining host specificity for *P. ambiguus.*

Humoral

Plasma cells ('type B cell') occur in the inflammatory tissue around the proboscis of *P. laevis* in rainbow trout (*Salmo gairdneri*), and they are probably responsible for the humoral response produced by the fish (Wanstall *et al.*, 1986). Even though immunoglobulins are relatively slow to develop in fish following an infection, and precipitins are rare (Taraschewski, 2000), antibodies precipitating to acanthocephalan antigens have been reported from sera (Harris, 1970; Szalai *et al.*, 1988) and intestinal mucus (Harris, 1972) of infected fishes. Sera from chub (*L. cephalus*) held at 10°C and with no history of exposure to *P. laevis* do not have anti-*P. laevis* precipitins, but precipitins were detected within 160 days after infection. Parenteral injection of *P. laevis* antigen also induces a similar response by 150 days after injection (Harris, 1972). Anti-*P. laevis* precipitins are not found in four other piscine species in which *P. laevis* occurs but, unlike in *L. cephalus*, does not reach maturity. This led Harris (1972) to speculate that the antigenic substance is produced only by mature worms.

Despite a humoral response, chub (*L. cephalus*) have large numbers of *P. laevis* in a variety of stages of development. Harris (1972) found no evidence of expulsion of worms, even from fish with mature worms.

Cross immunity

Concurrent infections with more than one species of acanthocephalan and/or with other helminths of other phyla are frequent. *P. bulbocolli* and *A. dirus* occur concurrently in rainbow darters (*E. caeruleum*), where they occupy sites in close proximity to one another. There appears to be no synergistic effect as both species cause damage as if they were in single-species infections (McDonough and Gleason, 1981). In contrast, numbers of the cestode *Proteocephalus exiguus* and *Neoechinorhynchus* sp. show an inverse relation in ciscos (*Coregonus artedii*), although the two helminth species occupy different intestinal sites. Cross (1934) believed that non-specific immunity limits either *P. exiguus* or the species of *Neoechinorhynchus*.

Very little is known about the nature of precipitins in serum of acanthocephalan-infected fishes, and seldom has specific immunoglobulin been demonstrated. Szalai *et al.* (1988), however, confirmed that anti-*Neoechinorhynchus carpiodi* precipitins in serum from infected quillback (*Carpiodes cyprinus*) were not complement-reactive protein or the alpha migrating factor. Partial characterization of chub (*L. cephalus*) antibody to *P. laevis* indicated that it is an immunoglobulin M (IgM) type (Harris, 1972). Consequently, precipitating antibody occurs in at least one acanthocephalan infection but its role in limiting infection and the degree of species specificity are not known.

In Vitro Cultivation

Studies on nutritional requirements, development and response to chemotherapeutic

agents would be greatly facilitated by successful *in vitro* cultivation. Hamers *et al.* (1991) obtained axenic growth of *P. ambiguus* taken from intermediate hosts, but it was substantially slower than *in vivo* growth and maximum survival was 63 days. In no case have immature worms been cultured to egg-producing maturity.

There is only one report of culturing acanthocephalans *in ovo*, a procedure highly successful for digenetic trematodes (Fried and Stableford, 1991). Young and Lewis (1977) obtained growth of *Corynosoma constrictum* cystacanths, normally infective to waterfowl, on the chicken chorioallantoic membrane; however, the worms survived for only 3 days. Cystacanths of two other species, also parasitic in birds, survived up to 9 days, but they did not grow.

Crompton and Lassiere (1987) reviewed reported maintenance techniques and listed medium constituents and protocols for those where there was some indication of success.

Nutrition, Physiology and Biochemistry

Feeding mechanisms

Most information regarding feeding, nutrition and metabolism in Acanthocephala is based on two species from mammals and one from waterfowl. Taraschewski (2000) reviewed these processes and integrated available knowledge from fishes.

Uptake of nutrients occurs through the tegument. Intimate contact between the acanthocephalan proboscis and neck and the flattening or erosion of host mucosal cells along the trunk of the worms lead to a frequent assumption that nutrients are obtained as a result of leakage from host tissue. Bullock (1963) suggested that, in addition to uptake of secretions from the host mucosa, worms absorb nutrients from the copious mucus frequently produced in response to infection and which contains end products of host digestion. It is known that at least some species obtain nutrients from dietary contents in the intestinal lumen of the host (Edmonds, 1965) and that hydrolytic enzyme activity occurs at the tegumental surface of the worm (Uglem and Read, 1973). Although cytological localization of surface hydrolases is unknown, phosphatase (Byram and Fisher, 1974) and aminopeptidase (Uglem and Read, 1973) activities are associated with the tegumental crypts (Fig. 13.3).

The tegumental crypts, formed by infolded plasma membrane, provide an extensive amplification of surface area for nutrient uptake, but Starling (1985) concluded that the crypts are unstirred regions comparable to intervillar spaces of vertebrates and, as such, might make a relatively minor contribution to uptake of solutes such as glucose and free amino acids. They should, however, greatly increase the facility with which products of hydrolytic enzymes are absorbed.

Nutritional requirements

In addition to dietary protein (Crompton *et al.*, 1983), hydrolysis of secretory digestive proteins, degradation of moribund mucosal cells and probably exchange between the mucosal epithelium and intestinal lumen are sources of free amino acids in the intestinal lumen of a vertebrate animal (Starling, 1985). Amino acids from host tissues contribute significantly to the pool available to acanthocephalans, which assimilate certain of these both by diffusion and by active transport (Uglem and Read, 1973). This exchange among host tissue, host dietary materials and worms *in vivo* and the inability to culture acanthocephalans satisfactorily *in vitro* have impeded attempts to define nutritional requirements for amino acids.

Taraschewski (2000) reached the following conclusions after reviewing lipid metabolism for acanthocephalans. Non-emulsified lipid from necrotic host tissue at the site of attachment is absorbed through the tegumental surface of the proboscis and neck of the worms. Apparently, these lipids are stored in the lemnisci. In contrast, emulsified free fatty acids and monoacylglycerols resulting from action of intestinal lipases in

the intestinal lumen of the host are absorbed along the trunk of the parasite. Clearly acanthocephalans contain large amounts of lipid and possess enzyme systems for lipid metabolism (Filipponi *et al.*, 1994; Weber *et al.*, 1995); however, use of these lipid deposits in energy metabolism is yet to be demonstrated.

The role of carbohydrates in nutritional requirements of acanthocephalans has been widely studied. Glucose is absorbed readily against a concentration gradient (Crompton and Lockwood, 1968), and the growth, fecundity and longevity of worms in hosts fed different dietary sugars, in various concentrations, have been determined (Parshad *et al.*, 1980; Crompton, 1991).

Energy metabolism

Glycogen and trehalose appear to be the principal endogenous carbohydrates in acanthocephalans. Their tissues are rich in glycogen, and depleted stores are rapidly replenished (Read and Rothman, 1958). Glycogen synthase occurs in both dephosphorylated and glucose-6-phosphate-dependent phosphorylated forms, which can apparently be interconverted (Starling, 1985). Trehalose is present in pseudocoelomic fluid, reproductive tissues and the body wall (McAlister and Fisher, 1972). Presumably it contributes to the osmolality of body tissues and extracellular fluids, but the role of trehalose is largely undetermined in acanthocephalans. It is unlikely to serve as a primary storage of carbohydrate for energy (Laurie, 1959). Starling and Fisher (1978) believed that trehalose may trap glucose within the acanthocephalan tegument after its absorption and carry glucose moieties to non-tegumental tissues.

Carbohydrate is the main source of energy, and it is reasonably clear that glucose and glycogen are metabolized via the conventional Embden–Meyerhof pathway to phosphoenol pyruvate. Further metabolism probably involves anaerobic reactions common to other helminths, but the steps are still uncertain (Starling, 1985). Lactate and succinate are the main end products, although Crompton and Ward (1967) found extensive ethanol excretion by *M. moniliformis*. Starling (1985) reviewed the glycolytic enzymes and metabolic end products of acanthocephalans.

Affinity for heavy metals and other elements

Acanthocephalans in some freshwater fishes contain concentrations of heavy metals, e.g. lead and cadmium (Sures, 2001), and other elements, e.g. calcium, potassium and magnesium (Sures *et al.*, 1999), in remarkably higher concentrations than in the tissues of the host. Concentrations of these substances may be tens of thousands of times higher in the acanthocephalan than in host tissue (Taraschewski, 2000). Such concentrations do not occur in other kinds of parasites in the same fish (Sures *et al.*, 1994) or in zebra mussels (*Dreissena polymorpha*). These findings led Sures and Siddall (2003) to suggest that acanthocephalan parasites of fishes might be more effective indicators of pollution due to heavy metals in aquatic ecosystems than are the frequently used zebra mussels.

The competition for essential minerals, including calcium, that occurs between *A. lucii* and its definitive host perch (*Perca fluviatilis*) suggests an adverse relationship between parasite and host. Such competition might even explain observations of skeletal deformation that are occasionally reported for acanthocephalan-infected animals (Sures, 2002). A direct link between accumulation of minerals by acanthocephalans and pathological changes in their hosts, however, is yet to be established.

Feeding-induced pathogenesis

Deep penetration by the proboscis results in fibrinous nodules, which may project several millimetres into the coelom (Fig. 13.6). Szalai and Dick (1987) reported extensive vascularization of such nodules in quillback (*C. cyprinus*) induced by *N. carpiodi* and demonstrated increased leakage of

proteins from blood in the region of the nodules. They suggested that the lesion might ensure a limited, but steady, supply of nutrients for the parasites.

P. laevis is another species that induces nodule formation (Fig. 13.8). Polzer and Taraschewski (1994) found that the worm releases an aminopeptidase and a trypsin-like collagenase into the culture medium. They concluded that acanthocephalan aminopeptidases have a nutritional role but that the collagenase facilitates rapid and deep penetration by the worm into the intestinal tissues. This suggests that proteolytic enzymes degrade peptides at the body surface. In some species, the histolytic action permits rapid and deep penetration, leading to formation of nutrient-supplying nodules.

Prevention and Control of Infection

Diagnosis

At least some species of fish respond to acanthocephalan infections (natural and in the laboratory) by production of specific antibodies (Harris, 1972). However, there is insufficient information for immunodiagnosis. Acanthocephalans can be diagnosed from eggs passed out in the faeces of the host, but this is not convenient and there is little necessity for the technique. Most infections are detected during post-mortem examination.

Chemotherapy

The antidiarrhoeic drug loperamid was efficacious in treating rainbow trout (*S. gairdneri* = *O. mykiss*) infected with *E. truttae*; doses of 50 mg/kg were administered on 3 consecutive days (Taraschewski *et al.*, 1990). This treatment might be useful in commercial cultivation of fishes.

Prophylaxis

Prevention of exposure is the most effective method of limiting acanthocephalan infections. These parasites are good colonizers and expand their ranges by movements of infected animals, but transfaunations due to anthropochore fish movements, including those that result from stocking and management of freshwater fishes, are of growing concern (Kennedy, 1993). Acanthocephalans can be introduced with either definitive hosts as a result of stocking (Malta *et al.*, 2001) or with intermediate hosts in the water supply (Valtonen and Koskivaara, 1994). If colonization of the habitat is successful, the resulting parasitism can be costly to aquaculture endeavours (Bristol *et al.*, 1984; Yasumoto and Nagasawa, 1996).

Summary and Conclusions

Acanthocephalans occur globally in most fishes, but epizootics and significant economic losses as a result of infection are unusual. Acanthocephalans require vertebrate animals for definitive hosts and arthropods for intermediate hosts. Isopods, amphipods and ostracods are the usual intermediate hosts for aquatic species. Infection occurs when a definitive host consumes the infective cystacanth stage contained in an arthropod or in a paratenic host. There is an increasing awareness that, in some cases, postcyclic transfer of adult worms from fish to fish can occur as a result of predation. Worms are typically recruited into fish populations during the spring, with maturation, egg production and transmission to intermediate hosts in the summer and early autumn. Adult worms usually live about one season.

Acanthocephalans attach to the intestine of definitive hosts by means of a spiny proboscis. Mucosal tissue is damaged at the attachment site, resulting in fibroplasia, which may extend through the submucosa and into the muscularis. Occasionally perforation of the gut wall occurs. The mucosal epithelium is frequently compressed or eroded along the trunk of the worm, and the tips of the villi may be absent. Destruction of intestinal villi and necrotic and degenerative changes in mucosal epithelium almost certainly reduce the absorptive efficiency of the fish intestine.

Some acanthocephalans are known to induce antibody formation in piscine species. Haemorrhage at the attachment site and copious amounts of mucus, from goblet-cell hyperplasia, in parasitized fishes provide an opportunity for antibody–worm interaction, but it is not known if protective immunity develops.

Acanthocephalans lack an alimentary tract and hence uptake of nutrients, derived both from leakage of host tissues and from dietary contents in the intestinal lumen of the host, is through the tegument. Hydrolytic enzyme activity at the tegumental surface probably assists in obtaining nutrients and in rapid penetration by the worm. Some acanthocephalans penetrate deeply and induce formation of a nodule, which extends into the coelom of the host. Such nodules are extensively vascularized. Increased leakage of proteins from the blood into the nodules ensures a steady supply of nutrients for the parasites.

Carbohydrate, in the form of glycogen, is the main substrate for energy, and glycolysis occurs in the presence or absence of oxygen. Lactate and succinate are the main end products, but large amounts of ethanol are excreted by at least one species.

All media in which significant growth has been achieved contain undefined components. Further research for a defined medium is necessary before appreciable progress can be made in determining nutritional requirements, especially for amino acids and lipids.

The consequences of acanthocephalan-induced reductions in energy efficiency and altered metabolism of hosts are likely to be focused more sharply with increasing emphasis on aquaculture. Suitable methods of *in vitro* cultivation are necessary to elucidate responses of worms to chemotherapeutic agents. Known effects detrimental to commercially cultured fishes are likely to stimulate research directed at testing for chemotherapeutic compounds. When efficacious drugs are found, their toxicity to fishes, aquatic invertebrates and human beings must be assessed.

Recent appreciation of the propensity of acanthocephalans to accumulate heavy metals and other elements is likely to stimulate research to evaluate their effectiveness as bioindicators of heavy-metal pollution. The broad spectrum of metals that can be accumulated, even if present in very low concentrations, might make acanthocephalans pre-eminent among organisms used as sentinels in aquaculture systems.

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14 Phylum Arthropoda

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Introduction

Arthropod parasites of fish have been recorded since the time of Aristotle (300 BC). He noted that parasitic isopods (almost certainly cymothoids) were common on the fins of fishes, and that 'lice' (probably ergasilids) occurred under the gills of 'chalkis' (possibly bleak) in such numbers that the fish were killed. He also mentioned a 'scorpion-like' parasite, possibly a pennellid, from the body of tuna and swordfish.

About 2000 species of parasitic arthropods have been described, the majority of which belong to the class Copepoda. Many others await description. Thatcher (1998) estimated that there were 9000 undescribed parasitic copepod species in the Amazon alone.

Some of the arthropods that affect wild fish are of commercial significance as they affect host survival or cause unsightly changes in the flesh. Others (e.g. the anchor worm, *Lernaea cyprinacea*) cause ongoing problems in aquaculture. The biology of pests in freshwater aquaculture is reasonably well known. Parasites in the sea, especially sea lice (*Lepeophtheirus salmonis*) and more recently cymothoid isopods, have come under intense scrutiny with the development of rearing fish in sea cages.

In this chapter, we discuss those arthropods for which pathological changes in fish have been described.

Copepoda: Ergasilidae

Introduction

Ergasilid copepods damage the gills and cause commercially significant epizootics in cultured and wild populations of fishes. *Ergasilus sieboldi* is widespread in temperate parts of Europe and Asia, *Ergasilus lizae* is important in mullet culture in the Mediterranean and Middle East, and *Pseudergasilus zacconis* infects cultured ayu, *Plecoglossus altivelis*, in Japan. In North America, Kelly and Allison (1962) implicated *E. lizae* in the death of largemouth bass (*Micropterus salmoides*) in a brackish lake and Hogans (1989) reported that *Ergasilus labracis* killed large numbers of Atlantic salmon (*Salmo salar*) parr (85–125 mm long) over a 4-day period in New Brunswick, Canada.

Host range, distribution and seasonality

Most species in the family Ergasilidae belong to the genus *Ergasilus*, of which 65 species

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are parasitic on freshwater fishes and 33 species on marine teleosts (Kabata, 1979).

Ergasilus sieboldi infests over 60 species of fish throughout much of the former USSR. The main hosts are *Tinca tinca*, *Esox lucius*, *Acerina cernua*, *Lucioperca lucioperca* and *Silurus glanis* (Dogiel *et al.*, 1961; Zmerzlaya, 1972). Several thousand parasites may occur on the gills of one fish. In Germany, 39 of 79 species of fishes are infected, the most important hosts being *Ergasilus lucius* and *Abramis brama* (see Dogiel *et al.*, 1961). *E. sieboldi* is common on British freshwater fishes (Abdelhalim *et al.*, 1991) and its distribution has probably been aided by the introduction of affected fish.

Neoergasilus japonicus, originally described from Taiwan in 1930, was first reported in Europe in the 1960s (Fig. 14.1). Over the next 20 years it spread across Europe.

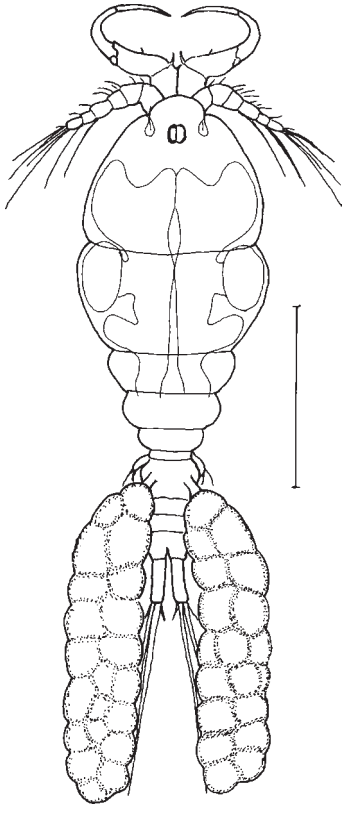


Fig. 14.1. *Neoergasilus japonicus,* adult female (after Harada, 1930). Bar = 250 µm.

It first appeared in Finland and Cuba in the 1980s and was first found in North America in the 1990s. It has low host specificity and now occurs on cyprinids and percids in the Palaearctic, plus centrarchids and ictalurids in North America (Hayden and Rogers, 1998; Hudson and Bowen, 2002). Another ergasilid, *Ergasilus centrarchidarum*, is believed to have been spread within the USA by anglers (Muzzall *et al.*, 1995).

Amongst the marine ergasilids, *E. labracis* infects a wide range of marine species, including salmonids (Hogans, 1989), and *Ergasilus luciopercarum* infects chinook salmon, rainbow and brown trout and yellow perch. In contrast, *Ergasilus nerkae* infects only coho salmon (Buttner and Hamilton, 1976) and ergasilids on Amazonian fishes also appear to be very host specific (Thatcher and Boeger, 1983).

Larvae of *E. sieboldi* occurred in Lake Arakul, former USSR, from the end of May to November, with two peaks in summer (August and September) (Kashkovsky and Kashkovskaya-Solomatova, 1986). Females that overwintered produced egg sacs in April (water temperature 5.7–7°C), and the first generation of females settled on the host in late June. A second generation appeared from mid-August to mid-September. There was no recruitment after October, when egg sac formation ceased (Zmerzlaya, 1972). According to Reichenbach-Klinke and Landolt (1973), there was no development if the water temperature dropped below 14°C. Egg sac formation is stimulated by temperature in spring and early summer, but in late summer and autumn photoperiod rather than temperature apparently limits development (Kuperman and Shulman, 1977). In Finland, the parasite was more abundant in oligotrophic than in eutrophic lakes (Tuuha *et al.*, 1992) and prevalence was markedly reduced in waters polluted by pulp and paper mill effluents (Pulkkinen and Valtonen, 1998).

At least two generations per year, including one that overwinters, were suggested by Tuuha *et al.* (1992) for *E. sieboldi*, *Ergasilus briani* and *Neoergasilus japonicus* in Finnish lakes.

E. labracis infected almost all the striped bass, *Morone saxatilis*, from lower

Chesapeake Bay, caught in a wide range of salinities (0.1 to 32 ppt). The parasite was present and reproduced year-round, with only a temporary slow-down in egg production during November and December. Laboratory-held eggs hatched at temperatures as low as 5°C (Paperna and Zwerner, 1976).

Peak prevalences of *E. briani* in central Finland (Tuuha *et al.*, 1992), *N. japonicus* in the Great Lakes (Hudson and Bowen, 2002) and *Ergasilus versicolor* on the US east coast (Rawson, 1977) occur in the summer.

Ergasilus lizae is a cosmopolitan species that is restricted largely to the Mugilidae (Paperna and Overstreet, 1981; Kabata, 1992), though it has been found on eels, tilapia and carp as well as mullet in aquaculture ponds with salinities from 0.2 to 21 ppt in Israel (Paperna, 1975, 1977, 1991). High water temperature and low salinity are associated with high levels of infection (Raibaut *et al.*, 1975; Rawson, 1977; Ben Hassine *et al.*, 1983). Females from silver mullet, *Mugil curema*, survived for over 4 h in salinities ranging from 0 to 37 ppt (Conroy and Conroy, 1986).

Systematics

To identify ergasilid copepods to genus, see Boxshall (2004). For specific keys see Roberts (1970) for North America, Do (1982) for Japan and Kabata (2003) for Britain. A key to 22 species of ergasilids from brackish water is given by Lin and Ho (1998). Keys to *Dermoergasilus*, an Indo-West Pacific genus, and *Therodamas*, a Neotropical genus, are given by El Rashidy and Boxshall (2001) and Amado and Rocha (1996), respectively. Hoffman (1998) lists species from North American freshwater fish. Amado and Rocha (2001) suggest that the morphology of the ventral plates is a useful guide for identifying males and premetamorphosed females of *Ergasilus* spp. taken in plankton samples.

Morphology and life cycle

Most ergasilid copepods are less modified than other fish-parasitic copepods and resemble free-living copepods in segmentation. Only fertilized females are parasitic; males and developmental stages live in the water column. The main characteristic of ergasilids is the large, recurved, second antennae, which are used for attachment. In *Ergasilus*, *Neoergasilus* and *Sinergasilus*, the second antennae end in a single, large, curved spine (Fig. 14.1); in *Thersitina* (syn. *Diergasilus*; see Ohtsuka *et al.*, 2004), they end in two spines and, in *Paraergasilus*, three spines. The a2 cuticle is swollen in genera such as *Dermoergasilus.*

The mature female of *E. sieboldi* is 1.5 mm long and has a cephalothorax formed from the cephalosome and the first leg-bearing segment. Four leg-bearing segments follow, the last intimately associated with the swollen genital segment, which gives rise to paired multiserial egg sacs, each with more than 100 eggs (Kabata, 1979; Grabda, 1991). Mouthparts are on the midventral surface of the cephalothorax, and consist of a two-segmented mandible with a falcate terminal segment, a short first maxilla with two distal setae and a falcate second maxilla with dentiform setae (Kabata, 1979).

The naupliar feeding apparatus is typical for planktotrophic copepod nauplii and the adult feeding apparatus (mandibles, maxillae 1 and 2) appears at the first copepodid stage (Abdelhalim *et al.*, 1991). The stomach extends anterior and posterior to the mouthparts, merging posteriorly with the straight intestine, which leads to the anus at the end of the abdomen between the paired uropods. During sexual maturation, uterine processes that contain oocytes fill a large part of the cephalothorax. The oviducts lead to the genital segment to join with paired cement glands.

Members of the family Therodamasidae have developed long 'necks' with mouthparts embedded in the fish (Figs 14.2 and 14.3), in a development strikingly similar to lernaeids and pennellids except that in therodamasids the neck is formed from the cephalosome whereas in lernaeids it is the thoracic segments that are elongated and in pennellids the genital segment.

The life cycles of *N. japonicus*, *E. sieboldi* and *E. briani* (Urawa *et al.*, 1980a,b;

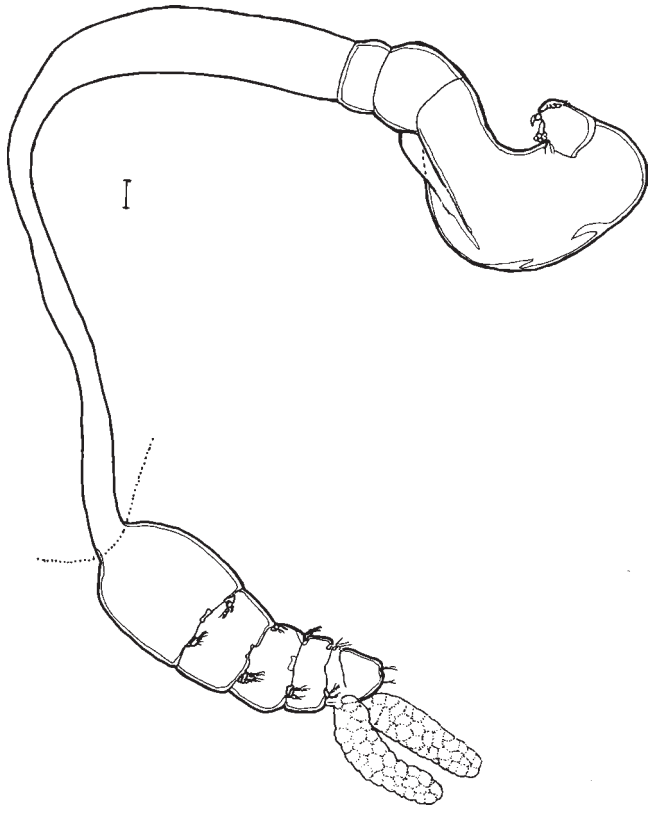


Fig. 14.2. *Therodamas tamarae* adult female, a long-necked ergasilid. Bar = 100 µm (after Amado and Rocha, 1996.)

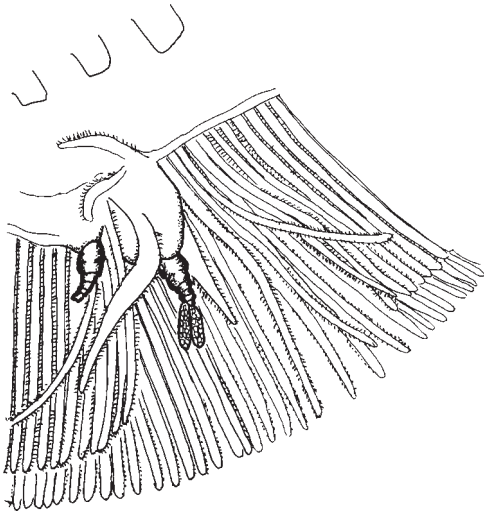


Fig. 14.3. *Plagioscion squamosissimus* gill containing two partly embedded *T. tamarae* (after Amado and Rocha, 1996).

Abdelhalim *et al.*, 1991; Alston *et al.*, 1996) consist of six naupliar, five copepodid and one adult stage. Sexual differentiation is evident in the fourth copepodid (Zmerzlaya, 1972; Abdelhalim *et al.*, 1991). It takes 22 days for the eggs of *E. sieboldi* to develop into free-living males and females at 17.7–20.1°C (Zmerzlaya, 1972). After fertilization, the female attaches to a fish. Zmerzlaya (1972) observed that overwintered females of *E. sieboldi* produced eggs in the spring, which meant that sperm was stored for several months.

Host–parasite relationships

Site and host selection, course of infection

E. sieboldi attaches near the base of the gill filament and prefers (in decreasing order) gill arches 3, 2, 4 and 1 (Abrosov and Bauer,

1959; Zmerzlaya, 1972). It generally infects lake-dwelling fish over 2 years old (Dogiel *et al.*, 1961). Females can leave a fish and transfer to another but ovigerous females apparently readily fall to the bottom (Zmerzlaya, 1972). Abrosov and Bauer (1959) kept whitefish in nets in different parts of a lake and found that those on the bottom and away from the shore became the most heavily infected.

E. lizae attach near the base of gill filaments of *M. cephalus* and prefer gill arches 2 and 3 (Rawson, 1977). *E. labracis* also attach near the base of the gill filament. Young females occur on gill arches 2 and 3 but most mature females are on gills 1 and 4 (Paperna and Zwerner, 1981). *Ergasilus anchoratus*, an abundant parasite on *Silurus asotus* in a Chinese reservoir, was distributed almost evenly over the gill arches without any observed gill arch preference (Nei, 2000).

Unlike most ergasilids, *N. japonicus* attaches primarily to the outside of the fish (Fig. 14.4), especially the fins, and is not found in the gill cavity (Harada, 1930; Hayden and Rogers, 1998). Again unlike most ergasilids, the parasitic female readily moves from host to host.

The numbers of *Ergasilus colomesus* increase with fish size (Thatcher and Boeger, 1983). Those of *E. centrarchidarum*, *E. lizae* and *Ergasilus auritus* decrease in large fish (Noble *et al.*, 1963; Joy, 1976; Cloutman and Becker, 1977); factors involved in this age immunity have still to be elucidated.

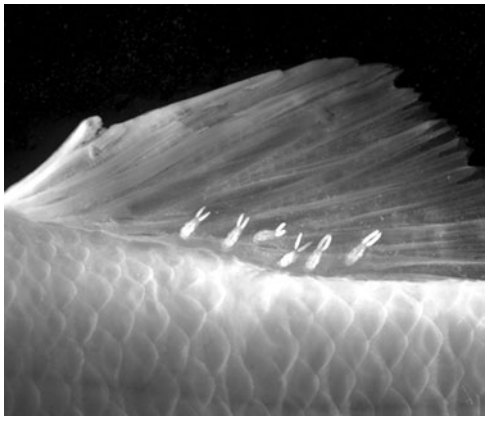


Fig. 14.4. *Pimephales promelas* dorsal fin with *Neoergasilus japonicus* females attached, an unusual site for an ergasilid (from Hudson and Bowen, 2002).

Clinical signs and histopathology

Mullets (*Liza macrolepis*) in a brackish-water pond in Taiwan died carrying 500 to 1000 *Diergasilus* sp. per fish (Lin and Ho, 1998). In Russia, fish such as *Tinca tinca* that are heavily infected with *E. sieboldi* die, especially in summer when water temperatures are high. There is extensive gill damage and severe haemorrhage, with inflammation and exsanguination associated with the attachment and feeding of the parasite. Blood vessels in the gill filaments are blocked and this leads to atrophy of gill tips (Dogiel *et al.*, 1961). In adjacent tissue, there are increased numbers of mucus cells, eosinophilic cells, rodlet cells and neutrophils (Dezfuli *et al.*, 2003). Similar histopathology is associated with *P. zacconis* (see Nakajima *et al.*, 1974). Infection of *A. brama* with *E. sieboldi* results in the disturbance of blood-cell maturation with lymphocytopenia and reactive granulocytosis in heterophil and basophil leucocytes (Einszporn-Orecka, 1973a,b). Rakova (see Dogiel *et al.*, 1961) reported a 20% drop in lymphocytes and an increase in monocytes, polymorphonuclear agranulocytes and neutrophils in *T. tinca* and *Leuciscus idus* infected with *E. sieboldi. M. cephalus* infected with several hundred *E. lizae* were emaciated (Paperna, 1975), and Atlantic salmon parr infected with *E. labracis* exhibited slow, weak swimming, gulping of air at the surface and darkening of the skin (Hogans, 1989). Paperna and Zwerner (1981) reported an increase in number of mucus cells, epithelial hyperplasia and infiltration of macrophages, lymphocytes and eosinophils in gill filaments of *Morone saxatilis* infected by *E. labracis.* Increase in number of mucus cells, fusion of lamellae and filaments due to epithelial proliferation, lymphocyte infiltration and exsanguination was reported by Rogers (1969) for *Ergasilus cyprinaceus.* A similar

effect was described by Thatcher and Boeger (1983) for *E. colomesus. Ergasilus manadensis* caused enlargement of the gill filaments, especially at the distal margin, necrotic patches and haemorrhages in a catfish (Seenappa and Venkateshappa, 2000). *Ergasilus australiensis* causes epithelial hyperplasia in the basal half of the gill filaments and pseudobranchs of *Acanthopagrus australis* (see Roubal, 1986b, 1989b).

Ergasilids that encircle a filament, such as the acusicolids *Ergasilus intermedius* and *Dermoergasilus acanthopagri*, produce a tourniquet effect, causing localized swelling, hyperplastic epithelium, which fuses adjacent lamellae, occlusion of the filament arterial vessels and necrosis of the tip of the filament (Roubal, 1987, 1989b; Thatcher, 1998; Ingram and Philbey, 1999).

Small ergasilids, such as *Paraergasilus acanthopagri*, attach to the nasal and opercular cavities using small spines, which cause localized epidermal proliferation (Roubal, 1989b).

In *Therodamas* spp., which burrow into the gill arch, adjacent gill filaments become stunted and deformed. The epidermis and dermis grow out, forming a fibrotic capsule from which the thorax protrudes (Amado and Rocha, 1996; Fig. 14.3).

Mechanism of disease

Histopathology associated with *E. sieboldi* is apparently caused by both attachment and feeding (Zmerzlaya, 1972), though most of the tissue proliferation associated with *E. labracis* and *Ergasilus mirabilis* was adjacent to the mouthparts and was not caused by attachment (Paperna and Zwerner, 1981; Oldewage and Van As, 1987).

Gill damage results in loss of gill surface area for respiration and leads to suffocation, particularly at high water temperatures (Grabda, 1991). Lopmann (1940) found that no more than 50% of gill surface area could be affected before the fish died. Ojha and Hughes (2001) estimated that one *Ergasilus begalensis* on the gill filament of the catfish *Wallego attu* caused a 30% reduction in laminar flow, resulting in a 68% reduction in oxygen uptake.

Effects on host

Heavy infections cause death. Sublethal infections result in decreased condition (Grabda, 1991; Schaperclaus *et al*., 1991). Infections of *T. tinca* and *Coregonus peled* with *E. sieboldi* were associated with reduced weight, reduced length, reduced condition, reduced fat and increased water content (Abrosov and Bauer, 1959; Dogiel *et al*., 1961; Kabata, 1970).

Concurrent infection

Concurrent infections are rare. No secondary bacterial infections were found in the gills of cyprinid fishes in Alabama infected with *E. cyprinaceus* (see Rogers, 1969). However, Reichenbach-Klinke and Landolt (1973) reported that wounds in pond-cultured *T. tinca* caused by *E. sieboldi* became secondarily infected with *Saprolegnia* sp.

Parasite nutrition and physiology

Halisch (1940) suggested that attached ergasilid copepods fed by external digestion and showed that female copepods confined in small volumes of water released factors that dissolved gelatin. The diet of *E. sieboldi* was shown by Einszporn (1964, 1965a,b) to consist of mucus, epithelium and blood. She proposed that blood was a major component of the diet. However, extrabuccal digestion is now considered a major part of feeding by ergasilids (Kabata, 1984). Donoghue (1989) found mucus to be the principal diet of *Thersitina gasterostei*.

Little is known about the nutrition and physiology of the planktonic free-living stages. They often contain bright pigments, particularly blue and also red and orange, which may help to protect tissues from photo-oxidation (Alston *et al*., 1996). The colours are lost from most species as the females mature.

Diagnosis

The parasites are readily seen with the naked eye or under a dissecting microscope.

Prevention and control

Though some ergasilid numbers apparently decrease as fish age, there is little evidence that resistance develops in infected fish; indeed, Donoghue (1986) found that *Pungitius pungitius* already infected by *T. gasterostei* were more likely to be reinfected by a second generation of the parasite than fish being exposed for the first time.

Fresh water and hyposaline sea water were ineffective against *E. lizae* and *E. intermedius* (Conroy and Conroy, 1986; Ingram and Philbey, 1999). Hogans (1989) used Neguvon (0.25 mg/l) to successfully treat *E. labracis* on Atlantic salmon parr. *Ergasilus ceylonensis* on the gills of the Asian cichlid *Etroplus suratensis* in Sri Lanka was treated successfully with 3–5 ppm potassium permanganate or 3–5 ppm 3,4-dichloropropioanilide but infected fish were unable to tolerate 50–500 ppm formalin, 5 ppm gammexane or 0.1–5.0 ppm fenitrothione (Wijeyaratne and Gunawardene, 1988). Ingram and Philbey (1999) used 2 mg/l Dipterex for 24 h to control *E. intermedius*. They found that 0.15 mg was ineffective, as also was 0.08 malachite green continuously.

Kabata (1985) suggested several treatments, including: (i) copper sulphate and ferric sulphate in a 5 : 2 ratio; (ii) Dipterex (Dylox, Chlorophor, Foschlor, Neguvon, Masoten) at 0.15 ppm for 6 h; (iii) Bromex (dimethyl-1,2-dibromo-2,2-dichloroethylphosphate) 0.125–0.15 ppm in water of greater than 400 ppm chlorinity (fish in fresh water are more sensitive, thus significantly reducing the safety margin); (iv) malathion (0,0-dimethyl-*S*-(1,2-dicarbethoxyethyl)-phosphorothioate) at 0.2 ppm for 6 h; and (v) the dyes brilliant green and violet K at 0.1–1 mg/l, which killed nauplii in 3–24 h. Schaperclaus *et al.* (1991) recommend a dip bath (10 min) in trichlorphon (5 mg/l) to free tench from *E. sieboldi*.

To control *Ergasilus celestis* in captive eels (*Anguilla rostrata*), Barker and Cone (2000) recommend a flow rate of more than 5 cm/s and a pH below 5.5.

Prophylactic measures against *E. sieboldi* suggested by Schaperclaus *et al.* (1991) include: strict quarantine to prevent the import of infected fish and water; net lakes to reduce levels of carrier fish such as *Abramis brama*; growth of aquatic vegetation to restrict open water; and replacement of tench by eel or common carp.

Copepoda: *Sarcotaces*

Introduction

These bizarre copepods cause cysts several centimetres long in the muscle under the skin (Fig. 14.5). The cysts are exposed when fish are filleted and cut cysts release copious black fluid over the fillets.

Sarcotaces verrucosus Olsson 1872, the type species, has a worldwide distribution, from abyssal depths to tropical reefs (Heegaard, 1947a; Kuitunen-Ekbaum, 1949; Moser *et al.*, 1985). Hosts include members of the families Moridae, Macrouridae, Scorpaenidae, Antennariidae, Triglidae, Acanthuridae, Serranidae and Pinguipedidae. Two other species, *Sarcotaces japonicus* and *Sarcotaces shiinoi*, were described from Japanese fish by Izawa (1974). The genus is usually included in the family Phylichthyidae; these are small copepods that parasitize the lateral line and other sensory canals of teleosts.

Morphology and life cycle

The female is up to 45 mm long in cold temperate waters, smaller in the tropics, the body broadly oval and the surface verrucose in bands corresponding to segments, with a double rosette around minute mouthparts and the tail pointed with a spine (Fig. 14.5). Most of the bulk of the body is formed from the gut, which typically contains a thick black fluid. The male is up to 3 mm, with 0 to 26 in a capsule with each female (Gonzalez and Tanzola, 2000). For further details see Lester and Roubal (1995).

Copepoda: *Lernaea*

Introduction

Lernaeids or anchor worms are common pests in freshwater aquaculture of cyprinids

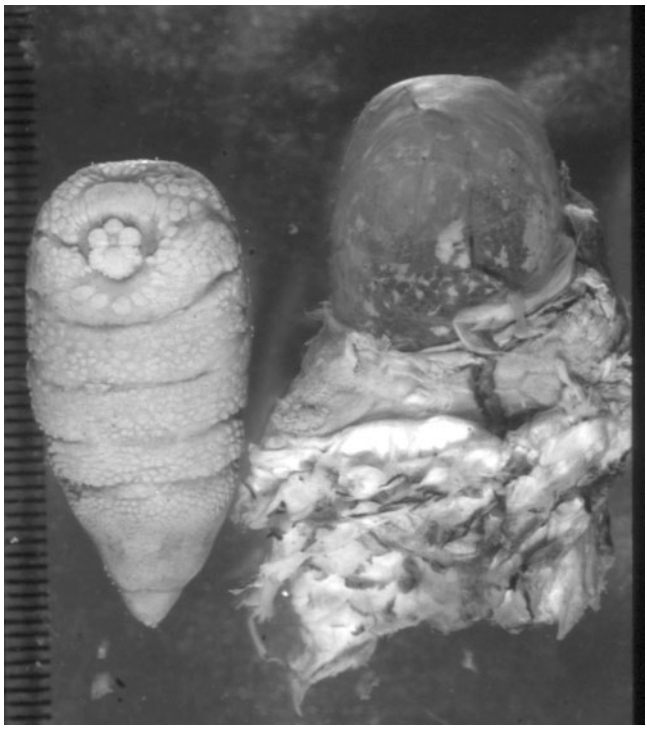


Fig. 14.5. *Sarcotaces verrucosus* female, ventral view, alongside host capsule (from *Mora moro,* Tasmania). Scale: units 1 mm.

and, to a lesser extent, of salmonids and other fish. Epizootics in cultured fish are often associated with high mortality. The parasites also cause problems in human-made lakes (Anon., 1980; Berry *et al.*, 1991) and in commercial aquaria (Shariff *et al.*, 1986; Dempster *et al.*, 1988). They are particularly pathogenic to small fish because of their relatively large size.

Host range, distribution, seasonality

Lernaeids are parasites of freshwater teleosts. *Lernaea cyprinacea* (Fig. 14.6A, B) has a worldwide distribution, typically on *Carassius carassius*, though it has been reported from 45 other species of cyprinids. It is also found on other Cypriniformes (Catostomidae, Cobitidae) and on fish belonging to the following orders: Acipenseriformes (Acipenseridae), Anguilliformes (Anguillidae), Channiformes (Channidae), Cyprinodontiformes (Cyprinodontidae, Poecillidae), Gadiformes (Gadidae), Gasterosteiformes (Gasterosteidae), Perciformes (Anabantidae, Apogonidae, Centrarchidae, Cichlidae, Gobiidae, Mastacembalidae, Osphronemidae, Percidae), Salmoniformes (Esocidae, Plecoglossidae, Salmonidae, Umbridae) and Siluriformes (Bagridae, Ictaluridae, Siluridae) (Kabata, 1979). In a tropical display aquarium in Malaysia, 23 out of 58 species of fish became infected (Shariff *et al.*, 1986). In North America, the parasite has also been reported on tadpoles (Tidd and Shields, 1963).

Distribution of the parasite is partly dictated by water temperature. Its optimum temperature is 26–28°C (Shields and Tidd, 1968). Thus, in temperate climates the parasite is most common in late summer. It is more prevalent in still or slowly flowing water than in fast-flowing streams (Hoffman, 1976; Bulow *et al.*, 1979) and less common during floods than in

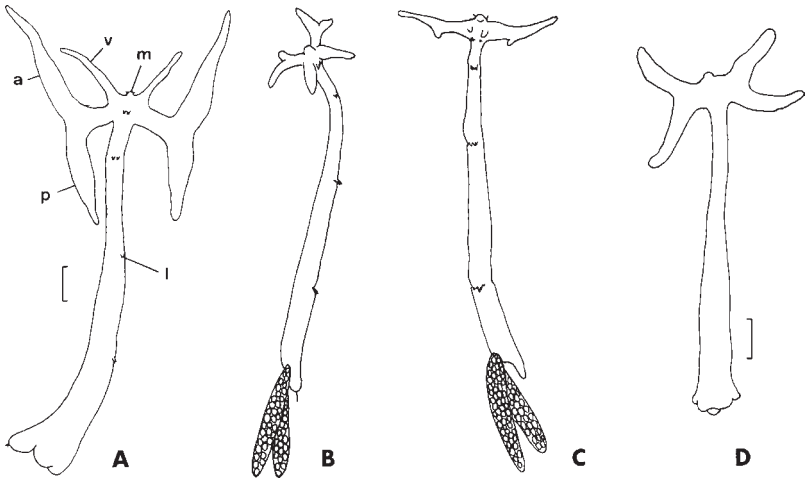


Fig. 14.6. *Lernaea* spp. morphology of female. A and B. *L. cyrinacea*. C. *L. polymorpha*. D. *L. cruciata*. a, anterior process of dorsal horn; l, 3rd pair of legs; m, mouth; p, posterior process of dorsal horn; v, ventral horn. Scale bars 1 mm. (A redrawn from Kabata, 1979; B and C redrawn from Shariff and Sommerville, 1986a; D redrawn from Kabata, 1988.)

droughts (Mederios and Maltchik, 1999). It has become widespread in Bulgarian carp farms since 1993 (Daskalov and Georgiev, 2001), presumably through human introductions.

In Arkansas, USA, heavy losses of channel catfish, *Ictalurus punctatus,* occurred on three farms that were also growing big-head carp (*Hypophthalmichthys nobilis*). By late summer, most of the catfish in affected ponds had died (Goodwin, 1999).

In South East Asia, big-head carp and silver carp (*Hypophthalmichthys molitrix*) carry *Lernaea polymorpha* (Fig. 14.6C; Shariff and Sommerville, 1986a). In India, *Lernaea bhadraensis* attacks *Catla catla* (Tamuli and Shanbhogue, 1996b).

Systematics

There are at least 110 species of lernaeids, all in fresh water. They belong to two subfamilies: the Lamprogleninae, which are confined to Africa and Asia, and the Lernaeinae, which are widespread (Ho, 1996). Kabata (1983) and Boxshall (2004) give keys to genera. Ho and Kim (1997) report ten species of lernaeid from fishes in Thailand. Species from North America are given by Hoffman (1998). New genera and species have recently been described from South America (Boxshall *et al.*, 1997; Thatcher and Williams, 1998).

In the genus *Lernaea*, there are over 40 species. Identification is based in part on the shape of the anchors, a difficult character, not only because of their inherent variability (compare Fig. 14.6A with Fig. 14.6B) but also because their shape is modified by bone or other resistance the anchors encounter during their development in the fish (Fryer, 1961; Thurston, 1969).

L. polymorpha (Fig. 14.6C), common in South East Asia, can be distinguished from *L. cyprinacea* by the T-bar shape of its anchor and the relatively short ventral horns, which grow to face each other (Shariff and Sommerville, 1990). A third South East Asian species, *Lernaea minuta*, can be distinguished from *L. cyprinacea* and *L. polymorpha* by body length and second copepodid stage (Kularatne *et al.*, 1994a).

Lernaea cruciata, a parasite of *Lepomis*, *Ambloplites*, *Micropterus* and *Morone* spp. in North America, lacks ventral horns (Kabata, 1988; Fig. 14.6D).

Morphology and life cycle

The adult female *Lernaea cyprinacea* has a small semispherical cephalothorax, which contains the mouth. Behind it is a well-developed holdfast, normally consisting of two bifurcate dorsal processes and two simple ventral processes (Fig. 14.6A). The elongate neck and trunk carry the four pairs of legs of the premetamorphosed female. The abdomen is short. *In situ*, the holdfast and part of the trunk are buried in the host while most of the trunk and the abdomen project into the water (Fig. 14.7).

L. cyprinacea has three free-living naupliar stages and five copepodid stages. The naupliar stages are non-feeding and moult to infective copepodids in about 4 days. Copepodids are usually on the gills and are relatively immobile, although they are not permanently attached. Copepodids finally moult to free-moving adult males or premetamorphosed females in 7+ days, depending on temperature (Shields, 1978; Shariff and Sommerville, 1986b). Adult males die within 24 h. Females are fertilized and either attack the same host or swim to another host, where they chew and bore their way into the host tissues as they metamorphose into adults. Within 1 day, before they have fully metamorphosed, they produce their first batch of eggs. These hatch 24 to 36 h later and the egg sacs are then shed. A new pair of egg sacs is produced within 1 to 3 days (Shields and Goode, 1978; Shariff and Sommerville, 1986b). The largest egg sacs are produced 5 to 10 days post-metamorphosis and the parasites die within 30 days at 28–32°C.

Development is greatly reduced at lower temperatures; at 20°C nauplii take 7 days to moult into copepodids and they cease development altogether below this temperature. Shields and Tidd (1968) found that some metamorphosed females survived at 10°C for over 3 months and grew slowly, though many were lost from the fish. Others were lost when the temperature was raised to 25°C, possibly because they had not burrowed deeply enough during the cold period. Of 71, only six survived to produce eggs. Nevertheless, in cold temperate climates, the parasites probably overwinter as metamorphosed females.

L. cyprinacea requires only one host to complete its life cycle, usually a cyprinid, though adult females have been found on many species of fish (see above) and copepodids also have relatively low host specificity. Copepodids have been found on the gills of other fishes, such as African catfish *Bagrus* spp., and even in the branchial

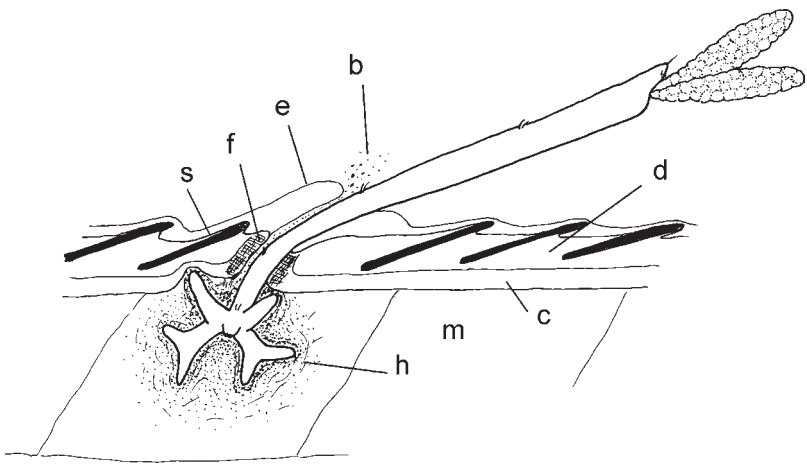


Fig. 14.7. *Lernaea cyprinacea*. Diagram of adult female *in situ*. b, blood seepage; c, stratum compactum of dermis; d, stratum spongiosum of dermis; e, epithelial collar; f, fibrous collar; h, area of muscle disorganization, fibrous granulation tissue and haemorrhage; m, muscle; s, scale.

chamber of tadpoles, *Rana* spp. (Fryer, 1966; Shields and Tidd, 1974). In a polyculture system in southern USA, channel catfish, *I. punctatus*, had eight to 50 copepodids on the surface of each gill filament and small numbers of adult females on the skin, whereas thousands of adult females were on the skin of cohabiting big-head carp *H. nobilis* (Goodwin, 1999).

L. polymorpha also requires only one host, *Aristichthys nobilis*, to complete its life cycle (Shariff and Roberts, 1989). Some lernaeids apparently use two. Copepodids of *Lernaea variabilis* are on short-nosed gars, *Lepisosteus platostomus*, whilst adult females occur on bluegills, *Lepomis pallidus* (see Wilson, 1917). In Africa, catfish, *Bagrus* spp., carry copepodids of both *Lernaea cyprinacea* and *Lernaea barnimiana* prior to the development of metamorphosed females on *Tilapia* sp. (Fryer, 1966; Thurston, 1969).

Host–parasite relationships

Site and host selection, course of infection

Larval lernaeids occur typically on the gills. Adult females usually attach on the body surface and lodge in the superficial layers of the body musculature. Those of *L. cyprinacea* attach all over the body of cyprinids in still water, whereas in moving water they are principally near the bases of fins. On rainbow trout, a third of them may attach to gills or the wall of buccal cavities (McNeil, 1961). On *Tilapia* sp. and *Anguilla anguilla*, they are found principally in the buccal cavity (Fryer, 1966; Ghittino, 1987).

Clinical signs and histopathology

Copepodids of *L. cyprinacea* on small cyprinids cause disruption and necrosis of the gill epithelium. Khalifa and Post (1976) reported that large numbers of larvae on the gills caused the death of fish. Fingerlings with more than six adult females became moribund (Daskalov *et al.*, 1999). In polyculture ponds, the grazing of large numbers of copepodids on the surface of each gill filament of channel catfish, *I. punctatus*, caused epithelial hyperplasia, telangiectasis, haemorrhage and death (Goodwin, 1999).

Penetration of metamorphosing females is generally associated with punctate haemorrhages, which increase to 5 mm across as the parasite grows (Khalifa and Post, 1976). Such lesions, both with and without parasites, are common in epizootics (Haley and Winn, 1959; Uehara *et al.*, 1984; Berry *et al.*, 1991).

Muscle necrosis is evident at the anterior end of penetrating parasites. Around the parasite's head is an area of acute inflammation, with many infiltrating leucocytes and sometimes giant cells (Daskalov *et al.*, 1999). Around its neck is a sheath of epithelium. Extravasated blood is frequently present between the parasite and the sheath. This may ooze into the water, together with cellular debris. Eventually a thick connective tissue sheath envelops the part of the parasite within the host and may extend from the fish to form a collar (Fig. 14.7; Khalifa and Post, 1976; Shields and Goode, 1978; Berry *et al.*, 1991). In *Catostomus commersoni* the collar contains large numbers of inflammatory cells, particularly neutrophils, eosinophilic blood granulocytes and lymphocyte-like cells (Lester and Daniels, 1976).

In amphibian tadpoles, the parasite caused more tissue destruction than normally observed in fish but stimulated less tissue response (Shields and Goode, 1978).

Daskalov *et al.* (1999) reported changes in the liver and kidneys of infected carp fingerlings, particularly cloudy swelling, granulation and vacuolation in the hepatocytes and, under the electron microscope, swollen mitochondria. Increased numbers of neutrophils and decreased numbers of lymphocytes were reported in the caudal vein of infected *Schizodon intermedius* from culture ponds by Silva-Souza *et al.* (2000).

L. cruciata in white bass (*Morone chrysops*) caused an initial mild necrosis, which initiated a mild oedema and infiltration of neutrophils around the anchor. This was followed by macrophage infiltration and phagocytosis of dead neutrophils and other cell debris. Fibroblasts then proliferated and eventually matured into fibrocytes,

with collagen deposition. They formed part of a chronic granuloma, which adhered tightly to the anchor processes and cephalothorax. Growing anchors were able to penetrate scales (Joy and Jones, 1973). Burrowing females in largemouth bass, *Micropterus salmoides*, were completely concealed beneath the scales, and caused local yellow-to-red discoloration. Host reaction was primarily a mononuclear infiltrate containing eosinophilic granular cells. This was mostly confined to the scale pocket containing the parasite (Noga, 1986).

Punctate haemorrhages appeared when female *L. polymorpha* began to penetrate bighead carp (*Aristichthys nobilis*). The transparent body of the parasite became milky and finally an ivory colour. Heavily infected fish became sluggish and died (Shariff and Roberts, 1989).

In naïve fish, penetrating females caused disruption of the epidermis and dermis, necrosis and haemorrhage. This was followed by acute inflammation and finally by a highly vascular chronic granulomatous fibrosis, whereby collagen fibres encapsulated the horns of the parasite. The penetration site was first sealed around the parasite by epidermal migration, then by inflammatory exudate and finally by a collar of fibrous tissue. In resistant fish, the breach in the epidermis was relatively small but there was extensive haemorrhage in the dermis. The thickened epidermis and oedematous dermis contained far more eosinophilic granule cells and lymphocytes than naïve fish, apparently the result of immune hypersensitivity (Shariff and Roberts, 1989).

L. cyprinacea in the eyes of trout caused blindness (Uzmann and Rayner, 1958). The holdfast in the anterior chamber of the eye of bighead carp caused extensive mechanical damage and a severe inflammatory response. The antlers were surrounded by bands of fibrous tissue, the cornea was opaque, the lens capsule ruptured and an inflammatory exudate of mononuclear cells and neutrophils filled the anterior chamber (Shariff, 1981).

In India, *C. catla* with heavy infections of *L. bhadraensis* were weak and emaciated and had a rough body surface. Haemorrhage, trauma and melanin deposition were evident at the attachment site. A thick fibrous capsule formed around parasites in some fish. In others, muscle was eroded down to the vertebral column and the fish became lethargic and died (Tamuli and Shanbhogue, 1996b).

Host immune response

Shields (1978) suspected that goldfish acquired immunity to *L. cyprinacea* because he was unable to reinfect fish that had once had a heavy infestation. Shields and Goode (1978) observed that half of the parasites on experimentally infected goldfish were rejected by the host 1 week after maturation of the first egg sacs. Wounds left by rejected females healed rapidly.

Shariff *et al.* (1986) found that, after an epizootic of *L. cyprinacea* in a display aquarium, 18 out of 23 fish species had apparently acquired resistance by the time of a second outbreak 6 months later. Few new infections developed in experimentally infected *Helostoma temmincki* that had recovered from an earlier infection. Fish that became infected lost their infections rapidly. Parasites on recovered fish produced fewer eggs and the resultant larvae were less infective than those from females on naïve fish (Woo and Shariff, 1990).

A similar phenomenon occurs in some other lernaeids. In an experimental infection of *L. polymorpha* on bighead carp, the prevalence first increased and then declined to zero after 4 months (Shariff and Sommerville, 1986c). Degenerating females were found in haemorrhagic ulcers of resistant fish (Shariff and Roberts, 1989). Fingerlings of Indian carp (*C. catla*) from which *L. bhadraensis* had been removed were re-exposed to the parasites and examined 30 and 45 days later. Fish that had had heavy infections (20+ per fish) had few new infections, whereas those that had been lightly infected (three per fish) were heavily parasitized (Tamuli and Shanbhogue, 1996c).

No immunity was acquired by Javanese carp, *Puntius gonionotus*, against subsequent infection by *L. minuta*, possibly because the parasite does not penetrate far

into the tissue and stimulates little or no inflammatory reaction (Kularatne *et al.*, 1994b).

Noga (1986) suggested that haemorrhagic ulcers in largemouth bass, *M. salmoides,* which contained degenerating *L. cruciata,* were the result of immune hypersensitivity.

Mechanism of disease

The mechanical destruction of epidermis and dermis caused by grazing copepodids stimulates inflammation (Shields and Tidd, 1974). When the parasites are abundant on the gills, this presumably precipitates respiratory distress through oedema, poor gas transfer and slowed blood circulation in the lamellae.

Host mortality caused by adult females is generally the result of the physical destruction of tissues. The parasites also induce pressure necrosis, and may secrete a histolytic and/or digestive enzyme (Shields and Goode, 1978; Shariff and Roberts, 1989). Small fish (4–6 cm) and tadpoles suffer more than large fish because the head of the parasite often enters the body cavity and penetrates the liver, or penetrates the brain, spinal cord or other vital organ. Some tadpoles apparently died from blood loss through haemorrhages caused by the penetration of metamorphosing females (Tidd and Shields, 1963).

Effects on host

L. cyprinacea hindered the feeding and growth of eels (*Anguilla anguilla*) in Italy (Ghittino, 1987). In Egypt, six or more parasites per fingerling slowed the growth of cultured *Cyprinus carpio* and *Ctenopharyngodon idella* (Faisal *et al.*, 1988). During an outbreak in a Malaysian aquarium, 7–9% of fish died (Shariff *et al.*, 1986). Mortality of small cyprinids, salmonids and tadpoles has frequently been associated with infection by this parasite (Tidd and Shields, 1963; Khalifa and Post, 1976; Anon, 1980).

The fins of bighead carp (*A. nobilis*) were continually moving once nauplii of *L. polymorpha* were added to the tank, suggesting that the parasites were attaching to and irritating them (Shariff and Roberts, 1989). In infested ponds, the carp were up to 35% lighter than carp in uninfested ponds (Shariff and Sommerville, 1986c).

Concurrent infection and cross immunity

Khalifa and Post (1976) observed unspecified bacterial and fungal infections in the wounds caused by the penetration of *L. cyprinacea* into cyprinids. Noga (1986) investigated early 'red-sore' lesions on the skin of largemouth bass (*M. salmoides*) and found that at least 35% of them contained the remains of *L. cruciata.* The parasites were almost all metamorphosing females. Bacteria, particularly *Aeromonas hydrophila*, were present in some of the early lesions and bacteria of several species predominated in more advanced lesions. He suggested that the wounds caused by the copepod allowed in bacteria and fungi and thus initiated red-sore disease.

Wilson (1917) observed that the gills of *Lepisosteus platostomus* carrying copepodids of *Lernaea variabilis* frequently did not have glochidia, and vice versa.

***In vitro* culture**

Egg sacs, either separate or still attached to excised females, develop normally in dishes in non-aerated water. Nauplii hatch and moult three times to the infective first copepodid in the dish (Grabda, 1963; Shields, 1978).

Parasite nutrition and physiology

Naupliar stages do not feed. Copepodids apparently graze on epidermis and dermis (Shields and Tidd, 1974). Metamorphosed females ingest tissue debris and erythrocytes (Khalifa and Post, 1976) released by mechanical damage from their mouthparts and possibly from the secretion of digestive enzymes (Shariff and Roberts, 1989).

The osmolarity of the haemolymph of attached metamorphosed females is similar

to that of the host, though attached parasites died within 24 h in 90% sea water. Attached females in 15% or 30% sea water survived for at least 6 days but failed to produce viable eggs. They resumed normal reproduction within 48 h of return to fresh water. In 10% sea water the parasite successfully completed its life cycle. Excised parasites showed little ability to osmoregulate (Shields and Sperber, 1974).

Diagnosis of infection

Adult females can be seen macroscopically; copepodids require the use of a dissecting microscope.

Prevention and control

Elimination of the parasite usually requires treatment over several weeks to break the life cycle at the larval stage, because embedded females are difficult to kill.

Organophosphate insecticides, particularly trichlorphon (Dipterex, Neguvon, Masoten), are commonly employed. As copepodids take 8–9 days to develop (at 27°C), treatment is carried out every 7 days to prevent reinfection until all the adult females have died (within 30 days at 27°C). Trichlorphon at 0.25 ppm will kill the copepodid stage but not the nauplii or adults (Sarig, 1971; Kabata, 1985). It is less effective at high temperatures. Bromex (dimethyl-1,2-bromo-2,2-dichlorethylphosphate) at 0.12–0.15 ppm kills both nauplii and copepodids (Sarig, 1971). Manal *et al.* (1995) successfully eradicated lernaeids from a farm using 0.01–0.02% malathion in three applications at 10-day intervals. The use of organophosphates creates problems with residues. Ghittino (1987), who used trichlorphon (0.25–0.3 ppm) in 3 to 6 weekly treatments for infested eels, discontinued treatment for at least 1 month before the eels were harvested.

Shariff *et al.* (1986) successfully treated infested fishes with 0.16 ppm Unden (2-isopropoxyphenyl-*N*-methylcarbamate) at weekly intervals for 4 weeks and then a final treatment of 0.16 ppm trichlorphon (Dipterex) in week 5. Nauplii were not affected by either drug. Unden initially immobilized copepodids within 2 h and killed them, but by the fourth treatment half the copepodids were still alive. Both drugs caused fish to cease feeding.

A safer non-residual chemical that has been used effectively is sodium chlorite (Dempster *et al.*, 1988). When the chlorite ion ClO_2^- was maintained at a concentration of 20–40 mg/l and the pH above 6, the chlorite eradicated *L. cyprinacea* from a commercial aquarium and was non-toxic to fish. As a side effect, it killed bacteria in the biological filters so water had to be exchanged during the first 2 weeks to keep the ammonia and nitrite levels down until chlorite-resistant strains of bacteria developed and the filters again became biologically active.

Hoffman and Lester (1987) reported that Dimilin (UniRoyal Chemical Co., USA), a growth regulator, at 0.03 ppm eradicated *L. cyprinacea* from golden shiner (*Notemigonus crysoleucas*). Other chemicals that have been used in the past are in Hoffman and Meyer (1974) and Hoffman (1976). Kabata (1985) gives details of herbal remedies, such as tea-seed cake, that have been used in China.

For small numbers of fish, adult female parasites can be removed by hand, larval stages killed chemically and the fish returned to clean water. Scott and Fogle (1983) bathed adult infested carp (*C. carpio*) for 5 min in 1% trichlorphon (Dipterex; 10 g active ingredient per litre), anaesthetized the fish, removed visible copepods and then put the fish in a clean tank. Thereafter the water was treated with 0.2–0.25 ppm trichlorphon monthly. Faisal *et al.* (1988) eradicated *L. cyprinacea* from carp brood stock by mechanically removing metamorphosed females and placing the fish in potassium permanganate (25 g/m^3) for 30 min to kill other stages. Vulpe *et al.* (2000) found that 1 g/l potassium permanganate for 30s killed most adult females. Tamuli and Shanbhogue (1996a) used 30 ppm potassium permanganate for 20 min twice per day to kill adult and juvenile

L. bhadraensis, though the infected fish (*C. catla*) became distressed, the parasite's eggs remained viable and free-living stages revived. They deparasitized individual fish by clipping off females or touching them with a fine brush dipped in concentrated potassium permanaganate (Tamuli and Shanbhogue, 1996c).

To avoid environmental, tissue residue and other problems associated with chemical treatments, several biological methods have been proposed. Shields (1978) observed that goldfish removed maturing parasites from each other. Tamuli and Shanbhogue (1995) found that tilapia, *Oreochromis mossambicus* reduced the prevalence of *Lernaea* on *C. catla*, whereas in the control tanks prevalence increased and high mortality occurred. Woo and Shariff (1990) suggested that no naïve fish be introduced (e.g. into a pond) for a period as the resistance that recovered fish acquire may be sufficient to break the life cycle.

To reduce the numbers of copepodids, Shields (1978) recommended increasing the rate of water exchange. Shariff and Sommerville (1986b) proposed that all fish should be removed from the pond for a minimum of 7 days (at 25–29°C) as all the nauplii and first copepodids (infective copepodids) will have died by the 7th day if no host is available. Kabata (1985) reported that the copepod *Mesocyclops* preyed on free-swimming larval *Lernaea* and suggested that planktonic predators could be used in biological control.

Copepoda: Caligidae

Introduction

Sea lice (family Caligidae) are among the most notorious pests affecting cultured marine fishes. The best-known representatives of this group are those infesting caged salmonids. Sea lice infestations in salmon farms in Norway and Scotland are estimated to have cost over 70 million ecu in 1997 (Costello and Boxshall, 2000). In 1995 in Canada, they are estimated to have cost CAN$20 million (Pike and Wadsworth, 1999). As evidence of their importance, there is a series of international conferences on sea lice, the proceedings of which have been published in special issues of international journals (*Pest Management Science* 58 (6) 2002 and *Aquaculture Research* 35 (8) 2004), and a conference on the use of cleaner fishes to combat sea lice has been held. In addition, an email newsgroup (Caligus@listserve.heanet.i.e) and an industry newsletter ('Caligus', available on the internet) have been established. A comprehensive review of the extensive literature on sea lice affecting salmonids is by Pike and Wadsworth (1999).

Atlantic salmon (*Salmo salar)* in the northern hemisphere are often infested with two species of sea lice: *Lepcophtheirus salmonis* (Fig. 14.8) and *Caligus elongatus* (Wootten *et al.*, 1982; Pike, 1989). Infestations cause erosion of skin, most often on or near the head. Heavily infected salmon die. Smolts newly introduced to sea water are the most susceptible (Wootten *et al.*, 1982). The first outbreaks of *L. salmonis* occurred in the 1960s within a few years of the first salmon farms in Norway. Outbreaks were recorded in Scotland a decade later (Wootten *et al.*, 1982). *L. salmonis* also causes lesions to pen-cultured salmonids in Japan (chum salmon, *Oncorhynchus keta*, pink salmon, *Oncorhynchus gorbuscha*, and masu salmon, *Oncorhynchus masou*) (Urawa, 1998; Nagasawa, 2004). Infestations of a second species of caligid in Japan, *Caligus orientalis*, have been known to kill pen-cultured rainbow trout (Urawa and Kato, 1991). In the southern hemisphere, caged coho salmon (*Oncorhynchus kisutch*) and rainbow trout in Chile were infected from the early 1980s with *Caligus teres*; from 1997, Atlantic salmon in Chile began to be plagued by another species, *Caligus rogercresseyi* (see Bravo, 2003). These problems in Chile present a sharp contrast with other parts of the southern hemisphere where salmonids are reared, such as Australia and New Zealand, where there are no reports of caligids causing serious problems (despite the reported presence of *C. elongatus*).

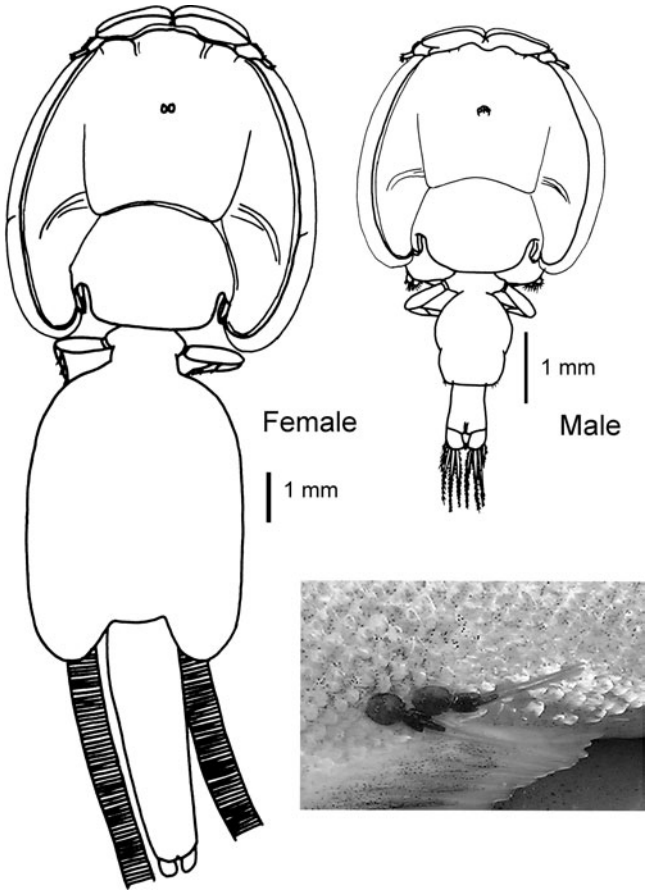


Fig. 14.8. Adult salmon lice, *Lepeophtheirus salmonis* (redrawn from Kim, 1998; photo C. Orr).

The cage culture of non-salmonids is also developing rapidly around the world, and sea lice are becoming increasingly associated with mortality and disease in these fishes, too. Ho (2000) listed nine caligids that have attracted attention in Asia (Table 14.1). Ho (2000) concluded that three species are potential major pathogens of cage aquaculture in Asia: *Caligus epidemicus* (Fig. 14.9), *C. orientalis* and *Caligus punctatus*. In Israel, *Pseudocaligus apodus* killed cultured mullet (Paperna and Lahav, 1974). In Australia, *C. elongatus* infects southern bluefin tuna (*Thunnus maccoyii*) after the tuna have been captured and transferred to sea cages (Rough, 2000). The copepod erodes epithelium and consequent damage to the eye leads to corneal ulceration and secondary bacterial infection. Cataracts and blindness interfere with feeding and result in further loss of condition.

Sea lice common on wild fishes occasionally cause disease. *C. epidemicus* (Fig. 14.9) killed a range of wild fishes in southern Australia in the late 1960s (Hewitt, 1971). This sea louse might have been translocated to southern Australia in ballast water from north-east Asia shortly before this outbreak, because the species was previously unknown in southern Australia and other organisms from north-east Asia that became established in southern Australia in the late 1950s and the 1960s were apparently introduced in ballast water.

Table 14.1. Caligids reported to have caused mortalities among non-salmonid fishes in Asia (from Ho, 2000).

Caligid species	Country	Hosts	Source
Caligus acanthopagri	Taiwan	*Acanthopagrus schlegeli* (black sea bream/porgy); *Epinephelus malabaricus* (Malabar rock cod); *Oreochromis mossambicus* (Mozambique tilapia); *Scatophagus argus* (common spade fish)	Lin *et al.*, 1994; Lin, 1996, cited by Ho, 2000
Caligus epidemicus	Taiwan	*A. schlegeli*; *Chanos chanos* (milkfish); *E. malabaricus*; *Lates calcarifer* (giant perch); *Liza macrolepis* (large scale mullet); *Mugil cephalus* (grey mullet); *O. mossambicus*; *Oreochromis aurea* (blue tilapia); *S. argus*; *Terapon jarbua* (three-striped tigerfish); *Trachinotus blochii* (snubnose pompano)	Lin and Ho, 1993; Lin, 1996, cited by Ho, 2000
Caligus orientalis	China and Taiwan	*A. schlegeli*; *C. chanos*; *E. malabaricus*; *L. calcarifer*; *L. macrolepis*; *M. cephalus*; *O. mossambicus*	Hwa, 1965; Lin, 1996, cited by Ho, 2000
Caligus patulus	Philippines	*C. chanos*	Lavina, 1977; Jones, 1980
Caligus punctatus	Taiwan	*A. schlegeli*; *C. chanos*; *E. malabaricus*; *Lateolabrax japonicus* (Japanese sea bass); *L. calcarifer*; *L. macrolepis*; *M. cephalus*; *O. mossambicus*; *O. aurea*; *T. jarbua*; *T. blochii*	Lin, 1996, cited by Ho, 2000
Caligus rotundigenitalis	Taiwan	*A. schlegeli*; *E. malabaricus*; *S. argus*	Lin *et al.*, 1994; Lin, 1996, cited by Ho, 2000
Caligus spinosus	Japan	*Seriola quinqueradiata* (yellowtail)	Fujita *et al.*, 1968
Lepeophtheirus longiventris	Japan	*Verasper variegatus* (spotted halibut)	K. Ogawa, personal communication, cited by Ho, 2000
Lepeophtherius paralichthydis	Japan	*Paralichthys olivaceus* (olive flounder)	K. Ogawa, personal communication, cited by Ho, 2000

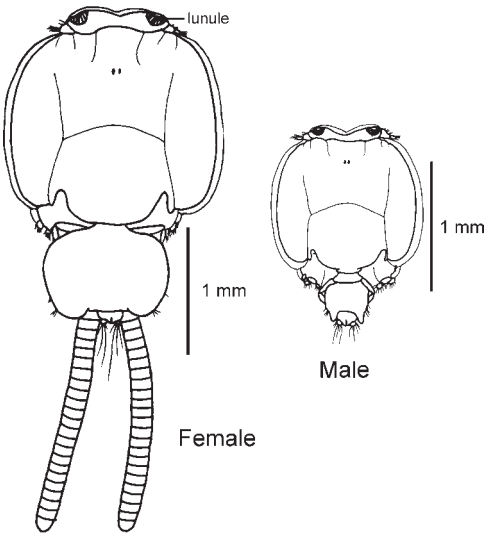


Fig. 14.9. Adult *Caligus epidemicus*. Note the sexual dimorphism, with the large genital segments in the female.

Host range of the parasite and seasonality

Most sea lice occur in tropical and warm temperate waters. *L. salmonis* (Fig. 14.8) is an exception in having a northern circumpolar distribution in cold temperate waters. Unlike other species of sea lice, it is largely restricted to the Salmonidae, especially the genera *Salmo*, *Salvelinus* and *Oncorhynchus* (see Kabata, 1979; Egidius, 1985). Other species of sea lice on salmonids have broad host ranges that include both non-salmonid teleosts and elasmobranch hosts (Johnson, 1998, and references therein). The prevalence and intensity of infection of *L. salmonis* on wild fish is occasionally high. White (1940) found grilse of *S. salar* entering the estuary of the Moser River, Nova Scotia, to be heavily infected and exhibiting severe lesions. Berland (1993) found the prevalence and intensity of *L. salmonis* and *C. elongatus* on wild salmon in west Norway to be low in 1973 and 1988, but high in 1992. *L. salmonis* is absent from sites with lowered salinity. Farms in Norway are in sites with a consistently higher salinity than in Scotland and have more severe *L. salmonis* infections (Pike, 1989). Salmon farms in the Bay of Fundy, Canada, are more adversely affected by *C. elongatus* than by *L. salmonis* (Hogans and Trudeau, 1989a,b). In the north Pacific, Nagasawa *et al*. (1993) found that 78% of *L. salmonis* were on pink salmon (*O. gorbuscha*), 15% on chinook salmon (*Oncorhynchus tschawytscha*) and the remainder on steelhead trout (*Oncorhynchus mykiss*), coho (*O. kisutch*), chum (*O. keta*) and sockeye (*Oncorhynchus nerka*) salmon.

Gravid females and other stages of *L. salmonis* occur year-round on salmon farms in northern Europe, with a succession of generations during the year (Wootten *et al.*, 1982). The greatest numbers of *L. salmonis* occurred in late summer and autumn, due to an accumulation of the parasite from successive generations. However, Tully (1989) found that the total intensity of infection did not rise cumulatively because mature lice disappeared from fish before maturation of the next generation was complete. Although reproduction is temperature-dependent in coldwater sites (Wootten *et al.*, 1982), there was little reproduction during the summer in Ireland until the onset of winter because high summer water temperatures adversely affected the parasite (Tully, 1989). Adult females from winter generations are significantly larger and produce larger egg sacs and more but smaller eggs than in summer (Ritchie *et al.*, 1993).

Lepeophtheirus pectoralis infects pleuronectids such as plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*) and dab (*Limanda limanda*). It is found in the north-east Atlantic Ocean, the western Baltic Sea and the White Sea (Boxshall, 1974b). Zeddam *et al*. (1988) have shown *Lepeophtheirus thompsoni* to infect only turbot (*Psetta maxima*) whereas *Lepeophtheirus europaensis* infected brill (*Scophthalmus rhombus*) and flounder (*P. flesus*). Meeüs *et al*. (1990) infected turbot with both *L. thompsoni* and *L. europaensis* and hybridized the two species of parasite experimentally. They attributed natural reproductive isolation to host specificity of infective stages and the low probability of heterospecific mating.

L. pectoralis has its greatest prevalence (> 95%) and intensity (five per fish) of

infection on flatfish in the summer (August–September) (Boxshall, 1974a). There is bimodal egg production in May and August, the first peak resulting from gravid females that have overwintered (parasite longevity of 10 months) but die in the spring after producing two to three pairs of egg strings. The resulting larvae produce a summer generation, which develops rapidly (but is shorter-lived than the winter generation), with a peak of egg production in August (Boxshall, 1974a).

C. elongatus is a cosmopolitan species and has been found on over 80 species of fishes in 17 orders and 43 families (including salmonids, pleuronectids, scombrids, clupeids, gadids and elasmobranchs). It is the most common species of parasitic copepod in British waters (Parker, 1969; Boxshall, 1974b; Kabata, 1979), being rare on wild salmon but more common on wild sea trout (*Salmo trutta*) (Wootten *et al.*, 1982) and has been recorded on cultured brook trout (*Salvelinus fontinalis*) and rainbow trout in eastern Canada (Hogans and Trudeau, 1989b).

In Scotland, *C. elongatus* infects sea-caged salmon year-round, with the greatest number in autumn, but with an apparent lack of well-marked successive generations (Wootten *et al.*, 1982). Although large invasions by larvae do occur, relatively few copepods seem to mature; the parasite may die or adult parasites may enter the plankton (Wootten *et al.*, 1982). Possible reservoirs of infection in wild fish include saithe (*Pollachius virens*) and herring (*Clupea harengus*) (Wootten *et al.*, 1982; MacKenzie and Morrison, 1989; Bruno and Stone, 1990).

The abundance of *C. elongatus* on cultured Atlantic salmon in the lower Bay of Fundy, Canada, is highest in late summer and autumn (average 17 per fish) and lowest in winter (one to three per fish) (Hogans and Trudeau, 1989b). Market-sized Atlantic salmon and smolt have similar numbers of parasites because the annual cycle of infection prevents accumulation in older fish (Hogans and Trudeau, 1989b). The generation time in laboratory culture of *C. elongatus* is 5 weeks at 10°C. This allows four to eight annual generations in the water temperature range of 5–14°C in the Bay of Fundy (Hogans and Trudeau, 1989a). The parasite leaves the host at temperatures lower than 6°C (Stuart, 1990). *Caligus curtus* was found rarely on these salmon, and was found usually on gadoid fish such as cod, haddock and pollack (Hogans and Trudeau, 1989b). *Caligus clemensi* is the only species in marine waters of British Columbia where it infects a wide range of clupeiform, perciform, gasterosteiform and gadiform fish in surface waters (Parker and Margolis, 1964).

C. epidemicus (Fig. 14.9) infects four species of marine bream, *A. australis*, *Acanthopagrus berda*, *Acanthopagrus butcheri* and *Acanthopagrus latus*, from around Australia (Byrnes and Rohde, 1992), as well as many other species of fishes. The parasite was abundant on *A. australis* in an estuary in northern New South Wales during one year (2.8 per fish) but not the next (0.5 per fish), although salinity and temperature were similar in both years (Roubal, 1990). An epizootic of *C. epidemicus* within the lower Mitchell River, Victoria, Australia, caused the death of bream (*A. butcheri*) and mullet (*Mugil cephalus*, *Aldrichetta forsteri*, *Liza argentea* and *Myxus elongatus*); the epizootic was associated with high salinity, up to 28 ppt, and temperature up to 21.8°C during a severe drought (Hewitt, 1971). *C. epidemicus* has also been recorded on the telson and legs of the prawn, *Penaeus monodon* (see Ruangpan and Kabata, 1984).

An epizootic of *Caligus pageti* on a fish farm in Egypt was associated with a high salinity of up to 45 ppt (Hewitt, 1971). *C. orientalis*, a parasite of cultured rainbow trout in Japan, is a euryhaline species that infects a wide range of other hosts, including *Hucho perryi*, *Tribolodon hakonensis*, *Hypomesus transpacificus* and *M. cephalus* (Urawa and Kato, 1991).

Systematics and taxonomic position

The family Caligidae currently contains 445 species in 33 genera; more than three-quarters of these species are members of the genera *Caligus* (239 species) and *Lepeophtheirus* (107 species) (Ho, 2000). The general body shape and relative size of

body tagma are the primary features used in identification, although setation of appendices is also helpful. A key to genera is given by Boxshall (2004). Specific keys are provided by Pillai (1885) for India, Kabata (1988) for Canada, Kabata (2003) for the UK and Ho and Lin (2004) for Taiwan.

Parasite morphology and life cycle

Adult caligids usually show sexual dimorphism; the female is usually larger than the male, and male appendages, particularly the first maxillae and second antennae, are modified to aid attachment during copulation. Adults of *L. salmonis* have the large, rounded, flat cephalothorax characteristic of most caligids. The female, 10–18 mm long, has a more prominent genital segment than the male (5–7 mm long) (Kabata, 1979; Fig. 14.10). Paired egg strings, up to 2 cm long and bearing a total of 700 eggs (197–1220; see Wootten *et al.*, 1982; Costello, 1993), are produced by the female from the posterior end of the genital segment. The limbs are similar in both sexes except the male has a striated ventral surface on the second antenna to enhance attachment to the posterodorsal surface of the female during mating. Males attempt to mate with adult females and with the two pre-adult stages (Wootten *et al.*, 1982), a phenomenon also reported for *Lepeophtheirus polyprioni* by Hewitt (1964). In experimental studies with *L. pectoralis*, Anstenrud (1990d) found that spermatophore transfer was successful only between males and adult females, not between males and pre-adult females.

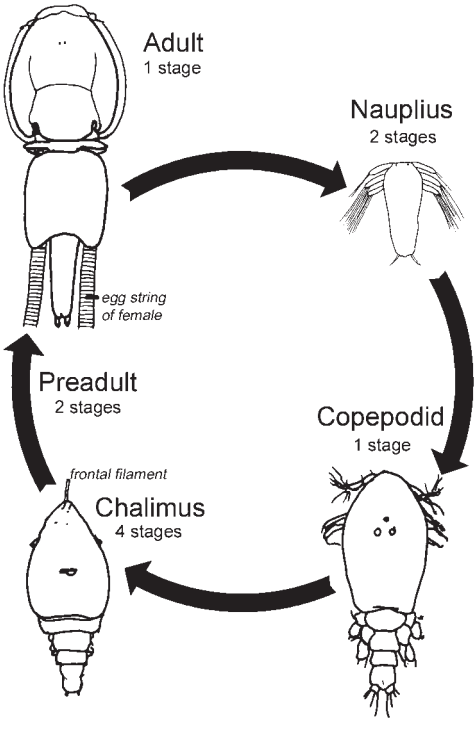


Fig. 14.10. Life cycle of the salmon louse *Lepeophtheirus salmonis* (redrawn from Johnson, 1998).

Caligus spp. have frontal lunules, which are absent in *Lepeophtheirus* spp. Frontal lunules are evident in the late chalimus stages of *Caligus*. Young stages of the parasites are difficult to identify, especially in mixed infections, though the chalimus stages of *C. elongatus* have a long frontal filament and paired eyespots, whereas *L. salmonis* has a short frontal filament and a single eyespot (Wootten *et al.*, 1982).

Adult females of *C. elongatus* are 6–8 mm long; males have a slimmer genital segment and are about 5 mm long. The parasite is golden-brown or yellow (Hogans and Trudeau, 1989b). Two egg sacs each contain about 30 eggs (MacKinnon, 1992).

The internal anatomy of the Caliginae, especially the reproductive system, has been given by Wilson (1905a) and Gnanamuthu (1950). The ultrastructure of sensory structures was described by Bron *et al.* (1993) and Gresty *et al.* (1993) and the frontal filament by Pike *et al.* (1993). Unlike in other copepods, the midgut is not divided into different zones (Nylund *et al.*, 1992). Lewis (1969) discussed the homology of the maxillae, and subsequent studies on the musculature of this and other appendages in *L. pectoralis* were given by Boxshall (1990). Locomotion of adult caligids was described by Kabata and Hewitt (1971).

Kabata (1972) proposed that the life cycle of caligid copepods consists of five phases and ten stages. The life cycle of

L. salmonis has the typical caligid complement of two free-living naupliar stages (N1 and N2), an infective copepodid stage (C), four attached chalimus stages (Ch1–Ch4), two free-living, pre-adult stages (PA1, PA2) and one adult (A) stage (Johnson and Albright, 1991a, b; Fig. 14.10). The natural lifespan of adult *L. salmonis* has not been determined (Pike and Wadsworth, 1999). Descriptions of the whole or part of the life cycle of *Lepeophtheirus* spp. can be found in White (1942), Lewis (1963), Boxshall (1974a), Johannessen (1978) and Schram (1993). Studies of varying completeness on the life cycles of *Caligus* spp. include Wilson (1905b), Heegaard (1947b), Hwa (1965), Izawa (1969), Hewitt (1971) and Jones (1980). However, nine stages (two N, one C, four Ch, one PA, one A) have been recorded for most *Caligus* spp., with 11 stages (six Ch) for *C. epidemicus* (see Lin and Ho, 1993) and eight (four Ch, no PA) for *C. punctatus* (see Kim, 1993) and *C. rogercresseyi* (see González and Carvajal, 2003).

Temperature and duration of development stages

The timing of the different stages of development is directly dependent on water temperature. The generation time for *L. salmonis* is 8–9 weeks at 6°C, 6 weeks at 9–12°C and 4 weeks at 18°C (Wootten *et al.*, 1982; Stuart, 1990). Up to four generations can occur between May and October in Scotland with a summer water temperature of 9–14°C (Wootten *et al.*, 1977, 1982). Tully (1989) found a generation time (ovigerous female to ovigerous female) of 56 days at 13.6°C (males took 52 days) in an experimental cage in Ireland; Johnson and Albright (1991a) reported a generation time of 7.5–8 weeks (at 10°C) in the laboratory. Duration of egg development varies from 17.5 days at 5°C to 5.5 days at 15°C, with durations at these respective temperatures of 52 h and 9.2 h for the N1 stage and 170 h and 35.6 h for the N2 stage. Durations for the copepodid (up to 10 days), Ch1 (5 days), Ch2 (5 days), Ch3 (9 days), Ch4 (6 days), PA1 (10 days) and PA2 (12 days) have also been recorded (Johnson and Albright, 1991b).

Acclimatization of ovigerous females to 11.5°C promoted more successful hatching at 22°C than did acclimatization at a lower temperature (Johannessen, 1978). Tully (1989) found that low water temperature promoted large body size, long developmental time and greater fecundity.

In *L. europaensis* the period from fertilized egg to first egg laying was 44 days (15°C), with a maximum lifespan of 135 days (Meeüs *et al.*, 1993).

In *C. elongatus*, the N1 and N2 stages last 15–30 h and 35 h, respectively, at 10°C, with no moults below 3°C, and the copepodid stage lasts 50 h at 13°C (Hogans and Trudeau, 1989b). The life cycle of *C. rogercresseyi* in Chile took 45 days at 10.3°C (July), 31–32 days at 12.4 and 12.8°C, respectively (April), and 26 days at 15.2°C (November) (González and Carvajal, 2003). The minimum temperature threshold, where there is no development of the parasite, was 4.2°C. In warmwater species, the developmental cycle of *C. epidemicus* from hatching to ovigerous female was 17 days at 24.5°C and 10–11 days for *C. pageti* (see Lin and Ho, 1993). Durations of developmental stages of *C. epidemicus* are N1 6 h, N2 14.5 h, C up to 3–4 days, Ch2–Ch6 each approximately 1 day (Lin and Ho, 1993).

Host–parasite relationships

Site/host selection and course of infection

Lepcophtheirus salmonis nauplii and copepodids are positively phototactic and exhibit a daily vertical migration, rising from the deeper waters to the surface during the day and sinking at night. Heuch *et al.* (1995) conclude that 'crossing over', as copepodids migrate upwards and salmon move downwards during daylight, allows transmission to take place. Nauplii and copepods also swim upwards in response to pressure, but they do not respond to chemical cues. A change in water flow or a mechanical vibration produces a burst in

swimming. On contacting a host, the copepodid grips the skin with its clawed antennae and examines the surface using the antennules, which bear high-threshold contact chemoreceptors. Non-salmonid hosts are rejected and the copepodids re-enter the water column (Bron *et al.*, 1993). On suitable salmonid hosts, the antennae penetrate the epidermis and dermis, and the anterior end of the cephalothoracic shield is pushed into the epidermis, causing it to separate from the basement membrane. An adhesive secretion is produced, which quickly hardens to form the frontal filament (Figs 14.10 and 14.11). The larva then moults into the first chalimus stage (Jones *et al.*, 1990; Bron *et al.*, 1991).

The chalimus stages typically attach to the dorsal and pectoral fins and around the anus of wild and caged fish (Wootten *et al.*, 1982), though under confined experimental conditions they will also attach in the buccal cavity and gills (Bron *et al.*, 1991; Johnson and Albright, 1991b).

Pre-adult and adult stages of *L. salmonis* move freely over the surface of the host and are capable of leaving the host to infect other fish. They are abundant on the head and dorsal surface and, especially in *O. gorbuscha* and *Oncorhynchus nerka*, on the posterior ventral surface (White, 1940; Wootten *et al.*, 1982; Nagasawa, 1987). They attach by suction, generated by the cephalothorax and sealed by its marginal membrane and the expanded base of the third pair of legs (Kabata and Hewitt, 1971).

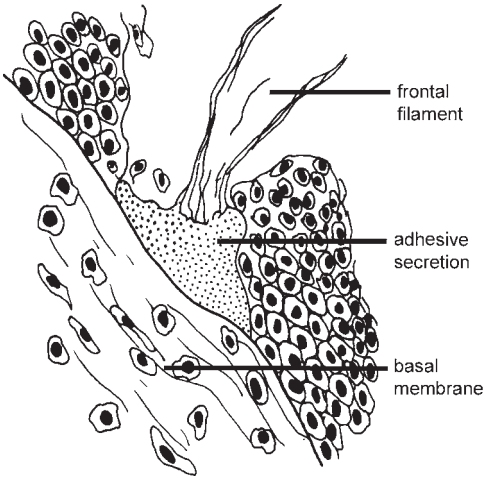


Fig. 14.11. *Lepeophtheirus salmonis*. Section through the attachment site of a copepodid recently attached to *Salmo salar*. (Redrawn from Bron *et al.*, 1991.)

Nauplii of *L. pectoralis* are positively phototactic. The copepodids are positively phototactic initially but later sink to the bottom, whereupon they display positive rheotaxis (Boxshall, 1976). On attachment to a fish, they evidently use chemical clues, as copepodids from adults on flounder preferentially settled on flounder whereas copepodids from plaice preferred plaice. Infection occurs on or near the base of the body fins. Mobile post-chalimus stages occur on the general body surface. Males, non-ovigerous females and juveniles of *L. pectoralis* attach to the body, fins and walls of the branchial chamber, but ovigerous females prefer the undersurface of pectoral and pelvic fins (Boxshall, 1976, 1977). *C. elongatus* adults and larvae attach to most areas of the body of salmon, but adults prefer the dorsal and lateral surfaces of the head, the anterior portion of the abdomen between the opercula and the base of the caudal fin (Hogans and Trudeau, 1989b). In *C. epidemicus* the copepodids and chalimus are found on the body and fins of bream, *A. australis*, whereas in *Caligus epinepheli*, chalimus stages occur on the gill arches and adults within the buccal cavity (Roubal, 1981). Adult *C. epidemicus* that had settled on the bottom of a container responded with rapid upward swimming when a shadow passed over the container (Lester and Roubal, 1995).

Fraile (1986) reported that larvae of *Caligus minimum* were attracted by the mucus of its host, the sea bass *Dicentrarchus labrax*. The parasite displayed positive phototaxis and positive rheotaxis in fast water currents but not in slow water currents. Young whiting in the shade of floating logs and medusae, primarily *Cyanea* spp. and *Rhizostoma* spp., in the sea off Plymouth, UK, were more frequently infected by *C. elongatus* (syn. *rapax*) than other whiting, suggesting that the copepodids

had a preference for these habitats (Russell, 1933; Shotter, 1973).

In north-west and north-east Ireland, all production of *L. salmonis* larvae is from wild stocks of *S. trutta*, but in the west 94% of *L. salmonis* larvae originated from farmed salmon (Tully and Whelan, 1992). Tully and Whelan (1992) estimated that 1–38 million larvae were produced per day from single salmon farms. Major gaps remain in our knowledge of the population dynamics of sea lice, especially in and around sea cages. This lack of data prompted Revie *et al.* (2003) to use statistical regression to determine which of 20 variables they recorded were key factors in explaining patterns of abundance of mobile *L. salmonis* across 40 salmon farms in Scotland. These authors identified six variables as significant: level of treatment; type of treatment; cage volume; current speed; loch flushing time; and sea louse abundance in the preceding 6 months. Factors that had been assumed to be critical, including stocking density, site biomass, water temperature and the presence of neighbours, were not found to be significant in this model. Revie *et al.* (2002) also examined the epizootiology of *C. elongatus* in the west of Scotland over 4 years, and determined that, in contrast with *L. salmonis*, infections were more common in the first year of production rather than in the second, and that fallowing had no effect on abundance.

Heuch *et al.* (2003) compared the epizootiological patterns of *L. salmonis* infections in farmed Atlantic salmon in Norway and Scotland over several years, and reported that infection levels of both chalimus and mobile stages were consistently higher in Scotland. These authors noted that this could be because the water bodies used for farming in Scotland are shallower and more enclosed; because pens are shallower and smaller; because sea water temperatures are higher; and because access to medications differ.

Fast *et al.* (2003) concluded that the high susceptibilities of Atlantic salmon and rainbow trout to infection with *L. salmonis* may be related to characteristics of the host mucus and the high degree at which the mucus of each stimulates the production of low molecular weight proteases in the sea lice. These authors found variation in the release of respective proteases and alkaline phosphatases in sea lice in response to mucus from these two species and the mucus of two others (coho salmon and winter flounder).

Glover *et al.* (2004) found that the strain of Atlantic salmon also influenced louse burdens: individuals of the wild Dale strain, when kept in tanks with four other stocks and then challenged with *L. salmonis*, had significantly lower louse density than did two other stocks (wild Vosso and Farm 2 strains).

Epizootiological models of sea lice have potential for assisting in farm management and louse control. Tucker *et al.* (2002) used laboratory trials to develop a simple mathematical model of a single cohort in the life cycle of *L. salmonis* and successfully predicted the timing and numbers of parasites present on laboratory salmon. Interestingly, these authors also found that the death rates of post-settlement stages of lice in experimental tanks were highly variable, despite the simplification of natural conditions.

Clinical signs, gross and histopathology

Initially, whitish spots across the neck and along the base of the dorsal fins indicate sites of feeding by *L. salmonis*. At higher levels of infection, these become skin lesions and then large open wounds (Fig. 14.12); there may be subepidermal haemorrhaging and erosion of the skin to expose the cranial bones (Wootten *et al.*, 1982). The large open wounds may be associated with secondary bacterial infections (Egidius, 1985). Secondary fungal infections may ensue if fish with exposed wounds are returned to fresh water (Hastein and Bergsjo, 1976). Oedema, hyperplasia, sloughing of epidermal cells and inflammation are caused by attachment and feeding of pre-adult and adult *L. salmonis* (Jonsdottir *et al.*, 1992). Chinook and coho salmon are more resistant to *L. salmonis* than Atlantic salmon and respond to infection by extensive



Fig. 14.12. *Lepeophtheirus salmonis* on Atlantic salmon *Salmo salar* showing severe erosion of the epidermal and deeper tissues of the head and operculum (photo courtesy of T. Hastein).

epithelial hyperplasia and inflammation (Johnson and Albright, 1992a). Jones *et al.* (1990) described the histopathology associated with early developmental stages of *L. salmonis*. Initial mechanical damage caused by copepodid and attachment and feeding by Ch1 and Ch2 causes a mild epidermal hyperplasia. Later stages cause damage by feeding, with a focus of irritation around the periphery of the lesion associated with the frontal filament. The greatest damage is associated with the remnant of the frontal filament following detachment by the Ch4 stage. Lesions, 0.5 cm in diameter, have an outer ring of heavily pigmented tissue and a depressed core of white skin. The basement membrane is reorganized over the remains of the filament and there is dermal fibrosis with inflammatory infiltration.

Damage caused by *L. pectoralis* is usually epidermal hyperplasia restricted to one side of the fin (Boxshall, 1977). Sessile stages such as adult females and, more significantly, Ch3 and Ch4 cause the greatest damage, which frequently extends to the dermis. Dermal insult leads to fibroplasia, cellular infiltration and eventually dense granulation tissue (Boxshall, 1977). Heavy infections by *L. pectoralis* result in lacerated and bleeding skin, with partial destruction of the pectoral fin (Mann, 1970).

C. elongatus leaves a 'grazing trail' over the body surface from the original point of attachment to its 'preferred site' (Hogans and Trudeau, 1989b). When several adults occur at one site, there is extensive damage to epidermal and dermal tissue.

C. orientalis causes skin ulcers on *Liza akame* (Urawa *et al.*, 1979), and *Caligus macarovi* leaves small round scars in the epidermis of *Cololabis saira* (see Hotta, 1962). *Caligus uruguayensis* in the mouth cavity of *Trichurus savala* is partly encapsulated by the epithelial and connective tissue hyperplasia that it stimulates (Radhakrishnan and Nair, 1981a). Adipose fins of *O. gorbuscha* were eroded by the attached chalimus stages of *Caligus clemensi* (see Parker and Margolis, 1964). In contrast, adults of *C. epidemicus* on *A. australis* cause little damage. Its copepodids erode the epidermis during initial settlement, and the chalimus stages are associated with only minor epidermal proliferation and inflammation in the dermis where the frontal filament is attached (Fig. 14.11). However, after the final chalimus moult, the cast-off frontal filament provokes a localized chronic inflammatory response, characterized by epidermal and fibroblast proliferation, lymphocyte and macrophage infiltration and giant cell formation (Roubal, 1994).

Innate immunity, acquired host immune response (humoral and cell-mediated), protective immunity, immunopathology

Salmon do not naturally produce an antibody response to *Lepeophtheirus salmonis* (Grayson *et al.*, 1991; MacKinnon, 1991). For most of the life cycle, the sea lice are not in intimate, fixed contact with host surfaces (Pike and Wadsworth, 1999). Even chalimi, which attach to a fixed position on hosts, are distanced from the skin (except when feeding) by the inanimate frontal filament.

A serum antibody response to an antigen (> 200 kDa), associated with the apices of gut epithelium folds in *L. salmonis*, was found in naturally infected Atlantic salmon (Grayson *et al.*, 1991). Grayson *et al.* (1991) also found different antigens in adult and chalimus stages of *L. salmonis*. Atlantic salmon immunized with crude extracts of either adult *C. elongatus* or *L. salmonis* produced humoral antibodies that reacted with antigens in these extracts, as well as fewer antigens from crude extracts of chalimus stages and eggs (Reilly and Mulcahy, 1993). As yet, none of these antigens have been shown to produce protective immunity in the salmon.

Coho salmon implanted with hydrocortisone (0.5 mg/g body weight) produced a diminished epithelial hyperplasia and inflammation and were more susceptible to infection by *L. salmonis* than were control coho salmon. This suggested by Johnson and Albright (1992b) that non-specific host defence mechanisms are important in resistance of coho to *L. salmonis*. Mustafa *et al.* (2000a) found that macrophage function (as measured by respiratory burst activity and phagocytosis rate) was significantly impaired in Atlantic salmon experimentally infected with pre-adult *L. salmonis*.

Mechanism of disease

The primary cause of pathology associated with adult caligid copepods results from parasite feeding; the extent of damage depends on the number of parasites. Five adult *L. salmonis* cause skin erosion on salmon smelts, but up to 2000 parasites have been recorded on a fish (Brandal and Egidius, 1977), indicating that fish can withstand significant erosion. F.R. Roubal (unpublished data) found that the sparid *A. australis* confined in 1 m^3 experimental sea cages and with infections of 6000 individuals of *C. epidemicus* per fish were in poor condition and had damaged fins and the body surface was covered with mucus.

The damage caused by the chalimus stages results from the attachment by the frontal filament and consequently the limited feeding radius (Boxshall, 1977). There is no evidence that enzymes are released on to the surface of the host.

Osmoregulatory failure through extensive skin damage appears to be the main cause of death, though secondary bacterial infection has also been suggested (Wootten *et al.*, 1982). Small host fish can die very rapidly without any appreciable disease (Ho, 2000). For example, larval rock cod (*Epinephelus malabaricus*) 2–3 cm long died within 3 min after attack by a single *C. epidemicus*; in contrast, 20-cm long rock cod did not die until 4 or 5 days later after being attacked by nearly 100 sea lice (Lin, 1996, cited by Ho, 2000). Twelve to 15 *C. elongatus* will kill salmon smolt, and 40–50 will kill an adult salmon (Stuart, 1990).

Wagner and McKinley's (2004) predictive feeding-rate model indicated that 15–25% of the tissue consumed by *L. salmonis* is blood. These authors note that at higher sublethal infection levels (> 0.5 sea lice/g) this consumption rate may cause anaemia, and that this would compound problems with osmotic balance.

Unidentified bacteria found in the gut of *L. salmonis* have been proposed as a source of disease in caged salmonids (Nylund *et al.*, 1991, 1992). *L. salmonis* may act as a vector for *Aeromonas salmonicida* and infectious salmon anaemia (Nylund *et al.*, 1993).

Mustafa *et al.* (2000b) found that 2-year-old rainbow trout challenged with the microsporidian *Loma salmonae* 28 days after exposure to sea lice developed 2.5 times the number of xenomas than control trout not exposed to sea lice. This increase corresponded with the suppressed macrophage function mentioned above.

Effects on host physiology

Sublethal infection by *L. salmonis* compromises the overall fitness of Atlantic salmon. Even when sea lice are not feeding, they cling to the host by digging into the epidermis, using claw-like antennae and maxillipeds. Hence, the mere presence of sea lice is enough to cause stress to fish (Ho, 2000).

A number of more recent studies demonstrate that experimental infection of Atlantic salmon with *L. salmonis* elevates levels of cortisol significantly compared with those in controls (for example, Bowers *et al.*, 2000; Mustafa *et al.*, 2000a). Fast *et al.* (2004) detected prostaglandin E2, a potent vasodilator that is thought to aid in parasite evasion of the host immune response, in secretions of *L. salmonis*; concentrations ranged from 0.2 to 12.3 ng per individual and varied with incubation temperature and time kept off the host. Critical swimming speeds in Atlantic salmon infected in the laboratory with high numbers of *L. salmonis* were significantly lower than both control salmon and salmon with low numbers of sea lice (Wagner *et al.*, 2003). In addition, after swimming, chloride levels in salmon with higher sea louse numbers were also significantly more increased than levels in control salmon and those with low numbers of sea lice.

***In vitro* culture**

Pike and Wadsworth (1999) decribe how egg sacs can be removed from mature gravid females, hatched in clean, filtered sea water and the nauplii allowed to moult to infective copepodids. Experimental hosts are exposed to copepodids in static, aerated water for several hours. Successful establishment is indicated by black pigment spots at the sites of infection.

Parasite nutrition, physiology and biochemical and molecular biology

Caligids feed by scraping the skin with a dentigerous bar, the strigil, located on the posterior lip of the mouth tube. The mandibles aid the passage of food into the mouth tube (Kabata, 1974). Naupliar stages lack a gut and anus, whereas the copepodid has a mouth cone but lacks the strigil (Johnson and Albright, 1991b). The first chalimus stage of *L. salmonis* has well-developed mouthparts and a functional alimentary canal and is the first feeding stage in the life cycle (Jones *et al.*, 1990; Bron *et al.*, 1991).

Mucus and epidermis appear to be the main diet (Boxshall, 1977; Wootten *et al.*, 1982). Blood was found in the gut of adult *Lepeophtheirus* spp. when blood vessels or haemorrhaging tissue occurred near the surface (Brandal *et al.*, 1976). *C. elongatus* has higher levels and a greater diversity of proteases in the gut than does *L. salmonis*, a difference attributed to the wider host range of the former (Ellis *et al.*, 1990). Lipase was found in the gut of *L. salmonis* by Grayson *et al.* (1991).

Kvamme *et al.* (2004) characterized five trypsin-like peptidase transcripts from *L. salmonis*, and found that their levels increased from planktonic to early host-attached stages and also from pre-adult to sexually mature stages. These authors also noted that the digestive functions of these five peptidases are indicated by their finding that they are all transcribed throughout the undifferentiated midgut.

The genetic population structure of *L. salmonis* has been investigated in some detail to evaluate the claim that individuals originating from farmed salmonids are responsible for the decline of populations of sea trout (*S. trutta*) since 1989 on the west coasts of both Scotland and Ireland. However, populations show varying degrees of polymorphism and hence there is currently no consensus as to whether sea lice from farms are reducing numbers of sea trout. Todd *et al.* (1997) predicted that the inclusion of a planktonic larval phase in the life cycle of *L. salmonis*, in addition to the high mobility of salmon, would enhance gene flow and preclude genetic differentiation of populations as a result of random drift alone. This was confirmed in their analysis of allozyme variation in two polymorphic loci of female sea lice from

sea trout, rainbow trout and caged Atlantic salmon from around the Scottish coast. Isdal *et al.* (1997) also examined allozyme data, from sea lice in Norway, but, in contrast with Todd *et al.* (1997), concluded that there were two distinct populations of *L. salmonis* in the north and south of the country.

Todd *et al.* (1997) also analysed patterns in randomly amplified polymorphic DNA (RAPD) and, in contrast with their allozyme results, found that there was some genetic differentiation of sea louse populations around the coasts of Scotland. *L. salmonis* populations sampled from wild salmon and sea trout were genetically homogeneous, but samples taken from farmed salmon and rainbow trout showed significant genetic differentiation, both between wild and farmed salmonids and among the various farms. There was also evidence of high levels of small-scale spatial or temporal heterogeneity of RAPD marker band frequencies shown for the one farm from which repeat samples were analysed. RAPD analysis also revealed that putative 'farm markers' were present in some individual parasites from west coast wild sea trout, indicating that their lice had most probably originated from salmon farms. The authors concluded that the observed range of phenotypes were produced by a combination of a founder effect on farms and strong selection pressures, perhaps in reaction to pesticides.

Dixon *et al.* (2004) further analysed RAPD fragments of *L. salmonis* from wild and cultured *S. salar* in Scotland and concluded that, although distinct clusters of populations were discernible, the genetic differentiation did not fit any geographical pattern. These authors suggest that this indicates that selection for chemical resistance may occur after dispersal.

Todd *et al.* (2004) assessed variation at six microsatellite loci among *L. salmonis* and found no significant differentiation among lice from wild and farmed salmonids in Scotland, wild sea-run brown trout in Norway and farmed Atlantic salmon in eastern Canada. These authors conclude that long-distance oceanic migration of wild hosts and larval interchange between farmed and wild host stocks are sufficient to prevent genetic divergence. In contrast, sea lice from farmed Atlantic salmon in Pacific Canada (off British Columbia) showed significant but low differentiation from this Atlantic population.

Bjørn *et al.* (2001) examined wild stocks of sea trout and Arctic charr feeding nearby and distant from Atlantic salmon farms in northern Norway for sea lice, and concluded that the burdens of lice on wild salmonids around farms were about ten times higher than those on salmonids away from farms, and also that the most heavily infected fish in farm areas returned to fresh waters prematurely.

Diagnosis of infection

Large female caligoids, though semi-transparent and often cryptically coloured, are usually visible to the discerning eye on the gills, fins or body of fish or in the buccal and opercular cavities, particularly when mobile. Copepodids and chalimus larvae are generally small (less than 4 mm long) and detection requires at least the use of a magnifying glass (Johnson, 1998). Sea lice still attached to the skin of freshly killed fish may also be detected by running a wet hand along the flanks of the body, and will be felt as a minor 'lump'.

Prevention and control

Integrated pest management schemes currently employed against sea lice in salmonid sea cages rely heavily on the use of chemotherapeutics. With the development of improved treatments and with the introduction of coordinated management systems for salmonid cages over wider areas, including programmed treatment and fallowing of sites, sea lice are now under greater control. However, with still such a limited range of approved chemicals, the increasing development of resistance to them remains a problem (Alderman, 2002). Cleaner wrasses offer additional relief in the form of

biological control, particularly in Norway, where they are used in over half of the salmon farms (Johnson, 1998).

Areas of promising research now under way include: the development of effective vaccines; selecting salmon stock with innate resistance to sea lice; the isolation of bacteria pathogenic specifically to sea lice; the development of photomechanical devices to trap sea lice; supplementing diets with nucleotides to increase resistance to sea lice (Burrells *et al.*, 2001); and the isolation of semiochemicals (behaviour-modifying odours) derived from salmon that could be used in traps for sea lice (see below).

The first chemotherapeutic agents used were organophosphates; although effective, they are also hazardous to use (Johnson, 1998; Alderman, 2002). Organophosphates inhibit acetylcholinesterase (AChE) activity in cholinergic nervous systems; they pose toxic hazards not only to fish stock but also to the environment. Canvas skirts must also be used around cages to contain treatment baths. Additionally, organophosphates have been linked with human health problems, and this has increased the pressure to find alternative medications. The first compound used was trichlorphon (Neguvon, Dipterex, Tugon, Dylox, Masoten); this was later superceded by dichlorvos (Nuvan 500EC, Aquaguard, Vapona, Apavap). A more potent (and toxic) organophosphate, azamethiphos (Salmosan, Alfacron), has now replaced dichlorvos (Alderman, 2002), as it does not cause cumulative AChE depression from repeated exposure and is better tolerated by fish. It kills 100% of post-chalimus stage lice at 0.01 mg/l (1 h), but, like other organophosphates, it shows little efficacy against attached chalimus stages (Roth *et al.*, 1996). Sea lice repeatedly exposed to organophosphates can develop resistance (Jones *et al.*, 1992; Fallang *et al.*, 2004). This means that stronger solutions have to be used to treat the parasite, with a concomitant reduction in the safety margin (Roth *et al.*, 1993).

An effective alternative to organophosphates is hydrogen peroxide (Salartect 350 and 500, Paramove 35 and 50), an oxidizing agent that removes lice by mechanical disruption. It breaks down rapidly and leaves no residues, so it is considered 'organic' or 'environmentally friendly'. Nevertheless, canvas skirts are still required on cages, and transport hazards may present difficulties, particularly for island-based fish farms where hydrogen peroxide tanks cannot be shipped on passenger ferries (Alderman, 2002). Exposure to 1.5 g/l hydrogen peroxide in a bath for 20 min removed 85–100% of lice, and was effective against mobile stages of sea lice (Thomassen, 1993). However, use at higher water temperatures (> 14°C) revealed toxicity problems for fish (Alderman, 2002). In addition, the only effect on chalimus is delayed development, and a high proportion of adult and pre-adult stages recover (Johnson *et al.*, 1993; Thomassen, 1993; McAndrew *et al.*, 1998). Treasurer *et al.* (2000) also found that sea lice from a farm with a history of using hydrogen peroxide had developed resistance. Exposure times of 60 min or at 10 g/l for 30 min caused damage to the gill epithelium (Thomassen, 1993).

Pyrethroid compounds such as pyrethrum (Py-Sal) have also been used for sea louse control (Johnson, 1998). These kill organisms by interfering with the closure of sodium channels in nerve membranes, and are relatively toxic to fish and invertebrates (Roth *et al.*, 1993). In the late 1990s, a synthetic pyrethroid, cypermethrin (Excis), became available, followed in Norway by deltamethrin (Alpha max Vet) (Alderman, 2002). These still require the use of canvas skirts and they are much more environmentally persistent.

Ivermectin, a member of the avermectin group of macrocyclic lactones, is a well-established insecticide with neurotoxic properties. Experiments at an oral dose of 0.2 mg/kg body weight after coating with 5% gelatin on food pellets effectively reduced the number of lice and consequential skin damage to Atlantic salmon, though there was a narrow safety margin (Palmer *et al.*, 1987). However, the manufacturer has not developed formulations for fish or obtained marketing authorization (Alderman, 2002). Nevertheless, oral formulations of this

chemical designed for treating pigs can be used legally under the veterinary prescription cascade system. Ivermectin requires a long withholding period, since, even under the cascade system, no residues can be present in fish at slaughter as no maximum residue level exists for fish (Alderman, 2002). Hoy *et al.* (1992) concluded that ivermectin was not well suited to oral treatment because high concentrations reached the central nervous system, and the drug was excreted slowly. Information about its toxicity to target and non-target marine organisms remains limited (Davies and Rodger, 2000).

Most recently, insect growth regulators have been developed as oral anti-sea louse agents, which can be applied in feed and for which marketing authorizations have been obtained (Alderman, 2002). Insect growth regulators are not readily water-soluble and are stable in light; they inhibit chitin synthesis and hence are not effective against adult lice. Diflurbenzuron (Dimilin, Lepsidon) has a half-life of 29 days at 10°C, pH 7.7, is effective at an oral dose of 75 mg/kg over 14 days, and is relatively non-toxic to fish (rainbow trout 96 h median lethal dose (LD_{50}) = 140 mg/l). Other effective insect growth regulators include teflubenzuron (Calicide, Ektobann), which was effective at a dose of 10 mg/kg/day for 7 consecutive days, both at 12–15°C (Branson *et al.*, 2000) and at 5.4–8.3°C (Ritchie *et al.*, 2002), and emamectin benzoate (Slice), which was effective at a dose of 50 µg/kg/day for 7 consecutive days (Stone *et al.*, 2000). However, a major disadvantage of insect growth regulators is their toxicity to marine crustaceans (Burridge *et al.*, 2004).

The use of quantities of such insecticidal compounds in sea cages, especially in enclosed sea lochs and fjords, presents difficult environmental problems. Whilst assessment for safety as a veterinary medicine requires a detailed consideration of aspects of environmental impact, this can only be generalized in nature. Impact assessment of individual fish farms and individual permits to discharge sea louse treatment chemicals (and most other aquaculture medicines) from the appropriate environment protection authority have become increasingly important and now play a considerable role in the availability of these products for use at each salmon farm site (Alderman, 2002).

The ability of *L. salmonis* to survive for several days in fresh water precludes fresh water as a treatment for this species. *C. elongatus* is more sensitive. The parasites on pond-reared sea drum, *Sciaenops ocellatus*, were controlled by a 20–30 min dip in fresh water, whereas treatment had been unsuccessful with 'Copper Control' (Argent; 8.5% copper chelated with mono- and triethanolamine) at 6 mg/l (18 h) and 150 mg/l (30 min), formalin at 10 mg/l (18 h) or 250 mg/l (30 min), or trichlorphon at 0.25 mg/l (18 h) (Landsberg *et al.*, 1991). *C. elongatus* is difficult to control in sea cages because the adult parasite readily leaves caged salmonids and lives in the water column or infects other fish in the vicinity (Wootten *et al.*, 1982).

Non-chemical control methods for sea lice are frequently part of integrated pest management, and these include fallowing, site selection, vaccination and the use of cleaner fishes as biological controls. Fallowing of salmon farms for periods greater than 30 days (7–8°C) may prevent the carry-over of infectious agents and allow the seabed to recover (Grant and Treasurer, 1993). In addition, cage sites can be selected where water currents are such that the infectious copepodid stages are flushed off site (Johnson, 1998).

Vaccines offer many advantages over chemotherapeutics, but none are yet available for the control of sea lice. The digestive processes and gut function of sea lice are poorly known, and so developing a vaccine from gut antigens of sea lice – as has been developed for other blood-feeding parasites (such as cattle tick) – is proving to be difficult (Raynard *et al.*, 2002). Nevertheless, this phenomenon is being pursued by industry. In the laboratory, the percentage of female *L. salmonis* carrying eggs was found to be lower on salmon injected with a crude extract of *L. salmonis* than it was on control salmon, and the average number of eggs produced by those copepods was also fewer (Grayson *et al.*, 1995).

Two semiochemicals linked to salmon (isophorone, 1-octen-3-ol) cause behavioural activation of adult male lice, including electrophysiological responses in their antennal nerves (Ingvarsdóttir *et al.*, 2002). Identification of such host odour cues in sea water may permit the development of slow-release louse traps.

Sea lice have been controlled by cleaner fish in European salmon farms for over a decade (Fig. 14.13). Over 100 years ago, Wilson (1905b) suggested that *Caligus*, *Lepeophtheirus* and allied genera could be controlled by the 'introduction of small fish. . . which will eat up the larvae of parasitic copepods'. Cleaner wrasse, particularly rockcook, *Centrolabrus exoletus*, and goldsinny, *Ctenolabrus rupestris*, are used to control sea louse infestation in salmon farms. In 1989, Norwegian farmers used 50,000 wrasses (65% goldsinny, 15% rockcook) to treat 2.3 million smolts in 115 cages. At ratios of goldsinny : salmon of 1 : 8 most lice were removed; a ratio of 1 : 158 resulted in more lice remaining but they caused no harmful effects; at 1 : 260 the wrasse were not effective (Bjordal, 1988, 1990; Costello and Bjordal, 1990). The number of mobile lice, and not chalimi, was lower on salmon in cages with wrasse (2–11 lice per fish) than in cages without wrasse (< 50 lice per fish) (Treasurer, 1993). Cages in which wrasse have been introduced require little or no chemical treatment to control sea lice. The wrasse were not hosts to *L. salmonis* and *Caligus centrodonti* found on wrasse were not found on salmon, indicating that copepod transfer between wrasse and salmon did not pose a problem (Bron and Treasurer, 1992). Wrasse were not carriers of the typical strain of *A. salmonicida* (see Treasurer, 1993), and the atypical strain found in some wrasse was non-pathogenic to salmon (Frerichs *et al.*, 1992).

Udonellid monogeneans, which are found on a number of sea lice, have been suggested as candidates for biological control, but they are probably unsuitable as they are ectocommensals that feed on the fish, not the lice.



Fig. 14.13. *Lepeophtheirus salmonis* being removed by a goldsinny wrasse *Ctenolabrus rupestris* from the head of *Salmo salar* (Photo courtesy of A. Bjorndal).

Copepoda: Pennellidae

Introduction

Pennellids are widespread and highly visible parasites of marine fishes. Most cause localized changes in adjacent tissues and some result in loss of condition or reduced gonad development.

Lernaeocera branchialis (Fig. 14.14) has been estimated to cause reductions of more than 1000 t/year in the gadoid catch around Scotland through loss of condition (Kabata, 1970). In mariculture, infected cod (*Gadus morhua*) farmed in sea cages in Newfoundland show poor weight gain and increased mortality (Khan *et al.*, 1990). *Lernaeocera lusci* has caused mortality in cultured sole (*Solea solea*) in Britain (Slinn, 1970; Kirmse, 1987).

Dark muscle lesions caused by *Pennella hawaiiensis* delayed the marketing of 169,000 t of boarfish (*Pentaceros richardsoni*) until the source of the lesion was identified (Kurochkin, 1985).

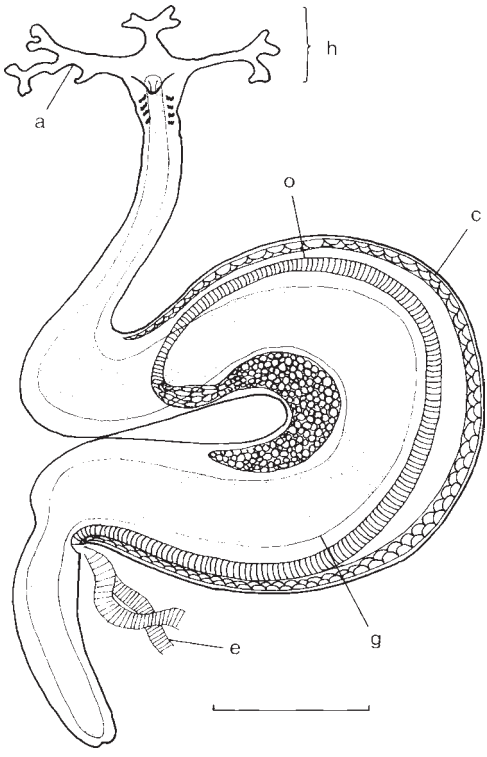


Fig. 14.14. *Lernaeocera branchialis*. Adult female (diagrammatic). a, antler; c, cement gland; e, egg string; g, gut; h, holdfast; o, oviduct. Bar = 5 mm. (Based on Sproston and Hartley, 1941b, and Capart, 1948.)

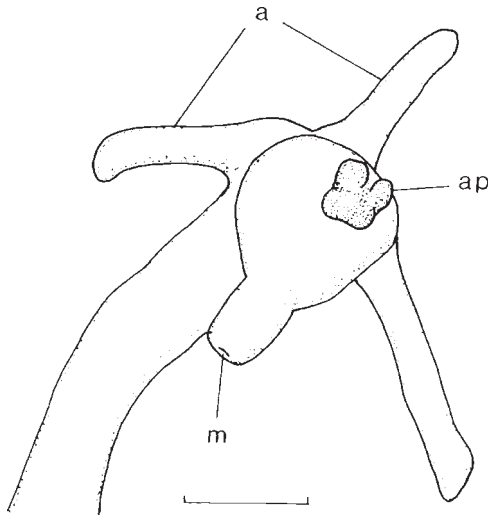


Fig. 14.15. *Lernaeocera lusci*, from *Trisopterus minutus*, showing the antennary processes (ap). a, antlers; m, mouth. Bar = 1 mm. (Drawn from Bastide-Guillaume *et al.*, 1987.)

Host range, distribution and seasonality

Lernaeocera species are restricted to the North Atlantic. Adult females of *L. branchialis* are found primarily on gadoids, particularly *G. morhua*, *G. ogac*, *Boreogadus saida*, *Pollachius pollachius*, *Merlangius merlangus* and *Eleginus navaga*, but also occur on fishes in other families, including Merluccidae (*Merluccius merluccius*), Serranidae (*D. labrax*, *Serranus cabrilla*) and Callionymidae (*Callionymus lyra*) (Kabata, 1979). They are often associated with coastal waters, possibly because here they come into contact with intermediate hosts carrying the juvenile and male parasites and because young cod are apparently more susceptible to infection that older cod (Khan, 1988). *L. branchialis* is absent from fish in low-salinity waters (Sundnes *et al.*, 1997; Koie, 1999, Mellergaard and Lang, 1999).

Adult female *L. lusci* (Fig. 14.15) occur primarily on the gadoid, *Trisopterus luscus*, and the goby, *Pomatoschistus minutus*, in north-west Europe, and on *Trisopterus minutus* and *M. merluccius* in the Mediterranean (Bastide-Guillaume *et al.*, 1987; Tirard *et al.*, 1993; Van Damme *et al.*, 1997). Slinn (1970) found juvenile stages on *S. solea*.

In the North Sea, juveniles and males of *L. branchialis* reach peak abundance on flounder (*Platichthys flesus*) in April/May, followed by a peak in abundance of females on cod and haddock in mid- to late summer (Stekhoven, 1936; Kabata, 1958). They also occur on lemon sole (*Microstomus kitt*) in the northern North Sea (Kabata, 1979). Off Newfoundland, juveniles and males are commonest on lumpfish (*Cyclopterus lumpus*) in July/August (Templeman *et al.*, 1976; Khan *et al.*, 1990) and the main transmission to cod occurs in the autumn, especially in inshore waters (Brattey, 1997). In the English Channel there is little seasonal change (Sproston and Hartley, 1941a). Like many parasites, the prevalence of pennellids

can vary greatly from year to year (Kabata, 1958; Hughes, 1973; Nagasawa *et al.*, 1988).

The pennellid *Haemobaphes dysphaerocephalus*, a parasite of the west coast of North America, was found on Atlantic salmon introduced into west coast sea cages (Kent *et al.*, 1997), an example of how exotic fish in aquaculture can pick up native parasites.

Systematics

A key to genera of pennellids based on adult females is in Boxshall (2004). Outlines of common genera are given in Figs 14.14–14.16. The two species of *Lernaeocera*, *L. branchialis* and *L. lusci*, are distinguished by the shape of the holdfast of adult females (Van Damme and Ollevier, 1995). *L. branchialis* (Fig. 14.14) has one dorsal and two lateral thoracic lobes, whereas the holdfast of *L. lusci* incorporates two additional lobes, the antennary processes, which arise on the dorsal side between the mouth and the holdfast proper (Fig. 14.15). A key to *Cardiodectes* is given by Bellwood (1981) and to pennellid species of India by Pillai (1985).

Males and copepodids can be recognized as members of the family Pennellidae from the characteristic second antennae, which are typically subchelate with a strong opposable claw. The chalimus stage of pennellids typically has a very short frontal filament, sometimes completely embedded in the tissues of the fish (Kabata, 1988). Schram (1979) gives features that distinguish juveniles of *Lernaeenicus sprattae* from those of *Lernaeocera branchialis.* Juvenile females of *Lernaeocera*, *Lernaeenicus*, *Trifur*, *Peniculus* and possibly *Pennella*, which are difficult to distinguish, could possibly be identified to genus from the ornamentation on the posterior wall of the mouth cone (Romero and Kuroki, 1986, 1989).

Morphology and life cycle

Adult female pennellids develop elaborate holdfasts and show little signs of segmentation (Figs 14.14 and 14.16). Their life cycles require either one or two hosts.

Lernaeenicus sprattae, a parasite of sprat (*Sprattus sprattus*) and pilchards (*Sardina pilchardus*), requires one host species. Eggs hatch into the first naupliar stage, which is free-swimming. After a day they moult into a second nauplius and a day later into a copepodid (Schram and Anstensrud, 1985). The copepodid is attracted to the sea surface at night and fastens to a sprat or young pilchard by its second antennae. It moults through four chalimus stages and then changes into a mobile male or pre-metamorphosis female. Fertilization occurs

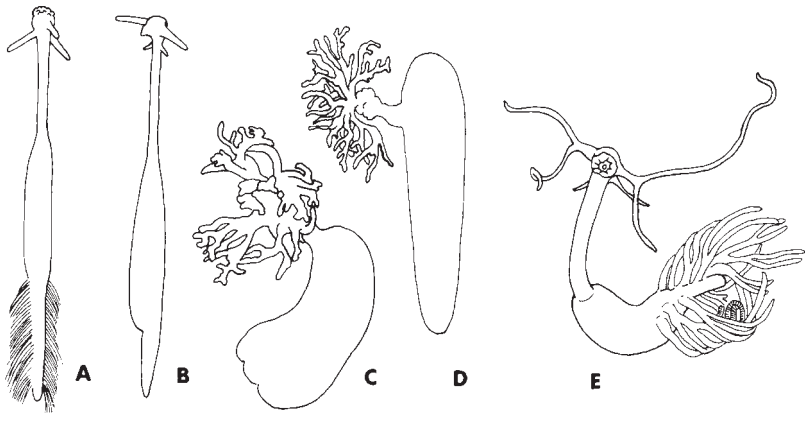


Fig. 14.16. Pennellidae, morphology of adult females. A. *Pennella*; B. *Lernaeenicus*; C. *Phrixocephalus*; D. *Peroderma*; E. *Lernaeolophus*. (A–D redrawn from Kabata, 1979, E from Grabda, 1991.)

on the fish; then the female may swim to a second fish or remain on the first fish to complete her metamorphosis (Schram, 1979). El Gharbi *et al.* (1985) found that in the Golfe du Lion, Mediterranean Sea, copepodids attached to larval pilchards on the breeding grounds during the winter. The chalimi developed on them as they moved inshore into lagoons during the spring. By summer, fertilized pre-metamorphosed females were ready to infest the same or another host and completed their development on that host as it moved back out to the breeding grounds in the autumn to complete the life cycle in about a year.

The egg string is a tube of eggs. After embryonic development, the intact egg leaves through a slit in the lateral wall of the egg string and soon after hatches to the first nauplius. An excised adult female spontaneously shed its empty egg strings and immediately produced new ones, which it filled with eggs over the next 24 h (Schram, 1979).

Some pennellids require two different species of host to complete their life cycles. *L. branchialis* uses a non-gadoid fish as its first host and then the female transfers to a gadoid to complete her development (though the non-gadoid dab, *Limanda limanda*, has been found to function as both intermediate and definitive host (Begg and Bruno, 1999)).

The adult female of *L. branchialis* is a dark red sigmoid worm, often with yellow egg strings (Fig. 14.14). Eggs hatch in 12 days at 10°C and release nauplii, which moult to metanauplii and then to infective copepodids within 2 days (Capart, 1948; Whitfield *et al.*, 1988). Copepodids search for a host for up to a week. They attach to gills of flounder (*P. flesus*) or lumpfish (*Cyclopterus lumpus*) and moult through four chalimus stages into adult males and subadult females. This takes about 25 days at 10°C (Whitfield *et al.*, 1988). Males, which develop faster than females, seek out female chalimi and attach to adjacent gill tissue. As soon as the female completes her final moult and while still attached (now by her second antennae), the male grips her with his second antennae and moves back until he is attached at the genital segment. He secretes two spermatophores, curls his abdomen under the female and attaches them over the openings of her two oviducts. This takes 2–3 min. Over the next few hours the spermatophores empty into the receptaculum seminis located just inside the genital opening (Capart, 1948) and then the spermatophore envelope is shed. He may fertilize the female a second time, or she may be fertilized by other males several more times before she leaves the flounder. Males remain on flounders for over 5 weeks after maturity and thus accumulate on their gills (Anstensrud, 1989, 1990a,b,c; Heuch and Schram, 1996).

The metamorphosing female becomes free-swimming for several days until it locates the second host and, after attaching to the abdomen, elongates by straightening folds in the cuticle (Smith and Whitfield, 1988). Four to 5 months later she starts to produce eggs and dies after 1.5 years (Kabata, 1958). The eggs, held close to the female in coiled strings by a mesentery-like membrane (Heegard, 1947b), appear to be fertilized as they are extruded (Wilson, 1917). A second batch of eggs has been produced *in vitro* by excised females (Heegaard, 1947b; Whitfield *et al.*, 1988), suggesting that several batches of eggs could be produced in the field. Khan (1988) found that egg strings were not lost by females *in situ* but increased in size from December to June and then shrank and eventually dropped off when the parasite died. Kabata (1958) suggested that eggs were laid more or less continuously, were liberated from the end of the egg string and sank to the bottom, where they hatched. Liberation of the egg does not appear to be a prerequisite for hatching, as nauplii hatch directly from the egg string *in vitro* (Sproston, 1942; Heegard, 1947b; Whitfield *et al.*, 1988).

In the southern North Sea, *L. branchialis* has one generation per year (on *Merlangius merlangus*), while *L. lusci* has two, first using sand gobies (*Pomatoschistus minutus*) as the definitive host and then a much larger population on sand gobies and bib (*Trisopterus luscus*) (Van Damme *et al.*, 1997).

Cardiodectes medusaeus (Fig. 14.17) uses an invertebrate first host and a

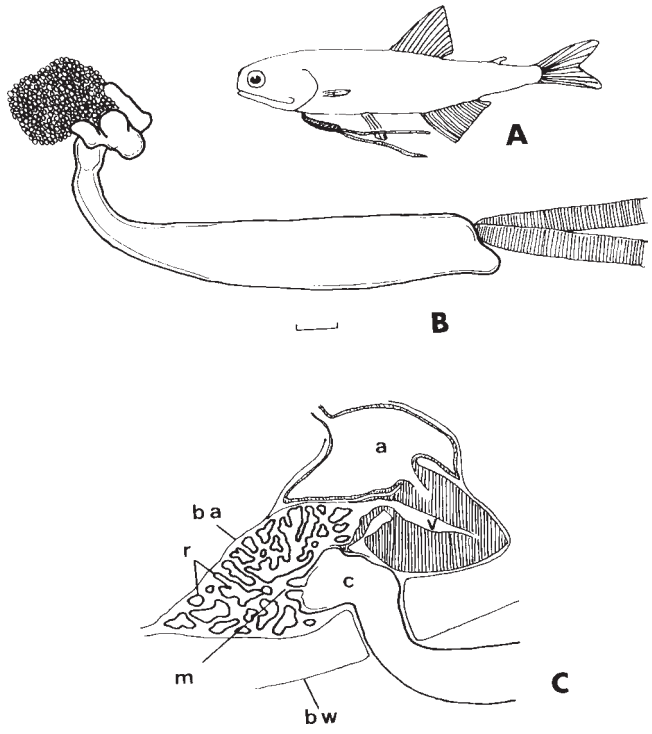


Fig. 14.17. *Cardiodectes medusaeus*. A. Adult female attached to myctophid (after Perkins, 1983). B. Entire parasite, scale bar 1 mm (after Shiino, 1958). C. Section though heart of myctophid with parasite embedded in the bulbus arteriosus. a, atrium; ba, bulbus arteriosus; bw, body wall; c, copepod; m, mouth; r, rhizoid holdfast processes; v, ventricle. (After Kabata, 1981.)

myctophid second. Unlike *L. sprattae* or *L. branchialis*, the egg hatches directly into an infective copepodid. The copepodid attaches to the mantle or gill of a pelagic gastropod (heteropod, pteropod or *Janthina*) and after three chalimus stages it moults to an adult male or a pre-metamorphosis female. Fertilized females leave the snail and seek out and attach to fish (Perkins, 1983).

Peniculisa shiinoi (Fig. 14.18), like *C. medusaeus*, has no free-swimming naupliar stage. The egg extrudes an embryo surrounded by the naupliar cuticle attached to the egg string by a short stalk. The embryo swells and a free-swimming copepodid hatches from it. As the copepodid did not attach to the definitive host, an intermediate host is suspected (Izawa, 1997).

Roth (1988) suggested that *Haemobaphes intermedius* (Fig. 14.19), a parasite of cottids, possibly completes its life cycle on one host, though Goater and Jepps (2002) found only females of another species, *Haemobaphes diceraus*, on shiner perch, *Cymatogaster aggregata*, and suggested that the male *Haemobaphes* sp. they found on pipefish, *Syngnathus griseolineatus*, may point to the intermediate host.

The life cycle of a *Pennella* species has yet to be elucidated. Four chalimus stages, males and immature females described by Wierzejski (1877) from cephalopods and attributed to '*Pennella varians*' are common on the gills of *Sepia* and *Loligo* in the Mediterranean (Rose and Hamon, 1953). In the eastern Atlantic, 100% of *Todaropsis eblanae* and 70% of *Illex coindetii* were infected, with up to 100+ parasites per squid (Pascual *et al*., 1997, 2001). It is not clear to which pennellid species these larval stages belong.

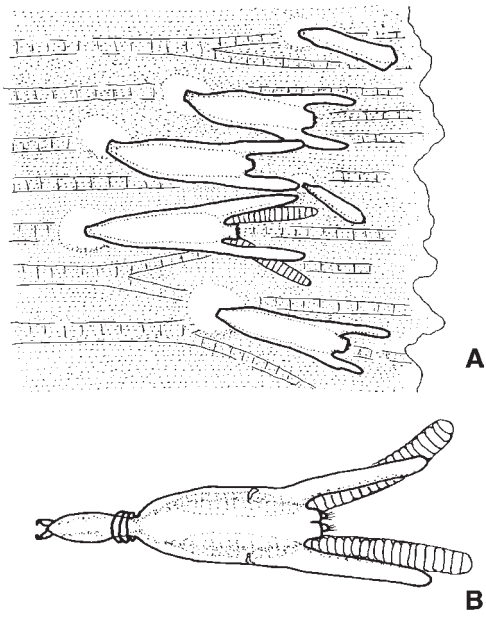


Fig. 14.18. *Peniculisa wilsoni*. A. Adult females attached to fin of *Diodon histrix*. B. Entire parasite (after Radhakrishnan, 1977).

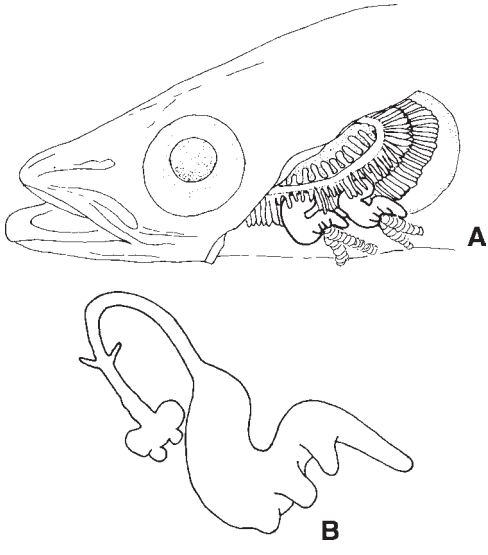


Fig. 14.19. *Haemobaphes diceraus*. A. Adult females attached to gill of *Theragra chalcogramma* (after Grabda, 1975, 1991). B. Entire parasite minus egg strings (after Kabata, 1979).

Host–parasite relationships

Site and host selection, course of infection

Pennellid juveniles and males attach to the surface of the skin or gills. Adult females penetrate into tissues and are usually attached near blood vessels. Some, such as *Peniculisa wilsoni* (Fig. 14.18), penetrate only a short distance, while others, such as *Lernaeenicus hemirhamphi*, burrow deeply, seeking any organ with a rich blood supply (Natarajan and Nair, 1973). In all cases, at least part of the body and egg strings protrude from the fish.

Juvenile *L. sprattae* occur primarily on the fins, particularly the pectoral fins, of pilchards and sprat. Copepodids initially attach anywhere on the body surface; then over the next 24 h they move to the fins before moulting into chalimi (Anstensrud and Schram, 1988). Adult females on pilchards tend to burrow into muscles, particularly near the base of the dorsal fin (El Gharbi *et al.*, 1985). They occasionally pierce the heart (Rousset and Raibaut, 1989). Females on sprat primarily attack the eye, particularly the upper rear quadrant (Anstensrud and Schram, 1988).

Juveniles and males of *L. branchialis* attach and grow near to the tips of the gill filaments of the first host. Metamorphosing females swim into the gill cavity of the second host, attach to gill filaments and work their way along the filament and down the arch (Kabata, 1958). They usually penetrate near the ventral end of the third or fourth arch, and develop their anchors and mouthparts in the wall of either the bulbus arteriosus (in North Sea gadoids; Stekhoven, 1936; Kabata, 1979) or the ventral aorta (in Newfoundland cod; Khan, 1988).

Maturing females of *Phrixocephalus cincinnatus* (Fig. 14.16) develop within the eye of flounder (*Atheresthes stomias*). The juvenile female breaks through the cornea, causing little damage, and develops internally, penetrating through the retina into the choroid, where it produces cephalic and thoracic lateral outgrowths. The holdfast is well formed before the genital area begins to grow and pushes out of the eye through the

cornea, possibly by pressure necrosis or by lytic secretions from its anus. Thoracic outgrowths of mature females spread throughout the vitreous humour (Kabata, 1969a).

Lernaeolophus sultanus (Fig. 14.16), a pennellid with a worldwide distribution parasitic on scombrids and other fishes, typically attaches to the palate. Its cephalic processes grow up into the eye socket, while its genital segment and abdomen lie free in the buccal cavity (Grabda, 1972). *Lernaeolophus aceratus* on wrasse attaches in the gill cavity near the dorsal end of the gill arches, and embeds its head between the liver and vertebral column (Ho and Honma, 1983).

Two pennellids insert their heads directly into blood vessels. *H. diceraus*, a relatively large pennellid (60 mm or more long), attaches in the gill cavity of *Theragra chalcogramma* (Fig. 14.19). Its S-shaped genital segment and abdomen lie on the posterior side of a gill arch between the two rows of gill filaments. The neck enters the lumen of the branchial artery and passes against the blood flow into the dorsal aorta and eventually into the conus arteriosus so that its head is adjacent to the valves of the ventricle. The length of the neck varies according to where the parasite settles in the gills (Grabda, 1975).

Adult female *Cardiodectes medusaeus* lie ventral to the heart of myctophids and penetrate into the bulbus arteriosus (Fig. 14.17). Metamorphosing females have been found encapsulated in the wall of the bulbus and lying free in the body cavity, suggesting that, like *P. cincinnatus*, this species is fully enclosed during development (Perkins, 1983; Boxshall, 2000).

Peroderma cylindricum (Fig. 14.16) embeds its rootlets in the anterior kidney of pilchards, *Sardinia pilchardus*. The trunk of the parasite, 12 mm long, lies in a muscle cavity with a small opening to the outside through which the egg strings pass.

Clinical signs and histopathology

The head and anterior thorax (cephalothorax) of most pennellids is buried deeply in the host tissues. The head is usually enveloped in a large chronic granuloma, composed of fibrous and necrotic tissue, with some vascularization and free blood near the mouth of the parasite. The fibrous tissue may form a hard cartilage-like capsule, as in *Cardiodectes longicervicus* (see Shiino, 1958). A sleeve of hyperplastic, fibrous connective tissue surrounds the thorax from the granuloma to the surface of the fish.

The posterior thorax or genital complex generally lies outside the host and is usually free of any host tissue response. However, in *Lernaeolophus aceratus* in the wrasse *Halichoeres tenuispinis*, the sleeve around the parasite extends outwards into the gill cavity so that it wraps around most of the parasite's trunk and leaves only the abdomen with its branching processes free (Ho and Honma, 1983).

Juvenile *L. branchialis* cause the ends of gill filaments of flounder to thicken and lamellae to fuse as a result of tissue proliferation (Kabata, 1958). Infections have been associated with a decrease in the condition factor (Moller and Anders, 1986). Adult female parasites on cod are associated with hyperactivity in the fish plus erratic swimming and a tendency to remain at the water surface.

The head of the adult female *L. branchialis* is encapsulated by a mass of vascularized fibrous tissue, which distends the wall of the bulbus arteriosus. As they grow, the antlers sometimes break into the lumen, but the mouthparts always appear to be lodged in the granuloma in the wall. Local tissue necrosis occurs around the head; adjacent muscles may be discoloured, fragile and necrotic. In multiple infections, parasites are sometimes shed, leaving open haemorrhagic lesions, which develop necrotic margins and caseous exudates (Stekhoven, 1936; Khan, 1988).

Peniculisa wilsoni, a parasite that tends to occur in groups on the fin of *Diodon hystrix* (Fig. 14.18), grips the fin ray with its second antennae. Dense fibrous tissue, partly calcified, develops around the distal segment of each antenna, fusing them to the bone. The ray element may break and become displaced within the tissue, and soft tissue around the site of attachment proliferates and grows into a conspicuous

tumour, which envelops the head and part of the thorax (Radhakrishnan, 1977; Radhakrishnan and Nair, 1981b,c).

Species that invade the eye stimulate granuloma formation but otherwise do surprisingly little damage while they are alive. In *Phrixocephalus cincinnatus* (Fig. 14.16), a haematoma forms around the holdfast and this displaces the retina towards lens. When the parasite dies, decomposition of the holdfast destroys the posterior chamber of the eye and sometimes also the lens (Kabata, 1969a).

The skull of fish with *Lernaeolophus sultanus* (Fig. 14.16) is eroded near the body of the parasite, the olfactory epithelium in the nasal cavity is disrupted by the holdfast and small haemorrhages occur in the tumour around the oral opening of the parasite (Grabda, 1972).

Gill filaments adjacent to *Haemobaphes diceraus* are usually shortened or absent (Fig. 14.19). Parasite and tissue response partly block the lumen of the branchial artery and cause damage to the inner layer of the conus arteriosus (Grabda, 1975). Parts of dead *Haemobaphes cyclopterina* were found in the heart and branchial artery of the flounder *Lepidorhombus whiffiagonis* by Bristow and Berland (1988). The lumen of the conus arteriosus of myctophids is greatly expanded by the two sets of anterior processes and the two pairs of lateral lobes of *C. medusaeus* (Fig. 14.17). The wall of the conus adjacent to the copepod is devoid of muscle and a fibrous collar surrounds the neck of the parasite at its point of exit on the fish (Moser and Taylor, 1978; Perkins, 1983).

Peroderma pacifica (Fig. 14.16), which is completely buried in host tissue except for its posterior tip, penetrates through to the vertebral column of its host, the codlet *Bregmaceros japonicus* (see Izawa, 1977). The muscle cavity in which *P. cylindricum* lies is lined by thin fibrous tissue. Normal kidney tissue exists only at the periphery of the root mass. The rootlets themselves are surrounded by a haematoma and fibroconnective tissue but there is little sign of inflammation (Fig. 14.20). The opening to the outside heals over after the death of the parasite but leaves a scar (Becheikh *et al.*, 1997).

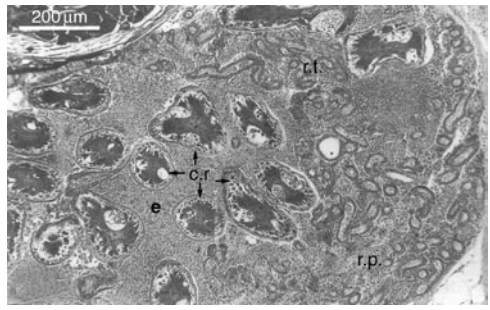


Fig. 14.20. *Peroderma cylindricum*. Section through holdfast embedded in kidney of *Sardinia pilchardus* showing an extensive haemotoma (e) around the rootlets (cr). rp, renal tubules. (Reproduced from Becheikh *et al.*, 1997.)

Host immune response

Though Khan (1988) found slight evidence of acquired resistance to *Lernaeocera branchialis* in that recently attached females were more likely to be shed if a parasite was already present than if the fish was uninfected, previous infections do not protect against reinfection (Stekhoven and Punt, 1937; Khan, 1988). Indeed, in cages, cod that had been previously infected had a significantly higher rate of new infections than previously uninfected fish (Lysne and Skorping, 2002). The reason is not clear but may be associated with different behaviours in the two groups of fish.

No antibody to *Lernaeenicus radiatus* was found in an infected sciaenid, *Leiostomus xanthurus*, by Thoney and Burreson (1988), using an ELISA test. Infection by *Phrixocephalus cincinnatus* actually suppresses parts of the cellular immune response in flounder (Faisal *et al.*, 1990).

Mechanism of disease

The disease caused by *L. branchialis* is primarily the result of anorexia, stress and blood loss. Sudden mortality occurs when part of the holdfast enters the vessel lumen, resulting in thrombi and blockage of major blood vessels (Khan, 1988). *C. medusaeus*, a parasite that lies within a blood vessel, does not cause such thrombi, perhaps because it

injects an anticoagulant into the bloodstream (Kabata, 1981, 1984).

Effects on host

Parasites attached to fins or in muscle seem to have little effect on the general health of the fish. For example, Nagasawa *et al.* (1985) and Watanabe *et al.* (1985) could detect no change in the condition or sexual maturity of saury (*Cololabis saira*) infected with *Pennella* sp. in the western Pacific. However, Hughes (1973) observed a 17% loss of weight in the same fish species infected by *Pennella* sp. in the eastern Pacific.

Pennellids in more critical sites appear to have a greater effect on their host. *Hemirhamphus xanthopterus* parasitized by *Lernaeenicus hemirhamphi* in the kidney and elsewhere had smaller and darker livers than normal. Their fat contents dropped from 15% wet weight to 8% in fish with two to five parasites. In contrast, the liver fat in fish with immature parasites increased to 20% (Natarajan and Nair, 1976), a response similar to that in gadoids with *Lernaeocera branchialis* described below.

Cardiodectes medusaeus in the conus arteriosus was associated with decreased body weight and increased otolith mass in two species of myctophids (Sakuma *et al.*, 1999), though in a third myctophid (*Stenobrachius leucopsarus*) infection was associated with an increased growth rate and sterilization (Moser and Taylor, 1978).

Fish infected with *L. branchialis* lose condition depending on intensity of infection, age of infection, fish age, availability of food and temperature (Mann, 1952; Sherman and Wise, 1961; Hislop and Shanks, 1981; Guillaume *et al.*, 1985). The parasite is particularly detrimental to its host if the host is young (Khan *et al.*, 1990). Up to 30% mortality occurred in young experimentally infected fish (Khan, 1988). Reminiscent of the myctophid above, for the first month of infection before emaciation sets in, fish show increased growth, increased haemoglobin and increased fat content compared with unparasitized fish (Kabata, 1958; Khan, 1988; Khan and Lee, 1989). It appears that parasitized cod compensate for the infection by consuming more feed (Khan *et al.*, 1993); nevertheless, fish with established infections eventually become anaemic. The severity of the anaemia increases with number of parasites (Khan *et al.*, 1990; Knudsen and Sundnes, 1998). Kabata (1958) recognized infected haddock (*Melanogrammus aeglefinus*) by their pallor, particularly in the skin around the eyes, and showed that their blood had decreased haemoglobin content. Mann (1952) found that the blood of infected whiting (*Merlangius merlangus*) had only 20–22% haemoglobin, compared with 38–40% in controls. There was a marginal decrease in erythrocyte count, and their oxygen uptake decreased by 30%. In hake (*Merluccius merluccius*) the erythrocyte count, haematocrit and haemoglobin content all decreased in relation to the number of parasites (Fig. 14.21; Guillaume *et al.*, 1985). Hake infected by *L. lusci* also show a reduced haematocrit (Van Damme *et al.*, 1994).

Naturally infected cod have lower survival rates after capture (Moller, 1984; Khan *et al.*, 1990). Jones and Taggart (1998) estimated that parasitized cod around Newfoundland suffered an 8% differentially higher mortality relative to non-parasitized cod. This was more evident in northern regions, suggesting that the physiological effects of the parasite may be reduced in warm waters where the cod grow quickly.

The total fat content of infected gadoids is less than that of uninfected fish (Kabata, 1958; Moller and Anders, 1986), particularly in whiting (Mann, 1970). The parasite also

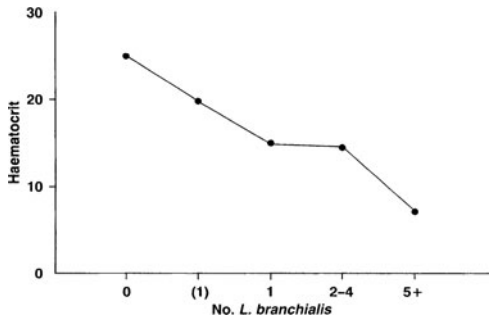


Fig. 14.21. Effect of *Lernaeocera branchialis* on the haematocrit of *Merluccius merluccius* (from data in Guillaume *et al.*, 1985).

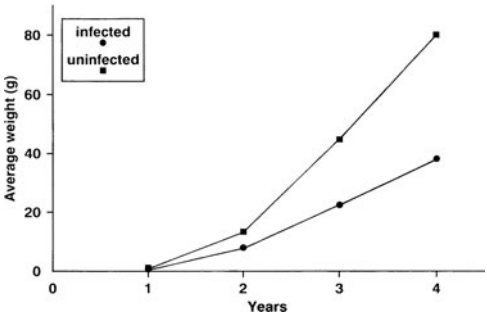


Fig. 14.22. Effect of *Haemobaphes diceraus* on the weight of *Limanda herzensteini* (from data in Nagasawa and Maruyama, 1987).

affects the development of the gonads. Hislop and Shanks (1981) estimated that *L. branchialis* was responsible for a 21% reduction in egg production in haddock. Khan (1988) found that infected cod had a gonadosomatic index about half that of controls and spawning was retarded.

Sole (*Limanda herzensteini*) infected with *Haemobaphes diceraus* in their branchial arteries were shorter than uninfected fish of the same age and lighter in weight (Nagasawa and Maruyama, 1987; Fig. 14.22). *Haemobaphes cyclopterina* in Arctic cod was associated with a lower condition factor, hepatic and gonadal indices and haematocrit. Females were especially affected (Khan *et al.*, 1997). Infection by *H. disphaerocephalus* in an unnatural host, cultured Atlantic salmon, was associated with anaemia and lethargy (Kent *et al.*, 1997).

Sardines (*Sardinia pilchardus*) infected with *P. cylindricum* have a lower condition factor, the degree related to the number of parasites. Gonad development is inhibited (Kabata, 1970) but this is apparently reversed when the parasite dies (Ben Souissi and Ben Hassine, 1991). Many sardines had two and some had three or four parasites (Ben Hassine *et al.*, 1990), suggesting that high mortality is not associated with the infection.

Concurrent infection

Bib (*Trisopterus luscus*) with *Lernaeocerea lusci* frequently had *Cryptocotyle lingua*, another coastal parasite (Evans *et al.*, 1983). Khan (1984) suggested that *L. branchialis* was absent from offshore cod (Newfoundland/Labrador) because of mortality associated with concurrent infection with the trypanosome *Trypanosoma murmanensis.* In the laboratory, cod with *L. branchialis* plus the trypanosome or plus exposure to fractions of crude oil had an increased mortality rate (Khan, 1984, 1988).

Parasite nutrition and physiology

Nauplii, metanauplii and early copepodid stages of *L. branchialis* and *Lernaeenicus sprattae* do not feed and can be readily raised in sea water (Heegaard, 1947b; Schram, 1979; Schram and Anstensrud, 1985).

Pennellid chalimus larvae presumably feed on epidermal cells like the chalimus of caligoids. Mature males of *Lernaeocera branchialis* feed on epidermis (Anstensrud, 1989).

Adult female pennellids generally feed on the blood and lymph of fish from haemorrhage and inflammation within the granuloma. Blood cells have been found within the gut of *L. sprattae*, *Phrixocephalus cincinnatus*, *Cardiodectes medusaeus* and *Lernaeolophius aceratus* by Rousset and Raibaut (1989), Kabata (1969a, 1981) and Ho and Honma (1983), respectively.

Adult females of *L. branchialis* feed intermittently. Capart (1948) concluded that digestion took about 8 days, during which the intestine, capacity 0.1 ml, changes colour from red to clear (Sproston and Hartley, 1941a). Digestion in this parasite, and in *Lernaelophus aceratus*, is associated with the release of large vesicles from the epithelium. These vesicles, which sometimes form chains, move into the lumen and burst, presumably to release digestive enzymes (Stekhoven and Punt, 1937; Capart, 1948; Ho and Honma, 1983). As *L. branchialis* age, they become darker, turning from red to black, possibly because of accumulated by-products from blood digestion. The anus appears to be nonfunctional (Sproston and Hartley, 1941b).

Heegaard (1947b) kept excised females alive and unfed for at least 2 months in sea water at 6–8°C, during which time they laid

eggs, which hatched. The parasite evidently has little ability to osmoregulate and is unable to survive in water of less than 16‰ salinity (Knudsen and Sundnes, 1998).

Many pennellids have rootlets and/or abdominal appendages, the functions of which are not clear. Monterosso (1926, in Becheikh *et al.*, 1997) suggested that the rootlets of *Peroderma cylindricum* and the abdominal processses in *Pennella* and *Lernaeolophus* species (Fig. 14.16) may be associated with respiration, as the parasites have well-developed mouthparts and apparently functioning guts. Nevertheless, the cuticle on the holdfast of *Pennella antarctica* and *Pennella elegans* contains pores, which may be involved in translocation of material (Wilson, 1917; Kannupandi, 1976), and Perkins (1985) found that the anterior processes of *C. medusaeus* contained masses of mitochondria, suggesting that small molecules might pass through the cuticle from the host blood.

Prevention and control

Slinn (1970) anaesthetized brood stock infected with *Lernaeocera lusci* and removed or damaged the developing female parasites with forceps. It may be possible to locate sea cages away from concentrations of intermediate hosts. In some circumstances, cleaner fishes may be useful as a *Peniculisa shiinoi* was recovered from the stomach of the tropical cleaner fish *Labroides dimidiatus* (see Williams and Williams, 1986).

Copepoda: Lernaeopodidae

Introduction

Lernaeopodids are highly modified copepods chiefly parasitic on marine teleosts. Adult females are attached permanently by a unique structure, the bulla, which is implanted in the host tissue. Dwarf, short-lived, adult males attach to the female.

Members of the freshwater genus *Salmincola*, especially *S. californiensis* (Fig. 14.23), can be a problem for farmed salmonids. Rainbow trout production at a Californian farm dropped from 600,000 lb to less than 100,000 lb per year due to the parasite. Apart from the direct detrimental effect on fish health, retail markets resisted heavily infested product and state restrictions limited the stocking of 'grubby' fish in reservoirs (Modin and Veek, 2002). In Japan, *Alella macrotrachelus* (Fig. 14.24) is reported to be one of the most harmful gill

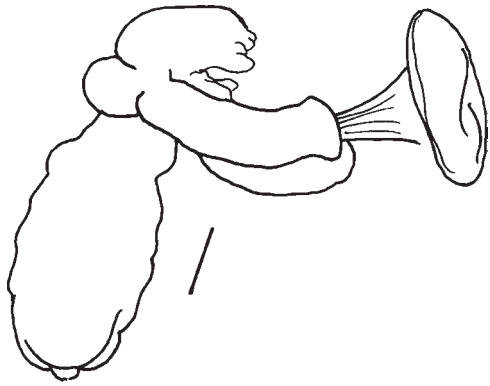


Fig. 14.23. *Salmincola californiensis* female (after Kabata and Cousens, 1973). Bar = 0.5 mm.

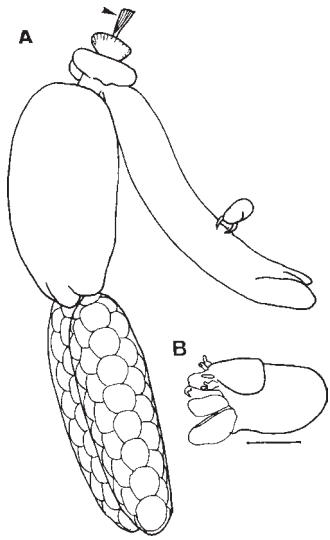


Fig. 14.24. *Alella macrotrachelus.* A. Adult female with small male attached to neck. Thorn-like bulla (arrow) arises from the second maxilla. B. Male. Bar = 0.2 mm.

parasites of cultivated sparids (Ueki and Sugiyama, 1979; Kawatow *et al.*, 1980).

Systematics, distribution, host range and seasonality

The family Lernaeopodidae contains over 260 species, 90% of which are marine. The family has five branches: *Salmincola*, *Lernaeopoda*, *Charopinus*, *Brachiella* and *Clavella*. The *Salmincola* branch, which is associated with freshwater fishes, is the most primitive and *Clavella* is the most advanced. The small *Charopinus* branch infects elasmobranchs (Kabata, 1984). For a key to genera, see Boxshall (2004). Pillai (1985) gives keys to Indian lernaeopodid species and Kabata (2003) keys to UK species.

Kabata (1966) proposed that the evolution of the freshwater forms was associated with Palaearctic salmonids and later spread to ecologically related fishes. There are no freshwater species in the southern continents.

Salmincola spp. are restricted largely to salmonids and coregonids (Hoffman, 1998). *S. californiensis* (Fig. 14.23) is a native of North American streams that empty into the northern Pacific Ocean (Kabata, 1969b). It infects all *Oncorhynchus* species and has been introduced into the eastern USA with the transport of live fish and eggs (Hoffman, 1998). It was imported into Iowa with farmed trout from Missouri (Sutherland and Wittrock, 1985).

Salmincola edwardsii has a Holarctic distribution and is restricted to charr (*Salvelinus* spp.), especially brook trout (*S. fontinalis*) in North America (Black *et al.*, 1983). It is generally found only on the gills (though Fryer (1981) found it exclusively on the fins of Arctic charr in Ennerdale Water, UK).

Salmincola salmonea occurs only on *S. salar* (Friend, 1941). Species that infect non-salmonid fish are *Salmincola lotae* on gadids and *Salmincola cottidarum* on several cottids (Lasee *et al.*, 1988).

Although *Salmincola* spp. are restricted largely to fresh water, Black *et al.* (1983) reported that *S. edwardsii*, *S. salmonea* and *S. californiensis* can survive on salmonids in the sea. Friend (1941) found that *S. salmonea* could survive for several months, and possibly up to 2 years, on Atlantic salmon at sea. The parasite was able to grow but not reproduce. New infection occurred only when the salmon returned to fresh water to spawn. The parasites that survived at sea were able to produce viable eggs and infect other salmon. Most *S. californiensis* died within 2 months following the transfer of its host, *O. nerka*, from fresh to sea water, but some survived for up to 1 year (Bailey *et al.*, 1989).

Alella macrotrachelus (Fig. 14.24) infect the gills of sparids in the Mediterranean, Japan and Australia, particularly in the summer, and apparently overwinter on the fish as ovigerous females (Cabral, 1983; Roubal, 1990). *Alella pagelli*, also from the gills of sparids, occurs in the North Sea, the Mediterranean and South Africa (Kabata, 1979). *Alella ditrematis* infects Japanese embiotocid fish and *Alella pterobrachiata* parasitizes *Epinephelus merra* in Australia (Ho, 1983).

The genus *Clavella* contains approximately 19 species (Kabata, 1979) that parasitize four orders of fishes in the Pacific and Atlantic Oceans. The prevalence and intensity of *Clavella adunca* (syn. *uncinata*) (Fig. 14.25) on whiting (*Merlangius merlangus*) in the Irish Sea decreased with age of fish and the movement of fish away from inshore areas (Shotter, 1971).

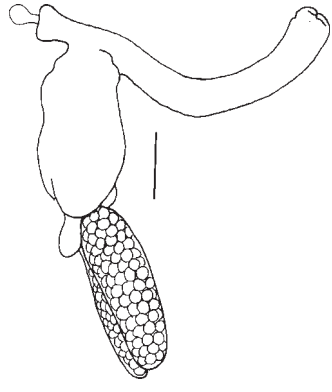


Fig. 14.25. *Clavella adunca* adult female. Bar = 1 mm. (After Kabata, 1979.)

Parasite morphology and life cycle

A characteristic feature of lernaeopodids is the bulla implanted in the fish by the female and to which her second maxillae are attached.

In the genus *Salmincola* the second maxillae of adult females are longer than the cephalothorax and meet distally, where they join the button-shaped bulla (Fig. 14.23). Paired, multiseriate egg sacs arise near the rounded posterior or distal end of the trunk. Females of *Alella* (Fig. 14.24) are similar except that the second maxillae are much shorter than the neck, and the bulla is thorn-shaped.

The bulla, produced by the maturing female in the frontal region of its head, consists of a stalk-like manubrium that expands into an anchor and is inserted permanently in the host tissue. Kabata and Cousens (1972) and Kabata (1981) recognized three types of bulla based on the internal arrangements of ducts. These types correspond to bullae from freshwater teleosts, marine teleosts and elasmobranchs. The mouthparts, located at the end of the neck, consist of large second antennae, small first antennae and maxillae and large maxillipeds. The mandibles are located within the mouth cone, as in other siphonostomes. The mouth leads to a short oesophagus, a long intestine and an anus.

The male salmincolid is tiny, lacks a bulla and attaches to the female usually near the genital openings by the second maxillae and maxillipeds (Wilson, 1911; Kabata and Cousens, 1973), though it may also be attached elsewhere (Fig. 14.24; Roubal, 1981). Male lernaeopodids have a blind-ending alimentary tract, which suggests that they do not feed once attached to the female (Wilson, 1915; Rigby and Tunnell, 1971).

Kabata (1981) recognized two types of life cycle within the lernaeopodid family. The most common type is illustrated by *Salmincola* and *Alella*, whereas the other, abbreviated type is exhibited by *Clavella*.

The life cycle of *S. californiensis* consists of six stages: one copepodid which hatches from the egg and is the infective stage, four chalimus (Ch1–Ch4) and one adult. Copepodids attach to the host by a frontal filament and moult to the Ch1 stage. They undergo three more moults. During the Ch4 stage the female develops a bulla in the anterior space on the cephalothorax previously occupied by the frontal filament and uses this to attach permanently to the host following the final moult. The preadult then metamorphoses, without moulting, to an adult. The young male does not reattach after moulting from the Ch4 stage but seeks out a female Ch4 and attaches to the genital region. Spermatophores are introduced into the orifices of the oviducts and, once inseminated, a cement substance seals the vaginal openings to prevent further copulation. The male dies after copulation (Kabata and Cousens, 1973). After 1–2 weeks the adult female produces a pair of egg sacs (60–300 eggs) and the copepodids hatch after 2–3 weeks. A second pair of egg sacs is produced 2–3 weeks after the first eggs hatch and the parasite dies after this second lot of eggs hatch. The life cycle may take 1–6 months depending on temperature and species. The parasites may overwinter as copepodids (Hoffman, 1977).

The moults occur rapidly and adult males are formed 4–5 days after infection. Adult females take a little longer to develop and start to form the bulla soon after their final moult at around 2 weeks (Kabata and Cousens, 1973). The life cycle of *S. edwardsii* is similar. The time for egg development was directly related to temperature (5 days at 8°C, 0.5 days at 20°C). Photoperiod had no effect. Male *S. edwardsii* apparently survive for only 3 days at 13°C (Conley and Curtis, 1994).

The life cycle is more prolonged in *S. salmonea*. Copepodids, which are released from the egg sac within a membrane and sink to the bottom, take 15 days to hatch at 12–13°C. It takes 5 to 6 months from infection to the production of new copepodids (Friend, 1941). Low water temperature reduces body size and the number of eggs produced (Johnston and Dykeman, 1987).

In *A. macrotrachelus* and *Tracheliastes maculatus* the egg hatches into a nauplius

rather than directly into a copepodid, though the nauplius moults within a few minutes to a copepodid. The parasite then moults through four chalimus stages to the adult (Kawatow *et al.*, 1980; Raibaut, 1985; Piasecki, 1989; Lester and Roubal, 1995).

In *C. adunca* the chalimus stages are fused. The nauplius moults to a copepodid, which attaches and becomes a 'pupa'. The pupa grows without moulting to an adult (Heegaard, 1947b; Shotter, 1971).

Host–parasite relationships

Site and host selection, course of infection

Salmincola copepodids spend some time swimming in short bursts (Kabata and Cousens, 1977), but most of their time is spent on the bottom, where shadows and shock waves trigger increased duration and speed of swimming (Poulin *et al.*, 1990; Conley and Curtis, 1993). Brook trout fry that remained motionless were less likely to acquire copepodids than more active fish. Infected fish became more active so tended to acquire even more parasites (Poulin *et al.*, 1991).

Copepodids move over the surface of the host using their second antennae and maxillae. The maxillipeds make a cavity in the host's surface and the large adhesive terminal plug of the frontal filament is inserted into the cavity (frequently on to underlying skeletal structures). The copepodid 'walks' backwards and uncoils the frontal filament; this evagination may take 3–5 h (Kabata and Cousens, 1973).

Following the chalimus stages, a young female inserts the bulla into a cavity in the host's surface and plants the second maxillae into it. The bulla is then expanded by secretory substances from the maxillary glands passing through the maxillae into the bulla (Kabata and Cousens, 1973).

The distribution of the parasite on the host is determined by the stage and density of the parasite as well as the species and size of the host. In sockeye salmon fry (*O. nerka*), chalimi of *S. californiensis* are found all over the body, whilst adults tend to be concentrated at the bases of the gills. In juvenile fish, adult copepods are primarily on the walls of the branchial chamber (Kabata and Cousens, 1977). In rainbow trout, chalimi attach to the base of the gill filaments but adult copepods attach to the tips of the filaments. At high levels of infection, adults are also found on the body surface and opercular and oral cavities (Sutherland and Wittrock, 1985). In brook trout (*Salvelinus fontinalis*) adult females of *Salmincola carpionis* are attached to the gill arches and neighbouring branchial region, whereas in white-spotted charr (*Salvelinus leucomaenas*) they attach more anteriorly (Nagasawa *et al.*, 1997). Adult *Salmincola edwardsii* on brook trout (*S. fontinalis*) most often occur on the gills but chalimi are found on the fins and elsewhere on the body (Black, 1982; Black *et al.*, 1983). Immature female parasites may move to the new location or conversely there may be selective mortality of parasites in non-preferred sites. Conley and Curtis (1994) noted that 80% of chalimi of *S. edwardsii* died in the first 9 days of infection, and Roubal (1981) observed that several copepodids of *A. macrotrachelus* may attach to the tip of a primary gill filament of *Acanthopagrus australis*, but only one adult female persists. Friend (1941) concluded that chalimi of *S. salmonea* first attached near the tip of the gill filaments and then, following the moult to the juvenile adult, the parasite moved towards the base of the filament.

Caillet and Raibaut (1979) found that copepodids of *Alella macrotrachelus* were morphologically alike but sexually predetermined. Male copepodids settle in the vicinity of adult females already on the gills.

Clavella adunca enters the mouth of adult whiting (*M. merlangus*) and cod (*G. morhua*) and settles on a gill arch, particularly the first. In juvenile whiting most parasites occur on the posterior opercular rim (Kabata, 1960; Shotter, 1976). Pelagic (larval) whiting are not infected but they become infected once they settle to the bottom (Kabata, 1960). Shotter (1971) reported that the nauplius was positively phototactic for 2–3 h post-hatching but then settled on the bottom of the container.

Clinical signs and effect on host

Heavy infections with *S. californiensis* were associated with 'runt' rainbow trout (Sutherland and Wittrock, 1985) and reduced their fecundity (Gall *et al.*, 1972). Growth rate of chinook salmon was reduced when an average of six parasites were on fish of 40 g or less (Johnson and Heindel, 2001).

Brook trout, 22-cm long with an average of 40 *S. edwardsii* in their gills, had one or both opercula folded underneath (Muzzall, 2000). Infected fish had a lower resistance to high water temperature than trout without the parasite (Allison and Latta, 1969; Vaughan and Coble, 1975). Duston and Cusack (2002) found that heavily infested fish had a poor appetite and failed to grow. Infestation at a local hatchery resulted in such mortality and poor egg production that it was uneconomical to retain brood stock after 2 years. Two hundred adult female parasites were the lethal limit for a 750-g fish.

In Japan, a 34 cm brook trout in poor condition was carrying 57 *S. carpionis* in the buccal cavity (Nagasawa *et al.*, 1998), and Sakhalin taimen (*Hucho perryi*) eventually died as a result of poor appetite associated with infection by *Salmincola stellatus* (Nagasawa *et al.*, 1994; Hiramatsu *et al.*, 2001).

Histopathology

The pathology associated with lernaeopodid copepods depends on the tissue infected, the species of parasite, its size and the type of bulla. In *S. californiensis* the 'burrowing' behaviour of young females prior to implantation of the bulla can lead to extensive damage and even perforation of the body wall into the viscera. Older parasites on the opercular wall produced pressure on the tips of the gill filaments, which resulted in a reduction in length of several adjacent primary gill filaments. In juvenile sockeye salmon, up to 25% of gill surface area was lost through such 'crypting' of the gill filaments (Kabata and Cousens, 1977). The same parasite causes crypting in *O. mykiss*. Sutherland and Wittrock (1985) attributed it to retarded filament growth rather than filament degeneration. The extent of crypting on the gills of Atlantic salmon caused by *S. salmonea* may be a measure of the period since infection; older infections being associated with deeper crypts than more recent infections (Friend, 1941).

The gill epithelium of *O. nerka* affected by *S. californiensis* shows hyperplasia and hypertrophy, leading to fusion of filaments; aneurysms also appear (Kabata and Cousens, 1977). Attachment by the larval frontal filament causes only a limited proliferative response, and insertion of the bulla into subepithelial sites causes only a mild reaction of the connective tissue. However, in muscular attachment sites, compaction and disintegration of myotomes occur, especially near the subanchoral surface of the bulla (Kabata and Cousens, 1977). When the parasite is attached to the operculum, there may be osteogenic activity in the vicinity of the bulla, leading to a build-up of spongy bone and eventually to bone degeneration (Kabata and Cousens, 1977). Sutherland and Wittrock (1985) found hyperplasia of the gill epithelium of *O. mykiss*, with fusion of up to 40 adjacent gill filaments, caused by *S. californiensis*. Extravasation and infiltration of eosinophilic granule cells was also noted. There was loss of epidermis where the bulla of *S. californiensis* penetrated into the pharyngeal tissue of *O. mykiss* and the lamina propria was replaced by infiltrating leucocytes. Distortion of gill filaments and pronounced epithelial hyperplasia was caused by *Salmincola yamame* (see Hoshina and Suenaga, 1954).

S. edwardsii in brook trout caused severe diffuse exuberant proliferation of gill epithelium, resulting in severe lamellar fusion and aneurysms (Duston and Cusack, 2002).

Adult female *A. macrotrachelus* affect gill filament length and surface area in *A. australis*, especially in young fish. Recently attached parasites are associated with the swelling of the tips of one or two filaments but, as the parasite grows, the longer neck permits feeding over a wider area. Filaments on adjacent hemibranchs and, in the case of small fish, adjacent holobranchs are affected (Roubal, 1986a, 1987). Muroga *et al.* (1981)

reported aneurysms in the secondary lamellae of *Acanthopagrus schlegeli* in the vicinity of attached *A. macrotrachelus*.

Introduction of the frontal filament during initial attachment by Ch1 to *A. australis* is associated with epithelial hyperplasia and cellular necrosis as a result of mechanical damage. Increased numbers of neutrophils are evident (Roubal, 1989a). Introduction of the bulla by maturing female parasites causes localized damage and epithelial hyperplasia. Infiltrating cells are dominated by lymphocytes and macrophage-like cells. Giant cells and foci of necrotic epithelial tissue are seen occasionally. When the bulla abuts on to the filament's cartilaginous supporting bar, there is a proliferation of chondrocytes, which engulfs the distal end of the bulla and ensures permanent attachment (Roubal, 1989a).

A small 'tumour of attachment' is caused when *C. adunca* attaches to the gill filaments of *G. morhua*, but the same parasite causes a large tumour when attached to the gill filaments of *Melanogrammus aeglefinus* (see Kabata, 1984).

Host immune response

Hucho perryi, a salmonid, produced an immunoglobulin M (IgM)-like antibody to *S. stellatus*, though this was not protective (Hiramatsu *et al.*, 2001). In chinook salmon there was no evidence of resistance associated with prior exposure to *S. californiensis* (Johnson and Heindel, 2001).

Mechanism of disease

In general, attachment causes greater damage to the host than does feeding (Kabata and Cousens, 1977; Kabata, 1984; Sutherland and Wittrock, 1985; Duston and Cusack, 2002). Attachment to the gill filaments, as well as pressure from parasites within the buccal cavity, damages the gills, leading to a loss of respiratory surface area. In *Alella* spp. both attachment and feeding determine the damage to the gills of *A. australis*, since the number and length of filaments affected are related to the length of the neck (age of parasite) and the size of the fish (Roubal, 1986a, 1987, 1989a).

In rare cases, attachment sites may also provide portals of entry for secondary invaders. Fungal infection of gill filaments infected by *S. salmonea* follows spawning and poor condition of Atlantic salmon (Friend, 1941).

***In vitro* culture**

Detached egg sacs will develop and hatch. Copepodids provided with a suitable host will develop further.

Nutrition and physiology

Lernaeopodids feed on mucus and epithelial tissue. The mandibles rake across the skin of the host and passage of food towards the mouth opening is then assisted by contraction of the mouth tube (Chandran and Nair, 1988). Adults and larvae of *S. californiensis* apparently feed by browsing on the host epithelium and, where blood vessels are close to the surface, blood is also ingested (Kabata and Cousens, 1977). Bare tips on gill filaments adjacent to where *S. salmonea* was attached were attributed to feeding behaviour (Friend, 1941).

The role of the bulla, apart from attachment, is unclear. Cousens showed that radioactive-labelled amino acids passed through the bulla into the parasite, but whether substances pass from the bulla into the host is unresolved (see Kabata, 1984). Substances secreted by the parasite may facilitate the passage of the bulla into the host tissue during attachment.

In a study on the effects of temperature on the size of aquatic ectotherms, Atkinson (1995) concluded that a high rearing temperature was associated with a reduced organism size. One of the few exceptions he found was *S. salmoneus*. He suggested that oxygen shortage was normally the limiting factor but in *S. salmoneus* the oxygen supply was 'enhanced by fish respiratory activity'.

Diagnosis of infection

The large female parasites are usually visible with the naked eye on the gills, fins or body of the fish or in the buccal and opercular cavities.

Prevention and control

Hoffman (1970) recommended manual removal. Chemicals such as 0.85% calcium chloride, 0.2% copper sulphate, 1.7% magnesium sulphate, 0.2% potassium chlorate and 1.2% sodium chloride were ineffective against adult *Salmincola* but were useful against copepodids at 3- or 4-day intervals as long as was deemed necessary (Hoffman, 1977). Manual removal together with ivermectin (22,23-dihydroavermectin) 0.2 mg/kg body weight weekly twice or three times by gavage after MS222 eliminated live *S. californiensis* from chinook salmon. All associated necrosis of gill tissue was resolved within 9 weeks. Emamectin benzoate 0.2% premix ('Slice') coated on to pellets and fed to brook trout (700–900 g) for 7 days resulted in a 40–60% reduction in the mean number of adult female *S. edwardsii*, whereas controls increased by 20%. The medicated fish also had better body weight and condition factor than controls (Duston and Cusack, 2002).

Bailey *et al.* (1989) suggested the removal of *S. californiensis* by sea water, but the ability of the parasite to survive for long periods in this environment makes such an approach impractical. McGladdery and Johnston (1988) suggested the physical removal of *S. salmoneus* and holding Atlantic salmon at 2–3°C to prevent reinfection. In California, maintenance of brook trout in the incoming water supply resulted in the disappearance of *S. californiensis* from rainbow trout in a commercial hatchery (Modin and Veek, 2002). They suggest that the parasites attached to the brook trout but were unable to complete their development on this host and died.

Hiramatsu *et al.* (2001) developed an antiserum to IgM antibody developed against *S. stellatus*. They used this in an immunoblot designed to test incoming fish for the parasite.

Copepoda: *Sphyrion*

Sphyrion lumpi (Fig. 14.26) commonly infects redfish (*Sebastes mentella*) in the North Atlantic. Holdfasts left by dead parasites cause abscesses 2 cm or more across in

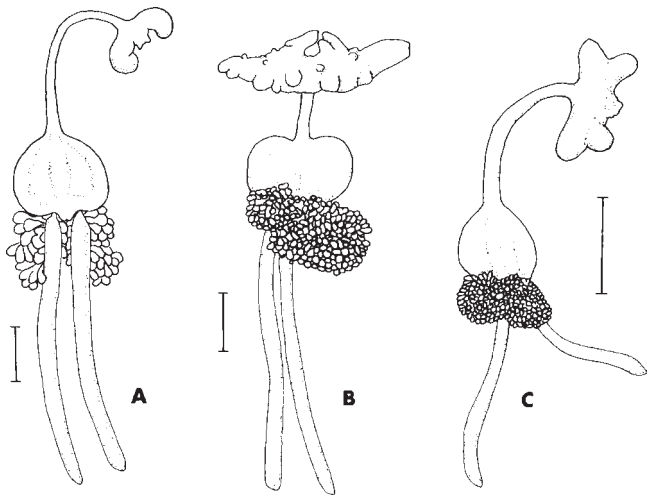


Fig. 14.26. *Sphyrion* species, adult females. A. *S. lumpi*. B. *S. laevigatum* from *Genypterus capensis*. C. *S. quadricornis* from *Coelorhynchus braueri*. Bars = 10 mm. (A after Grabda, 1991, B and C after Gayevskaya and Kovalaeva, 1984.)

the flesh. In Germany, regulations prohibit the sale for human consumption of fillets with more than 5% affected by the parasite (Jungnitz, 1989). Heavily infected fish go to fishmeal or pet food. The parasite is estimated to increase the cost of processing redfish fillets by 80% (Hargis, 1958). The parasite also occurs in 18 other species of deepwater pelagic fish in the temperate north and south Atlantic Ocean, including other scorpaenids, gadids, morids, macrourids and a pleuronectid (Ho, 1989; Moran *et al.*, 1996; Boxshall, 1998). A larger species, *Sphyrion laevigatum* (Fig. 14.26), found in the Southern Ocean, leaves deep scars over 6 cm long in the flesh of *Genypterus blacodes* (see Brickle *et al.*, 2003).

For other details of *Sphyrion* spp., see Lester and Roubal (1995).

Branchiura

Introduction

Argulids have been recognized as pests of cultured trout in Europe and carp in China since the 17th century (Wilson, 1902; Kabata, 1985). They cause mortalities of fish in aquaria, fish ponds, lakes and estuaries. Epizootics of *Argulus foliaceus* in a Scottish loch twice closed an angling fishery for rainbow trout, apparently destroying all the trout the first time (Northcott *et al.*, 1997). A similar infestation was thought to be the cause of the collapse of a rainbow trout fishery in a reservoir in Northern Ireland and prevented the development of a rainbow trout fishery in the Azores (Menezes *et al.*, 1990; Gault *et al.*, 2002). Infested carp and goldfish acquire secondary infections of fungi and bacteria, which reduce their commercial value (Shimura, 1983a). Though less common in the sea, argulids occasionally cause problems in sea-caged salmonids (Stuart, 1990; Jafri and Ahmed, 1994).

Host range, distribution and seasonality

Most branchiurans are freshwater parasites, while a few are found in estuarine or marine environments (Kabata, 1984). The three best-known species are *A. foliaceus*, *Argulus japonicus* and *Argulus coregoni*, all freshwater.

A. foliaceus is common on carp in Europe and Asia. It occurs in cold temperate climates and infests a wide range of hosts, including Cyprinidae, Salmonidae, Gobiidae, Gasterosteidae, Acipenseridae, frogs and toads (Yamaguti, 1963). It prefers eutrophic rather than oligotrophic lakes (Valtonen *et al.*, 1987).

A. japonicus has a worldwide distribution. It was introduced with aquarium fish from the Orient and is now common wherever goldfish are found (Cressey, 1978). In North America it infests primarily goldfish but has been found on *Cyprinus* and *Ictalurus* (see Amin, 1981). In Europe it infests *Carassius*, *Cyprinus* and other genera including *Esox*, *Perca*, *Tinca* and *Sardinus*. Its distribution overlaps with that of *A. foliaceus*, but it is generally found in warmer waters (Stammer, 1959).

A. coregoni occurs primarily on salmonids and also infests cyprinids and other hosts. It occurs in rivers and large lakes, generally in cooler water than *A. foliaceus*, in Europe, China, Japan and the USA (Gurney, 1948; Yamaguti, 1963; Pasternak *et al.*, 2004). It has not been reported from Canada (Kabata, 1988).

These three species reach peak abundance during the summer and autumn. In severe climates they overwinter as eggs (Razmashin and Shirshov, 1981; Shimura, 1983a; Hakalahti and Valtonen, 2003). In more moderate climates, *A. foliaceus* survive the winter as adults (Kimura, 1970) and may breed all year (Bower-Shore, 1940). In South Africa, gravid females of *A. japonicus* have been found during the winter (Shafir and Van As, 1986).

Argulus alosae, an estuarine species, was found on cultured salmon, *S. salar*, and trout, *O. mykiss*, in three marine fish farms in eastern Canada. Fish mortality associated with the infestation occurred when the water temperature rose above 16°C. The trout were more severely affected than the salmon (Stuart, 1990; S.E. McGladdery, personal communication).

Dolops spp. occur in Africa and South America (Fryer, 1968b). *Dolops ranarum* infests a wide variety of hosts. Its females overwinter on *Oreochromis* and *Clarias* species (Avenant and Van As, 1986). *Chonopeltis* spp. occur only in Africa.

Systematics

Branchiura differ from Copepoda in that they have compound eyes, continue to moult after maturity, lay eggs singly and develop without a true naupliar stage. Spermatophores are not normally produced and, when they are, they contain products from both testes. Argulids are closely related to pentastomes (Abele *et al.*, 1989; Martin and Davis, 2001).

Of the 150 or so species of Branchiura, about 100 belong to the genus *Argulus* (Kabata, 1985). For keys to Branchiura and a guide to the early taxonomic literature, see Yamaguti (1963). More recent keys include Cressey (1978) for species from the north-eastern USA, Kabata (1988) for species in Canada, Fryer (1982) for those in Great Britain and Rushton-Mellor (1994) for *Argulus* spp. in Africa.

Parasite morphology and life cycle

The most obvious feature of argulids is the sucker-like first maxillae (Fig. 14.27; Overstreet *et al.*, 1992). These are mobile structures on a slender stalk. The parasite can detach them from the fish alternately and so 'walk' about the fish surface.

The two large compound eyes are important for host detection. Mikheev *et al.* (2000) report that in the light female *A. foliaceus* use ambush behaviour, whereas in the dark they swim more and explore an area three

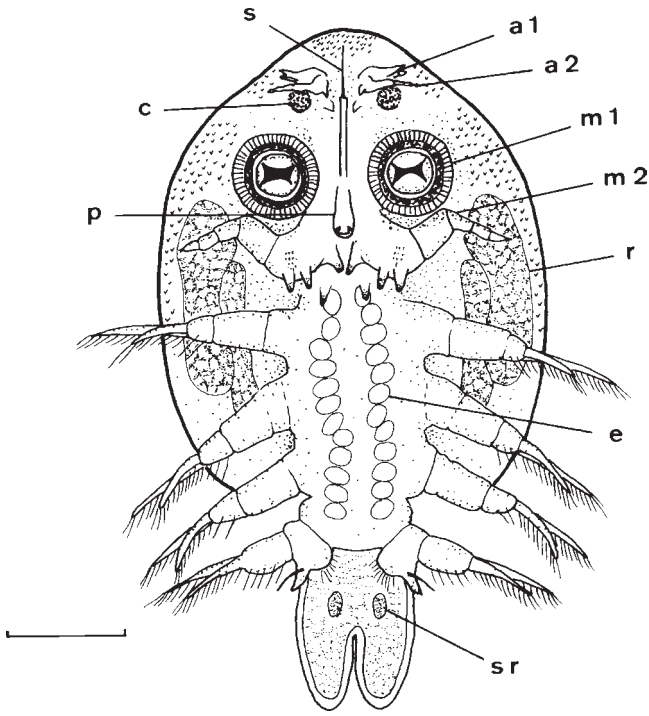


Fig. 14.27. *Argulus monodi*, adult female. a1 and a2, first and second antennae; c, compound eye; e, egg; m1 and m2, first and second maxillae; p, proboscis; r, respiratory area; s, stylet; sr, seminal receptacle. Bar = 1 mm. (After Fryer, 1959.)

to four times larger. Fish such as perch reduce their swimming speed in the dark, making them more susceptible than fish such as roach, which do not. However, in the light, roach are brighter and more reflective so they become more susceptible. Highly reflective walls of aquaria confuse the parasite (Mikheev *et al.*, 1998).

On either side of the carapace are two respiratory areas, where the cuticle is thin, permeable, devoid of spines and adjacent to a blood sinus (Martin, 1932; Sutherland and Wittrock, 1986).

The proboscis is formed from the bases of the mandibles and the interlocked labrum and labium. At the entrance to the mouth are two conical projections, the 'labial spines' or 'siphons', which have a pore at the tip. They probably secrete enzymes that aid digestion. Within the proboscis lie two large serrate mandibles. These can be everted through the mouth and presumably lacerate host tissue. Three delicate lamellae guard the opening of the oesophagus. They are finely serrated and act as a filter fine enough to exclude whole cells (Martin, 1932). Under the scanning electron microscope, the surface of the labrum appears smooth and the surface of the labium is covered in scattered rows of blunt teeth (Shimura, 1983b; Sutherland and Wittrock, 1986).

The proboscis of *D. ranarum* is relatively short and lacks the two labial spines. In place of the lamellae at the mouth of the oesophagus are densely packed setae (Avenant-Oldewage and Van As, 1990).

The stylet in argulids lies anterior to the mouth tube and is separate from the digestive tract (Fig. 14.27, s). It is a delicate structure, consisting of a long tapering hollow spine and a broad sheath into which the spine can be retracted. Near the tip of the stylet are two openings, one of which may be secretory and the other sensory (Shimura, 1983b).

Females grow larger than males. They can usually be recognized well before they are mature by the prominent seminal receptacles (spermathecae) on the abdomen (Fig. 14.27, sr). The basal segments of the second, third and fourth swimming legs of the male are modified for clasping the female. During copulation the male *A. foliaceus* attaches to the dorsal side of the female and clasps her last pair of legs between his last two pairs, interlocking his legs using a socket on the basal segment of the last leg and a peg on the basal segment of the third leg. The abdomen of the male is twisted under that of the female first to one side and then to the other. In this way the male genital opening is brought into direct contact with the spermathecae of the female (Martin, 1932). The spermathecal spine of the female is apparently inserted into the genital atrium of the male and pierces the wall of the ejaculatory duct. Sperm are then pumped from the ejaculatory duct into the spermatheca (Avenant, 1993). Unlike most argulids, males of *D. ranarum* produce spermatophores (Fryer, 1960). Eggs are fertilized at the time of deposition (Wilson, 1902), the ovum shell being pierced by a spine in *D. ranarum* (Fryer, 1960).

Adult argulids can survive for several days away from fish. Females generally lay their eggs on an inert object one at a time, side by side in single, double or triple columns, after which they reattach to fish and feed again (Shafir and Van As, 1986).

Eggs of *A. foliaceus* hatch in 17 days at 23°C and 30 days at 20°C. The entire life cycle takes 55 days at 20°C (Rahman, 1995). Eggs of *A. japonicus* hatch after 10 days at 35°C and after 61 days at 15°C. Hatching is asynchronous and unrelated to the position of the egg in the egg mass (Shafir and Van As, 1986), whereas in *D. ranarum* eggs at the periphery of the egg mass hatch sooner than those in the centre (Fryer, 1964).

Eggs of *A. coregoni* laid in August hatch in September, while those laid in September, October and November hatch in May, June and July the following year. Thus there are two generations per year (cf. three generations in *A. foliaceus* and *A. japonicus*) (Shimura, 1983a). Ultraviolet (UV) enhances hatching in *A. foliaceus*. Eggs buried under sediment may delay hatching for at least a year (Mikheev *et al.*, 2001).

Eggs of *Argulus americanus* hatch in 35–37 days at 15–16°C; hatched females become gravid by day 49 (Shimura and

Asai, 1984). Eggs of *Argulus varians*, a marine species from *Sphaeroides testudineus*, hatch in 25 days at 25°C (Bouchet, 1985).

The stage that hatches from the egg is immediately parasitic and, other than in *Chonopeltis* (see below), usually attaches to the host species on which it will mature. The hatchlings of *A. foliaceus*, *A. japonicus*, *A. coregoni*, *A. americanus*, *Argulus catostomi*, *Argulus lepidostei* and *Argulus maculosus* are copepodid-like, with long second antennae and mandibular palps (Fig. 14.28). After several days they moult to the second stage, which resembles an adult without suckers. After a further five to six moults (eight in *A. coregoni* and *A. foliaceus*) at intervals of 2 to 6 days, they mature in about 4 weeks, depending on the temperature (Wilson, 1902; Hindle, 1949; Schluter, 1979; Shimura, 1981; Rushton-Mellor and Boxshall, 1994).

Eggs of *Argulus funduli*, *Argulus megalops*, *Argulus puthenveliensis*, *Argulus stizostethii* and *D. ranarum* hatch into juvenile adults (Wilson, 1902; Fryer, 1968b; Shimura, 1981).

The hatchling of *Chonopeltis* species bears some resemblance to the copepodid-like larva of argulids. However, its three pairs of segmented appendages, second antennae and first and second maxillae lack setae and are modified for grasping. The first maxillae are the largest, and are tipped by two barbed spines, one lying in a groove of the other, which deeply penetrate into the tissues of the host. Larvae of *Chonopeltis australis* and *Chonopeltis brevis* use a small catfish or cyprinid as an intermediate host and attach under the operculum. Adults are parasitic on the body or fins of other cyprinids. Their mode of transfer from host to host is not clear, as neither larvae nor adults are able to swim (Fryer, 1956, 1968b; Nierkerk and Kok, 1989).

Host–parasite relationships

Site and host selection, course of infection

A. foliaceus and *A. japonicus* attach primarily to the caudal peduncle of carp in culture ponds (Bazal *et al.*, 1969). Site preference is less marked for *A. foliaceus* on *Xiphophorus helleri*. At 28°C most parasites are on the flank, caudal fin and pectoral fins; at lower

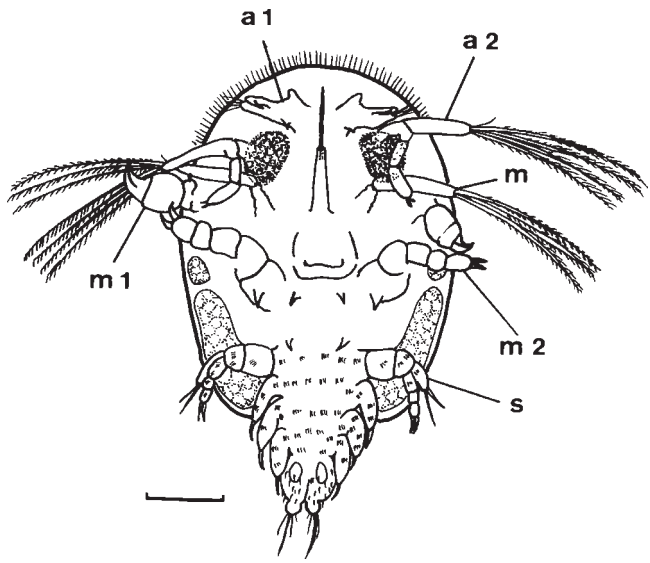


Fig. 14.28. *Argulus coregoni* first stage larva. a1 and a2, first and second antennae; m, mandibular palp; m1 and m2, first and second maxillae; s, first swimming leg. Bar = 0.1 mm. (After Shimura, 1981.)

temperatures many of the large parasites (over 2.8 mm) were on the surface of the operculum (Schluter, 1978). Small individuals of *A. coregoni* attach all over *O. masou.* Large individuals prefer the skin behind the bases of the pectoral and pelvic fins and, to a lesser extent, the adipose fin. The parasites spread all over dead fish, suggesting that site specificity is related to water flow. Few occur in the buccal or gill cavities except for recently hatched parasites (Shimura, 1983a). The branchial cavity is the primary site for attachment of many branchiurans, including *A. catostomi*, *Argulus amazonicus*, *Argulus juparanaensis*, *D. ranarum*, *Dolops geayi* and some *Chonopeltis* species (Wilson, 1902; Fryer, 1968b; Malta, 1982; Malta and Silva, 1986).

Argulus canadensis attach more frequently on fish that already have *Argulus* than on uninfected fish (Poulin and FitzGerald, 1989c). The factors relating to this behaviour are not known.

Immature stages of *A. foliaceus* tend to stay in the same position on the fish for about 20 days until they mature unless mechanically disturbed or the host dies, in which case they move to another location or another fish. Adults are more mobile (Rahman, 1995).

Clinical signs and histopathology

Infested fish are lethargic, stay in the corners of tanks, cease feeding and lose condition (Hindle, 1949; Das *et al.*, 1980). Carp with *A. foliaceus* will initially try to remove the parasite by rubbing against the substrate. In chronic heavy infections, the skin becomes opaque and the fins frayed and the fish is listless (Stammer, 1959). Rainbow trout with *A. foliaceus* swim in tight circles at the start of an epizootic. Trout carrying up to 1500 lice will have fin damage and severe scale loss, especially on the dorsal surface (Northcott *et al.*, 1997).

Allum and Hugghins (1959) reported an epizootic of *Argulus biramosus* (= *Argulus appendiculosus*) on catostomids, cyprinids, ictalurids and other fishes in two lakes in the USA. The 300–400 argulids per fish were principally attached near the bases of dorsal, anal and caudal fins. The skin was inflamed, the scales were loosened and, in severe cases, the fins were frayed and almost gone. In Pakistan, 15- to 25-cm long *Labeo rohita*, *C. catla* and *Cirrhinus mrigala* infested with 70 to 100 *Argulus indicus* or *A. japonicus* were swimming near the surface with blood oozing from minute punctures left by the lice. Fish died daily (Jafri and Ahmed, 1994).

The first feeding sites of argulids are often marked by haemorrhagic spots. Under low magnification they appear as craters formed by hyperplasia of the epidermis at the margins of the wound (Fig. 14.29). Histologically, the craters may be restricted to the epidermis, especially on large fish with a thick epidermis, or they may penetrate through to the stratum spongiosum of the dermis and even to the stratum compactum beneath. The dermis becomes oedematous. Mucus and club cells are absent from any epidermis remaining in the crater but are abundant in tissue at the margin of the crater. In terminal cases the epithelium over the whole fish becomes thin and may be missing from parts of the body and fins (Stammer, 1959).

Bower-Shore (1940) noted that, 6 months after an *A. foliaceus* had fed on a small carp, the site was still plainly visible as a pale area. With the same parasite and same host, Al Hamdanne and Al Taee (1995) noted intensive melanin pigmentation in the damaged epidermis.

Most damage done by *D. ranarum* apparently comes not from the mouthparts but from the first maxillae, which are hook-like rather than suckers. The hooks are driven into the host's integument, causing haemorrhage when removed (Avenant, 1994). Similarly, the claw-like first maxillae of copepodid and juvenile *A. foliaceus* penetrate deep into the tissue (Rahman, 1996).

Mechanism of disease

Though there are reports of fish particularly sensitive to *Argulus* infection and which either thrash wildly (Kroger and Guthrie, 1972a) or become immobile after a single

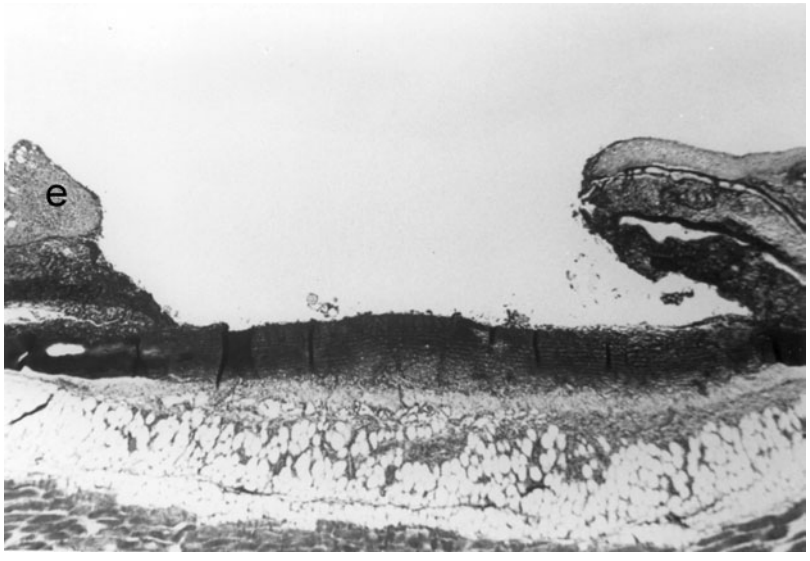


Fig. 14.29. *Argulus* sp. lesion on the sciaenid *Cynoscion regalis* showing thickened epidermis (e) around the border and erosion down to the stratum compactum of the dermis (from Noga *et al.,* 1991).

puncture, fish can generally carry many *Argulus* with little sign of disease (Stammer, 1959). Mortalities are usually associated with hundreds of *Argulus* per fish and these counts may be underestimates because argulids rapidly leave a host when the host is caught (Shimura, 1983a; Stuart, 1990). Such mortalities are probably due to the breakdown of the epithelial integrity and the resultant loss of ionic and osmotic homeostasis. Furthermore, open lesions in the dermis allow fungal and bacterial infections to establish. These, together with anorexia, contribute to the mortality.

Effects on host

Infected fish appear to be stressed and may repeatedly rub themselves against the substrate (Shimura, 1983a). The presence of *A. japonicus* increased the rate of epithelial apoptosis in cortisol-injected carp (Salm *et al.*, 2000). A single *A. indicus* in association with *Saprolegnia* sp. seemed to greatly reduce the growth rate of carp fingerlings (Singhal *et al.*, 1990). Fingerlings exposed to a short (1 week) infection by *A. foliaceus* remained stunted for over 60 days (Rahman, 1996). Rainbow trout appear to be excellent hosts for *A. foliaceus* when the conditions are right for the parasite to multiply (Menezes *et al.*, 1990). One trout can carry up to 3000 lice (Gault *et al.*, 2002).

Young *Oncorhynchus masou* infested with about 250 *A. coregoni* per fish became anaemic. Leucocyte counts, plasma protein, cholesterol and calcium were also lowered (Shimura *et al.*, 1983b).

Three species of sticklebacks (Gasterosteidae) held in aquaria had a significantly higher mortality in tanks with *A. canadensis* than in tanks without the parasite (Poulin and FitzGerald, 1987). Sticklebacks in infested tanks tended to keep away from the bottom (Poulin and FitzGerald, 1989a). They also formed larger shoals than in uninfested tanks and this behavioural change decreased the average number of attempted attachments received by individual fish (Poulin and FitzGerald, 1989b). Parasitized sticklebacks swam erratically and unparasitized fish did not join schools of parasitized fish (Dugatkin *et al.*, 1994).

Antigenicity

There is no evidence of acquired immunity to the parasites. Inflammation at the feeding

sites is not a major component of the histological changes, indicating that the secretions of the parasite have low antigenicity. Ruane *et al.* (1995) produced antibody to *A. foliaceus* in rainbow trout after injecting homogenized tissue, though there was no suggestion that this was protective.

Concurrent infection

Argulid infections are often concurrent with saprolegniosis, which becomes the ultimate cause of death (Bower-Shore, 1940; Allum and Hugghins, 1959; Stammer, 1959; Rahman, 1996). Argulids have been shown to be mechanical vectors for the virus that causes spring viraemia of carp (SVCV) (Ahne, 1985). They may facilitate the entry of bacteria as *O. masou* exposed to water contaminated with *Aeromonas salmonicida* had a higher mortality rate in tanks that also contained small numbers of *Argulus coregoni* (10 per fish) than in tanks without the parasite, though there was little correlation between the location of furunculosis lesions and the sites of attachment of the crustacean (Shimura *et al.*, 1983a).

Argulids are intermediate hosts for fish-parasitic nematodes belonging to the Anguillicolidae, Skrjabillanidae and Dracunculoidea (Moravec, 1978; Tikhomirova, 1983; Molnar and Szekely, 1998; Moravec *et al.*, 1999). They also carry epiphytes (Van As and Viljoen, 1984; Viljoen and Van As, 1985; Sutherland and Wittrock, 1986; Poly, 1998), though these are not apparently infective to the fish. Rushton-Mellor and Whitfield (1993) report a disease of the carapace in *A. foliaceus*.

Parasite nutrition and physiology

Argulids use the stylet to probe the tissue before feeding. They suck up the products of extracellular digestion, which apparently results from the secretions of the stylet and the labial spines and macerations by the mandibles. *Dolops ranarum* apparently feeds by a rolling movement of the mandibles, which eventually exposes the dermis (Avenant, 1994).

Shimura and Inoue (1984) injected an extract of the mouthparts and stylet of *A. coregoni* into the muscle of rainbow trout and observed a strong haemorrhagic response. They suggested that the response facilitated the ingestion of blood by the parasite. Bower-Shore (1940) used histological stains to show traces of haemoglobin in the gut of *A. foliaceus*, though no intact blood cells were found (Minchin, 1909; Stammer, 1959).

Chonopeltis australis and *C. brevis* feed on mucus. They have brush-like structures on the labium, and *C. australis* has a radula-like disc with spines just inside the mouth, all presumably to aid in mucus collection (Nierkerk and Van As, 1986; Nierkerk and Kok, 1989). *D. ranarum* feeds on blood. It produces a deep wound, apparently using the first maxillae (which are not sucking discs in this genus) as the mandibles are relatively small (Fryer, 1968b; Avenant-Oldewage and Van As, 1990).

Respiration in argulids probably takes place not only through the 'respiratory areas' but also through the abdominal lobes. The lobes have a brisk blood circulation and are very large in *Chonopeltis schoutedeni*, a parasite of benthic fish in muddy areas. *D. ranarum* has haemoglobin as a respiratory pigment and can survive in waters with low oxygen (Fryer, 1968b).

Diagnosis of infection

Argulus spp. can be seen scuttling over the surface of the fish with the naked eye.

Prevention and control

Insecticides are commonly used to treat fish with argulosis. Gemmexane (hexachlorocyclohexane, benzene hexachloride (BHC) or 666) is particularly effective (Hindle, 1949; Singhal *et al.*, 1986), though it is highly toxic to humans and some fish (Siluridae) and not easily degraded. Ponds are treated with 0.1–0.2 ppm in two or three applications at weekly intervals. The parasite

develops resistance to it fairly quickly (Kabata, 1985).

Other chemical treatments include: pyrethrum (20–100 ppm for 10–20 min); malathion (0.25 ppm for 6 h); dipterex (100 ppm for 1 h, longer at lower dose); trichlorfon (Tr-technical grade or Tr-crystalline (Neubert, 1984) at 0.25–5 ppm in fresh water for several hours, or 0.5 ppm in sea water for 14 to 16 h weekly for 3 weeks); Neguvon (up to 50,000 ppm for short baths, 0.25 ppm for long treatments); and the antimalarial drugs quinine hydrochloride (13.5 ppm) or atebrine (10 ppm) for several days (Kabata, 1985). DTHP (0.0-dimethyl 2,2,2-trichloro-l-hydroxyethylphosphonate) was used by Puffer and Beal (1981) at 2.5 mg/l for 1 h. Padmavathi and Prasad (1998) compared dichlorvos (Nuvan) with quinalphos (Ekalux, 25% W/V 0.0-diethyl-0-quinoxalinyl-(2)-thionophosphate, Sandoz India Ltd.) to control *A. japonicus* in carp ponds and recommended using 0.0025 ppm Ekalux together with 10 ppm NaCl four times at weekly intervals. Emamectin benzoate ('Slice') in feed (50 mg/kg/day) controlled *A. coregoni* on rainbow trout (Hakalahti *et al.*, 2004). Powdered quicklime 10 kg/acre (2.6 ppm) spread over the surface three times over 10 days and potassium permanganate 5 kg/acre (1.3 ppm) over 3 days both controlled an infestation of *A. indicus*/*A. japonicus* in Pakistan (Jafri and Ahmed, 1994). Details of other treatments that sometimes work, such as formalin, are given by Kabata (1985).

To prevent the introduction of argulids into hatcheries, incoming water can be filtered or taken from a source free of fish. New fish should be quarantined and, if necessary, treated, either using chemicals or by physically removing the *Argulus* with forceps.

For biological control in muddy ponds, remove all hard objects from the pond and introduce a substrate such as wooden slats to which parasite eggs are attached (Kabata, 1985). The slats can be removed weekly and eggs laid on them destroyed by drying. Shimura (1983a) hung transparent acrylic plates vertically in Japanese fish ponds to collect eggs. Gault *et al.* (2002) used opaque corrugated polypropylene boards 1 m^2 floating in a reservoir in Northern Ireland. Changing the boards every 2 weeks reduced the prevalence of *A. foliaceus* on rainbow trout.

To avoid problems with *A. foliaceus* in south-western Siberia, Razmashin and Shirshov (1981) suggested that lakes should not be stocked with coregonid fry until the water had been at 14°C or more for 4–5 days. By then, overwintering eggs would have hatched and the larvae would have perished. To control *A. coregoni* in tanks, Hakalahti and Valtonen (2003) recommend bathing and moving fish and then drying out the empty tanks to kill the eggs.

Argulids are eaten by *Gambusia*, angelfish (*Pteropyllum scalare*) and sticklebacks (Gasterosteidae) (Kabata, 1970, 1985; Poulin and FitzGerald, 1987). Predation on *A. catostomi* by small dace and roach was proposed by Wilson (1902) to control its numbers on fish in a dam in Massachussetts.

Isopoda

Introduction

The suborder Cymothoida contains about 500 species that parasitize fish, all in the superfamily Cymothooidea, in the families Corallanidae, Aegidae, Cymothoidae and Gnathiidae (Bunkley Williams and Williams, 1998a; Brandt and Poore, 2003). In this review we first deal with the Cymothoidae and then with other Flabellifera (Corallanidae, Aegidae and Cirolanoidea) and conclude with Gnathiidae.

CYMOTHOIDAE

Of the 300 or so cymothoids described, over 80% are from tropical and subtropical seas, many from the Indo-Malayan archipelago (Avdeev, 1985). A few occur in fresh water, especially in South America, though none has been found in fresh water in North America or Europe. All cymothoids appear to be parasitic on fish.

The skin parasite *Nerocila orbignyi* and the gill parasite *Mothocya parvostis* have been associated with poor fish growth and increased fish mortality in cultured *Dicentrarchus labrax* in the Mediterranean and cultured *Girella punctata* in Japan, respectively (Hatai and Yasumoto, 1982a; Bragoni *et al.*, 1983, 1984). Salmon farms in Chile and Australia are plagued by the buccal parasites *Ceratothoa gaudichaudii* and *Ceratothoa* cf. *imbricata* (Schafer *et al.*, 1989; N.L. Bruce, personal communication).

Host range

N. orbignyi occurs on at least ten families of fishes, including Mugilidae, Sparidae, Carangidae, Molidae and Holocephalidae. The parasite is found in the Mediterranean, in the tropical and southern Atlantic and in Australasia (Trilles, 1975; Bruce, 1987). It is the most abundant species of cymothoid on the north-west shelf of Africa (Rokicki, 1997). *Ceratothoa oestroides*, a common parasite in the Mediterranean, is found in Sparidae, Carangidae, Clupeidae, Maenidae, Scorpaenidae and Mugilidae. Paired parasites are commonly found in market-sized bass in sea cages. Of other common species, *Olencira praegustator* parasitizes menhaden along the east coast of the USA from New Jersey to Florida (Kroger and Guthrie, 1972b) and *N. acuminata* is found in up to 40 species of fishes along the west coast of the USA (Brusca, 1981).

Host specificity tends to be low in species of *Nerocila* and high in *Mothocya* and *Renocila* (Bruce, 1990). In temperate waters *Anilocra* spp. appear to have low specificity; *Anilocra physodes*, from the north-east Atlantic and Mediterranean, is reported from 25 genera of fishes in 13 families (Trilles, 1975). In the tropics, not only do *Anilocra* spp. show high host specificity (Williams and Williams, 1981), but the species of host preferred may vary with locality (Williams *et al.*, 1982). On the Great Barrier Reef at Heron Island, adult *Anilocra pomacentri* parasitize the pomacentrid *Chromis nitida* (see Adlard, 1990). At Lizard Island they are found on two *Pomacentrus* spp. and at Palm Island, midway between the two sites, they are found on only three other pomacentrid species, though the first three pomacentrids occur there (Bruce, 1986b).

Host range is extended in aquaculture, where fish not naturally infected will become infected with parasites from wild fish that are attracted to sea cages. *Ceratothoa gaudichaudii* occurs along the South Atlantic and Pacific coasts of the Americas. The normal host appears to be *Trachurus murphyi* in Chile (Sievers *et al.*, 1997), though in aquaculture the parasite is on coho and Atlantic salmon, but not rainbow trout (Gonzalez *et al.*, 1997). Prevalence of *C. gaudichaudii* has decreased in Chilean aquaculture in recent years, apparently because of an absence of *T. murphyi* (caused by El Niño and overfishing) (G. Sievers, personal communication). Recently, a new parasite, *Anilocra montii*, has been found in the buccal and gill chambers of Chilean sea-caged coho and rainbow trout (Thatcher and Blumenfeldt, 2001). Its natural host is unknown. *N. orbignyi*, a parasite of Mugilidae and other fish, and *Emetha audouini*, normally a parasite of Sparidae and Centracanthidae, attack sea bass (*D. labrax*) in cages and as yet are not found on sea bass in the wild (Trilles, 1968; Papapanagiotou *et al.*, 1999).

Cymothoids are chiefly parasites of marine teleosts. The few records from elasmobranchs and invertebrates may represent trawl transfers, though the large isopod *Elthusa splendida* from the coast of Brazil (Sadowsky and Soares Moreira, 1981) has been found only in the mouth of the dogfish, *Squalus cubensis*. In South America, at least eight genera, comprising the subfamily Artystonenae, occur in freshwater teleosts (Thatcher, 2000) and the genus *Ichthyoxenus* occurs in freshwater teleosts in Asia.

Systematics

Trilles (1991) recognized at least 334 species of cymothoids. Identification to species is difficult because of unclear early

literature and the inherent variability in the animals. Bruce (1986a), for example, listed 22 published records of '*Irona melanosticta*', all of which were incorrect. Keys to selected genera and species are available (Schultz, 1969; Kussakin, 1979; Brusca, 1981; Brusca and Iverson, 1985; Bruce, 1986a, 1987; Bruce and Bowman, 1989; Kensley and Schotte, 1989; Williams and Williams, 1992). Bruce (1990) gives a review of marine species that have been assigned to the genus *Livoneca.* Hoffman (1998) has a key to ten species from North American freshwater fishes. Charfi-Cheikhrouha *et al.* (2000) gives a key to eight species from Tunisia, and Horton (2000) contains a key to seven species from the north-east Atlantic. Thatcher (2000) summarizes 45 cymothoids from South American fishes.

Parasite morphology and life cycle

The chief morphological features used in identification are illustrated in Fig. 14.30.

Females are normally broader than males. The width/length ratio × 100 (the Montalenti index; Montalenti, 1948) has been used to sex cymothoids, though some species, such as *C. gaudichaudii*, do not show such sexual dimorphism (Szidat, 1966). Some females from gill chambers are bilaterally asymmetrical, a feature that is presumably environmentally determined, though *Mothocya epimerica* occurs more frequently on the left side of its host, *Atherina boyeri*, leading Bello *et al.* (1997) to suggest that the isopod is genetically predetermined for left curvature.

Females have two gonopores on the ventral surface near the bases of the sixth pair of legs and develop plate-like oostegites, which grow from the bases of the legs and which interleave to cover the marsupium. Males have two penes near the midline between the seventh (last) pair of legs. Usually the endopods of their second pleopods are drawn out on the inner side to form an 'appendix masculina'.

To copulate, the male of *A. physodes* moves beneath the female and turns over (Legrand, 1952). The male of *Elthusa vulgaris* moves from the gills to the buccal cavity to reach the female (Brusca, 1978b). Copulation occurs before the oostegites cover the gonopores (Szidat, 1966; Brusca, 1978a).

It has been known for over a century that cymothoids are protandrous hermaphrodites (Bullar, 1876; Mayer, 1879; Legrand, 1951, 1952; Fryer, 1968a). Sex reversal occurs in a single moult in *Nerocila acuminata* at one of several moults (Brusca, 1978a). Inversion depends on the inhibitory influence of the female partner. The inhibition seems

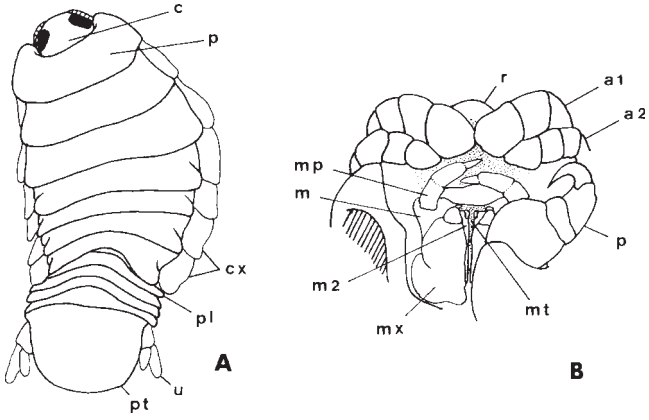


Fig. 14.30. Cymothoid morphology. A. *Mothocya rosea* dorsal view. c, cephalon; cx, coxae; p, first perionite; pt, pleotelson; u, uropod rami. (After Bruce, 1986a.) B. *Ceratothoa guttata* mouthparts. a1, a2, first and second antennae; m, mandible; m2, second maxilla; mp, mandibular palp; mt, mouth; mx, maxilliped; p, first pereopod (leg); pl, pleonite; r, rostrum. (After Bruce and Bowman, 1989.)

less effective in those species where the male retains swimming ability. Raibaut and Trilles (1993) suggest that the female cymothoid 'almost certainly secretes a sexual pheromone, transported by the blood of the fish which acts on the neurosecretory system of the male inducing it to secrete a masculinizing hormone'. When the female dies, the inhibition disappears.

Mothocya bohlkeorum and *A. prionuri* probably have multiple broods, as large mature females are found with and without oostegites (Williams and Williams, 1982, 1986). During its lifespan of less than a year, *A. pomacentri* has three broods (Adlard and Lester, 1995).

Fertilized ova pass into the marsupium of the female cymothoid, where they embryonate, hatch and moult once or twice (Hatai and Yasumoto, 1980; L.-T. Chang, personal communication). The resultant 'pullus II' or 'manca' has six rather than seven pairs of legs, two large compound eyes and setose pleopods, uropods and telson. They leave the marsupium in groups and swim rapidly, frequently against the current and towards the light, at least for the first day (Sandifer and Kerby, 1983; Williams, L.B. and Williams, 1985; Segal, 1987; Adlard and Lester, 1995). They attach to a variety of fish (Lindsay and Moran, 1976; Waugh *et al.*, 1989) and attempt to feed over the next 2 to 3 days. Mancae of *E. vulgaris* appear to be attracted by fish mucus (Moser and Sakanari, 1985), while those of *A. pomacentri* only feed on *Chromis nitida* less than 30 mm long, apparently because the mancae are unable to dislodge scales of larger fish to reach the muscle beneath. Muscle fibres are visible in the gut of feeding mancae within 2 days and blood cells within 3 days (Adlard and Lester, 1995). Mancae of *N. acuminata* feed on fish and then drop off and moult on the bottom of the tank (Segal, 1987). Like other isopods, they moult in two halves, the posterior half first.

In skin-inhabiting forms, the stages following mancae have not been confirmed experimentally. In species such as *Anilocra* spp., the males retain their swimming capability and micropredatory habit, and are rarely found on host fish. Males of *O. praegustator* and *Livoneca redmanii* (as *Litoneca ovalis*) are rare on fish in the Delaware estuary, though they are common in plankton tows, 47 males of the first species being taken in one 5 min tow (Kroger and Guthrie, 1972b; Lindsay and Moran, 1976). Even in more sedentary species, such as the gill-inhabiting male of *Livoneca papernea*, the male retains its swimming ability (Colorni *et al.*, 1997). These males only lose their ability to swim when they start to change into females. In many other species, mature males are found permanently attached to fish alongside females. Adult females are permanently attached non-swimmers.

The buccal-inhabiting *Ceratothoa oestroides* can apparently develop on a single host and does not need to transfer between hosts unless the host dies (Mladineo, 2003). Mancae of this species attached to fish and migrated under the operculum within 2 h. Only two isopods persist in a fish; additional isopods are lost, indicating a strong competitive mechanism (Mladineo and Valic, 2002). Mancae of the burrowing *Ichthyoxenus fushanensis* attach to many species of fish but only burrow into *Varicorhinus barbatulus*. Those that fail to enter a body cavity die within a week (Tsai and Dai, 1999).

Though there are exceptions (Hamann, 1997), the length of females is usually strongly correlated with host length (Montalenti, 1948; Menzies *et al.*, 1955; Weinstein and Heck, 1977; Maxwell, 1982; Alvarez and Flores, 1997), suggesting early and long infection. Some males show a similar correlation. Trilles (1964a) used the strength of the male correlation in six species as an indication of whether males moved between hosts and hence of their swimming ability. Williams and Bunkley-Williams (2000) suggest that female isopods in the mouth or gill cavities always grow quickly to fill available space. External isopods are not space-limited and their lengths do not always correlate with host length. Certainly, experimental studies on *C. oestroides* suggest that the host species or size has a major influence on development time. Mancae of *C. oestroides* on *Sparus aurata* fry developed into males within 2 months but had still not matured into females after

4 months; yet a male on 1-year-old *Diplodus annularis* had apparently developed into a female and been fertilized and a new generation of mancae was produced all within 29 days (Mladineo, 2003).

Estimates of the lifespan of adult females vary from up to the life of the fish, e.g. 9 years for *C. imbricatus* (see Maxwell, 1982) to 1 year or less for *L. redmanii, N. orbignyi* and *A. pomacentri* (Sadzikowski and Wallace, 1974; Bragoni *et al.*, 1984; Adlard and Lester, 1995). In salmon (*S. salar*) in southern Chile, *C. gaudichaudii* appears to have a 2-year life cycle (Inostroza *et al.*, 1993) and thus is rarely found to mature on salmon, which are kept in cages for only 1.5 to 1.7 years, though it will mature on brood stock kept for a longer time (Sievers *et al.*, 1997; G. Sievers, personal communication).

Host–parasite relationships

Site and host selection

Adult females either are attached to the skin, gills and buccal cavity or burrow into the fish and develop in a pouch. In all cases, they almost invariably show strong site specificity. Many species on the skin are above the eye, below the eye or on the caudal peduncle. Those in the gills are frequently asymmetrically shaped to fit the curve of the gill chamber.

Adult females of buccal species are usually attached to the floor of the mouth, though some, such as the giant isopods *E. splendida* and *Elthusa neocyttus*, are attached to the roof (Sadowsky and Soares Moreira, 1981; Stephenson, 1987). *Olencira praegustator* is twisted, the anterior part attaching to the roof and the posterior to the side (Turner and Roe, 1967). *Ceratothoa oestroides* sometimes attaches to the tongue, but more often to the roof (Vu-Tan-Tue, 1963; Trilles, 1964b).

Most buccal isopods face towards the mouth opening, sometimes being heavily pigmented anteriorly and colourless posteriorly (Colorni *et al.*, 1997). A few face towards the throat, such as *Enispa convexa* and *Asotana magnifica* (Menzies *et al.*, 1955; Thatcher, 1988). *Elthusa splendida* faces sometimes anteriorly and sometimes posteriorly (Sadowsky and Soares Moreira, 1981), and in *O. praegustator* immature females face posteriorly while adults face anteriorly (Kroger and Guthrie, 1972b). *Ceratothora gaudichaudii* attaches to the inner surface of the mouth or, less frequently, the gills (Sievers *et al.*, 1995).

Pouch-dwelling forms normally penetrate somewhere on the ventral surface of the fish and project into the body cavity, leaving their pleopods in contact with the outside.

Juvenile stages frequently show little host specificity and will feed on a range of fishes (Lindsay and Moran, 1976; Segal, 1987; Waugh *et al.*, 1989; Adlard and Lester, 1994). Juveniles of *E. vulgaris* on their final host attached first to the body and then over the next few hours to days crawled into the gill cavity (Brusca, 1978b).

The prevalence of the parasites may be underestimated in trawl samples, as Robinson (1981) showed that small males of *E. vulgaris* rapidly abandoned fish in trawls.

Clinical signs and pathology

BEHAVIOUR. Fish frequently react violently to mancae and juveniles that are attempting to attach. Sandifer and Kerby (1983) found that mancae from *L. redmanii* caused striped bass (*Morone saxatilis*) 60–70 mm long to leap and thrash about the tank. Segal (1987) described how seven species of fish when attacked broke surface, swam rapidly, thrashed their bodies, rubbed against objects and dived into the sand to dislodge the parasites. He also observed that the reactions were more violent if the fish had already experienced a manca attack. Mladineo and Valic (2002) remarked that fish fry seemed to be frightened of the mancae of *C. oestroides*. Adlard and Lester (1994) reported that *Chromis nitida* attacked by mancae of *A. pomacentri* rubbed so violently against objects that skin haemorrhages appeared. Longer-term behavioural changes were reported by Guthrie and Kroger (1974), who found that menhaden parasitized by *O. praegustator* tended to stay in nursery areas and had difficulty avoiding surface trawls. Adult isopods generally cause little

irritation except when they move, as during copulation (Legrand, 1952).

GROSS PATHOLOGY. There is rarely an open wound associated with permanently attached parasites. Adult cymothoids feed intermittently and the feeding lesions heal over between meals, a phenomenon first noticed by Trilles (1968) and confirmed by Romestand (1979). Where damage is reported, such as the damage to scales, epidermis, dermis and muscle of anemone fish under adult *Renocila heterozota* (see Bowman and Mariscal, 1968), the observation may have been made shortly after the parasite had fed.

Healing lesions have been reported from under the mouthparts of several skin-dwelling isopods. Morton (1974) found that *Nerocila phaeopleura* on the caudal peduncle of *Sardinella* was associated with a shield-shaped wound. Williams and Williams (1981) observed that *Anilocra myripristis*, attached near the eye of a soldier fish, *Myripristis jacobus*, caused erosion of scales and loss of pigment, presumably a result of dermis removal and repair. Beneath *Anilocra haemuli*, the skin of *Haemulon* spp. had turned from brown to orange and some bone deformation was observed. *Nerocila acuminata* on the caudal peduncle of a serranid was associated with skin lesions up to 0.5 mm across, which Rand (1986) concluded were probably old feeding wounds. He also observed loss of pigment in skin around the pereopods.

Isopods in the gill cavity are frequently associated with loss of gill filaments as a result of the feeding actions of the parasites and irritation from their presence (Menzies *et al.*, 1955; Turner and Roe, 1967; Kroger and Guthrie, 1972b; Guthrie and Kroger, 1974; Sadzikowski and Wallace, 1974; Williams and Williams, 1982, 1985a, Colorni *et al.*, 1997). Filaments take longer to regenerate than dermis and damage to filaments has been used to indicate former infestation (Stephenson, 1976). Just the presence of the parasites causes pressure atrophy. In *Illisha melastoma* with *Joryma brachysoma*, many gill rakers and gill filaments were atrophied (Ravichandran *et al.*, 1999). In *Gymnothorax eurostus* almost all the gill filaments were missing from gill chambers occupied by *Ichthyoxenus puhi*; this extreme damage was presumably why only one gill cavity per fish was found to be parasitized (Bowman, 1960).

Cuna insularis in the gill chamber of *Abudefduf saxatilis* in the West Indies eroded the wall to such an extent that it exposed the heart (Williams and Williams, 1985a).

The damage done by isopods in the buccal cavity is usually less noticeable but more chronic. Weinstein and Heck (1977) reported a little scar tissue on the tongue under *Cymothoa excisa.* Sadowsky and Soares Moreira (1981) observed a darkened area beneath the mouth of *Elthusa splendida* but no break in the skin. Stephenson (1987) noticed slight skin abrasion together with a patch of clotted blood under the mouthparts of *E. neocyttus.* Thatcher (1988) noted only a small indentation in the floor of the mouths of piranhas with *Asotana magnifica.*

However, some species cause the tongue to degenerate. *C. excisa* and *Cymothoa exigua* caused partial and total degeneration, respectively, in lutjanids (Weinstein and Heck, 1977; Brusca and Gilligan, 1983). Romestand and Trilles (1977b) described the regression of the tongue of the sparid *Boops boops* infected with *Ceratothoa oestroides.* The tongue first became narrow and then shorter until 50% of the tongue was lost.

B. boops with either *C. oestroides* or *Ceratothoa parallela* attached to the roof of the mouth developed a ventrally curved rather than flat vomer bone and a large patch of vomerine teeth, which are not found in unparasitized fish (Vu-Tan-Tue, 1963; Romestand and Trilles, 1977b). Similar changes occurred in *Spicara* spp. with the same parasites (Trilles, 1964b), and in *Maena smaris* with *Emetha audouini* (Romestand, 1979).

The pleopods of buccal parasites often contact the gill arches and their continuous beating irritates the tissue. The presence of *C. imbricatus* causes a callus-like thickening (dystrophic calcification) over the inner edges of the gill rakers and branchial arch in *Trachurus declivis* (Stephenson, 1976). *Livoneca papernea* also causes gill rakers of infected atherinids to become thicker,

shorter, deformed and calcified (Colorni *et al.*, 1997). *Joryma brachysoma* in the buccal cavity of *I. mellastoma* causes callus-like thickenings on the gill arch (Ravichandran *et al.*, 1999).

The pouch of pouch-dwelling genera is formed from an invagination of the skin. It develops over several months as the growing isopod pushes its way into the body wall. The pouch is kept open to the outside by the hindmost legs and the continual movement of pleopods (Huizinga, 1972). When *Artystone minima* penetrates near a pectoral fin, the fin is eventually lost (Thatcher and Carvalho, 1988). The parasite lies at right angles to the main axis of the fish and, though it does not penetrate the peritoneum, it damages the liver, stomach and intestine by pressure atrophy (Schaefer, 1993). Species of the South American freshwater genera *Riggia* and *Artystone* penetrate directly into the peritoneal cavity and do not lie in pouches, nor do they elicit much other response from the host. They usually lie near the liver with their posterior extremity projecting out of the entrance hole. They cause pressure atrophy of the liver but do not stimulate an inflammatory reaction or encapsulation by the host. There is also no evidence of secondary infection at the entrance (Thatcher, 2000).

Mancae and juveniles are aggressive feeders. Within 48 h of attachment, mancae of *Anilocra pomacentri* produce an ulcer up to 2.5 mm across, which penetrates through the epidermis and dermis and into the muscle (Adlard and Lester, 1995). A larva of *Artystone trysibia* in the orbital cavity of experimentally exposed catfish causes severe haemorrhage, which destroys the eye (Huizinga, 1972). Juveniles of *E. audouini* cause deep ulcerations in the head, expose muscle and cause haemorrrhages and ocular injuries in sea bass, *Dicentrarchus labrax*, in cages (Papapanagiotou *et al.*, 1999). Juveniles of *C. gaudichaudii* attack net-caged salmon and cause ulcerations and haemorrhage (Fig. 14.31).

HISTOPATHOLOGY. Inflammation, if present, is typically localized and adjacent to the mouthparts. Adult females of *Anilocra physodes* on *M. maena* were associated with loss of scales and dermal layers and an influx of erythrocytes, eosinophils and lymphocytes into adjacent epidermis, dermis and superficial layers of muscle (Romestand and Trilles, 1977b).

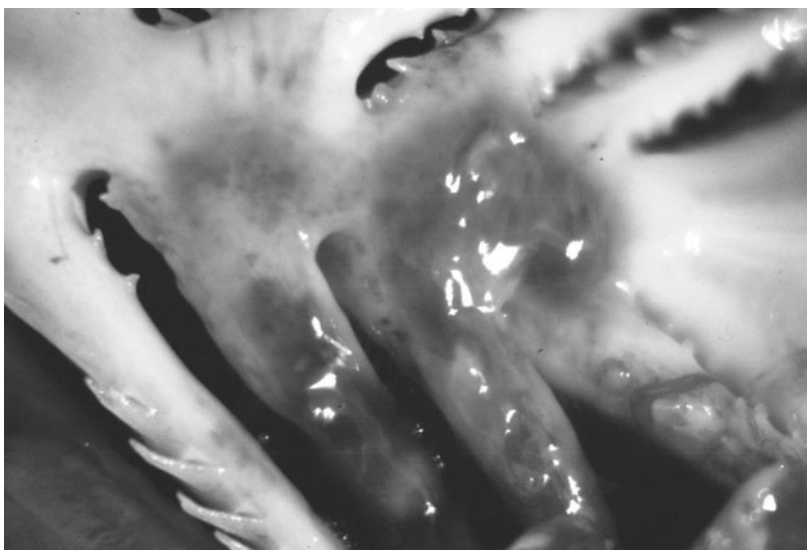


Fig. 14.31. *Salmo salar* buccal area. Ulceration and haemorrhage caused by *Ceratothoa gaudichaudii* in Chile (from Sievers *et al.*, 1996).

The regression of the tongue of *B. boops* parasitized by *C. oestroides* involves osteoclasts and granuloma formation (Romestand and Trilles, 1977b). In those species that form pouches within the fish, such as *Ourozeuktes* and *lchthyoxenus*, the wall of the pouch is thin and membranous, apart from near the mouthparts, where it is roughened and thickened. It is formed from skin components and contains epidermis, fibrous connective tissue, muscle and, in some cases, pigment cells and scale primordia (Hale, 1929; Akhmerov, 1939; Huizinga, 1972; Avdeev, 1983).

Host immune response

Precipitating antibodies to the haemolymph of *Anilocra*, *Nerocila*, *Mothocya* and especially the buccal isopods *Emetha* and *Ceratothoa* spp. were demonstrated in the blood of uninfected wild-caught fish by Romestand and Trilles (1975). These had low specificity. It was suggested that the antibodies were partly protective as the fish species that produced them usually carried only one or two isopods whereas *Mugil cephalus*, which had no demonstrable antibody, carried up to 12 *N. orbignyi* per fish.

Effects on hosts

Romestand and Trilles (1977a) and Romestand (1979) found that *A. physodes* and *Ceratothoa* spp. caused the haematocrit and erythrocyte count to decrease and the erythroblast count and spleen size to increase in three species of fishes. Infected fish also had reduced lipid in the serum and liver (Romestand, 1979). *N. orbignyi* on caged sea bass caused hypochromic macrocytic anaemia, a decrease in blood protein, blood lipids and triglycerides and an increase in urea. The conditions were reversed after the parasites were lost (Bragoni *et al.*, 1983). Caged bass with *C. oestroides* also developed anaemia (Horton and Okamura, 2003). *I. melastoma* with *J. brachysoma* had extremely pale gills, indicating anaemia (Ravichandran *et al.*, 1999).

In wild populations of fish, some cymothoids apparently have little effect on host weight, examples being *Cymothoa excisa* in the buccal cavity of lutjanids (Weinstein and Heck, 1977), *Ceratothoa imbricatus* in jack mackerel (Maxwell, 1982), *L. papernea* on an atherinid (Colorni *et al.*, 1997) and *Mothocya epimerica* on smelt (Leonardos and Trilles, 2003). Others are believed to cause a loss of weight. These parasites include: *Leuciscus waleckii* with *Ichthyoxenus amurensis* (see Petrushevsky and Shulman, 1961), *Hemirhamphus sajori* with *Irona melanosticta* (probably *Mothocya sajori*; see Bruce, 1986a; Hattori and Seki, 1956), *Morone americana* with *Livoneca redmanii* (see Sadzikowski and Wallace, 1974), *Girella tricuspidata* with more than two *C. imbricatus* (see Lanzing and O'Connor, 1975), *Pagellus erythrinus* with *A. physodes* (see Romestand and Trilles, 1979), *B. boops* with *C. oestroides* or *C. parallela* (see Romestand and Trilles, 1979), *D. labrax* with *N. orbignyi* (see Bragoni *et al.*, 1983) and *Pachymetopon blochii* with *Anilocra capensis* (see Wright *et al.*, 2001). Weight reduction in bluefish with *L. ovalis* was 3% or 0% according to Marks *et al.* (1996) and Landau *et al.* (1995), respectively.

When age is taken into account, several workers have shown that parasitized fish grow more slowly than uninfected fish (Sadzikowsky and Wallace, 1974; Romestand, 1979; Romestand and Trilles, 1979; Hatai and Yasumoto, 1981, 1982a; Adlard and Lester, 1994; Lester and Roubal, 1995). This is particularly evident in aquaculture. In the Mediterranean, *C. oestroides* reduces the growth rate of cultured sea bass (*D. labrax*), especially of large fingerlings (Fig. 14.32; Sarusic, 1999; Horton and Okamura, 2001; Mladineo, 2002). In Chile, Atlantic salmon (*S. salar*) with fewer than three *C. gaudichaudii* gained a fifth more weight than those with more than eight parasites. Weight loss was thought to be mostly due to damage on the gills when the parasites settled (Sievers *et al.*, 1996).

Krykhtin estimated that 13% of the coregonids in the Amur River, Russia, died before reaching marketable size as a result of *Ichthyoxenus amurensis* (see Petrushevsky and Shulman, 1961). Bragoni *et al.* (1984) found that 7% and 18% of

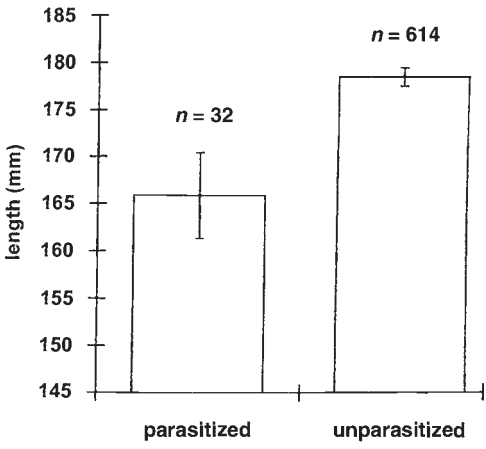


Fig. 14.32. Effect of *Ceratothoa oestroides* on growth of sea bass *Dicentrarchus labrax* in Turkey (from Horton and Okamura, 2001).

mortalities in two cages of sea bass were due to *N. orbignyi*, which had apparently been carried into the vicinity by migrating mullet. Other adult isopods seem to have little direct effect on mortality, but the larvae of many species are highly pathogenic to small fish (Lindsay and Moran, 1976; Sandifer and Kerby, 1983; Segal, 1987; Adlard and Lester, 1994).

Isopods are an emerging problem in fishes in Mediterranean sea cages. In Greece, eight to ten manca of *E. audouini* in buccal and branchial cavities of 30-g sea bass (*D. labrax*) caused 10% mortality (Papapanagiotou *et al.*, 1999). Mancae and juveniles of *C. parallela*, apparently from wild sparids, attacked 2-g fry of *Sparus aurata* and caused 50% mortality in 2 months (Papapanagiotou and Trilles, 2001). In Croatia, Sarusic (1999) reported 10–20% loss of fingerlings of bass and bream from damage to gills caused by juveniles of *C. oestroides*.

Hattori and Seki (1956) found that the drop in condition factor of adult female *H. sajori* parasitized by *Mothocya sajori* caused a decrease in the size of the gonad. Adlard and Lester (1994) observed that few oocytes were produced by *C. nitida* carrying *A. pomacentri*. Their computer simulation of the parasitized population showed that the reduction in fecundity had a greater impact on host numbers than the increased mortality caused by feeding manca stages. Presumably the relatively large amounts of blood taken by the parasites produce a nutritional drain on the host and this inhibits full development of the ovaries.

Concurrent infection

The rapid healing of skin feeding sites is probably the reason why few secondary infections occur, though Rand (1986) noticed a mixed population of Gram negative bacteria near the edge of one lesion, and Ravichandran *et al.* (2001) found that feeding lesions of *N. phaeopleura* had high *Vibrio* counts. Lawler *et al.* (1974) in Mississippi found lymphocystis in the gills of 21 perch, 20 of which had *L. redmanii*. They concluded that the isopod was probably not a vector but that it irritated the skin and thus allowed the virus to enter. *C. gaudichaudii* is possibly involved in the transmission of *Piscirickettsia salmonis* (see Kent and Poppe, 1998).

Fish with isopods are less able to withstand other forms of stress. Keys (1928) observed that killifishes, *Fundulus parvipinnis*, with *Elthusa californico* died more quickly under low oxygen conditions or in low salinity than uninfested fish. Lewis and Hettler (1968) found that menhaden with *Oleucira praegustator* with related gill damage did not survive well at 34°C compared with non-parasitized fish.

In vitro culture

Eggs removed from brood pouches of *M. parvostis* were raised in sea water and hatched to produce larvae infective to fish (Hatai and Yasumoto, 1980).

Parasite nutrition and physiology

Cymothoids feed on blood and macerated tissue. They use their sturdy maxillipeds to tear into the host to release blood which they suck up with their muscular

oesophagus. An anticoagulant is produced by the lateral oesophageal glands (Romestand and Trilles, 1976; Romestand, 1979). Haematin, a by-product of blood digestion, was found in the intestinal caeca (hepatopancreas) though not in the intestine or posterior intestinal diverticula of *A. physodes* (Romestand, 1979).

Cymothoids feed intermittently (Trilles, 1968; Romestand, 1979), sometimes only after a long interval. The intake of nutrients is cyclic and closely tied to the moult cycle and sexual development. Adult females release mancae, moult and then feed (Trilles and Hipeau-Jacquotte, 1996). Female *C. oestroides* feeds after the larvae have left the brood pouch and before its ovary enlarges for the next brood. No significant feeding is thought to occur at other times. The intestinal caeca, swollen and dark brown at the start of the reproductive cycle, gradually become clearer and smaller as eggs develop (Romestand *et al.*, 1982). In *A. pomacentri*, 3 days after larvae were released the parent moulted and shortly thereafter host blood was observed within the three pairs of intestinal caeca. A new brood of eggs appeared in the brood pouch 18 days later without further ingestion of blood. The entire reproductive cycle took 2 months (Adlard and Lester, 1995).

Prevention and control

Formalin was used to treat cultured pompano infested with cymothoids (Brusca, 1981), though neither it nor antibiotics were effective against mancae of *C. parallela* (see Papapanagiotou and Trilles, 2001). Hatai and Yasumoto (1980) used trichlorphon at 100 ppm for 5 min to remove *M. parvostis* from *Seriola quinqueradiata.* Another pesticide, methyl isoxathion, was used by Hatai and Yasumoto (1982b) at a dose of 1 ppm for 2 to 5 min to remove the same parasite from *G. punctata.* Athanassopoulou *et al.* (2001) recommended 0.05 mg/l of pyrethroid (Deltamethrin) for 2 h. The drug has already proved to be useful against salmon lice. Sievers *et al.* (1995) found 60 min baths with 300 ppm trichlorfon (Neguvon) or 3 ppm dichlorvos (Nuvan 1000) were 100% effective against *C. gaudichaudii*. Salmon cultured in sea cages in Tasmania are given freshwater baths in the summer to control amoebic gill disease. This also controls the isopods (*C.* cf. *imbricata*), which tend to attach in late summer but leave the fish in freshwater baths. Alternatively, one treatment with dichlorvos in late summer (1.5 ppm at 15.5°C or 2.0 ppm at 11°C for 1 h) has proved effective (C. Foster, personal communication).

Bragoni *et al.* (1984) recommended using fine mesh nets in the vicinity of aquaculture cages to keep out mullet, believed to be the source of *N. orbignyi* infection in cultured bass, *D. labrax.* To avoid *Emetha audouini*, cages containing sea bass were moved away from the shore into deep water with a strong current (Papapanagiotou *et al.*, 1999).

Williams and Williams (1979) and Bunkley Williams and Williams (1998b) observed that the cleaner shrimp *Periclimenes pedersoni* removed juvenile *Anilocra* sp. from French grunt, *Haemulon flavolineatum*, in the laboratory. The cleaner shrimp removed isopods almost immediately and returned to the aquarium floor to eat them. Williams *et al.* (1994) hypothesized that heavy infection of a sea-caged *Caranx hippos* with *Cymothoa oestrum* was the result of the fish being unable to get to a cleaning station. Thorsen and Trilles (2002) observed that *A. capensis* (as *A. physodes*) was less abundant on reefs with abundant cleaner fish (*Coris julis* and *Thalassoma pavo*) than on reefs with few cleaner fish.

OTHER FLABELLIFERA

Members of the Aegidae, Corallanidae and Cirolanidae are sometimes found in association with fish. They differ from the cymothoids in that the last four pairs of legs are not prehensile. The first three pairs may be strongly prehensile, as in the Aegidae. Aegids do not appear to be protandrous hermaphrodites (Wagele, 1990). For keys to families and genera, see Delaney and Brusca (1985), Delaney (1989) and Kensley and Schotte (1989).

The non-cymothoid Flabellifera are mostly free-living. A few, particularly in the Aegidae, appear to be parasitic. On the Great Barrier Reef two species, an aegid, *Aega lethrina*, and a corallanid, *Argathona macronema*, are common in the nasal passages of serranids and lutjanids (Bruce, 1983). Some Flabellifera are micropredators, which temporarily attach to fish to feed. The aegid *Rocinela belliceps* attacks, takes blood and kills young pink salmon, *Oncorhynchus gorbuscha*, on the Pacific coast of the USA (Novotny and Mahnken, 1971). *Rocinella angustata* from Alaskan sablefish readily attack *Sebastes* and *Blepsias* spp. under laboratory conditions. They mostly attack at night and those on the body remain for 4 to 14 days, after which their intestines contain haemoglobin. After they leave the fish, the intestine clears within a day and they reattach within 1 to 4 days. They actively swim to fish (Wing and Moles, 1995). *Aega antarctica* is a very inactive species and usually crawls on to a flatfish to feed. A single meal can take from 6 to 10 months to digest (Wagele, 1990).

The aegid *Alitropus typus* is widespread in the tropical Indo-Pacific in brackish water. It attacks the gills, fins and body surface of many fishes, including *Chanos chanos*, *Channa striatus* and species of *Tilapia*, *Mugil*, *Gerres, Lebistes* and *Oreochromis* (Nair and Nair, 1983; Kabata, 1985; Ho and Tonguthai, 1992). In the Nabagonga River, Bangladesh, farmers with cages reported that fish culture is impossible in early spring because the fish are attacked by large numbers of isopods, resulting in anaemia and death within 24 h. Once the monsoon arrives, the parasite disappears. The large amount of aquatic vegetation in the river may be related to the abundance of the parasite (McAndrew, 2002). In the laboratory, young isopods attach to *C. striatus* for 1–2 days, during which they feed. They then leave the fish for 1–2 days, reattach, feed again and leave the fish a second time. Infected fish develop macrocytic hypochromic anaemia during the feeding periods and begin to recover afterwards. Small fish in the field die from the attacks (Nair and Nair, 1983). In aquaria 15–20 isopods will kill 50–80 mm tilapia within 5 to 6 h (Chinabut, 2002). Fenitrothion, dichlorvos, malathion, ediphenphos and phosphomidon will kill 50% of the parasites in 2 days at dosages of 0.001, 0.009, 0.05, 0.28 and 5.19 ppm, respectively (Nair and Nair, 1982). Chinabut (2002) suggests stocking an isopod predator, such as a plankton-eating fish or cleaner fish, in reservoirs to reduce the prevalence of young isopods.

In Western Australia, *Aega cyclops* is commonly observed in wild dhufish, *Glaucosoma hebraicum.* They typically emerge from fish nostrils during freshwater treatment following post-capture anaesthesia. The treatment is fresh water with 300 mg/l 2-phenoxyethanol for 1.5 h (Pironet and Jones, 2000).

The blind aegid, *Syscenus infelix*, attaches behind the dorsal fin of benthopelagic rat-tails (macrourids). It erodes the skin and results in a pale area except for black pigment around the mouthparts and along the line of dactyls. Fish with healing scars are observed. It appears to attach to fish early in life and remain for an extended period (Ross *et al.*, 2001). Another aegid with a long life history is *A. antarctica*. It was estimated that mature males are more than 5 years old and spawning females more than 10 years old. The sexes are separate and not protandrous hermaphrodites like cymothoids (Wagele, 1990).

No cirolanids are true parasites, though *Cirolana* spp. are widespread scavengers and are sometimes encountered deep in the tissues of dead and dying teleosts and elasmobranchs (Bird, 1981; Berland, 1983). In southern California fish in cages died within a few hours after sunset from attacks by *Cirolana diminuta* and an ostracod *Vargula tsujii*. The isopods cause extensive gill damage. To avoid these micropredators, fish swim well off the bottom at night, bury themselves in sand or secrete a protective envelope (Stepien and Brusca, 1985). *Cirolana kiliani* introduced into a Dutch commercial aquarium irritated and injured fish to such an extent that grouper and eel were driven out of their hiding places at night and had to move off the bottom. Food items became rapidly infested, causing them to be rejected

by the fish and the starving fish eventually contracted *Amyloodinium*. There may be 110,000 *C. kiliani*/m^2 in the coral on the bottom of the tanks. Trichlorfon 0.6 ppm for 6 h eradicated the micropredators (Dral *et al.*, 2001).

ISOPODA: GNATHIIDAE

Introduction

Gnathiids are blood-feeding marine isopods (with five pairs of legs), which have been reported to cause mortality in captured eels (*Anguilla anguilla*), sea-caged mullet (*Crenimugil crenilabis*) and sea-caged snapper (*Pagrus major*) (Paperna and Overstreet, 1981; Mugridge and Stallybrass, 1983; Patarnello *et al.*, 1995).

Gnathiids infest many species of marine and estuarine teleosts and elasmobranchs. In the laboratory, larvae of *Paragnathia formica* infect fishes of the families Ammodytidae, Anguillidae, Gasterosteidae, Labridae, Cyprinidae, Triglidae, Cottidae, Gobiidae, Pleuronectidae, Trachinidae and Callionymidae and even a freshwater amphibian (*Rana temporaria*). They do not attack several invertebrates or humans (Monod, 1926). Some species of *Gnathia* infest only elasmobranchs (Paperna and Por, 1977; Honma *et al.*, 1991). The group is believed to have radiated in the cold waters of the southern hemisphere (Cohen and Poore, 1994).

The 160 or so species described are based on the characters of the free-living adult males. The third-stage larvae from fish can be kept *in vitro* in sea water until they moult into adults, the males of which can then be identified (Hesse, 1855; Stoll, 1962; Paperna and Por, 1977; Wagele, 1988, 1990; Smit and Basson, 2002).

Morphology and life cycle

Eggs of *P. formica* and *Gnathia calva* hatch into the first of three larval stages, all of which are parasitic (Stoll, 1962; Wagele, 1988; Figs 14.33 and 14.34). Unfed larvae

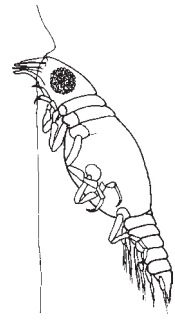


Fig. 14.33. *Paragnathia formica* attached to host (after Monod, 1926).



Fig. 14.34. *Ganthia* sp. praniza larvae on *Mugil subviridis* (photograph courtesy of I. Paperna).

are referred to as 'zuphea'. Zuphea swim very rapidly, using their pleopods. If the first zuphea contacts a fish, it attaches and feeds, becoming the first 'praniza' stage. After engorging, it leaves the fish, moults on the bottom to second zuphea and then reattaches and repeats the cycle twice more (Fig. 14.35). The third and last praniza leaves the fish and moults into an adult female or male. Additional moult stages have been reported. The eggs of *Gnathia piscivora* hatch into a stage that does not feed but moults after 7 days into the first zuphea, which is parasitic (Paperna and Por, 1977), and the last praniza stage in *G. calva* may moult first into a premale if a male is already present (Wagele, 1988). Adult gnathiids do not feed but live in small colonies of several females and usually a single male on the seabed in burrows, crevices, dead barnacles or sponges.

The duration of the life cycle varies widely between species. It takes about a year in *P. formica*; the first two praniza last 6 to 7 weeks each, the third up to 4 months and adults live for 12 to 20 months (Stoll, 1962; Amanieu, 1963). In *G. calva*, an Antarctic

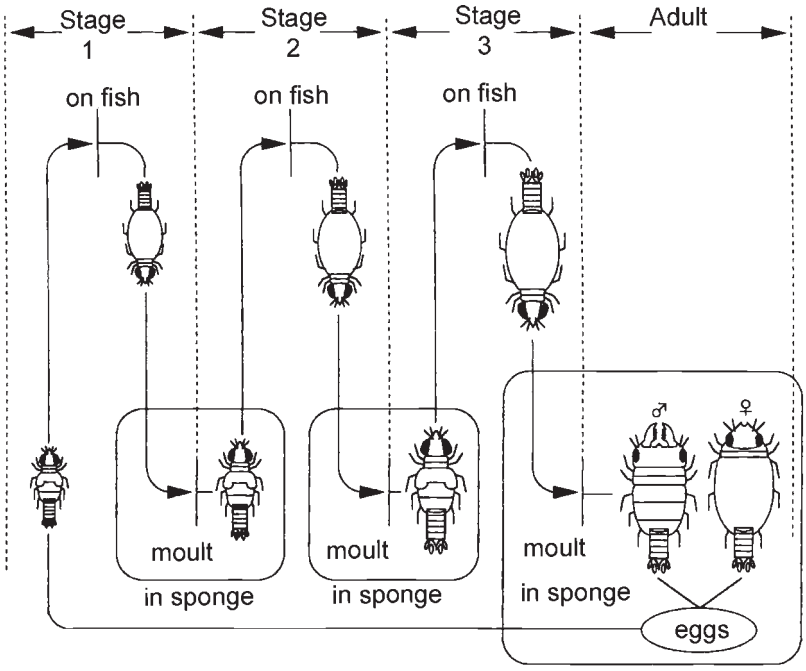


Fig. 14.35. Diagrammatic life cycle of a gnathiid isopod (after Tanaka, 1998).

species, the life cycle takes several years. The eggs are incubated for about a year and the larvae take 4 to 5 years to reach maturity. Males may survive a further 2 years as the guardian of a harem (Wagele, 1988). In contrast, the life cycles of the temperate water species *Gnathia africana* and *Elaphognathia cornigera* take 2 months at 18–25°C, with about 8 days between each moult (Tanaka and Aoki, 2000; Smit *et al.*, 2003). The duration of the third praniza stage of *G. piscivora* at 24°C was 7–10 days (Paperna and Por, 1977). Males of *P. formica* live twice as long as females and mate with females of the next generation (Upton, 1987a; Tinsley and Reilly, 2002).

Host–parasite relationships

Site and host selection, course of infection

Praniza larvae that attack elasmobranchs attach mainly to gills and gill septa (Paperna and Por, 1977; Lester and Sewell, 1989; Honma and Chiba, 1991) or around the cloaca (Heupel and Bennett, 1999). Those that attack teleosts will attach almost anywhere if scales are absent or poorly developed; otherwise they attach to the fins, gills or buccal cavity (Monod, 1926; Mugridge and Stallybrass, 1983). Larvae from elasmobranchs moult only into adults (Smit and Basson, 2002; R.J.G. Lester personal observations). Where praniza 1 and 2 are found is a mystery.

Some gnathiids have a clear diurnal rhythm, attacking fish only during the day or only at dusk or at night and hiding on the bottom at other times (Paperna and Por, 1977; Grutter *et al.*, 2000). They can be a particular problem in aquaria (Marino *et al.*, 2004).

Clinical signs and histopathology

Fish behave erratically to dislodge newly attached parasites, though this reaction lasts only a few minutes. The mouthparts of the parasite penetrate the epidermis and cause haemorrhage from vessels in the dermis. Some species feed for only a few hours. In Israel, fish in cages or entangled in gill

nets developed dermal haemorrhages after attack by praniza. They were stressed and became anaemic (Paperna and Por, 1977). In aquaria, heavily infected fish became anaemic (Jones and Grutter, 2005), localized pigment loss occurred in the skin and there were necrosis and fusion in the gills (Marino *et al.*, 2004).

The penetration site on a young eel parasitized 4 days previously was raised 0.5 mm diameter as a result of epithelial hyperplasia and an accumulation of lymphocytes and eosinophils (Monod, 1926). The feeding site of *Gnathia* sp. on the gill of an epaulette shark was associated with disruption of lamellae and loss of epithelium (Heupel and Bennett, 1999). There was little evidence of inflammation, possibly because the parasite had been attached for only a short time.

Some gnathiids on rays remain in position for days, if not weeks, and inflammation develops around their feeding site. The sting rays *Dasyatis akajei* and *Dasyatis matsubarai* with praniza in Japan show reddish marks at the site. The adjacent dermis is vacuolated and filled with masses of intact and lysing erythrocytes. Macrophages, neutrophils, lymphocytes and thrombocytes are abundant (Honma and Chiba, 1991; Honma *et al.*, 1991).

Parasite nutrition

The praniza is attached by its hooked rostrum, the hooked gnathopods (= 'pylopod' of Wagele, a modified first pair of legs) and the hooked and serrated but immobile mandibles. Skin penetration is performed by the maxillules, which make bursts of rapid tearing movements. A strong muscular oesophagus draws blood into the proventriculus, apparently aided by salivary glands that produce anticoagulants. The anterior hind gut is rapidly filled, aided by the unfolding of the bellows-like cuticle of the midpereon. Digestion takes place in the two sac-like digestive glands (Juilfs and Wagele, 1987; Giannetto *et al.*, 2003). Symbiotic bacteria up to 10 μm long occur in the rectal vesicle of *G. calva* (Juilfs and Wagele, 1987).

Gnathia maxillaris on blennies take 2–24 h to feed (Monod, 1926; Davies, 1981). The feeding times for the three praniza stages of *G. africana* are approximately 2, 3 and 10 h (Smit *et al.*, 2003). *G. piscivora* that attach to the skin leave the host within 2–4 h, while those that attach to gills and the walls of the gill cavity remain for 1 or more days (Paperna and Por, 1977; Paperna and Overstreet, 1981).

Concurrent infection

Foraminiferans, especially *Cibicides wuellerstorfi*, occur on the dorsal and ventral surfaces of the head of male *Gnathia stygia*, presumably as a result of a poorly groomed head projecting from the burrow (Svavarsson and Davidsdottir, 1994). *Gnathia maxillaris* that fed on blennies infected with *Haemogregarina bigemina* developed gametocytes in the hind gut, suggesting that they could be a vector for the blood parasite (Davies *et al.*, 1994). Davies and Smit (2001) suggested that transmission might be through ingestion of infected isopods.

Prevention and control

Free-swimming gnathiids are eaten by small fish (Tanaka, 2002). Gnathiids on fish are taken by the cleaner fish *Labroides dimidiatus*. In the Caribbean, a cleaner had over 100 gnathiids in its stomach (Williams and Williams, 1986). On the Great Barrier Reef, *L. dimidiatus* reduced gnathiid abundance on teleosts by 75% (Grutter, 1999). Large cleaners ate larger gnathiids (Grutter, 2000) and parasitized fish were more attracted to cleaners than unparasitized fish (Grutter, 2001). Cleaner shrimps also remove gnathiids (Becker and Grutter, 2004). In the UK, the abundance of *P. formica* was apparently limited by the availability of well-drained burrows for adults and resting larvae, and in Japan numbers of *E. cornigera* were related to the availability of the sponge *Halichondria okadai* (Upton, 1987b; Tanaka and Aoki, 1998). In aquaculture, the numbers of *Paragnathia* sp. on *A. anguilla* were controlled

by changing the incoming water from sea to fresh (Mugridge and Stallybrass, 1983).

Cirripedia: *Anelasma*

Introduction

Lepadid barnacles of the genus *Anelasma* parasitize deepwater sharks of the subfamily Etmopterinae worldwide (Yano and Musick, 2000). The parasites typically attach to the body behind one of the dorsal spines, to the dorsal fins or sometimes to other fins, the claspers or elsewhere on the body. They usually occur in pairs of similar size side by side. One species has been described, *Anelasma squalicola* (Loven, 1844).

Parasite morphology and life cycle

The parasite has a purplish-black flexible mantle with roots growing out from the creamy yellow peduncle into the muscles of the fish. The exposed region, partly covered by the mantle, can be over 36 mm and contains a prosbosciform mouth, six pairs of biramous appendages, a penis and egg sacs (Figs 14.36 and 14.37). The soft peduncle may make up to half of the animal and contains the ovaries. Egg sacs are usually white, though orange egg sacs were reported by Long and Waggoner (1993).

Eggs are found throughout the year. They hatch to release naupliar larvae, which contain yolk, have no eyespot and no functional mouth and are poor swimmers (Frost, 1928; Hickling, 1963). Presumably nauplii moult to infective cyprids (Yano and Musick, 2000).

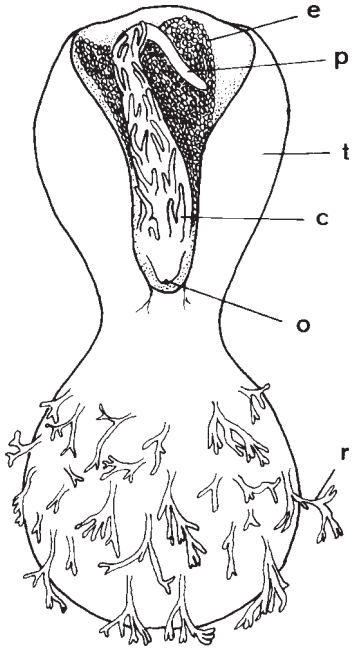


Fig. 14.36. *Anelasma squalicola*, anterior view. c, biramous appendage (cirrus); e, egg sac; o, mouth; p, penis; r, roots; t, mantle (after Johnstone and Frost, 1927).

Host–parasite relationships

Parasite prevalence peaked on fish (*Etmopterus spinax*) of 25 to 29 cm and then decreased. The low prevalence in large fish plus the absence of evidence of parasite loss suggested to Hickling (1963) that parasitized fish had a higher mortality rate than uninfected fish.

The parasite projects from the skin at an angle. A granuloma develops around its peduncle to cause an obvious swelling. Adjacent muscle is replaced by fibrous connective tissue. The epithelium around the parasite is intact and epithelial tongues grow down into the cavity. The parasite's 'roots' may extend 10 mm into the muscle; adjacent to them are degenerating muscle fibres, an increased number of blood vessels and fibrous tissue typical of chronic inflammation (Johnstone and Frost, 1927).

Though the livers of infested fish 41 to 44 cm long were 10% lighter than those of uninfested fish of the same length (Hickling, 1963), the main effect of the parasites seems to be on the reproductive organs. Hickling (1963) found that, in mature female *E. spinax*, 98% of those parasitized had inactive ovaries, compared with 42% in non-parasitized fish. In mature males, 86% had 'reduced' testes, compared with 0.05% in unparasitized fish. Yano and Musick (2000) reported that the ova of a



Fig. 14.37. *Anelasma* sp. A pair in pockets anterior to the dorsal fin of *Etmopterus granulosus* from Tasmania. The mantle has been partly withdrawn to reveal the visceral mass and white egg sacs. In the left animal, the cirri (arrow) are extended. The mouth is near the base of the cirri close to the surface of the fish.

parasitized *Etmopterus unicolor* female were much smaller and whiter than the large yellow ova of non-parasitized fish. The testes of infected *E. unicolor* and *Etmopterus granulosus* were much smaller than those of unparasitized fish, though clasper length was unaffected.

Parasite nutrition, physiology

The thin cuticle over the peduncle, the branching root system and the physiological changes in infected hosts strongly suggest that the parasite absorbs nutrient from the host. Furthermore, the lack of spines or setae on the legs suggests that these do not carry out the normal cirripedian function of filtering water for food particles. However, the well-developed mouth and digestive gland in young parasites and the degenerate digestive tubules in older animals suggest that the main mode of nutrition may change during development, as proposed by Broch in 1918 (see Johnstone and Frost, 1927).

Summary, Conclusions and Recommendations

The increase in marine aquaculture has provided much new information about the biology of parasitic Crustacea. This is especially true of the parasitic isopods. Conclusions from classic earlier work on isopods, particularly the French school, including Monod, Legrand, Trilles and Romestand, have been vindicated and sometimes extended. However, many questions remain.

One aspect that has become particularly striking is the apparent interaction between parasitic crustaceans. Males of the gnathiid isopod *G. calva* are inhibited from developing in the presence of another male. Males of many cymothoids are inhibited from changing into a female if a female is present elsewhere on or in the fish. Often only two isopods persist, a male and a female. Similarly, the parasitic barnacle, *Anelasma*, is usually found in pairs, side by side. If a pheromone is involved in limiting these numbers, is it transmitted though the blood of the host, as suggested by Raibaut and Trilles (1993), or carried in the water? There must be a strong mechanism or mechanisms operating to produce these results. Its elucidation may provide an additional tool in controlling parasitic disease.

Lernaea cyprinacea has been a problem in aquaculture for centuries and is likely to be around for many more years. Details of its biology that continue to remain clouded include the host specificity of the copepodid stage. Is it weak, as suggested by Grabda (1963), or strong, as suggested by the data of Haley and Winn (1959)? Wilson (1917) stated that females were fertilized at the last copepodid stage, but Grabda (1963) found no copepodids with spermatophores, only

pre-metamorphosed females. Fryer (1966) suggested that *Lernaea bagri* were fertilized not in the gills but on the final host as he recovered lernaeid males from a plankton sample. It is not known whether males feed.

The taxonomy and life cycles of *Pennella* spp. need further attention. It should be relatively straightforward now to match sequences from the ribosomal DNA of adult pennellids to those from juvenile pennellids, which are abundant on the gills of Atlantic squid. The sequences will also give a basis to revise the genus. Perkins (1985) has shown that the complex head structures of pennellids may serve for much more than attachment. A good laboratory model is needed to further explore their function. Similarly, the function of the abdominal processes of *Pennella* and *Sphyrion* species are unclear. In *Sphyrion*, do contractions of the prominent dorsoventral muscles cause haemolymph to flow into the branched appendages?

Sphyrion lumpi has a low host specificity and thus may lend itself to ready establishment of a laboratory population to investigate its physiology. Its life cycle, how it penetrates into the fish and whether it only feeds in the initial phases of infection are all unclear. A short laboratory study plus analysis of field data will reveal how long females survive and how long the scars persist. Analysis of growth rings on scales or otoliths of infected and uninfected fish may indicate whether the parasite has any effect on the growth rate of the fish. *Sarcotaces verrucosus* is another copepod with low host specificity. Its life cycle, mode of nutrition and effect on the fish need elucidation. Laboratory infections will elucidate the reason for the strong host specificity in *Lernaeocera* species, particularly in view of Slinn's (1970) observation that *L. lusci* matured on its first host under experimental conditions.

The parasitic barnacle *A. squalicola* continues to intrigue but we are not much closer to understanding its biology, especially its mode of nutrition, than we were in 1927.

The taxonomy of parasitic isopods, which are an emerging problem in aquaculture in the tropics and subtropics, needs revision, particularly the genera *Ceratothoa, Cymothoa* and related forms.

One or a small number of parasites frequently kill small fish, often without evidence of much tissue damage. For example, small numbers of argulids, cymothoids and caligoids may all kill small fish without noticeable histopathology. The fish are often highly agitated, suggesting neural disturbance, i.e. pain, leading to rapid muscular exercise, from which the fish do not recover. Better understanding of the phenomenon may help to avoid mortalities from such parasites.

Control

In wild fish stocks, the main tool we have for controlling infection is fishing pressure. An increase of this will decrease both the population density and the average age of the fish and this may be sufficient to eliminate the parasite (Forrester, 1956). In many cases increasing fishing pressure is not an option. In other cases, it needs to be applied selectively, perhaps to a reservoir host or only to areas where the parasite is abundant.

In aquaculture, there are more opportunities for parasite control. There are also more opportunities for normally benign parasites to cause disease. It is likely that only a rolling combination of methods will limit the effects of the highly evolved multicellular organisms that are the parasitic Crustacea. Husbandry methods, such as quarantine procedures and adjustment of water flow, are widely employed. However, information such as the factors that control the numbers of argulids in natural fish populations is currently unknown. Multispecies systems, such as wrasse in salmon farms to control sea lice, may have applicability in the control of parasites such as argulids and lernaeids.

Chemicals are widely used to control infections. The rapidity with which lernaeids, argulids and, to a lesser extent, caligoids develop resistance to insecticides suggests that we need to look for alternative ways of controlling infections, especially as some

naupliar stages seem to be totally resistant. For lernaeids, the demonstration that fish can acquire immunity, though in what form is not yet known, opens up what may be the best long-term solution: the enhancement of resistance in fish. This may be through controlled exposures to the parasite, vaccination or genetic transformation, as discussed below.

The copepodid stages of species of lernaeids and pennellids develop on gills. It is not known whether immunity to this stage develops in fish. If it can be stimulated, either naturally or through vaccination, it could help to control the parasite. The high rejection rate of metamorphosing females of *L. branchialis* in multiple infections suggests a hypersensitive reaction. If one is present, it may lead to ways to protect the fish immunologically.

Fish antibodies themselves may not be very effective in removing the parasite, even if active against a hidden antigen, because the parasites have evolved ways to deal with teleost antibodies. We may be more successful if we introduce a gene from a vertebrate that manufactures a different type of antibody. Genetic transformation opens up other possibilities. The epidermal cells, the first point of contact for a parasitic copepod, may be modified to produce a secretion distasteful to the copepod or to block receptors on the copepod used in host recognition. The transformation may be applied topically by dipping, perhaps using an epithelial virus as a vector, or by producing transgenic offspring, in which all the epithelial cells would be modified. The large number of eggs produced by many fish makes them an ideal candidate for electroporation and biolistic methods of introducing DNA. These methods have already been used to introduce other genes, such as the gene for growth hormone. In the first edition of this volume, we proposed that, within a decade, developments in genetic transformation would lead to effective control of at least *Lepeophtheirus salmonis* in *Salmo salar*. This clearly has not occurred. It is now evident that controlling this and other parasitic crustaceans requires an evolving battery of chemical and biological weapons in an integrated programme of pest management, such as is applied in Ireland (McMahon, 2000) and proposed elsewhere (Mordue and Pike, 2002).

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15 Phylum Annelida: Hirudinea as Vectors and Disease Agents

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Introduction

Leeches are the only important fish pathogens in the phylum Annelida. Both freshwater and marine leeches have worldwide distribution and they occur in a diversity of habitats. Leeches can potentially affect the health of fishes in a variety of ways. Their most important role is as vectors of potentially pathogenic organisms. Both freshwater and marine leeches are known to transmit haemoflagellates of the genera *Trypanosoma* and *Cryptobia* (= *Trypanoplasma*) and the intracellular haemogregarines and piroplasmas to fish. There is accumulating evidence that leeches can also transmit viruses and bacteria. In addition, leeches may affect the host by the sheer amount of blood withdrawn during feeding. The feeding or attachment wounds caused by leeches may also serve as sites for secondary pathogenic invaders.

Aquatic leeches also serve as second intermediate hosts for Digenea (Chapter 10), harbouring metacercaria of a number of different species. Most of these worms are adults in waterfowl, but some mature in freshwater fishes. However, none of these Digenea has been implicated in pathology of fishes so they are not considered further in this review. Additional information on the role of leeches as intermediate hosts is in Sawyer (1986) or papers by Vojtek *et al*. (1967), Spelling and Young (1986a,b,c) and McCarthy (1990).

Extensive reviews on the general biology of leeches have been published (Mann, 1961; Sawyer, 1986). Pathological implications of marine leeches have been reviewed by Rohde (1984), and the role of leeches in the pathology of fish cultured in the tropics has been reviewed by Kabata (1985).

Host Range

Leeches that parasitize fishes are primarily in the families Glossiphoniidae and Piscicolidae. Glossiphoniid leeches occur on a wide variety of freshwater fishes, as well as other freshwater aquatic vertebrates. Piscicolid leeches occur on both freshwater and marine fishes. Freshwater fish leeches are most abundant in temperate lakes, ponds and streams, and occur worldwide on all continents except Antarctica. Marine leeches are known from all seas, but are most abundant in polar to temperate regions. They occur primarily on demersal species of all major fish groups, including lampreys, elasmobranchs and teleosts.

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Systematic and Taxonomic Position

Historically, leeches have been placed in the class Hirudinea within the phylum Annelida, subphylum Clitellata, and equal in rank to the class Oligochaeta. However, recent evidence from molecular phylogenetic studies suggests that leeches are specialized oligochaetes. Siddall *et al.* (2001), using sequence data from nuclear 18S ribosomal DNA (rDNA) and mitochondrial cytochrome *c* oxidase subunit I from 101 annelid species, found support for the hypothesis that Branchiobdellida, Acanthobdellida and Hirudinea, together with the oligochaete family Lumbriculidae, constitute a clade within Oligochaeta. Results supported synonymy of Clitellata and the more commonly used Oligochaeta, and suggested that leeches, branchiobdellidans and acanthobdellidans should be regarded as orders of oligochaetes equal to their closest relatives, the order Lumbriculida (Fig. 15.1).

The systematic relationships within the leeches and the evolution of blood feeding have also received much attention in recent years with the development of molecular phylogenetic methods (Apakupakul *et al.*, 1999; Trontelj *et al.*, 1999). Molecular data support many, but not all, of the traditional groupings that were based on morphology. Leeches are usually divided into two main groups, the Rhynchobdellida, which possess a protrusible proboscis, and the Arhynchobdellida, which lack a proboscis but have armed or unarmed jaws. Molecular data support monophyly of the Arhynchobdellida (Siddall *et al.*, 2001; Borda and Siddall, 2003), but many of the traditional groups within the Arhynchobdellida are not monophyletic (Fig. 15.2). For example, there is no support for monophyly of the families Haemadipsidae, Haemopidae or Hirudinidae or for the genus *Hirudo* (Borda and Siddall, 2003).

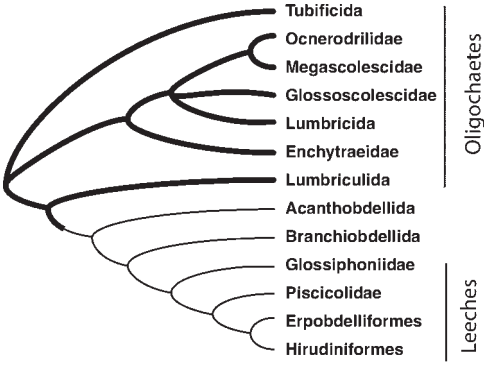


Fig. 15.1. Phylogenetic tree based on DNA sequence data showing that Acanthobdellida, Branchiobdellida and leeches are a clade within the Oligochaeta. Redrawn from Siddall *et al.*, 2001, with permission of *Molecular Phylogenetics and Evolution*.

Within the Rhynchobdellida, molecular phylogenetic analyses support monophyly for the families Glossiphoniidae (Light and Siddall, 1999; Siddall *et al.*, 2001) and Piscicolidae (Siddall *et al.*, 2001), but not for the Rhynchobdellida as a whole because Piscicolidae is a sister taxon to the Arhynchobdellida, not to Glossiphoniidae (Siddall *et al.*, 2001; Borda and Siddall, 2003). This latter result may be due, in part, to the poor taxon sampling in the family Piscicolidae, where sequences have been obtained for only a few species. Epshtein *et al.* (1994) proposed a large number of tribes within the family Piscicolidae based on morphology, but these need to be verified with molecular phylogenetic studies.

Molecular data have provided insight into taxonomic relationships for a few arhynchobdellid families that are problematic based on morphology, but have suggested that other, long-standing groups are now problematic (Borda and Siddall, 2003). Additional taxon sampling will probably alter some hypotheses suggested by the few studies conducted to date on all leech groups and, thus, formal taxonomic revisions have not been proposed by most authors.

Sanguivory is a common feature of many leeches and of all leeches that are parasitic on fish. Evolution of this behaviour has long been of interest. Two previous hypotheses speculated that there were independent origins of blood feeding in the Rhynchobdellida and Arhynchobdellida (Sawyer, 1986; Siddall and Burreson, 1996). More recent phylogenetic studies hypothesize that the ancestral leech was sanguivorous and that behaviour has been

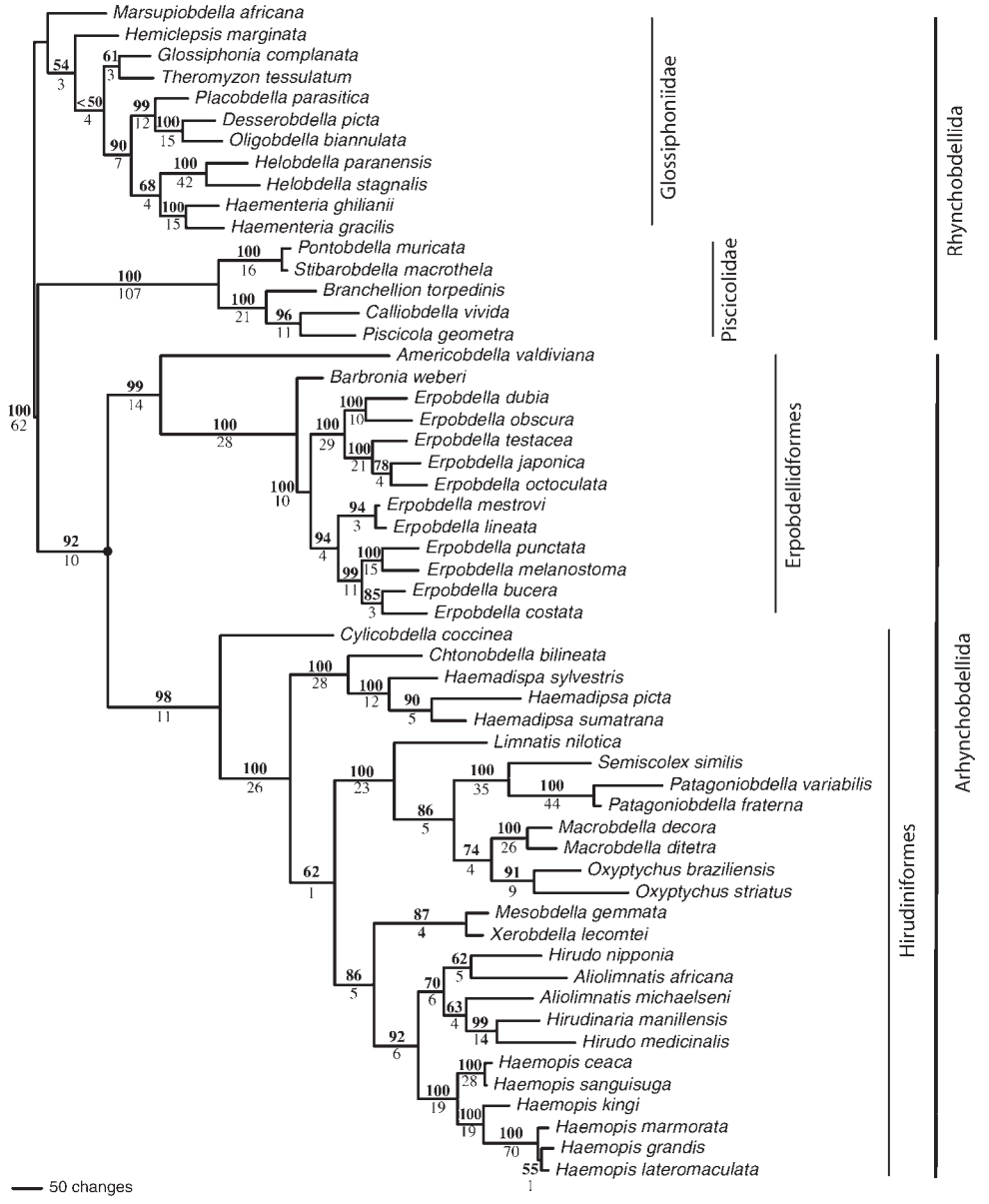


Fig. 15.2. Phylogenetic tree of the leeches demonstrating monophyly of the Glossiponiidae and Piscicolidae, but not of the Rhynchobdellida. From Borda and Siddall, 2003, courtesy of *Molecular Phylogenetics and Evolution*.

retained in all Piscicolidae and most Glossiphoniidae (Borda and Siddall, 2003). However, the ancestral feeding preference of the Arhynchobdellida cannot be determined from recent phylogenetic analyses (Borda and Siddall, 2003). Thus, it is not clear whether the ancestor was sanguivorous, with subsequent loss of blood feeding in the Erpobdellidormes, Haemopidae and other some groups, or whether the ancestor was predacious with reacquisition of sanguivory in the Hirudiniformes.

Parasite Morphology and Life Cycle

Leeches that are important in fish pathology are in the suborder Rhynchobdellida, families Glossiphoniidae and Piscicolidae. Body shapes and generalized internal anatomy of representative members of these two families are shown in Fig. 15.3.

Identification keys to freshwater leeches of the world and to marine leeches of the North Atlantic are in Sawyer (1986). Although dated, this is still the best key available for most areas of the world. Earlier keys to European and North American freshwater leeches and to the marine leech genera of the world are in Mann (1961). A key to genera of the family Piscicolidae can be found in Soós (1965). Keys to North American freshwater leeches are also in Klemm (1982) and Davies (1991), and keys to freshwater leeches of the former USSR are in Lukin (1976) and Epshtein (1987, Piscicolidae only). A key to marine leeches of the Indian Ocean can be found in Sanjeeva Raj (1974).

Leeches that feed on blood of fishes may be either temporary or semi-permanent parasites. Temporary parasites usually leave the host soon after a blood meal. They usually seek a sheltered location among vegetation or under stones to digest the blood meal. However, some estuarine and marine leeches that inhabit soft substrate areas utilize the hard carapace of crustaceans for attachment after leaving the fish host. Semi-permanent parasites remain on the fish and take successive blood meals; they leave the host only to deposit cocoons. Leeches are hermaphroditic and mating may involve copulation through the female gonopore or hypodermic implantation of spermatophores anywhere on the body. Mating may occur on or off the fish host, but cocoons are never deposited on the fish. The cocoons of piscicolid leeches, which usually adhere to a hard substrate, such as vegetation, rocks, shells, the carapace of live crustaceans or even fish eggs, typically contain a single egg. However, some species of *Malmiana* may deposit up to five eggs in each cocoon. Cocoons are left unattended and newly hatched piscicolid leeches must find a fish host on their own; juvenile leeches can usually survive for a week or more before their first blood meal. Hatching time is usually temperature-dependent, but some species may aestivate in the cocoon until appropriate environmental conditions reoccur. Unlike the piscicolids, glossiphoniid leeches brood their eggs in fragile cocoons, which, depending on the species, may be attached either to the substrate or to the ventral body surface of the leech. Newly hatched leeches attach to the parental ventral surface and are carried to a host for their first blood meal.

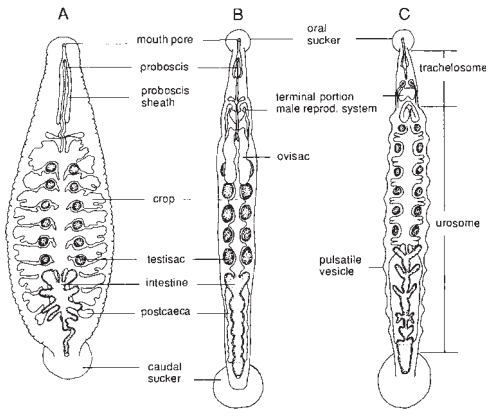


Fig. 15.3. Semi-diagrammatic representation of some rhynchobdellid leeches illustrating the main morphological features. A. Family Glossiphoniidae. B. Family Piscicolidae, subfamily Platybdellinae. C. Family Piscicolidae, subfamily Piscicolinae.

The annual cycle of piscicolid leeches is extremely variable and depends on habitat, geographical location and host biology. Most marine piscicolid leeches appear to have a synchronized annual cycle (Khan and Paul, 1995), with adult leeches dying after cocoon deposition. However, some, such as *Malmiana virida*, have successive generations and are present throughout the year (Burreson, 1977). *Piscicola geometra*, a widespread freshwater piscicolid leech, deposits cocoons in late winter and adults die by April. There are successive generations through the summer, but reproduction stops in September. Adults survive the winter to deposit cocoons in late winter (Malecha, 1984). The close tie that can occur between the leech life cycle and host occurrence is

perhaps best demonstrated by *Piscicola salmositica*, which parasitizes spawning salmon when they return from the sea to rivers of the Pacific North-west of North America. Leeches occur in high abundance in the rivers when salmon return in September and through December each year (Becker and Katz, 1965b). They feed on the salmon during autumn and then deposit cocoons before they die in December. The cocoons do not hatch until the following September, just as the annual salmon run begins again and approximately 300 days after cocoons were deposited. There does not seem to be any temperature or light cue to hatching (Bower and Thompson, 1987), so the timing may be innate and have evolved over time.

Based on the biology of *Hemiclepsis marginata*, the annual cycle of glossiphoniid leeches is similar to that of *P. geometra* and is cued by water temperature. Cocoons of *H. marginata* are deposited in late spring/early summer when water temperature reaches 15°C. Adult leeches brood the young, carry them to their first host after hatching and then die. Leeches feed and grow through the summer and autumn, overwinter under vegetation, and resume feeding in the spring prior to reproduction (Needham, 1969).

Leeches as Pathogens

Host–parasite relationships

Clinical signs and gross pathology

Leeches alone are generally not considered important fish pathogens. Effects are usually localized and restricted to attachment and/or feeding sites on the skin, fins, gills or mouth. The muscular caudal sucker used for attachment usually causes little damage; however, leeches that are semi-permanent parasites may elicit a substantial host tissue response at the attachment site. Rhynchobdellid leeches feed on host blood or tissue fluid by means of a protrusible proboscis, which is inserted into host tissue and this feeding activity may produce localized petechial haemorrhage (Sloan *et al.*, 1984; Jones and Woo, 1990b). Even though pathology is usually localized, heavy infestations can result in severe epidermal erosion and even mortality because of large amounts of blood loss or secondary effects of multiple feeding wounds. Thus, large numbers of leeches in aquaculture facilities should always be a cause for concern. Fish can tolerate a high burden of leeches with little apparent effect, but pathology may depend on the relative size of the leech compared with the fish. Kabata (1985), without providing details, reported that the presence of 100 leeches on a single fish in Africa resulted in no serious harm to the host. Other reports, however, suggest that leeches may be pathogenic or provide portals of entry through their feeding or attachment wounds for secondary pathogens. For example, general statements by Bauer (1961), Markevich (1963) and Bauer *et al.* (1973) suggest that *P. geometra* can severely affect carp and other species in rearing ponds in Eastern Europe by causing severe emaciation, small bleeding ulcers and secondary invasion by bacteria and fungi.

One of the earliest accounts of a leech causing mortality is the report by Badham (1916) involving the leech *Austrobdella translucens* and the sand whiting, *Sillago ciliata*, in Australia. A few dozen whiting were periodically stocked with other species in a saltwater pond over a number of years and on each occasion the whiting were killed by the leeches. The fish developed large ulcerated patches on the skin and badly infested fish harboured as many as 100 leeches on the fins and body surface. *A. translucens* was specific for sand whiting and other species of fish in the pond apparently survived well, suggesting that mortality of whiting was not the result of unfavourable water quality but from the effects of the leeches.

Leeches have recently been implicated in pathology of tank-reared orange-spotted grouper, *Epinephelus coioides*, in the Philippines. Heavy infestations of *Zeylanicobdella arugamensis* occurred in multiple patches of several hundred leeches on and at the bases of the fins and in the skin folds of the lower jaw (Fig. 15.4). The affected



Fig. 15.4. Heavy infestation of *Zeylanicobdella arugamensis* on cultured orange-spotted grouper in the Philippines. A. Entire fish showing clusters of hundreds of leeches (arrows). B. Close-up of the anal fin of fish shown in A illustrating masses of leeches (arrows). Photos courtesy of Erlinda Cruz-Lacierda, South-East Asian Fisheries Development Center, Iliolo, Philippines.

fish had multiple areas of haemorrhage and hyperplasia at attachment and feeding sites (Cruz-Lacierda *et al.*, 2000), but there was no report of mortality.

There have been a number of reports of leeches causing pathology in feral freshwater and estuarine fishes in North America. An epizootic of *Piscicola punctata* caused pathology in the bigmouth buffalo, *Ictiobus cyprinellus*, in the Rock River near Rockford, Illinois, USA (Thompson, 1927). During February and March of 1926, almost every fish was heavily infested with the leech; intensity ranged up to 50 leeches per fish taken from the river channel, and over 100 leeches per fish collected from backwaters and sloughs. It is interesting that the leech had not been observed during the previous two winters, even though sampling had been intensive, and fishermen reported no similar infestation in the last 50 years. Leeches were removed by fishermen prior to delivery to the retail market, but the raw and bleeding scars around the attachment sites made the fish difficult to sell. There was no mention of host mortality in the report. Leeches left the fish by the end of March when water temperature increased above 0°C. In late April fungal infections were observed on wounds apparently made by leeches. This leech outbreak was unusual, but there were no obvious, unusual environmental conditions known to be present that would have caused such an occurrence. Undoubtedly, a number of factors contributed to the greater than normal reproductive success and hence the high abundance.

Another unusual occurrence of fish mortality caused by leeches involved adult brook trout, *Salvelinus fontinalis*, and freshwater leeches in a small, shallow lake in Maine (Rupp and Meyer, 1954). During periods of hot weather, water temperature in the lake can become critically high and trout congregate around cooler underwater springs in shallow water. The congregating trout encouraged poaching and bird predation, so brush was placed in the lake to provide cover for the fish. Unfortunately, the brush provided an ideal habitat for two hirudinid leeches, *Macrobdella decora* and *Haemopis grandis*. These leeches are not normally associated with fish, but on one occasion 50 to 60 large trout were observed 'being fiercely attacked by hordes' of leeches from the brush. Despite continual harassment by the leeches, the trout remained congregated in the spring. On one occasion over 20 dead trout were reported during a 3-day period. One captured, dying trout had six leeches attached to the gill arches, isthmus and fin bases; one *M. decora* had rasped an opening through the body wall and into the ventral aorta. It appeared certain to the investigators that the fish was dying from loss of blood. This unusual occurrence was possible only because of high concentrations of fish and leeches in close proximity for prolonged periods.

Freshwater leeches have also caused mortality in fish hatcheries. A heavy infestation of *P. salmositica* caused mortality as high as 25% in sac fry of pink salmon, *Oncorhynchus gorbuscha*, in the state of Washington (Earp and Schwab, 1954).

Leeches invaded the hatchery through the freshwater supply from a nearby stream and attacked fry as they hatched in trays. Once attached to the fry, leeches quickly became gorged with blood and the fry invariably died, apparently from blood loss. Adult salmon migrants in the supply stream also had heavy infestations of *P. salmositica* and indications were that some fish died before spawning.

Leeches that are semi-permanent parasites on fishes, such as the freshwater and estuarine leech *Myzobdella lugubris*, tend to remain attached at a single site. They often cause very localized histopathological changes, including cellular infiltration, erosion of the integument under the attachment site and hyperplasia of the epidermis around the caudal sucker. Localized subcutaneous haemorrhages often occur at leech feeding sites. Paperna and Zwerner (1974) reported removing over 500 *M. lugubris* from a single moribund white catfish, *Ictalurus catus*, in the York River estuary in Virginia, USA. Leeches were in the mouth and under the operculum and externally on the skin fold behind the lower jaw and at the bases of the fins. Extensive histopathological changes caused by the leech included inflammation, displacement and erosion of the dermis and hyperplasia of the epithelium. All pathological changes were attributed to leeches and it was concluded that leeches were at least a major contributing factor to the distressed condition of the fish. The same leech was also implicated in an epidemic of oral ulcerations in adult largemouth bass, *Micropterus salmoides*, from Currituck Sound, North Carolina, USA (Noga *et al.*, 1990). A systematic survey was not conducted, but sport fishermen reports suggested that 90% of legal-size fish might have been affected. These fish had large ulcerations on the tongue and buccal cavity, often extending to underlying musculature; leeches were always present in or near the wounds. Localized pathology from *M. lugubris* also has been reported in logperch, *Percina caprodes*, and brown bullhead, *Ictalurus nebulosus*, in Ontario, Canada (Appy and Cone, 1982), and in mullet, *Mugil* sp. (Paperna and Overstreet, 1981). Heavy infestations of *M. lugubris* were reported in intensive striped bass (*Morone saxatilis*) culture facilities on Chesapeake Bay, Maryland, USA, but there was no mention of pathology (Woods *et al.*, 1990).

Similar pathological effects caused by *Austrobdella bilobata* have been reported in yellowfin bream, *Acanthopagrus australis*, in New South Wales, Australia (Roubal, 1986). Khan (1982) described subcutaneous lesions caused by *Johanssonia arctica* in Atlantic cod, *Gadus morhua.*

Effects on host physiology

Leeches have only rarely been implicated as serious pathogens of fishes. Their effects are usually localized to the feeding or attachment sites and only become serious when infestation is high. Documented fish mortality caused by leeches is extremely rare and appears to occur only when there is close, prolonged contact between large numbers of leeches and fish. Although a single leech may withdraw only a small amount of blood, it nevertheless weakens the host (Meyer, 1946a). The subtle effect of blood loss was elegantly demonstrated by Mace and Davis (1972), who studied the energetics of parasitism by the leech *Malmiana brunnea* on the shorthorn sculpin, *Myoxocephalus scorpius*, in Newfoundland, Canada. Energy budgets were developed for leeches and fish, and growth was measured in two groups of unparasitized fish for 5 weeks. Growth of a parasitized group was significantly lower than the expected growth and the difference was attributed to the energy requirements of the leeches. It was concluded that the additional energy consumption of the host because of the leeches was approximately 750 cal/g of leech per week. According to Mace and Davis (1972), a well-adapted host–parasite relationship should exert a metabolic demand that is little more than the energy requirements of the parasite. However, tissue damage, harmful metabolites and/or hormone leakage cause increased energy loss. They concluded that energy loss in sculpins was not entirely because of the energy requirements of the leech, but probably also because of increased physiological

strain from leech saliva secretions or undetected mechanical irritation of feeding leeches.

Diagnosis of infection

Leeches can be difficult to collect and to identify. They often leave the host after feeding and, therefore, may go undetected even when abundance is high. They are usually sufficiently large to be detected by the naked eye and occur on the body surface and fins or in the gill cavity or mouth. If possible, leeches should be collected by gently dislodging the caudal sucker with forceps and placing them in a dish containing water. For proper identification it is important to observe leeches alive and to note as many external characters as possible. It may be necessary to relax leeches in weak alcohol or another narcotizing agent prior to examination. Careful observation should be made of pigmentation colour and pattern, number and arrangement of eyes on the oral sucker, ocelli on the body and caudal sucker, number of lateral pulsatile vesicles, if present, and arrangements of papillae, tubercles or other obvious external characters. Leeches that have been fixed unrelaxed are almost impossible to identify because they usually contract strongly or curl into a tight ball, making observation of important characters difficult. Leeches relaxed prior to fixation in formalin will usually retain their pigmentation and eyes for long periods; however, pigmentation fades rapidly after transfer to alcohol. Leeches that have been fixed and then preserved in alcohol are often difficult to identify, especially to species. Generic determination of many leeches, especially piscicolids, may depend on internal anatomy, which can only be determined with serial sections. All these difficulties combine to make identification of leeches, even for experts, problematic.

Leeches as Vectors of Pathogens

Leeches are much more important as vectors of fish pathogens than as pathogens. Haematozoa of fishes are all transmitted by rhynchobdellid leeches and, although less well documented, leeches may transmit viruses and bacteria as well. As vectors for other organisms, leeches must feed on at least two different host individuals; they must acquire the organisms by feeding on an infected host and they must then leave that host and attach to and feed on a different host individual. Thus, leeches that are semi-permanent parasites tend to be less efficient vectors. A good example is *M. lugubris*, which is common in fresh water in the USA and in south-eastern estuaries. This leech occurs on a wide variety of hosts, many of which harbour haematozoa (E.M. Burreson, unpublished data), but it has never been implicated as a vector. Another example is *Oceanobdella pallida*, which occurs in the mouth of English sole, *Parophrys vetulus*, in the north-eastern Pacific. English sole are commonly infected with *Trypanosoma pacifica* off Oregon (Burreson and Pratt, 1972) and developmental stages have been observed in the crop of the leech, but never in the proboscis sheath. All attempts to transmit *T. pacifica* to uninfected sole with *O. pallida* failed because leeches removed from infected hosts could not be induced to reattach to other hosts (Burreson, 1975). The leech appears to be a semi-permanent parasite and is probably not the vector for *T. pacifica*. These results illustrate that the presence of developmental stages of haematozoa in a leech does not necessarily indicate that the leech is a vector.

Vectors for fish pathogens are restricted to the rhynchobdellid families Glossiphoniidae and Piscicolidae; however, within each family, behaviour of individual species and not taxonomic grouping determines their role as a vector. Thus, vectors are distributed throughout most subfamilies. Nevertheless, there appears to be a general trend of semi-permanent parasitism within the piscicolid subfamily Platybdellinae and a general trend of leaving the host after the blood meal in the subfamily Piscicolinae and possibly also the subfamily Pontobdellinae. Thus, one expects more vectors in the subfamily Piscicolinae than in the Platybdellinae, and this seems to be true, although it must be

noted that vectors have been identified for only a small number of fish haematozoa. Of the 21 genera in the subfamily Platybdellinae (Sawyer, 1986), only one genus, *Malmiana*, is known to have species that are vectors (Burreson, 1982; Siddall and Desser, 1993). However, Sloan *et al*. (1984) reported that 71.0% of the *Notostomum cyclostoma* (Johansson) (subfamily Platybdellinae) examined in northern British Columbia, Canada, harboured heavy infections of *Cryptobia* in the proboscis sheath and one leech had trypanosomes in the proboscis sheath. This leech is undoubtedly a vector, but the fish host of the flagellates is unknown. In addition, Siddall and Burreson (1994) found developmental stages of a haemogregarine in the salivary cells of *Platybdella* sp. from the Bering Sea, suggesting that it is a vector. Of the 15 genera in the subfamily Piscicolinae, five genera, *Calliobdella*, *Johanssonia*, *Cystobranchus*, *Piscicola* and *Orientobdella* have been documented as vectors, and *Calliobdella* and *Piscicola* have more than one species known to be vectors. Of the four genera in the subfamily Pontobdellinae (Sawyer, 1986), only one genus and species, *Pontobdella muricata*, has been definitively shown to be a vector, although *Oxytonostoma typica* is the likely vector of *Haemogregarina delagei* in *Raja erinacea* in New Brunswick, Canada (Siddall and Desser, 2001).

Leeches as vectors of viruses

There have only been two reports implicating fish leeches as vectors of fish viruses. Ahne (1985) demonstrated that *P. geometra* mechanically transmitted spring viraemia of carp virus (SVCV, *Rhabdovirus carpio*) to carp, *Cyprinus carpio*. Leeches acquired the virus from infected carp during the first feeding and transmitted the virus to uninfected carp during two successive feedings. However, leeches eventually lost the virus and carp used for the fourth feeding did not become infected. Ahne concluded that SVCV did not replicate in *P. geometra* and that the leech served only as a mechanical vector.

Mulcahy *et al*. (1990), on the other hand, present evidence that infectious haemopoietic necrosis (IHN) virus may replicate in the leech *P. salmositica*. They isolated IHN virus from *P. salmositica* collected on sockeye salmon, *Oncorhynchus nerka*, and from the stream bed of the Cedar River, Washington, USA. Leeches from salmon were isolated in laboratory containers and, over a 16-day period, the virus titre increased from 4.0×10 plaque-forming units (pfu) per gram to 6.5×10^3 pfu/g. This increase suggests that the virus may be replicating in the leeches; however, viral replication was not demonstrated with certainty. Prevalence of IHN virus in leeches from the stream bed decreased from 57.0% to 20.0% over 72 days, suggesting that the leeches were gradually losing the virus. Although no experiments were conducted using infected leeches to transmit the virus to uninfected fish, the authors concluded that the leeches probably increase the infection rate of the virus in spawning sockeye salmon. The virus apparently is not transmitted vertically in the leeches, as the virus was not isolated from leech cocoons.

Leeches as vectors of bacteria

Although there has been speculation that leeches are capable of transmitting pathogenic bacteria to fishes, there have been few cases of transmission actually documented (Cusack and Cone, 1986). Negele (1975) reported that *P. geometra* transmitted *Aeromonas hydrophila* to fish during the feeding process, but gave no details. Bragg *et al*. (1989) isolated a bacterium from the leech *Batracobdelloides tricarinata* that was biochemically and serologically identical to *Streptococcus* sp. pathogenic to rainbow trout. The authors proposed that the leech was a possible reservoir for the bacterium, but no transmission experiments were conducted. Dombrowski (1953) reported that *P. geometra* transmitted the pathogenic *Pseudomonas punctata* to carp.

Leeches, including the important fish parasites *P. geometra* and *H. marginata*,

harbour endosymbiotic bacteria in specialized anterior crop diverticula called mycetomes or oesophageal diverticula (Jennings and Van Der Lande, 1967; Sawyer, 1986; Siddall *et al.*, 2003). These bacteria aid in digestion of blood by providing normally deficient digestive enzymes; however, they may be mistaken for bacteria that are pathogenic to fish.

Leeches as vectors of haematozoic protists

According to Laveran and Mesnil (1912), Leydig first observed flagellates in the leeches *Piscicola* and *Pontobdella* in 1857, and Doflein in 1901 first suggested that leeches were vectors of fish haemoflagellates. This speculation was confirmed by a number of separate studies in the early 1900s (Lom, 1979). The leeches that have been ascertained to be vectors of fish haematozoa through experimental transmission studies are listed in Table 15.1. Although a large number of haemoflagellates have been described from fishes (Lom, 1979; Woo, 1987), very few vectors have been identified. Even less is known about leeches as vectors of intraerythrocytic parasites of fish. Although leeches have long been suspected as vectors of these parasites in fishes, the first life cycles were not confirmed until the early 1980s (Khan, 1980; Lainson, 1981). Much more research remains to be done, but it is apparent, especially from the work by Khan in Newfoundland, that very few species of leeches in a given area transmit many different haematozoa.

Of the leech vectors listed in Table 15.1, five of them are especially important, either because they have a wide geographical and host range or because they parasitize commercially important hosts. These are *H. marginata*, *P. geometra*, *J. arctica*, *Calliobdella vivida* and *P. salmositica*; each will be discussed in detail in the following sections.

Hemiclepsis marginata (*O.F. Müller*)

H. marginata transmits *Cryptobia borreli*, a known pathogen in European freshwater fish (Dyková and Lom, 1979). The life cycle of *C. borreli* and vector role of *H. marginata* were elucidated by Robertson (1911). This leech also transmits *Cryptobia ompoki* and *Trypanosoma punctati* in India (Shanavas *et al.*, 1989; Shanavas, 1991) and at least three trypanosomes in Europe, *Trypanosoma cobitis* in the stone loach, *Nemacheilus barbatulus* and other hosts (Letch, 1979, 1980), *Trypanosoma tincae* in the tench, *Tinca tinca* (see Needham, 1969) and *Trypanosoma granulosum* in eels, *Anguilla anguilla* (see Brumpt, 1906a,b). In addition, developmental stages of *Trypanosoma danilewskyi* were found in the proboscis sheath of *H. marginata*; however, experimental transmission to the fish was not attempted (Qadri, 1962).

H. marginata is widely distributed, from the UK through most of Europe (Harding, 1910; Wilkialis, 1970; Elliott and Mann, 1979) and Asia (Shulman, 1961; Khalifa, 1985; Sawyer, 1986; Singhal *et al.*, 1986; Shanavas *et al.*, 1989). It is abundant in small, hard-water ponds or slow-moving streams that contain abundant vegetation, especially large sheath-bearing plants; it does not occur in rapidly flowing water (Mann, 1955; Sawyer, 1986). Although apparently widespread, both Robertson (1911) and Needham (1969) had difficulty collecting *H. marginata*. From late spring to early autumn, *H. marginata* is primarily in the leaf bases of large marginal reeds; it is rarely found on fish. During winter *H. marginata* occurs among the plant rhizomes (Needham, 1969).

Long known as a fish parasite, *H. marginata* has been shown to feed on a wide variety of freshwater fishes under laboratory conditions (Robertson, 1911). Letch and Ball (1979) suggested that, because of habitat similarities and the inactive nature of the fish, *H. marginata* feeds primarily on the stone loach, *N. barbatulus*, bullhead, *Cottus gobio*, and gudgeon, *Gobio gobio*, under natural conditions in England. Fish hosts in culture ponds in India are listed in Singhal *et al.* (1986). After feeding, the leech leaves the host and seeks a concealed area among plant leaves to digest its blood meal.

Table 15.1. Leech vectors of fish haematozoa.

Leech species	Distribution and hosts	Haematozoa transmitted
Freshwater		
Glossiphoniidae		
Hemiclepsis marginata (O.F. Müller)	Western Europe to north-east Asia, India (Sawyer, 1986). Not host specific (Robertson, 1911; Letch and Ball, 1979)	*Trypanosoma punctati* (Shanavas, 1991); *Trypanosoma cobitis*, *Trypanosoma granulosum* and probably other trypanosomes (Brumpt, 1906a, b; Needham, 1969; Letch, 1980); *Cryptobia borreli* (Robertson, 1911), *Cryptobia ompoki* (Shanavas *et al.*, 1989)
Haementeria '*lutzi*' Pinto (according to Sawyer, 1986, this leech is *species inquirendae*)	Brazil on *Synbranchus marmoratus* (Lainson, 1981)	*Trypanosoma bourouli*, *Cyrilia gomesi* (Lainson, 1981)
Desserobdella phalera (Graf)	USA and Canada (Klemm, 1982) on bowfin, *Amia calva*, and largemouth bass, *Micropterus salmoides*, and other fishes (Jones and Woo, 1990a)	*Trypanosoma phalera* (Jones and Woo, 1990b)
Batracobdelloides tricarinata (Blanchard)	Africa on *Clarias lazera* (Negm-Eldin, 1997)	*Trypanosoma mukasai*, *Babesiosoma mariae* and *Cyrilia nili* (Negm-Eldin, 1997; Negm-Eldin and Davies, 1998)
Piscicolidae		
Piscicola geometra L.	Eurasia, North America (?). Not host specific (Sawyer, 1986; Epshtein, 1987; Madill, 1988).	*Cryptobia borreli*, *Trypanosoma danilewskyi* and possibly other trypanosomes (Brumpt, 1906b; Khaibulaev and Guseinov, 1982; Kruse *et al.*, 1989).
Piscicola salmositica Meyer	North-western North America, primarily on salmonids (*Oncorhynchus* spp.) and sculpins (*Cottus* spp.) (Becker and Katz, 1965b)	*Cryptobia salmositica* (Becker and Katz, 1965a)
Cystobranchus meyeri Hayunga and Grey	Eastern North America (range undetermined) on a variety of freshwater fishes (Hayunga and Grey, 1976)	*Cryptobia cataractae* (Putz, 1972a,b). Leech vector misidentified by Putz as *Cystobranchus virginicus* Hoffman

Continued

Table 15.1. *Continued*. Leech vectors of fish haematozoa.

Leech species	Distribution and hosts	Haematozoa transmitted
Marine		
Piscicolidae		
Pontobdella muricata L.	North-east Atlantic Ocean, Mediterranean Sea, on skates (*Raja* spp.) (Sawyer, 1986)	*Trypanosoma rajae* (Brumpt, 1906a; Robertson, 1907, 1909; Neumann, 1909)
Johanssonia arctica (Johansson)	Arctic seas, North Atlantic, north-east Pacific to California. Not host specific (Meyer and Khan, 1979; Khan, 1982; Madill, 1988; E.M. Burreson, unpublished data)	*Trypanosoma murmanensis*, *Haemohormidium terraenovae*, *Haemohormidium beckeri*, *Haemogregarina uncinata* (Khan, 1976, 1978a, 1980, 1984)
Calliobdella punctata Beneden and Hesse	North-east Atlantic Ocean. Distribution and hosts (*Enophrys bubalis*, *Blennius pholis*) poorly known (Sawyer, 1986)	*Trypanosoma cotti* (Brumpt, 1906a; Khan, 1978b)
Calliobdella vivida (Verrill)	East and Gulf coasts of North America, primarily in estuaries. Not host specific (Sawyer *et al*., 1975; Appy and Dadswell, 1981; Burreson and Zwerner, 1982; Madill, 1988)	*Cryptobia bullocki* (Burreson, 1982)
Malmiana diminuta Burreson	North-east Pacific. Not host specific (Burreson, 1977)	*Cryptobia* (?) *beckeri* in cabezon, *Scorpaenichthys marmoratus* (Burreson, 1979)
Malmiana scorpii (Malm)	North Atlantic on *Myoxocephalus octodecemspinosus* and *Myoxocephalus scorpius* (Khan and Meyer, 1976)	*Haemogregarina myoxocephali* (Siddall and Desser, 1993)

H. marginata is in the family Glossiphoniidae, subfamily Glossiphoniinae. Mature adults are up to 30 mm long by 7 mm wide; their colour is green to pale yellow, with seven longitudinal rows of yellow spots (Mann, 1961). There are usually two pairs of eyes on the head region, but the anterior pair may be coalesced. This species can easily be separated from other glossiphoniids by the expanded head and oral sucker region (Fig. 15.5), which, at rest, is wider than the body segments immediately posterior to it (Mann, 1961; Sawyer, 1986). A key to the recognized European glossiphoniids is in Sawyer (1986).

An annual life cycle for *H. marginata* has been proposed by Needham (1969). Eggs are laid in late spring and early summer when water temperature rises to 15°C. Cocoons are deposited on a substrate, usually the protective shelter of leaf bases of reeds, and are brooded by the adult. When eggs hatch, young leeches attach to the venter of the adult and are eventually carried to a fish host. On the host the young leeches leave the adult and take their first blood meal. The adult leech soon dies, and the young leave the fish host and return to the shelter of the reeds to digest their blood meal. A period of subsequent feeding and growth occurs through summer and early autumn. During winter, feeding is reduced or may cease, but large leeches can survive 10 months without feeding (Sawyer, 1986). As the water temperature increases in early spring, feeding activity increases prior to the breeding cycle. *H. marginata* is apparently relatively easy to maintain in the laboratory if vegetation is provided for cover.

Piscicola geometra *L.*

On the basis of flagellate developmental stages in the crop, *P. geometra* has long been implicated as a vector for fish trypanosomes and cryptobiids (Léger, 1904; Keysselitz, 1906;

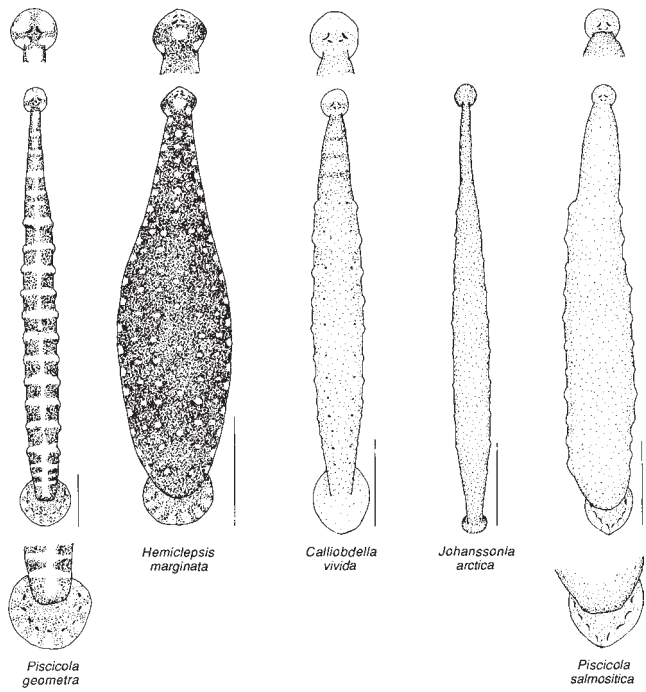


Fig. 15.5. Important leech vectors of pathogenic haematozoa in fishes. Vertical line beside each leech is 5.0 mm. See text for identification characters. *Hemiclepsis marginata* redrawn from Mann, 1961, courtesy of Pergamon Press; *Piscicola salmositica* redrawn from Klemm, 1982, courtesy of US Environmental Protection Agency.

Lom, 1979). However, none of the early researchers clearly documented transmission of either trypanosomes or cryptobiids to uninfected fishes through the feeding activity of *P. geometra*. For example, Brumpt (1905) discussed development of cryptobiids in *P. geometra* and *H. marginata* and stated that successful transmission was achieved using leeches, but he did not specifically state that transmission was successful using *P. geometra*. Keysselitz (1906) did not observe developmental stages of cryptobiids in the proboscis sheath of *P. geometra* and was unable to actually transmit the parasite to uninfected fish via leeches because of a lack of uninfected hosts. Brumpt (1906a) stated that *T. granulosum* developed in *P. geometra* for about 15 days, but all flagellates eventually died. Lom (1979) reported that *Trypanosoma danilewskyi* developed in *P. geometra*, but made no mention of successful transmission. Khaibulaev (1970) reported development of both trypanosomes and cryptobiids in *P. geometra*, but his paper is unaccompanied by figures and should be treated with scepticism. Needham (1969) reviewed the photographs in Khaibulaev's doctoral dissertation and reported that the flagellates observed in *P. geometra* appeared to be leech spermatozoa. In any case, Khaibulaev did not report transmission of flagellates to uninfected fish through the feeding activities of *P. geometra*. The only documented experimental transmission of *C. borreli* to uninfected fish through the feeding activity of *P. geometra* is the report by Kruse *et al.* (1989). *Cryptobia* infections in carp, *C. carpio*, were detected 6 days after feeding by infected leeches. They also confirmed the observation of Keysselitz (1906) that flagellates did not invade the proboscis sheath. The only evidence that *P. geometra* transmits trypanosomes to fish is the report by Khaibulaev and Guseinov (1982). They were apparently able to transmit *T. danilewskyi* to *Lebistes reticulatus*, *Trypanosoma percae* to *Cobitis taenia* and *Trypanosoma scardinii* to *Pseudorasbora parva* through the feeding activity of *P. geometra*.

P. geometra is a serious pest in commercial fish ponds (Bauer, 1961; Bielecki, 1988), but its vector relationships, especially for trypanosomes, remain unclear. Flagellates develop much higher abundance and persist for much longer in *H. marginata* than in *P. geometra* (Lom, 1979); in addition, *C. borreli* invades the proboscis sheath of *H. marginata*, but not *P. geometra*. These observations, all of which need confirmation, suggest that *H. marginata* is the natural vector for European freshwater haemoflagellates and that *P. geometra* has only secondarily acquired the ability to transmit flagellates and is less efficient.

P. geometra occurs throughout most of Eurasia (Shulman, 1961; Elliot and Mann, 1979; Sawyer, 1986; Epshtein, 1987) and may be circumpolar. There has been speculation (Klemm, 1982) that *Piscicola milneri* (Verrill), which is widely distributed in northern North America, is identical to *P. geometra*, and Sawyer (1986) states that *P. milneri* is inadequately distinguished from *P. geometra*. Nevertheless, Madill (1988) and Davies (1991) list both species in North America. *P. geometra* appears to be tolerant of diverse environmental conditions and has been collected in a wide range of habitats, including estuaries of the Caspian and Baltic Seas (Epshtein, 1987). However, *P. geometra* requires cool water that is relatively highly oxygenated and it is most abundant in rapidly flowing streams and large lakes where fish and vegetation are abundant (Mann, 1961; Sawyer, 1986). *P. geometra* is not found in stagnant ponds or slow-moving streams, the preferred habitat of *H. marginata*.

Over 30 species of fish have been recorded as hosts for *P. geometra*. Leeches feed anywhere on the body, including skin, fins, gills and mouth cavity, and they may remain on the host for successive blood meals. However, most leeches leave the host within 7 days after feeding (Sawyer, 1986).

P. geometra is in the family Piscicolidae, subfamily Piscicolinae. It is a cylindrical, elongate leech up to 50 mm in length, but usually 15–30 mm long with a caudal sucker diameter 1.5–2.0 times the maximum body width. The urosome diameter is nearly constant throughout its length. The body colour may be brownish olive, greenish or grey with segmental, unpigmented transverse bands and usually a longitudinal, mid-dorsal

unpigmented stripe. Two pairs of eyes are present on the oral sucker (Fig. 15.5) and 12 to 14 ocelli usually occur on the caudal sucker, alternating with radiating pigment bands. Recent keys that include *P. geometra* are in Sawyer (1986), Epshtein (1987) and Bielecki (1997).

The life cycle of *P. geometra* was studied extensively by Malecha (1984) in northern France. The lifespan of an individual leech is about 7 to 9 months and three to four generations are produced each year. The overwintering generation deposits cocoons during March and adults die between April and June. Cocoons are deposited on any hard substrate, including vegetation. Cocoons hatch in late April and early May and leeches reach sexual maturity during June. Adults reproduce more or less continuously through August, producing two or three generations. The winter generation emerges from cocoons in September and individuals feed on fish for much of the winter. No reproduction occurs during this period and leeches may become quite large compared with the summer generations. Reproduction eventually begins in late February and March, when cocoons that will become the next summer generation are deposited.

Johanssonia arctica (*Johansson*)

J. arctica is important as a vector for a variety of haematozoa in northern seas. It transmits *Trypanosoma murmanensis* to a number of commercially important hosts, including Atlantic cod, *G. morhua*, American plaice, *Hippoglossoides platessoides*, and yellowtail flounder, *Limanda ferruginea* in the northwest Atlantic (Khan, 1976, 1977, 1991). It also transmits the intraerythrocyte parasites *Haemohormidium beckeri* in perciform fishes (Khan, 1980) and *Haemohormidium terraenovae* in pleuronectiform fishes (Khan, 1984). In addition, *J. arctica* may transmit *Haemogregarina uncinata*; developmental stages were observed in the leech crop, but transmission was not demonstrated because of a lack of uninfected hosts (Khan, 1978a). Khan (1982) has speculated that *J. arctica* also transmits an undescribed cryptobiid. *J. arctica* is the only known vector for haematozoa in the Newfoundland region, even though it has a rich leech fauna (Khan and Meyer, 1976; Meyer and Khan, 1979; Appy and Dadswell, 1981). Early developmental stages of *H. beckeri* were observed (Khan, 1980) in another leech, *Platybdella olriki*, but the presence of developmental stages alone is not sufficient evidence of a vector role. Studies by Siddall and Desser (1992a, 1993) on the developmental sequence of *Haemogregarina myoxocephali* in *Malmiana scorpii* strongly suggest that this leech is the vector for the parasite; however, actual transmission to fish has not been attempted.

J. arctica is commonly encountered in Arctic seas (Epshtein, 1961, 1962), in the north-western Atlantic Ocean (Meyer and Khan, 1979; Khan, 1982) and in deep waters in the north-eastern Pacific Ocean as far south as California (E.M. Burreson, unpublished data). In Newfoundland, leeches have been collected in nature from Atlantic cod, *G. morhua*, polka-dot sea snail, *Liparis cyclostigma*, and American plaice, *H. platessoides*. In laboratory studies, 19 additional hosts were identified, but leeches seemed to prefer American plaice and Atlantic cod (Khan, 1982). Preferred feeding sites are the head of Atlantic cod and the dorsal and ventral fins of American plaice, although leeches also feed on other regions of the body.

J. arctica is in the family Piscicolidae, subfamily Piscicolinae. The species was originally described by Johansson (1899) from Greenland as *Oxytonostoma arctica*. Epshtein (1968) transferred the species to *Johanssonia* Selensky. Individuals are subcylindrical, up to 29 mm total length, with a narrow body and an especially narrow neck region. Unengorged leeches can appear almost thread-like. The body is devoid of pigmentation and lacks eyes on the oral sucker and ocelli on the body or caudal sucker; the integument is thin and transparent. The body has 11 pairs of very small, lateral, pulsatile vesicles and 12 longitudinal rows of small papillae; both vesicles and papillae may be difficult to discern in preserved specimens. The oral sucker, attached strongly eccentrically, is wider than the maximum body width of unengorged individuals,

an unusual character. The caudal sucker is relatively small and approximately the same size as the oral sucker (Fig. 15.5). Recent keys that include *J. arctica* are in Appy and Dadswell (1981) and in Sawyer (1986). An excellent photograph of *J. arctica* appears in Sawyer (1986); the drawings of the suckers in Appy and Dadswell (1981) are more representative of the actual leech than those in Meyer and Khan (1979).

The life cycle of *J. arctica* in Newfoundland waters has been well studied (Khan, 1982; Khan and Paul, 1995). The leech needs a stable physical environment, with relatively constant temperatures of –1 to 2°C, and its life cycle involves little seasonality. Laboratory experiments show that leeches cannot tolerate water temperature greater than 5°C. Leeches mature after four blood meals, and mating and cocoon deposition take place on the spider crab, *Chionoecetes opilio*, and occasionally on the toad crab, *Hyas coarctatus*, or the sea spider, *Nymphon* sp. In the north-eastern Pacific, cocoons of *J. arctica* have been observed on the tanner crab, *Chionoecetes tanneri* (E.M. Burreson, unpublished data). In the laboratory, engorged leeches, both mature and immature, rapidly leave the fish host and attach to crabs, which serve as a hard substrate for attachment in an otherwise muddy environment. Cocoons are deposited on the ventral femoral surfaces of the first and second walking legs. One naturally infested crab harboured 1246 cocoons (Khan, 1982). At ambient temperatures of –1 to 2°C, cocoons hatched in 176 to 262 days. In laboratory experiments, newly hatched leeches held at 0 to 1°C began depositing cocoons after the fourth blood meal and deposited from two to 26 cocoons after each feeding through eight blood meals, at which time the leeches were 2 years old. Average clutch size was 16 cocoons and a mean of 62 cocoons per leech was produced over the 2-year period. In nature, young leeches were observed throughout the year, but were most abundant from May through October.

Calliobdella vivida (*Verrill*)

C. vivida transmits *Cryptobia bullocki*, which has been shown to be pathogenic to summer flounder, *Paralichthys dentatus*, a species of commercial and recreational importance along the east coast of the USA (Burreson, 1982; Burreson and Zwerner, 1984). *Trypanosoma burresoni* in American eels, *Anguilla rostrata*, is also transmitted by *C. vivida* in Chesapeake Bay (E.M. Burreson, unpublished data) and it is likely that *C. vivida* is also the vector for *Haemogregarina platessae*, although no experimental transmission studies have been conducted on this parasite.

C. vivida occurs during winter in estuaries and is known from Louisiana to western Florida in the Gulf of Mexico and along the east coast of North America from Georgia to Newfoundland (Sawyer *et al.*, 1975; Appy and Dadswell, 1981; Madill, 1988). Although extensive collections have not been conducted in all localities, the leech appears to be most abundant in the large estuaries of Virginia, North Carolina and South Carolina. Leeches are most commonly collected at salinities between 10 and 22 ppt, but individuals can withstand oceanic salinity and pond water for long periods (Sawyer and Hammond, 1973; Burreson and Zwerner, 1982).

The host range of *C. vivida* is extremely wide. In Chesapeake Bay, *C. vivida* was collected from nine different hosts and fed on another five host species in the laboratory (Burreson and Zwerner, 1982). No host was found to which *C. vivida* would not attach and feed. Common hosts in Chesapeake Bay include summer flounder, *P. dentatus*, hogchoker, *Trinectes maculatus*, menhaden, *Brevoortia tyrannus*, and oyster toadfish, *Opsanus tau*. On the basis of studies in other areas and of the host distribution of *C. bullocki*, *C. vivida* seems to prefer pleuronectiform fishes and menhaden (Laird and Bullock, 1969; Becker and Overstreet, 1979). Leeches feed anywhere on the body, including the fins.

C. vivida is in the family Piscicolidae, subfamily Piscicolinae. The species was originally described, inadequately, by Verrill (1872), as *Cystobranchus vividus*, but no type material has been found. Sawyer and Chamberlain (1972) described *Calliobdella carolinensis* from South Carolina estuaries,

but later, on the basis of further collections, Sawyer *et al*. (1975) considered *C. carolinensis* to be a junior synonym of *C. vividus*. However, they transferred the species to the genus *Calliobdella* as *C. vivida*. The species was thoroughly described by Sawyer and Chamberlain (1972). The subcylindrical body is not distinctly divided into trachelosome and urosome; total length can be up to 40 mm, but most mature individuals are approximately 18 to 30 mm long. The oral sucker is well developed and has two pairs of concentric eyes (Fig. 15.5). The caudal sucker is slightly wider than the maximum body width, is attached strongly eccentrically and lacks ocelli. Pigmentation varies from none to faint, segmental, transverse bands, usually reddish brown in colour, especially obvious on the trachelosome. Paired, segmental, punctiform ocelli are often visible dorsolaterally and ventrolaterally on the body. Eleven pairs of pulsatile vesicles are usually obvious on the lateral margins of the urosome. Keys that include *C. vivida* are in Appy and Dadswell (1981) and Sawyer (1986).

The life cycle of *C. vivida* has been studied through field collections and laboratory experiments by Sawyer and Hammond (1973) in South Carolina and by Burreson and Zwerner (1982) in Chesapeake Bay. In Chesapeake Bay leeches are abundant from December through March, when water temperature averages 4 to 8°C. Leeches begin to deposit cocoons as early as February, but most cocoon deposition occurs in late April and early May when water temperature is about 15 to 17°C. In the laboratory a single large leech deposited 51 cocoons over a 6-day period (Burreson and Zwerner, 1982). Leeches die after depositing cocoons and no leech has been collected in nature after June. Eggs oversummer in the cocoon and begin hatching in the autumn, when water temperature decreases to between 15 and 18°C, usually late October to early November in Chesapeake Bay, but as late as December in South Carolina. Unfed *C. vivida* are strong swimmers and are routinely collected in plankton nets.

The marked seasonality of *C. vivida* has implications for its role as a vector. Most fish species in Chesapeake Bay are migratory and leave the Bay during autumn to spawn on the continental shelf or overwinter south of Cape Hatteras, North Carolina; they return to the Bay in late spring. Thus, most fish are migrating out of the Bay at the time *C. vivida* is hatching from the cocoon and fish are re-entering the Bay as leeches are dying after cocoon deposition. Thus, most fish species are exposed to *C. vivida* for only short periods and the prevalence of *C. bullocki* is low in migratory fishes. Resident fish, such as hogchoker, oyster toadfish and juvenile summer flounder, are exposed to leeches throughout the winter and harbour higher prevalences of *C. bullocki*.

Piscicola salmositica *Meyer*

P. salmositica is the vector for *Cryptobia salmositica*, an important pathogen in Pacific salmon of the north-west coast of North America (Wood, 1979; Bower and Margolis, 1984) and in rainbow/steelhead trout (Wales and Wolf, 1955; Woo, 1979; Mundie and Traber, 1983). The elucidation of the vector role of *P. salmositica* by Becker and Katz (1965a) was the first documentation of a freshwater leech transmitting haematozoa to fish in North America.

P. salmositica is restricted to the northern Pacific coast of North America, where it occurs in moderate to rapidly flowing streams with low water temperatures, high dissolved oxygen content and gravel beds. It has been collected from northern California to central British Columbia (Becker and Katz, 1965b). In California it is known from the Eel River and from Fall Creek, a tributary of the Klamath River. The coastal Alsea and Nehalem Rivers and Eagle Creek, a tributary of the Willamette River, have yielded specimens in Oregon and leeches have been collected from most of the coastal rivers in Washington and from the Columbia River system. Leeches have been collected from streams on both the east and west coast of Vancouver Island and from tributaries in the Fraser River system in British

Columbia, Canada. The report of *P. salmositica* on cut-throat trout in Wyoming by Cope (1958) needs confirmation.

Hosts for *P. salmositica* include most of the Pacific salmon, *Oncorhynchus* spp., including *O. kisutch*, *O. nerka*, *O. tshawytscha*, *O. gorbuscha* and *O. keta*, rainbow/steelhead trout, *O. mykiss* (*Salmo gairdneri*), and the sculpins *Cottus rhotheus* and *C. gulosus*. However, on the basis of the host range of *C. salmositica*, the host range for *P. salmositica* is probably much greater than indicated by actual collections (Becker and Katz, 1965c; Becker, 1980; Bower and Margolis, 1984). Leeches usually feed in the axillae of the pectoral or pelvic fins or on gill lamellae.

P. salmositica is in the family Piscicolidae, subfamily Piscicolinae; it was thoroughly described by Meyer (1946b). The body is subcylindrical and the general form is not as elongate as that of most other members of the genus (Fig. 15.5). The urosome is rather broad throughout and contains 11 pairs of lateral pulsatile vesicles; total length is up to about 40 mm. Pigmentation is uniformly dark grey to black. The oral sucker has two pairs of eyes, a large, crescentiform anterior pair and a smaller dash-like posterior pair. The caudal sucker is relatively small, not as wide as the maximum body width, and harbours eight to ten strongly crescentiform ocelli. The posterior margin of the caudal sucker may be angular rather than discoidal (Fig. 15.5). Keys that include *P. salmositica* are Klemm (1982) and Sawyer (1986).

The biology of *P. salmositica* was studied by Becker and Katz (1965b) in the vicinity of the Green River salmon hatchery located on Soos Creek, a tributary of the Green River near Auburn, Washington. The leech exhibits a definite seasonal cycle tied to spawning migrations of the host salmon. Leeches, small and few in number, first appear in late September attached to spawning chinook salmon. The leech becomes more abundant and individual leeches are larger during the coho salmon migration in November and December. Mean number of leeches per host for ten hosts was as high as 40 in December 1961, and the total leech population in Soos Creek was estimated at over 1 million leeches (Becker and Katz, 1965b). As they mature, leeches attach to the underside of stones, mate, deposit cocoons and die. The salmon spawning migrations terminate in February and leeches disappear by March. On the basis of field studies, it was concluded that cocoons hatched in 7 days, an unusually short time period, especially at ambient temperatures below 10°C. This short hatching time is contrary to results in laboratory experiments, in which leeches took 300 days to hatch (Bower and Thompson, 1987). Becker and Katz (1965b) concluded that only small leeches survived the summer, although none was ever collected, and it was these leeches that attached to the first returning salmon each autumn. On the basis of research with other species that demonstrate marked seasonality (Burreson and Zwerner, 1982), it is more likely that only eggs survive the summer period and that they hatch in the autumn when water temperature decreases. This view was supported by Bower and Margolis (1984), who did not find leeches during summer, but research (Bower and Thompson, 1987), in which leeches hatched during autumn even when held at constant temperature, suggests that hatching may not be environmentally controlled.

Newly hatched leeches may feed on the resident torrent sculpin, *C. rhotheus*, known to serve as a reservoir for *C. salmositica*, prior to the arrival of the salmon. In this manner leeches may already be infected with *C. salmositica* when the first migrating salmon enter the streams. Leeches may also periodically feed on sculpins during the winter. Thus, the wide host range of *P. salmositica* facilitates the transfer of *C. salmositica* to salmon.

Prevention and Control

Much of the research on control of leeches has been oriented towards *P. geometra* in Eastern European fish ponds and has been summarized by Bauer (1961), Bauer

et al. (1973), Negele (1975), Kabata (1985) and Bielecki (1988). Therapeutic agents listed in these papers and summarized below may not be approved for food fish in all countries.

Prevention is the best control and, according to Bauer *et al.* (1973), the presence of leeches in a culture pond is a sign of neglect, primarily the failure to remove aquatic vegetation, which provides an ideal substrate for cocoon deposition. Sand or gravel filters can prevent the introduction of leeches with the water supply. In carp culture in Eastern Europe, leeches must be removed from fish before they are transferred to the wintering ponds. The following baths have been shown to be effective against *P. geometra*: (i) 2.5% sodium chloride for 1 h. Leeches leave the fish host, but are not killed; care must be taken not to reintroduce them to the ponds (Kabata, 1985); (ii) 200 g quicklime (calcium oxide) per 100 l of water for 5 s. Concentration and time are critical, too high a concentration or too long a time can cause death of the fish. The quicklime must be fresh and free of impurities (Negele, 1975); (iii) 0.2% solution of lysol or 0.4% solution of priasol (creosote derivative) for 5 to 15 s (Conroy and Herman, 1970); (iv) 0.005% solution of cupric chloride for 15 min. The solution should be changed after each group of 15 to 20 kg of fish (Bauer *et al.*, 1973); and (v) the proprietary varieties of Dylox are successful leech therapeutic agents. For example, 2.5% Masoten for 5 min is effective, but old baths are toxic to fish (Negele, 1975).

Varieties of Dylox have also been utilized to treat ponds infested with leeches. Neguvon at 1 ppm for 5 days is effective against adult leeches, but this concentration does not kill embryos in cocoons. A concentration of 10 ppt is necessary to kill embryos, but this concentration is also toxic to fish (Prost *et al.*, 1974). Masoten is effective in ponds at a concentration of 1 g/4 m^3, but it is apparently harmful to zooplankton as well (Negele, 1975). Chlorofos has been used at 2.0 ppm for 4 days (Kabata, 1985). Correct dosage may be difficult to calculate in ponds where the water volume is not known.

Massive infestations of *P. geometra* require that the pond be drained and disinfected. Although desiccation for 24 h kills embryos in cocoons (Prost *et al.*, 1974), it is very difficult to effectively dry a pond. Thus, disinfection with quicklime (calcium oxide) is a common prophylactic measure. Normal concentrations are 2000 to 3000 kg/ha, but much less can be used if the calcium oxide purity is at least 93%. It is critical that the calcium oxide concentration is high enough to yield a pH of at least 8.6 to ensure death of both adults and embryos in cocoons (Prost *et al.*, 1974). The general procedure is to drain the pond, spread the lime evenly over the pond bottom, and then flood the pond for 7 to 10 days (Kabata, 1985).

H. marginata has also been the subject of chemotherapeutic studies in fish culture ponds in India. A number of different treatments are effective (Singhal *et al.*, 1986). A concentration of 0.001 mg/l glacial acetic acid followed by 10 mg/l potassium permanganate, with an immersion time of 5 min each, reduced *H. marginata* infection prevalence in treatment aquaria by 80% compared with untreated control fish. A single treatment of 5 mg/l gammexane (1-6-hexachlorocyclohexane) sprayed over the surface of the culture ponds removed most of the leeches from the fish, but it was not reported whether the leeches actually died from the treatment. A 15 min immersion in 30 mg/l sodium chloride solution caused the leeches to detach from the fish hosts and was found to be the most effective treatment.

Because of its importance as a vector and parasite in salmonids in hatcheries, *P. salmositica* has been the subject of a number of studies on control. In an outbreak of *P. salmositica* on pink salmon fry, none of the chemicals commonly employed to control bacterial and other parasitic diseases (formalin, roccal and pyridylmercuric acetate) had any effect on the leeches (Earp and Schwab, 1954). However, the leeches were sensitive to undiluted sea water (salinity of at least 28 ppt); 1 h exposures killed the leeches, but did not harm the salmon eggs or fry.

Meyer (1969) conducted a study to examine the efficacy of three organophosphate insecticides, Dylox, Baygon and Baytex, on leeches held in small beakers. The effects of Baygon and Baytex were only temporary, but Dylox caused permanent damage and a concentration of 0.5 ppm killed *P. salmositica* in 96 h. Another fish leech, *M. lugubris*, was more sensitive to Dylox; a concentration of 0.125 ppm killed it in 96 h.

Bower and co-workers have investigated the use of chlorine for controlling *P. salmositica*. They found a wide range of chlorine concentrations and exposure times that would kill juvenile leeches, but adult leeches were more resistant (Bower *et al.*, 1985). In a subsequent paper, Bower and Thompson (1987) found cocoons of *P. salmositica* to be surprisingly resistant to various chemical treatments. High concentrations of an iodophore disinfectant and of chlorine (two to 13 times higher than concentrations lethal to juvenile leeches) had little effect on embryos in cocoons. A chlorine concentration that was lethal to leech embryos was also potentially harmful to fish. Hatching was prevented by freezing cocoons, in or out of water, or by desiccation for at least 7 days; even desiccation for 24 h reduced hatching by 50%.

The freshwater and estuarine leech *M. lugubris* has been the subject of chemotherapeutic studies in striped bass (*M. saxatilis*) intensive culture facilities located on Chesapeake Bay, Maryland, USA (Woods *et al.*, 1990). Dylox at a concentration of 40 ppm caused detachment of all leeches from fish after 3 h static baths at 11.5°C. Potassium permanganate at 10 ppm was not effective at that temperature and Dylox was not effective at temperatures below 10°C.

There is only one report on control of marine leeches. A 50 ppm formalin bath for 1 h was effective in controlling *Z. arugamensis* on orange-spotted grouper in the Philippines (Cruz-Lacierda *et al.*, 2000). Freshwater baths may also be effective for marine leeches if the fish host can tolerate the bath.

Summary and Conclusions

Leeches can be directly pathogenic to fishes, especially if conditions allow close, continuous contact between large numbers of leeches and their hosts. Thus, the presence of leeches in aquaculture facilities should always be cause for concern. However, the most important role of aquatic leeches is as vectors of pathogenic organisms. The study of leeches as vectors of pathogenic viruses and bacteria is still in its infancy and should be a productive area of research in the future. Even though the role of leeches as vectors for the various haematozoa groups is now well documented, vectors have been identified for only a very few of the myriad species known to inhabit the blood of fishes, especially for the intraerythrocytic protozoa. Current knowledge suggests that in a given area only one or two leech species transmit a wide variety of blood parasites, but vectors are known for so few haematozoa that this may not be a valid conclusion.

One of the intriguing questions remaining to be answered is the vector specificity of fish haematozoa. It has been generally assumed that vector specificity is high and that a haematozoan species utilizes only a single leech species as a vector. However, this assumption is contradicted by *C. borreli*, which appears to utilize two vectors that belong to different families, *H. marginata*, a glossiphoniid, and *P. geometra*, a piscicolid. Rigorous comparative studies are needed to confirm the vector relationships of both *P. geometra* and *H. marginata* and to ascertain that they are transmitting the same species of flagellates. Experimental studies with frog and turtle trypanosomes (Siddall and Desser, 1992b) identify multiple leech vectors, although in both cases one of the leech species appears to be a more important vector than the other species. These results raise many interesting questions about the evolution of the vector–parasite relationships.

There are regions with an abundant leech and haematozoa fauna where important research could be conducted to determine if fish haematozoa species can be transmitted by more than one leech. For example,

developmental stages of various haematozoa were reported from many different leech species in Newfoundland (Khan *et al*., 1991), but experiments are necessary to demonstrate whether other leeches can actually transmit haematozoa known to utilize *J. arctica* as a vector. Similar experiments should be conducted to determine whether *M. lugubris* can also transmit haematozoa known to utilize *C. vivida* in estuaries of the south-eastern USA. It is disappointing that few studies have been conducted on the vector role of fish leeches over the last decade.

With the advent of molecular phylogenetic techniques, an emerging area of interest is phylogenetic analysis of leeches. Recent studies have already suggested that leeches are just specialized oligochaetes (Siddall *et al*., 2001) and have also questioned some of the traditional classification based on morphology. Increased taxon sampling is needed before revised classifications can be proposed, but such studies are under way. Phylogenetic studies have resulted in an increased interest in fish leech taxonomy and recent studies have reported new species, even in areas that were thought to be well studied (e.g. Bielecki, 1997; Burreson and Williams, 2004). Unfortunately, the fish leech fauna in many parts of the world is still poorly known. Our knowledge is especially inadequate for the marine fauna of the southern hemisphere, but, even in areas where the fauna is relatively well known, taxonomic confusion persists (Barta and Sawyer, 1990).

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16 Fish-borne Parasitic Zoonoses

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Introduction

Numerous marine and freshwater fishes serve as sources of medically important parasitic zoonoses. Some can cause severe clinical manifestations, even death. Previously, these infections were only prevalent in a few countries where the local population eat raw fish or fish products. However, in recent years, such eating habits have become popular worldwide. Therefore, fish-borne diseases are more widely distributed. Unfortunately, there is still no efficient/cost-effective method to screen fish on a large scale.

Generally, fish can be either an intermediate host of parasites involving a human as the definitive host or a carrier of larvae of animal parasites that only invade human tissues for a limited period without undergoing further development. The latter are considered incidental infections. The natural definitive hosts for the parasites are usually marine mammals or birds. However, larval stages of a few fish-borne parasites can mature in both animals and humans.

This chapter provides an in-depth review on the pathogenicity, symptoms, methods of transmission, diagnosis, epidemiology and treatment of the major fish-borne diseases. The basic morphological features of the parasites, however, are only briefly described.

Trematodiasis

Fish-borne trematodiasis is especially important in South East Asia, the Far East and regions where people are dependent on freshwater fish as the major source of protein. Infections by both large and small digenetic trematodes are common. Although the diseases *per se* are seldom fatal, they can cause morbidity and serious complications. The route of infection is by ingesting metacercariae located in muscles and subcutaneous and other tissues of fish.

Clonorchiasis

Clonorchiasis is caused by *Clonorchis sinensis* (Cobbold, 1875) Looss, 1907, which is located in the bile duct of humans. The adult worm measures 8–15 mm × about 4 mm and is characterized by the presence of two highly branched testes and a prominent seminal receptacle (Fig. 16.1). The worm also matures in cats, dogs, pigs and rats. Although this trematode has also been referred to as *Opisthorchis* (see Muller, 1975), the old nomenclature is retained here because of its wider acceptance by the medical profession. Furthermore, in Asia, clonorchiasis is considered as distinct from opisthorchiasis (which will be described later).

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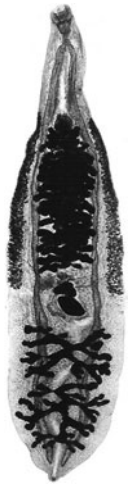


Fig. 16.1. *Clonorchis sinensis* whole worm (original, × 6).

Epidemiology

Clonorchiasis is still regularly encountered in Hong Kong, southern China, Taiwan and South Korea.

In Hong Kong, no natural transmission of the parasite occurs. The snail intermediate hosts cannot propagate in the local fish ponds, which have high salinities. Also, in recent years, many ponds have been developed into apartment blocks. This disease is due to the consumption of infected cyprinid fish imported from the Chinese mainland. Duchastel (1984) reported that 13.4% of 25,095 Hong Kong residents who applied to emigrate to Canada during 1979–1981 had ova in stool samples. Most applicants were students under 25 years old. Li (1991) reported that the infection rates in the Chinese population varied from 1 to 48%. A national survey in China during 1988–1992 showed that the infection occurred in 22 provinces (Yu *et al.*, 1994).

In Taiwan, there were three endemic areas: Miao-li in the north, Sun-moon in the middle and Mei-nung in the south. However, recent surveys have shown that the areas may be more widespread due to an increase in consumption of raw fish. The infection seemed to be more common in Hakkanase and farmers than in other groups (Chen, 1991a).

In South Korea, stool samples of 13,373 people living within 6 km of some rivers had an overall infection rate of 21.5% (Seo *et al.*, 1981). The rate was as high as 40% in the Nakdong River basin. Soh (1984) reported that the rate was more than 10% in endemic areas. Rim (1986) extensively reviewed the epidemiology of the disease in South Korea and other countries. The rate has dropped recently. Lee, S.K. *et al.* (1994) examined faecal specimens of 52,552 patients in a Seoul hospital during 1984–1992 and reported an infection rate of 3.2%.

The southern Chinese commonly acquire the infection by eating rice congee laden with pieces of raw or poorly cooked carp. Raw carp is also consumed during special festivals, e.g. Chinese New Year. The Japanese like to eat raw carp (particularly *Cyprinus carpio*, *Carassius carassius*) and Koreans also consume raw fish, especially during drinking parties.

Biology of parasite

Adult worms in the biliary passage lay operculated ova, which are passed into the external environment via the intestine. When laid, each ovum (26–30 μm × 15–17 μm) is fully embryonated with a ciliated miracidium (Fig. 16.2). The ovum is characterized by a small protuberance on the posterior extremity. In water, the miracidium hatches and enters the first intermediate host (freshwater snails) either by direct penetration or by ingestion. Inside the snail, the miracidium develops into a sporocyst, then a redia and finally the free-swimming cercaria, which is released. The cercaria penetrates the skin of the second intermediate host (freshwater fish) to become the metacercaria. The infective metacercaria (135–145 μm × 90–100 μm) is enclosed by a thick cyst wall. It is located mainly in muscles and subcutaneous tissues but sometimes also on the scales, fins and gills of fish (Fig. 16.3).

The important molluscan hosts are *Parafossarulus manchouricus*, *Bithynia* (= *Alocima*) *longicornis* and *Bulimus fuchsiana*. Other susceptible species include *Semisulcospira libertina*, *Thiara granifera*, *Assiminea lutea* and *Melanoides tuberculata*.

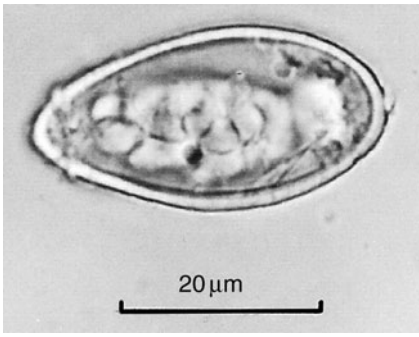


Fig. 16.2. Egg of *Clonorchis sinensis* recovered from faeces (original).

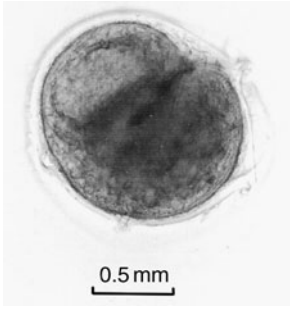


Fig. 16.3. Metacercariae of *Clonorchis sinensis* (original).

Many freshwater fishes, and in particular members of Cyprinidae, serve as the second intermediate host. Yoshimura (1965) listed 81 species that are susceptible to *C. sinensis*, including 71 species in the Cyprinidae, two species each in the Ophiocephalidae and Eleotridae and one species each in the Bagridae, Cyprinodontidae, Clupeidae, Osmeridae, Cichlidae and Gobiidae. In China, 43 species of freshwater fishes (Cyprinidae, Cyprinodontidae, Ophiocephalidae, Eleotridae and Gobidae) have been found to be infected. Among these, the most heavily and widely distributed species are *Pseudorasbora parva*, *Rhodeus ocellatus* and *Cultriculus kneri*. However, the major species which are commonly eaten raw are *Ctenophargyndon idellus*, *Hypothalmicthys molitrix*, *Mylopharyngodon aethiops* and *Culter aburnus*. In southern China, the traditional practice of building outside toilets above carp ponds and the use of night soil for pond enrichment sustain the infection in the cultured fish population (Fig. 16.4). In Taiwan, *Tilapia mossambica* and *Ophiocephalus maculatus* are two common species eaten raw. In South Korea, 36 species of fish belonging to Cyprinidae, Bargridae and

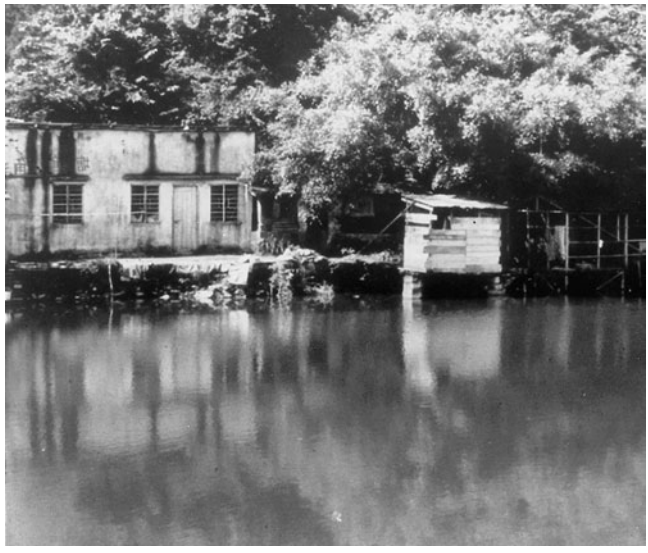


Fig. 16.4. A carp pond in Hong Kong, showing an outside toilet over pond (original).

Clupeidae can serve as the second intermediate host. The important species are: *P. parva*, *Sarcocheilichthys sinensis*, *Hemibarbus labeo*, *Acanthorhodeus gracilis*, *Acanthorhodeus taenianalis*, *Puntungia herzi*, *Pseudogobio esocinus*, *Gnathopogon* spp., *Acheilognathus intermedia* and *C. kneri* (see Rim, 1986; Soh and Min, 1990). In Japan, Komiya and Suzuki (1964) reported that metacercariae were frequently found in *P. parva*, *Sarcocheilichthys variegates*, *Acheilognathus lanceolata* and *Tribolodon hakonensis*. However, the importance of these fishes as sources of infection in recent years is not known.

Pathogenesis

The pathology of clonorchiasis was studied extensively (Ong, 1962; Hou and Pang, 1964; Gibson and Sun, 1965; Mcfadzean and Yeung, 1966; Chan and Teoh, 1967). The infection has also been implicated in recurrent pyogenic cholangitis (Cook *et al.*, 1954; Ong, 1962), cholangiohepatitis (Fung, 1961) and cholangiocarcinoma (Hou, 1956; Belamaric, 1973; Abdel-Rahim, 2001; Watanapa and Watanapa, 2002).

The histopathology of clonorchiasis was first described in detail by Hou (1955). After ingestion, the excysted metacercaria reach the bile duct by direct migration from the intestine. The adult worms ingest blood in the bile ducts. Hepatic parenchymal damage and portal hypertension are usually absent in light and uncomplicated infections. The lesions are usually localized in the distal biliary passages, particularly those in the left lobe of the liver. The extent of the lesions is dependent on worm burden and on the presence of a secondary bacterial infection. The latter complication commonly leads to biliary obstruction due to extensive adenomatous proliferation, calculi and cholangitis. During the acute stage of infection, the mucosa of bile ducts is oedematous, with desquamation of the epithelium. Later, due to inflammatory responses, the bile ducts become thickened and crypt formation occurs. Metaplasia of the epithelial cells follows, resulting in proliferation of glandular-like structures in the mucosa which are responsible for producing mucin in the bile. During chronic and heavy infections, continuous hyperplasia may cause formation of adenomatous tissue. This is probably elicited by continuous mechanical and chemical stimulation (Flavell, 1981). Recurrent attacks of suppurative cholangitis may occur due to biliary obstruction by the trematodes. Atrophy of the biliary epithelium and underlying hepatic cells may also develop.

Clonorchiasis may also cause pancreatitis (Hou, 1955). Hou reported that as many as 50 worms were recovered from pancreatic ducts. The main ducts were dilated. A limited degree of periductal fibrosis and adenomatous tissue occurred but with considerable squamous metaplasia. Mcfadzean and Yeung (1966) reported that, in 110 Chinese patients in Hong Kong suffering from acute pancreatitis of unknown aetiology, 83% were infected with *C. sinensis*.

Hou (1956) noted a close correlation between clonorchiasis and primary liver carcinoma. The close association between this trematode infection and cholangiocarcinoma was further elaborated by Belamaric (1973), Purtillo (1976) and Schwartz (1980). Kim (1984) suggested that exogenous carcinogenic promoters, nutritional, immunological and genetic factors, probably induce goblet-cell metaplasia and dysplasia of the bile-duct epithelial cells, resulting in carcinoma. Ona and Dytoc (1991) reported two cases with unusual manifestations. Watanapa and Watanapa (2002), however, after reviewing the literature, concluded that *C. sinensis* is only a probable cause of cholangiocarcinoma.

Symptoms

In endemic areas, patients with an enlarged liver and a history of eating raw or undercooked freshwater fish should be examined for clonorchiasis. In light and acute infections, more than half of the patients are usually asymptomatic. However, some show general malaise, abdominal discomfort, occasional diarrhoea, slight fever, eosinophilia (5–20%), gall-bladder syndrome, paroxymal epigastric pain, mild

jaundice and hepatomegaly. In heavy and chronic infections, loss of weight, lassitude, mental depression, anorexia, paraesthesia, palpitation, tachycardia, headache, jaundice, tenderness of liver, tetanic cramps, tremors and toxaemia may occur (Belding, 1965; Kim *et al.*, 1982; Tierney *et al.*, 2001).

Laboratory diagnosis

The traditional laboratory diagnostic method is by stool examination. Rim (1988) reviewed some alternative methods. Although the infection can readily be detected by finding the characteristic ova in faecal samples, serological tests are sometimes necessary when there is obstruction of biliary canals. Various serodiagnostic tests, which are based on antibody detection, have been used. However, no attempt has been made to detect circulating antigens that can be used to diagnose acute infections.

Three clinical stages are classified according to the pattern of antibody responses at various times post-infection. In experimentally infected cats, during the acute stage of infection, the major circulating antibodies are immunoglobulin M(IgM), IgA and IgG. Peak antibody titres for the first two immunoglobulins occur at 3 and 11 weeks post-infection. In the subacute stage, only IgA and IgG predominate. In the chronic stage, only IgG is present (Chen *et al.*, 1989; Yen *et al.*, 1992).

Isolation of specific antigens

Despite numerous attempts, a highly specific and sensitive antigen for detecting clonorchiasis is still not available. Takei and Chun (1976) produced fractions from crude somatic extracts using gel filtration on a Sephadex G-100 column, followed by disc electrophoresis. A fraction, KD1, containing protein and carbohydrate was produced and tested, using serum samples from 32 clonorchiasis patients. All tests were positive. Negative reactions were observed in samples from uninfected persons and patients with schistosomiasis, ascariasis, filariasis and hookworms. Sawada *et al.* (1976) purified antigens by using Biogel-A filtration. Using indirect haemagglutination (IHA), a fraction, K6-H, was tested on serum samples of the following patients: 38 with clonorchiasis, three with paragonimiasis, five with schistosomiasis, ten with ascariasis and five with ancylostomiasis. Positive reactions were only observed in the samples from clonorchiasis patients.

Immunoaffinity purification of somatic worm extracts and characterization of antigens of ova and metacercariae were carried out by Lee *et al.* (1988). Ova antigens varied from 15 to 20 kDa, metacercaria antigens from 15 to 100 kDa and adult antigens from 11 to 80 kDa. An enzyme-linked immunosorbent assay (ELISA) was more sensitive to the adult crude antigens than to those of metacercariae. Negative reactions were observed when egg antigens were used.

Kim (1998) isolated a 7 kDa antigen (CsE) from the excretory/secretory (E/S) products but it also reacted with *Metagonimus yokogawai* antibodies. Yong *et al.* (1998) produced a recombinant antigen, pBCs31, of 28 kDa, which showed high specificity in ELISA. Chung *et al.* (2000) partially purified a 17 kDa cysteine proteinase from adults by Sephacryl S-200 gel filtration and anion exchange chromatography but it was not specific. Using molecular methods, Kim *et al.* (2001a) cloned and characterized a proline-rich antigen, CsPRA, which was suggested to show high sensitivity and specificity.

Immunoassays

A large number of assays have been tested. The complement fixation test (CFT) was first used by Ryoji (1922) and subsequently by Ito (1925), Chung *et al.* (1955), Sadun *et al.* (1959) and Wykoff (1959). The precipitation test, gel diffusion (GD), immunoelectrophoresis (IE), indirect fluorescent antibody test (IFAT) and IHA have also been tried but the results were not satisfactory (Mori, 1957; Pacheco *et al.*, 1960; Phillipson and Mcfadzean, 1962; Sun and Gibson, 1969; Cho and Soh, 1974, 1976; Im, 1974; Lee, 1975; Kwon *et al.*, 1984). Yen and Chen (1989) evaluated the more sensitive counter-immunoelectrophoresis (CIE)

method on 70 clonorchiasis patients and seven cases of other parasitic infections. Antibody was detected in all clonorchiasis patients. Cross reactions, however, were also observed in sera from patients with toxocariasis and angiostrongyliasis.

Using the charcoal granule agglutination test, Hu *et al.* (1989) observed positive reactions in 83.5% of 79 clonorchiasis sera and 19.5% of 41 sera from cases of other parasitic diseases.

The ELISA has become the most commonly used method to diagnose clonorchiasis in humans and animals (Jin *et al.*, 1983; Yang *et al.*, 1983, 1984; Hahm *et al.*, 1984; Han *et al.*, 1986; Sirisinha *et al.*, 1990; Yong *et al.*, 1991). Hahm *et al.* (1984) reported that ELISA detected 78% of 55 known clonorchiasis sera, whereas IFAT and CFT detected 62% and 56%, respectively.

Significant cross reactions were observed between *Clonorchis* and *Opisthorchis* in ELISA. Immunoblotting and radioimmunoprecipitation showed that a 89 kDa metabolic antigen that was predominant in *Opisthorchis viverrini* reacted with antisera from both groups of patients. However, a 16 kDa antigen from *O. viverrini* appeared to be more specific (Sirisinha *et al.*, 1990).

A monoclonal antibody (mAb), CsHyb 065-23, was used in an ELISA inhibition test. Of known clonorchiasis cases, 78.5% tested positive. False positives were not found in sera from 16 paragonimiasis patients and 28 negative controls (Yong *et al.*, 1991). Nevertheless, the level of sensitivity of the test is too low for differential diagnosis.

Dot-immunogold-silver staining (dot-IGSS) was tested on 40 and 35 confirmed cases, which were all positive (Wu *et al.*, 1993; Liu *et al.*, 1995).

ELISA has also been employed to monitor serum antibodies in patients after treatment with praziquantel. In five of ten patients, the titres returned to normal after 1 year (Chen *et al.*, 1987a,b).

Recently, cystatin-capture ELISA, which binds cysteine proteinases of adult worms, showed a higher degree of specificity than conventional ELISA (Kim *et al.*, 2001b).

Diagnosis by imaging

Computed tomography scanning (CT), magnetic resonance imaging (MRI) and ultrasound can also be used to diagnose clonorchiasis. In a study involving 17 patients with clonorchiasis alone and 25 patients with clonorchiasis and hepatobiliary malignancies, CT showed a diffuse, minimal or mild dilatation of the intrahepatic bile ducts in 14 cases. However, a diffuse dilatation of the ducts was observed in all patients with both parasite and biliary malignancies (Choi *et al.*, 1989).

In a study of 947 clonorchiasis patients using ultrasound, gall stones were seen in 85 cases, common bile duct stones in three and a hepatic stone in one (Hon *et al.*, 1989). Lim *et al.* (1989) presented sonographic findings in 59 proven cases.

In heavy infections, intraoperative cholangiography can show multiple filling defects in the common bile duct, common hepatic duct and left hepatic duct (Nishioka and Donnelly, 1990).

Treatment

There was no effective drug to treat clonorchiasis until praziquantel became available. Chen (1991b) reported a 96% cure rate among 356 patients with praziquantel (60 mg/kg of body weight, divided into three doses in 1–2 days).

Control

The feasibility of using ^{60}Co gamma irradiation to control the infectivity of metacercariae was explored (Duan *et al.*, 1993). The median lethal dose (LD_{50}) of irradiation for metacercariae in fish was 0.05 kGy and the minimal effective dose was 0.15 kGy.

Opisthorchiasis

Opisthorchiasis is a zoonosis that is mainly caused by *O. viverrini* (Poirrier, 1886) Stiles and Hassall, 1869 and *Opisthorchis felineus* (Rivolta, 1884) Blanchard, 1895. The natural definitive host for the former species is the civet cat (*Felis viverrini*). Cats, dogs,

foxes and pigs are the common hosts for the latter.

The pathogenesis and biology of the two trematodes are similar to those of *C. sinensis*. A mini-review of Opisthorchiidae was given by King and Scholz (2001).

OPISTHORCHIS VIVERRINI

Human infection is prevalent in South East Asia, especially in northern Thailand, where it is not only a medical problem but also an economic impediment. The latter includes the high cost of mass treatment and obstructing water resources development.

The adult worms (5.5–9.6 mm × 0.8–1.7 mm) live in the biliary passage. This species is distinguished from *C. sinensis* by having lobed testes and a different pattern of flame cells in cercariae and metacercariae.

Epidemiology

According to Harinasuta (1984), 5–6 million people in northern and north-eastern Thailand were infected with this trematode. The infection rates varied from 10 to 90%. Males were more commonly infected than females and prevalence was higher in adults. Kasuya *et al*. (1989) found that 7.5% of 491 primary schoolchildren in Chiangmai were infected. Maleewong *et al*. (1992c) reported that the prevalence was 66% in 2412 persons in north-east Thailand. In Laos, the prevalence rates in two rural and one urban community were 52.9%, 55% and 60.7%, respectively (Kobayashi *et al*., 2000).

A control programme for opisthorchiasis has been conducted in northern and north-eastern Thailand. It was implemented by establishing a strong educational programme to change eating habits, and by giving a free annual treatment of a 40 mg/kg dose of praziquantel (Sormani, 1990; Jongsuksuntigul and Imsomboon, 1998).

The disease can be acquired by eating a local dish called 'koi-pla' that contains infected raw fish. The high prevalence of this infection is mainly due to unhygienic eating, the defecation habits of the local inhabitants and the common occurrence of the snail intermediate hosts. The infection is more commonly acquired at the end of the rainy season and the beginning of the dry season (September to February) when fish can be caught easily (Wykoff *et al*., 1965).

Biology of parasite

The adult worms, located in the biliary passage, lay thousands of operculated ova per day. The ova (29 μm × 16 μm) are discharged to the outside via the intestine. They are more elongated than those of *C. sinensis* and without a clear shoulder at the operculum.

Freshwater snails are the first intermediate host. The developmental stages of the parasite in snails are similar to those of *C. sinensis*. In Thailand, *Bithynia goniomphalus* is the important snail host in the north-east, *Bithynia funiculata* in the north and *Bithynia laevis* in the central region. The infection rate in snails in endemic areas is about 0.5% (Harinasuta, 1984).

The metacercariae (220 μm × 116 μm) are located in the muscles of freshwater fish, which are the second intermediate hosts. The important cyprinid fish hosts in northern Thailand are *Cyclocheilichhys siaja*, *Hampala dispar, Puntius orphoides* and *Puntius leiacanthus*. In highly endemic regions, the infection rates in fish can be 50–90% (Harinasuta, 1984; Sithithaworn *et al*., 1997; Waikagul, 1998; Sukontason *et al*., 1999). High metacercaria burdens occur in the late rainy season and winter (July to January).

Pathogenesis

Opisthorchiasis is a slow disabling disease (Riganti *et al*., 1989). In highly endemic regions, recurrence of the infection is common, resulting in heavy worm burdens. Similar to clonorchiasis, the presence of a large number of worms in the bile duct and its canaliculi, the gall bladder and hepatic ducts can elicit adenomatous hyperplasia of the biliary epithelium, resulting in thickening of the walls. In chronic infections, obstruction of the biliary system occurs. A heavy infection can be fatal because it leads to cirrhosis of the liver, cholangiocarcinoma or liver carcinoma and hepatic

cyst formation. Using ultrasound, cholangiocarcinoma was shown in eight of 87 patients with hepatomegaly or opisthorchiasis. This was confirmed by CT and endoscopic retrograde cholangiopancreatography (Vatanasapt *et al.*, 1990). Pungpak *et al.* (1990) suggested that measuring tumour markers, CA125 and CA19-9, may be useful in early detection of opisthorchiasis-associated cholangiocarcinoma. Prempracha *et al.* (1994) induced the carcinoma in *O. viverrini*-infected hamsters by exposure to dimethylnitrosamine. Haswell-Elkins *et al.* (1994) and Abdel-Rahim (2001) reviewed the possible mechanisms for parasitic infections to induce hepatic neoplasia. Watanapa and Watanapa (2002) believed that *O. viverrini* is a definite cause of cholangiocarcinoma.

Abnormalities have been observed in the high-density lipoprotein content of serum from opisthorchiasis patients. These are in the form of low cholesterol and cholesteryl ester concentrations but high triglycerides. This indicates a disturbance in the synthesis of the high-density lipoprotein or the removal of lipid content in lipoproteins (Changbrunrung *et al.*, 1988).

Symptoms

Clinical manifestations cannot be observed in light infections (fewer than 1000 eggs/g of faeces). In moderate or heavy infections, there may be a slight fever, eosinophilia, diarrhoea, flatulent dyspnoea, transient urticaria, weakness, anaemia, pain and 'hot sensation' over the liver, which can be slightly enlarged, and jaundice. In chronic heavy infections, symptoms of gastroenteritis may occur, together with cachexia, oedema and malnutrition (Belding, 1965; Harinasuta, 1984; Tierney *et al.*, 2001).

Laboratory diagnosis

Although faecal examination is the traditional diagnostic method, serodiagnostic tests have also been used.

Aqueous somatic extracts of whole worm, Triton X-100 and deoxycholate extracts of surface and tegument as well as E/S antigens were analysed, using radiolabelling, immunoprecipitation and polyacrylamide gel electrophoresis. An 89 kDa protein was the dominant antigen in the E/S products (Wongratanacheewin and Sirisinha, 1987; Wongratanacheewin *et al.*, 1988; Amonpunt *et al.*, 1991).

A 'sandwich' ELISA method was used to detect parasite antigens in faecal samples (Chaicumpa *et al.*, 1992; Sirisinha *et al.*, 1992, 1995). An IgG monoclonal antibody, which was specific to the 89 kDa antigen, was first captured on a microtitre well by rabbit anti-mouse IgG. As little as 0.1 ng of parasitic antigens could be detected. A specific DNA probe constructed from a repetitive DNA containing 340 base pairs was used in a dot-blot hybridization of the parasitic DNA. The probe detected DNA released from as few as five ova or even the presence of metacercariae in fish (Sirisinha *et al.*, 1991). Using the PCR method and a pair of specific primers, Wongratanacheewin *et al.* (2001) detected a single egg in artificially inoculated faeces. The primers were based on the pOV-Ab-specific DNA probe sequence with a 330 base-pair product. The ribosomal RNA gene from this trematode has been cloned and characterized (Korbsrisate *et al.*, 1992).

Treatment

Praziquantel, at a dosage of 25 mg/kg of body weight three times a day for 1–2 days, is effective, with a cure rate of over 95% (Tierney *et al.*, 2001). For mass treatment programmes, a single dose (40 mg/kg) is recommended (Harinasuta, 1984).

OPISTHORCHIS FELINEUS

Opisthorchiasis caused by *O. felineus* occurs in Poland, central Eastern Europe and Siberia. The highly endemic areas also include the Dnieper, Donetz and Desna basins. The cat is the common host.

Adult worms are usually located in the distal but sometimes in the proximal bile ducts. This species resembles *O. viverrini* except that its testes are less lobate and situated further from the ovary. The appearance and distribution of the vitellaria are also

different. The two species are also distinguished by the pattern of flame cells in the cercariae and metacercariae (Belding, 1965).

Epidemiology

The disease usually occurs in villages and localities near rivers or reservoirs in Siberia and Central Europe where fish are eaten raw. In the Ukraine, most infections are acquired by eating fish on the first day of salting (see Muller, 1975). However, the prevalence of this infection in humans is usually lower than that of *O. viverrini*.

Biology of parasite

The biology of this species is similar to that of *O. viverrini*. The first intermediate host is *Bithynia leachii* and cyprinids are the second intermediate hosts. The important fish hosts are *C. carpio*, *Idus melanotus*, *Abramis brama*, *Barbus barbus*, *Tinca tinca*, *Blicca bjoerkna*, *Leuciscus rutilis* and *Scardinus erythrophthalmus.*

Pathogenesis, symptoms and treatment

Hepatic lesions, symptoms and treatment are similar to those of *O. viverrini*.

Laboratory diagnosis

Faecal examination is the common method. The ovum is morphologically similar to that of *O. viverrini*. Serodiagnosis has also been employed. Teplukhin *et al.* (1987) used IHA and somatic or E/S antigens to detect antibodies. They concluded that somatic antigens were 1.5 times more sensitive than E/S antigens. Gitsu *et al.* (1987) reported that ELISA detected 31.8% of 323 patients passing fewer than 10 eggs/g of faeces and 86.3% with more than 1000 eggs/g.

Small trematodes

Besides *O. viverrini*, infections by numerous species of small trematodes (0.5–3 mm in length), especially members of the Heterophyidae, are also common in South East Asia and the Far East (northern Thailand, Laos, Cambodia, Vietnam, Indonesia, the Philippines and South Korea). Infections have also been reported in Hawaii, Egypt, Israel, Romania, Greece and eastern Siberia. Some important species are *Heterophyes nocens*, *Heterophyes continua*, *Heterophyes heterophyes*, *Heterophyes dispar*, *Heterophyopis continua*, *Haplorchis taichui*, *Haplorchis pumilo*, *M. yokogawai*, *Metagonimus takashii*, *Pyidiopsis summa*, *Diorchitrema* (= *Stellantchasmus*) *falcatus*, *Stictodora fascatum*, *Centrocestus armatus*, *Echinostoma hortense*, *Echinostoma cinetorchis*, *Echinochasmus japonicus*, etc. (see Belding, 1965; Muller, 1975; Chai *et al.*, 1988; Chai and Lee, 1991).

Transmission is by eating raw or newly salted/pickled fish. *H. heterophyes* infection is common in Egypt, where the pickled mullet (*Mugil cephalus*) is traditionally eaten at the feast of Sham-al Nessim (Muller, 1975).

An epidemiological study by Ahn *et al.* (1987) on *M. yokogawai* in South Korea showed that 6.6% of faecal samples from 7357 inhabitants of the eastern coast of Kanywon Province were positive for the infection. The infection rate was higher in females than in males. Metacercariae were found in 12 of 50 *Plecoglossus altivelis* caught in local streams.

Snails are the first intermediate host of these trematodes, e.g. *Tarebia granifera* for *Haplorchis* spp.; *Pirenella conica*, *Cerithidea cingulata microptera* for *Heterophyes* spp.; *S. libertina*, *T. granifera* for *Metagonimus* spp. Inside the snail, the development of the trematode usually includes sporocysts, one or two generations of rediae and cercariae.

The metacercariae can infect various species of freshwater or brackish-water fishes. Members of Cyprinidae, Silurdiae and Coltidae commonly serve as the second intermediate host. Examples of the fish host are: for *Heterophyes* spp., *M. cephalus*, *Mugil japonicus*, *Tilapia nilotica*, *Acanthogobius*, *Sciaena aquilla*, *Solea vulgaris*; for *Metagonimus* spp., *Leuciscus hakuensis*, *Odontobutis obscurcus*, *P. altivelis*, *Salmo perryi*, *P. parva*, *C. carassius*; and for *H. pumilo*, *Arius manilensis*.

Humans acquire the infections by eating poorly cooked or raw fish and the

metacercariae develop into adults in the intestine. Concurrent infections of several species of the small-sized trematodes are common. Therefore, the clinical manifestations of each species are not well documented. In general, the parasites only cause mild intestinal symptoms, such as tenderness, chronic bloody diarrhoea, colicky pains, vomiting, etc.

In heavy *Heterophyes* infections, superficial necrosis of the intestinal mucosa may occur. Occasionally, ova may enter the mesenteric circulation, producing granulomatous lesions in the myocardium and brain.

An extensive review on the distribution and prevalence of 19 species of food-borne intestinal trematodes, including *Heterophyes* and *Metagonimus* spp., in South Korea has been given by Chai *et. al.* (1998) and Chai and Lee (2002).

Diagnosis is by finding the characteristic ova of the various species in faecal samples. However, it is extremely difficult to differentiate them morphologically. Lee, S.C. *et al.* (1993) attempted to diagnose metagonimiasis using ELISA.

The treatment method is similar to that for opisthorchiasis.

Cestodiasis

There are relatively few cases of fish-borne cestode infections in humans. The cestodes that mature in the small intestine of humans are not very pathogenic and the diseases are never fatal. Diphyllobothriasis is the major cestodiasis transmitted by freshwater, marine and anadromous fishes.

Diphyllobothriasis

The disease is caused by pseudophyllid cestodes belonging to the genus *Diphyllobothrium*. The genus is characterized by a holdfast structure at the anterior extremity, called the bothrium (Fig. 16.5), and a highly coiled uterus with a uterine pore in the mature proglottids. The adult worm grows

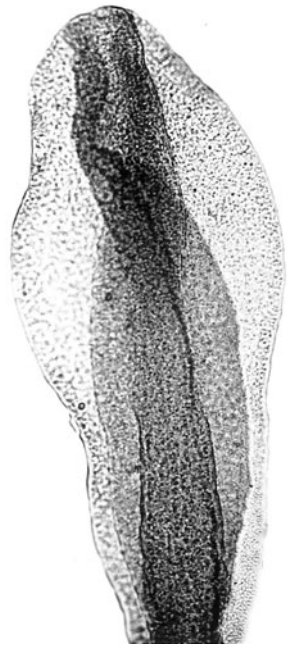


Fig. 16.5. Anterior extremity of *Diphyllobothrium latum,* showing the holdfast structure (bothrium) (× 50, original).

to 3–10 m in length, comprising about 4000 proglottids (each 2–4 mm × 10–12 mm). Most human infections are accidental because the natural definitive hosts of the genus are birds, bears, seals, dogs, walruses, etc. The method of infection is by ingesting raw or smoked fish that harbours the plerocercoid larva. However, there was one case that was presumably due to eating raw euphausiid crustaceans (Fukumoto *et al.*, 1988a).

An update on the foci, patterns, sources, etc. of the disease in humans was given by Dick *et al.* (2001).

The following are some major species of *Diphyllobothrium* that have been found in humans: in North America and Europe, *D. latum* (Linnaeus, 1758) Luhe, 1910; in Greenland and Iceland, *D. cordatum* Leuckart, 1863; on the Pacific coast of South America, *D. pacifica* (Nybelin, 1931) Margolis, 1956; in Alaska, *D. dendriticum* Nitzsch, 1824, *D. alascense* Rausch and Williamson, 1958, *D. lanceolatum* (Krabbe, 1865) Rausch and Hillard, 1970, *D. ursi* Rausch 1954, *D. dalliae* Rausch, 1956; in Japan, *D. nihonkaiense*

Yamane *et al.* 1986, *D. hians* (Diesing, 1850) Markowski, 1952, *D. cameroni* Rausch, 1969, *D. yonagoense* Yamane *et al.*, 1981, *D. scoticum* (Rennie and Reid, 1912) Markowski, 1952.

Several species have been reported in humans in Japan; two were considered new (Yamane *et al.*, 1981, 1986; Fukumoto *et al.*, 1988a; Kamo *et al.*, 1988a,b). Oshima (1984) suggested that the parasite from cherry salmon (*Oncorhynchus masou*) in Japan was probably not conspecific with *D. latum* described in North America and Europe. The mature proglottid of the worms in Japan shows a horizontal cirrus sac and a small seminal vesicle, whereas in *D. latum* the cirrus sac is oblique. Clinically, megaloblastic anaemia is often concomitant in European infections but not in Japanese cases.

However, the taxonomic status of many species within this genus is uncertain. The species are often distinguished on questionable morphological characters. Dick *et al.* (1991), Karlstedt *et al.* (1992) and Matsura *et al.* (1992) noted that it is extremely difficult to separate the different species by classical methods. The possibility of using DNA probes for species identification should be explored.

Epidemiology

Sporadic cases of diphyllobothriasis occur worldwide. According to Oshima (1984), the first scientific report on diphyllobothriasis in Japan was concerned with the self-infection of Professor Iijima of Tokyo University. He swallowed several plerocercoids recovered from salmon at the Tone River (Iijima, 1889). The incidence of diphyllobothriasis started to decline from 1920 and, by the end of the 1950s, no new cases were reported. However, from the 1960s onwards, the disease reappeared along the coast of the Sea of Japan, from Hokkaido Island to Shimane Prefecture. About 100 cases are reported annually. Outbreaks usually occur at the beginning of April and subside by August, i.e. about 1 month after the fishing season for cherry salmon (Oshima and Wakai, 1983).

In Hokkaido, cherry salmon is the major source of the infection, with the humpback salmon playing a minor role. In a survey undertaken between 1977 and 1982, the infection rate of cherry salmon varied from 15.9 to 48.4% and the mean number of worms per infected fish was two to three. Since 1955, the catches of cherry salmon have markedly increased, from several hundred tons before the Second World War to about 4000 tons annually. With increasing affluence, more Japanese can afford to eat raw cherry salmon fillet as 'sushi' or 'sashimi'. This socioeconomic change mainly accounts for the resurgence of diphyllobothriasis in the country. However, there is evidence that the cherry salmon in Japan are not infected prior to their seaward migration. The majority of the migrating fish in the Sea of Japan actually originated from the Siberian coastal area, e.g. Saghalin and Kamchatka Peninsula (Oshima and Wakai, 1983).

Other cases in Japan were also reported (Fukumoto *et al.*, 1988a; Hirai *et al.*, 1988; Kamo *et al.*, 1988a,b). Recently, five cases of *D. nihonkaiense* infections (after eating cherry salmon) were found (Ando *et al.*, 2001).

In South Korea, *D. latum* infection is rare. Two cases of *D. latum parvum* type were recorded by Lee, S.H. *et al.* (1994). Chung *et al.* (1997) reported five cases through eating raw redlip mullet, *Liza haematocheila*. Lee *et al.* (2001) reported that, among four family members who had eaten cherry salmon, two were infected.

In Peru, 136 cases of diphyllobothriasis caused by *D. pacifica* were reported between 1962 and 1976 (Arambulo and Thakur, 1990). The infection is commonly acquired by eating a popular local dish called 'ceviche', which consists of raw marine fish marinated with lime juice.

Sagua *et al.* (2001) attempted to correlate increases in new cases of *D. pacifica* in northern Chile with the cyclical effects of El Niño, as well as environmental contamination.

Infections also occur in Baltic countries and Russia where raw or smoked fish is eaten. Slices of raw fish, known as 'strogonina', are the main source of infection. A study undertaken in the region near

the Krasnoyark reservoir on River Enisel, Russia, between 1984 and 1985 showed that 7.7% of 679 villagers were passing *D. latum* ova, as were 27% of 34 dogs. Plerocercoids were harboured in 87–95% of *Esox lucius* and 11.4–17.5% of *Perca fluviatilis.* This focus of infection occurred about 10 years after the construction of the reservoir (Plyuscheva *et al*., 1987a). Plyuscheva *et al*. (1987b) discussed the role of anthropogenic factors in the establishment of infection foci in reservoirs. They suggested that foci will not be established in heavily polluted industrial areas, where the intermediate hosts cannot survive.

In the western coastal region of the Okhotsk Sea, 1% of 1846 human faecal samples taken in the Khabarorsk Territory were positive for *D. latum* ova. Plerocercoids were found in 27.3% of *Oncorhynchus keta*, 23.5% of *Oncorhynchus gorbuscha* and 30.7% of *Salvelinus leucomaenis* (see Dovgalev, 1988).

Epidemiological data were also given by Curtis and Bylund (1991), Muratov *et al*. (1992) and Revenga (1993).

Biology of parasite

Only the life cycle of *D. latum* is described briefly. The adult worm lives in the small intestine, where it produces unembryonated ova. Each ovum, which is operculated and with a small knob at the opposing end, measures about 55–76 μm × 40–60 μm (Fig. 16.6). In water, it embryonates into a ciliated larva (coracidium), which bears three pairs of hooks. After hatching, the free-swimming coracidium is ingested by copepods (e.g. *Cyclops* spp., *Diaptomus* spp.), the first intermediate host. The procercoid develops in the haemocoel. It has three pairs of hooks at an expanded posterior region called the cercomere.

In the second intermediate host (fish), the procercoid transforms into a worm-like, wrinkled, plerocercoid stage (metacestode, 10–20 mm in length) in muscles, viscera and connective tissues. The plerocercoid is the infective stage and is found in plankton-feeding as well as carnivorous fishes. The latter act as paratenic hosts. Members of the Salmonidae, Percidae and various species common in lakes and reservoirs can be infected, e.g. *E. lucius*, *Stizostedion vitreum*, *Stizostedion canadense*, *Lota maculosa*, *Lota lota*, *Perca flavescens*, *Oncorhynchus nerka*, *O. keta*, *O. gorbuscha*, *Salmo gairdneri*, *S. leucomaenis*, *Salvelinus malma*, *Salvelinus namaycush*, etc.

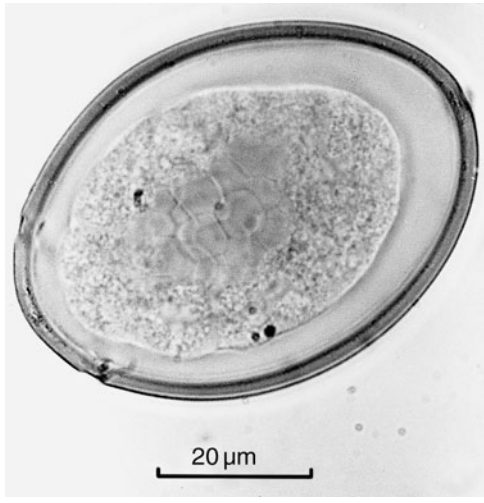


Fig. 16.6. Egg of *Diphyllobothrium latum* (original).

Pathogenesis

The adult worm attaches to the mucosa of the ileum and occasionally, the jejunum. Although a catarrhal condition may be produced in the mucosa, the parasite generally elicits little pathogenicity. For unknown reasons, the worm depletes vitamin B_{12} from its definitive host for its own extensive growth and development.

Symptoms

Most infections are asymptomatic but sometimes epigastric pains, cramp-like abdominal pains, diarrhoea or constipation, nausea, vomiting, loss of weight, anorexia and eosinophilia may occur. The major clinical manifestation in some patients is pernicious anaemia due to vitamin B_{12} deficiency, and there is central nervous system involvement.

Typically, this includes paraesthesia, numbness, impaired vibration sense and weakness. Folate absorption may also be decreased.

A 13–20-fold increase in lysozyme activity has been found in the urine of patients (Gosteva *et al.*, 1991).

Laboratory diagnosis

A brief review on diagnosis was given by Kamo (1988). The traditional method is by stool examination. The operculated ova are relatively large and can easily be seen.

Serodiagnosis is seldom used. Antigenic cross reactivities between the various species have not been fully established. Fukumoto *et al.* (1988b) compared the antigenic structures between *D. latum* and *D. nihonkaiense* by immunoelectrophoresis. Some differences were observed. Using heterologous antiserum, *D. latum* and *D. nihonkaiense* were found to share only six bands. The number of characteristic bands for the former was 21, whereas for the latter it was 14.

Treatment

The drugs of choice are niclosamide (Yomesan), bithionol (Bitin) and paramomycin sulphate. The dosages for the first two drugs are: Yomesan 1 g given early in the morning and then 1 g after 1 h; bithionol, 50–60 mg/kg of body weight in a single dose.

Hayashi and Kamo (1983) found that the administration of paramomycin sulphate would instantly immobilize the worm and destroy the tegument, especially in the anterior region. This would loosen the holdfast structure. A single dose of 50 mg/kg of body weight can result in 100% efficacy.

A single dose of 7–25 mg/kg with praziquantel was effective in treating *D. nihonkaiense* infection (Ohnishi and Murata, 1993). The anaemia and neurological symptoms elicited by the parasite would respond to vitamin B_{12}.

Nematodiasis

Fish-borne nematodiases are generally caused by the incidental infection of humans with nematodes whose natural definitive hosts are marine mammals, birds, pigs or other animals. Freshwater, brackish or marine fishes are the second intermediate host. In most infections, the worms can only survive for a limited period after the initial invasion of the gastrointestinal tract. The method of infection is by ingesting the infective-stage larvae, which can be located in the muscles, intestine or viscera of fish. Unlike cestodiasis, some nematode infections can be fatal.

Capillariasis

Capillariasis is caused by *Capillaria philippinensis* (Chitwood *et al.*, 1968). This species is most unusual because it can mature either in humans or in experimentally infected monkeys and birds. Moreover, the worm can either produce eggs or larvae. The disease was first known to occur in humans in 1963, when the nematode was found at the autopsy of a Filipino in Manila (Chitwood *et al.*, 1964). *C. philippinensis* has since been recognized as a medically important parasite.

Updates on the disease were given by Cross (1990, 1998, 2000). *C. philippinensis* is a trichuroid nematode with a narrow and filiform anterior region. The male worms measure 1.5–3.9 mm in length; the females are 2.5–5.3 mm in length. A trichuroid nematode is also characterized by a glandular structure called the stichosome, which is located posterior to the muscular oesophagus. Most known species have extraordinary life cycles. However, *C. philippinensis* is the only known capillarid that is transmitted to humans via fish. The life cycle has only been established by experimental infections of monkeys, Mongolian gerbils, fish and birds (Cross *et al.*, 1972; Bhaibulaya and Indra-Ngarm, 1979). Presumably the natural cycle is similar (Cross and Basaca-Sevilla, 1989).

Epidemiology

Capillariasis was originally presumed to be an indigenous disease of the Philippines,

where a major outbreak was recorded in 1967. However, subsequently, the disease was also found in Thailand, Japan, Taiwan, Indonesia, Korea, Iran, Egypt and India (Nawa *et al.*, 1988; Cross and Basaca-Servilla, 1991; Chichino *et al.*, 1992; Chunlertrith *et al.*, 1992; Lee, S.H. *et al.*, 1993; Kang *et al.*, 1994; Ahmed *et al.*, 1999). The single cases found in Spain, Italy and the UK were suspected to have originated from Indonesia, Columbia and Egypt (Cross, 1998; Austin *et al.*, 1999). A new focus of capillariasis was recently found in Compostela Valley Province in the Philippines, where 16 of 72 stool samples were positive (Belizario *et al.*, 2000).

In the Philippines, capillariasis was first reported from several islands, i.e. Luzon, Bohol, Mindanao and Leyte. In 1967, the number of confirmed new cases was 1037, with 77 deaths, but, by 1989, the incidence decreased to nine, with no deaths. This was probably due to the improved recognition of the disease and the establishment of an effective therapeutic regimen, using mebendazole. More cases were reported during the rainy season (from May to November). The infection rate was significantly higher in males than in females. Of 1849 cases, 70% were male and the prevalence was highest in the 20–29 age group. This is probably attributable to the fact that, in the endemic areas, most men are farmers who occasionally fish in lagoons, lakes and rivers. They frequently eat part of their catch raw as lunch. Young men also like to gather at nights in their village to drink 'basi' (Philippine gin) and eat raw food (Cross and Basaca-Sevilla, 1989).

Only small fish (6–7 cm in length) are eaten raw. They are known locally as bagsit (*Hypseleotris bipartite*), bagsan (*Ambassis miops*) and bacto (*Eleotris melanosoma*). Larger fish are cooked before eating.

In Thailand, more than 100 cases were reported in the 1980s. All involved fishermen and farmers in the central, northern and north-eastern parts of the country. The inhabitants in these regions enjoy eating a special dish that consists of chopped, raw, freshwater fish, seasoned with lime juice, red peppers and juice from fermented fish (Cross and Bhaibulaya, 1983).

The few cases reported elsewhere all had a history of eating raw fish.

Biology of parasite

Several species of monkey (*Macaca cyclopis*, *Macaca fascicularis* and *Macaca mulatta*) and Mongolian gerbils (*Meriones unguiculatus*) were successfully infected in the laboratory. Small wild rats (*Rattus* sp.) are also susceptible, but the patent period is short (Cross and Bhaibulaya, 1983).

Fish-eating birds, however, are presumed to be the natural definitive host. *Amauronis phoenicuris*, *Ardeiola bacchus*, *Bulbulcus ibis*, *Nyticorax nyticorax* and *Ixobrychus sinensis* were successfully infected under laboratory conditions (Bhaibulaya and Indra-Ngarm, 1979; Cross *et al.*, 1979). A natural infection was observed in yellow bittern in the Philippines (Cross and Basaca-Sevilla, 1989).

Adult worms are located in the lumen of the small intestine (Fig. 16.7). The female worms produce first-stage larvae as early as 13–14 days post-infection. The first-generation larvae are retained in the intestine, where they develop into a second generation of males and females in about 10 days. Most females in the second generation produce eggs (36–45 μm × 20 μm), which are peanut-shaped, with a thin shell and flattened bioperculated plugs (Fig. 16.8). The eggs are passed out with the faeces. But some females in the population are ovoviviparous, producing larvae, and this results in autoinfection. Cross *et al.* (1978) suggested that autoinfection is an integral part of the life cycle, both initially and in maintaining the infections. However, the genetic and molecular mechanisms that control such a unique developmental process have not been studied.

Embryonation of the eggs occurs in water and, upon ingestion by freshwater or brackish-water fish, first-stage larvae hatch in the intestine. The worms grow to maximal length (250–300 μm) without moulting in about 3 weeks. The fully grown larvae are infective to the definitive host.

The following freshwater and brackish-water fishes were infected in the

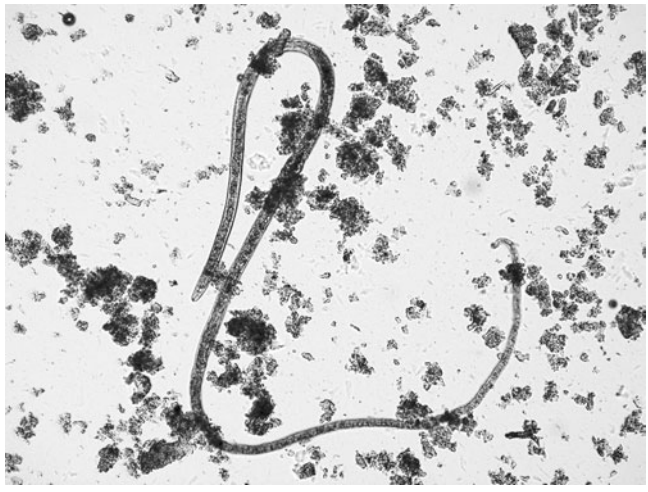


Fig. 16.7. Female *Capillaria philippinensis* (without eggs, × 160) (courtesy of Dr John Cross).



Fig. 16.8. Egg of *Capillaria philippinensis* showing two polar plugs (× 400, courtesy of Dr John Cross).

laboratory: *H. bipartite*, *A. miops*, *E. melanosoma*, *Scyopterus* sp., *Poecilia reticulata*, *C. carpio*, *Puntius gonionotus*, *Aplocheilus panchax*, *Gambusia holbrooki*, *Rasbora borapetenis* and *Trichopsis vittatus* (see Bhaibulaya *et al.*, 1979; Cross and Bhaibulaya, 1983).

Pathogenesis

Although capillariasis is basically an intestinal disease, untreated infections can be fatal. This is in part due to autoinfection, which is an inherent feature of the life cycle. Hence the parasite population can be increased dramatically within a short period. At the necropsy of two gerbils (each given two larvae) on 46 and 47 days post-infection, 2520 and 5353 worms were recovered (Cross and Bhaibulaya, 1983). The presence of a large number of parasites invariably disturbs the normal gastrointestinal functions, resulting in extensive pathophysiological lesions.

The lesions are usually more prominent in the jejunum. Atrophy of the crypts of Lieberkühn occurs and the villi are denuded and flattened. The lamina propria is mainly infiltrated with mononuclear leukocytes. Ultrastructural studies have shown a complete loss of adhesion specialization and widespread separation of the jejunal epithelial cells, with microulceration and compressive degeneration (Sun *et al.*, 1974).

The pathophysiological process during the acute phase of infection seems to be caused by alterations in the gastrointestinal functions. Toxins or other secretions released by the worms may be responsible for the diarrhoea and deficiencies in digestion and absorption, as well as the increased permeability of the intestinal mucosa to protein loss from the plasma. Since an increase in IgE has been observed (Rosenberg *et al.*, 1970), changes in the gastrointestinal functions may be due to anaphylactic response (Watten *et al.*, 1972).

Symptoms

The onset is marked by borborygmi and diffuse abdominal pain. This is followed by intermittent diarrhoea, two to five times a day, especially after every meal. The stool is watery, greenish yellow in colour with a foul odour. The volume may reach 2 l/day in severely ill patients. A marked protein-losing enteropathy and steatorrhoea generally result in the loss of large quantities of protein, fat and minerals in the stool. With the onset of diarrhoea, rapid weight loss occurs. Muscle wasting, weakness, emaciation, abdominal distension and oedema are prominent. Distant heart sounds, hypotension and other cardiac abnormalities may be present in some patients. Death due to cardiac failure may occur 2 weeks to 2 months after the initial onset of symptoms.

Laboratory studies have shown a decrease in the excretion of xylose, hypokalaemia, hypocalcaemia and hypoproteinaemia. An increase in IgE is common (Watten *et al.*, 1972; Cross and Basaca-Sevilla, 1989).

Laboratory diagnosis

The standard laboratory diagnostic method is by stool examination to search for the characteristic eggs, larvae and adults. Several stool samples may sometimes be required to detect the infection. Duodenal aspiration can be used to recover the worms.

IHA and double diffusion tests using antigens prepared from a chicken capillarid (*Capillaria obsignata*) have been evaluated for serodiagnosis but the test lacks both specificity and sensitivity (Banzon *et al.*, 1975). An ELISA test using somatic antigens of adult *C. philippinensis* was also unsatisfactory (Cross and Chi, 1978).

Treatment

Mebendazole is the drug of choice. The dosage is 400 mg/day (in divided doses) for 20–30 days. Few relapses occur with this regimen. Albendazole is also effective at the same regimen as mebendazole (Cross and Basaca-Sevilla, 1987). However, treatment with thiabendazole may result in relapses (Cross, 2000).

Gnathostomiasis

Gnathostomiasis is caused by members of the genus *Gnathostoma* that undergo visceral larval migration. Four species have been found in humans, i.e. *G. hispidum* Fedchenko, 1872. *G. spinigerum* Owen, 1836, *G. doloresi* Tubangui, 1925 and *G. nipponicum* Yamaguti, 1941. Gnathostomatid nematodes are characterized by a head bulb and a fluid-filled cephalic–cervical system. The wall of the head bulb is lined with circular musculature (Ko *et al.*, 1980). Both the head bulb and the body cuticle bear rows of spines (Fig. 16.9).

The definitive hosts are pigs, cats (and other felids), wild boars and weasels. They serve as the source for human infections. The adult worm is located in a tumour-like structure in the stomach or oesophagus; the head of the worm is embedded inside the wall. The infective third-stage larva undergoes extensive tissue migration if ingested by humans. The infection may be fatal.

Epidemiology

The disease occurs in South East Asia, China, Japan, Korea, the Indian subcontinent, the

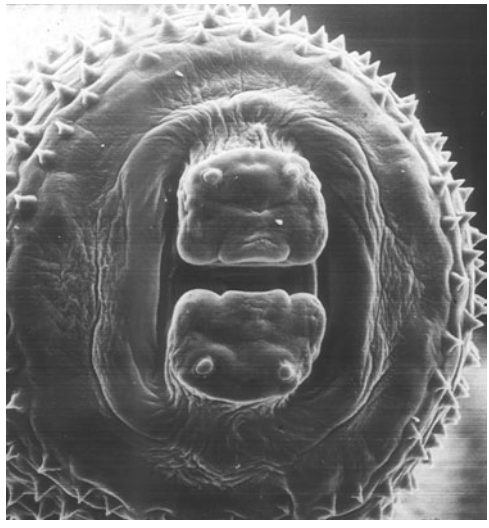


Fig. 16.9. *En face* view of head of young male adult *Gnathostoma spinigerum* from stomach of experimentally infected cat, showing the large pseudolabia and cephalic spines (× 400, original).

Middle East and Mexico. However, due to the nature of the infection and the lack of a specific seroepidemiological method, large-scale epidemiological studies have seldom been carried out.

In Thailand, gnathostomiasis, which is caused by *G. spinigerum*, is endemic in several provinces in the central region, where local people eat raw fish dishes known as 'hu-sae', 'som-fak' and 'pla-som'. Daengsvang *et al.* (1964) examined seven species of freshwater fishes and found the infection rates varied from 2.3 to 16.7%. The rates (33–83%) appeared to be markedly higher in four species of snakes.

During 1985–1988, more than 800 suspected cases were encountered annually at two major hospitals in Bangkok. Also during 1987–1989, *G. spinigerum* larvae were found in about 80% of freshwater eels (*Fluta alba*) purchased from a local market (Setasuban *et al.*, 1991). A survey undertaken by Nuamtanong *et al.* (1998) in central Thailand showed that the infection rates in 788 eels varied from 10 to 68.7%. The seasonal infection rates in eels bought at Bangkok were also studied by Rojekittikhun *et al.* (1998) and Saksirisampant *et al.* (2002).

A survey in 1965–1970 showed that 4% of cats and 1.1% of dogs in Bangkok were infected (Harinasuta, 1984). Most human cases were encountered during the rainy season (May to September). About 96% of the patients drank untreated water from ponds or canals, which were also suspected to be possible sources of infection (Punyagupta and Bunnag, 1991).

In Hong Kong, although the occurrence of gnathostomiasis has not been officially documented, two (4%) of the 49 parasitic cases referred to the author's laboratory (1987–1990) were due to this infection. One patient probably acquired the infection in Thailand. The second patient had eaten raw fish in a local restaurant. Of the 28 freshwater catfish (*Clarias fuscus*) that were purchased from local markets and examined by the pepsin digestion method, 11% were found to be infected with *G. spinigerum*; 106 third-stage larvae were recovered. Larvae were also found in *Clarias batrachus*, *Ophiocephalus* spp., frogs and snakes (R.C. Ko, unpublished data). An extensive survey was undertaken by Koga *et al.* (1996) on the presence of *Gnathostoma* in 689 freshwater fish in the south-eastern Yangtze Valley, China, during 1989–1990: 55 larvae were recovered.

In Japan, gnathostomiasis was rare and caused by *G. spinigerum*. However, since the 1980s, sporadic cases of *G. hispidum* infection have been reported in urban areas. The Japanese are fond of eating a freshwater loach (*Misgurnus anguillicaudatus*), which is sometimes swallowed live. Loaches have disappeared from the rice paddies in Japan due to extensive applications of agricultural chemicals. As a result, the fish has to be imported from China, Taiwan and South Korea, where it is commonly infected with *G. hispidum*. About 10% of the loaches were infected, each fish with six or seven larvae (Oshima, 1984).

Two cases of *G. doloresi* infection were first reported in humans in Japan. Both patients had a history of eating raw fish (Ogata *et al.*, 1988). Nawa *et al.* (1997) found 25 cases in Miyazaki Prefecture, most of the cutaneous type. Oyamada *et al.* (1997) examined 977 gobiid fish and found only one species, *Chaenogobius urotaenia*, that carried *G. nipponicum* larvae.

A survey in Bangladesh (1968–1970) showed that six species of fish, one species of frog and one species of snake were infected with *G. spinigerum* larvae. The rates of infection in fish varied from 15 to 60% (Bashirullah, 1972).

In Ho Chi Minh City, Vietnam, Le and Rojekittikhun (2000) examined 1081 eels and reported that the average rate of *G. spinigerum* infection was 0.11. However, the authors only used the insensitive tissue press method to detect the larvae.

In Mexico, gnathostomiasis was first recorded in 1970. Since then, the number of patients in various parts of the country seems to be increasing (Ogata *et al.*, 1998; Rojas-Molina *et al.*, 1999). In Culiacan, Sinaloa, 300 cases of cutaneous larva migrans were recorded between 1992 and 1995 (Diaz-Camacho *et al.*, 1998).

Biology of parasite

Only the biology of *G. spinigerum* and *G. hispidum* is briefly described.

The adult worms are located in the stomach wall of the definitive host, where unembryonated ova are deposited and passed to the outside via the intestine. The ovum (62–79 μm × 36–42 μm) is characterized by a polar thickening (Fig. 16.10). Embryonation occurs in fresh water and, upon ingestion by copepods, the hatched first-stage larva penetrates the digestive tract and reaches the haemocoel, where it develops into the second-stage larva. When the infected copepod is ingested by the second intermediate host (a freshwater fish or frog), the second-stage larva enters the muscle and moults into the third-stage worm, which is eventually encysted. A fully grown third-stage larva (4–5 mm) is infective to the definitive host (Fig. 16.11). Other fish, amphibians, snakes, mice, rats or chickens can serve as the paratenic host, where third-stage larvae are encysted in muscles.

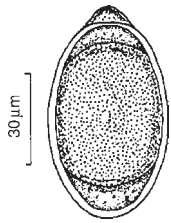


Fig. 16.10. Egg of *Gnathostoma spinigerum* (original).

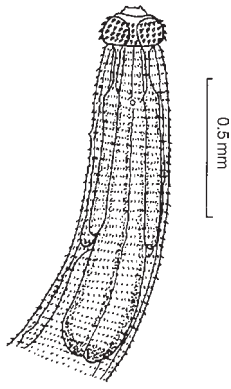


Fig. 16.11. Anterior region of third-stage larva of *Gnathostoma spinigerum* from catfish (*Clarias fuscus*) (original).

Daengsvang (1968) fed experimentally infected copepods to frogs, lizards, chicks, rats, mice, tree shrews and pigs and found advanced third-stage larvae in the liver, muscle and other organs. He also recovered larvae from two newborn mice after 22 pregnant females were fed with infected copepods.

The parasite has a low host specificity. Many species of copepods, amphibians, reptiles, freshwater fish and birds can serve as the intermediate and paratenic hosts. The following copepods have been commonly used in experimental studies: *Cyclops strenus*, *Cyclops vicinus*, *Eucyclops serrulatus* and *Mesocyclops leuckarti*.

The common fish hosts are *Ophiocephalus argus*, *Ophiocephalus* (= *Channa*) *striata*, *Ophiocephalus micropeltes*, *O. maculatus*, *Mogurnda obscura*, *M. anguillicaudatus*, *C. fuscus*, *C. batrachus*, *Anguilla japonica*, *Parasilurus asotus*, *Cyprinus auratus*, *C. carpio*, *Acanthogobius flavimanus*, *Heteropneustes fossilis* and *F. alba*.

Pathogenesis

If humans ingest the third-stage larva from fish, frogs or snakes, the worm undergoes visceral migration. A similar migration will also occur upon ingestion of infected copepods (Daengsvang, 1968). The degree of pathogenesis, however, is dependent on the extent of tissue invasion by the larvae (Sagara, 1953; Nishibuko, 1961).

Takakura (1988) showed that, in experimentally infected rats, *G. hispidum* larvae entered the abdominal cavity by penetrating the intestinal wall within 12 h post-infection. The worms then invaded the liver. About 3 weeks post-infection, they entered the muscles of the inner abdominal wall.

The pathogenesis of gnathostomiasis is probably partly caused by toxins secreted by the migrating larvae. These include acetylcholine, proteolytic enzymes, a haemolytic compound and hyaluronidase-like substances (see Muller, 1975).

The migrating larvae in subcutaneous tissues cause creeping eruptions along

their route. Intermittent swellings as a result of erythematous oedema are present. Invasion of the liver can disturb the normal function of this organ and the invasion of the eye, bladder or uterus can cause permanent impairment of vision, iritis, haemorrhages, orbital cellulitis, haematuria and leucorrhoea (Daengsvang, 1949). If the larvae enter the brain and spinal cord, eosinophilic encephalomyelitis will occur (Chitanondh and Rosen, 1967). Abscesses of the breast, mastoid region and subcutaneous tissues have also been reported.

Typical lesions of the infection usually show areas of degeneration, necrosis and perivascular oedema, with heavy cellular infiltrations of polymorphs. Haemorrhagic exudates, mononuclear leukocytes and fibroplasia also occur.

Symptoms

The disease is characterized by eosinophilia (5–90%) and the presence of migratory swellings of different sizes, which can be located on the chest, abdomen, head, eyelid and other parts of the body. The swelling is sometimes itchy, irritating and tender but shows no pitting on pressure. Involvement of the eyes may show iritis, ecchymosis, subconjunctival haemorrhages and orbital cellulitis. Involvement of the lungs will lead to mild coughing and difficulty in breathing. Involvement of the central nervous system will result in meningeal irritation, headache, convulsion, hypoaesthesia, paresis, paraplegia, coma and sometimes death. Only occasionally a slight fever occurs.

When there is central nervous system involvement, the symptoms of gnathostomiasis can be distinguished from those of angiostrongyliasis (a neurotropic nematodiasis also endemic in South East Asia). The former disease is usually in the form of eosinophilic myeloencephalitis, characterized by transverse or ascending myelitis following the symptoms of nerve root pain. The cerebral spinal fluid (CSF) is bloody or xanthochromic (Punyagupta, 1979). Also, due to the visceral migration of the larvae, there are signs and symptoms referable to other organs (besides the central nervous system), which have not been seen in angiostrongyliasis (Chitanondh and Rosen, 1967). Angiostrongyliasis is in the form of eosinophilic meningoencephalitis and the CSF is clear or turbid (Ko *et al.*, 1987).

Laboratory diagnosis

Although third-stage larvae can be surgically removed from the superficial swellings, serodiagnosis should serve as the key diagnostic method. However, in ELISA and IFAT, there are significant cross reactions between the crude antigens and heterologous antiserum of *G. spinigerum*, *G. hispidum* and *Angiostrongylus cantonensis* (Ko *et al.*, 1987). Therefore, before serological methods can be used for differential diagnosis, highly specific antigens must first be isolated from *Gnathostoma* spp.

Mimori *et al.* (1987) used ELISA and crude *G. doloresi* antigens to test the sera samples of known gnathostomiasis cases in Ecuador. Sera from 15 of 16 patients and five of 18 controls were found to be positive. Tada *et al.* (1987) reported a close correlation between the results obtained by *G. doloresi* and *G. hispidum* antigens in ELISA when they were used to screen sera samples from 30 patients. However, *G. doloresi* antigens have been reported to be less specific than those of *G. spinigerum* (Anantaphruti, 1989a,b).

According to Chaicumpa *et al.* (1991) and Nopparatana *et al.* (1991), a specific 24 kDa protein antigen has been isolated from the third-stage larvae of *G. spinigerum* by gel filtration, chromatofocusing and anion exchange chromatography. The antigen, which has a pI of 8.5, was used in the indirect ELISA to screen sera samples from four parasitologically confirmed patients, 15 clinically diagnosed patients, 136 patients with other parasitic diseases and 25 controls. The sensitivity, specificity and predicative values of the assay were 100%.

Using immunoblotting, Akao *et al.* (1989) studied sera samples from nine patients who were infected with *G. hispidum*. An epitope having a M_r of 31,500 was prominent in all samples.

Attempts have also been undertaken to detect circulating antigens. Tuntipopipat *et al.* (1989) successfully detected antigens in the CSF using a sandwich ELISA (using antibodies from rabbits immunized with E/S antigens from third-stage larvae). With a biotin–streptavidin procedure, antigens as low as 2 ng/ml could be detected. However, of the 11 patients showing symptoms of cerebral involvement, antigens were only detected in the CSF of one patient, whereas IgG antibodies were detected in nine patients. One other patient had immune complexes in the CSF.

A two-site ELISA was developed to detect circulating antigens in mice (Maleewong *et al.*, 1992b).

Treatment

The worms can be removed surgically when they are close to the skin. Thiabendazole, at a dosage of 2 g daily for 20 days, may help to alleviate some symptoms (Harinasuta, 1984). Albendazole has also been tried but the results were unsatisfactory (Suntharasamai *et al.*, 1990; Maleewong *et al.*, 1992a). However, according to Tierney *et al.* (2001), albendazole at dosages of 400 or 800 mg daily for 21 days may be effective. Ivermectin (single dose) has also been suggested to be effective (Nontasut *et al.*, 2000).

Anisakiasis

Anisakiasis refers to infection by larval ascaridoid nematodes whose normal definitive hosts are marine mammals. The genera involved are *Anisakis*, *Pseudoterranova* and *Contracaecum*. Larvae from squids and marine fish can invade the gastrointestinal tract of humans, causing an eosinophilic granuloma syndrome. In Europe, it has also been referred to as the 'herring worm' disease. The first human infection was documented in the Netherlands in 1955 and then later in Japan (van Thiel *et al.*, 1960; Asami *et al.*, 1965). However, in the Netherlands, since the passage of legislation against eating raw herring and requiring fish to be frozen prior to sale, anisakiasis has almost disappeared. Freezing fish for 24 h or heating processed fish to 65°C can kill the larvae. Also, the gutting of fish soon after they are caught prevents the migration of larvae to muscles.

The disease was reviewed extensively by Oshima (1972) and an update on the bibliography was given by Huang and Bussieras (1988). In Japan, more than 2000 cases of anisakiasis have been noted. Sporadic cases are also known to occur in North America and other parts of the world (Chitwood, 1970, 1975; Little and MacPhail, 1972; Suzuki *et al.*, 1972; Kates *et al.*, 1973; Little and Most, 1973; Lichtenfels and Brancato, 1976; Oshima, 1984; Huber *et al.*, 1989; Kowalowska-Grochouska *et al.*, 1989; Kim *et al.*, 1997; Machi *et al.*, 1997; Takabe *et al.*, 1998).

The taxonomy and morphogenesis of the anisakid nematodes are confusing. This is probably due to the fact that their life cycles have not been elucidated under experimental conditions. The identification of the adults and larvae from fish and squid is presently based on the classical morphometric method, which has great limitations. However, the larvae of the following species are considered to be the major aetiological agents: *Anisakis simplex* (Rudolphi, 1809), *Anisakis typical* (Diesing, 1861), *Anisakis physeteris* (Baylis, 1923), *Pseudoterranova decipiens* (Krabbe, 1878) and *Contracaecum osculatum* (Rudolphi, 1802). The worms are characterized by three lips and a tooth at the anterior extremity. Zhu *et al.* (1998) delineated the larvae of *A. simplex*, *Hysterothylacium aduncum* and *C. osculatum* by nuclear ribosomal DNA sequences, PCR-based restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism methods.

Genetic markers based on PCR-RFLP analysis and allozyme electrophoresis are useful in the identification of the sibling species of *A. simplex* (D'Amelio *et al.*, 1999; Paggi *et al.*, 2001). Using these methods, the larva obtained by endoscopy from a man in southern Italy was successfully identified as *Anisakis pegreffii*.

There are three types of *Anisakis* larvae, i.e. type I, type II and type III. The type I larva, measuring 19–36 mm in length, has a short tail (0.08–0.16 mm). The body length of type II is similar (19.0–25.5 mm) but the tail is longer (0.18–0.32 mm in length). The type III larva, measuring 23.8–38.4 mm in length, has the widest body (0.65–0.97 mm in width). The larva of *Pseudoterranova* is referred to as type A and that of *Contracaecum* as type B (Oshima, 1972).

Attempts have been undertaken to differentiate the various larvae using molecular biology methods. An analysis of RFLPs was applied to distinguish type I, type II and type B larvae. Different patterns of RFLPs of genomic DNA were observed. The patterns of the two different paratenic host-derived DNA were the same in hybridized fragments generated by endonucleases (Sugane *et al.*, 1989).

Epidemiology

More than 100 species of fish that the Japanese like to eat raw can harbour anisakid larvae. In Japan, eating raw fish is part of the culture and a traditional way of life. Hence, anisakiasis still occurs regularly.

The following Japanese dishes include raw sea food: 'sashimi' is raw fish or squid fillet eaten with shoyu and wasabi; 'sunomono' is pickled fillet of fish or squid with vinegar; 'isushi' is pickled rice with raw fillet of chum salmon, masu, cod and cod roes.

Anisakiasis due to type I larvae has been reported throughout Japan, from Hokkaido Island to Okinawa. The most commonly infected fishes are the common mackerel, Pacific pollack, cod, various salmon, herring and tuna. Slightly salted roes of cod and herring are also sources of infection. The majority of worms removed from the stomach wall of patients by gastrofibroscopy were type I larvae (Oshima, 1984).

Extensive surveys on the prevalence of anisakid larvae in fishes, squids and marine mammals have been undertaken in Japan (see Oshima, 1972). A study was carried out by Kobayashi *et al.* (1966) and Koyama *et al.* (1969) to determine the prevalence of infection in 15 species of fishes and five species of squids that were commonly available at the Tokyo metropolitan fish market. The prevalence rates of type I larva were 100% in *Theragra chalcogramma* (Pacific pollack) and *O. masou* (masu salmon), 83% in *Pneumatophorus japonicus japonicus* (common mackerel) and 50% in *Todarodes pacificus* (squid). These are the species that are most commonly eaten raw by the Japanese.

In Hokkaido, the incidence of regional enteritis has been observed to increase from October to March and decrease during summer (Ishikura, 1968). This seems to coincide with the fishing of Pacific pollack. However, in other parts of Japan, seasonal variations in the incidence of anisakiasis have not been observed.

Seasonal variations have been observed in the infection of squids (*T. pacificus*) with type I and type II larvae. Squids collected in June usually have the lowest prevalence and intensity of infection (Yamaguchi *et al.*, 1968; Kagei, 1969; Kosugi *et al.*, 1970). This was attributed to the seasonal migration of squids along the coast of Japan.

In Japan, anisakiasis appears to be more common in males than in females. Of 95 patients seen by Yokogawa and Yoshimura (1967), 65 were male. This is probably because men frequently consume raw fish at drinking parties.

In the USA, a study has shown that all of 171 mature herring (*Clupea harengus pallasi*) from the Puget Sound, Washington, were infected with *Anisakis* and *Contracaecum* larvae. The average worm burden was 27 per fish and 36% of fish harboured worms in muscles. The prevalence in the Pacific herring is markedly higher than that recorded previously. The increase is probably related to an increase in the number of marine mammals in Puget Sound and along the coastline after the enactment of the Marine Mammal Protection Act of 1972 (Adams *et al.*, 1990). Despite the common occurrence of anisakid larvae in fish in the USA, only a handful of anisakiasis cases have been reported. Most Americans do not eat raw fish or squid.

In China, Ma *et al.* (1997) studied the prevalence of anisakid larvae in marine fish and cephalopods from the Bohai Sea. A total of 5992 *A. simplex* larvae were recovered from 121 of 290 fish examined (belonging to 15 species) and eight of 108 squids.

In Mexico, five fish species (*Lutjanus synagris*, *Gerres cinereus*, *Sypyraena barracuda*, *Epinehelus morio*, *Haemulon plumieriwere*) commonly used for preparing a popular dish (cebiche) made with raw fish were examined. *Pseudoterranova* sp. was found in *E. morio* and *S. barracuda*, with a prevalence of 83 and 33%. *Contracaecum* sp. was found in *G. cinereus*, with a prevalence of 57% (Laffon-Leal *et al.*, 2000).

In Spain, a high prevalence of anisakid larvae has also been found in *Micromesistius poutassou*, *Merlucius merlucius* and *Scomber scombrus*. These fishes are common items in the Spanish diet (de-la-Torre-Molina *et al.*, 2000; Valero *et al.*, 2000).

Biology of parasite

The life cycle of anisakid nematodes has not been convincingly elucidated. It is too expensive to infect marine mammals experimentally. The following brief account is based on the reports by Oshima (1972) and Smith (1983).

Adult worms live in the stomach of the definitive host. The ellipsoidal eggs have a thick shell, measuring about 46–58 μm × 41–53 μm. They are passed to the outside with the faeces. Embryonation of the egg occurs in the sea. The egg hatches to release the second-stage larva, which is ingested by a euphausiid crustacean. In the haemocoel of the euphausiid, the second-stage larva moults to the third-stage worm, which can grow to a length of about 30 mm. Upon ingestion by a fish or squid (paratenic host), third-stage larvae penetrate the gastrointestinal tract and enter the viscera or muscles. The odontocete marine mammals acquire the infection by eating infected fish or squids. The worm attaches to the stomach wall and moults within 3–5 days to the fourth-stage larva. After further moulting, the worm develops to the adult stage.

Oshima (1972) provided a detailed list of fish in which anisakid larvae have been found. He listed 123 species for type I larvae, 25 for type II, two for type III, eight for type A and four for type B. These include *Oncorhynchus* spp., *T. chalcogramma*, *Gadus macrocephalus*, *P. japonicus*, *Clupea pallasi*, *Sarda orientalis*, *Epinephelus* spp., *Sebastes* spp., *Fugu* spp., *Osmerus dentex*, *Hexagrammos otakii*, *Sebasticus albofasciatus*, etc.

The common cetacean hosts are *Stenella caeruleo-alba*, *Phocaena phocoena*, *Phocoenoides dalli* and the pinniped hosts are *Phoca vitulina*, *Callorhinus ursinus* and *Enmetopias jubata* (Oshima, 1972).

The common euphausiid hosts are *Thysanoessa raschii*, *Thysanoessa longipes* and *Euphausia pacifica*.

Stromnes and Andersen (2000) compared the seasonal variation of the third-stage larvae of *A. simplex* in *Gadus morhua*, *Pollachius virens* and *Sebastes marinus* in Norway. The most distinct variation was observed in the first species. This 'spring rise' could be attributed to the increased supply of parasite eggs from northward-migrating whales and the spring bloom of plankton.

Iglesias *et al.* (2001) successfully cultured third-stage larvae of *A. simplex* to the adult stage in RPMI 1640 medium with fetal bovine serum and pepsin.

Pathogenesis

The pathology of anisakiasis in humans and experimental animals has been studied extensively (Meyers, 1963; Ashby *et al.*, 1964; Kuiper, 1964; Yokogawa *et al.*, 1965; Usutani, 1966; Yoshimura, 1966; Oyangi, 1967; Young and Lowe, 1969; Gibson, 1970; Ruitenberg *et al.*, 1971; Kikuchi *et al.*, 1972; Oshima, 1972; Jones *et al.*, 1990).

Anisakid larvae can penetrate into the stomach wall of rats within 1–4 h post-infection. Meyers (1963) recovered type I larvae from the mesentery, pancreas, liver and other tissues of experimentally infected guinea pigs. The degeneration of larvae has been observed in the submucosa of rabbits and dogs 10 days post-infection

(Kuiper, 1964; Usutani, 1966; Oyangi, 1967; Ruitenberg *et al.*, 1971).

The human cases diagnosed in Europe mainly involved the intestine, whereas in Japan gastric and intestinal infections are equally common. Lesions of human anisakiasis are classified into five types (Oshima, 1972). The foreign-body response type is characterized by cellular infiltrations, consisting mainly of neutrophils, with a few eosinophils and giant cells. This type is caused by a primary anisakid infection without any presentation.

The phlegmonous type is characterized by extensive oedematous thickening in the submucosa, with heavy infiltrations of eosinophils, lymphocytes, monocytes, neutrophils and plasma cells. Haemorrhages and fibrin exudation are also present. Such lesions are common in acute intestinal cases within 1 week of infection.

The abscess type is marked by the presence of numerous eosinophils, histiocytes and lymphocytes around the worms, surrounded by a granulomatous zone. Necrosis, haemorrhages, fibrin exudation or fibrinoid degeneration occur near the inner region of the surrounding granuloma. Such lesions occur in chronic cases.

The abscess–granulomatous type shows a reduction of the central abscess around highly degenerated worms, which is circumscribed by conspicuous granulation tissue with slight collagenization. Eosinophilic infiltration is less extensive than in the abscess type. This type occurs in infections of more than 6 months.

In the granulomatous type, the abscess is completely replaced by granulomatous tissue with eosinophilic infiltration. Occasionally, remnants of degenerated worm tissue can be seen. Such lesions are rare.

Recently, *A. simplex* has been emerging as an important food allergen. Many persons who had eaten fish showed hypersensitive symptoms, anaphylaxis and chronic urticaria, with high levels of specific IgE and positive skin-prick test results to the worm (Daschner *et al.*, 2000; Sanchez-Velasco *et al.*, 2000; Gracia-Bara *et al.*, 2001; Audicana *et al.*, 2002).

Symptoms

Acute gastric infections are manifested by gastric pain, nausea and vomiting 4–6 h after ingesting raw infected seafood. During the chronic phase, vague epigastric pain, nausea and vomiting may last from several weeks to 2 years. In more than 50% of the recorded cases, eosinophils varied from 4 to 41%. Occult blood may occur in the gastric juice and stools.

The onset of intestinal anisakiasis occurs within 7 days after ingesting infected raw seafood. The symptoms are severe pain in the lower abdomen, with nausea and vomiting. The pressure point, unlike appendicitis, is vague and there is no muscular rigidity. Marked leucocytosis is common but eosinophilia is rare. Straw-coloured ascites may be present.

Laboratory diagnosis

Fibre-optic gastroscopy is most useful to diagnose gastric anisakiasis. The diagnosis of intestinal cases, however, relies mainly on clinical symptoms and a history of eating raw seafood. X-rays, after administration of a barium meal, may show segmented movement of the barium and jagged stricture of the regional intestine, with proximal enlargement and a radiolucent area (Oshima, 1972; Higashi *et al.*, 1987). Ultrasound has also been used to diagnose intestinal anisakiasis (Shirahama *et al.*, 1992).

Many serodiagnostic methods have been tried, e.g. skin test, CFT, IFAT and ELISA (Yoshimura, 1966; Kikuchi *et al.*, 1970; Ruitenberg, 1970; Suzuki *et al.*, 1970; Boczon, 1988). The major problem is the failure to isolate specific antigens. Kennedy *et al.* (1988) reported extensive antigenic relationships between the somatic and E/S antigens of *A. simplex* and those of other ascarids, such as *Ascaris suum*, *Ascaris lumbricoides* and *Toxocara canis*. Rodero *et al.* (2001) attempted to purify *A. simplex* antigens by affinity chromatography.

E/S antigens were used in immunoblots to diagnose human anisakiasis (Akao *et al.*, 1990; Petithory *et al.*, 1991; Iglesias *et al.*, 1993).

Monoclonal antibodies

Takahasi *et al.* (1986) developed two mAbs, An1 and An2, from type I larvae. Using SDS-PAGE and Western blotting, An1 precipitated a band of 34 kDa and An2 bands of 40 and 42 kDa, respectively. An2 was also detected in the E/S antigens using ELISA. Yagihashi *et al.* (1990) reported that serum from patients reacted strongly to An2 in micro-ELISA.

Five mAbs (UA2, UA3, UA5, UA6, UA8) were produced by Iglesias *et al.* (1997). Lorenzo *et al.* (1999) found that 'UA2R' antigens (two proteins with molecular mass (MM) 48 and 67 kDa) were recognized by mAb UA2. 'UA3R' antigens (two proteins with MM 139 and 154 kDa) were recognized by mAb UA3. Ninety-five per cent of Japanese anisakiasis patients and 84% of the allergy patients showed detectable IgG_1 antibodies to the UA3R antigens. All allergy patients showed IgE antibodies to these antigens.

IgE

Specific IgE was found in anisakiasis patients (Kasuya and Koga, 1992). Garcia *et al.* (1997) found that IgE immunoblotting was useful in diagnosing *A. simplex* allergy. Daschner *et al.* (1999) considered that a rise of total and specific IgE in the first month after an allergic reaction is a good indication of gastro-allergic anisakiasis, even if the parasite cannot be seen by gastroscopy. Using ethanol precipitation and reversed-phase HPLC, Moneo *et al.* (2000) isolated an E/S protein of 24 kDa (named Ani s1) that is IgE-binding. Caballero and Moneo (2002) suggested that hypersensitivity and intestinal anisakiasis can be diagnosed by determining the specific IgE directed towards this major allergen.

Fish screening

Although a reliable serological method has not been developed to mass-screen fish for anisakid worms, Huber *et al.* (1989) briefly reported on the use of ELISA to detect antibodies against *A. simplex* in the saithe fish, *P. virens*. A good correlation was observed between antibody titres against E/S antigens and the number of anisakid larvae in fish. However, it is not known whether a cross-reactive study has been carried out by these authors and whether the fish used were also naturally infected with other species of parasites.

Treatment

Removing worms by gastrofibrescopy is the method of choice in acute gastric infection. For chronic infections, a partial resection of a small area of the lesion has been recommended.

For intestinal anisakiasis, only conservative treatment can be used. However, colonoscopic removal of worms was successful (Minamoto *et al.*, 1991).

Control

Fish-borne parasitic zoonoses can be controlled by requiring all fish or fish products to be deep-frozen or well cooked before consumption. Guidelines can be posted on a website. Irradiating fish with high levels of gamma-radiation (200 Gy) can reduce the infectivity of larval parasites in muscles and minimize the risk of transmission (Chai *et al.*, 1995). However, this may not be a cost effective measure for large-scale or routine operation. The high cost of the commercial irradiator and the problems of disposing of radioactive wastes are the limiting factors.

Conclusions

Since the biology and pathogenesis of many fish-borne parasites still remain unknown, it is possible that new zoonoses may be discovered if clinicians and public health workers are better informed about parasitic diseases.

Theoretically, fish-borne parasitic zoonoses can easily be prevented by refraining from eating raw seafood. However, in many parts of the world, such an eating habit represents an established way of life or part of the inherent culture. It cannot be

easily changed, even by the implementation of a strong education programme or the passage of legislation. Therefore, these diseases will remain as public health problems and there is a need to undertake regular epidemiological studies. These studies, however, cannot be carried out effectively without the development of more cost effective, sensitive and specific diagnostic methods that can be used in large-scale screening of fish. The use of molecular biological techniques can also help to clarify species of dubious validity and to trace the source of infection. Stronger support for this neglected area of research is required.

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17 Parasitic Diseases of Shellfish

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Introduction

Numerous species of parasites have been described from various shellfish, especially representatives of the Mollusca and Crustacea (see Lauckner, 1983; Sparks, 1985; Sindermann and Lightner, 1988; Sindermann, 1990). Some parasites have had a serious impact on wild populations and shellfish aquaculture production. This chapter is confined to parasites that cause significant disease in economically important shellfish that are utilized for either aquaculture or commercial harvest. These pathogenic parasites are grouped taxonomically. However, the systematics of protozoa (protistans) is currently in the process of revision (Patterson, 2000; Cox, 2002; Cavalier-Smith and Chao, 2003). Because no widely accepted phylogeny has been established, parasitic protozoa will be grouped according to the hierarchy used in both volumes edited by Lee *et al.* (2000). In that publication, Perkins (2000b) tentatively included species in the genera *Bonamia* and *Mikrocytos* in the phylum Haplosporidia. As discussed below, subsequent analysis has verified that *Bonamia* spp. and *Mikrocytos roughleyi* are Haplosporidia but that *Mikrocytos mackini* is not and has unknown taxonomic affiliations. In addition, several other pathogenic protozoa (three species unofficially grouped as Paramyxea that parasitize oysters; and a recently encountered pathogen of pandalid shrimp) that were not mentioned in Lee *et al.* (2000) and have unknown taxonomic affiliations are discussed prior to presenting the metazoans that are problematic for shellfish.

Protozoa Related to Multicellular Groups

Microsporida

Introduction

Many diverse species of microsporidians (Fig. 17.1) (genera *Agmasoma*, *Ameson*, *Nadelspora*, *Nosema*, *Pleistophora*, *Thelohania* and *Microsporidium* – unofficial generic group), have been described from shrimps, crabs and freshwater crayfish worldwide (Sparks, 1985; Sindermann, 1990). The majority of these parasites are detected in low prevalences (< 1%) in wild populations. Although the economic impact of most species of Microsporida on crustacean fisheries is unknown, some species are perceived to have adverse economic impacts. Also, these parasites occasionally cause serious disease epizootics in penaeid aquaculture (Lightner, 1996).

Host range

Ameson (= *Nosema*) *nelsoni* infects at least six species of shrimp throughout the Gulf of

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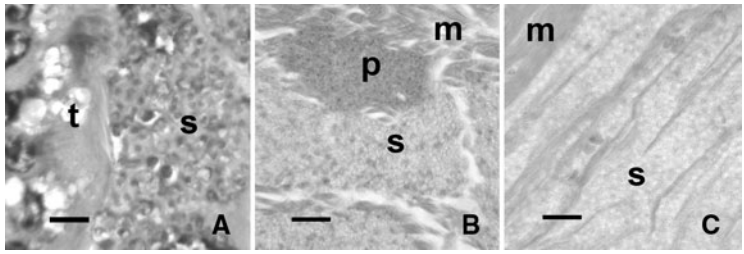


Fig. 17.1. Histological images of plasmodia (p) and developing spores (s) of unidentified Microsporida in crustaceans in British Columbia, Canada. A. Infection congesting the connective tissue between the hepatopancreas tubules (t) of a *Pandalus platyceros* in which the muscle tissue was not infected. B. Infection replacing the skeletal muscle (m) tissue of a *Pandalus jordani* in which the hepatopancreas was not infected. C. Similar infection to B in a *Cancer magister*. All bars = 10 μm.

Mexico and north along the Atlantic coast of the USA to Georgia (Sparks, 1985). It is a common pathogen and has caused significant financial losses to the bait and food shrimp industries (Sindermann, 1990). Microsporidosis in captive–wild *Penaeus* brood stock (infections not apparent at time of collection) resulted in losses of up to 20% (Lightner, 1988). Also, prevalences (16% and 15%, respectively) of *A. nelsoni*, in pond-reared brown shrimp from Texas and in white shrimp in a net-enclosed bay in Florida suggest a potential threat to shrimp reared in extensive culture (Lightner, 1975).

Ameson (= *Nosema*) *michaelis* is widely distributed at low prevalences in blue crab on the Gulf and Atlantic coasts of the USA (Sparks, 1985). Diseased blue crabs (*Callinectes sapidus*) often inhabit sheltered areas near the shore and experience high mortalities when stressed (Overstreet, 1988). However, unlike *Ameson* in shrimp, the transmission of *A. michaelis* is direct, i.e. by ingestion of infected tissue (Sparks, 1985; Overstreet, 1988). Some authors indicated that this parasite was a significant factor in blue crab mortality and thus a potential threat to the industry. However, more information is needed on pathogenicity, geographical distribution and prevalence in various populations before its economic significance can be established (Sparks, 1985).

Nadelspora canceri occurs in Dungeness crab (*Cancer magister*) along the US Pacific Coast from Bodega Bay, California, to Gray's Harbor, Washington, with prevalences lowest in open oceans (0.3%) and highest in estuaries (usually about 14% but up to 41.2% in one location) (Childers *et al.*, 1996). Like *A. michaelis*, *N. canceri* was also directly transmitted to juvenile and adult crabs in the laboratory by allowing them to ingest infected tissue and to megalope and early juvenile crabs by placing them in a suspension of 10^6 spores/ml (Olson *et al.*, 1994).

An unidentified microsporidian in the hepatopancreatocytes of tiger shrimp (*Penaeus monodon*) was associated with low production, slow growth rates and occasional mortalities in brackish-water pond culture in Malaysia (Anderson *et al.*, 1989). Also, unidentified Microsporida have been presumed to cause high mortalities in freshwater crayfish in Western Europe and England (Pixell Goodrich, 1956).

Morphology

Each species of Microsporida is characterized by the number of spores per sporont, the spore size, the tissues infected and, to some extent, the host species (Sparks, 1985; Lightner, 1996). Although the spores of most Microsporida are ovoid and relatively small (about 3 to 5 μm in length), the spores of *N. canceri* are unique in being exceptionally long (about 10 μm) and needle-shaped (0.2 to 0.3 μm in diameter), tapering to a posterior pointed end (Olson *et al.*, 1994).

Host–parasite relationships

Microsporidians replace host tissue with spores as they grow, without invoking host inflammatory responses. Infected individuals exhibit poor stress resistance and poor stamina and are thus prone to loss by predation and to poor survival following capture and handling. Infection of the gonad by some species renders infected individuals sterile and may cause feminization of infected male penaeids (Lightner, 1996).

Diagnosis of infection

Infected tissue, especially muscle, is eventually replaced by spores, giving it an opaque appearance. Due to this white discoloration, heavy infections are apparent and justify the common names of 'cotton', 'milk' or 'cooked' shrimp and crabs. In addition, the cuticle of some crustaceans may have blue-black discoloration due to expansion of cuticular melanophores (Lightner, 1996). The fluorescent technique described by Weir and Sullivan (1989) for screening for Microsporida in histological sections may be useful for detecting light infections. A molecular probe has been developed for the detection of *Agmasoma* sp. in *Penaeus* spp. (Pasharawipas *et al.*, 1994).

Prevention and control

The only known method of prevention is removal and destruction (freezing may not destroy spores) of infected individuals (Lightner, 1988; Overstreet, 1988). The intermediate hosts (fin fish) should be excluded from culture systems and water supplies (e.g. *Ameson penaei* became infective for pink shrimp following passage through the gut of a shrimp predator, the spotted sea trout (*Cynoscion nebulosus*) (Lightner, 1988)). A single treatment of buquinolate (used to treat coccidiosis in boiler chickens) prevented microsporidosis caused by *A. michaelis* in most exposed blue crab (Overstreet, 1975). Lightner (1988) suggested that Fumidil B (an antibiotic used to control microsporidosis in honeybees) and benomyl (a systemic fungicide used to control microsporidosis in the lucerne weevil) may be suitable treatments for this disease in penaeid shrimp. However, Overstreet (1975) found that Fumidil B seemed to exacerbate *A. michaelis* infection in blue crabs and benomyl was not as effective as buquinolate and apparently killed some crabs.

Alveolates

Apicomplexa

Introduction

Many species of gregarines and coccidians have been described from shellfish. Because information to date indicates that most of these species are relatively benign in pathogenicity, they will not be mentioned here. However, two species of coccidia have been associated with pathology.

Coccidians (family Eimeriidae) have been described from the kidneys and less frequently other organs of bivalves. Although they were all designated as species of *Pseudoklossia* (Upton, 2000), only the first (type) species and one other appear to be heteroxenous. Because the other species undergo monoxenous development in their molluscan host, Desser and Bower (1997) proposed the creation of a new genus, *Margolisiella*, to accommodate these parasites. Disease concerns were associated with *Margolisiella kabatai* (Fig. 17.2A) in Pacific littleneck clams (*Protothaca staminea*) that were found on the surface of the substrate in Washington State (Morado *et al.*, 1984) and *Margolisiella* (= *Pseudoklossia*) *haliotis*, which can occur in extremely heavy infections in the kidneys of abalone (*Haliotis* spp.) from California (Friedman *et al.*, 1995).

In addition to gregarines and coccidians, Levine (1978) proposed that *Perkinsus marinus* (= *Dermocystidium marinum* = *Labyrinthomyxa marina*), a pathogen of eastern (American) oyster (*Crassostrea virginica*), also be included within the phylum Apicomplexa. Subsequent taxonomic analysis based on nucleotide sequences indicated that this parasite may be more closely related to the Dinoflagellida (Perkins, 1996;

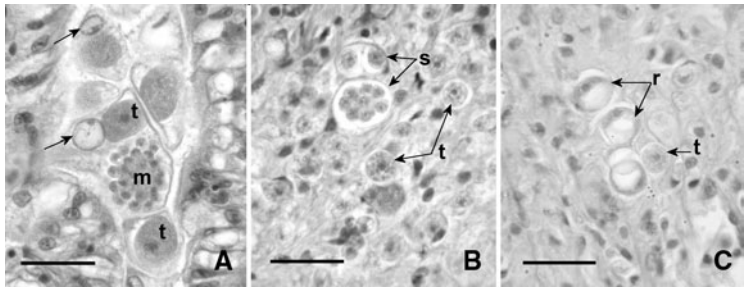


Fig. 17.2. Histological images of Apicomplexa in molluscs from British Columbia, Canada. A. Mature microgamont (m) with peripherally arranged microgametes and trophozoites (t) of *Margolisiella kabati* in the cytoplasm of renal epithelial cells with hypertrophied nuclei (arrows) in *Protothaca staminea*. B and C. Trophozoites (t), mature trophozoites (signet-ring stage, r) and two schizonts (s) consisting of two and eight trophozoites, respectively, of *Perkinsus qugwadi* in the connective tissue of the gonad of *Patinopecten yessoensis*. All bars = 20 μm.

Reece *et al.*, 1997b; Siddall *et al.*, 1997). Norén *et al.* (1999) proposed that perkinsids, which share features with both dinoflagellates and apicomplexans, be assigned to the phylum Perkinsozoa, equivalent to other alveolate phyla. However, Perkins (2000a) tentatively suggested maintaining the link with the Apicomplexa because molecular phylogeny assays have not yet been applied to parasitic dinoflagellates or to more primitive Apicomplexa, which seem to have morphological features akin to those of *Perkinsus* spp. Nevertheless, the genus *Perkinsus* (Fig. 17.2B, C) incorporates several species that are highly pathogenic to molluscs and are thus described in further detail.

Host range

Members of the genus *Perkinsus* (order Perkinsorida, family Perkinsidae) have been detected in over 67 species of molluscs (primarily bivalves) from temperate to tropical regions of the Atlantic and Pacific Oceans and the Mediterranean Sea (Perkins, 1996). Although several species have been named (see related pathogens below), the best known and first named species, *P. marinus*, is one of the prime challenges to the productivity of the eastern oyster, including the devastation of the once profitable oyster industry in Chesapeake Bay, USA, and has caused up to 50% mortality in areas of the Gulf of Mexico (Andrews, 1988a; Burreson and Ragone Calvo, 1996; Ford, 1996; Cook *et al.*, 1998). Also, mass mortalities of eastern oysters (30 to 34 million oysters or 90 to 99% of the stock) imported into Pearl Harbour, Hawaii, were attributed to this pathogen (Kern *et al.*, 1973). In addition to mortalities, meat yields are drastically reduced by high levels of infection, and infections may reach 100% in eastern oysters exposed to two consecutive summers of *P. marinus* activity (Andrews and Ray, 1988; Crosby and Roberts, 1990).

P. marinus occurs along the east coast of the USA from Massachusetts to Florida, along the Gulf of Mexico to Venezuela and in Puerto Rico, Cuba and Brazil. However, the development of *P. marinus* is correlated with salinity and temperature (Crosby and Roberts, 1990; Ford, 1992, 1996). The parasite is most virulent in eastern oysters at salinities above 15 ppt during periods of elevated water temperatures (above 20°C for at least 1 month) (Chu and Greene, 1989). Thus, the disease is prominent for about half the year in high-salinity areas of Chesapeake Bay and active for most of the year in the Gulf of Mexico (Lauckner, 1983). Also, Delaware Bay is periodically free of the disease, owing to: (i) poor propagation of the parasite due to cool temperatures; and (ii) an embargo placed on importation of eastern oysters from more southern areas (Andrews, 1988a).

Most described species of *Perkinsus* lack distinctive morphological features that

can be used for specific identification. As indicated below, at least one other species is known from within the geographical range of *P. marinus* and both species have been reported from the same bivalve hosts, including the eastern oyster and the sympatric clams *Macoma balthica*, *Macoma mitchelli* and *Mercenaria mercenaria* (Coss *et al.*, 2001). In addition, *P. marinus* was reported to develop in the ectoparasitic snail *Boonea impressa* (White *et al.*, 1987). However, the host range of *P. marinus* has probably not been fully identified. The recent development of molecular diagnostic tools for the detection and identification of *Perkinsus* spp. should facilitate the identification of host species susceptible to *P. marinus*.

Morphology and life cycle

The disease caused by *P. marinus* is popularly known as 'dermo', and fungal terminology, stemming from previous taxonomic interpretations, is still occasionally used for various stages in the life cycle (Fig. 17.3). In the eastern oyster, the trophozoite (aplanospore or meront) ranges in size from 2 to 20 μm, with the smaller forms often observed within the phagosome of a haemocyte. At maturity, the trophozoite (10 to 20 μm in diameter) has an eccentric vacuole (often containing a refringent vacuoplast), which may comprise 90% of the cell volume. The peripheral location of the nucleus produces the characteristic signet-ring configuration. The mature trophozoite undergoes schizogony by successive bipartitioning of the protoplast (alternating karyokinesis and cytokinesis) to form a schizont (sporangium, tomont or 'rosette') stage (ranging from 15 to 100 μm but usually less than 25 μm in diameter) containing from two to 64 (usually eight to 32) trophozoites (coccoid or cuneiform and 2 to 4 μm in the longest axis), which are released when the schizont ruptures (Perkins, 1996).

At death and decomposition of an infected host or when infected tissue is placed in anaerobic fluid thioglycollate medium supplemented with salt and antimicrobial agents (described by Ray (1966a) and now

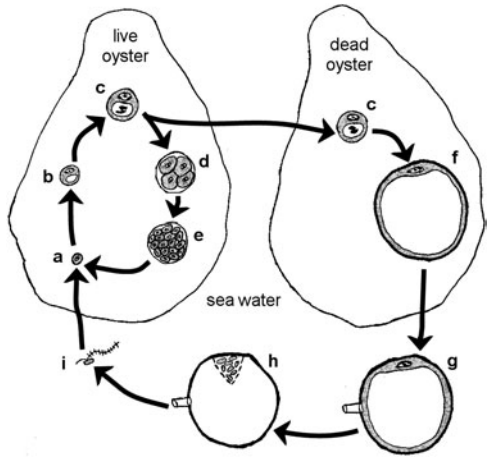


Fig. 17.3. Diagrammatic developmental cycle of *Perkinsus marinus* within living and dead *Crassostrea virginica* and free in the marine environment as adapted from Perkins (1996). Within the living oyster, immature trophozoites (a) grow and develop a cytoplasmic vacuole (b). Mature trophozoites (c) have a large eccentric vacuole containing a vacuoplast, thereby displacing the nucleus to the cell periphery (signet-ring stage). Palintomy (d, e) occurs and results in the formation of a schizont (e) from which the immature trophozoites (about four to 64 per schizont) escape through a tear in the wall. When the oyster dies and the tissue becomes anoxic, the mature trophozoite (c) develops into a large prezoosporangium (f). On liberation into the marine environment, the prezoosporangium develops a discharge tube (g) and undergoes palintomy (h), resulting in the formation of numerous biflagellated zoospores (i) capable of initiating a new infection.

called RFTM), the trophozoites transform into prezoosporangia (hypnospores), which enlarge to diameters usually between 30 and 80 μm, with extremes of 480 μm (Perkins, 1996). Upon release into sea water (aerobic conditions), the prezoosporangia (from either dead oysters or RFTM) differentiate into flask-shaped zoosporangia, within which numerous biflagellated zoospores develop in about 4 days. The biflagellated zoospore (ovoid body 4 to 6 μm by 2 to 3 μm) has a row of long filamentous mastigonemes (tinsels) along the length of the anterior flagellum and a naked posterior flagellum. It also has an apical complex, consisting of a conoid, polar ring, up to 39

subplasmalemma microtubules, rhoptries and micronemes (Perkins, 1996). Zoospores escape from the zoosporangium via the discharge tube and may initiate infection in the epithelium of gill, mantle or gut, where they become trophozoites (Perkins, 1996). Apparently, trophozoites contained within haemocytes that underwent diapedesis are infective and represent an alternate means by which the infection is transmitted.

Hoese (1964) speculated that dissemination of *P. marinus* might be achieved through scavengers because he detected prezoosporangia in the faeces of fishes, oyster drills and crabs that fed on dead or moribund infected oysters. He was able to infect eastern oysters with this material. In addition, White *et al.* (1987) speculated that *P. marinus* might also be transmitted by an ectoparasitic pyramidellid snail, *B. impressa*, which directly injected *P. marinus* into the oyster mantle during feeding or *P. marinus* was transferred through the water between the snail and oyster, perhaps entering the feeding wound made by the snail.

Extensive data on the influence of environmental conditions on the prevalence and intensity of infection and the disease process have been integrated into mathematical models of host–parasite–environmental interactions (Hofmann *et al.*, 1995; Powell *et al.*, 1996; Ragone Calvo *et al.*, 2001). The models simulate infection cycles within the oyster and in oyster populations under different environmental conditions and forecast conditions that can initiate and end epizootics in oyster populations.

Host–parasite relationships

Gross signs of 'dermo' are severe emaciation, gaping, pale appearance of the digestive gland, shrinkage of the mantle away from the outer edge of the shell, inhibition of gonadal development, retarded growth and occasionally presence of pus-like pockets (Lauckner, 1983; Sindermann, 1990). In the early stages of infection, many *P. marinus* trophozoites are engulfed by haemocytes and the infection spreads systemically throughout the oyster. Although the extent of the haemocytic response is variable, several layers of haemocytes can encapsulate trophozoites. Also, host cell destruction appeared limited to the immediate vicinity of the pathogen. Advanced infections were characterized by haemocyte activation and recruitment, with concomitant exuberant production of haemocyte-derived oxygen intermediates (oxyradicals), which may be associated with the pathogenesis of the disease (Anderson *et al.*, 1992).

Foci of infection or abscesses containing thousands of *P. marinus* and host debris may attain several hundred micrometres in diameter during later stages of infection. In addition, the pathogen often occludes haemolymph sinuses. Although the epithelium and adductor muscle are invaded, they do not appear to be damaged until late in the infection. By the time the eastern oyster becomes moribund, large numbers of *P. marinus* have accumulated in all tissues. Paynter and Burreson (1991) have indicated that, in Chesapeake Bay, groups of eastern oysters, which incurred high prevalences and intensities of infection, exhibited low mortalities during their first year but suffered high mortalities during the following year. Bushek and Allen (1996a,b) observed variations in the virulence of *P. marinus* to genotypically different stocks of eastern oysters and proposed that different strains of *P. marinus* may vary in virulence or different oyster stocks may vary in resistance to infection.

In vitro *propagation*

P. marinus is one of the few shellfish pathogens that can be maintained by continuous *in vitro* propagation of the trophozoite (La Peyre, 1996; Casas *et al.*, 2002b). In addition to having biological characteristics similar to the histozoic stages of *P. marinus* (i.e. morphology, antigenicity, biochemistry and development in thioglycollate medium, as described by Ray (1966a)), some cultured isolates were infective to eastern oysters.

The transformation of the trophozoites into prezoosporangia in RFTM (Ray, 1966a) is frequently referred to as a culture technique. However, prezoosporangia have poor survival in RFTM. The subsequent

transformation of the prezoosporangia into zoospore-producing zoosporangia can be achieved by transferring the prezoosporangia from RFTM to sea water. The use of RFTM is now considered to be part of a diagnostic technique, as described below.

Diagnosis of infection

In addition to routine histopathological examination of oyster tissues for the detection of *Perkinsus*, other diagnostic techniques have been developed. The RFTM procedure indicated above involves the incubation of test mollusc tissues in fluid thioglycollate medium as modified by Ray (1966a) for about 1 week at room temperature, which induces the development of prezoosporangia. When the sample is stained with dilute Lugol's iodine solution, the prezoosporangia readily stand out as dark brown to blue-black spheres. A semi-quantitative estimate of disease intensity was determined by the apparent percentage of squashed mantle or rectal tissue that contained *P. marinus* prezoosporangia (Andrews, 1988a; Choi *et al.*, 1989). Gauthier and Fisher (1990) demonstrated that haemolymph could be assayed by RFTM to produce a sensitive, reliable and completely quantitative method of estimating the intensity of infection. Although this method is inadequate for detecting light infections, it does not require that the oyster be sacrificed (Bushek *et al.*, 1994). Because RFTM is not species specific, it has been used to detect other species of *Perkinsus* in various Mollusca (see below).

Monoclonal and polyclonal antibodies produced against the prezoosporangia can be used in ELISA or immunofluorescent assays for identification and quantification of *P. marinus*. The various antibodies show differences in cross reactivity with other life stages of *P. marinus* and with other species of *Perkinsus* (Choi *et al.*, 1991; Dungan and Roberson, 1993).

Molecular techniques, including specific and sensitive semi-quantitative and competitive polymerase chain reaction (PCR) and multiplex PCR (simultaneous testing of two or more pathogens in a single test reaction), based on the sequence of the small subunit ribosomal RNA (SSU rRNA) gene, have been developed (De la Herrán *et al.*, 2000; Penna *et al.*, 2001). The use of PCR primers to amplify up to six polymorphic loci of genomic DNA from cultured *P. marinus* indicated that, *in vitro, P. marinus* is diploid and that oysters may be infected by multiple strains of this parasite (Reece *et al.*, 1997a, b). Allelic and genotypic frequencies differed significantly among isolates from regions of the north-east and south-east US Atlantic coast and the coast of the Gulf of Mexico. The inter- and intraspecific genetic variation among *Perkinsus* species has provided the opportunity to design genus- and species-specific molecular diagnostic assays (Casas *et al.*, 2002a; Dungan *et al.*, 2002; Murrell *et al.*, 2002). In addition, Yarnall *et al.* (2000) developed a quantitative competitive PCR that proved to be more sensitive than the RFTM tissue assay. However, before molecular analysis (e.g. PCR) can be recommended as the method of choice for disease diagnosis, more research is necessary to validate the various molecular diagnostic assays and compare them with standard diagnostic techniques (Burreson, 2000).

Prevention and control

Continuous bath treatment with low levels of cyclohexamide (1 µg/ml/week for 45 days) prolonged the life of laboratory stocks of eastern oysters infected with *P. marinus* (Ray, 1966b). However, chemical treatment is impractical in the field. Andrews (1988a), Andrews and Ray (1988) and Sindermann (1990) indicated that control of the disease depends on isolation and manipulation of seed stock and recommended the following procedures: (i) avoid use of infected seed stocks; (ii) plant oysters thinly on beds; (iii) isolate newly planted beds (0.4 km) from infected eastern oysters; (iv) continually monitor eastern oysters (especially oysters at 2 years of age or older in the late summer or early autumn) for the disease, using RFTM; (v) harvest early if beds become infected; and (vi) fallow beds after harvest to allow all infected oysters to die before

replanting. Goggin *et al.* (1990) further recommended that the spread of *Perkinsus* sp. from shellfish processing plants could be prevented by not returning untreated mollusc tissues to the sea.

Although *P. marinus* persisted in eastern oysters held at low salinities (6 ppt), it was less virulent at salinities below 9 ppt (Ragone and Burreson, 1993). The occurrence of disease only at higher salinities has been used in management practices (Paynter and Burreson, 1991). In Chesapeake Bay, uninfected eastern oyster seed are acquired from areas of low salinity, which are not suitable for oyster culture because oyster growth and condition are reduced by low salinity. In the Gulf of Mexico, where warmer temperatures allow the infection to remain active year-round, freshwater diversions into high-salinity bays have been proposed in order to revive or enhance areas that are marginally productive for eastern oysters (Andrews and Ray, 1988). The possibility of breeding eastern oysters that are resistant to *P. marinus* is under investigation (Gaffney and Bushek, 1996). Also, the introduction of a non-endemic species that is more tolerant of *P. marinus* (Meyers *et al.*, 1991) is being considered as a method for the recovery of stable oyster production in areas of Chesapeake Bay where native eastern oysters have been eliminated (Mann *et al.*, 1991).

Related pathogens

Prezoosporangia of *Perkinsus* sp. have been detected by RFTM in many species of Mollusca from temperate to tropical waters of the world. For example, in Australia, *Perkinsus* spp. were detected in at least 30 species of Mollusca (Lester *et al.*, 1990). Although *Perkinsus* sp. was associated with giant clam (*Tridacna gigas*) mortalities (Alder and Braley, 1989) and lesions in the tissues of pearl oysters (*Pinctada maxima*) (Norton *et al.*, 1993), many *Perkinsus* sp. infections seem to have no detectable adverse affects on their hosts (Goggin *et al.*, 1990).

In addition to *P. marinus*, six other species have been named. The most distinctive species is *Perkinsus qugwadi*, considered enzootic in British Columbia, Canada, but only known from Japanese scallops (*Patinopecten yessoensis*) that were introduced into Canada from Japan for culture purposes (Blackbourn *et al.*, 1998). Scallops native to enzootic areas (*Chlamys rubida* and *Chlamys hastata*) were resistant to infection, while mortalities among cultured Japanese scallops often exceeded 90% (Bower *et al.*, 1999). Unlike all other *Perkinsus* spp., *P. qugwadi*: (i) proliferated and was pathogenic at cool temperatures (8–15°C); (ii) developed zoospores within tissues of juvenile living hosts instead of outside the host; and (iii) did not produce prezoosporangia in RFTM or stain blue-black with Lugol's iodine (Bower *et al.*, 1998). In addition to these differences, phylogenetic analyses based on the internal transcribed spacer (ITS) regions of rRNA of *P. qugwadi* consistently place this species at the base of a clade containing the other *Perkinsus* spp. (Coss *et al.*, 2001; Casas *et al.*, 2002a,b; Dungan *et al.*, 2002).

The second named species was *Perkinsus olseni*, first reported as a pathogen of abalone (*Haliotis rubra*) in Australia (Lester and Davis, 1981). This species is now reported from three other species of abalone (*Haliotis laevigata*, *Haliotis cyclobates* and *Haliotis scalaris*) along the southern coast of Australia and is often associated with significant mortalities. It is also believed to occur in a wide variety of molluscan species from the Great Barrier Reef but was not detected in abalone from that area (Goggin and Lester, 1995). *Perkinsus olseni* was experimentally transmitted and highly infectious to a range of molluscs under laboratory conditions (Goggin *et al.*, 1989).

The third species to be named was *Perkinsus atlanticus*, a pathogen of native clams (*Ruditapes* (= *Tapes* = *Venerupis*) *decussatus*, *Ruditapes* (= *Tapes*) *semidecussatus*, *Ruditapes pullastra*, *Venerupis aurea*, *Venerupis pullastra*) and the introduced Manila clam (*Venerupis* (= *Tapes* = *Ruditapes*) *philippinarum*) along the coasts of Portugal, Spain (Galicia and Huelva areas) and the Mediterranean Sea (Azevedo, 1989; Rodríguez *et al.*, 1994; Ordás *et al.*, 2001; Casas *et al.*, 2002a).

In the late 1990s, a *Perkinsus* sp. was associated with significant mortalities of

native stocks of Manila clams in Korea, Japan and China (Choi *et al.*, 2002). Hamaguchi *et al.* (1998) found that the nucleotide sequence of two internal transcribed spacers (ITS1 and ITS2) and the 5.8 S region of the SSU rRNA of the *Perkinsus* sp. from Manila clams in Japan were almost identical to those of *P. atlanticus* and *P. olseni* and suggested that the parasite in Japan may be *P. atlanticus*. Several other investigations found similar results (Goggin, 1994; Robledo *et al.*, 2000; Casas *et al.*, 2002a). However, in all investigations, the gene sequences of the *P. atlanticus* and *P. olseni* isolates were distinct from those of *P. marinus* (from the oyster *C. virginica* from Virginia, USA). Based on similarity (98–99%) in the sequences of the non-transcribed spacer (NTS) region, Murrell *et al.* (2002) proposed that *P. olseni* and *P. atlanticus* be synonymized, with the name *P. olseni* having priority. If this synonymy is upheld, *P. olseni* will have a wide host range (infecting gastropods as well as bivalves) and a wide geographical range (including the coasts of Australia, New Zealand, Japan, Korea and Europe). The wide variability in the pathogenicity of this parasite may be attributed to either differences in strains of the parasite or differences in host responses.

The validity of another species, *Perkinsus karlssoni*, has been refuted. *P. karlssoni* was identified as a pathogen of cultured bay scallops, *Argopecten irradians*, being conditioned for spawning under hatchery conditions in Atlantic Canada (McGladdery *et al.*, 1991; Whyte *et al.*, 1994). This parasite was described because a Lugol-positive organism was detected in diseased scallop tissues incubated in RFTM. However, diagnosis by RFTM alone is controversial and Goggin *et al.* (1996) surmised that the description was based on a contaminant biflagellate organism. This species may be reinstated if further RFTM-positive prezoosporangia are obtained and phylogenetic analyses of sequence data place the species within the *Perkinsus* clade.

Another species, *Perkinsus chesapeaki*, has been isolated from the gills of soft-shell clams (*Mya arenaria*) from the same location (Chesapeake Bay, USA) as *P. marinus* (McLaughlin *et al.*, 2000). Although the life-cycle stages and zoosporulation process were similar to those described for other *Perkinsus* spp., *P. chesapeaki* was identified based on minor differences in the morphology of the zoospore. Also, the genetic sequence of the SSU rRNA of this isolate was found to be distinct from that of *P. marinus* (Casas *et al.*, 2002a; Dungan *et al.*, 2002). McLaughlin and Faisal (2001) reported a difference in the production of extracellular proteins by *P. chesapeaki* and *P. marinus*, which may help to explain the difference in pathology observed in infected soft-shell clams and the eastern oysters, respectively.

More recently, *Perkinsus andrewsi* was described from Baltic clams (*M. balthica*) from the east coast of the USA. The species identification was based on sequence data from the SSU rRNA locus, which differed from those of *P. marinus*, *P. atlanticus*, *P. olseni* and *P. qugwadi* (Coss *et al.*, 2001). DNA analysis (using PCR assays on regions of the SSU rRNA loci (mainly ITS1 and ITS2)) indicated that *P. andrewsi* can coexist with *P. marinus* in Baltic clams and other sympatric clams (*M. mitchelli* and *M. mercenaria*) and in the eastern oyster (Coss *et al.*, 2001). Subsequent analysis of the ITS regions of several species of *Perkinsus* (including several isolates of some species) consistently grouped *P. chesapeaki* and *P. andrewsi* (Casas *et al.*, 2002a; Murrell *et al.*, 2002). Analysis of the ITS sequence from cloned isolates of *Perkinsus* sp. from Baltic clams and another sympatric clam (*Tagelus plebeius*) from Chesapeake Bay suggested that the minor variations among ITS sequences of *P. chesapeaki* and *P. andrewsi* indicate true polymorphism within a single parasite species (Dungan *et al.*, 2002). If these species are synonymous, *P. chesapeaki* will have precedence over *P. andrewsi*.

The current major limitations to identifying the various species of *Perkinsus* are the absence of significant morphological differences among known species and the broad host range encountered for isolates tested in the laboratory and assayed from the field. A conservative perspective suggests that only four of the named species may be valid (i.e. *P. marinus*, *P. olseni*, *P. qugwadi* and *P. chesapeaki*). If this perspective is

correct, then the host and geographical range is very broad for at least one of these species (i.e. *P. olseni*) and at least two other species can infect the same hosts in the same geographical area (i.e. *P. marinus* and *P. chesapeaki*). These characteristics and the lack of distinctive morphological features render the identity of *Perkinsus* spp. encountered in the field and the specific identity of all new isolates open to question. Because of the significant negative economic impact caused by some of these parasites, it is important to be able to differentiate between pathogenic and supposedly non-pathogenic species or to determine which species are pathogenic for which hosts. Investigations into the genetic sequence and associated biology of various isolates and the continuing development of molecular assays will address this problem in the future.

Ciliophora

Two closely related genera of holotrich ciliates (class Oligohymenophorea, subclass Scuticociliatia, order Philasterida, family Orchitophryidae) periodically cause high mortalities among Crustacea in captivity. *Mesanophrys* (= *Paranophrys* = *Anophrys*) spp. have been observed in the haemolymph of Dungeness crabs (*C. magister*) and red rock crabs (*Cancer productus*) on the west coast of North America (Fig. 17.4A), in blue crabs (*C. sapidus*) on the east coast of the USA and in edible crabs (*Cancer pagurus*) and green crabs (*Carcinus maenas*) in Europe. *Mugardia* (= *Paranophrys* = *Anophrys*) sp. occurs in lobsters (*Homarus americanus*) on the east coast of North America (Sindermann, 1990; Morado and Small, 1995). Although the ciliates are rare in the haemolymph of most wild-caught Crustacea, *Mugardia* sp. was observed in stained hepatopancrease smears from all 89 lobsters from ten locations along the coast of Maine in November 1990 (Sherburne and Bean, 1991). The ciliate is infectious and lethal for animals held in artificial enclosures. Presumptive diagnosis of these ciliates is made by observing numerous ciliates of typical elongate form and reduced numbers of haemocytes in the

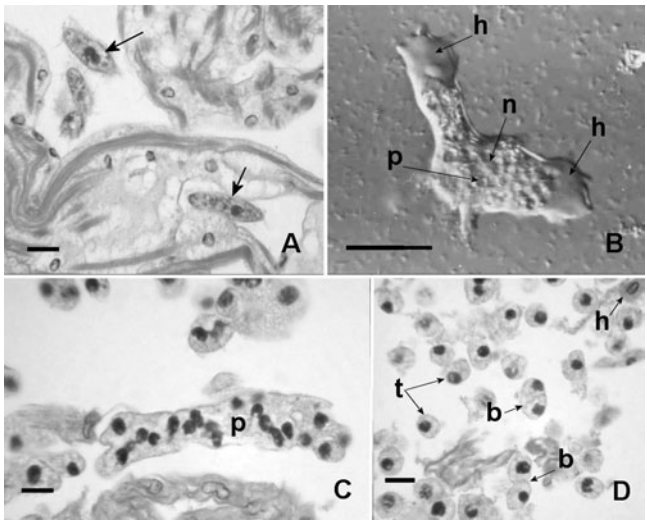


Fig. 17.4. A. Histological images through *Mesanophrys pugettensis* (arrows) in the haemal sinuses of the heart of *Cancer magister* from British Columbia, Canada. B. Wet-mount preparation (Nomarski optics) of *Paramoeba invadens* (hyaline region (h), nucleus (n) and parasome (p)) from *in vitro* culture isolated from *Strongylocentrotus droebachiensis* in Nova Scotia, Canada (courtesy of R.E. Scheibling). C and D. Histological section through *Hematodinium* sp. (plasmodium (p), trophozoites (t) and binary fission in trophozoites (b)) in the heart sinus (haemocyte (h)) of *Chionoecetes tanneri* from British Columbia, Canada. All bars = 10 μm.

haemolymph. Ciliates can also be observed in histological sections of the soft tissues, especially the heart (Fig. 17.4A) and gills and may be associated with tissue destruction, especially of the intestine (Sherburne and Bean, 1991). Protargol-stained preparations are required for specific identification (Armstrong *et al.*, 1981). Because most reports of infection pertained to injured crabs and lobsters being held in enclosures, lowering densities (i.e. less stress of crowding) and reducing mechanical damage during holding may be beneficial (Sindermann and Lightner, 1988).

Dinozoa (Dinoflagellida)

Introduction

The parasitic Dinoflagellida in the genus *Hematodinium* spp. (order Syndiniales) are significant pathogens of commercially harvested crabs and lobsters (Shields, 1994).

Host range

The first reported and type species, *Hematodinium perezi*, was originally described from the haemolymph of crabs (*C. maenas* and *Liocarcinus* (= *Portunus*) *depurator*) from European waters (Chatton and Poisson, 1930) and was more recently reported to cause high mortalities in *C. pagurus* and *Necora puber* in France (Wilhelm and Mialhe, 1996). On the western side of the North Atlantic Ocean, from New Jersey to the western coast of Florida and in the Gulf of Mexico to southern Texas, a *Hematodinium* sp. that is believed to be the same parasite was reported from other species of crabs, including the blue crab, *C. sapidus* (Couch, 1983). Based on results from epizootiology studies, Messick and Shields (2000) suggested that this parasite represented a significant threat to blue crab populations in high-salinity estuaries along the Atlantic and Gulf coast of the USA. The second species, *Hematodinium australis*, occurs in *Portunus pelagicus*, *Scylla serrata* and possibly *Trapezia* spp. from the coast of Queensland, Australia (Hudson and Shields, 1994).

Based on differences in nucleotide sequence, two additional unnamed species have been documented (Hudson and Adlard, 1996). *Hematodinium* sp. causes an astringent aftertaste (bitter crab syndrome) and mortalities in Tanner crabs (*Chionoecetes bairdi* and *Chionoecetes opilio*) along the coast of Alaska (Meyers *et al.*, 1996). Meyers *et al.* (1990) conservatively estimated that the total economic loss to fishermen due to rejected diseased crabs was about 5% of the catch for the 1988/89 season. In addition, data from the commercial Tanner crab fishery suggested that there was an increase in prevalence and spread of the disease to new areas. A similar parasite was found in *C. opilio* from the coast of Newfoundland (Pestal *et al.*, 2003) and in *Chionoecetes tanneri* from coastal British Columbia (Bower *et al.*, 2003). The other *Hematodinium* sp. occurs in the Norway lobster (*Nephrops norvegicus*) off the west coast of Scotland and in the Irish Sea (Field and Appleton, 1995). Severe infection has an adverse effect on meat quality, noted by fishermen and processors. Peak infections of 70% were found in some trawl samples, which seasonally coincided with the annual moult. The decrease in Norway lobster abundance in the last decade may in part reflect the higher level of infection by *Hematodinium* sp. during this time (Field *et al.*, 1998).

Parasite morphology

Superficially, *Hematodinium* spp. appear similar, with only slight differences in size for the two named species (Hudson and Shields, 1994). The most abundant form is a round trophozoite (about 6 to 18 μm in diameter) with a single dinokaryon nucleus, which is characteristic of the dinoflagellates (Fig. 17.4D). Binucleate cells and multinucleate ovoid to vermiform plasmodia (usually containing fewer than about 20 nuclei (Fig. 17.4C)) are occasionally observed in the haemal sinuses (Couch, 1983; Hudson and Shields, 1994). A flagellated dinospore occurs during the terminal stages of infection

and is rarely observed in some crustacean hosts. Two sizes of motile dinospores were observed in tissues of *C. bairdi* in Alaskan waters during August and September (Love *et al.*, 1993). A common ultrastructural characteristic is the presence of cytoplasmic organelles called trichocysts in mature trophozoites and dinospores (Hudson and Shields, 1994).

Host–parasite relationships

The haemolymph of heavily infected crustaceans is opalescent or milky, slow to clot, devoid of haemocytes and filled with non-motile trophozoites. Also, there may be total lysis of hepatopancreatic tubules and partial destruction of muscle fibres (Couch, 1983). These crustaceans are lethargic and have a short survival time in captivity. The main cause of death may be the disruption of gas transport and tissue anoxia caused by proliferation of large numbers of dinoflagellate cells in the haemolymph. Alternatively, Shields *et al.* (2003) indicated that infected blue crabs probably die from metabolic exhaustion, as indicated by reduced levels of haemolymph proteins, haemocyanin and glycogen. Meyers *et al.* (1987) suggested that the bitter flavour in cooked infected Tanner crabs is the result of either the dinoflagellate itself or its metabolite(s).

Infected blue crabs were found only in areas above 11 ppt salinity and in all seasons except late winter and early spring (Messick and Shields, 2000). A reduction in prevalence of the disease in the winter was also reported in Tanner crabs. Laboratory transmission by injection indicated that both types of dinospores from Tanner crabs (Eaton *et al.*, 1991) and trophozoites and plasmodia from blue crabs (Messick and Shields, 2000) are infectious to their respective host crabs. However, *Hematodinium* spp. have not been transmitted by feeding (predation or cannibalism) or cohabitation, but moulting may predispose crabs to invasion (Messick and Shields, 2000). The complete life cycle of all *Hematodinium* spp. in the field is poorly understood.

Diagnosis of infection

The non-motile trophozoites are evident as numerous spheres (6 to 18 μm in diameter) in wet mount preparations of haemolymph from discoloured crustacea examined microscopically (× 100 magnification). *Hematodinium* spp. are also apparent in histological sections (Figs. 17.4C, D). However, the paucity of morphological characteristics for species identification has resulted in the development of PCR techniques based on the nucleotide sequences of parts of the SSU rDNA gene (Hudson and Adlard, 1996). Gruebl *et al.* (2002) described an 18S rRNA gene-targeted PCR-based diagnostic technique capable of detecting one *Hematodinium* sp. in 300,000 blue crab haemocytes. The partial sequences of the 18S rDNA gene of *Hematodinium* sp. from blue crabs deposited in GenBank (accession numbers AF421184 and AF286023) are nearly identical to the equivalent sequences of the parasite from *C. tanneri* in British Columbia, which is believed to be a different species. Because of this close association, this region of the genome will not be useful in differentiating between species. The development of specific molecular tools to differentiate between species will probably necessitate the analysis of more divergent genes, such as the ITS regions of the SSU rDNA. Currently such gene sequences are not available for most *Hematodinium* spp. from various crustaceans around the world.

Field and Appleton (1996) developed an indirect fluorescent antibody test (IFAT) to detect *Hematodinium* sp. in the haemolymph and tissues of Norway lobsters. This technique was more sensitive than gross observations and wet-mount examinations and was capable of detecting low-level haemolymph infections as well as previously undiagnosable tissue infections. However, the species specificity of this assay has not been assessed.

Prevention and control

The management of the Tanner crab fishery to avoid product quality problems (bitter crab syndrome) with infections of *Hematodinium*

sp. may be possible by harvesting Tanner crabs in the winter, when fewer crabs are severely parasitized and meats are more marketable. Also, proper disposal of infected Tanner crabs is essential in controlling dissemination of the parasite (Meyers *et al.*, 1990).

Stramenopiles

Residual heterotrophic Stramenopiles – Labyrinthulida

Introduction

Only one named species of Labyrinthulida has been documented as a pathogen of economically important shellfish. *Labyrinthuloides haliotidis*, an achlorophyllous, eukaryotic protist, is pathogenic to small, juvenile, northern abalone (*Haliotis kamtschatkana*) and small, juvenile, red abalone (*Haliotis rufescens*) (Bower, 1987a).

Host range

To date, *L. haliotidis* has only been observed in small abalone (less than 1 cm in shell length) from an abalone culture facility in British Columbia. Within 2 weeks of first being detected in a raceway, over 90% of the small abalone succumbed to infection and the disease quickly spread between raceways. The high mortalities caused by *L. haliotidis* were one of the reasons why this particular abalone culture facility is no longer in operation. Small abalone that are susceptible to infection are rarely found in the field. Thus, the geographical distribution of this parasite and its effect on wild stocks are not known.

Parasite morphology and life cycle

The trophozoite of *L. haliotidis* was spheroid (5 to 9 μm in diameter) and had a unique organelle of this group called the sagenogenetosome (Fig. 17.5A). Several sagenogenetosomes were scattered on the surface of each organism, forming openings in the thin laminated cell wall, and each produced the ectoplasmic net upon which the parasite moved (0.3 ± 0.16 μm/min) and obtained nutrients. Following removal from sources of nutrients (i.e. placement in sterile sea water), the trophozoite underwent synchronous multiple fission to form a zoosporoblast (6 to 10 μm in diameter) containing about ten developing zoospores (Fig. 17.5B). The uninucleate, ovate, motile, biflagellated zoospores (Fig. 17.5C) escaped through a rupture in the zoosporoblast wall (Bower, 1987a). When the zoospore contacted a hard surface or after about 24 h of active swimming in sea water, both flagella were shed. The resulting cell was morphologically similar to the trophozoite and survived in sterile sea water for at least 2 years (Bower, 1987a).

Trophozoites that developed from zoospores were infective to small abalone.

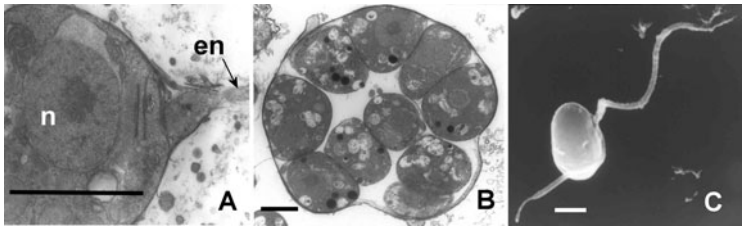


Fig. 17.5. Electron micrographs of *Labyrinthuloides haliotidis* from British Columbia, Canada. A. Trophozoite within the muscle tissue of a juvenile abalone (*Haliotis kamtschatkana*) showing the nucleus (n) and the ectoplasmic net (en) originating from the sagenogenetosome. B. Zoosporoblast from sea water containing well-developed zoospores. C. Zoospore illustrating the subapical attachment site of the two flagella, the coarse texture of the longer anterior flagellum, where debris has attached to the mastigonemes, and the thin tapered tip of the short posterior flagellum. All bars = 2 μm.

Within 4 h of contact, sagenogenetosomes were evident and host epithelial cells adjacent to the parasite were lysed. By 24 h post-exposure, the ectoplasmic net was well developed, the parasite had moved into the head and foot tissues of the abalone and dividing forms were observed (Bower *et al.*, 1989b). Within 10 days after exposure to about 10^4 parasites in 20 ml of seawater, about 90% of the abalone (less than 4.0 mm in shell length and 140 days of age) died with numerous parasites throughout the head and foot (Bower, 1987b). As dead abalone decomposed, trophozoites released from the tissues developed into zoosporoblasts, which produced zoospores within about 24 to 72 h. Parasites released from infected abalone were infective to other abalone on contact. Although alternative hosts have not been described, *L. haliotidis* can utilize diverse sources of nutrients but seems incapable of coexisting with bacteria.

Host–parasite relationships

The tissues of heavily infected abalone were slightly swollen, with a loss of integrity. Prevalence and intensity decreased and time to death increased as the abalone increased in age and size. Abalone greater that 15 mm in shell length could not be infected even when injected intramuscularly with about 1.5×10^4 *L. haliotidis*. The mechanism of defence against this parasite is not known. There was no indication of a haemocytic response in young susceptible abalone. Possibly the resistance of older abalone corresponded to the development of cellular or humoral defence mechanisms as the abalone matured (Bower, 1987b).

Small juvenile Japanese scallops (*P. yessoensis*) and juvenile Pacific oysters (*Crassostrea gigas*), both less than 8 months of age, were resistant to infection. However, two oysters with badly cracked shells became infected, suggesting that *L. haliotidis* was capable of utilizing oyster tissue for growth and multiplication if it was able to gain access to the soft tissues of the oyster (Bower, 1987b).

In vitro *culture and propagation*

Aseptic cultures of *L. haliotidis* grew well on several different liquid media and agar-based solid media. An *in vitro* life cycle could be produced by placing the parasite alternately in minimum essential medium with 10% fetal calf serum (where rapid production of trophozoites occurred through binary fission) and in sterile sea water (where the trophozoites transformed into zoosporoblasts and zoospores were produced) (Bower, 1987c). Cultured *L. haliotidis* were infective to small abalone (Bower, 1987b; Bower *et al.*, 1989b). The trophozoite also grew on pine pollen (*Pinus contorta*) in sea water but failed to produce zoosporoblasts and zoospores (Bower, 1987c).

Diagnosis of infection

The spheroid parasite is readily observed with light microscopy ($\times$ 100 magnification) in the head and foot of small infected abalone squashed in sea water between a glass slide and a cover slip. *L. haliotidis* is also evident in histological sections prepared using routine procedures. However, due to the morphological similarities of *L. haliotidis* to other thraustochytrids, identification of the parasite outside its host is impossible by microscopy. A direct fluorescent antibody technique showed promise in facilitating the detection of this parasite (Bower *et al.*, 1989a). However, this technique has not been fully tested to verify its specificity. A partial sequence of the SSU rRNA has been identified and used to assess phyletic relationships (Leipe *et al.*, 1996). However, this information has not been utilized in the development of a diagnostic tool.

Prevention and control

The source of infection in the abalone hatchery was not established. Transmission of the disease between raceways could be prevented by employing sanitary techniques. The parasite was destroyed in 20 min when exposed to 25 mg/l of chlorine in sea water. The fungicide cyclohexamide at 1–2 mg/l for 23 h per day on 5 consecutive days

cured infected abalone. However, this treatment had the disadvantages of: (i) being detrimental to diatoms upon which the abalone fed; (ii) being ineffective against non-growing but infective zoospores such that reinfection occurred within 2 to 3 weeks following treatment; and (iii) inducing resistant forms (as few as three successive treatments resulted in the production of forms twice as resistant to cyclohexamide) (Bower, 1989). Ozone treatment of incoming water may only be efficacious if ozone exposure is greater than 0.97 mg ozone/l for 25 min (Bower *et al.*, 1989c).

Amoeboid protists

Introduction

Two species in the order Euamoebida and family Paramoebidae are significant pathogens of shellfish. *Paramoeba perniciosa* is the cause of 'grey crab disease' or paramoebiasis in the blue crab (*C. sapidus*) and is infectious to other crustaceans. *Paramoeba invadens* is pathogenic to sea urchins (*Strongylocentrotus droebachiensis*).

Host range

P. perniciosa has been reported in blue crabs along the east coast of the USA from Connecticut to Florida, including the high-salinity areas of Chincoteague Bay and Chesapeake Bay, where it periodically causes mass mortalities and has caused ongoing low-level mortalities since 1967 (Couch, 1983; Sparks, 1985). Epizootics with high mortalities (about 17%) were reported from Chincoteague Bay in early summer and mortalities (20–30%) were observed in shedding tanks (for production of newly moulted softshell crab) (Johnson, 1988). It has also been reported from the rock crab *Cancer irroratus*, the exotic European green crab *C. maenas* and the American lobster *H. americanus* (Couch, 1983).

P. invadens was associated with mass mortalities of the sea urchin along the Atlantic coast of Nova Scotia in the early 1980s (Jones, 1985; Jones *et al.*, 1985). From 1980 to 1983 sea urchin mortalities were estimated to be at least 245,000 t (Miller, 1985). No mortalities were observed in other echinoderms, including other echinoids, asteroids and ophiuroids from the same area (Scheibling and Stephenson, 1984). However, the transformation of echinoid-dominated 'barren grounds' into kelp beds provided increased areas for American lobster (*H. americanus*) recruitment and thus increased lobster productivity (Wharton and Mann, 1981).

Morphology and life cycle

P. perniciosa is round to elongate, with linguiform lobopodia, and can be differentiated into small (3 to 12 μm) and large (15 to 35 μm) forms (Couch, 1983). Each amoeba contains a vesicular nucleus with a large central endosome and a 'second nucleus', 'Nebenkörper' or elongate parasome (1 to 4 μm) with a Feulgen-positive middle bar and two opposing basophilic polar caps. *P. invadens* is similar in size (20–35 μm in length and 8–15 μm in width) but is more elongated in shape, with a length/width ratio of about 2, and has digitiform pseudopodia (Fig. 17.4B). Also, the parasome (2 to 3 μm in size) has Feulgen-positive poles but no Feulgen-positive central band (Jones, 1985). Due to the unusual ultrastructure of the parasome, which is characteristic for *Paramoeba* spp., Perkins and Castagna (1971) proposed that the parasome may be a discrete organism of unknown taxonomic affinities and not an organelle of the amoeba.

The mode of transmission in the field has not been fully elucidated for either amoeba. Some attempts to infect blue crabs by injecting *P. perniciosa* or feeding infected crab tissues failed (Couch, 1983). However, Johnson (1977) observed the disease in two blue crabs 34 and 39 days post-inoculation with infected haemolymph and Sparks (1985) claimed that the disease was transmitted by consumption of moribund or dead infected blue crab. The transmission of *P. invadens* was direct and the infection was waterborne (Jones, 1985).

Host–parasite relationships

P. perniciosa, a parasite of the connective tissues and haemal spaces, occurs along the

midgut, antennal gland and Y organ in light infections. Haemal spaces in gills are usually invaded in medium and heavy infections and, in the terminal phase, the infection becomes systemic (Johnson, 1977; Sparks, 1985). In heavy infections, pathological changes caused by large numbers of amoeba include: tissue displacement; probable lysis of some types of tissue, including haemocytes; and significant decreases in protein, haemocyanin (the oxygen-binding and transport molecule of crustaceans) and glucose (Pauley *et al.*, 1975; Johnson, 1977). Sparks (1985) suggested that the probable cause of death was a combination of anoxia and nutrient deficiency. Terminal infections are usually observed during the late spring to early autumn, but infected blue crabs are found throughout the year (Johnson, 1988).

Most infected blue crabs demonstrate a defence response, which is manifested usually as phagocytosis of amoeba by hyaline haemocytes and infrequently as encapsulation of amoeba by haemocytes, but destruction of amoeba by humoral factors also occurred (Johnson, 1977). Occasionally a blue crab would overcome the infection.

In vitro *propagation*

P. perniciosa could not be cultured in various media, including a medium that supported continuous growth of *Paramoeba eilhardi*, a free-living species originally isolated from algal material. However, *P. perniciosa* survived for about 2 weeks in 10% calf serum agar overlaid with sterile sea water and incubated at 18°C (Sprague *et al.*, 1969). Unlike *P. perniciosa, P. invadens* was easily cultured on malt–yeast–seawater agar and on non-nutrient agar with marine bacteria as a food source. However, there was some loss of virulence after 15 weeks in monoxenic culture and 58 weeks in polyxenic culture (Jellett and Scheibling, 1988).

Clinical signs and diagnosis of infection

In blue crabs, signs of infection include a greyish discoloration of the ventral exoskeleton, general sluggishness, reduced or absence of clotting of the haemolymph and poor survival subsequent to handling or holding in tanks (Sparks, 1985). Infection is easily diagnosed only in the terminal phase, when numerous *P. perniciosa* and virtually no haemocytes are present in circulating haemolymph. Characteristic signs of paramoebiasis in sea urchins included muscle necrosis, general infiltration of coelomocytes, reddish-brown discoloration, poor attachment to substrate and high mortalities (Jones, 1985; Jellett *et al.*, 1988). Amoeba of both species can be observed with phase contrast either live or fixed in 5% formalin sea water and stained with dilute methylene blue. Smears can be stored following fixation in Bouin's, Davidson's, Hollande's or 10% formalin solutions and staining with iron haematoxylin or Giemsa's stain. Before amoebae appear in the circulation, they may be observed in squashes of subepithelial connective tissue examined with phase contrast if the infection is sufficiently advanced (Johnson, 1988). Infection can also be diagnosed by histological examination.

Haplosporidia (Haplospora)

Introduction

Included within the Haplosporidia are several species that are significant pathogens of oysters. Species of Haplosporidia were first described from oysters in the early 1960s and since that time the group and its affiliations have undergone several changes in classification. Initially these parasites were included in an order that was changed from Haplosporida to Balanosporida in the phylum Ascetospora (Sprague, 1979; Levine *et al.*, 1980), Perkins (1990) indicating that they were sufficiently distinct to warrant the status of phylum with the name Haplosporidia. Subsequently, Siddall *et al.* (1995) indicated that the haplosporidians are more closely related to alveolates (ciliates, dinoflagellates and apicomplexans) than to other spore-forming protozoans based on sequence comparisons of the 16S SSU rDNA gene. More recently, Cavalier-Smith and Chao (2003) indicated that the order status should be reinstated under the recently

created phylum Cercozoa. The proposal by Cavalier-Smith and Chao (2003) has not yet received wide acceptance and thus the phylum status of this group as indicated by Lee *et al*. (2000) is used here.

In addition to challenges in determining higher taxonomic affiliations, the sorting of species at the genus level has also undergone modifications. The initial grouping only included spore-forming endoparasites but differentiation between genera proved problematic, with species being transferred between *Haplosporidium* and *Minchinia* (Lauckner, 1983). Although the visibility and morphology of spore ornamentations (e.g. the presence of prominent extensions (tails) on the spore wall, visible by light microscopy) were proposed as features for differentiating between these two genera (Perkins, 2000b), recent phylogenetic analysis by Reece *et al*. (2003) supported an earlier proposal that composition of the spore ornamentation is a key characteristic (not the morphology of spore ornamentation). Specifically, species with spore ornamentation composed of epispore cytoplasm are *Minchinia* and those within it composed of spore wall material are *Haplosporidium* (Azevedo, 2001).

Recently, oyster pathogens in the genus *Bonamia*, which are not known to produce spores, were incorporated into the phylum Haplosporidia based on molecular phylogenetic analysis (Carnegie *et al.*, 2000). The following review focuses on the most significant representatives of the spore-forming and non-spore-forming members of this phylum. Specifically, *Haplosporidium nelsoni* (commonly known as MSX from the original assignation of multinucleate sphere unknown) and then *Bonamia ostreae* will be discussed. In addition, other significant species in each genus are mentioned under the headings 'Related pathogens'.

HAPLOSPORIDIUM NELSONI

Host range and economic significance

Catastrophic epizootics caused by *H. nelsoni* were first encountered in the late 1950s in eastern oysters (*C. virginica*) from Delaware Bay (Haskin *et al.*, 1966). This parasite (Fig. 17.6A, B, C) currently occurs along the

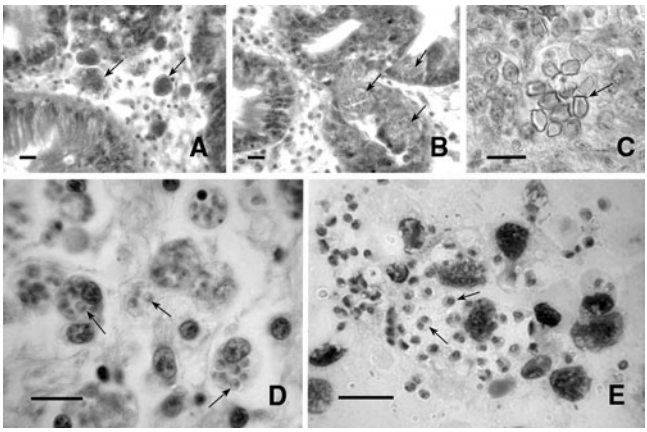


Fig. 17.6. Histological images (A to D) and tissue imprint (E) of Haplosporidia in oysters. A to C. Developmental stages of *Haplosporidium nelsoni* in *Crassostrea virginica* from Nova Scotia, Canada. A. Plasmodia (arrows) in the connective tissue of the digestive gland between the tubules. B. Sporocysts (arrows) within the epithelium of the digestive-gland tubules. C. Spores with a prominent operculum (arrow) in the disrupted tissue of a digestive-gland tubule (courtesy of M. Maillet). D and E. *Bonamia ostreae* from heavily infected *Ostrea edulis* from France. D. Numerous *B. ostreae* (arrows) located within haemocytes in the connective tissue between the tubules of the digestive gland. E. Imprint of connective tissue containing many *B. ostreae* (arrows), some with two nuclei, freed from ruptured haemocytes. All bars = 10 μm.

eastern coast of the USA from Florida to the Piscataqua River estuary in Maine/New Hampshire. A recent (autumn of 2002) epizootic, with localized high mortalities (about 80%), occurred in Bras d'Or Lakes, Nova Scotia, Canada (Stephenson *et al.*, 2003). However, the parasite has not yet been detected in oyster stocks between the southern end of Maine and Bras d'Or Lakes.

Sporogonic and/or plasmodial stages of haplosporidians that resemble *H. nelsoni* were observed in the Pacific oyster (*C. gigas*) from California and Washington State, USA, Taiwan, Korea and Japan (Friedman, 1996). With the development of molecular diagnostic techniques, reports of *H. nelsoni* in Pacific oysters have been confirmed from California, USA, France, Korea and Japan (Burreson *et al.*, 2000; Renault *et al.*, 2000; Kamaishi and Yoshinaga, 2002). Effects of *H. nelsoni* on Pacific oysters have not been described, but some authors speculate that it may be pathogenic, especially for juvenile oysters. However, haplosporidosis has not been associated with mortality of this oyster species.

In 1957, 85% mortality (with 50% dead within 6 weeks) occurred among eastern oysters planted in Delaware Bay. The high mortality represented a loss in production from 7.5 million lbs of shucked meats prior to the enzootic to about 100,000 lbs of production in 1960, and production has not significantly recovered (Lauckner, 1983; Sindermann, 1990). Average mortalities in eastern oysters have been estimated at 50 to 60% in the first year with a 50% further loss in the second year of oyster grow-out. Also, eastern oyster culture in the lower Chesapeake Bay was abandoned for at least 25 years due to this disease (Andrews, 1988b).

Morphology and life cycle

Despite more than 40 years of intensive research, the complete life cycle, the mode of infection and several aspects of the general biology remain obscure (Ford, 1992). In eastern oysters, spheroid plasmodia (4 to 30 μm in diameter; Fig. 17.6A) are usually multinucleate (up to about 60 nuclei per plasmodium, with each nucleus about 2 μm in diameter). Smaller plasmodia are formed by cytoplasmic cleavage of the larger ones. They are first observed in the gills, palps and suprabranchial chambers but subsequently occur in the vesicular connective tissues adjacent to the digestive tract, and eventually become systemic (Lauckner, 1983). Plasmodia and prespore stages (Fig. 17.6B) are most frequently observed, while sporocysts containing mature spores (Fig. 17.6C) are rare in adult oysters (< 0.01%). However, sporulation occurs in at least 75–85% of the infected young oysters (< 1 year) in Delaware Bay (Burreson, 1994). Sporulation, when present, occurs in the epithelial cells of the digestive gland tubules (Fig. 17.6B). Spores are operculate and measure 7.5 μm by 5.4 μm unfixed (Couch *et al.*, 1966; Rosenfield *et al.*, 1969). Ford and Haskin (1982) and Perkins (1993) noted that the parasite could not be transmitted in the laboratory with either infected tissues or spore suspensions and foci of infection persisted in areas where eastern oysters were sparse. Although no hosts other than oysters have been found (Ford, 1992), there is speculation that an intermediate host is required for the completion of the life cycle of *H. nelsoni* (Ford and Tripp, 1996).

Extensive epizootiological data indicate that infections acquired in the early summer become patent in July and mortalities begin in early August and peak in September, with a subsequent decline to low levels by November (Andrews, 1982). A few mortalities occur in late winter, followed by increased mortalities in June and July of the second year resulting from infections acquired during the late summer and autumn of the previous year. Essentially the disease is regulated by temperature, with both parasite and host being inactive below 5°C. Between 5 and 20°C, the parasite multiplies faster than the host can contain it. Above 20°C, resistant oysters can inhibit parasite multiplication or undergo remission (Sindermann, 1990). Levels of *H. nelsoni* have fluctuated in a cyclic pattern, with peaks in prevalence every 6 to 8 years and reduced parasite activity following very cold winters (Ford and Haskin, 1982). In addition to temperature, salinity is also known to affect

the pathogenicity of *H. nelsoni*. The disease is restricted to salinities over 15 ppt (*H. nelsoni* cannot survive below 10 ppt), rapid and high mortalities occur at about 20 ppt and the disease may be limited by salinities between 30 and 35 ppt (Andrews, 1988b). Extensive data on the influence of environmental conditions on the prevalence and intensity of infection and the disease process have been integrated into a mathematical model of host–parasite–environmental interactions (Ford *et al.*, 1999; Paraso *et al.*, 1999; Powell *et al.*, 1999).

Host–parasite relationships

H. nelsoni is highly virulent for eastern oysters and the occurrence of moribund oysters with relatively light infections suggests a toxic effect (Andrews, 1988b). The course of the infection seemed dependent on the history of exposure in eastern oyster stocks (Farley, 1975). In susceptible populations, the prevalence of infection can reach 100%, with mortalities ranging between 40 and 80% (Andrews, 1988b). However, in enzootic areas such as Delaware Bay, natural selection has increased the proportion of disease-resistant eastern oysters and mortalities were about half those of naïve stocks (Ford and Haskin, 1982). Further development of high disease resistance in wild oyster populations was attributed to drought conditions in the mid-1980s, which caused a salinity increase in the usually lower-salinity areas of the upper Delaware Bay, thereby allowing incursion of *H. nelsoni*, with resulting high mortalities and thus selection for disease resistance in the brood stock (Ford, 2002).

During their second year, eastern oysters that survived the infection were able to suppress or rid themselves of the parasite in the late spring as temperatures approached 20°C (Ford and Haskin, 1982). Remission was characterized by diminution of infection and localization of parasites to external epithelium, with diapedesis resulting in the deposition of moribund parasites and necrotic tissues against the shell, followed by external conchiolinous encapsulation (Farley, 1968). Ford and Haskin (1982) indicated that resistance to mortalities was not correlated with an ability to prevent infection but with restriction of parasites to localized non-lethal lesions. Chintala and Fisher (1991) proposed that lectins in the haemolymph could be related to disease resistance or affected by *H. nelsoni* infection.

Parasitism was associated with reduced meat yield, impaired gonadal development and lower fecundity (Barber *et al.*, 1988). The greatest effect on reproduction occurred when gametes were in the formative stage rather than after they matured (Ford *et al.*, 1990a). There was also a threefold increase in the proportion of females among infected oysters, which Ford *et al.* (1990a) suggested was due to inhibition of the development of male more than female gametes. However, infected oysters that underwent temperature-associated remission during the summer developed mature gonads and spawned before new or recurrent infections proliferated in the autumn (Ford and Figueras, 1988).

Propagation

H. nelsoni has not been cultured *in vitro* and controlled transmission has not been achieved. Even transplantation of infected tissues was unsuccessful (Lauckner, 1983). Enriched suspensions of *H. nelsoni* plasmodial stages can be obtained using the 'panning' technique described by Ford *et al.* (1990b).

Clinical signs and diagnosis of infection

The only specific but rare sign of this disease is a whitish discoloration of the digestive gland tubules due to the presence of mature spores. Other non-specific signs are: emaciation, mantle recession, failure of shell growth, retracted mantle and, rarely, brown patches of periostracum opposite lesions on the mantle surface (Lauckner, 1983; Andrews, 1988b). Histological examination is used to confirm the presence of infection, and heavy infections can be detected by microscopic examination of stained haemolymph smears (Andrews, 1988b; Burreson *et al.*, 1988; Ford and Kanaley, 1988). When mature spores are present, the sporoplasm specifically stains

bright red with a modified Ziehl–Neelsen carbol fuchsin technique (Farley, 1965). The location of the spore stages in the epithelium of the digestive gland tubules can be used to differentiate *H. nelsoni* from a closely related and potentially cohabiting species *Haplosporidium costale* with spores that occur in the connective tissue between the tubules. If spores are not present (usually the case for adult oysters infected with *H. nelsoni* and during early developmental stages of *H. costale*), the plasmodial stages of the two species cannot be differentiated by histological examination. Although Barrow and Taylor (1966) and Burreson (1988) illustrated the potential use of immunoassays for detecting infection and possibly for identifying alternative hosts, molecular diagnostic techniques have proven to be more widely used.

An SSU rRNA gene of *H. nelsoni* was sequenced (Fong *et al.*, 1993) and DNA probes that targeted this region were tested for sensitivity and specificity (Stokes and Burreson 1995; Stokes *et al.*, 1995). In addition to being used by various researchers to identify *H. nelsoni* in eastern oysters, DNA sequence equivalency (tested by PCR amplification of genomic DNA and *in situ* hybridization) provided conclusive evidence that the haplosporidian in Pacific oysters from other areas of the world was *H. nelsoni* (Burreson *et al.*, 2000; Renault *et al.*, 2000; Kamaishi and Yoshinaga, 2002). The primer sequence was also used to develop a competitive, quantitative PCR assay for this parasite (Day *et al.*, 2000). The subsequent identification of the SSU rRNA gene for *H. costale* (Ko *et al.*, 1995) and the development of specific PCR assays confirmed by *in situ* hybridization have led to the differential diagnosis of mixed *H. costale* and *H. nelsoni* infections in eastern oysters (Stokes and Burreson, 2001). Also, multiplex PCR (simultaneous testing of two or more pathogens in a single test reaction) was developed for *H. nelsoni* and two other cohabiting parasites, *H. costale* and *P. marinus* (Penna *et al.*, 2001). Unfortunately, attempts to use PCR technology to identify the complete life cycle of *H. nelsoni* were unsuccessful (Stokes *et al.*, 1999).

Prevention and control

Reduced salinities (< 10 ppt) adversely affected the pathogenicity and survival of the parasite in oysters (Haskin and Ford, 1982). Thus, management strategies depend, in large measure, on avoiding the disease by culturing oysters in areas of low salinity and/or altering the time at which oysters are moved to enzootic areas of high salinity to take advantage of better growth. Continuous monitoring and early diagnosis of infections are important because they allow mortality to be predicted so that growers and managers can make informed decisions on when or whether to plant and harvest (Ford and Haskin, 1988). The recently developed mathematical model of host–parasite–environmental interactions has been used to simulate infection cycles within the oyster and in oyster populations under different environmental conditions to forecast conditions that can initiate and end epizootics in oyster populations (Ford *et al.*, 1999; Paraso *et al.*, 1999; Powell *et al.*, 1999).

Excellent survival has been achieved in enzootic areas using eastern oysters that were experimentally selected for disease resistance (Ford *et al.*, 1990a). Barber *et al.* (1991) indicated that resistance in the selected strain may be the result of physiological responses that inhibit parasite development and basic metabolic adjustments to parasitism. Much of the physiological response may be derived from an increased number of haemocytes, which plug lesions, remove debris and repair tissue, thereby helping resistant oysters to survive infection (Ford *et al.*, 1993). Because of economic limitations, resistant oysters have not yet been produced in commercial quantities (Andrews, 1988b). However, triploid eastern oysters seem more resistant to the disease than diploid cohorts (Matthiessen and Davis, 1992). The increased resistance in triploids may provide a viable alternative for the eastern oyster culture industry in areas where the disease occurs. Also, natural selection in Delaware Bay seems to have resulted in wild oysters that are more resistant to disease than oysters from populations that have not undergone such long-term, intensive, selection processes (Ford, 2002).

The possibility of growing non-native oyster species that appear to be more resistant to *H. nelsoni*, such as the Pacific oyster and Suminoe oyster (*Crassostrea ariakensis* = *Crassostrea rivularis*) are being assessed.

Related pathogens

In addition to numerous reports of unidentified species of *Haplosporidium* or *Minchinia* in marine invertebrates (Burreson and Ford, 2004), three named species occur in bivalves of economic importance.

H. costale (commonly referred to as SSO, an acronym for seaside organism) has been detected in eastern oysters along the east coast of North America but has caused significant disease only in high-salinity (> 25 ppt) areas from Delaware to Virginia (Andrews, 1988c). It can be differentiated from *H. nelsoni* by: (i) a smaller spore size (3.1 μm by 2.6 μm); (ii) occurrence of sporulation throughout all connective tissue and not in the epithelium of the digestive gland; (iii) antigenic differences; and (iv) species-specific molecular diagnosis, as indicated above. Initially thought to have a regular and clearly defined life cycle (a 4- to 6-week period of disease, sporulation and concurrent mortalities in May and June, followed by an 8- to 10-month prepatent period in newly exposed oysters), the application of molecular diagnostic tools has revealed unseasonably advanced infections in the autumn (Stokes and Burreson, 2001). Also, mixed infections with *H. nelsoni* are more frequent than originally thought. *H. costale* is not as serious a pathogen as *H. nelsoni* and losses can be minimized by harvesting oysters at 18 to 24 months of age (Andrews, 1988c).

Haplosporidium (= *Minchinia*) *armoricana* causes brown meat disease in flat oysters (*Ostrea edulis*) in Brittany (France) to Spain and in the Netherlands among flat oysters imported from Brittany (van Banning, 1985a; Azevedo *et al.*, 1999). Numerous operculate spores (5.0 to 5.5 μm by 4.0 to 4.5 μm) with two long projections (70 to 100 μm) in sporocysts (35 to 50 μm in diameter) throughout the connective tissue result in brownish discoloration of heavily infected flat oysters. Although the disease is fatal, the prevalence of infection to date has been low (< 1%) with an insignificant impact on the flat oyster culture industry in Europe (van Banning, 1979; Lauckner, 1983).

Minchinia (= *Haplosporidium*) *tapetis*, was described from European littleneck clams (*R.* (= *T.*) *decussatus*) in Portugal and France (Lauckner, 1983; Chagot *et al.*, 1987; Azevedo, 2001). Slightly ovoid spores (4 to 6 μm in diameter) were observed in the connective tissue of the gills, mantle and ventral to the digestive gland tubules. Reported prevalences of infection were low (4%) and pathogenicity was minimal.

BONAMIA OSTREAE

Host range and economic significance

B. ostreae (commonly called a microcell because of its small size; Fig. 17.6D, E) is a lethal pathogen of flat oysters (*O. edulis*), in which it causes a disease called bonamiasis (Pichot *et al.*, 1980). However, other oyster species, including Australian flat oysters (*Ostrea angasi*), New Zealand dredge oysters (*Ostrea chilensis* (= *Tiostrea chilensis* = *Tiostrea lutaria*)), *Ostrea puelchana* and Suminoe oysters (*C. rivularis*) were experimentally infected (Cochennec *et al.*, 1998). The Pacific oyster (*C. gigas*), mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) and clams (*R. decussatus* and *V.* (= *R.*) *philippinarum*) could not be naturally or experimentally infected and these bivalves did not appear to act as vectors or intermediate hosts for *B. ostreae* (Culloty *et al.*, 1999). This parasite occurs along the Atlantic coast of Europe from Spain to Denmark, Great Britain (excluding Scotland), Ireland and Italy (OIE, 2003a). *B. ostreae* also occurs in some introduced flat oyster populations on the west (California and Washington) and east (Maine) coasts of the USA (Zabaleta and Barber, 1996). Evidence suggests that *B. ostreae* was inadvertently introduced into Europe, Maine and Washington from California by the translocation of infected flat oysters in the late 1970s (Elston *et al.*, 1986; Friedman and Perkins,

1994; Cigarría and Elston, 1997). The source of infection for the introduced flat oysters in California is not known.

This parasite was first associated with mortalities in Brittany, France, in 1979 and the disease quickly spread throughout the major flat oyster culture areas in Europe. Average losses were about 80% or higher (Grizel, 1986; Hudson and Hill, 1991; van Banning, 1991). In conjunction with the protist *Marteilia refringens* (see below), *B. ostreae* reduced flat oyster production in France from 20,000 t per year in the 1970s to 1800 t in 1995 (Boudry *et al.*, 1996). Production of flat oyster has not recovered, and the Pacific oyster (*C. gigas*) is now the main species of oyster cultured in Europe.

Morphology and life cycle

Two morphological forms of *B. ostreae* have been identified (Lauckner, 1983; Grizel *et al.*, 1988). The most frequently observed 'dense forms' (2 to 3 μm in diameter) have basophilic, dense cytoplasm with a pale halo around the nucleus. This form is usually free (extracellular) in tissues altered by the disease and may represent the transmission stage. The slightly larger and less dense 'clear forms' (2.5 to 5 μm in diameter) may be the trophozoite, schizogonic stage and typically occur within the cytoplasm of haemocytes and in branchial epithelial cells (Montes *et al.*, 1994). The usual mode of multiplication in the oyster is by simple binary fission. However, Brehélin *et al.* (1982) described a true plasmodial multinucleate stage with three to five nuclei and about 6 μm in diameter.

Unlike the spore-forming haplosporidians, *B. ostreae* can be directly transmitted between flat oysters, and lethal infections usually develop within 3 to 6 months after exposure (Grizel *et al.*, 1988; Sindermann, 1990). Transmission occurs year-round, with the highest prevalence of infection found during the summer. Van Banning (1990) suggested that an infectious phase may occur in the ovarian tissue of flat oysters.

Host–parasite relationships

Bonamiasis is usually systemic because *B. ostreae* normally resides within haemocytes and has not been reported in other host cells. Infections are often accompanied by dense, focal haemocyte infiltration into the connective tissue of the gill and mantle and around the gut, and may result in tissue lesions (Cochennec-Laureau *et al.*, 2003a). Many of the infiltrating haemocytes contain several microcells (Fig. 17.6D), which are often in cytoplasmic vacuoles. As the infection progresses, infected haemocytes occur in the vascular sinuses, and microcells may be released by lysis of haemocytes and found free in necrotic tissues (Balouet *et al.*, 1983). Two years of age appeared to be critical for disease development in oysters, and infection level was statistically independent of oyster gonadal development and sex (Culloty and Mulcahy, 1996).

In vitro tests were used to determine that haemocytes of Pacific oysters were able to bind more *B. ostreae* than were haemocytes of flat oysters (Fisher, 1988), but haemocyte infection rates were similar for both species (Mourton *et al.*, 1992). The apparent inability of flat oyster haemocytes to inactivate the parasites once they are ingested may explain differences in susceptibility and disease development in oysters (Chagot *et al.*, 1992; Xue and Renault, 2000).

Propagation

B. ostreae is readily propagated *in vivo* by injection of infected haemocytes or purified parasite suspensions and by cohabitation of diseased and uninfected oysters (Hervio *et al.*, 1995). Comps (1983) reported *in vitro* proliferation of *B. ostreae* in the presence of flat oyster cells after 48 h of incubation but the viability of the cultures over longer periods was not indicated.

Diagnosis of infection

Microcells are detected by histological examination (Grizel *et al.*, 1988). Although many infected oysters appear normal, others may have yellow discoloration and/or extensive lesions (i.e. perforated ulcers) on the

gills and mantle. The isolation and purification of *B. ostreae* from infected flat oysters (Mialhe *et al.*, 1988a) have led to the production of monoclonal antibodies (Rogier *et al.*, 1991) and the development of an IFAT (Boulo *et al.*, 1989) and of an ELISA diagnostic technique with 90% reliability in comparison with standard histopathological light microscopic examinations (Cochennec *et al.*, 1992). Because classical histological (Fig. 17.6D) and heart smear (Fig. 17.6E) techniques are unreliable for detecting light infections (Culloty *et al.*, 2003) and immunoassays (ELISA kits) are no longer commercially available, molecular diagnostic techniques were developed.

A PCR reaction specific for an rDNA amplicon (528 base pairs (bp) spanning 341 bp of 18S rDNA and 187 bp of ITS1) with a gene sequence resembling that belonging to members of the phylum Haplosporidia was identified and found to detect the parasite in naturally infected *O. edulis* in Maine, USA (Carnegie *et al.*, 2000). This PCR assay proved to be more sensitive, more specific and less ambiguous than standard histological and cytological (tissue imprint) techniques. Another DNA probe identified from the same area of the genome also detected another species of *Bonamia* (see *B. exitiosus* below) and *H. nelsoni* (Cochennec *et al.*, 2000).

Prevention and control

Following the recognition of bonamiasis in Europe, measures such as the destruction of infected stocks and restricting movement of flat oysters were implemented (van Banning, 1985b; Grizel *et al.*, 1986; Hudson and Hill, 1991). In many instances, these measures were employed too late to prevent the spread of the pathogen. Studies in the Netherlands indicated that *B. ostreae* persisted in low levels for at least 6 years in areas where flat oysters were virtually eradicated (van Banning, 1987). Mortalities due to bonamiasis were reduced by using suspension culture and lower stocking densities and marketing the oysters at a relatively young age (after 15 to 18 months of culture) (Lama and Montes, 1993; Montes *et al.*, 2003).

Alternative resistant species, such as Pacific oysters, are now being cultured in areas where flat oyster populations were devastated by bonamiasis. However, flat oyster production has marginally persisted in a few areas of France in which the seeding of young oysters was reduced from 5 to between 1 and 2 t/ha, and by the use of 'deep water', where the parasite is apparently not transmitted (Grizel *et al.*, 1986). Also, the absence of infection in juveniles has allowed the use of oyster seed produced in areas where *B. ostreae* occurs (Grizel *et al.*, 1988). Selecting for disease-resistant flat oysters is showing some success (Culloty *et al.*, 2001). However, there is evidence from DNA microsatellite loci analysis that a population bottleneck has occurred during the selection process in some stocks of bonamiasis-resistant *O. edulis*. The small effective number of breeders is expected to lead to increasing inbreeding and have important consequences for the future management of at least three selected bonamiasis-resistant populations (Launey *et al.*, 2001).

Related pathogens

Bonamia exitiosus has devastated dredge oysters (*O.* (= *T.*) *chilensis* (= *lutaria*)) populations in the Foveaux Strait south of South Island, New Zealand (Hine *et al.*, 2001b). Stocks of dredge oysters were reduced by 67% in 1990 and by 91% in 1992 from levels recorded in 1975. The commercial dredge oyster fishery was closed in 1993, with severe economic impacts on South Island coastal communities (Doonan *et al.*, 1994). Like *B. ostreae*, *B. exitiosus* resides in haemocytes, is small in size (2 to 7 μm) and has light and dense forms, which vary in prevalence seasonally (Hine, 1991a,b). However, *B. exitiosus* can de differentiated from *B. ostreae* by antigenic features (Mialhe *et al.*, 1988b), divergent regions in the SSU rDNA sequence and ultrastructural differences in

the dense forms. Dense forms of *B. exitiosus* are less dense and slightly larger in size (3.0 ± 0.3 μm mean diameter in comparison with 2.4 ± 0.5 μm for *B. ostreae*), have more haplosporosomes, mitochondrial profiles and lipoid bodies per ultrastructure section, have smaller tubulovesicular mitochondria and have nuclear membrane-bound Golgi/nuclear cup complexes and a vacuolated stage that are lacking in *B. ostreae* (Hine *et al.*, 2001b). Ultrastructural examination of cytoplasmic structures in *B. exitiosus* led Hine and Wesney (1992) to suggest that the haplosporosome-like bodies may be a sign of disease within the microcells. In part, this suggestion was derived because of the similarities: (i) between haplosporosomes and virus-like particles; and (ii) between haplosporogenesis and virus production within host cells. As with *B. ostreae*, molecular diagnostic assays (*in situ* hybridization and PCR amplification) were more sensitive in the detection of *B. exitiosus* than traditional techniques (microscopic examination of heart imprints and histology) (Diggles *et al.*, 2003).

The microcell that causes winter mortalities in Sydney rock oysters (*Saccostrea glomerata* (= *commercialis*)) in New South Wales, Australia, initially described as *Mikrocytos roughleyi* (Farley *et al.*, 1988), is now believed to be a species of *Bonamia* (Cochennec-Laureau *et al.*, 2003b). The morphological features that separate this parasite from the other *Bonamia* spp. have not been identified. Like other *Bonamia*, this microcell also occurs within haemocytes and is associated with focal abscess-type lesions in the gill, connective and gonadal tissues and the alimentary tract. Disease is associated with low temperatures and high salinities (30–35 ppt). It can kill up to 70% of mature Sydney rock oysters in their third winter before marketing and mortalities seem to be highest in autumns and winters with low rainfall (Wolf, 1979). The high mortalities can be reduced by harvesting large oysters before the winter and by overwintering smaller oysters on up-river leases where lower salinities and higher racks protect them from the disease (Anderson, 1990).

Unknown Taxonomic Affiliations

Mikrocytos mackini

Like *Bonamia* spp. described above, *M. mackini* (Fig. 17.7A, B, C) is commonly referred to as a microcell (2 to 4 μm in diameter) and is infective to at least four species of oysters (Pacific oysters, *C. gigas*; eastern oysters, *C. virginica*; flat oysters, *O. edulis*; and Olympia oysters, *Ostrea conchaphila*). However, *M. mackini* is not related to *Bonamia* spp. and its taxonomic affiliations remain unknown. This parasite has been reported from oysters in the southern part of British Columbia, Canada and adjacent areas of Washington, USA. Disease caused by *M. mackini* appears to be restricted to older oysters (over 2 years) in some locations in British Columbia and mortalities (recorded as high as 30% in older oysters at low tide levels) occur in the spring (April and May) after a 3–4-month period when temperatures are less than 10°C. The requirement for cool temperatures and the long prepatent period may explain why the disease only occurs during the spring and seems to be confined to oysters cultured in more northerly locations. *M. mackini* can de differentiated from *Bonamia* spp. by its location in vesicular connective tissue cells, in adductor muscle myocytes and, less frequently, in haemocytes, and by the apparent lack of mitochondria and haplosporosomes. Also, *M. mackini* seems to have a very unique way of obtaining energy from its host cell. Hine *et al.* (2001a) depicted tube-like structures extending into the cytoplasm of *M. mackini* from the mitochondria of its host cell (Fig. 17.7A, B). *C. gigas* seems to be more resistant to the disease than the other species of oysters challenged experimentally under laboratory and field conditions (Bower *et al.*, 1997). Hybridomas that produce monoclonal antibodies specific for *M. mackini* were produced (Hervio *et al.*, 1996), but this product has not been developed into an immunodiagnostic assay. Carnegie *et al.* (2003) described PCR and fluorescent *in situ* hybridization assays for *M. mackini*, based on the SSU rDNA, which detected three to four times more infections than standard histopathology.

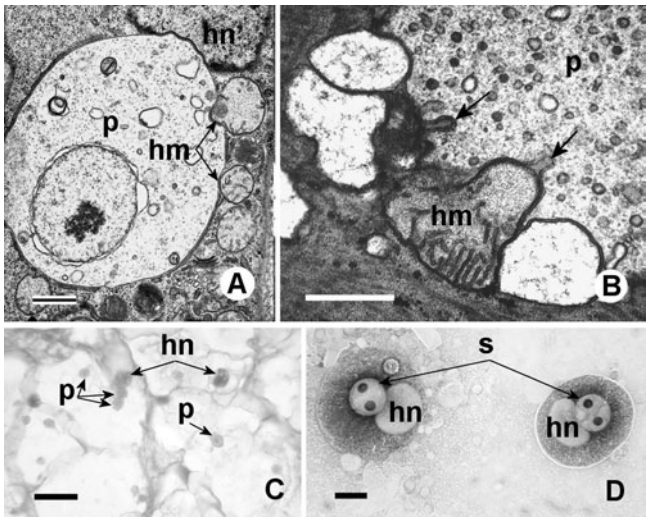


Fig. 17.7. Transmission electron micrographs (A and B) and a histological image (C) of *Mikrocytos mackini* in the cells of *Crassostrea gigas* and *Ostrea edulis,* respectively, from British Columbia, Canada. A. Protist (p) against the host cell nucleus (hn) and two closely associated host mitochondria (hm). B. Higher magnification of a host mitochondrium (hm) with tube-like structures (arrows) extending into the cytoplasm of *M. mackini* (p). C. Several *M. mackini* (p) in the cytoplasm of vesicular connective-tissue cells (hn, nuclei of host cells) of the labial palps of *O. edulis*. D. Tissue imprint of the gonad of *Crassostrea gigas* from Japan with a sporangiosorus (s) of *Marteilioides chungmuensis* in each ovum against the host cell nucleus (hn). Each sporangiosorus contains two sporonts and each sporont contains one basophilic developing spore. A and B bars = 0.5 µm, C and D bars = 10 µm.

Mortalities caused by *M. mackini* can be circumvented by well-timed plantings and harvests of Pacific oysters in relation to season and tide levels (Bower, 1988).

Paramyxea

Introduction

These spore-forming bivalve pathogens were initially assigned to the phylum Ascetospora in the same class (Stellatosporea) as the haplosporidians (Levine *et al.*, 1980). Because of significant morphological and developmental differences, Desportes (1984) moved them to the class Paramyxea and order Marteiliida, and Desportes and Perkins (1990) suggested that the class Paramyxea be raised to the rank of phylum. Based on an SSU rDNA gene sequence that was very different from all known sequences of eukaryotic organisms, including myxosporeans and haplosporeans, Berthe *et al.* (2000) supported this phylum designation. These parasites are characterized by the presence of several cells enclosed inside one another, which arose by a process of internal cleavage ('endogenous budding') within a stem cell. Included in this group are pathogens in two genera, *Marteilia* (several species) and *Marteilioides* (two species), that have had a significant impact on bivalve production in different areas of the world. Each genus will be presented separately.

MARTEILIA SPP.

Host range and economic significance

Species of *Marteilia* produced disease of economic concern on the coast of Europe, eastern Australia and Florida, USA. In Europe, especially along the Atlantic coast of France, *M. refringens*, commonly known as Aber disease or digestive-gland disease, caused recurrent serious mortalities (from 1967 to about 1977) in flat oysters (Grizel

et al., 1974; Sindermann, 1990). Alderman (1979) indicated that the decline (to about 47%) of flat oyster production in France over 6 years (from 18,000 t in 1969 to 8400 t in 1975) was a direct result of the spread of this parasite. To date, *M. refringens* has been detected in flat oysters from the coasts of France, Portugal, Spain, Italy, Greece and Morocco (OIE, 2003b). In addition to flat oysters, *M. refringens* was reported from blue mussels (*M. edulis*), European cockles (*Cardium edule*) and imported Pacific oysters (*C. gigas*), and was experimentally infective to New Zealand dredge oysters (*O.* (= *T.*) *chilensis* (= *lutaria*)) and Australian flat oysters (*O. angasi*). Interestingly, negligible pathology was found in most of these other species (Cahour, 1979; Grizel *et al.*, 1983; Bougrier *et al.*, 1986). As the impact of Aber disease seemed to be subsiding in the late 1970s, the flat oyster industry in Europe was struck by another devastating disease caused by the microcell *B. ostreae* (see above), from which it has not yet recovered.

In addition to *M. refringens*, two other species, *Marteilia maurini* in 'gallo' mussels (*M. galloprovincialis*) and blue mussels from France, Spain and the Persian Gulf (Comps *et al.*, 1981), and *Marteilia lengehi* from oysters (*Saccostrea* (= *Crassostrea*) *cucullata*) from the Persian Gulf (Comps, 1976), have been described. Due to the morphological similarity between these marteilias, the validity of the latter two species is questioned. Nevertheless, in some areas, mortalities of mussels attributed to *M. maurini* are significant, especially for 'gallo' mussels from rías in Galicia, northwest Spain (Villalba *et al.*, 1997). High prevalences (37 to 70%) have also been reported in blue mussels from the north coast of Brittany (Auffret and Poder, 1983).

On the east coast of Australia, in subtropical and tropical regions of southern Queensland and northern New South Wales, another species, *Marteilia sydneyi*, the cause of QX disease, was associated with high mortalities (often exceeding 80%) in Sydney rock oysters (*S.* (= *Crassostrea*) *glomerata* (= *commercialis*)) (Lester, 1986). The same, or a very similar, species of marteilid has been reported in the black-lip oyster (*Saccostrea* (= *Crassostrea*) *echinata*) in Australia but has not been associated with overt pathology (Hine and Thorne, 2000).

On the coast of Florida, another *Marteilia* sp. has been implicated in mass mortalities approaching 100% of the calico scallop (*Argopecten gibbus*). The fishery, which produced 11–40 million lbs of adductor muscle meats annually prior to December 1988, was destroyed within 1 month, with mortalities spread over a 2500 square mile area. As of the spring of 1992, the scallops had not returned to commercially harvestable quantities (Moyer *et al.*, 1993).

Parasite morphology and life cycle

Infections by all *Marteilia* spp. are presumably initiated by a primary cell or stem cell (5 to 8 μm in diameter) in the epithelial cells of the gut or gills (Grizel *et al.*, 1974). The early development of *M. sydneyi* in the gill and palp epithelium of the Sydney rock oyster was revealed by Kleeman *et al.* (2002a) using molecular *in situ* hybridization techniques. The primary uninucleate cell contained a secondary uninucleate daughter cell in a vacuole within its cytoplasm. The daughter cell divided by binary fission to produce four daughter cells within the enlarged primary (stem) cell and within each daughter cell a uninucleate cell developed by internal cleavage. The primary cell degenerated to release the daughter cells, which became new primary cells. For *M. sydneyi*, initial proliferation in the epithelial cells of the gills and palps was followed by systemic dissemination to the digestive-gland tubules, where the basal membrane of the tubules was penetrated and the parasites became established as nurse cells at the base of the epithelial cells. Nurse cells containing daughter cells proliferated and eventually degraded. Daughter cells of *M. sydneyi* and *M. refringens* in the digestive gland tubules become sporangiosori, described as 'primary cells' by Perkins and Wolf (1976). Sporulation occurred within the sporangiosorus via a unique process of internal cleavages (endosporulation) to produce cells within cells (Fig. 17.8).

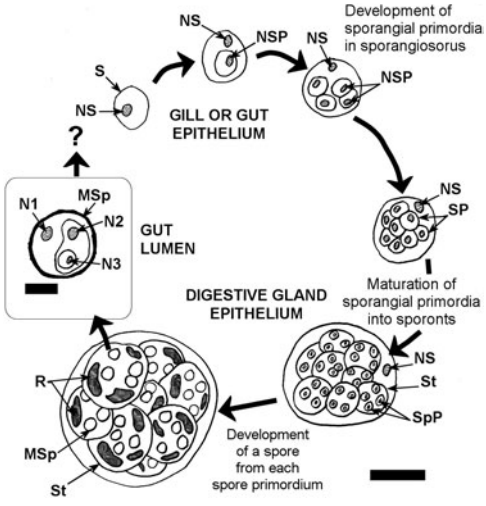


Fig. 17.8. Schematic drawing to scale of the sporulation process of *Marteilia* spp. based on descriptions by Grizel *et al*. (1974), Perkins (1976) and Kleeman *et al*. (2002a). S, sporangiosorus (or primary cell); NS, nucleus of sporangiosorus; SP, sporangial primordium (or secondary cell); NSP, nucleus of sporangial primordium; St, sporont; SpP, spore primordium (or tertiary cell); MSp, mature spore; R, refringent bodies; N1, nucleus of outermost sporoplasm; N2, nucleus of middle sporoplasm; N3, nucleus of innermost sporoplasm. Bar = 10 μm and 2 μm for inset of spore.

At the initiation of sporulation, uninucleate segments become delimited within the cytoplasm of the sporangiosorus to form the sporangial primordia (secondary cells). Eventually, eight to 16 sporangial primordia (each about 12 μm in diameter at maturity) form within the sporangiosorus, which retains its nucleus and enlarges to about 30 μm in diameter. Each sporangial primordium matures into a sporont containing two to four spore primordia (tertiary cells), which mature into spores (Fig. 17.8). Each spore contains three uninucleate sporoplasms of graded sizes, with each of the smaller sporoplasms being enclosed within the cytoplasm of the next largest one (i.e. consecutive internal cleavage of two sporoplasms within the spore primordium) (Perkins, 1976). A continuous spore wall with no operculum occurs around each spheroid mature spore, which measures 3.5 to 4.5 μm in diameter. As the spore matures, light-refractile inclusion bodies appear in the sporont cytoplasm surrounding the spores. The specific name of *M. refringens* was derived from these refringent inclusion bodies. Mature spores are shed into the tubule lumen for evacuation from the oyster and infected oysters may shed large numbers of spores before oyster death.

Marteilia sydneyi can be differentiated from *M. refringens* by: (i) the formation of eight to 16 sporangial primordia in each sporangiosorus instead of eight for *M. refringens*; (ii) each sporont contains two or infrequently three, rather than four, spores; and (iii) the heavy layer of concentric membranes surrounding mature spores of *M. sydneyi* is lacking around *M. refringens* spores (Perkins and Wolf, 1976).

The development of *M. refringens* and *M. sydneyi* was directly related to water temperature, with the disease being most severe towards the end of summer, which coincides with the sporulation process. Transplantation experiments in France suggested that new infections of *M. refringens* were acquired from early May to early September (Grizel, 1979). However, similar experiments in Australia indicated that oysters may be exposed to infection over a very short interval (possibly only a few weeks per year) during the summer (Lester, 1986; Wesche, 1995). Warm temperatures favoured parasite development and, at lower temperatures, host mortality was retarded and parasite development suppressed. Young plasmodia may persist throughout the winter and reinitiate clinical infections the following spring (Lauckner, 1983).

The mechanism of infection has not been determined for any species of *Marteilia*. Experimental attempts to transmit the disease to oysters in the laboratory met with failure, although field exposures were successful (Roubal *et al*., 1989; Berthe *et al*., 1998). As with *Haplosporidium* spp., an intermediate host is suspected (Perkins, 1993). Audemard *et al*. (2002) detected *M. refringens* with molecular tools in the gonad of the copepod *Paracartia grani* and speculated on its involvement in the life cycle of this parasite.

Host–parasite relationships

Signs of disease in oysters include a poor condition index, with glycogen loss (emaciation), discoloration of the digestive gland, cessation of growth, tissue necrosis and mortalities (Sindermann, 1990). The pathogenesis of *M. refringens* remains obscure due to the lack of consistent correlation between the degree of infection and mortality (Lauckner, 1983). Some flat oysters kept in high-prevalence areas for extended periods showed characteristic signs of disease without notable numbers of parasites, while other flat oysters heavily infected with young sporangiosori and mature spores exhibited virtually no histological alterations. To explain these inconsistencies, Balouet (1979) and van Banning (1979) suggested that either: (i) the parasite produced toxins inconsistently; (ii) the parasite required the synergistic effect of another, as yet unidentified, pathogen; (iii) an intermediate host was required to amplify parasite abundance; and/or (iv) unfavourable environmental conditions (e.g. physicochemical factors in sea water) played prominent roles in determining the apparent pathogenicity of *M. refringens*. Anderson *et al.* (1994a) determined that fluctuations in pH, salinity and water temperature in close proximity to the Sydney rock oysters did not correlate with epizootics of *M. sydneyi*.

Diagnosis of infection

Because there are no specific clinical signs, infection can best be confirmed by histological examination (Grizel, 1979; Kleeman *et al.*, 2002a). A diagnostic feature is the presence of *Marteilia* spp. in histological sections of the digestive gland tubule epithelium and occasionally in the gills and palps (Sindermann, 1990). Gutiérrez (1977) described a modified staining technique for enhancing the detection of the parasite in paraffin-embedded histological sections. An IFAT, based on the polyclonal antibodies that were specific for sporulating stages of *M. sydneyi*, failed to detect presporulation stages of *M. sydneyi* in the connective tissue of recently infected oysters (Anderson *et al.*, 1994b). Subsequent identity of segment sequences within the SSU rDNA of both *M. sydneyi* (Anderson *et al.*, 1995; Kleeman and Adlard, 2000) and *M. refringens* (Le Roux *et al.*, 1999; Pernas *et al.*, 2001) was used in the development of PCR and *in situ* hybridization assays. Assays developed from the ITS1 by Kleeman and Adlard (2000) proved specific to *M. sydneyi* when tested for their potential to cross react with related species of Paramyxea (Kleeman *et al.*, 2002b). Although the 'Smart 2 probe' identified by Le Roux *et al.* (1999) cross-reacted with various species of Paramyxea (Kleeman *et al.*, 2002b), this probe provided a stronger signal in the detection of sporont stages and was more reliable in the detection of mature spores of *M. sydneyi* than the ITS1 probe. Thus, Kleeman *et al.* (2002b) indicated that the Smart 2 probe was preferred for use in the screening or surveillance of oyster populations and that the ITS1 probe should be used as one means of confirming the specific identity of the pathogen as *M. sydneyi*.

Prevention and control

Similar farm management practices were employed to reduce the risk of infecting oysters with *M. sydneyi* in Australia and *M. refringens* in Europe (Lester, 1986; Sindermann, 1990). During the summer, oysters were not planted in areas of risk and young oysters were held in high-salinity water, where they grow more slowly but remain free of infection until after the risk of infection has passed. Also, large oysters were harvested prior to the onset of the transmission period. In Europe, Pacific oysters, which seem to be resistant to the disease, are cultured in most areas affected by *M. refringens*.

MARTEILIOIDES SPP.

Protozoa in the genus *Marteilioides* can be differentiated from *Marteilia* spp. in that two sporonts usually develop in each sporangiosorus, each sporont produces a single pluricellular spore and the mature spore contains two concentric cells rather than three. The *Marteilioides* species of greatest

concern is *Marteilioides chungmuensis*, which causes a nodular appearance (like multiple tumours) in the gonad of Pacific oysters in western Japan and southern Korea. Surveys in Gokasho Bay, Japan, in 1996 and 1997 revealed prevalences in cultured female oysters between 18 and 20% from autumn to spring, with up to 52% infected in July (Imanaka *et al.*, 2001). In Gosung Bay, Korea, surveys conducted in 2000 did not detect *M. chungmuensis* between February and May and prevalence peaked at about 16% in December (Ngo *et al.*, 2003). A similar-looking parasite was reported from the ova of black-lip oysters (*Saccostrea echinata*) from Western Australia and Northern Territory, Australia (Hine and Thorne, 2000).

M. chungmuensis infects the cytoplasm of oocytes (Fig. 17.7D) and can affect large areas of the reproductive follicles, causing irregular enlargement of the infected gonadal tissues (Itoh *et al.*, 2002). This parasite may cause a significant reduction in the reproductive output of an infected female oyster. Also, *M. chungmuensis* can have a serious economic impact because infected oysters lose their marketability due to the unaesthetic appearance caused by the disease. Basic biological information pertaining to the complete life cycle of this parasite, including the route of infection, early infective and multiplication stages in the oyster and method of transmission, remains unknown (Imanaka *et al.*, 2001). The recent identification of a partial sequence of the SSU rDNA and development of specific molecular diagnostic assays will be used to elucidate the life cycle of *M. chungmuensis* and to determine the phylogenetic position of this parasite (Itoh *et al.*, 2003).

A related species, *Marteilioides branchialis*, in conjunction with *M. sydneyi*, was associated with significant economic losses among Sydney rock oysters (Anderson and Lester, 1992). *M. branchialis* was differentiated from *M. chungmuensis* by sporulation in the cytoplasm of epithelial cells, connective tissue cells and occasionally haemocyte accumulations within lesions on gill lamellae and not in ova.

Spot prawn parasite

A parasite in pandalid shrimp (*Pandalus platyceros* and *Pandalus borealis*) reported from the west coast of northern North America was initially identified as *Hematodinium*-like (Meyers *et al.*, 1994). However, a few inconsistent morphological features (massive plasmodia and binary fission of the trophozoites, with mitosis unlike that of parasitic dinoflagellates; Fig. 17.9), antigenic characteristics that were inconsistent with those of other parasitic dinoflagellates and genetic analysis that consistently grouped this parasite with members of the phylum Haplosporidia clearly indicated that this lookalike pathogen was not related to the Dinoflagellida (Bower and Meyer, 2002). The identity of this shrimp parasite remains unknown because none of the morphological features found to date can be used to affiliate it with the Haplosporidia.

Phylum Annelida

The cosmopolitan spionid polychaetes include several species (most in the genera *Polydora* and *Boccardia*) that burrow into the shells of living molluscs. Spionid polychaetes are filter feeders and do not derive nutrients from their host; however, the burrows that they create in mollusc shells can be problematic. Due to the overall low economic significance of this group of parasites, the taxonomic problems, as indicated by Lauckner (1983), will not be reiterated here. Instead, instances where these polychaetes have had an impact on commercial stocks of molluscs in various parts of the world will be mentioned.

In European waters, mortalities and loss of market quality of blue mussels were caused by *Polydora ciliata* (Lauckner, 1983). The burrows excavated by *P. ciliata* in blue mussel shells not only caused unsightly blisters containing compacted mud but also resulted in significant reductions in shell strength, thereby increasing susceptibility to predation by birds and shore crabs (Kent, 1981). Nacreous blisters

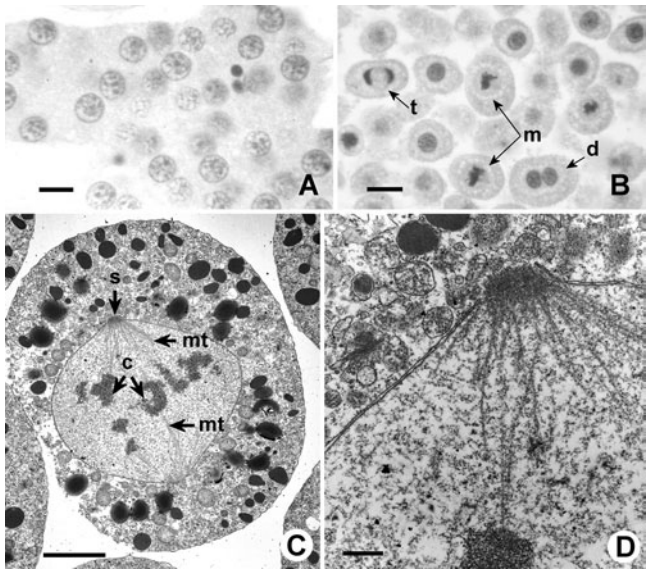


Fig. 17.9. Histological images (A and B) and electron micrographs (C and D) of an unnamed protist with unknown taxonomic affiliations from *Pandalus platyceros* in British Columbia, Canada. A. Plasmodium with numerous nuclei. B. Trophozoites in the process of binary fission showing metaphase (m), late telophase (t) and one cell in which the nucleus has recently divided (d). C. Trophozoite in late metaphase with an intact nuclear membrane surrounding condensed chromosomes (c), which are connected by microtubules (mt) to spindle-pole bodies (s) emerging through the nuclear envelope. D. A higher magnification of C illustrating the microtubules connecting to the spindle-pole body at a gap in the nuclear membrane. A and B bars = 10 µ m, C bar = 2.5 µm and D bar = 0.5 µm.

produced by blue mussels in response to *P. ciliata* may result in atrophy and detachment of the adductor muscle and possibly interference with gamete production when the calcareous ridges occur adjacent to these organs (Lauckner, 1983).

On the east and south coasts of North America, *Polydora websteri* may cause unsightly mud blisters in the shell and yellowish abscesses in the adductor muscle (when the burrow comes in contact with the muscle tissue) of eastern oysters (Lauckner, 1983). Prevalence and intensity vary considerably with local ecological conditions, but there is a general tendency for infection to be more severe on the south and south-east coasts. Infection rarely causes mortalities and infected oysters can be marketed. However, mud blisters may interfere with shucking and this reduces the commercial value of oysters to be served on the half-shell. Similar conditions caused by unidentified species of *Polydora* were observed in Pacific oysters cultured on the south coast of Brazil and in Baja California, Mexico (Caceres-Martinez *et al*., 1998).

In British Columbia, Canada, stunting and high mortalities caused by high intensities of *P. websteri* (burrows too numerous and interwoven to count in shells of dead scallops) have precluded the culture of introduced Japanese scallops in a few localities (Bower, 1990). However, *P. websteri* only occurred in low intensities (fewer than ten per shell) and had no apparent effect on Pacific oysters and giant rock scallops (*Crassedoma giganteum*) cultured in the same localities (S.M. Bower, unpublished data).

In southern Australia, five species of polydorid polychaetes (*Polydora haswelli*, *Polydora hoplura*, *P. websteri*, *Boccardia chilensis* and *Boccardia polybranchia*) were observed in up to 95% of blue mussels. Although the intensity of infection was generally low, about 15% of the blue mussels from two localities had serious shell damage

attributed to polydora. The most heavily infested blue mussels were from bottom samples (Pregenzer, 1983). Also, spionid polychaete infestations along the east coast of Australia caused Sydney rock oyster aquaculture to change from bottom culture to an intertidal stick-and-tray culture system (Anderson, 1990; Handley, 1997).

Phylum Trematoda

Numerous species of digenean trematodes have been described from various shellfish worldwide. In general, the trematodes that cause the greatest economic impact are species in the families Bucephalidae and Fellodistomidae that utilize bivalves as primary hosts. In such instances, miracidia are infective to bivalves and the larval trematode life stages of sporocyst and development of cercariae occur within the tissues of the bivalve. Four cases in which trematodes from other families were reported to cause pathology are noted.

Family Bucephalidae

Introduction

Numerous species of Bucephalidae (suborder Gasterostomata) have been described from marine and freshwater fishes and the larval forms have been reported from bivalves worldwide. However, few experimental life cycle studies have been conducted. Thus, the taxonomy of many larval Bucephalidae in bivalves remains obscure (Lauckner, 1983). For simplicity, the Bucephalidae will be considered as a group, with examples of certain species presented where appropriate.

Host range and economic significance

Larval bucephalids infecting commercially important scallops, oysters and mussels are possibly the most deleterious metazoan parasites of marine bivalves (Lauckner, 1983). Examples have been reported from various locations: (i) scallops (*Pecten alba*) from Bass Strait, Australia, parasitized by *Bucephalus* sp. (prevalence of 31%) were castrated and had significant adductor muscle (only part of this scallop that is marketed) atrophy (Sanders and Lester, 1981); (ii) *Bucephalus longicornutus* caused castration and significant mortalities of infected dredge oyster (*O. chilensis* (= *lutaria*)) under laboratory conditions with suspected impact on wild stocks in New Zealand (Millar, 1963; Howell, 1967); and (iii) weakness and gaping caused by *Prosorhynchus squamatus* (Fig. 17.10) in blue mussels from northwestern Europe, Britain, Iceland and the White Sea, Russia, reduced product value during shipping and marketing (Coustau *et al.*, 1990). In 1997, *P. squamatus* was encountered for the first time in mussels from Atlantic Canada and a similar-looking parasite was detected in a few mussels from the Pacific coast of Canada. Surprisingly, parasitic castration of blue mussels caused by *P. squamatus* (Coustau *et al.*, 1993) was

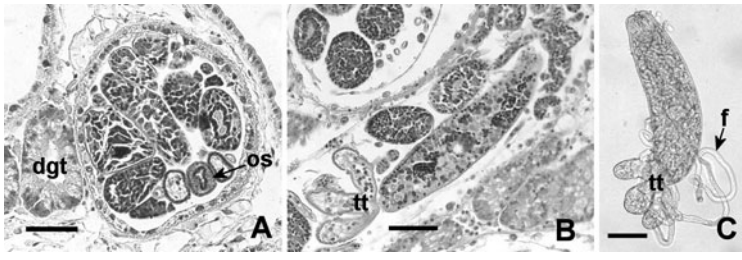


Fig. 17.10. Histological images (A and B) and a wet mount (C) of *Prosorhynchus squamatus* from *Mytilus edulis* in Nova Scotia, Canada (courtesy of S.E. McGladdery). A. Anterior end of sporocyst sectioned through oral sucker (os) adjacent to digestive-gland tubule (dgt). B. Sporocyst containing cercaria sectioned through the trilobate tail (tt). C. Cercaria with trilobate tail (tt) and curled furcae (f). All bars = 50 µm.

once thought to be beneficial for blue mussel culture, because parasitized blue mussels do not spawn and appear to remain in good condition during the summer spawning season. However, consumption of trematode-infested molluscs may be hazardous to humans, due to accumulation of toxic metabolites (butyric and other short-chain fatty acids) resulting from degeneration of the host's neutral fats by parasite-secreted enzymes (Cheng, 1967; Lauckner, 1983).

Morphology and life cycle

Bucephalids have fairly uniform life-cycle patterns. Sporocysts (Fig. 17.10A) and cercariae (Fig. 17.10B, C) occur in bivalves. Metacercariae occur in various parts of the central and peripheral nervous systems or in internal organs and musculature of teleost fish, and adults inhabit the alimentary tract of piscivorous fish (Lauckner, 1983). In bivalves, the large, dichotomously branching sporocyst forms a dense interwoven network, which infiltrates practically every organ, especially the gonad. Infection is terminal following growth into and occlusion of the haemal sinuses and the gradual destruction and replacement of molluscan tissue by the sporocyst. Prevalence of infection usually increases with bivalve age (Matthews, 1974). Cercariae (Fig. 17.10C), often several hundred at a time, are forcibly discharged through the bivalve's exhalant siphon. Although they are not active swimmers, transmission to the intermediate host is aided by the long, extendable and retractile furcae (Matthews, 1974; Lauckner, 1983).

Host–parasite relationships

Bucephalid sporocysts and cercariae cause castration of infected bivalves, tissue necrosis and debilitation, expressed as a significant reduction in tolerance of environmental stress (Lauckner, 1983). Despite the severe pathology associated with *Bucephalus* sp. infection in eastern oysters, there is usually little host response to the parasite, but massive biochemical alterations have been observed (Lauckner, 1983).

Prevention and control

Haplosporidian hyperparasites have been described from *Bucephalus* sp. parasitic in eastern oysters (Lauckner, 1983) and from *B. longicornutus* parasitic in dredge oysters (Howell, 1967). Although both hyperparasites are pathogenic for the bucephalids, Howell (1967) concluded that ecological conditions as well as the difficulty of collecting large numbers of infective spores precluded the effective use of the hyperparasites as biological controls.

Coustau *et al.* (1990) showed that blue mussels are more susceptible to *P. squamatus* than hybrids of blue mussels and 'gallo' mussels and suggested that it may be possible to select for a mussel stock that is resistant to this parasite.

Family Fellodistomidae

Although numerous species of this family parasitize many marine pelecypods as primary hosts and secondary hosts worldwide (Lauckner, 1983; Wolf *et al.*, 1987), *Proctoeces maculatus*, which infects blue mussels as well as other mollusca, has the greatest economic impact. Thus, this section presents information only on *P. maculatus*.

P. maculatus from shellfish and fin fish appear in the literature under a variety of synonyms, and life stages have been described from a wide variety of bivalves and gastropods (Bray, 1983). Metacercariae occur in various mollusca (including species of Amphineura, Gastropoda, Cephalopoda and Lamellibranchiata), Polychaeta (Annelida) and Echinoidea (Echinodermata). Adults have been reported in mollusc-eating fishes (mainly labrids and sparids) in tropical and subtropical areas, as well as in some Gastropoda, Lamellibranchiata and Polychaeta. However, sporocysts have only been reported from blue mussels, 'gallo' mussels, and hooked mussels (*Ischadium recurvum*). The wide host tolerance, global distribution in tropical and temperate marine waters and morphometric variability led Lauckner (1983) to speculate that more than one species of trematode may have been included

in *P. maculatus*. Thus, this species (group) requires further study, using biochemical and DNA analysis as well as life cycle studies in the laboratory.

P. maculatus was reported in up to 46% of blue mussels and 'gallo' mussels on both sides of the North Atlantic Ocean and in the Mediterranean and Black Seas (Lauckner, 1983). In 'gallo' mussels from the Black Sea, up to 28,000 sporocysts per mussel, comprising 20% of the biomass of the soft tissues, were observed (Machkevski, 1985). In Italy, extensive mortalities in cultured mussels were attributed to this parasite, which was thought to have been introduced via a depuration plant located nearby (Munford *et al.*, 1981).

In mussels, sporocysts of *P. maculatus* usually occur in the vascular system of the mantle (Lauckner, 1983). Infection causes an alteration in haemolymph components, a sharp decrease in energy stores, a reduction in growth rate and weakness in respect of valve closure and attachment to the substrate (Mulvey and Feng, 1981; Machkevski, 1985, 1988). In heavily infected mussels, sporocysts developing in the mantle can seriously reduce the glycogen content of the tissues and efficiency of the circulatory system. This results in disturbances to gametogenesis and possibly castration and death (Mulvey and Feng, 1981; Machkevski and Shchepkina, 1985; Feng, 1988). Mussels may also serve as a final host for *P. maculatus* (Lauckner, 1983). Progenetic development (Fig. 17.11) represents a mechanism by which *P. maculatus* could become established in new localities as a result of moving infected stocks. *P. maculatus* probably represents a threat to mussel culture worldwide. However, Lauckner (1983) indicated that the hazard would be minimal due to the supposed narrow range of ecological conditions under which *P. maculatus* is capable of disseminating.

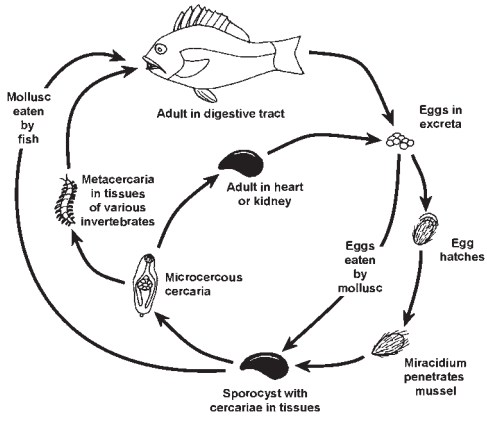


Fig. 17.11. Life-cycle alternatives of the Trematoda *Proctoeces maculatus*. The occurrence of a progenetic cycle (adult stages of *P. maculatus* in the heart or kidney of the blue mussel (*Mytilus edulis*)) eliminates the requirement of a final (definitive) fish host for the completion of the life cycle.

Other pathogenic Trematoda

In addition to the Bucephalidae and *P. maculatus*, several other digenetic trematodes have been reported as pathogens of shellfish. Some of the more prominent examples are as follows:

1. In the North Sea and adjacent areas, reduced byssal production and impaired shell cleaning were reported in young blue mussels infected with metacercariae of the bird trematode *Himasthla elongata* (family Echinostomatidae) (Lauckner, 1984). Also, *H. elongata* and *Renicola roscovita* were thought to have an impact on European cockle populations on the German North Sea coast (Lauckner, 1983).

2. On the west coast of Sweden, high intensities of *Cercaria cerastodermae* (family Monorchiidae) in about 20% of the cockles (*C.* (= *Cerastoderma*) *edule*) led to severe tissue damage, impairment of burrowing and eventual mortalities (Jonsson and André, 1992).

3. Several species of gymnophallid metacercariae, which occur between the mantle and shell of various lamellibranchs on both sides of the North Atlantic Ocean, are reported to cause soft-tissue pathology, induction of pearl formation and shell deformities (Lauckner, 1983).

As aquaculture operations expand and diversify, diseases caused by various

trematodes will probably be encountered. However, the requirement of at least two different hosts for completing the life cycle in most species renders these parasites vulnerable to control once the life cycles have been identified. Aquaculture practices alone may be sufficient to create an unfavourable environment for the completion of a trematode's life cycle, as illustrated by the reduced prevalence of *R. roscovita* in farmed (4 to 12%) as opposed to natural populations (96 to 100%) of blue mussels from the west coast of Sweden (Svärdh and Thulin, 1985).

Phylum Cestoda

Metacestodes (larval cestodes) have been reported from a wide variety of aquatic invertebrates. Among marketed shellfish, metacestode infections are economically insignificant. Nevertheless, there are a few isolated instances of high prevalences and intensities of metacestodes in bivalves and crustacea from various subtropical and tropical areas of the world (Lauckner, 1983; Sparks, 1985; Sindermann, 1990). Metacestodes of *Echeneibothrium* spp. were associated with unusual behaviour of Pacific littleneck clams (*P.* (= *Venerupis*) *staminea*) and fringed littleneck clams (*Protothaca laciniata*) in California (Warner and Katkansky, 1969) and caused histopathology and gonad atrophy in Atlantic calico scallops (*A. gibbus*) in North Carolina (Singhas *et al.*, 1993). In most cases, the final hosts of the cestodes are fishes, mainly elasmobranchs.

Phylum Nematoda

Nematodes are uncommon as parasites of shellfish (Lauckner, 1983; Sindermann, 1990). However, the exceptions are all larval stages and include the following:

1. Various species of the gnathostomid genus *Echinocephalus* from oysters, scallops and abalone from tropical and subtropical marine waters. Although the pathology in the bivalve hosts is minimal, there is concern that at least some species may have public health significance as potential invaders of the human digestive tract. The species (*Echinocephalus pseudouncinatus*) in pink abalone (*Haliotis corrugata*) from California causes blisters and weakens the foot as a holdfast organ in heavily infected specimens (Sindermann, 1990).

2. An ascaridoid *Sulcascaris sulcata* is widespread in warm seas and has a considerable host range, including scallops and clams (Lauckner, 1983; Sindermann, 1990). Although *S. sulcata* is a minor pathogen for its hosts, significant economic impact occurred on the east coast of North America where a haplosporidian hyperparasite (*Urosporidium spisuli*) caused the usually white to yellowish coloured worm to become dark brown. The epizootic spread of the hyperparasite in *S. sulcata* parasitizing Atlantic surf clams (*Spisula solidissima*) in the mid-1970s caused considerable economic concern for aesthetic reasons (Payne *et al.*, 1980).

3. *Angiostrongylus cantonensis*, the rat lungworm that causes human eosinophilic meningoencephalitis in parts of Asia, can utilize eastern oysters and quahogs (*M. mercenaria*) as aberrant intermediate hosts under experimental conditions (Sparks, 1985). These findings could be significant for some of the Pacific Islands where the rat lungworm occurs and oysters and clams may be eaten raw or poorly cooked (Lauckner, 1983).

4. The 'codworm' *Phocanema decipiens* in the North Atlantic has been observed in blue mussels and softshell clams (*M. arenaria*), which may serve as paratenic hosts for this parasite (Lauckner, 1983).

Phylum Arthropoda

The pathogenic arthropods all belong to the class Crustacea (subclass Copepoda, mainly in the order Cyclopoida and subclass Malacostraca, order Isopoda). Because the economic significance of all species is either disputable or confined to small local areas, these pathogens are only briefly mentioned.

Subclass Copepoda

The cycloid copepods presumed to cause the most significant mortalities among shellfish belong to the genus *Mytilicola*. These copepods have a direct life cycle and reside in the intestinal tract of a wide variety of bivalves (Dare, 1982; Gee and Davey, 1986). Prevalence and intensity of *Mytilicola intestinalis* in mussels in Europe can be high. For example, in Cornwall, UK, the prevalence in mussels from some localities only fell below 90% during the early summer months and intensity of infection often exceeded 30 copepods per mussel (Davey, 1989). Several workers concluded that some of the periodic mass mortalities in cultured mussels in Europe were attributable to *M. intestinalis* (Sparks, 1985; Blateau *et al.*, 1990). However, these conclusions: (i) were not substantiated by statistical analysis; (ii) were not supported by experimental evidence; and (iii) did not rule out the possibility that microscopic pathogens were responsible for the mortalities (Lauckner, 1983). From the results of a 10-year study conducted in Cornwall, England, Davey (1989) concluded that *M. intestinalis* is not a harmful parasite. Nevertheless, more work is required before the pest status of *M. intestinalis* can be fully appreciated, especially in respect of its synergistic relations with other pathogens and/or pollutants (Davey and Gee, 1988).

A parasitic copepod, *Pectenophilus ornatus*, of unknown taxonomic affinity and originally thought to be a species of rhizocephalan in the subclass Cirripedia, is considered a serious pest of commercial scallop production in Japan (Nagasawa *et al.*, 1991). The bright yellowish or orange female (up to 8 mm wide, consisting mainly of a brood pouch with no appendages) attaches to the gills and feeds on the haemolymph of commercially valuable scallops (*P. yessoensis* and *Chlamys* spp.). Heavy intensities of infection (greater than 20 *P. ornatus* per scallop) have detrimental effects on the condition of cultured scallops and the parasite also reduces market acceptability (Nagasawa and Nagata, 1992).

Subclass Malacostraca

Members of the family Bopyridae within the order Isopoda are common parasites of the branchial chamber of many species of shrimp worldwide. Infected shrimp are conspicuous due to the protruding lump on the lateral aspect of the carapace of the cephalothorax caused by the presence of the bopyrid (Sparks, 1985). Although the prevalence of bopyrids is usually low (< 5%), a few instances of high prevalences and associated pathology have been noted. Japanese red prawns (*Penaeopsis akayebi*) were frequently infected (up to 70%) with *Epipenaeon japonicus*, with associated gonad reduction or castration in some male prawns (Sindermann, 1990). In the Gulf of Carpentaria, Australia, the bopyrid *Epipenaeon ingens* infects up to 25% of the grooved tiger prawns (*Penaeus semisulcatus*), which it castrates and whose growth and geographical distribution it alters in comparison with those of uninfected prawns (Somers and Kirkwood, 1991).

Conclusions

A wide variety of parasites have been identified as causing significant economic losses in shellfish production worldwide. Many of these pathogens have the potential of causing significant losses either in endemic areas or if they inadvertently become established in other areas. In the past, transplants of commercial shellfish have been notorious for the accidental introduction of associated parasites (Sindermann, 1990, 1993). In order to avoid future disasters, all movements of shellfish must be conducted with caution. Equally essential is the acquisition of information on agents of disease, including parasites, such that risks associated with impending movements and aquaculture practices can be accurately assessed. This information should also prove useful for treating or controlling a disease in the event that an accidental introduction occurs.

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18 The Immune System of Fish

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Introduction

Vertebrates are distinguished from invertebrates by an internal skeleton of cartilage or bone. The subphylum Vertebrata includes the jawless fish (Agnatha), such as hagfish and lamprey, the Placodermi, which are the earliest group of jawed fish, the cartilaginous fish (Chondrichthyes), such as sharks and rays, the bony fish (Osteichthyes), such as sturgeon, trout, carp and *Tilapia*, the amphibians, the reptiles, the birds and the mammals. Fish are the oldest animal group with an immune system showing clear similarities with the defence systems of mammals and birds. The defence system is organized on two levels: (i) an innate (inborn) defence system; and (ii) an acquired (adaptive) defence system. Protection based upon innate immunity has a general character and does not depend upon recognition of distinctive molecular structures of the invading organisms. Moreover, this component of the system can act rapidly (minutes to hours) and is relatively temperature independent. The acquired component is characterized by specific antigen recognition and memory development. Specific responses usually require between weeks and months to build up adequate protection against pathogens. Moreover, the appearance of specific receptors, such as immunoglobulin (Ig) as well as other members of the 'Ig superfamily', is observed for the first time in this animal group. Classic Ig molecules are lacking in the invertebrate phyla. This chapter provides a general overview of the defence mechanisms in fish. Most data are derived from bony fish, but some interesting differences with cartilaginous or jawless fish will be discussed. It is known from fossil remains that the earliest fish appeared some 350–400 million years ago. This implies that unique specializations in the defence systems of certain fish species may have developed over the years.

Innate Defence Mechanisms

Epithelial barriers

The first line of defence includes structures that form stable physical and/or chemical barriers against invading microorganisms. The epithelial surfaces (e.g. skin, gills and gut) are examples of these barriers. It is of prime importance for the fish to maintain the integrity of its covering epithelia because they are important in defence and for osmoregulation. Hence wound healing is a remarkably rapid process in fish. Normal epithelia are covered by a mucus layer, which is secreted by goblet cells. The most

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important function of mucus is to prevent the attachment of bacteria, fungi, parasites and viruses to epithelial surfaces. Moreover, mucus also contains antimicrobial activities. The genes for antibacterial peptides, such as pleurocidin, have been cloned from the winter flounder (*Pleuronestes americanus* Walbaum); the peptide is found in skin and intestine. The gene is first expressed at 13 days post-hatch, suggesting that this factor plays an important role in the early life of the fish before acquired immunity can develop (Douglas *et al.*, 2001).

Lectins

Lectins (or natural agglutinins) in fish can be detected as natural precipitins or agglutinins. They are usually cross-linking carbohydrate moieties on the surface of xenogeneic erythrocytes or bacteria. They are probably important in neutralizing bacterial components (e.g. exotoxins) or in immobilizing microorganisms and hence will facilitate phagocytosis (Fletcher, 1982). Fish lectins are not structurally related to Ig, but resemble plant or invertebrate agglutinins. Fish lectins have been found in coho salmon (*Oncorhynchus kisutch*) eggs (Yousif *et al.*, 1995), rainbow trout (*Oncorhynchus mykiss*) serum (Hoover *et al.*, 1998) and mucus of ayu (*Plecoglossus altivelis*) (Itami *et al.*, 1993). A mannose-binding lectin, isolated from Atlantic salmon (*Salmo salar*) serum, has been shown to opsonize a virulent *Aeromonas salmonicida* strain and lectin-coated bacteria can induce macrophages to kill them (Ottinger *et al.*, 1999).

Lysozyme

This enzyme is found in fish mucus, serum and eggs (Ellis, 1999) and is able to digest the peptidoglycan layer of bacterial cell walls. Lysozyme is produced by macrophages and neutrophilic granulocytes (Murray and Fletcher, 1976) and is bactericidal even for serious pathogens such as *Aeromonas salmonicida* and *Aeromonas hydrophila* (Ellis, 1999).

C-reactive protein

In teleost fish, C-reactive protein (CRP) is a serum component that increases rapidly upon exposure to bacterial endotoxins (Ingram, 1980) or experimental infection with bacterial pathogens (Murai *et al.*, 1990). CRP reacts with polysaccharide structures at the cell surface of microorganisms. It has lectin-like properties and can act as an opsonin to enhance phagocytosis or to activate the complement system after binding to the bacterium *Vibrio anguillarum* (Nakanishi *et al.*, 1991). CRP from rainbow trout (*O. mykiss*) was isolated and characterized as a 66 kDa glycoprotein that contains two protein subunits (Murai *et al.*, 1990).

Interferon

Interferon (IFN) is a cytokine that is produced by many cell types in response to viral infections. It increases the resistance of host cells to different viruses by inducing the expression of proteins that inhibit the translation of viral mRNA. IFN in teleosts is species-specific, e.g. IFN produced by rainbow trout does not protect cyprinid cells *in vitro*. *In vivo* synthesis of IFN during a viral infection peaks after 2–3 days and usually precedes the virus-neutralizing effects of circulating antibodies, which appear 1 or 2 weeks later (De Kinkelin *et al.*, 1977). It is interesting that type I and type II IFN can be distinguished in rainbow trout based upon acid stability (pH 2) and relative temperature resistance (60°C) (Secombes, 1991). Today IFN activity has been demonstrated in a number of fish species, e.g. rainbow trout, Atlantic salmon and halibut (*Hippoglossus hippoglossus* L.) (Robertsen, 1999).

Complement

The complement system consists of a group of protein and non-protein components that are involved in both innate defence mechanisms and specific adaptive immunity. The complement system can be activated along two major routes: (i) the classical pathway,

which is stimulated by antigen-antibody complexes; and (ii) the alternative pathway, which is started by contact with microbial cell-wall polysaccharides (lipopolysaccharide (LPS), zymosan) and rabbit erythrocytes. In both cases, the activation results in the opsonization and/or lysis of foreign cells. Yano (1996) shows that most classes of fishes, including jawless fishes, possess a lytic complement system. Nanoka *et al.* (1981, 1984) isolated C3 and C5 from rainbow trout plasma. Yano (1996) has shown that C1–C9 are present in carp (*Cyprinus carpio*) plasma. These studies suggest that all the mammalian complement factors (C1–C9, B, D) are present in fish blood and that both pathways operate in fish. Woo (1992) has shown that the alternative pathway is the protective mechanism against haemoflagellate parasites (*Cryptobia*) in naïve fish. The classical pathway turned out to be important in acquired immunity after survival of parasitic infections or against bacteria, such as *V. anguillarum* (Boesen *et al.*, 1999). Yano (1996) suggested that the alternative pathway in fish is more active than in mammals. However, this may also depend on water temperature (season), age or condition of the animals.

Inflammation

Inflammation is a local reaction upon tissue damage, e.g. caused by invading microorganisms. The initiation of inflammation is highly complex and multifactorial. Many soluble factors (clotting system, kinin system, complement system) and cells (thrombocytes, granulocytes and macrophages) play a role (Secombes, 1996). Characteristics of the process include local vasodilatation and an influx of granulocytes and monocytes/macrophages. The massive influx of cells confers some degree of protection by 'walling off' an infected area from the rest of the body. Histopathological studies in fish provide evidence for inflammatory responses in bacterial, viral, fungal, protozoan and metazoan parasitic infections (Roberts, 1978). Acute inflammation responses in bony fish are comparable with those in mammals (Finn and Nielsen, 1971). Granulocyte infiltration appears 12–24 h after injection of bacteria or Freund's complete adjuvant in rainbow trout. The infiltrating cells (granulocytes and macrophages) increase in numbers till day 2–4. The macrophages are stimulated to secrete interleukin-1 (IL-1) and eicosanoids, which attract and activate other leukocytes, including lymphocytes (Secombes *et al.*, 1999). These events can be seen as an example of the interaction between the innate and acquired immune systems in fish.

Phagocytic cells

Macrophages and neutrophilic granulocytes in fish are the principal phagocytic cells (Secombes and Fletcher, 1992; Verburg-Van Kemenade *et al.*, 1994). These cells recognize evolutionarily conserved epitopes present on microorganisms, using so-called 'pattern recognition receptors' (PRRs). Different types of PRRs have been described for fish, including Toll-like receptors (Bricknell and Dalmo, 2005). Upon stimulation through PRRs, these cells phagocytize antigenic material and/or exert cytotoxic activity. The killing of intracellular or extracellular pathogens is based upon the release of a number of oxygen radical species and nitric oxide (NO) (Campos-Perez *et al.*, 2000; Saeij *et al.*, 2002). Phagocytosis of antigenic material by macrophages is not only an activity of the non-specific innate defence system but is also the initial step in the specific adaptive immune response (see Fig. 18.4). As in mammals, we are probably dealing with subpopulations of mononuclear phagocytes that differ in function. In this respect, it is interesting to note that macrophages from immune fish are more active in phagocytosis than those from control animals. This is probably due to opsonization of the antigen by antibodies or to metabolic activation of the macrophages (Griffin, 1983). Sakai (1984) has even suggested that salmonid macrophages have Fc and C3 receptors on their surface facilitating the binding and subsequent phagocytosis of opsonized material. Most macrophages from the hind

gut of carp bind purified Ig, which is an indication for Fc receptors on these cells (Koumans-Van Diepen *et al.*, 1994). This is another example of cooperation between the innate immune system (phagocytes) and the acquired immune system (Ig molecules).

Non-specific cytotoxic cells

Studies in channel catfish (*Ictalurus punctatus*) reveal the presence of non-specific cytotoxic cells (NCC) in these bony fish (Graves *et al.*, 1984). The monocyte-like NCC show a clear *in vitro* lytic activity against certain transformed mammalian cell lines. NCC have been shown in the blood, spleen and head kidney of several teleost fishes (Manning, 1994). These cells are the teleost equivalent of mammalian natural killer (NK) cells (Evans and Jaso-Friedmann, 1992). They are probably involved in killing protozoan parasites and virus-infected cells.

Lymphoid Cells and Organs

Lymphocytes are cells essential to the acquired immune response because they express the Ig and T-cell receptor (TCR) molecules as antigen-specific recognition units. Lymphocyte heterogeneity (T- and B-cells) in fishes has been demonstrated in hapten-carrier studies (Stolen and Mäkela, 1975), by using monoclonal antibodies (Secombes *et al.*, 1983) and by functional tests for cell cooperation (Miller *et al.*, 1987). The main lymphoid organs in cartilaginous fish (Fig. 18.1A) are the thymus, Leydig organ, epigonal organ, kidney and spleen (Fänge, 1982). There are also indications for substantial gut-associated tissue in these animals (Tomonaga *et al.*, 1986). Teleosts do not have a Leydig organ or epigonal tissue. However, the thymus, head kidney (pronephros), trunk kidney (mesonephros), spleen and intestine contain high numbers of leucocytes (Fänge, 1982; Rombout *et al.*, 1986, 1989a; Fig. 18.1B). Considerable numbers of leucocytes are also found in the skin and gills (Iger and Wendelaar Bonga, 1994), which indicates that a mucosal immune system is well developed in fish. Bone marrow, the bursa of Fabricius, Peyer's patches and lymph nodes, which are present in birds and/or mammals, are not found in fish. Most observations indicate that the spleen of bony fish is an erythropoietic and secondary lymphoid organ (Van Muiswinkel *et al.*, 1991), whereas the thymus is a primary lymphoid organ, mainly involved in T-cell differentiation (Zapata *et al.*, 1996). The kidney (pronephros and mesonephros) is probably analogous to mammalian bone marrow (Lamers, 1985; Zapata *et al.*, 1996). Therefore, it may function as a primary organ (blood-cell formation, B-lymphocyte differentiation) but also as a secondary organ (memory-cell and plasma-cell development).

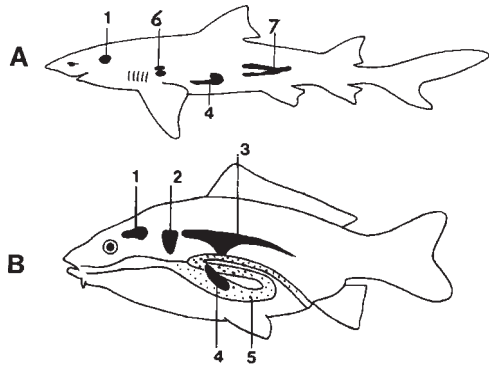


Fig. 18.1. The main lymphoid organs in cartilaginous fish (A) and bony fish (B). 1, thymus; 2, head kidney (pronephros); 3, trunk kidney (mesonephros); 4, spleen; 5, intestine; 6, Leydig organ; 7, epigonal organ. (A from Fänge, 1982, and B from Lamers, 1985, slightly modified.)

Antigen Recognition and Presentation

Ig structure

The major Ig in bony fish consists of heavy (H) chains and light (L) chains and hence is similar to that in other vertebrates. The native Ig molecule (Fig. 18.2) of fish is usually a tetramer with four structural units (H2L2)4. It contains $4 \times 2 = 8$ antigen-binding sites and has a molecular weight between 600 and 900 kDa (Pilström *et al.*, 1998). The molecule is usually called IgM because of

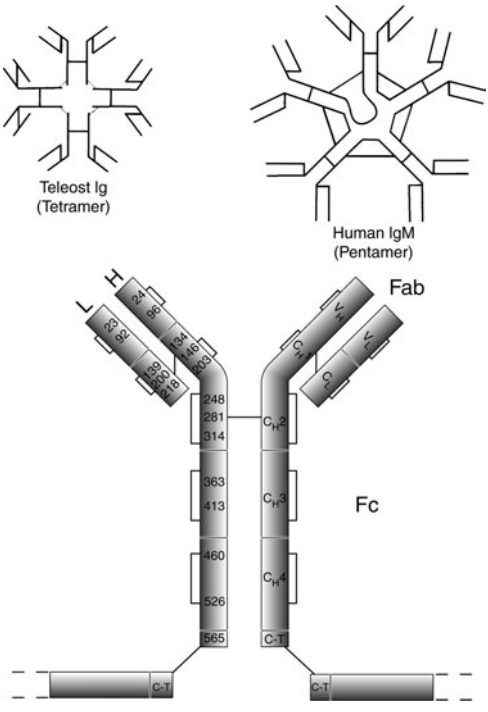


Fig. 18.2. Schematic representation of secreted teleost and human IgM. The teleost Ig molecule (upper left) is composed of equimolar amounts of heavy (H) and light (L) chains. These are assembled to produce a tetrameric molecule, as opposed to the pentameric human IgM (upper right). Each monomer (lower centre) possesses five domain heavy chains ($V_H + C_H1–4$) and two domain light chains ($V_L + C_L$). The brackets and lines depict potential intradomain and interchain disulphides. Numerals refer to positions of cysteine residues. Fab, fraction antigen-binding; Fc, fraction cristallizable; C-T, C-terminal tailpiece. (From Kaattari and Piganelli, 1996, with permission.)

its high molecular weight and polymeric structure. However, the mammalian IgM is a pentamer with five structural units (H2L2)5. The amino acid sequence of the four constant domains in the H chain (CH) shows 24% homology with the mouse μ chain (Ghaffari and Lobb, 1989). Interestingly enough, the variable heavy (VH) genes of channel catfish (*I. punctatus*) (Ghaffari and Lobb, 1989) or rainbow trout (*O. mykiss*) (Matsunaga *et al.*, 1990) show much higher amino acid sequence identity (45–60%) with mammals than the C domain genes. In other words, the antigen-binding Fab part of the Ig molecule is better conserved in evolution than the so-called constant part of the same molecule.

Ig isotypes

Most authors agree that cartilaginous fishes (e.g. sharks) have both pentameric and monomeric serum Ig (Frommel *et al.*, 1971). In both Ig types, the H chain corresponds with the mammalian μ chain. Although low-molecular-weight Ig has been reported in teleosts (Lobb and Clem, 1981a), no structural or functional equivalency to mammalian IgG has been found (Wilson and Warr, 1992). On the other hand, convincing evidence for the existence of IgM isotypes was found in molecular studies on the C genes of sharks (Kokubu *et al.*, 1987) and in biochemical studies on the Ig H chains in teleosts (Lobb and Olson, 1988). Moreover, the existence of a separate mucosal Ig (sub)class in bile and mucus of sheepshead (*Archosarchus probatocephalus*) (Lobb and Clem, 1981b) and carp (Rombout *et al.*, 1993) has been described. Interestingly enough, a novel chimeric Ig heavy chain sharing similarities with IgD has been found in channel catfish (Wilson *et al.*, 1997) and Atlantic salmon (Hordvik *et al.*, 1999). At least two distinct light-chain types (F and G) were found in channel catfish. This distinction was based upon differences in molecular weight, antigenic structure and peptide mapping (Lobb *et al.*, 1984).

Antibody repertoire

The mechanism by which antibody diversity is generated in mammals is well known and involves recombination of various Ig gene segments (V, D, J and C) during differentiation from haemopoietic stem cell to B lymphocyte (Tonegawa, 1983). Studies in sharks (*Heterodontus*) show a remarkable Ig gene organization (Hinds and Litman, 1986; Hinds-Frey *et al.*, 1993). In contrast to mammals, we see high numbers (≥ 200) of closely linked clusters of V, D, J and C segments in genomic DNA of these marine

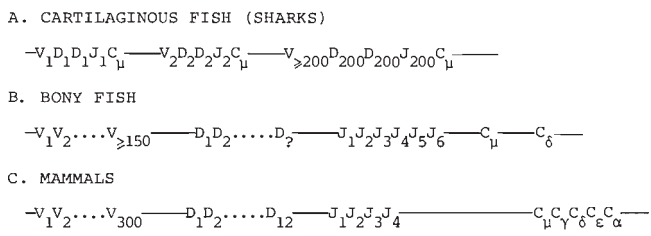


Fig. 18.3. Schematic presentation of Ig heavy-chain loci in germline DNA of cartilaginous fish, bony fish and mammals. V, variable gene segment; D, diversity gene segment; J, joining gene segment; C, constant gene segment. The V, D, J and C gene segments are recombined during B-cell development in bony fish and mammals. In cartilaginous fish this process has taken place already at the early germ-line stage.

fish (Fig. 18.3). Inter-cluster rearrangements are not thought to occur during B-cell development, which will limit antibody diversity in these 'primitive' fish. In bony fish, the organization of V, D, J and C segments is almost identical to that in mammals (Fig. 18.3). There are at least 150 V, two or more D, at least six J and a single Cμ region (Matsunaga and Törmänen 1990; Ghaffari and Lobb, 1991; Marchalonis *et al.*, 1998), which means that there are numerous possibilities for recombination during B-cell development in bony fish. However, an Ig class switch (change of H chain) has not been observed. This may explain why somatic hypermutation and the subsequent selection of high-affinity B-cell clones is restricted in bony fish (Du Pasquier, 1982; Kaattari, 1992).

T-cell receptors

We know from molecular studies in mammals that Ig and TCRs are related protein molecules that are characterized by an extreme variation in antigen-binding sites based upon rearrangements of V, J, C and sometimes D region gene segments in the genome of early B- or T-cells. In mammals, two antigen-specific TCR types (αβ and γδ) are present. TCR-α and β chain gene sequences have been described in teleosts (Partula *et al.*, 1996; Hordvik *et al.*, 1999; Wilson *et al.*, 1998). The isolation of TCR-γδ chains in cartilaginous fish (Rast *et al.*, 1995), as well as CD3-like polypeptides in sturgeons (*Acipenser rhutenus*) (B.Y. Alabyev, personal communication), suggests the existence of different and functional TCR–CD3 complex types in fish.

Major histocompatibility complex

Studies in mammals and birds have shown that the gene products of the major histocompatibility complex (MHC) play a key role in the regulation of the immune response (Klein and Horejsi, 1999). The MHC incorporates a group of closely linked genes, which show a high degree of polymorphism. They code for membrane glycoproteins, which are divided into class I and II. Class I molecules are present in all nucleated cells, whereas class II molecules are more or less restricted to cells of the immune system, i.e. lymphocytes and antigen-presenting macrophages. These MHC molecules also play an important role in the development of the T-cell repertoire (self-tolerance) and in antigen presentation (Fig. 18.4). In the last 15 years, an impressive amount of information has became available on class I and class II loci in bony fish. Using the polymerase chain reaction, Hashimoto *et al.* (1990) were able to demonstrate that the genome of carp contains nucleotide sequences that show considerable homology with MHC class I and II sequences in humans and mice. Subsequently, classical class I and class II genes have been found in zebrafish (*Danio rerio*) (Bingulac-Popovic *et al.*, 1997), carp (Stet *et al.*, 1993, 1997), rainbow trout (Hansen *et al.*, 1999), medaka (*Oryzias latipes*) (Naruse *et al.*, 2000) and

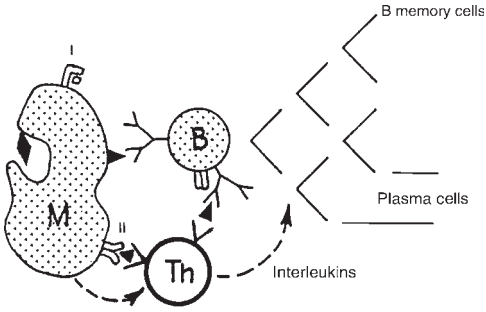


Fig. 18.4. Schematic diagram of cell interactions during the humoral response in vertebrates. Specialized macrophages (M) are able to trap and process foreign molecules or particles, i.e. antigen (◆). These macrophages will present relatively small antigenic determinants (▶) associated with MHC class II molecules to lymphoid cells. Subsequently, specific T-helper cells (Th) are activated by interaction with the antigenic determinant and factors secreted by the macrophages (interleukin-1). The activated Th-cells stimulate the differentiation and proliferation of effector cells as B-lymphocytes (B) and cytotoxic T-cells (not shown in this scheme) by secretion of different factors (e.g. interleukin-2). Depending on the circumstances, B-cells will develop into long-lived B memory cells or short-lived plasma cells. These plasma cells secrete huge amounts of specific antibodies (immunoglobulins), which will bind or kill invading microorganisms, showing the corresponding determinant. <, Proliferation; →, interleukins; I, MHC class I molecule (immunoglobulin); II, MHC class II molecule; >-, B-cell receptor; V, cell receptor.

Atlantic salmon (Grimholt *et al.*, 2002). An important observation in all bony fish studied is the fact that – in contrast to the situation in mammals – the class I loci are not linked to class II loci, but are found on different linkage groups. In other words, a classical MHC does not exist in fish. Several suggestions have been made to explain the absence of linkage between these class I and class II genes (Kuroda *et al.*, 2002). For example, duplication of parts of a chromosome bearing the MHC could have taken place, followed by translocation and subsequent loss of certain loci, or class II loci were translocated from a prototype MHC to other chromosomes in the ancestor of fish. Stet *et al.* (2003) suggested that the classical class I and II genes were unlinked in fishes and this could provide an evolutionary advantage. The offspring in mammals are endowed with only four possible MHC genotypes (haplotypes) per family. Local populations will usually show only a small number of MHC haplotypes. This limited diversity becomes risky when environmental circumstances change or new diseases arise. This risk can be counteracted by investing a relative large amount of resources (care, energy) in a relatively small number of offspring. Fish, on the other hand, usually have high numbers of offspring, up to thousands or even millions (McLarney, 1987). Mortality in fishes can be over 80% in the early life history, which can be due to predation but diseases will probably play a role as well. Fish with unlinked major histocompatibility (MH) genes have the ability to endow their offspring with high numbers of genotypes, which will increase the chance that at least some individuals will survive.

The Humoral and Cellular Response

Cell cooperation

The acquired response in fish shows the expected characteristics of specificity and memory. At the start of the humoral response, it takes some time before the first specific antibodies appear in the circulation. This lag phase is needed for antigen processing and cell cooperation between distinct leucocyte populations (accessory cells, B- and T-cells). Accessory cells (monocytes and macrophages) process different antigens and present the processed antigenic determinants in association with MHC class II molecules to lymphocytes (Fig. 18.4). We know from mammalian studies that the TCR on the cell membrane of a T-helper (Th) cell is important for the recognition of the antigenic determinant. Also, other molecules, such as CD3 and CD4, are essential as co-receptors. Activated macrophages secrete IL-1, which is essential for the induction of the response by activating Th cells. Th cells regulate the proliferation and differentiation of B-cells into antibody-secreting

plasma cells by producing IL-2 and other interleukins (Fig. 18.4). Most of these B, Th and accessory cell functions have been verified by using monoclonal antibodies and functional *in vitro* tests for channel catfish (Miller *et al.*, 1985; 1987) or carp (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984).

Cytokines

Surprisingly, the apparently old and conserved cytokine system exhibits low degrees of homology among vertebrate species when its ligands are compared at the level of amino acid sequences (approximately 30% homology between the human and teleost forms of IL-1β). On the other hand, the secondary and tertiary structure of the IL-1 molecule appears to be quite conserved. Secombes *et al.* (1998) have shown that the trout IL-1 sequence can be superimposed on the human crystal structure for IL-1β. It would appear that, in an evolutionary context, the conservation of the three-dimensional structure is more important for cytokine function than its primary sequence. In recent years, a variety of cytokine sequences have been elucidated for several fish species. Fibroblast growth factor (FGF) and some CC and CXC chemokines have been cloned from a number of fish species (Secombes *et al.*, 1999; Laing and Secombes, 2004). Several isoforms of the anti-inflammatory cytokine transforming growth factor-β (TGF-β) have been described for fish and isoforms of the pro-inflammatory cytokines IL-1β and tumour necrosis factor-β (TNF-β) sequences have been published (Secombes *et al.*, 1999). The first teleost sequence for IL-1β was published for rainbow trout by Zou *et al.* (1999), followed by the IL-1β sequence for common carp (Fujiki *et al.*, 2000), sea bass (*Dicentrarchus labrax*) (Scapigliati *et al.*, 2001) and gilthead sea bream (*Sparus aurata*) (Pelegrin *et al.*, 2001). For both rainbow trout (Pleguezuelos *et al.*, 2000) and carp (Engelsma *et al.*, 2001; Huising *et al.*, 2004), a second IL-1β sequence was found. An explanation for the existence of two related but distinct forms may be the tetraploidization event, which occurred independently in the two species during evolution.

Cytokine receptors

In addition to the IL-1β sequences, the IL-1 receptors type I (Holland *et al.*, 2000) and type II (Sangrador-Vegas *et al.*, 2000) were published for rainbow trout. Elegant three-dimensional models of IL-1β and IL-1 receptor type I from rainbow trout and sea bass were predicted by comparison with those available from humans and mice (Scapigliati *et al.*, 2004; Fig. 18.5). The multiple forms of IL-1β and the presence of both types of receptors indicate that the complexity of the IL-1 system in teleost fish is similar to that in mammals.

Cytokine function

A biological role for carp IL-1β is strongly supported by the observation of a transient *in vivo* expression of this interleukin during days 1–4 of *Trypanoplasma borelli* infection (Saeij *et al.*, 2003b). Functional aspects of TNF-α action in fish were demonstrated using human recombinant TNF-α in rainbow trout macrophages (Knight *et al.*, 1998) and assaying for hepatocyte serum amyloid



Fig. 18.5. Molecular complex of rainbow trout interleukin-1β (IL-1) and IL receptor type I (IL-1R). The interaction of IL-1 with its receptor has been simulated on the basis of the experimental structure of the human IL-1β–IL-1R complex. (From Scapigliati *et al.*, 2004, with permission.)

A expression (Jørgensen *et al.*, 2000). TNF-α sequences have been published for Japanese flounder (*Paralichthys olivaceus*) (Hirono *et al.*, 2000), rainbow trout (Laing *et al.*, 2001) and carp (Saeij *et al.*, 2003a). While most teleost cytokine sequences are available, functional information on cytokines in neuroendocrine communication in teleosts is still limited. IL-1β is the best-studied teleost cytokine and it has considerable importance and potency in the communication between the neuroendocrine system and the immune system (see section on Stress).

Humoral immunity

The kinetics of the humoral response in bony fish have been studied in detail (Sailendri and Muthukkaruppan, 1975; Rijkers, 1982; Kaattari and Piganelli, 1996). It is important to realize that, following immunization, the length of the lag phase, exponential phase and decay phase may be influenced by several factors, such as water temperature, type of antigen, antigen dose, route of application, age and species involved (see also sections on memory, vaccination and temperature). Injection of an optimal dose of sheep red blood cells (SRBC) into carp (24°C) evokes peak numbers of antibody (Ab)-forming cells in spleen and kidney after 9–10 days (Rijkers *et al.*, 1980a), but rainbow trout (12–17°C) need 14–15 days for the same response (Chiller *et al.*, 1969). Recent studies in European eel (*Anguilla anguilla* L.) (Esteve-Gassent *et al.*, 2003) kept at 26°C have shown that the antibody response to *Vibrio vulnificus* in mucus is faster (peak days 3–4) than in serum (peak day 7 or later). The graph shown in Fig. 18.6 is a theoretical presentation of the humoral serum response of cyprinid fish to SRBC at 20°C. The first Ab producing plasma cells appear in the spleen and kidney around 1 week after immunization, followed by a peak in the second week. Circulating Ab-titres peak later, due to the relatively long half-life of the Ig molecule (Harrell *et al.*, 1975). After a second contact with the same antigen, the lag phase is shorter and the response is accelerated. Moreover, higher numbers of plasma cells or titres are reached. However, an Ig isotype switch is not observed and the increase in antibody affinity is limited when compared with that in mammals (Arkoosh and Kaattari, 1991; Kaattari, 1992).

Cellular immunity

Cellular immunity in fish has been studied *in vitro* using mixed leukocyte reactions (MLR), cytokine production and stimulation of DNA synthesis by T-cell mitogens or

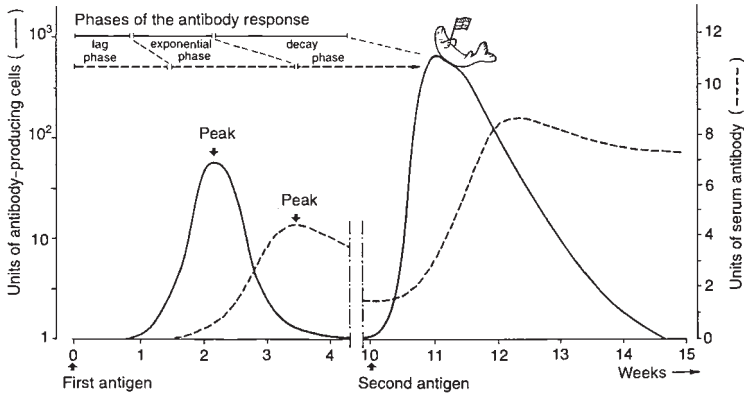


Fig. 18.6. A schematic representation of the primary and secondary humoral response in bony fish (from Lamers, 1985, with permission).

antigens (Kaastrup *et al.*, 1988; Secombes, 1991). *In vivo* studies include delayed-type hypersensitivity reactions and graft rejection (Rijkers, 1980; Manning and Nakanishi, 1996). The *in vivo* kinetics of specific cellular responses has been extensively studied by following the fate of transplanted scales or skin (Perey *et al.*, 1968; Borysenko and Hildemann, 1970; Rijkers and Van Muiswinkel, 1977). The cellular reactions that occur at the grafting site are essentially the same as in mammals. The graft-invading host cells are lymphocytes and macrophages. Jawless and cartilaginous fishes reject first-set grafts in a chronic way (median survival time (MST) of the graft ≥ 30 days). The more advanced bony fishes show an acute type of rejection (MST ≤ 20 days). Second-set grafts are rejected more rapidly than first-set grafts (Fig. 18.7). Specific cytotoxicity has also been shown in *in vitro* approaches by using modified autologous cells as targets (Verlhac *et al.*, 1990). It was demonstrated that the response of primed leucocytes to autologous trinitro phenol TNP-modified target cells was considerably greater than against allogeneic TNP-modified cells, suggesting that MHC restriction was involved. In mammals, cytotoxic T-cells recognize antigen in association with self (MHC class I) surface molecules.

Immunological Memory

An important feature of the immune system is the capacity to develop immunological memory. A first contact with an antigen usually induces relatively short-lived effector cells (activated Th, plasma cells or cytotoxic T-cells). There are also long-lived memory cells among the progeny of the original non-primed lymphocytes. These memory cells retain the capacity to be stimulated by

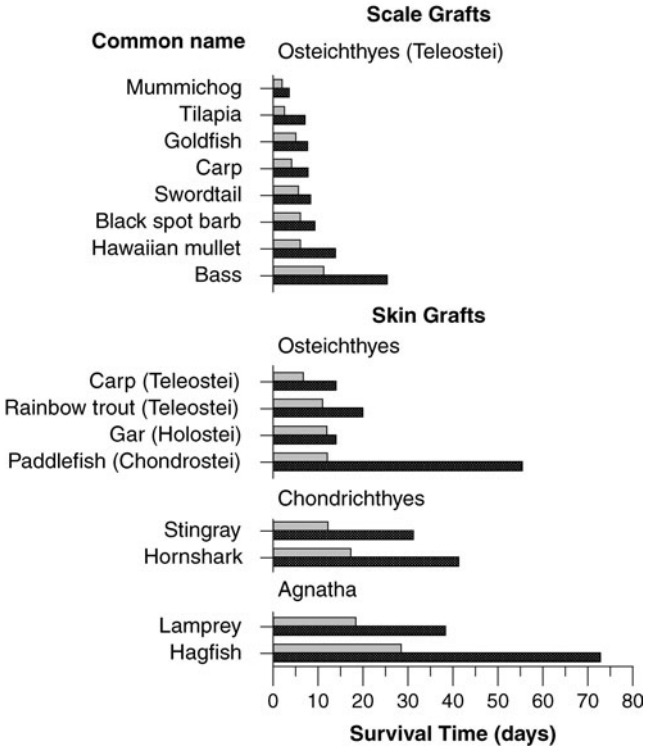


Fig. 18.7. Survival times of scale and skin allografts in different groups of fish. Dark columns: first-set grafts; grey columns: second-set grafts. (From Manning and Nakanishi, 1996, with permission.)

the antigen (Fig. 18.4). The development of immunological memory is often examined indirectly by monitoring the secondary response. In the case of positive memory, this response will be faster and more vigorous than the primary response (Figs 18.4 and 18.6). The height of the secondary response is dependent on the amount and antigenicity of the priming antigen. A relatively low priming dose is usually optimal for memory development in carp (Rijkers *et al.*, 1980b; Lamers *et al.*, 1985). In carp, the ratio between secondary and primary antibody responses never reached the high levels of that in mammals (5–20-fold in carp and up to 100-fold in mammals). Immunological memory has also been demonstrated in both vaccine-challenged and infected–recovered brook charr (*Salvelinus fontinalis*). Ardelli and Woo (1997) described rapid increases in complement-fixing antibody titres after challenge with *Cryptobia salmositica*. The parasites were lysed when they were incubated with immune *S. fontinalis* plasma and complement, which confirms that complement-fixing antibodies can play an important role in protection. The existence of immunological memory in rainbow trout has been demonstrated *in vitro* as well (Marsden *et al.*, 1995). Separated T- and B-cells from rainbow trout that were injected previously with *A. salmonicida* (the causative agent of furunculosis in salmonids), appeared to proliferate in response to various antigen preparations of *A. salmonicida*. All primed cell populations demonstrated enhanced responses to these antigens *in vitro*. This indicates the existence of T- and B-cell memory in vaccinated individuals. Elegant studies in rainbow trout also showed that the B precursor cell frequency in fish immunized with the hapten-carrier TNP-keyhole limpet haemocyanin increased about 15-fold (Arkoosh and Kaattari, 1991). The same authors also showed that there was no evidence for antibody affinity maturation during the primary or secondary response against this T-dependent antigen. This would indicate that memory in fish is probably due to an expansion of the antigen-specific precursor cell pool (Fig. 18.8). Several differences between the secondary responses of mammals and teleosts have been found. One distinction that can be made is that the ratio between the secondary and the primary response is much higher in mammals than in teleosts (which can be expressed as the 'memory factor' (MF)). The MF in mice injected with *Salmonella* flagellar antigen was, for example, 100 (Nossal *et al.*, 1965), whereas in carp injected with *A. hydrophila* the maximum MF was 6.1 (Lamers *et al.*, 1985).

During the secondary response the dominant Ig isotype in mammals is IgG. It is not surprising that IgG is absent in fish, since teleosts possess only the IgM isotype. Isotype switching is triggered in mammals during the secondary response, in contrast to teleosts, where this phenomenon has not been demonstrated. A temperature dependence of the secondary response in carp has been observed (Rijkers *et al.*, 1980a). In mammals, this is not the case as endotherms are not dependent on the environmental temperature. In teleosts, B-cell immunological memory is probably due to an increase in the antigen-sensitive precursor pool without any of the accompanying characteristics observed in mammals (such as a switch in isotype). In mammals, there is an increase in both the precursor pools and clone sizes after initial antigen priming (Kaattari, 1992; Fig. 18.8). Affinity maturation and somatic mutation have not been found in rainbow trout (Arkoosh and Kaattari, 1991). However, Fiebig *et al.* (1977) showed that, although minor increases in the absolute affinity of the carp Ig occurred during an immune response, the functional affinity increased logarithmically. This means that only a minor binding-site affinity increase is required to generate major functional affinity increases. Somatic mutation is involved in affinity maturation in mammals. Some affinity maturation takes place in teleosts, and this indicates that somatic mutation may occur in teleosts as well. It is interesting to note that, at least in sharks, evidence for the occurrence of somatic mutation has been found (Hinds-Frey *et al.*, 1993).

If it is known where antigen is processed and presented, then perhaps the location of memory formation in the organs

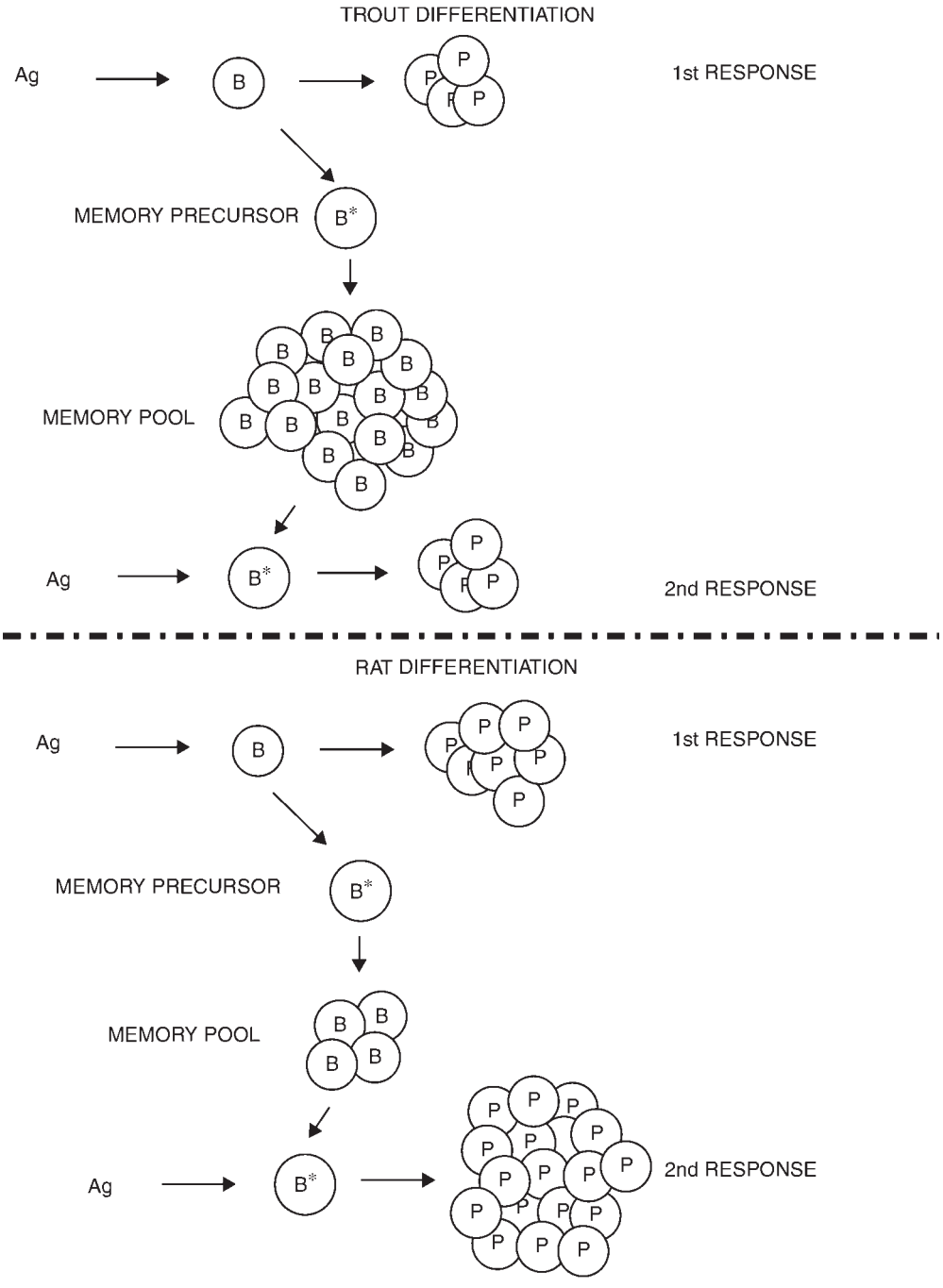


Fig. 18.8. The development of memory B-cell populations in teleost fish (rainbow trout) and mammals (rat). Note the difference in size of the memory pool between the trout and the rat. B*, memory B cell; B, B cell; P, plasma cell; Ag, antigen. (Slightly modified after Kaattari, 1992.)

can be identified. An antigen-localization study was carried out in carp, using *A. hydrophila* as an antigen (Lamers and De Haas, 1985). The presence of the antigen in the lymphoid organs was examined up to 12 months after injection. The antigen was at first present in splenic ellipsoids and in solitary phagocytic cells in the spleen, head and trunk kidney. Two weeks later, the antigen was gradually more concentrated in or near the melano-macrophage centres (MMC) in the spleen (Fig. 18.9), head and trunk kidney. After 1 month, the antigen was detected attached to cells in and around the MMC, where it remained for at least a year. It has been suggested that the MMC could be the location for the immune response and/or formation of immunological memory (Secombes *et al.*, 1982; Van Muiswinkel *et al.*, 1991). Analogies have been drawn between the MMC of teleosts and the germinal centres of mammals (Agius, 1980). The MMC consists of groups of dark pigment-containing cells, which are present at the bifurcation of large blood vessels or near the ellipsoids. Reticular cell and macrophage populations were found among the MMC of the spleen (and in the head kidney) and within the peri-ellipsoidal macrophage sheaths of the spleen as well (McL. Press *et al.*, 1994). Aggregations of lymphoid cells can occasionally be seen, especially after immunization (Secombes *et al.*, 1982). Large clusters of Ig^+ cells (probably B-cells) were observed in Atlantic salmon 3 months after vaccination and were associated with MMC (McL. Press *et al.*, 1994). These observations support the idea that B memory-cell development could take place near or in the MMC of fish.

Vaccination

General aspects

Fish farming has grown significantly during the last 30 years. Fish like trout, *Tilapia* and salmon are often kept at high population densities. This increases the risk of dramatic disease outbreaks. Although antibiotics can be used for the treatment of bacterial diseases, this also has some drawbacks. Repeated use can induce drug resistance in microorganisms or suppress the immune system of fish (Rijkers *et al.*, 1980c). Moreover, harmful residues may be present in the fish sold for human consumption. Hence, it is not surprising that there is an increasing interest in protecting fish by vaccination. There are several reviews or books on fish vaccination (Lamers, 1985; Ellis, 1988; Gudding

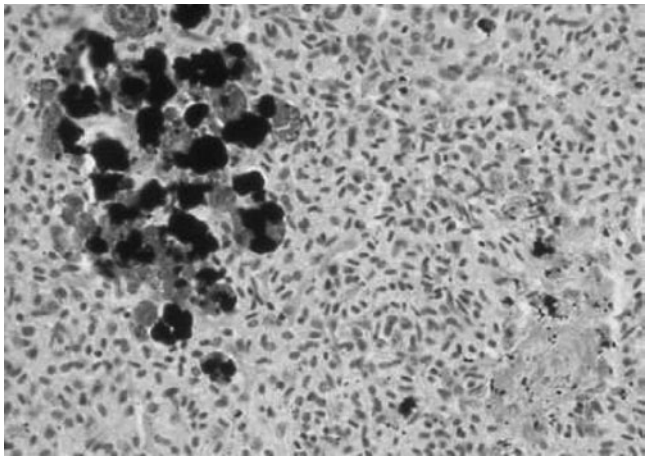


Fig. 18.9. Section of a melano-macrophage centre (MMC) in the spleen of adult rosy barb (*Barbus conchonius*). The dark staining (left) is characteristic for the pigment-containing macrophages in the MMC.

et al., 1997). In addition to the usual vaccination method by injection, new procedures for bath or immersion methods have been developed. These impose less stress on fish and are almost as effective as injection.

Oral vaccines

Oral vaccination usually evokes only minimal immune responses in the host. It is not easy to explain this phenomenon. Stroband and Van Der Veen (1981) showed that the intestine in almost all fish is divided into three different segments. The first or proximal segment is involved in the digestion and absorption of lipids and proteins. The second segment contains epithelial cells with pinocytotic activity and the third segment or end-gut probably plays a role in osmoregulation (Fig. 18.10). In a study by Rombout and Van Den Berg (1989), it was shown that the second gut segment is important for antigen transport and antigen processing by macrophages. Numerous lymphoid cells are also present in this gut segment (Rombout *et al.*, 1989a). These cells probably play a role in local (mucosal) responses. Repeated oral administration of bacterial antigen resulted in antibodies in skin mucus and bile, but not in serum (Rombout *et al.*, 1989b). It is expected that encapsulation of vaccines is needed to prevent digestion in the first part of the gut and to ensure that the essential antigenic determinants reach the second gut segment in a non-degraded and immunostimulatory form (Joosten *et al.*, 1995). This approach should allow the development of new and effective oral vaccines in the future.

DNA technology

In recent years, various vectors have been used to produce large quantities of antigens by recombinant DNA technology. In aquaculture, research on recombinant vaccines has focused mainly on viral vaccines, because traditional production of viruses in cell culture systems is relatively expensive. Glycoproteins of viruses causing viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) in rainbow trout elicit protective antibodies (Lorenzen and Olesen, 1997). Genetic immunization using naked DNA is the most recent approach in vaccine development. Intramuscular injection of plasmid DNA containing genes encoding glycoproteins or nucleocaspid protein in rainbow trout protected against challenge by VHS and IHN (Lorenzen *et al.*, 2002).



Fig. 18.10. Uptake of horseradish peroxidase by epithelial cells in the second intestinal segment of 20-day-old grasscarp larva (*Ctenopharyngodon idella* Val.). Note that the enzyme activity is absent in the first (left) and third (right) segment of the gut. The circle in the lumen is an empty *Artemia salina* eggshell, which was part of the food used. (Courtesy H.W.J. Stroband.)

Environmental Effects

Temperature

In cold-blooded animals such as fish, the metabolic activity is directly influenced by the ambient water temperature. The effects of temperature on antibody synthesis have been known for a long time (Bisset, 1948). The summer flounder (*Paralichthys dentatus*) needs water temperatures above 18°C for an effective Ab response against parasitic haemoflagellates (Sypek and Burreson, 1983). The relationship between temperature and the humoral response in carp is shown in Fig. 18.11 (Rijkers *et al.*, 1980a). This relationship matches the effect of temperature on allograft survival in goldfish (*Carassius auratus*) (Hildemann and Cooper, 1963). Avtalion (1981) studied the effects of temperature on antibody production in carp and *Tilapia* against bovine serum albumin and hapten-carriers. They showed that

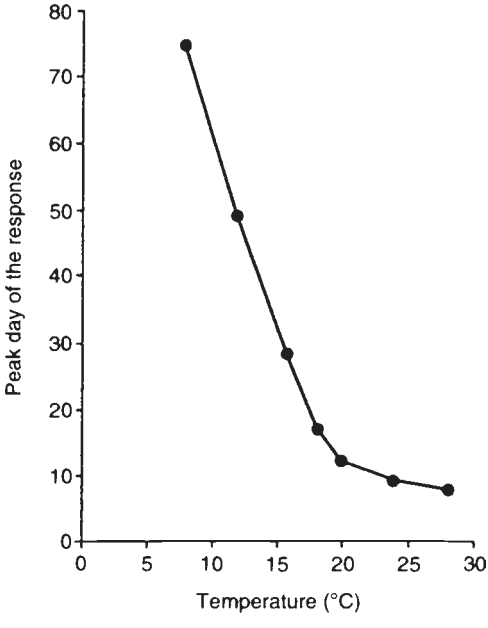


Fig. 18.11. The relationship between the water temperature and the speed (peak day) of the humoral response in carp (from Rijkers *et al.*, 1980a, with permission).

synthesis and release of antibody could take place at low temperatures (≤ 12°C) if fish were kept at high temperatures (25°C) during the early phase of the response. It was suggested that antigen processing and subsequent cooperation between macrophages, Th- and B-cells is a temperature-sensitive event, which lasts 3–4 days in these warm-water fish. The temperature sensitivity of T-cells was confirmed by Miller and Clem (1984), who showed that low temperatures inhibited the generation of putative carrier-specific memory Th-cells from virgin Th-cells. Cytokine production in rainbow trout is also inhibited at non-permissive temperatures. Again, it is the T-cell that is the temperature-sensitive cell, the macrophage function itself remaining intact at low temperatures when suitably stimulated with macrophage-activating factor (MAF) (Hardie *et al.*, 1995). It is possible that innate immunity may compensate for the loss of acquired immunity at lower temperatures. For example, low temperatures inhibiting the mitogenic effect of phytohaemagglutinin (PHA) on carp T-cells were found to enhance NCC activity (Le Morvan-Rocher *et al.*, 1995). Recent work on rainbow trout (Nikoskelainen *et al.*, 2004) showed that innate immunity (respiratory burst activity, lytic activity of total and alternative complement pathways) was still working but responding at a lower level in animals acclimatized to lower temperatures (5–10°C). The normal function of fish lymphocytes at different temperatures is highly dependent on homoviscous adaptation of membrane lipids (Abruzzini *et al.*, 1982). It is likely that the fatty acid composition (unsaturated versus saturated) determines the fluidity and permeability of membranes, as well as the activity of membrane-associated receptors and enzymes. Sheldon and Blazer (1991), working with channel catfish at optimal (28°C) and suboptimal (19°C) temperatures, observed a positive correlation between the bactericidal activity of macrophages and the level of highly unsaturated fatty acids in the diet. This opens new perspectives for the improvement of disease resistance of fish at lower temperatures.

Stress

It is obvious that several human activities affect fish welfare, e.g. commercial and sports fisheries, aquaculture, ornamental fish keeping and scientific research. Tissue damage, physical exhaustion and severe oxygen deficit can occur during handling or capture. Moreover, pain and stress can be expected when a fish is killed. It is inevitable that fish are exposed to stress induced by aquaculture practices such as crowding, transport, handling and impaired water quality. In fishes, as in mammals, the stress response comprises activation of the sympathetic nervous system, as well as of the hypothalamus–pituitary–interrenal (HPI) axis; the interrenal tissue in the head kidney of fish contains the equivalents (cortisol-producing cells and chromaffin cells) of the mammalian adrenals (Wendelaar Bonga, 1997). In response to hypothalamic release of corticotrophin-releasing hormone (CRH) and thyrotrophin-releasing hormone (TRH), the pituitary enhances synthesis of Pro-opiomelanocortin POMC and release of its cleavage products. Adrenocorticotrophic hormone (ACTH) is a potent stimulator of cortisol production by the interrenal steroid-producing cells. Cortisol has both glucocorticoid and mineralocorticoid actions in fish (the type of response to cortisol is receptor-dependent).

Endocrine-immune interactions

As the head kidney combines glucocorticoid and catecholamine production with important immune features, e.g. lymphopoiesis and antibody production, the potential for paracrine modulation of immune responses by stress hormones is indicated. Effects of cortisol on the immune system of fish are generally similar to those in mammals. Numerous studies suggest that prolonged stress causes lymphocyte depletion in peripheral blood and lymphoid organs (Zapata *et al.*, 1992). Circulating lymphocyte populations decrease in number while neutrophilic granulocytes remain constant or increase (Ellsaesser and Clem, 1986; Ainsworth *et al.*, 1991). Lymphocyte proliferation is decreased after injection with cortisol (Espelid *et al.*, 1996) and *in vitro* antibody responses are impaired after cortisol administration (Carlson *et al.*, 1993). Reports on the effects of stress or cortisol on respiratory burst and phagocytosis are conflicting, but may reflect differences between species as well as differences in methodologies. Receptors for glucocorticoids were demonstrated in salmon and carp leukocytes (Maule and Schreck, 1990; Weyts, 1998). In carp a differential effect of cortisol was demonstrated on lymphocytes and neutrophilic granulocytes under *in vitro* conditions. Activated B-cells harvested from the blood are easily triggered to enter cortisol-induced apoptosis (Weyts, 1998). Moreover, compared with B-cells from head kidney and spleen, circulating B-cells are most affected by cortisol (Verburg-Van Kemenade *et al.*, 1999). In contrast to the sensitivity of B-cells to apoptosis signals, carp neutrophilic granulocytes are rescued from apoptosis by cortisol (Weyts, 1998), demonstrating dual actions of glucocorticoids in fish. Not surprisingly, there are indications that not only mammalian but also fish leukocytes produce HPI-axis hormones. Ottaviani *et al.* (1998) demonstrated in goldfish the presence of immunoreactive CRH in the thymus. Channel catfish leukocytes (Peripheral blood leucocytes (PBL); B- and T-cell lines) secrete ACTH (Arnold and Rice, 2000), both constitutive and CRH-driven. Thus, although research in this field is only starting, we anticipate that 'stress hormones' in fish are produced by leukocytes to allow for bidirectional communication between the neuroendocrine system and the immune system (Fig. 18.12).

Genetic Aspects and Disease Resistance

The identification of genes involved in the regulation of defence mechanisms is important for understanding and perhaps improving disease resistance in fish. Studies with several mammalian species have shown that the products of the MHC genes play a key role in the regulation of the immune response (Klein and Horejsi, 1999).

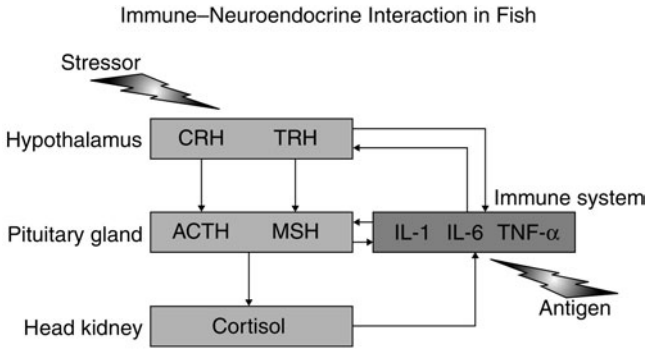


Fig. 18.12. Interaction between the stress response and the immune response in fish. During the stress response, neuropeptides, including corticotrophin-releasing hormone (CRH) and thyrotrophin-releasing hormone (TRH), control the release of pituitary hormones involved in the regulation of cortisol (ACTH, adrenocorticotrophic hormone; MSH, melanophore-stimulating hormone). The head kidney of fish contains equivalents (e.g. cortisol-producing interrenal cells) of the mammalian adrenal. High levels of cortisol may affect the expression of cytokine genes in cells of the immune system. Cytokines (e.g. IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumour necrosis factor) play an important role in the regulation of the immune response, but are also known to interact with the hypothalamus–pituitary–interrenal (HPI)-axis. Administration of IL-1 in experimental fish can activate CRH neurons and stimulates the release of CRH, illustrating immune–neuroendocrine interaction (J. Metz, G. Flik and S.E. Wendelaar Bonga, personal communication).

Moreover, an association has been established between certain MHC alleles and the susceptibility for specific diseases in birds and mammals (De Vries *et al.*, 1979; Svejgaard *et al.*, 1982). The increasing knowledge of the MHC in fish will certainly be important for our ideas about regulation of the immune response in fish (see also the section on antigen recognition and presentation). Several examples of genetic differences in disease resistance in fish have been described (Chevassus and Dorson, 1990; Houghton *et al.*, 1991; Wiegertjes *et al.*, 1993), but well-defined genetic markers are still scarce. In a study with captive-bred chinook salmon (*Oncorhynchus tshawytscha*), it was shown that outbred and/or heterozygous (MHC genes) animals were usually more resistant to *V. anguillarum*, IHN virus and the parasite that causes whirling disease than inbred or homozygous fish (Arkush *et al.*, 2002). Only a few studies have addressed the functional aspects of MHC molecules in fish. Grimholt *et al.* (2003) showed a significant association between resistance to disease (infectious salmon anaemia virus (ISAV) and *A. salmonicida*) and MH gene polymorphism in Atlantic salmon. These observations underline the importance of genetic variation in a population of fish.

Conclusions

During the last 20–30 years, considerable progress has been made in describing and understanding the immune system of fish. Antigenic stimulation in fish evokes responses that are comparable to those in warm-blooded vertebrates. An effective innate immune system is present and acquired immune responses show the expected characteristics of specificity and memory. However, an isotype switch or affinity maturation in Ig is usually absent. There are clear influences of environmental factors, such as temperature and stress conditions. Our knowledge of the immune system of fish can be used for evaluation of the health status of fish under different conditions, but can also be used for vaccination and breeding for disease resistance in aquaculture.

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19 Immunocompetent Cells and Their Mediators in Fin Fish

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Introduction

Much progress has been made in recent years, particularly in the identification of cytokines and chemokines; however, the immune system of fin fish is still not as well characterized as that of more recently evolved vertebrates, such as mammals. Although the chapter will focus on teleosts and cartilaginous fishes, much of what is discussed will be done with reference to the more well-defined immune system of mammals.

The immune system has two basic components that respond to foreign antigens: (i) the humoral response, involving production of immunoglobulins (antibodies) by plasma cells; and (ii) the cell-mediated response, mediated by T-lymphocytes. In addition, there are also cell populations responsible for non-specific and specific immune defences and they include lymphocytes, monocytes/macrophages, granulocytes and thrombocytes. The lymphocytes are classified as immunoglobulin positive B-cells and immunoglobulin-negative T-cells. Other cell types include mast cells, non-specific cytotoxic cells and dendritic cells. In addition, the tissues of the spleen and kidney of fish also contain a distinct population of cells, known as melanomacrophages. All of the cell types described are generally identified based on their morphological and functional characteristics, as well as by their receptors (Miller *et al.*, 1998).

Cells of the Immune System

Mononuclear cells

Monocytes

Monocytes are found mainly in the blood, and in small numbers in lymph nodes, the spleen, bone marrow and connective tissue in higher vertebrates. These cells migrate from the blood when they receive the appropriate stimuli and enter tissues. They are generally regarded as immature until they leave the circulation, enter the tissue and develop into mature, phagocytic macrophages. Monocytes are much larger than lymphocytes, and in mammals they are described as having large ovoid or horse-shaped nuclei that occupy half of the cell. True monocytes (Figs 19.1 and 19.2, M) have been described from a few species of fishes. Generally, most studies do not differentiate between the monocyte and the phagocytic macrophage. Monocytes have been clearly demonstrated in plaice (*Hippoglossoides platessoides*) and, although

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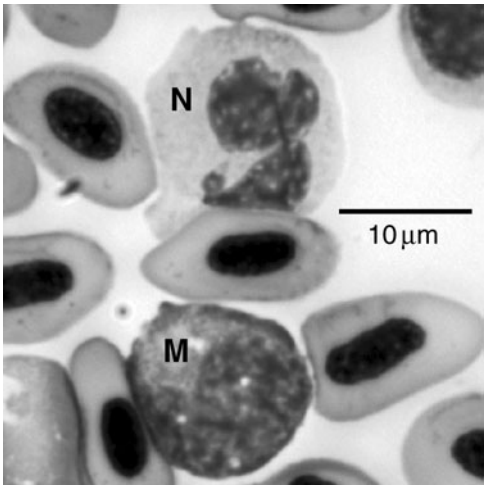


Fig. 19.1. Blood smear from an Atlantic salmon (*Salmo salar*) stained with Wright–Giemsa stain – monocyte (M) and neutrophil (N); bar = 10 μm, magnification × 1000 (original – courtesy of A. Chin).

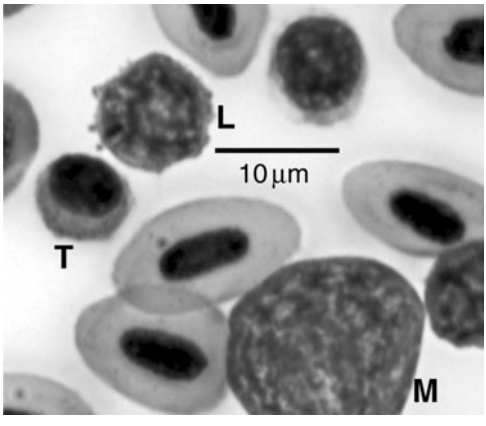


Fig. 19.2. Blood smear from an Atlantic salmon (*Salmo salar*) stained with Wright–Giemsa stain – lympocyte (L), monocyte (M) and round-form thrombocyte (T); bar = 10 μm, magnification × 1000 (original – courtesy of A. Chin).

scarce, are found in the blood and kidney. They are morphologically similar to mammalian monocytes and are capable of phagocytosis. The monocytes of gilthead sea bream (*Sparus auratus*) are large cells with an indented nucleus. The cytoplasm contained numerous vesicles of different sizes with a few lysosomes (Zuasti and Ferrer, 1989). Similarly, carp (*Cyprinus carpio*) cells with monocyte/macrophage morphology contained abundant organelles, with varying-size granules and occasional phagolysosomes (Bielek, 1988). The cytochemical features of the monocyte–macrophage have been described in sea bream. The lysosomes of macrophages are not acid-phosphatase-positive. In another study of sea bream, a monocyte–macrophage cell with acid and alkaline phosphatase and peroxidase has been described (Meseguer *et al.*, 1994).

Macrophages

Until recently, macrophages have been considered to be the primary cell type serving as an antigen-presenting cell. In mammals, mononuclear phagocytes originate in the bone marrow, circulate for a short time in the blood as monocytes and then move into tissues, where they develop into macrophages. Tissue macrophages (fixed or wandering) are derived primarily from blood monocytes, but they may also form by proliferation in the tissues as well. When in other tissues, macrophages are often called alveolar phagocytes (lungs), microglia (central nervous system), reticular cells (bone marrow and lymphoid organs) or Kupffer cells (liver).

In mammals, macrophages have a unique surface glycoprotein, known as F4/80 (160 kDa) (Hirsch *et al.*, 1981). They are larger than B- or T-cells and are characterized by a large number of lysosomes. Mammalian macrophages also have receptors for class II major histocompatibility complex (MHC) antigens, FcRs, complement (C3b) and lymphokines. In contrast to T-cells, macrophages do not possess specific antigen receptors but they bind, ingest and degrade virtually any type of antigenic material. They produce some of the major components of complement, interferons, prostaglandins and cytokines, such as interleukin-1 (IL-1) (see later section). In the nurse shark (*Nebrius ferrugineus*), the neutrophil, not the macrophage, carries the receptor for F_c (McKinney and Flajnik, 1997). The F_CR falls

into two general classifications – those involved in effector functions and those that transport immunoglobulin (Ig). The F_CRs that mediate phagocytosis in human macrophages are classified as FcγRI, FcγRIIA and FcγRIII (Ravetch, 1997). F_CRs exist for every antibody class: FcγRs bind IgG, FcαRs bind IgA, FcεRs bind IgE, FcμRs bind IgM and FcδRs bind IgD (Fridman *et al.*, 1991). The FcεRI receptor was cloned from carp and was expressed in leucocytes (Fujiki *et al.*, 2000b). In channel catfish (*Ictalurus punctatus*), natural killer (NK)-like cells express a putative FcμR receptor for IgM.

Macrophages in fish are typically found in connective and other tissues and are not normally seen as circulating leukocytes. Similarly to mammals, macrophages of fish are capable of phagocytosis and are important scavengers of necrotic tissue. Kupffer cells have been identified in the liver of polka-dot catfish (*Pimelodus masculatus*) and are described as being attached by desmosomes to the endothelial cells lining the sinusoids (Ferri and Sesso, 1981). Macrophages are also in the gastrointestinal tract of catfish, and they have been suggested to be the primary mediators of intestinal mucosal immunity (Herbert *et al.*, 2002). In carp, macrophages and neutrophilic granulocytes produced an IL-1-like molecule, with T-cell-proliferating potency. It shares structural similarity with mammalian IL-1 (Verburg-van Kemenade *et al.*, 1995).

The response of goldfish macrophages to various pathogens has been well studied. The biochemical events that lead to induced nitric oxide production by teleost macrophages are similar to those of mammals. For example, cultured goldfish macrophages produce nitric oxide in response to macrophage-activating factors, secreted by mitogen-stimulated leukocytes, or bacterial lipopolysaccharide (LPS) (Neumann *et al.*, 1995). Inducible nitric oxide synthase (iNOS) appears to be a functional enzyme in goldfish macrophages, as there is increased iNOS expression post-stimulation with bacteria LPS (Laing *et al.*, 1996). An interesting study by Barreda *et al.* (2000) suggested that, unlike mammals, fish macrophages develop through alternative pathways rather than the classical hematopoietic pathway. Three distinct macrophage subpopulations were identified from a goldfish cell line that appeared to develop from differentiation of monocyte-type cells and from direct differentiation in the absence of a monocyte-like stage. The mature macrophage-like cells could proliferate and distinct macrophage subpopulations, designated R1, R2 and R3, with distinct functions were identified (Neumann *et al.*, 2000). The R1-type macrophages contained acid phosphatase, but not myeloperoxidase or non-specific esterase. The R2-type cell had a morphological resemblance to mature mammalian tissue macrophages, and contained acid phosphatase, myeloperoxidase and non-specific esterase. The R3-type macrophage, described as a round cell, had an eccentrically placed nucleus, which closely resembled mammalian macrophages. These subpopulations of macrophages also exhibited distinct functional responses. After activation with macrophage activation factors (MAF) and/or bacterial LPS, the R2-type macrophages produced nitric oxide, while R3 produced little or no nitric oxide. A new class of low-molecular-weight, cysteine-rich, regulatory growth factors, designated granulins, was characterized from carp (*C. carpio*) and goldfish (*Carassius auratus*) (Belcourt *et al.*, 1995). This growth factor was localized, using immunocytochemistry, to macrophages/monocytes.

Melano-macrophage centres

In addition to phagocytic macrophages fishes also have aggregates of macrophages, more commonly called melano-macrophage centres (MMCs) (Agius and Roberts, 2003). MMCs are localized accumulations of macrophages that contain haemosiderin, lipofuschin and ceroid, as well as melanin-staining pigments. These structures have also been described from other poikilotherms, such as reptiles (i.e. turtles) and amphibians (Christiansen *et al.*, 1996). The MMCs are generally found in the spleen, kidney and liver, but have been found in other locations, particularly during inflammation. The size and location of these centres vary with the species of fish. They are considered good

indicators of fish health because the size, number and pigmentation vary depending on the health of the fish (Gogal *et al.*, 1999). Granulin-1 isolated from carp has immunoreactivity in the MMCs of the spleen and head kidney (Belcourt *et al.*, 1995). Excessive copper storage in the liver of white perch (*Morone americana*) has been associated with enlarged MMCs (Bunton *et al.*, 1987). In the turbot (*Scophthalmus maximus*), Ig^+ cells are mainly associated with MMCs in the spleen and kidney (Fournier-Betz *et al.*, 2000). In tilapia (*Oreochromis niloticus*) injected intraperitoneally with azathioprine, an anti-neoplastic drug and therapeutic immunosuppressant, MMCs were larger and in greater abundance in fish given a high dose compared with controls (Gogal *et al.*, 1999). In flounder (*Platichthys flesus*) exposed to pollutants for 3 years, MMCs in the liver were in much higher densities in exposed fish compared with the controls (Vethaak *et al.*, 1996), while guppies (*Poecilia reticulatus*) aged 1 year or older showed marked accumulation of melanin in MCCs of the kidney, and the amount present increased with age (Woodhead *et al.*, 1983).

Thrombocytes (platelets)

Platelets in the blood of fish may be small cytoplasmic fragments with diameters less than half that of a red blood cell. In mammals, they are derived from large megakaryocytes in the bone marrow and possess small granules, surface receptors (FcRs) for IgG and IgE and class I MHC antigens. Platelets are not only important for blood clotting (as producers of thromboplastin), but they are also critically involved in hypersensitivity reactions and the inflammatory process. This cell type releases permeability-increasing substances, such as histamine, following aggregation and degranulation. Platelets can be induced to aggregate and degranulate by platelet-activating factor, immune complexes and complement components (C3a and C5a). Platelet-activating factor is produced by a variety of cells, for example, basophils and mast cells, following interaction with allergens or immune complexes and complement factors.

The origin of thrombocytes in fish is not known but it is generally accepted that they are produced in the spleen. Similarly to mammals, thrombocytes of fish are involved in clotting and are also capable of phagocytosis. Very few studies have been conducted to characterize platelets morphologically (Figs 19.2 and 19.3, T). In one study, the thrombocytes of *S. auratus* were described as having numerous vacuoles and abundant glycogen in the cytoplasm (Zuasti and Ferrer, 1989). The platelet-derived growth factor receptor and colony-stimulating factor receptor 1 gene were cloned from the pufferfish, *Fugu rubripes* (Williams *et al.*, 2002), and the platelet-derived growth factor-α gene was cloned from the zebrafish (*Danio rerio*) (Liu, L. *et al.*, 2002).

Granulocytic cells

Granulocytes are leukocytes that are characterized by the presence of cytoplasmic granules. This group is subdivided into three types in mammals, based on the nature of the granules present: (i) basophils, containing basophilic granules; (ii) eosinophils,

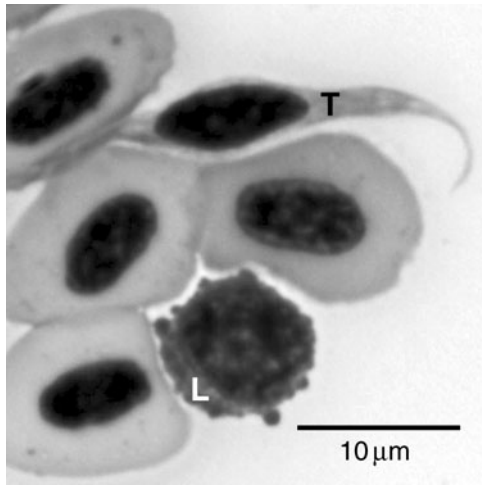


Fig. 19.3. Blood smear from an Atlantic salmon (*Salmo salar*) stained with Wright–Giemsa stain – lymphocyte (L) and spindle-form thrombocyte (T); bar = 10 µm, magnification × 1000 (original – courtesy of A. Chin).

having eosinophilic granules; and (iii) neutrophils, which lack specific granules. In mammals they develop in the bone marrow, but development in fish is variable. Granulopoiesis in cartilaginous fishes occurs in the spleen and lymphomyeloid tissues (epigonal organ and organ of Leydig), while the spleen and kidney are the granulopoietic organs in bony fishes. In some elasmobranchs, granulopoiesis aggregates are found in the central nervous system. Human eosinophils, basophils and mast cells share a number of recruitment pathways with one another and with other cell types. However, each also possesses unique adhesion and migration responses, which may contribute to their preferential accumulation. The behaviour of these cell types is not identical, however. All three are linked to allergic reactions and yet their localization within a given tissue can be distinct.

Neutrophils

The granular cytoplasm in neutrophils does not stain well with either acidic or basic dyes at neutral pH. In mammals, mature neutrophils are characterized by distinct lobed nuclei and are commonly referred to as polymorphonuclear neutrophils (PMNs). The percentage of circulating neutrophils in fish is generally smaller than in mammals (60%), because they tend to have larger numbers of circulating lymphocytes. The PMNs in mammals are derived from pluripotent stem cells, as are the other blood cells, but in fish the head kidney is the major haemopoietic organ. The main function of neutrophils is phagocytosis of foreign, aberrant or dead cells, as well as pinocytosis of immune complexes. In mammals, neutrophils also exhibit antibody-dependent cell-mediated cytotoxicity (ADCC) and are capable of rapid activation and mobilization in response to chemotactic stimuli, such as bacterial products or activated components of complement (C5a). Once activated, a variety of receptors (Fc, C3b, C3d and C5a) is displayed. During the early stages of inflammation, as a result of infection and/or immune complexes, neutrophils are the most prominent cell type at the inflammatory site and bind, ingest or lyse the foreign target (Miller *et al.*, 1998).

The neutrophils of fish vary in size (8–15 μm in diameter) and are round to oval, and the shape of the nucleus is variable. The cytoplasm ranges in colour (in Romanowsky stain) from whitish to whitish grey. Trout appear to be the only species that have the typical polymorphonuclear nucleus (Fig. 19.1, N), which in other species ranges from round to lobed. The granules of fish neutrophils also vary in size, shape, colour and chemical composition. Fish neutrophils have phagocytic ability and appear similar to those of mammals in that chemotaxis, adherence, ingestion or engulfment and then destruction of the foreign microorganism occur (Sepulcre *et al.*, 2002). In the head kidney of gilthead sea bream, *S. auratus*, neutrophils are the most abundant cell type. In this fish, the most distinguishing characteristic of the neutrophil is an eccentric and slightly segmented nucleus and cytoplasm containing numerous homogeneously dense granules (Zuasti and Ferrer, 1989). The cytoplasmic granules are positive for alkaline phosphatase and peroxidase, but are acid-phosphatase-negative (Meseguer *et al.*, 1994).

Eosinophils

Eosinophils contain an abundance of highly basic proteins within their granules, which confer their affinity for acid dyes (Gleich and Loegering, 1984). In mammals, these cells are characterized by bilobed nuclei, abundant ribosomes and mitochondria, and they account for 3–5% of the leukocyte population. However, certain allergic conditions and some parasitic infections cause a dramatic increase in the number of eosinophils (Butterworth and David, 1981). These cells, like neutrophils, have phagocytic abilities, and engulf and remove immune complexes (Bodammer, 1986). However, it is generally accepted that the major function of eosinophils is secretion of their granular contents in response to parasitic infections. Mammalian eosinophils possess FcRs and can mediate ADCC and respond to chemotactic factors released by T-cells. As indicated later, basophils and mast cells secrete histamine, which is a mediator of type I hypersensitivity reactions, and eosinophils

release histaminase to inactivate the histamine (McEwen, 1992).

Eosinophils in fishes are involved in immunity against parasitic infection. Eosinophils adhere to parasites to kill them, neutralize secreted parasite products and attract leukocytes to the infected area. In *S. auratus*, eosinophils in the head kidney have lobed nuclei with two types of granules in the cytoplasm (Zuasti and Ferrer, 1989). The eosinophils of gilthead sea bream (*S. auratus*) are alkaline phosphatase- and peroxidase-positive at pH 11.0. Eosinophils have also been described from the peripheral blood of the nurse shark (*Ginglymostoma cirratum*) (Hyder *et al.*, 1983). Eosinophils of carp (*C. carpio*) have an increased polarization response when exposed to extracts of the tapeworm *Bothriocephalus acheilognathi* (Nie and Hoole, 2000).

Basophils and mast cells

Basophils comprise less than 1% of white blood cells. Both blood basophils and their tissue counterparts, mast cells, possess prominent, randomly distributed, basophilic (because of their affinity for the dark aniline dyes) granules. These granules contain eosinophil chemotactic factors and pharmacological mediators of type I hypersensitivity. They release their contents when stimulated by allergens and cross-link these molecules, which have become attached to the basophil through high-affinity FcRs. Mammalian basophils also possess FcRs for IgG and receptors for the complement components C3a, C3b and C5a, suggesting a capability to exhibit ADCC, phagocytosis and chemotaxis (Denburg, 1999).

Basophils are scarce in the head kidney of the sea bass (*Dicentrarchus labrax*), and they have similar characteristics to those from other species (Meseguer *et al.*, 1990). Similarly, basophils are also scarce in gilthead sea bream, *S. auratus* (Zuasti and Ferrer, 1989). In experimental peritoneal inflammation induced in goldfish (*C. auratus*), the most striking feature in the head kidney during inflammation was a severe depletion of cells with basophilic granules. It was suggested that the head kidney of goldfish is involved in the inflammatory process (Bielek *et al.*, 1999). The granules of basophils in carp are larger than those of eosinophils and they have a lamellar structure (Khamidov and Nishanbaev, 1975).

Mammalian mast cells originate from distinct bone-marrow haematopoetic progenitors, which circulate before entering tissues, where they subsequently differentiate into mature mast cells (Saito *et al.*, 1996; Denburg, 1999). The granules of mammalian mast cells contain histamine as well as glycosaminoglycan and proteins. These cells are activated by local tissue damage and degranulate to release histamine, which causes dilatation and increased permeability of small blood vessels. This 'degranulation' manifests itself as redness and oedema. In addition to histamine, mast cells also release heparin, proteases and chemotactic factors (Daffern *et al.*, 1995). Once activated, they also produce secondary mediators, such as prostaglandins, blood platelet-activating factor, leukotrienes and cytokines (Saito *et al.*, 1996). Histamine is important in anaphylactic hypersensitivity reactions (type I hypersensitivity). Once a type I reaction develops, IgE is produced and mast cells become sensitized through binding of the immunoglobulin to the specific receptors on mast cells (Columbo *et al.*, 1992). Secondary exposure to the antigen leads to binding between antigen and immunoglobulin, leading to the degranulation process.

Mast cells are generally quite similar to basophils in morphology, expressed receptors and function. The presence of true mast cells in fish has been questioned by several investigators. Mast cells in fish are often referred to as 'eosinophilic granular cells' (ESGs). Some ESGs found in the gills, skin and alimentary canals of fishes have been considered analogous to mast cells, in terms of structure and function (Hollan and Rowley, 1988; Vallejo and Ellis, 1989). However, in one study, no histamine was found in ESGs, although they did contain aryl sulphatase B and peroxidase activity, particularly in granules. These cells had phagocytic activity (Sire and Vermier, 1995). ESGs have been described from the connective tissues in the gut, gills, pseudobranch,

nares and heart of teleosts. Similarly to eosinophils, basophils and mast cells of fish are important against parasitic infections in the intestine, gills and skin. Eosinophils appear to have opposing functions – they are the main effector cells of parasitic infections and, yet they modulate type I hypersensitivity reactions (Kay, 1985). The brains of several teleost fishes were examined by using light, fluorescence and electron microscopy. Of 56 fish examined, mast cells were detected in only four of the cases. The mast cells were concentrated in the meninges of one pike (*Esox lucius*) and in the meninges and in blood vessels of the hypothalamus of two trout (*Salmo trutta fario*). In the hypothalamus of the carp (*C. carpio*), an infiltration of mast cells was detectable in the meninges and in blood vessels and capillaries between neuronal and glial fibres and sub- and intra-ependymally. In contrast to what was observed in the pike, the granules of mast cells in the carp changed, suggesting that those in carp released their contents. Junctions between the mast cells and capillary walls or ependymal cells are characterized by structures indicating an enhanced cytopempsis (Weiss, 1979).

Dendritic cells

Information on structural and functional aspects of follicular dendritic cells is mostly based on studies in mammals, particularly human and rodent species. In birds, follicular dendritic cells are present and functional. However, it is difficult to state if follicular dendritic cells exist in lower vertebrates. A dendritic cell is a type of antigen-presenting cell (APC) that has long membrane processes, reminiscent of the dendrites of nerve cells. Dendritic cells are unique APCs because they are the only ones able to induce primary immune responses, thus permitting establishment of immunological memory (Hart, 1997). After 'capturing' antigen in tissues, these cells migrate to lymphoid organs and present antigen to lymphocytes. Dendritic cell progenitors in the bone marrow give rise to circulating precursors, which reside as immature cells in tissues and have high phagocytic capacity. Following tissue damage, immature dendritic cells capture antigen and subsequently migrate to the lymphoid organs, where they select rare antigen-specific T-cells, thereby initiating immune responses (Banchereau *et al.*, 2000). Dendritic cells present antigen to CD4+ T-helper cells, which in turn regulate the immune effectors, including antigen-specific CD8+ cytotoxic T-cells and B-cells, as well as non-antigen-specific macrophages, eosinophils and NK cells (Lambrecht *et al.*, 1998).

Mammalian dendritic cells are found in non-lymphoid and lymphoid organs and tissues, as well as in blood and lymph. Non-lymphoid dendritic cells include Langerhans cells (in epidermis) and interstitial dendritic cells (in heart, lungs, liver, kidney and gastrointestinal tract) (Steinman, 1991). The function of non-lymphoid dendritic cells is to capture antigen and carry it to the regional lymph nodes. As mammalian non-lymphoid dendritic cells enter the blood and lymph, they change morphologically and become 'veiled' cells (Banchereau *et al.*, 2000).

These lymphoid dendritic cells are interdigitating and follicular cells. The interdigitating cells are in T-cell-rich regions of the lymphatic organs, where they interact with T-cells to form large multicellular aggregates, which are thought to enhance antigen–MHC presentation to T-cells. Lymph follicles are rich in B-cells and, similarly to interdigitating cells, the follicular cells are thought to trap antigen, thus facilitating B-cell activation. Follicular dendritic cells express high levels of membrane receptors for antibody and complement (Szakal *et al.*, 1989).

Follicular dendritic cells have not been demonstrated in fish; however, there are indications that interdigitating cells are present in the thymus of sea bass. Organs containing lymphoid cells in fish are in the spleen and kidney, but the degree of organization of lymphoid tissue varies between species. Trapping of heterologous immune complexes or heat-aggregated antigen was observed in the spleen and pronephros of carp (*C. carpio*) and plaice (*Pleuronectes platessa*). In the spleen, immune complexes were trapped by cells with dendritic extensions in areas described as ellipsoids. Although it was shown that trapping required antibody,

it was not clear how the immune complexes were trapped. The significance of immune-complex trapping in fish is not clear, but, analogously to higher vertebrates, it may have a role in the establishment of the secondary antibody response (Gudding *et al.*, 1999). Members of the Ikaros multigene family of zinc finger proteins are expressed in a tissue-specific manner and most are critical determinants in the development of both B- and T-lymphocytes, as well as NK cells and dendritic cells. Studies conducted in the clearnose skate (*Raja eglanteria*) demonstrate specific expression patterns for two of the Ikaros genes (Haire *et al.*, 2000). The expression pattern was similar to that observed in mammals.

Lymphoid cells

Lymphocytes occur in large numbers in the lymph of the thoracic duct in mammals. Similarly, lymphocytes in fish are numerous in lymph, particularly in lymph of the neural lymphatic duct. A lymphatic system has been described for most teleosts, although in some fishes the presence of a true lymphatic system has been challenged (Vogel, 1985).

Lymphocytes are ovoid cells and, depending on the species of fish, they range in size (8 to 12 μm in diameter). As in mammals, fish have T-cells and B-cells and they are morphologically indistinguishable using light microscopy. However, both classes of lymphocytes have characteristically large nuclei, which fill almost the entire cell (Figs 9.2 and 9.3, L). Lymphocytes are mobile cells and they circulate throughout the body. In mammals, approximately 20–30% of circulating lymphocytes are antigen-reactive B-cells, 65–75% are T-cells and fewer than 5% are null cells. In contrast, fish have 12×10^3 lymphocytes/mm^3, while mammals have a much lower number, at about 2×10^3 lymphocytes/mm^3 (Ellis, 1986). The lymphocytes of *S. auratus* have different sizes, with small microvilli on the cell surface and scanty cytoplasm (Zuasti and Ferrer, 1989).

With the use of immunological assays, cell separation techniques and the development of distinct clonal leukocyte cell lines, the existence of T-, B- and NK cells have been confirmed in fish. In mammals, T-lymphocytes are formed in the bone marrow, migrate to the thymus and then undergo differentiation, division and development into mature antigen-reactive T-cells. The mature T-cells in mammals migrate to secondary lymphoid organs, such as the spleen, lymph nodes and mucosal-associated lymphoid tissue (MALT). MALT includes the intestinal villi, tonsils, the appendix and Peyer's patches.

A thymus gland also exists in all classes of fishes except the Agnatha (e.g. hagfishes, lamprey). In contrast to mammals, fish lack bone marrow and lymph nodes, and the head kidney functions as the major lymphoid organ, with the thymus, spleen and MALT functioning as secondary organs. After maturation in the thymus, lymphocytes in fish migrate to the spleen. Fish T-cells are still indirectly defined as Ig-negative lymphocytes because definitive surface markers have not been identified. Ig-negative lymphocytes respond in mixed leukocyte cultures (Scapigliati *et al.*, 2002), proliferate specifically to autologously processed and presented antigen (Jansson *et al.*, 2003), provide helper function for *in vitro* antibody responses and produce interleukin-like factors upon activation (Secombes *et al.*, 2001). Recent identification of teleost T-cell receptor alpha and beta genes has now permitted the unequivocal genetic demonstration that some of these Ig$^-$ cells are bona fide T-cells (Miller *et al.*, 1998).

The B-lymphocytes are derived from the bursa of Fabricius in birds and the bone marrow in mammals. In fish, these cells are formed in the kidney. As in higher vertebrates, B-lymphocytes can be distinguished by their surface immunoglobulin and they secrete antibody in response to antigenic stimuli. B-cells are directly defined by monoclonal antibodies to teleost Ig and identification of Ig heavy (H) and light (L) chain genes. As in mammals, fish B-cells show Ig H-chain gene rearrangements and allelic exclusion, produce both membrane-bound and secreted forms of Ig and transduce intracellular proliferative signals upon anti-Ig cross-linking. It has also been found

that some fish B-cells express a unique chimeric Ig chain with sequence homology to mammalian Ig (Wilson and Warr, 1992).

Both classes of lymphocyte recognize, bind and interact with antigens via unique antigen receptors on their membrane surfaces. A single lymphocyte or a clone of identical lymphocytes possesses monospecific receptors that essentially bind a single type of antigenic determinant (an epitope) (Townsend and Bodmer, 1989; Kantor, 1991).

B-lymphocytes

In mammals, subpopulations of B-lymphocytes have been identified. B-1 cells can be distinguished from B-2 cells because they express CD5, are capable of self-replenishing, have a specific tissue distribution (i.e. abundant in peritoneal and pleural cavity) and produce autoantibodies (Kantor, 1991; Herzenberg *et al.*, 1996). The CD antigens are cell-membrane molecules used to differentiate human leukocyte subpopulations. The CD antigens are identified based on the reaction of lymphocytes with monoclonal antibodies. Monoclonal antibodies that identify the same membrane molecule are grouped into a common cluster of differentiation (CD). B-1 cells form a minor population of the total splenic B-cell pool, are absent from lymph nodes and have distinct physiological properties from those of conventional B (B-2) cells. The bone marrow does not reconstitute B-1 cells, whereas either fetal liver (presumably precursors) or B-1 cells from the peritoneum (mature cells) can reconstitute them. B-1 cells are thought to be major contributors of IgM in the serum and IL-5 receptor (IL-5R) expression is found on B-1 cells that reside in the peritoneum (Hitoshi *et al.*, 1990).

The membrane antigen of the B-cell is immunoglobulin, a serum glycoprotein that is composed of two H and two L chains. B-cells are marked by copies of the same immunoglobulin molecule, which serves as an antigen receptor and subsequently determines the specificity of antibodies secreted by plasma cells. B-cells also express other non-immunoglobulin cell surface markers, including a complement receptor, a receptor for the F_c portion of immunoglobulin molecules, as well as class II MHC (Accolla *et al.*, 1991). In mammals, pre-B-cells are recognized by diffuse H chains of immunoglobulins (IgM class). As these B-cells differentiate and develop into mature antigen-reactive cells, they begin to express membrane-bound monomeric IgM at the cell surface. Later, immunoglobulins of other classes (i.e. IgG, IgD, IgE, IgA) may also reside on cell membranes (Tonegawa, 1983).

Plasma cells

Plasma cells are scattered throughout the splenic white pulp in fish. These cells are fully differentiated antibody-synthesizing B-lymphocytes. Mammalian plasma cells possess a unique cell antigen not found on their B-cell precursor; however, unlike B-cells, they do not have MHC antigens or surface immunoglobulin receptors. In mammals, the antibody secreted by a single plasma cell (or specific clone) may change, that is, the class gene in the cell may undergo rearrangements to express a new H chain isotype without altering the specificity of the antibody (Tonegawa, 1983). However, the antigen-binding specificity is identical to that of the receptor expressed by the original B-cell. It is not clear if this phenomenon occurs in fish (see later section). The plasma cell of *S. auratus* is described as having a well-developed granular ectoplasmic reticulum (Zuasti and Ferrer, 1989). Channel catfish B-lymphocytes proliferate in response to membrane IgM cross-linking. Similarly to what happens in mammals, there is a rapid increase in intracellular calcium levels early in the activation process. The B-cell does not exhibit such changes with LPS, a mammalian B-cell mitogen (van Ginkel *et al.*, 1994). However, there was a rapid induction of protein phosphorylation on activation.

In mammals, the clones of memory cells produced during the first exposure to antigen, which display the same antigen recognition specificity as their lymphocyte precursors, can develop into a second wave of plasma cells and memory cells upon subsequent encounter with the same antigen. This second wave of plasma cells is

responsible for the more rapid and intense response to the previously encountered foreign antigen. The identification of a subpopulation of memory cells in fish is a matter of some dispute. Generally, heightened secondary antibody responses are used as the most indicative measure of B-cell memory. Characteristics of fish memory generation include increased antibody titres (Miller and Clem, 1984a; Arkoosh and Kaattari, 1991), accelerated antibody responses (Arkoosh and Kaattari, 1991) and increased sensitivity to antigen (Arkoosh and Kaattari, 1991). However, the processes of isotype switching and affinity maturation do not appear to occur to the same degree as in mammals. Most studies suggest that a fish memory B-cell is not a physiologically distinct cell. Studies suggest that increased antibody response is not only due to increased antibody secretions, but also to an increase in the antigen-sensitive precursor pool (Rijker *et al.*, 1980; Arkoosh and Kaattari, 1991).

T-lymphocytes

In mammals, T-lymphocytes are formed in the bone marrow and then migrate to the thymus to differentiate, divide and become mature antigen-reactive cells. Fish have a well-developed thymus and can elicit cell-mediated immunity, which involves thymus-derived (T) lymphocytes. T-cells recognize antigens only on the surface of other cells that express cell-surface antigens encoded by MHC. They can be further subdivided based on their effector functions, as either cytotoxic or helper cells. Unlike B-cells, which express and secrete immunoglobulins, T-cells express a specific membrane-bound receptor called the T-cell receptor (TCR). The TCR is a member of the immunoglobulin superfamily and is a disulphide-linked glycoprotein heterodimer, which in mammals is expressed in association with CD3 (Ashwell and Klusner, 1990). Once mature, T-cells migrate to secondary lymphoid organs and are responsible for expression of cellular immunity. T-lymphocytes (in mammals) are divided into different subclasses of regulators (T_H cells and T_S cells) and effectors (T_D cells and T_C cells), which are differentiated based on their cell-surface antigens. A recent study isolated and identified all four TCR (α, β, γ, δ) genes in the Japanese flounder (*Paralichthys olivaceus*), providing unequivocal evidence that T-cells exist in teleosts (Nam *et al.*, 2003).

T-lymphocytes participate in both humoral and cell-mediated immune responses. In mammals, helper (T_H) and suppressor (T_S) T-cells contribute to the regulation of humoral immunity, while cytotoxic (T_C) T-cells and T-cell mediators of delayed-type hypersensitivity (DTH) (T_D) are involved in cell-mediated immunity. These specific cell-mediated immune responses are mediated by two fundamentally different effector T-lymphocyte subsets, which are discriminated on the basis of the cluster designation (CD) antigens they display. In mammals, mature T-cells leave the thymus and they express either CD2, CD3, CD4 or CD2, CD3, CD8 phenotypes (Van Ewijk, 1991). However, a very small number of cells express neither CD4 nor CD8. T-cells that display CD8 surface markers recognize and react specifically with antigen on target cells, causing their lysis, and are referred to as cytotoxic T-cells (T_C). A second type of T lymphocyte, referred to as a T_D (DTH) cell, is phenotypically CD4 and secretes lymphokines (e.g. IL-1) when stimulated by antigen. T_D cells differ from T_C cells in that they do not specifically kill (i.e. lyse) other cells, but instead recruit cells, such as macrophages, and control their activity. Both T_C cells and T_D cells show a memory response and are regulated by T_H (CD4) and T_S (CD8) cell populations. Most T-cell populations express either CD4 or CD8 cell antigens and are thus MHC-restricted. However, there are a few T-cell populations that do not express CD4 or CD8 and are thus not MHC-restricted.

In fish, a cell-mediated immune response has been demonstrated *in vitro* using a mixed leukocyte reaction (Stuge *et al.*, 1997), cytokine production and cell proliferation induced by mammalian T-cell mitogens (conconavalin A, phytohaemagglutinin) or antigens. *In vivo* studies have demonstrated graft rejection and DTH reactions (Nakanishi and Ototake, 1999). Also, antibody responses to T-dependent antigens in fish require

APC/T-cell/B-cell collaboration, which occurs in an MHC-restricted fashion (Townsend and Bodmer, 1989). T-like lymphocytes in fish do not have surface immunoglobulin, but respond to the mitogen conconavalin A (a mammalian T-cell mitogen). In a mixed leukocyte response, they proliferate with monocytes and accessory cells and they display MHC restriction. Monoclonal antibodies that recognize antigenic determinants expressed by T-cells allowed lymphocyte subpopulations involved in allograft rejection of muscle transplants to be identified in *D. labrax* (Abelli *et al.*, 1999). A study by Partula *et al.* (1995) isolated complementary DNA (cDNA) clones from rainbow trout (*Onchorhynchus mykiss*) that have sequences very similar to amphibian, avian and mammalian TCR-β chains. Putative TCR-α and TCR-β cDNAs were cloned from Atlantic cod (*Gadus morhua*). A monoclonal antibody, DL15, that is specific for thymocytes and peripheral T-cells of sea bass (*D. labrax*) was used to purify immunoreactive cells from blood and gut-associated lymphoid tissue. Using reverse-transcriptase PCR (RT-PCR), a cDNA was amplified from DL15-purified cells from gut-assoicated lymphoid tissue that had a high similarity to the TCR constant region, suggesting that these cells are T-lymphocytes (Scapigliati *et al.*, 2000). The lymphocyte subpopulations involved in allograft rejection of muscle transplants in *D. labrax* were studied. Immunohistochemical studies showed a high density of lymphocytes in these allografts, and the results indicated that they were predominantly T-cells (Abelli *et al.*, 1999).

In mammals, cytotoxic T-lymphocytes display antigen specificity and are usually detectable in the body only after multiplication and following antigen exposure. Extensive studies suggest that recognition of antigen by most T_C lymphocyte populations is class I MHC-restricted and these cells express CD8 differentiation antigens. However, a subset appears to exhibit class II MHC restriction and express CD4 differentiation antigens, which are characteristic of T_H cells. Regardless of which antigen is expressed, both sets recognize antigen by means of the TCR (Townsend and Bodmer, 1989).

As mentioned previously, some unique populations of T_C lymphocytes may not be MHC-restricted. These cells lyse a broad spectrum of tumour cells bearing different MHC antigens. In these cases, a tumour antigen is recognized by clone-specific T_C cells using the TCR; however, association with MHC is not required for lysis. Also, these non-MHC-restricted cytotoxic T-cell populations do not express CD4 or CD8 differentiation antigens.

The generation of T_D cells provides a second antigen-specific cell-mediated mechanism of resisting infection that is distinct from direct cytolysis by T_C cells. T_D cells are involved in host defence against intracellular pathogens, such as viruses, bacteria, fungi and protozoa. DTH responses may also occur whenever the body encounters histoincompatible cells or self-cells that have been altered/transformed such that they are recognized as foreign. T_D cells are capable of recruiting leukocytes from various parts of the body to a site that they have recognized as non-self, and help defend the host by secreting lymphokines. The relative ability of T_D cells to protect the body is mainly due to their capacity to produce and release lymphokines. Lymphokines can recruit, activate and regulate non-specific effector cells that have the potential to combat infectious agents. T-lymphocytes (CD4 phenotype) that are active in DTH reactions are quite different from cytotoxic T-cells (CD8) involved in lytic reactions because they recruit potential killers, such as macrophages, and control their activity (Townsend and Bodmer, 1989).

Natural killer cells

Human NK cells are bone-marrow-derived lymphocytes that share a common progenitor with T-cells, do not express antigen-specific cell-surface receptors and comprise 10–15% of all circulating lymphocytes (Middleton *et al.*, 2002). Owing to their early production of cytokines and chemokines and their ability to lyse target cells without prior sensitization (hence the term ‘natural killer’ cells), NK cells are crucial components of the innate immune system, providing a first

line of defence against infectious agents. NK cells also exert cytotoxicity by recognizing and inducing lysis of antibody-coated target cells (ADCC) through antibody binding to the receptor CD16 (Fcγ receptor III). Whereas they express CD16 and CD56, they lack the expression of CD3 and CD4. Approximately half of human NK cells express CD8 but only the α/α form. NK cells can be activated non--specifically by several cytokines, including IL-2, IL-15 and IL-18 (Seaman *et al.*, 2000). There are two subsets of NK cells – the $CD56^{dim}$ NK subset is more naturally cytotoxic – whereas the $CD56^{bright}$ subset has the capacity to produce abundant cytokines but has low natural cytotoxicity (Ogata *et al.*, 2001). Class I MHC glycoproteins are important in controlling the effector functions of both cytotoxic and NK cells. However, unlike T-cells, which recognize antigen as peptide fragments bound to MHC molecules, NK cells become functional in the absence of class I MHC proteins on target cells.

The NK cells in mammals are large granular lymphocytes (LGL) that have reniform nuclei and cytotoxic ability. These cells lack immunoglobulin or T-cell receptors but can recognize and destroy some tumour cells without MHC restriction. They are commonly found in the blood, as well as in the spleen and liver. NK cells are not phagocytic and lack the immunological specificity and memory displayed by T_C cells. These cells have the innate ability to recognize and destroy various tumour cells, virus-infected cells and some microorganisms. They are found in the spleen and lymph nodes, gall bladder, liver, intestine (particularly the lamina propria), lungs, respiratory tract and uterus. The NK cell has many regulatory functions, including regulation of growth and differentiation of haemopoietic stem cells and regulation of cell-mediated immunity, as well as antibody responses. NK cells also act as natural suppressor cells and are capable of producing cytokines. NK cells participate in control of infections caused by viruses, bacteria, fungi and intra- and extracellular parasites. They also inhibit the development of primary tumours and metastases and are involved in organ transplant rejection.

The evolutionary precursors to mammalian NK cells in teleost fishes are called non-specific cytotoxic cells (NCC) (Jaso-Friedmann *et al.*, 2001). Teleosts appear to have NK-like cells, and little is known of the immune function of these cells. They have been most extensively studied in catfish and are described as agranular with numerous surface villi, and the nuclei are more pleomorphic and smaller than those in mammals. These cells are located in the kidney, liver and spleen and appear to be involved in defence against protozoan parasites. In teleost fishes, NCC are important mediators of innate immunity against bacterial and parasitic infections and tumour growth (Ruiz *et al.*, 2001). Unlike mammalian NK cells and T-cells, activated NCC do not express membrane FasL (Fas ligand) (Evans *et al.*, 2001).

Mediators of the Immune System

Chemokines

Chemokines are small (70–100 amino acids (aa)), inducible, structurally related proteins that guide cells expressing the complementary chemokine receptors to sites of immune response and inflammation. They have been identified and studied extensively in mammals, but little is known of their presence in other vertebrate groups. Based on the position of the first two conserved cysteines, chemokines are classified into four subfamilies: C, CC, CXC and CX3C. Recently, there have been a number of cytokine homologues cloned from teleost fishes, including a number that resemble chemokines, but there have been few functional assays to determine the validity of the chemokine/cytokine cloned. Lally *et al.* (2003) cloned a CK-1 chemokine from rainbow trout, *O. mykiss*. A recombinant CK-1 was produced and it attracted peripheral blood leucocytes (PBL) in a micro-chemotaxis chamber. The tissue distribution of CK-1 was assessed using Northern blotting of PCR products, produced using RT-PCR. Rainbow trout CK-1 is in the liver and gut and, when injected with

phytohaemagglutinin in addition to PBL, it is induced in the head kidney. Alabyev *et al.* (2000) cloned a CXCR4 chemokine from the sterlet, *Acipenser ruthenus*. CXCR4 and its ligand, SDF-1, are known to be essential for haematopoiesis, organogenesis and immunomodulation in mammals. A CC cytokine was cloned from carp (*C. carpio*) (Fujiki *et al.*, 1999) and a cytokine that is a member of the CXC cytokine group was cloned from zebrafish (*D. rerio*). CXC resembles a mammalian BRAK chemokine (Long *et al.*, 2000). Also, several chemokine receptor sequences have been discovered in trout (Daniels *et al.*, 1999).

Cytokines

These are secreted by stimulated immunocompetent cells (e.g. macrophages) and they encompass regulatory proteins, commonly called growth factors (CSF), interleukins, lymphokines, monokines and interferons (IFN). Classifications of cytokines in higher vertebrates have been based on the structural motifs they adopt. The largest group of cytokines has an antiparallel 4-α-helical bundle structure and is subdivided into short-chain or long-chain helical bundles. Short-chain cytokines include IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13 and IFN-γ and large-chain cytokines include IL-6, IL-12, IL-11, IL-10 and IFNα/β. Another group of cytokines are classified as having long-chain β-sheet structures. In mammals, these include the tumour necrosis factor (TNF) family of cytokines (i.e. TNF-α, TNF-β, CD40, CD27 and Fas ligands) (Nicola, 1994). Fish cytokines have adopted this categorization (Secombes *et al.*, 2001).

Cytokines regulate the intensity and duration of an immune response by stimulating or inhibiting the proliferation of various immune cells or their secretion of antibodies or other cytokines. To date, cytokines with activities similar to IFN, IL-1, IL-2, 1 L-4, chemokines, macrophage migration inhibition factor (MIF), macrophage-activating factor (MAF) and CSF have been described from rainbow trout (*O. mykiss*), carp (*C. carpio*), Atlantic salmon (*Salmo salar*), sea bass (*D. labrax*) and flatfish (*P. olivaceus*) (Hardie *et al.*, 1994; Secombes *et al.*, 1994; Fujiki *et al.*, 2000a; Hirono *et al.*, 2000; Nam *et al.*, 2000; Laing *et al.*, 2001; Scapigliati *et al.*, 2002).

Several immune system genes were identified from rainbow trout (*O. mykiss*), including IL-1 receptor-related protein and two invariant-chain-like proteins, TNF-α decoy receptor and a novel chemokine, trout chemokine 2, which is most similar to the glycosylation stalk of fractalkine, a mammalian CX(3)C chemokine (Liu, L. *et al.*, 2002). No functional studies were performed.

Interleukin-1

Interleukins are specifically secreted by leukocytes and function in cell growth and differentiation. IL-1 is the term for two polypeptide mediators, known as IL-1α and IL-1β (Dinarello, 1997). In mammals, IL-1 affects cells of haemopoietic origin, from immature precursors to differentiated leukocytes, vessel wall elements and cells of mesenchymal, nervous and epithelial origin. The activity of IL-1 overlaps largely with that of TNF and other cytokines (Dinarello, 1997).

When mammalian macrophages and monocytes are appropriately stimulated, they secrete increasing amounts of IL-1. A number of nucleated cells are also capable of producing low levels of IL-1, including dendritic cells, Langerhans cells, endothelial cells, epithelial cells, neutrophils, B-lymphocytes and LGL. Macrophages may be stimulated to produce IL-1 by contact with activated lymphocytes (as seen in cytotoxic and DTH reactions). However, all cells capable of producing IL-1 can be stimulated by a variety of T and B lymphokines, mitogens and adjuvants, as well as by latex or silica particles (Dinarello, 1997).

Both IL-1α and IL-1β stimulate T-cells to produce lymphokines (IL-2, 3, 4 and 5) and IFN-γ, as well as chemotactic factors. During cytotoxic and DTH responses, IL-1 promotes both proliferation and differentiation of T_C or T_D lymphocytes. For a T-cell to be activated, it must be in contact with the antigen/MHC complex (on the APC), as well as IL-1. Contact with IL-1 by T-cells

promotes production of IL-2 by T_H cells and the expression of IL-2 receptors. Thus, T_H cells may serve as an IL-1 target, and, later, as both an IL-2 target and producer. Moreover, this cytokine promotes proliferation, differentiation and antibody production by B-cells, as well as promoting expression of their membrane immunoglobulin receptors (Dinarello, 1997).

The influence of IL-1 extends beyond lymphocytes. Monocytes, macrophages, neutrophils and NK cells are also mobilized and activated by IL-1. In mammals, during an inflammatory response, IL-1 can act as an endogenous pyrogen responsible for fever production and bring about changes in plasma levels of metals and acute-phase proteins. IL-1 can also produce an influx of leukocytes to the inflammatory site (Dinarello, 1997).

It has been known for some time that IL-1 activity exists in fish. Recently, this cytokine has been cloned in several teleost species. The most important structural difference between IL-1 of fish and that of mammals is the lack of a clear IL-1β-converting enzyme cleavage site. Thus, it is not known if the fish molecule is processed to a mature peptide. There is amino acid sequence evidence for the existence of IL-1β in fish (see Secombes *et al.*, 2001). The known IL-1-like activity in fish, together with the induction of the IL-1β transcript in leukocytes (after *in vitro* stimulation with LPS or *in vivo* challenge with bacteria), suggests that IL-1β is involved in the immune response of fish (Engelsma *et al.*, 2003).

IL-1β was cloned from sea bass (*D. labrax*) and there are two incompletely spliced transcripts; Southern blot analysis suggests the presence of only one gene copy (Buonocore *et al.*, 2003). The carp IL-1β gene has been cloned and identified. Its recombinant protein significantly stimulates proliferation of head kidney and spleen cells from carp (Mathew *et al.*, 2002).

Carp (*C. carpio*) full-length cDNAs, analogous to IL-1β, and the gamma subunit of the high-affinity F_c receptor for IgE ($F_c\varepsilon RI\gamma$) have been cloned. Higher amounts of IL-1β expressed in sodium alginate stimulate peritoneal cells and head kidney cells and this is associated with inflammatory response. The $F_c\varepsilon RI\gamma$ expressed in leucocytes indicates that the F_c receptor is functional in the carp immune system (Fujiki *et al.*, 2000b).

Specific gene expression of the pro-inflammatory cytokine IL-1β and the type II IL-1β receptor (IL-1R11 decoy receptor) was studied in the skin of rainbow trout fry during infection with the ectoparasite monogean *Gyrodactylus derjavini.* Generally, low levels of specific IL-1β, IL1β11 and IL-1R11 gene transcripts were found in infected hosts. In contrast, a clear induction of both IL-1β isoforms was observed during the initial phases of primary *G. derjavini* infections. A less obvious induction of IL-1β expression was seen in secondary infections imposed just after recovery from the primary infection (Lindenstrom *et al.*, 2003).

Interleukin-2 and interleukin-15

IL-2 was discovered as a T-cell proliferative factor purified from mitogen-stimulated lymphocyte cultures. This interleukin controls the amplification of naïve T-cells by initially stimulating growth following antigen activation, and later promotes the activation-induced cell death (Fehniger *et al.*, 2002). IL-2 also has effects on several other immune cells, including NK cells, B-cells, monocyte/macrophages and neutrophils. IL-15 was identified by two independent groups based upon its ability to stimulate proliferation of the IL-2-dependent T-cell line in the presence of neutralizing anti-IL-2 antibodies. Interleukin-15 mediates functions similar to those of IL-2 *in vitro* because signalling by IL-2 and IL-15 occurs via shared IL-2/IL-15Rβ and γ_c receptor subunits. They have different *in vivo* functions (Fehniger *et al.*, 2002).

IL-2 is a cytokine that is a potent growth and differentiation factor for T-cells. It also stimulates NK cells and lymphocyte-activated killer cells as well as exhibiting B-cell growth factor activity. In mammals, cells bearing the CD4 phenotype (i.e. T_H) produce, secrete and respond to IL-2; however, resting T-cells do not produce IL-2. Once these cells are activated, IL-2 production begins within

minutes and is detectable extracellularly several hours later. In mammals, IL-1 and a variety of neurohormones and surface-active compounds can enhance IL-2 production. Very low levels of IL-2 may be produced in several diseases where T-cell activity is depressed (Fehniger *et al.*, 2002).

In mammals, all subsets of T-lymphocytes, B-lymphocytes, macrophages and LGL develop IL-2 receptors and respond to IL-2. IL-2 promotes the production of IFN-γ, additional interleukins, CSF and lymphotoxin (produced by T_H/T_D cells) and enhances the cytotoxic activity of T_C cells. It also stimulates clonal expansion of B-lymphocytes, increases antibody formation and induces macrophages to become more cytotoxic (Fehniger *et al.*, 2002).

There is indirect evidence that IL-2 is present in fish. Normally, to test for IL-2 activity in lower vertebrates, leukocytes are stimulated with T-cell mitogens (e.g. conconavalin A or phytohaemagglutinin under *in vitro* conditions) and the activity of IL-2 is tested in supernatants. Using this approach, effects analogous to IL-2 have been noted in carp (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984; Ghanmi *et al.*, 1993).

Interleukin-8

IL-8 belongs to the CXC chemokine subfamily. It can activate and attract migratory neutrophils to an inflammation site. This cytokine has multiple functions, including inducement of lysosomal enzyme release from neutrophils, expression of adhesion molecules on neutrophils, chemotactic activity for basophils and T-lymphocytes. It was formerly called monocyte-derived neutrophil chemotactic factor (Mukaida *et al.*, 1998).

In fish, basal expression is most prominent in immune organs like the anterior kidney and spleen. Anterior kidney phagocyte-enriched leucocyte suspensions express IL-8 and this expression is up-regulated by brief (4 h) stimulation with PMA, but not LPS. Neutrophilic granulocyte-enriched leucocytes display chemotaxis to human recombinant IL-8, thus confirming CXC chemokine-mediated chemotaxis of neutrophilic granules in teleost fish, which is similar to the mammalian situation (Huising *et al.*, 2003). The IL-8 gene has been cloned from dogfish (*Triakis scyllia*) (Inoue *et al.*, 2003), rainbow trout (*O. mykiss*) (Laing *et al.*, 2002; Sangrador-Vegas *et al.*, 2002; Zhang *et al.*, 2002; Fujiki *et al.*, 2003) and flounder (*P. olivaceus*) (Lee *et al.*, 2001). In flounder injected with LPS, IL-8 was detected in the head kidney and spleen. It was also detected in LPS-stimulated leucocytes, but not in tissues of leucocytes of non-stimulated flounder.

Tumour necrosis factor

TNF-α is secreted by macrophages in response to inflammation and infection. It has a cytotoxic effect on tumour cells, but not on normal cells. It also induces numerous cell types to secrete various cytokines involved in the inflammatory response. TNF-β is secreted by T_H cells (TH1 subset) and T_C cells. It kills tumour cells and, similarly to TNF-α, it enhances the phagocytic activity of macrophages and neutrophils.

A TNF-α-like factor was produced by rainbow trout macrophages that had sequence similarity to mammalian TNF-α. Supernatants from LPS-stimulated trout macrophage cultures significantly enhanced neutrophil migration and macrophage respiratory-burst activity. These supernatants were also highly toxic to murine L929 cells, a cell line sensitive to mammalian TNF-α (Qin *et al.*, 2001). Two TNF-α genes were cloned and sequenced from carp (*C. carpio*) (Saeij *et al.*, 2003). *In vitro* stimulation of carp head-kidney phagocytes induced expression of TNF-α. A TNF-α-like gene was also identified in channel catfish (*I. punctatus*) (Zou *et al.*, 2003). This TNF was expressed in proliferating macrophage and T-cell lines, but not in B-cell or fibroblast lines. In another study in rainbow trout, head-kidney leucocytes and a macrophage cell line were exposed to two recombinant TNF molecules. Exposure of cells induced expression of a number of genes, including IL-1β, TNF-1, TNF-2, IL-8 and COX2 (Zou *et al.*, 2003).

Interferons

Interferons are divided into two major groups, type I and type II. Members of the type I IFN family have homology to each other, bind to the same cell surface receptors and have overlapping functions (Oritani *et al.*, 2001). The type I interferon family is comprised of IFN-α, IFN-β, IFN-ω and IFN-τ. IFN-α and IFN-ω are produced by leucocytes, while IFN-β is a fibroblast product. Although only small amounts of IFN are produced under normal healthy conditions, these can be dramatically increased during a viral infection. Some growth factors and cytokines also induce IFN-α/β production. IFN-α/β affects proliferation, differentiation and function of various cell types in the immune system. For example, type I IFNs suppress the antigen-specific or mitogen-induced proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes, while they induce IL-15 production in macrophages and IL-15 can enhance T-cell growth. In contrast, IFN-α/β has been reported to augment the lytic activity of NK cells and cytotoxic T-lymphocytes. Enhanced expression of cell-surface antigens of the MHC represents one important mechanism for IFN's influence on immunity. IFN-α/β further modulates the immune system functions by influencing the production of pro-inflammatory cytokines, such as IL-1, TNF-α and IL-8. Expression of the nitric oxide synthetase gene in macrophages is also IFN-sensitive (see Oritani *et al.*, 2001).

An IFN gene has been cloned and characterized from the zebrafish (*D. rerio*). Treatment with the known IFN inducer, polyinosinic acid-polycytidylic acid (poly-(I)-poly-(C)), increases IFN mRNA transcripts in zebrafish. Also, a putative IFN from puffer (*F. rubripes*) was present when the zebrafish IFN was used to BLAST (basic local alignment search tool) the puffer genome (Altmann *et al.*, 2003). Fish are known to produce substances that exhibit IFN activity, but IFN has not been cloned or purified (Renault *et al.*, 1991). However, IFN-induced genes have been cloned from several species of fish. Following viral infection or exposure to double-stranded RNA, catfish cells produce a soluble factor that functions similarly to mammalian IFN (Long *et al.*, 2004). Channel catfish ovary cells were injected with a reovirus or double-stranded RNA and up-regulation of IFN mRNA was observed. In addition, an antiviral factor was found in the culture medium. A recombinant catfish IFN was produced in COS-7 cells and had antiviral activity in ovary cells.

Transforming growth factor

Transforming growth factor-β (TGF-β) is a cytokine secreted by platelets, macrophages and lymphocytes. This cytokine chemotactically attracts monocytes and macrophages, induces IL-1 production by macrophages, inhibits proliferation of epithelial, endothelial, lymphoid and haematopoietic cells, limits inflammatory response and promotes wound healing. It also induces class switch to IgA.

TGF-β1 was detected in the liver, brain, muscle, kidney, heart, gills and spleen of sea bream (*S. auratus*), as well as in the head kidney, macrophages and blood leukocytes (Tafalla *et al.*, 2003). Also, a TGF was isolated and cloned from hybrid striped bass anterior kidney mononuclear cells. Higher levels were detected in mononuclear cells of peripheral blood than from either kidney or spleen (Harms *et al.*, 2000). A TGF-β was also cloned from *O. mykiss* (Daniels and Secombes, 1999).

Immunoglobulin

IgM is the main immunoglobulin in teleosts, although IgD has been recently described from the Japanese flounder (*P. olivaceus*) (Hirono *et al.*, 2003). The piscine immunoglobulin is classified as an IgM-like molecule that has a tetrameric structure (i.e. four basic units joined together), as opposed to the pentameric structure of IgM in higher vertebrates. The monomer is the basic unit of an immunoglobulin and it is composed of two H (each chain approximately 70,000 Da) and two L chains (each chain approximately 25,000 Da). Each immunoglobulin molecule has two H and two L chains per monomer and four monomers per molecule; thus the

approximate molecular mass of an immunoglobulin with an IgM structure is 760,000 Da. Each monomer is joined to the others by disulphide bonds. In some cases, fish immunoglobulins have a J-chain-like protein, which in mammals is involved in the polymerization of the monomers into the multimer (Weinheimer *et al*., 1971; Mestecky *et al*., 1975; McCumber and Clem, 1976; Hagiwara *et al*., 1985). The J chain has not been detected in the gar (*Lepisosteus osseus*), paddlefish (*Polydon spathula*), pike (*E. lucius*) or chum salmon (*Oncorhynchus keta*) (Weinheimer *et al*., 1971; Mestecky *et al*., 1975; Kobayashi *et al*., 1982). Gel electrophoresis assays are commonly used to detect the presence of the J chain. It was suggested that the J chain was undetectable in some fishes due to an unusual net charge on the J polypeptide that distorts its mobility in an electric field (McCumber and Clem, 1976). The IgM of channel catfish (*I. punctatus*) has been relatively well studied. It consists of ~700,000 Da H chains and ~22,000–25,000 Da L chains. The basic repeating structural unit is H_2L_2, referred to as the monomeric form, which contains two antigen-binding sites (Warr and Marchalonis, 1987).

Fish antibody molecules are of lower affinity (a measure of the binding strength between one binding site on an antibody and an antigenic determinant) and more diversity than those of mammals and birds. Although fish are generally considered to have only one class of immunoglobulin, the immunoglobulins display some heterogeneity. For example, the Chondrichthyes (i.e. sharks and rays) have a pentameric IgM-like immunoglobulin and among the Osteichthyes (i.e. salmonids and cyprinids) tetramers, dimers and monomers exist, some among the same species. In addition, some teleosts appear to have antibodies that differ in the structure of the disulphide bonds and the size of the heavy chain, as well as having antigenically different antibody subpopulations. However, affinity or avidity maturation, immunoglobulin class switching and the presence of a secretory piece on immunoglobulin found in secretions have not been demonstrated in fishes (Wilson and Warr, 1992).

Conclusions and Future Directions

Although considerable progress has been made in the understanding of the immune system of fin fish, there are still obvious gaps in our knowledge. There has been considerable success in the cloning and sequencing of immune-system-related genes, particularly chemokines and cytokines; however, functional studies need to be undertaken. For example, the majority of the chemokines and cytokines have not been cloned or purified. Although knowing a gene is present is useful information, it is imperative that studies be undertaken to determine if these genes are functional in the various fish species. Also, the genes for many of the TCRs have been cloned and yet it still remains to be determined if fish T-cells express surface receptors similar to those observed in mammals. Finally, affinity or avidity maturation, immunoglobulin class switching and the presence of true memory cells are other areas that should be studied.

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20 Molecular Approaches and Techniques

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Introduction

Molecular genetic techniques have in the last 15 years gained widespread use in the study of fish parasites. These techniques have been particularly effective in unravelling the complex life histories of various parasitic forms, and have played a key role in determining the precise taxonomic relationships between parasite species that are difficult to ascertain using traditional morphological techniques. In some instances, these studies have provided key insights into evolutionary biology as a whole. In addition, molecular tools have contributed to our understanding of the cellular biology of parasites, and have proved indispensable in the more practical realm of disease diagnosis and vaccine development.

The present chapter focuses primarily on the use of two methods, namely, the polymerase chain reaction (PCR) and DNA sequencing, in the analysis of fish parasites. This emphasis reflects the widespread use of these techniques in diagnostics, epidemiology and systematics. Specific applications are described for Myxozoa, Microspora, Ciliates, kinetoplastids, Icthyosporea and helminths. Along with PCR and DNA sequence analysis (which rely on DNA–DNA interactions in solution), solid-phase hybridization methods are also discussed, with particular emphasis on *in situ* hybridization as a means of identifying parasites in host tissues. Finally, gene cloning methods and their use in the rapidly emerging fields of genomics and vaccine development are described.

PCR and DNA Sequence Analysis: an Overview

PCR is among the most widely used methods in biology today. Briefly, the technique utilizes short oligonucleotide primers that flank a given region of DNA to amplify large amounts (μg–mg quantities) of a specific target sequence in successive rounds of primer annealing and strand elongation (DNA synthesis) catalysed by thermostable DNA polymerase enzymes. Once made, amplified DNA fragments are purified, using agarose gel electrophoresis, and either directly sequenced or introduced into a standard plasmid cloning vector for subsequent analysis (Sambrook *et al.*, 1989; Fig. 20.1).

Perhaps the most common targets for amplification in both diagnostic testing and phylogenetic studies are the 18S small subunit ribosomal RNA genes (SSU rDNAs) and the internally transcribed spacer regions of the rDNA loci (ITS domains) (Sogin and Silberman, 1998; Van de Peer *et al.*, 2000; Fig. 20.2). These rDNA genes evolve at a slow rate in eukaryotes and thus serve as reliable markers for the identification of

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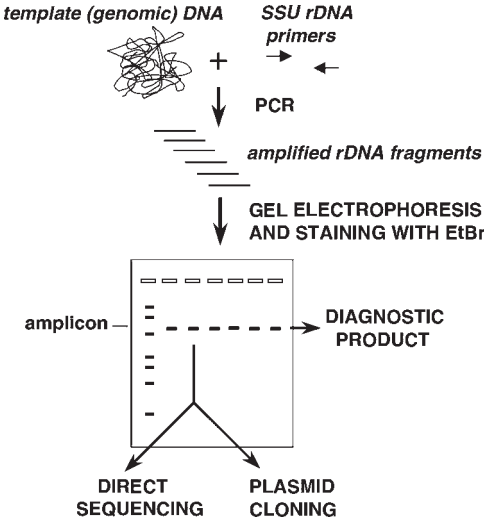


Fig. 20.1. Applications of PCR and DNA sequencing in molecular parasitology. Amplification of a DNA fragment from small subunit ribosomal RNA genes (SSU rDNA) is illustrated. Genomic DNA from infected host tissue (or from the agent itself) is used as a template for PCR amplification, along with forward and reverse primers complementary to regions of the rDNA sequence. Primers can be directed towards regions that are conserved among broad classes of organisms or towards sequences found only in particular species. Production of an amplicon of the expected size, following agarose gel electrophoresis and staining with ethidium bromide, is often diagnostic for the presence of a particular agent. A DNA size standard is depicted in the left lane of the gel. Definitive diagnosis can be achieved by sequencing of the purified PCR fragment (either directly or after insertion into a plasmid DNA cloning vector). Alternatively, the sequence can be used to infer evolutionary relatedness between the identified organism and other species. In the case of protein-coding genes, amplified PCR fragments can be used for a variety of other purposes, including the production of subunit vaccine antigens in heterologous cell types (typically yeast or *Escherichia coli*).

species. Indeed, the conserved nature of SSU rDNAs makes them ideal molecular 'clocks' for estimating evolutionary distances between widely diverged taxa. In contrast, the more rapidly evolving ITS domains are used to discriminate closely related species or, in some instances, strain differences within species. For some types of analyses, rDNA sequences alone are not sufficient and amplification of protein-coding genes becomes necessary.

For diagnostic testing, genomic DNA from the parasite in question is used as a template for PCR amplification, with primers that recognize sequences common to particular species or conserved amongst broader taxonomic groups. The choice often depends on the likelihood that a particular organism is present. In most instances, amplification does not require a highly purified template, owing to the sensitivity and specificity of PCR. Indeed, bulk DNA from infected tissues is often used as starting material for these reactions, since contaminating DNA from the host generally does not interfere with amplification of target sequences from the infecting agent. Resulting products (referred to as amplicons) are then fractionated using agarose gel electrophoresis and visualized under ultraviolet (UV) light, following staining with the fluorescent dye ethidium bromide. The presence of an amplicon of the expected size is generally sufficient to identify a particular agent, assuming proper controls are included. More stringent diagnostic tests include additional steps, such as digestion of purified amplicons with restriction endonucleases that recognize specific sequences within the target DNA (see below, PCR-restriction fragment length polymorphism (RFLP)). Restriction digests result in a complex pattern of bands (on agarose gel electrophoresis), which lend added confidence to the presumptive diagnosis. Clearly, the most definitive test and the one used in most phylogenetic studies involves the sequencing of amplicons to determine their primary structures. Following agarose gel electrophoresis, individual bands containing amplified DNA fragments are cut from the gel. The DNA is then purified on silica-based resins and either directly sequenced or introduced into plasmid cloning vectors for subsequent analysis (Fig. 20.1).

Although sequencing is still performed within individual laboratories, a more common practice is to outsource the DNA to a commercial or academic centre that employs



Fig. 20.2. Organization of ribosomal RNA loci. Small subunit (18S) and large subunit (28S) rRNA genes are separated by internally transcribed spacer regions (ITS1 and ITS2). The rDNA genes are expressed as a single large transcript that extends from the externally transcribed spacer region (ETS) just proximal to the small subunit gene, through the end of the large subunit gene. The transcript is processed into separate 18S, 5.8S and 28S rRNAs, which are then incorporated into the ribosome. The variable region at the proximal end of the large subunit gene (D1/D2), as well as the intergeneic non-transcribed spacer regions (IGS1 and IGS2) are sometimes used as targets for phylogenetic analyses.

standard dideoxy chain termination methods of sequence analysis (Sambrook *et al.*, 1989). Sequences are provided for the end-user in electronic form, which are then used to search comprehensive databases (e.g. GenBank; http://www.ncbi.nlm.nih.gov/) using standard algorithms such as the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990). An exact (or near) match is considered diagnostic for a given species. In the event that a novel sequence is found, a variety of computational methods (cluster analysis, maximum parsimony, maximum likelihood, neighbour-joining, etc.) can be used to define the taxonomic relationship(s) between the new or presumptive species and its closest relatives (see Swofford *et al.*, 1996). Comparisons of many sequences within and among taxonomic groups allow the establishment of phylogenetic trees. Inferred relationships are further strengthened by the inclusion of protein-coding gene sequences (in addition to rDNA) in such comparisons.

Applications of PCR and DNA Sequence Analysis in Diagnostics, Epidemiology and Systematics of Fish Parasites

Myxozoa

Analyses of SSU rDNA sequences have been particularly informative in studies of the Myxozoa (Chapter 8). Classification schemes based on SSU rDNAs separate these organisms into two classes, the Myxosporea, which alternate between vertebrate (primarily fish) and annelid hosts, and the Malacosporea, which alternate between fish and bryozoan hosts (Kent *et al.*, 2001). Partial or full-length 18S rDNA sequences are now available for a wide range of myxozoan species (Kent *et al.*, 2001) and have been used in the development of PCR-based diagnostic tests for detection of these organisms in fishes and in the aquatic environment. Such tests have clear advantages in terms of speed, sensitivity and specificity relative to more classical methods of detection, and have been applied to a number of species that are of importance to commercial aquaculture. For example, primer pairs that specifically amplify rDNA sequences of the enteropathogen *Ceratomyxa shasta* have been used to develop a simple diagnostic test for this pathogen in salmonids (Fig. 20.3). One such pair can be used to detect as little as 50 fg of purified *C. shasta* genomic DNA. The test is able to detect organisms in gill tissues of rainbow trout (*Oncorhynchus mykiss*) within 2 days of exposure of naïve fish to the actinosporean stage of the parasite (Palenzuela *et al.*, 1999). Diagnostic tests for several other important myxozoans affecting farm-raised fish are reviewed briefly.

A PCR-based test that amplifies a 104 bp SSU rDNA fragment from *Henneguya ictaluri* has proved extremely useful in detecting the proliferative gill disease agent of channel catfish (*Ictalurus punctatus*). The primers used for this test are highly specific for *H. ictaluri* and do not amplify detectable fragments from genomic DNA of other Myxozoa (including *Henneguya exilis*,

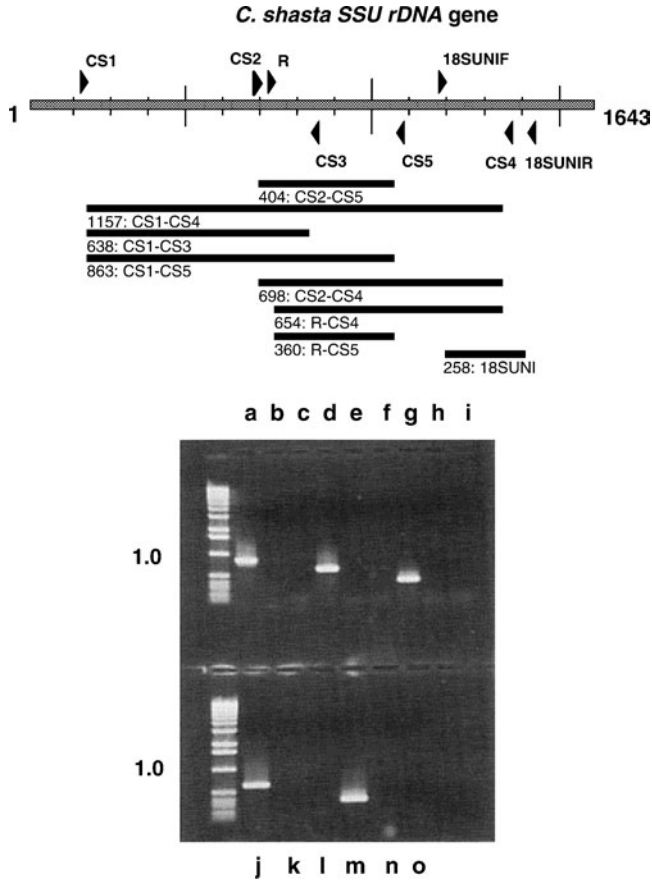


Fig. 20.3. (Top panel) SSU rRNA gene primer map for *C. shasta*. Predicted amplicon sizes for different combinations of the primers (Cs1, Cs2, R, Cs3, Cs4, Cs5, 18SUNIF and 18SUNIR are shown in base pairs. Bottom panel shows specificity of different primer pairs for *C. shasta* DNA. Amplicons of the predicted size were obtained with DNA from *C. shasta* spores (lanes a, d, g, j and m), but not rainbow trout (lanes b, e, h, k and n) or *Henneguya salminicola* (c, f, i, l and o). Primer pairs were Cs1–Cs5 (lanes a–c); Cs1–Cs3 (lanes d–f); Cs2–Cs5 (lanes g–i); R–Cs4 (lanes j–l); and r–Cs5 (lanes m–o). (From Palenzuela *et al.*, 1999 with permission from *Diseases of Aquatic Organisms*.)

Aurantiactinomyxon mississippiensis and *Helioactinomyxon* sp.) shed by the alternative invertebrate host, *Dero digitata* (Hanson *et al.*, 2001). In addition to *H. ictaluri*, SSU rDNA sequences have been determined for several other freshwater and marine species of *Henneguya* (Kent *et al.*, 2001).

Both single-round and nested PCR tests have been developed for detection of the whirling disease agent, *Myxobolus cerebralis*, in salmonids. Both tests have been shown to be more reliable indicators of infection (particularly at low parasite load) than the more standard pepsin–trypsin digest method, which relies on microscopic detection of parasites released from enzymatically digested tissue (Andree *et al.*, 1997, 1998; Schisle *et al.*, 2001). PCR-based tests also provide definitive diagnoses of *M. cerebralis* in host tissues, in contrast to less specific microscopic methods (Frasca *et al.*, 1999). Single-round PCR tests conducted on large numbers of free-ranging and hatchery-reared fish have suggested that this agent may be present in locations once thought to be free of the parasite

(Schisle *et al.*, 2001). Nested PCR, which is typically more sensitive than single-round reactions owing to successive rounds of amplification with different sets of primer pairs, can detect the equivalent of one sporoplasm of *M. cerebralis* per sample of infected tissue. The 415 bp amplicon generated by this method can also be detected in tissue samples as early as 1 week following exposure of rainbow trout to the actinosporean stage (Andree *et al.*, 1998). In comparative studies of SSU and ITS rDNA sequences from parasites collected in different geographical locations, relatively little sequence divergence was found among isolates from the USA and Germany (Andree *et al.*, 1999a). This lack of divergence provides support for the suggestion that *M. cerebralis* was introduced relatively recently into the USA from Europe. The parasite was first reported in North America in 1958. Similar analyses of 18S rDNA from ten different species of *Myxobolus* placed these into three distinct clusters that were independent of spore morphology, spore size and host specificity. The phenotypic trait most closely correlated with the rRNA-based clusters appeared to be tissue tropism, as these organisms infect muscle, neuronal or enteric sites (Andree *et al.*, 1999b). Additional applications of SSU rDNA analysis to the Myxobolidae include the identification of distinct species among the morphologically similar types *Myxobolus elegans* (Kashkovsky) and *Myxobolus hungaricus* (Jaczo) (Eszterbauer, 2002).

PCR-based (SSU rDNA) tests have also been developed for *Kudoa* spp., an emerging group of disease-causing agents of marine fishes. Such tests have permitted the diagnosis of *Kudoa thyrsites* in net-pen-reared Atlantic salmon, where it causes myoliquefaction and problems with flesh quality. PCR-based tests have suggested that tube-snouts (*Aulorhynchus flavidus*) are reservoirs for infection of commercial salmon fisheries (Shaw *et al.*, 1997). Tube-snouts were often found in the vicinity of net pens, and a high prevalence of *K. thyrsites* infection (up to 100%) was shown in fish collected near Vancouver, British Columbia (Shaw *et al.*, 1997). The genus *Kudoa* is found worldwide, and SSU rDNA analysis has permitted definitive diagnosis of particular species (for example, *K. thyrsites* and *K. amamiensis*) across a broad geographical range, including waters off eastern Australia (Whipps *et al.*, 2003). At least six species of *Kudoa* have been inferred based on SSU rDNA analysis (*K. thyrsites*, *K. paniformis*, *K. ciliatae*, *K. crumena*, *K. amamiensis*, and *K. miniauriculata*) (Kent *et al.*, 2001). The total number of species remains unknown, although analyses of infected tissues of a bullseye puffer (*Sphoeroides annulatus*) and sweeper (*Pempheris ypsilychnus*) have suggested the existence of at least two more species, namely, *Kudoa dianae* and *Kudoa minithyrsites*, respectively (Dykova *et al.*, 2002; Whipps *et al.*, 2003).

PCR-based diagnostic tests have been applied to less prevalent myxozoan species, such as *Parvicapsula minibicornis* and *Enteromyxum scophthalmi*. Epidemiological studies in migrating sockeye salmon in the Fraser River (British Columbia, Canada) found that the infection rate with *P. minibicornis* increased dramatically as fish entered their spawning grounds, with prevalence reaching ≥ 95% while it was 0% for fish while at sea. Infections were also detected in other salmon species (Jones *et al.*, 2003). In the case of *E. scopthalmi*, SSU rDNA analysis has suggested that this parasite represents an entirely new genus (Palenzuela *et al.*, 2002). *E. scophthalmi* has been identified as an enteric pathogen that can be lethal to cultured turbot (*Psetta maxima*). Based on rDNA analysis, it appears taxonomically related to *Myxidium leei*, which had previously been ascribed to the genus *Myxidium* on morphological grounds. It has been suggested that *M. leei* be renamed *Enteromyxum leei* and grouped with *E. scopthalmi* as members of the new genus, *Enteromyxum*, consisting of histozoic and enteric pathogens of marine fin fishes (Palenzuela *et al.*, 2002).

In addition to Myxosporea, PCR-based tests have been developed for Malacosporea, in particular *Tetracapsuloides bryosalmonae*. This is the causative agent of proliferative kidney disease (PKD) in salmonids (Saulnier and de Kinkelin, 1997;

Kent *et al.*, 1998; Hedrick *et al.*, 2004). Such tests have been used to detect parasites in infected fish and to distinguish *T. bryosalmonae* from other myxozoa within the class Malacosporea, including the worm-like parasite *Buddenbrokia plumatellae* and an as yet unidentified species belonging to what may be a third genus (Canning *et al.*, 2002; Hedrick *et al.*, 2004).

In addition to their practical application in diagnostics, molecular genetic analyses have played critical roles in unravelling the complex life cycles of myxosporeans. Comparisons of 18S rDNA sequences provided the first direct evidence that organisms previously considered to be members of different taxonomic classes (that is, Myxosporea and Actinosporea) were in fact different forms of the same organism alternating between vertebrate and invertebrate hosts (Andree *et al.*, 1997; Bartholomew *et al.*, 1997). This was demonstrated at about the same time for both *C. shasta* and *M. cerebralis*. In the case of *C. shasta*, primers specific for the parasite were able to detect relevant rDNA sequences in *Manayunkia speciosa*, a polychaete worm that was also capable of transmitting *Ceratomyxa* infection to susceptible fish (Bartholomew *et al.*, 1997). Annelids that could not transmit infections had no detectable *C. shasta* sequences. Similarly, 18S rDNA sequences from *M. cerebralis* were detectable using PCR in both infected fish (harbouring the myxosporean stage) and aquatic oligochaete worms (harbouring the triactinomyxon stage of the parasite) (Andree *et al.*, 1997). Further studies using this technique showed that 2–3% of tubifex worms in areas that are endemic for whirling disease actually carry the parasite (Rognlie and Knapp, 1998). Such worms (*Tubifex tubifex*) appear to be persistently infected, and can release the triactinomyxon stage periodically into the environment (Gilbert and Granath, 2001). Primers specific for rDNA sequences of *H. ictaluri* detected the actinosporean stage of the parasite (*Aurantiactinomyxon ictaluri*) in the aquatic oligochaete *D. digitata* (Pote *et al.*, 2000). Analogous PCR-based studies have shown that the malacosporean *T. bryosalmonae* is present in a range of bryozoan host species collected in diverse habitats, with temporal and spatial variations in the incidence of infection (Okamura *et al.*, 2001).

Finally, classification of the Myxozoa in respect of higher-order taxonomy has relied heavily on gene sequence data. Based on morphological criteria alone, the phylum was difficult to classify, and the Myxozoa were originally placed with the cnidarians. While there is some support for this hypothesis based on SSU rDNA sequence analysis, a number of studies suggest that they either fall within or are sister to the Bilateria (see Kent *et al.*, 2001; Canning and Okamura, 2004). This view is bolstered by the analysis of protein-coding genes, in particular *Hox* genes whose sequences in Myxozoa align with those of bilaterians (Ferrier and Holland, 2001; Finnerty, 2001). However, a more recent analysis of a gene that encodes a cathepsin-like protease has suggested that the Myxozoa may actually predate the bilaterians (Kelley *et al.*, 2003).

Microspora

The microsporidia are an extremely interesting group of intracellular parasites that infect vertebrate and invertebrate hosts (Chapter 7). More than 150 species are known to infect fishes (Lom and Nilsen, 2003). Microsporidia may cause either destruction of the host cell cytoplasm or hypertrophy and the formation of an enlarged host cell/parasite complex referred to as a xenoma. In fish, microsporidia are found in different host tissues, including gills, muscle, nerve, blood, gonads, liver and intestine, where they cause severe disease. Although morphological criteria have been used to classify the microsporidia, SSU rDNA sequence analyses have been far more informative in respect of phylogenetic relationships within this group. While a detailed phylogenetic tree for microsporidia is still emerging, sequence data are available for 12 of the 15 microsporidian genera known to infect fish. SSU sequence comparisons suggest that these cluster into five taxonomic groups,

four of which form a single clade. The outlying group (*Nucleospora*) is only distantly related and probably represents an entirely different taxonomic order (Nilsen, 2000). Supporting this argument is the fact that SSU ribosomal genes from *Nucleospora salmonis* and species belonging to the main clade share only ~ 60% sequence identity overall (Lom and Nilsen, 2003).

The high degree of conservation of SSU sequences sometimes obscures relationships between closely related species within the individual microsporidian group. However, ITS regions (which vary in length by a factor of 10 among different microsporidia), as well as protein-coding genes, often provide the necessary data to resolve the relationships between individual species (Lom and Nilsen, 2003). Interestingly, within the principal clade of microsporidia infecting teleosts, a number of species appear to be specific for hosts other than fish (including crustaceans, insects and humans). If this host specificity can be verified, similarities in the SSU sequences between fish-specific parasites and the non-fish species would suggest that the switch to non-fish hosts is a fairly recent event (Lom and Nilsen, 2003).

At the higher taxonomic level, initial studies of SSU rDNA sequences suggested that microsporidia evolved early in the eukaryotic lineage and were considered to be primitive protozoans (Vossbrinck *et al.*, 1987). Nevertheless, accumulating data from both SSU and protein-coding genes, including tubulin (Edlind *et al.*, 1996; Keeling and Doolittle, 1996), HSP70 (Germot *et al.*, 1997; Hirt *et al.*, 1997; Peyretaillade *et al.*, 1998) and RNA polymerase II (Cheney *et al.*, 2001), have indicated that the microsporidia are related to fungi and have evolved more recently than previously thought.

Apart from their use in phylogenetic studies, PCR-based analyses of SSU rDNAs have been useful as diagnostic tools for the detection of microsporidian infections in fishes. Single and nested PCR tests have been developed for *Microsporidium seriolae* (Bell *et al.*, 1999), *Pleistophora anguillarum* (Hung *et al.*, 1998), *Loma salmonae* (Docker *et al.*, 1997), and *N. salmonis* (Barlough *et al.* 1995; Gresoviac *et al.*, 2000). These tests are highly sensitive and can detect parasites in host tissues at the pre-spore stage, and as early as 3 days post-infection (Hung *et al.*, 1998; Bell *et al.*, 1999). In *N. salmonis*, diagnostic PCR tests have been used to look for divergence among parasites infecting salmonids and other fish hosts in different geographical locations. Sequences derived from amplified rRNA genes (along with their respective ITS domains) have been shown to be highly conserved, suggesting that *N. salmonis* forms a relatively homogeneous group across a broad geographical range (Gresoviac *et al.*, 2000). A rapid assay for differentiating rDNA sequences from microsporidia of fish, insects and shrimps was developed by Pomport-Castillon *et al.* (1997). In this assay, fragments of SSU and ITS sequences, amplified using PCR, are subjected to restriction endonuclease digestion to generate RFLPs, which are screened visually on agarose gels. The technique, alternatively known as riboprinting or PCR-RFLP (Graham, 1997), avoids the requirement for sequencing DNA fragments from individual isolates (Fig. 20.4).

Ciliates

SSU rDNA sequence analysis has been applied to the two most important parasitic ciliates of commercially raised fish, namely, *Ichthyophthirius multifiliis*, the causative agent of white-spot disease in freshwater fish, and *Cryptocaryon irritans*, its saltwater counterpart (Chapter 4). These species were long considered to be taxonomically related because of their similar life cycles and morphologies. Nevertheless, rDNA sequence analysis has indicated that *I. multifiliis* and *C. irritans* are highly diverged. *I. multifiliis* is one of two genera within the group Ophryoglenina that is sister to the tetrahymenines (class Oligohymenophorea) (Wright and Lynn, 1995). *C. irritans*, on the other hand, is only distantly related and is in a different class, the Prostomatea (order Prorodontida) (Diggles and Adlard, 1995; Wright and Colorni, 2002). Analysis of rDNA sequences from individual isolates of *Cryptocaryon* from Australia (Diggles and

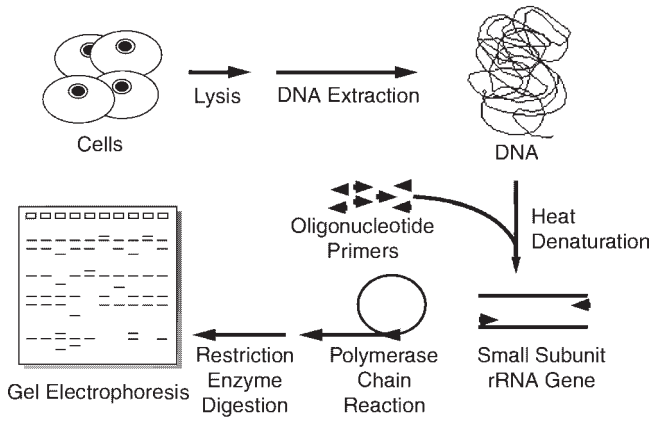


Fig. 20.4. Riboprinting. Specific primers (in this example for SSU rDNA) are used to amplify regions of DNA by PCR. PCR products are then digested with a given restriction endonuclease. Sequence polymorphisms among individual organisms, strains or species are manifested as different patterns of bands on agarose gels. (From Clark, 1997. With permission from *Journal of Eukaryotic Microbiology*.)

Adlard, 1997) and Taiwan (Yambot *et al.*, 2003) suggests the existence of strains that may occupy different ecological niches. Because *C. irritans* appears capable of changing its morphological and developmental characteristics, rDNA analysis is likely to provide the most reliable marker for strain differences in this species (Yambot *et al.*, 2003).

In the case of *Ichthyophthirius*, rDNA sequence analysis has been used to estimate the age of the ciliate phylum and, by inference, the 'crown' group eukaryotes as a whole. The rate of nucleotide substitutions within the rDNA of ciliates was estimated by comparison of SSU sequences of *Ichthyophthirius* and its closest free-living relative, *Ophryolgena*, using the earliest emergence of freshwater fish in the fossil record as a reference point. Based on this, the ciliates are predicted to have arisen in the Palaeoproterozoic, roughly 2 billion years ago (Wright and Lynn, 1997). The prediction is based on a number of assumptions, and suggests that the 'crown' group of eukaryotes (Knoll, 1992) evolved much earlier than previously thought.

A more practical application of sequence data has been the development of a PCR assay that distinguishes *I. multifiliis* from *Tetrahymena corlissi* in mixed cultures, using primers specific for regions of the SSU rDNAs of these organisms (Pugovkin *et al.*, 2001). The test is designed to ensure that *I. multifiliis* maintained in artificial media is free of contaminating *T. corlissi*.

Kinetoplastids

The kinetoplastids (Chapter 3) are a group of flagellated protozoa (phylum Euglenozoa) that share a common structural feature, the kinetoplast, which lies within the single mitochondrion at the base of the flagellum(a). This structure comprises a network of circular DNA molecules containing genes for mitochondrial proteins. However, unlike standard protein coding genes, those within the kinetoplast undergo an unusual process of post-transcriptional RNA editing in order that their sequences can be translated in an appropriate fashion (Maslov *et al.*, 1994; Vickerman and Coombs, 1999). The kinetoplastids consist of two or more suborders, the most widely recognized being Trypanosomatina, a group of exclusively parasitic forms that have a single flagellum (including the well-known *Trypanosoma* and *Leishmania* species), and Bodonina, which include free-living heterotrophic, ectocommensal and parasitic forms with two flagella. A number of species within each group are parasitic on/in

fishes. With the inception of SSU rDNA sequence analysis, the taxonomic relationships of such species within the larger kinetoplastid order have changed dramatically relative to the more classical scheme based on morphological features alone (Maslov *et al.*, 1996, 2001; Hollar *et al.*, 1998; Callahan *et al.*, 2002). Nevertheless, even molecular approaches have left a comprehensive phylogenic tree for the kinetoplastids in doubt, due in part to the paucity of data for many members of the group (Maslov *et al.*, 2001). In this regard, a recent study that included *Ichthyobodo necator* and a number of other important fish parasites has suggested a novel taxonomic order for the kinetoplastids (Callahan *et al.*, 2002). While previous work had indicated the existence of two well-defined suborders (consisting of Trypanosomatina and Bodonina), the inclusion of SSU rDNA sequences from *I. necator* splits the order into two different sublineages, one consisting of *Ichthyobodo* itself, and the other containing all other kinetoplastid species (Callahan *et al.*, 2002). Whether this alternative view prevails remains to be determined. However, even for the previous trees, 18S rDNA sequence analysis clearly indicates that several genera (as defined morphologically) within the suborders Trypanosomatina and Bodonina are polyphyletic, indicating that a reordering of these taxa will almost certainly occur (Maslov *et al.*, 2001).

Classification schemes for the kinetoplastids are interesting not just from the standpoint of taxonomy, but in terms of how parasitism evolved. In the bonodids, free-living and parasitic species intermingle within distinct lineages, and current schema (which include the trypanosomatids) suggest that a parasitic life style may have evolved independently a number of times during the evolution of the kinetoplastids (Maslov *et al.*, 2001).

Ichthyosporea (Mesomycetozoea)

Molecular genetic analyses have been instrumental in the classification of Ichthyosporea, a discrete taxon consisting primarily of protozoan parasites of fishes and crustaceans. Known alternatively as DRIPs (an acronym representing the genera *Dermocystidium*, the rosette agent (recently *Sphaerothecum destruens* Arkush *et al.*, 2003), *Ichthyophonus* and *Psorospermium*), this group maintained an enigmatic standing prior to phylogenetic analyses of rDNA and mitochondrial protein-coding genes. Based on SSU rDNA sequencing, these organisms (along with additional genera, such as *Rhinosporidium*) are clearly members of a distinct class of organisms that arose near the metazoan–protozoan boundary (Ragan *et al.*, 1996; Herr *et al.*, 1999). Indeed, their position in the descent leading from single-celled protists to metazoa is extremely interesting from an evolutionary standpoint, and prompted the recent sequencing of the entire mitochondrial genome of *Amoebidium parasiticum*, an ichthyosporean that can be readily cultured. Analysis of the content and architecture of the mitochondrial genome of *A. parasiticum*, when compared with mitochondrial DNA (mtDNA) from choanoflagellates (represented by *Monosiga brevicollis*) and Metazoa, indicates that these three lineages comprise a monophyletic assemblage (termed Holozoa) separate from the other major eukaryotic clades, including fungi. Based on likelihood tests of tree topologies generated from comparisons of 11 conserved mitochondrial proteins, the Holozoa appear to have evolved from a common ancestor, with Ichthyosporea emerging first, and Metazoa and choanoflagellates later as branching sister taxa (Lang *et al.*, 2002; Burger *et al.*, 2003). A few Ichthyosporea have evolved into fish parasites, thus falling within the lineage that led directly to the formation of sponges and other extant animals, and were among the first organisms to separate this lineage from the fungi. Given their phylogenetic position, Ichthyosporea have been renamed Mesomycetozoea (Herr *et al.*, 1999), and have been further subdivided into the orders Ichthyophonida and Dermocystida based on gene sequence, life style and morphological criteria (see Mendoza *et al.*, 2002). Although computational analyses of sequence data for the nuclear genes

encoding elongation factor 1-α from Ichthyosporea, animals and fungi do not provide a clear picture of the relationships between these organisms, they clearly group all three within a separate clade, known formally as the Opisthokonta (Ragan *et al.*, 2003).

Analyses of the mtDNAs of the Holozoa provide additional, interesting information regarding the evolution of the genome structure of this organelle. While Metazoa have relatively uniform compact (13–19 kb) mitochondrial genomes, consisting of closed circular DNAs present as monomers and concatenated oligomers, mtDNAs in other eukaryotic clades (including plants, fungi and protozoa) vary greatly in terms of their size and overall structures. Interestingly, the circular mtDNAs of choanoflagellates are up to four times as large as those in Metazoa and have roughly twice the number of protein-coding genes. Mitochondrial DNAs of Ichthyosporea are even larger (up to ten times the size), and consist of several hundred linear chromosomes that share elaborate terminal repeat structures (Burger *et al.*, 2003). The differences in mtDNA structure within various branches of the Holozoa would clearly suggest that compaction of the mitochondrial genomes seen in animals occurred at or near the time of emergence of a multicellular body plan.

Helminths

Nucleotide sequence analyses of rDNA and protein-coding genes have been widely applied in epidemiological and phylogenetic studies of parasitic helminths of fish. These approaches are particularly useful in uncovering cryptic or sibling species whose members are more or less morphologically similar. In addition, they relate to important issues in parasite evolution, including the idea that host switching plays a role in the speciation of at least some taxa. Examples of the use of these techniques are summarized briefly below, with primary emphasis on species that have an impact on wild and commercial fisheries.

Monogenean parasites (Chapter 9) belonging to the genus *Gyrodactylus* have been classified into 400 species on morphological grounds, although the true number may exceed 20,000 (Zietara and Lumme, 2002). A number of these species are pathogenic to fishes and have serious effects on wild populations, particularly those of Norwegian Atlantic salmon. Genetic markers that can distinguish species and/or intraspecific (i.e. strain) differences that relate to pathogenicity have long been sought. Because of its economic impact, considerable emphasis has been placed on *Gyrodactylus salaris*. The parasite has been identified in rivers and streams across Northern Europe, where it infects both Atlantic salmon (*Salmo salar* L.), and farmed rainbow trout. Disease outbreaks in specific locations have suggested that pathogenic strains of *G. salaris* may exist. However, pathogenicity has not been linked to any particular morphological trait(s). Indeed, while intraspecific differences in host adaptation appear to exist, the genus as a whole is morphologically quite similar. At the molecular level, neither SSU nor ITS rDNA sequence analysis has been particularly useful for discriminating strains. For example, ITS sequences are identical between *G. salaris* isolates obtained from different host species across a wide geographical range and fail to discriminate *G. salaris* from *Gyrodactylus thymalli*, its avirulent sister group that is specific for European grayling (*Thymallus thymallus*) (Cunningham, 1997; Zietara and Lumme, 2002). However, analyses of the intergenic spacer (IGS) regions of rDNA, as well as the mitochondrial cytochrome c oxidase I (COI) gene, have been far more revealing (Sterud *et al.*, 2002; Cunningham *et al.*, 2003; Hansen *et al.*, 2003; Meinilä *et al.*, 2004). While these studies have failed to yield markers for pathogenic strains, they provide considerable insight into the phylogeny of *G. salaris* and its sister group *G. thymalli*, and suggest an interesting hypothesis regarding mechanisms of speciation within the genus. Analysis of IGS rDNA sequences of *Gyrodactylus* collected at different locations from different types of fish show clear differences in the number and sequence of individual 23 bp repeats within the two

repetitive regions of the IGS domain (Sterud *et al.*, 2002; Cunningham *et al.*, 2003). While IGS elements were too conserved to make inferences regarding the geographical spread of the parasite, analysis of these regions showed unequivocal separation of *G. salaris* and *G. thymalli* and presumptive divergence of *G. salaris* into forms that infect either salmon or trout (Cunningham *et al.*, 2003). Two independent studies of the mitochondrial COI gene were equally informative. While they differed in their precise conclusions, each study identified multiple clades of both *G. salaris* and *G. thymalli* (Hansen *et al.*, 2003; Meinilä *et al.*, 2004). Furthermore, consistent with IGS sequence analysis, phylogenetic trees based on mitochondrial COI data showed that these clades are specific for either salmon (*G. salaris*), both trout and salmon (*G. salaris*) or grayling exclusively (*G. thymalli*) (Hansen *et al.*, 2003; Meinilä *et al.*, 2004). The fact that neither *G. salaris* nor *G. thymalli* appears to be monophyletic leaves open the question of whether they constitute: (i) a single polytypic species; (ii) two polytypic species; or (iii) a complex of more than two sibling species (Hansen *et al.*, 2003). Irrespective of the alternatives, the linkage between parasite clades and host species suggests that host switching (either in addition to or separate from geographical isolation) may underlie speciation within the gyrodactylids (Brooks and McLennan, 1993; Cribb *et al.*, 2002; Huyse and Volckaert, 2002; Poulin, 2002; Meinilä *et al.*, 2004).

Molecular genetic analyses of other parasitic helminths of fishes also suggest co-speciation of parasites and their hosts. For example, tapeworms (Chapter 11) of the genus *Bothriocephalus* infect a broad range of fish hosts that belong to distantly related taxa. Nevertheless, individual parasite species appear to be restricted to a given fish host. Since host species in this case extend across a broad taxonomic range, it becomes possible to examine whether particular hosts and their parasites evolved in parallel (through co-evolution) or whether switching to different hosts led to the emergence of different bothriocephalids. Verneau *et al.* (1997a,b) employed two complementary approaches (namely, DNA/DNA hybridization and ITS rDNA sequence analysis) to determine phylogenetic relationships among seven marine bothriocephalid species, and compared these with the lineages of their fish hosts. The study showed little congruence between the branching patterns of the different bothriocephalid species and their host species. Indeed, it appears that the majority of the parasites (six of seven species) have diverged recently, and it seems clear that switching events (leading to speciation) have occurred since the emergence of at least some host groups.

Along with marine bothriocephalids, sequence analysis of the ITS rDNA domains of the freshwater tapeworm *Bothriocephalus acheilognathi* has revealed the existence of multiple genotypes (distinguished on the basis of microsatellite repeats within the ITS2 region) that exhibit degrees of host specificity as well (Luo *et al.*, 2002). The apparent increase in the number of fish species infected by *B. acheilognathi* has suggested that colonization and host switching may have occurred recently (Luo *et al.*, 2002). The absence of widespread co-speciation during the evolution of *Lamellodiscus* (Monogenea) and their fish hosts, the Sparidae, has also been inferred based on molecular genetic data. In this case, phylogenetic trees built on SSU rDNA sequences (for *Lamellodiscus*) and both 16S and cytochrome-b mitochondrial gene sequences (for the Sparidae) were examined for co-evolution using computational methods, all of which agreed that widespread co-speciation in this host–parasite system is unlikely to have occurred (Desdevises *et al.*, 2002). In contrast, comparative phylogenies of helminths and their fish hosts have suggested co-speciation in evolution in at least two cases, namely, the genus *Diplozoon*, a class of monogeneans that interacts with cyprinid species (Sicard *et al.*, 2001), and the Tetraphyllidea, a group of tapeworms that infect elasmobranchs (Olson *et al.*, 1999).

In addition to its use in systematics and evolutionary biology, PCR amplification of rDNA sequences has served as an important tool for identifying species and for

determining fish hosts parasitized by worms such as digenean trematodes (Chapter 10). In this regard, ITS2 rDNA sequences were used to infer a three-host life cycle for *Bivesicula claviformis*, a digenean found in the intestines of warmwater fishes (Cribb *et al.*, 1998). Amplification of ITS2 rDNA from immature bivesiculids found in labrid species, such as *Thalassoma lunare*, identified these parasites as *B. claviformis*. Previously, this trematode had been found (as adults) only in large carnivorous serranids (namely, *Epinephelus fasciatus*) in the Great Barrier Reef, and was assumed to have a two-host life cycle. Since the diet of *E. fasciatus* includes labrid fish, it is likely that the larger carnivorous fish become infected by feeding on the intermediate host (*T. lunare*) (Cribb *et al.*, 1998). In other studies, ITS1 rDNA sequences have been used to identify encysted metacercariae in molluscs (*Cerastoderma edule*) collected from the Atlantic Ocean off the Iberian coast as those of *Monorchis parvus*. The adult stage infects *Diplodus sargus* and other fish species in the Mediterranean Sea. In the absence of rDNA sequence data, the encysted form had been identified only as *Cercaria cerastodermae* I (Bartoli *et al.*, 2000). The study also provided a means to distinguish *M. parvus* from the related species *Monorchis monorchis*, on the basis of ITS1 rDNA sequence data (Cribb *et al.*, 1998).

Application of PCR and DNA sequence analysis has been useful in identification and epidemiological studies of other trematodes, most notably *Bolbophorus*, which can be highly pathogenic to farm-raised fish. Since the late 1990s, catfish farms in southwestern Louisiana and the Mississippi delta region of the USA have experienced epidemics of metacercarial infections with high mortalities, especially among young fish. These mortalities were originally thought to have a viral etiology but have now been ascribed to *Bolbophorus* species, which can be distinguished on the basis of rDNA and mitochondrial COI gene sequence analysis (Levy *et al.*, 2002; Overstreet *et al.*, 2002). The principal agent responsible for these outbreaks is probably *Bolbophorus damnificus* (Overstreet *et al.*, 2002), with the possible involvement of a second unnamed species termed *Bolbophorus* sp. type 2 (Levy *et al.*, 2002). Using specific primers that generate 820 and 420 bp amplicons from *B. damnificus* and *Bolbophorus* sp. type 2, respectively, both species were shown to be present in the American white pelican, *Pelecanus erythrorhynchos* suggesting that the pelican is the definitive host (Levy *et al.*, 2002; Overstreet *et al.*, 2002). Similarly, both species were shown to be present in field-collected ramshorn snails (*Planorbella trivolvis*), considered the first intermediate host (Levy *et al.*, 2002). Interestingly, only *B. damnificus* was identified in field isolates of infected channel catfish, which presumably acts as a second intermediate host. Although *Bolbophorus* sp. type 2 has been shown to be pathogenic to a variety of fish species in direct challenge studies, it remains to be determined whether it causes disease in the commercially important catfish, *I. punctatus* (Levy *et al.*, 2002). Given the extreme differences in morphologies of the different stages of these parasites, molecular genetic analyses were indispensable in correctly matching parasite species with their respective hosts. Dzikowski *et al.* (2003) have used a similar approach to distinguish the North American species *B. damnificus* and *Bolbophorus* sp. type 2 from Old World species that affect cyprinid fish, namely, *Bolbophorus confusus* and *Bolbophorus levantinus*. Using species-specific primers that amplified 820 and 420 bp fragments of SSU rDNA from *B. confusus* and *B. levantinus*, respectively, it was possible to generate a preliminary phylogenetic tree relating the Old and New World species based on sequence comparisons. In this analysis, *B. damnificus* was more closely related to *B. levantinus* than to *Bolbophorus* sp. type 2. It is not understood why two species from different continents (and different hosts) are more closely related than species from the same continent. More in-depth sequencing of rDNA and protein-coding genes is clearly warranted to determine whether the inferred relationship is correct.

Marine and freshwater nematodes (Chapter 12) have also been targeted for

genetic analysis. A number of anisakid species (in particular, *Anisakis simplex*) are recognized as zoonotic agents in humans who consume undercooked seafood. Simple PCR-based assays that can be used to detect and distinguish species of anisakids infecting marine and freshwater fishes, mammals and fish-eating birds have now been developed (Zhu *et al.*, 1998; D'Amelio *et al.*, 2000; Kijewska *et al.*, 2002). These assays rely on RFLPs in a region of the rDNA containing ITS1, the 5.8S rRNA gene, ITS2 and ~ 70 bp of the 28S rRNA gene. Upon amplification of this region with broadly specific primers spanning the ITS1 and ITS2 domains of anisakid rDNA, batteries of restriction enzymes generate discrete patterns of fragments from different parasite species, including possible cryptic species of *A. simplex* (Zhu *et al.*, 1998; D'Amelio *et al.*, 2000; Kijewska *et al.*, 2002). These types of PCR-RFLP assays will undoubtedly be extremely useful in future diagnostic and epidemiological studies of marine and freshwater ascarids.

Arbitrary primed PCR: RAPD analysis

In addition to the direct sequencing of rDNA and protein-coding genes, intra- and interspecific differences within and between taxa can also be detected using arbitrary primed PCR. Referred to alternatively as random amplified polymorphic DNA (RAPD) analysis, this relatively simple method relies on the ability of arbitrary PCR primers to generate visibly different patterns of amplified DNA from templates representing different species (or strains of the same species) following electrophoresis on agarose gels. Initially either single or pairs of random PCR primers are used to amplify total DNA from a given organism, under conditions of low annealing temperature, which allow some mismatch between the primers and their target sequences within the genome. Following the initial rounds of PCR, the annealing temperature is raised and additional cycles are carried out to generate PCR fragments that can be visualized using ethidium staining on agarose gels. The complexity of eukaryotic genomes ensures that multiple fragments (RAPD markers) are generated with most arbitrary primer pairs, and that the patterns of bands in each case are distinct. Such patterns establish a fingerprint of DNA for an individual, with certain (fixed) markers being indicative of species. RAPD PCR provides a nimble way to study genetic diversity within populations, and has been particularly useful in uncovering sibling or cryptic species. Its advantages include the fact that it does not require prior knowledge of the nucleotide sequence of any gene and that it can generate a large number of genetic markers for establishing the population structure for a given species. RAPD analysis has been applied to two different types of helminths infecting fish, namely, *G. salaris* and *Hysterothylacium fabri* (an anisakid nematode). In the case of *G. salaris*, certain primers revealed clear polymorphisms in populations of worms taken from Atlantic salmon in different Norwegian rivers (Cunningham and Mo, 1997). In a more detailed study with *H. fabri*, RAPD PCR was used to generate more than 400 genetic markers, of which > 90% were polymorphic (Martín-Sánchez *et al.*, 2003). An analysis of genetic diversity and population structure using this approach suggested that *H. fabri* represents a species complex comprising at least three sister groups having little host specificity (Martín-Sánchez *et al.*, 2003). RAPD PCR analysis has also suggested the existence of bottlenecks of genetic diversity in *I. multifiliis* grown in different species of host fish (M. Gray, D.M. Cassidy-Hanley and T.G. Clark, unpublished; Fig. 20.5).

Solid-phase hybridization techniques

Along with solution-based assays, such as PCR, solid-phase methods, which rely on nucleic acid hybridization with labelled probes, provide additional means for detection and identification of pathogens (Fig. 20.6). As with PCR, such methods are highly specific, owing to the discrete interaction of nucleic acid probes with their

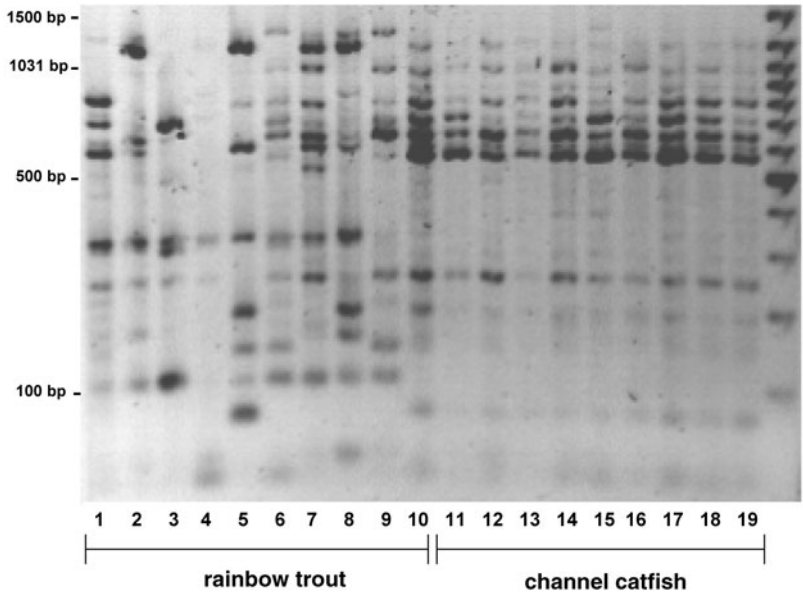


Fig. 20.5. RAPD PCR analysis of *Ichthyophthirius multifiliis*. DNA from individual (cloned) parasites collected from rainbow trout following a natural outbreak of 'white spot' disease in upstate New York was amplified,using a single pair of random PCR primers (lanes 1–10). The infection was passaged on channel catfish and individual parasites were again harvested and analysed by RAPD PCR with the same set of random primers (lanes 11–19). While DNA fingerprints from parasites collected from the initial infection on rainbow trout were quite varied, the patterns generated with parasites taken from channel catfish were largely homogeneous. The lane on the extreme right represents a DNA size standard.

cognate sequences. With most solid-phase techniques, DNA or RNA is purified from the target organism prior to annealing with a labelled probe that is specific for a given gene or sequence. In such cases, the purified DNA/RNA is either directly bound to a support (dot blots, slot blots, etc.) or fractionated using gel electrophoresis prior to immobilization on nylon or nitrocellulose membranes (Southern and Northern blotting analysis). Once attached, the target DNA/RNA is reacted with the probe under conditions that favour specific hybridization and discourage non-specific binding to non-target sequences or to the support itself. A second approach involves *in situ* hybridization (ISH), in which pathogen-specific probes are reacted directly with histological sections suspected to contain a given agent (Wilcox, 1993). Prior to screening, the sectioned material is treated so that endogenous nucleic acids are available for binding to the probe. Following hybridization, signals generated by the probe are used to detect and localize the pathogen (where the probe is labelled with a fluorochrome, the method is referred to as fluorescence *in situ* hybridization (FISH)).

ISH techniques for the detection of fish parasites have been applied to both Myxozoa and Microsporea. Antonio *et al.* (1998) used them to detect *M. cerebralis* in fixed tissues of rainbow trout and its intermediate host, *T. tubifex*. Three different oligonucleotide primers intended originally for PCR amplification of *M. cerebralis* SSU rDNA were end-labelled with the steroid hapten digoxigenin (DIG) and hybridized to fixed tissue sections that had been dewaxed and proteinase K-treated to expose parasite DNA. After high-stringency washing, probes were detected using alkaline phosphatase-conjugated anti-DIG antibodies and appropriate colorimetric (NBT/BCIP)

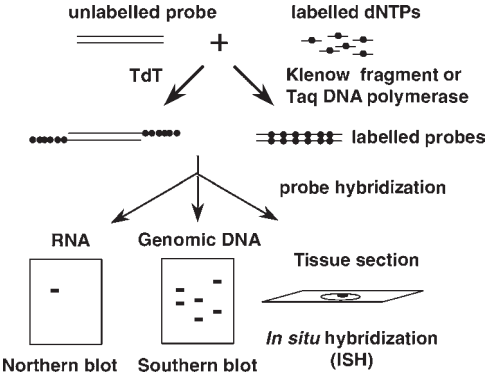


Fig. 20.6. Solid-phase hybridization with labelled nucleic acid probes. DNA probes are constructed from either PCR-generated or cloned fragments of DNA. Probes are labelled with tagged deoxyribonucleotides (dNTPs), using any of a number of different methods. Shown here are end-labelling with terminal deoxynucleotide transferase (TdT) and continuous labelling of both DNA strands in synthesis reactions with either the Klenow fragment of DNA polymerase or with thermostable Taq DNA polymerase (that is, by PCR). Commonly used tags include the steroid hapten digoxigenin, biotin and radioactive ^{32}P. Once labelled, probes are denatured to separate the DNA strands and then hybridized to immobilized RNA (Northern blot), genomic DNA (Southern blot) or histological sections (ISH) under low stringency conditions that favour annealing. The probe is then washed off the substrate under high-stringency conditions, which disrupt non-specific interactions (including mismatched hybrids), leaving only the probe bound to its complementary target sequence. Radioactive hybrids are detected using X-ray film, while biotin and digoxigenin probes are detected with streptavidin–enzyme conjugates and anti-digoxigenin antibody conjugates, respectively.

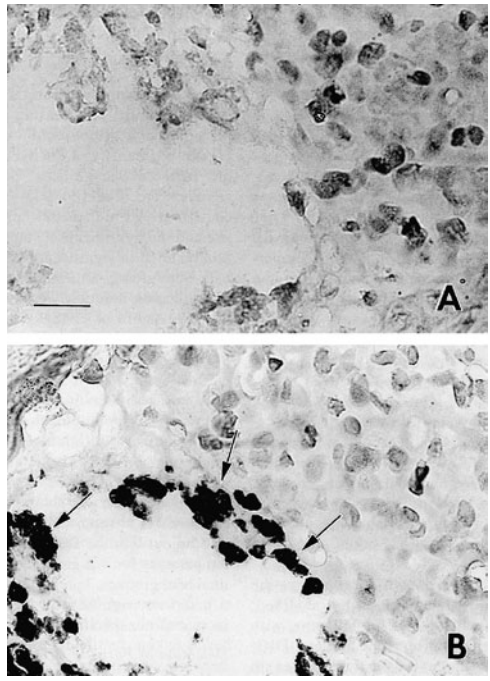


Fig. 20.7. Localization of *M. cerebralis* in host tissues by *in situ* hybridization. Panels (A) and (B) show sections of cranial cartilage from rainbow trout infected with *M. cerebralis* and processed for *in situ* hybridization 3 months post-exposure. Oligonucleotide probes were labelled at their 3′ ends with dUTP–digoxigenin. Preincubation of sections with excess unlabelled probe eliminated tissue staining (panel A). Strong staining of developmental stages of the parasite was visible in host tissues using the labelled probe by itself (arrows, panel B). (From Antonio *et al.*, 1998 with permission from *Journal of Aquatic Animal Health*.)

substrates. Developmental and sporogonic stages of the parasite were easily detected in fish and oligochaete tissues (Fig. 20.7). The probes were specific for *M. cerebralis* and did not generate positive signals in tissues from fish infected with other Myxozoa, including *C. shasta*, *T. briosalmonae* and *Myxobolus* sp. (Antonio *et al.*, 1998). Morris *et al.* (2000) applied similar methods for detection of the PKX agent, *T. bryosalmonae*, using oligonucleotide probes that were also developed for PCR. *T. bryosalmonae*-specific probes were end-labelled with DIG and detected with anti-DIG monoclonal antibodies linked to alkaline phosphatase. A modification of this method, which reduces the workup time from 2 days to about 5 h, involves the use of biotinylated probes and a streptavidin peroxidase conjugate. The ability to detect the parasite in tissues suggests that the gill is an important portal of entry for *T. bryosalmonae* and that the parasite spreads directly to the kidneys prior to the involvement of other tissues (Morris *et al.*, 2000).

Detection of the microsporidia *L. salmonae* and *Tetramicra brevifilum* has also been described using ISH techniques

(Sánchez *et al.*, 1999, 2001; Leiro *et al.*, 2001). In the case of *Loma*, a 272 bp single-stranded probe corresponding to the ITS and portions of the small- and large-subunit rDNA was constructed by PCR and labelled during synthesis with digoxigenin-11-deoxyuridine triphosphate (dUTP) in reactions that contained only a forward primer (Sánchez *et al.*, 1999, 2001). When applied to sections of fixed tissue from infected rainbow trout, the probe detected parasite xenomas with high sensitivity and specificity (Sánchez *et al.*, 1999, 2001). Using similar methods, a 1.2 kb digoxigenin-tagged probe, generated using PCR from SSU rDNA of *Tetramicra*; allowed definitive diagnosis of this agent in xenomas of infected turbot (*Scophthalmus maximus*) (Leiro *et al.*, 2001). Although patent infections with *T. brevifilum* are easily visualized in host tissues using conventional light microscopy, identification and localization of immature stages (or mild infections) are more difficult. ISH, on the other hand, has permitted detection of *L. salmonae* during its earliest stages of infection where it is localized to the gut epithelium of infected fish. With time, parasites are visible in the lamina propria and then blood cells within the heart, where they appear to undergo merogony prior to their final development in the gills (Sánchez *et al.*, 2001). This is the first description of early stages of infection in fish exposed to *L. salmonae*.

Applications of Southern and Northern blotting analyses in fish parasitology are described below in connection with the development of vaccines against *I. multifiliis*. However, another solid-phase technique, which combines PCR with hybridization of target and probe sequences in a microtitre plate format, has been developed for marine copepods (Kiesling *et al.*, 2002). The technique, which was developed to distinguish copepod species within the marine environment, significantly reduced the number of individual PCR reactions that would be required using more conventional methods. With this streamlined approach, a series of species-specific oligonucleotide probes was constructed against the highly variable D1/D2 region at the 5′ end of copepod 28S rDNA. After the addition of poly-dT tails, the probes were covalently attached to the wells of microtitre plates, where they became available for hybridization with labelled probes. The probes consisted of amplicons generated from single copepods (adult and naupliar stages) labelled during synthesis with biotin. In all cases, amplicons were produced using the same (universal) primers that recognize conserved sequences delimiting the variable D1/D2 region. Following hybridization, the probe was detected with a streptavidin–horse radish peroxidase conjugate. Because a single set of primers was used to amplify all unknown samples, the requirement for a large number of PCR reactions for each sample, using multiple species-specific primers, was avoided (Kiesling *et al.*, 2002). While the copepods identified in this study were not necessarily pathogenic, the procedure could easily be adapted and used as a means of identifying parasitic species within the environment.

Gene Cloning

The isolation of genes encoding specific proteins has become an indispensable tool for studying the biology of living species and is often a key step in the development of therapeutic and prophylactic reagents against pathogens. Procedures for gene purification generally take one of two routes. The first involves construction and plating of libraries of genomic or complementary DNA (cDNA) from the pathogen, followed by the identification and isolation of clones of interest by hybridization with specific nucleic acid probes (or by cross-reactivity with antibody probes in the case of expression libraries). The alternative approach involves amplification of known (or suspected) protein-coding regions by PCR, using genomic DNA as the template and degenerate oligonucleotide primers that target conserved regions of coding sequence. Once desired genes have been isolated, corresponding protein products can be synthesized using any of a number of heterologous

protein expression systems (e.g. *Escherichia coli* (Swartz, 2001), yeast (Gellissen, 2000), *Tetrahymena* (Gaertig *et al.*, 1999), insect (Jarvis *et al.*, 1996) and mammalian tissue culture cells (Fussenegger *et al.*, 1999)). The resulting recombinant products can then be used for a variety of purposes, ranging from X-ray crystallography and drug design to the development of recombinant subunit vaccines. In systems that allow reverse genetics, gene isolation often permits one to conduct functional assays of specific proteins by 'knocking out' the corresponding gene *in vivo,* using either targeted deletion via homologous recombination (Gong and Rong, 2003) or interfering RNA technology (RNAi) (Zanmore, 2002). The creation of 'knockout' strains can also serve in the construction of rationally attenuated vaccine strains, particularly in cases where gene deletion results in altered virulence (Kit *et al.*, 1985; Charles and Dougan, 1990; Uzonna *et al.*, 2004).

Along with conventional gene isolation methods, robotic systems are now available that permit high-throughput sequencing of large numbers of cloned inserts from DNA libraries. This approach has opened up an entirely new method for gene discovery, in which computational methods can be applied on a genome-wide scale. High-throughput, single-pass sequencing of cloned cDNAs (to create expressed sequence tags (ESTs)) (Gill and Sanseau, 2000) offers further opportunities, along with microarrays (Freeman, 2003), to examine patterns of gene expression at different stages of parasite life cycles or in response to changes in environmental conditions. High-throughput methods are particularly useful in systems that do not allow straightforward genetics, and they offer a powerful approach towards the discovery of novel targets for diagnosis, drug treatment and vaccine development. While the application of genomics to fish disease is still in its infancy, a number of pilot EST projects are under way for a diverse group of parasitic agents that affect wild and farm-raised fish. These include *Clonorchis sinensis* (a digenean trematode that is an important zoonotic agent in China, Korea and South East Asia) (Lee *et al.*, 2003), *Trypanosoma carassii* (a kinetoplastid that infects cyprinid fishes) (Aguero *et al.*, 2002), *Lepeophtheirus salmonis* (an ectoparasitic copepod that affects salmonids) (Johnson *et al.*, 2002) and *I. multifiliis* (the causative agent of 'white spot' in freshwater fishes) (T.G. Clark, unpublished). The GenBank dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/) currently lists 1921 EST sequences for *T. carassii*, 512 for *I. multifiliis* and 248 for *C. sinensis*. An additional 392 ESTs have been sequenced for *L. salmonis* (Johnson *et al.*, 2002). Of the 1921 ESTs for *T. carassii*, the vast majority (> 90%) had no significant match to other known protein-coding sequences at the time they were submitted to GenBank (Aguero *et al.*, 2002). Similarly, ~ 33% of the EST sequences identified in the *C. sinensis* library did not have significant database matches (Lee *et al.*, 2003). Such results clearly attest to the power of this approach for gene discovery. While the numbers of gene and EST sequences for fish parasites are relatively low (particularly when compared with agents that infect mammalian hosts), one can only expect that they will increase dramatically as work in this area expands and as the cost of high-throughput sequencing continues to decline.

Apart from large-scale data collection, the construction of genomic and/or cDNA libraries has made possible in-depth analyses of a number of important gene products from agents that parasitize fish. These include several trypsin-like enzymes from *L. salmonis* (Johnson *et al.*, 2002), transsialidase-like and mucin-like proteins from *T. carassii* (Aguero *et al.*, 2002) and a family of surface membrane antigens involved in protective immunity against *I. multifiliis* (Clark *et al.*, 1992, 1999; Lin *et al.*, 2002b). The latter case provides an example of how gene cloning can be used in vaccine development and is discussed in detail below. The use of expression libraries for the cloning of genes from the PKX agent of salmonids (*T. bryosalmonae*) has also been described (Saulnier *et al.*, 1996).

The alternative approach of gene cloning using PCR has been applied to a variety of fish parasites. For example,

full-length coding sequences for genes encoding abundant glycosyl phosphatidyl inositol (GPI)-anchored surface proteins from *Ichthyophthirius* and a cathepsin Z-like enzyme from *M. cerebralis* have been described (Lin *et al.*, 2002b; Kelley *et al.*, 2003). In both instances, fragments of the relevant coding sequences are amplified using PCR. The degenerate primers are designed against conserved regions of the corresponding proteins. After sequencing the truncated fragments, missing regions are obtained using rapid amplification of cDNA ends (RACE), a procedure that allows amplification of the 5′ and 3′ regions of the coding sequences of genes, using cDNA as a template (Frohman, 1990). In RACE, a first-strand cDNA is synthesized from mRNA, using oligo-dT as the primer, and missing 3′ sequences are amplified in PCR reactions, which utilize a forward primer based on a known sequence (derived from the previously amplified gene fragment) and oligo-dT as the reverse primer. Missing 5′ sequences are obtained after addition of a homopolymeric tail (e.g. poly-G) to the 5′ end of the first-strand cDNA, using terminal deoxynucleotidyl transferase. The relevant 5′ sequence is obtained by PCR, using a reverse primer of known sequence and a forward primer complementary to the homopolymeric tail. A variation on 5′ RACE, known as SMART (switching mechanism at 5′ end of RNA transcript) RACE, allows the addition of a specific sequence rather than a homopolymeric tail at the 5′ end of the cDNA and strongly favors the production of full-length products (Chenchik *et al.*, 1998). A variety of modifications of these procedures are also available (Schaefer, 1995; Das *et al.*, 2001). In the case of the cathepsin Z-like gene from *M. cerebralis*, a phylogenetic analysis comparing its deduced sequence with cathepsin proteases of other eukaryotic species has suggested that the myxozoan protein is among the earliest of the Z-group enzymes to have evolved. This analysis has implications for the origins of the Myxozoa (see above and Chapter 8) (Kelley *et al.*, 2003).

Additional protein-coding sequences have been amplified using PCR in connection with numerous other phylogenetic studies. As indicated above, these include full or partial sequences for homeobox-containing proteins, tubulins, ribosomal proteins, heat-shock proteins and mitochondrial proteins from Myxozoa, microsporidia, flatworms and a variety of other parasitic forms.

Molecular Parasitology and Vaccine Development

Fish are capable of mounting effective immune responses to parasitic protozoa, including *Ichthyophthirius* (Chapter 4). As with a variety of other parasites, however, *Ichthyophthirius* cannot be easily cultured, and it has long been recognized that development of an effective vaccine against this agent will require the expression of protective antigens in recombinant form. Immunological studies have shown that immunity against *I. multifiliis* is largely humoral and involves an antibody response to abundant parasite membrane proteins, known as immobilization antigens (i-antigens) (Clark *et al.*, 1995; Clark and Dickerson, 1997). Antibodies against these proteins immobilize swimming (theront and tomont) stages of the parasite *in vitro* and offer passive protection against infection when administered parenterally to naïve fish. Purified i-antigens confer active immunity as well (Wang *et al.*, 2002). However, inability to grow the parasite on a large-scale precludes the use of the native antigens for commercial vaccine development. This has led to efforts to express these proteins as recombinant antigens in heterologous systems.

These efforts began with the isolation of a 1.2 kb cDNA containing the preponderance of the coding sequence for a 48 kDa i-antigen from parasite isolate G1 (i-antigen serotype A). This sequence was obtained by screening a trophont cDNA library with an oligonucleotide probe based on the N-terminal sequence of the purified antigen itself (Clark *et al.*, 1992). The library was constructed using a standard approach, in which poly-A^+ trophont mRNA was used as the template for first-strand cDNA

synthesis, together with oligo-dT as the primer and AMV reverse transcriptase to make the first strand. Second-strand synthesis was carried out with DNA polymerase, and the resulting double-stranded product was introduced into a lambda phage vector. After plating on *E. coli*, the library was screened with the ^{32}P-labelled probe, and a phage plaque containing the 1.2 kb cDNA was isolated. This cDNA had the deduced sequence for a nearly full-length protein, 394 amino acids in length, that was rich in cysteine and had five tandemly repetitive amino acid sequence domains of ~ 80 residues each.

Northern and Southern hybridization studies were then carried out to examine the expression and organization of the corresponding gene (Fig. 20.8). In Northern blotting analysis, either total or poly-A$^+$ RNA was fractionated on agarose gels and blotted onto nitrocelluose or nylon filters under denaturing conditions. Membranes were then incubated with a labelled nucleic acid probe specific for the gene (cDNA) of interest. The probe was permitted to anneal with its target sequence under conditions of low stringency (high salt, low temperature) and the filter was washed at high stringency (low salt, high temperature) to remove weakly bound probe. As shown in Fig. 20.8, when the 1.2 kb cDNA was used to screen Northern blots of *I. multifiliiis* poly-A$^+$ RNA, the probe recognized two transcripts of 1.6 and 1.9 kb. This was consistent with Western blotting studies, which revealed two closely related i-antigens of 48 and 60 kDa in the G1 parasite strain. Steady-state levels of RNA transcripts for these proteins were shown to be very high (equivalent to ~ 6% of total poly-A$^+$ RNA) and were developmentally regulated through the parasite life cycle (with maximal levels of expression occurring at the infective theront stage) (Clark *et al.*, 1992). Southern blotting analysis, using the 1.2 kb cDNA as probe, also revealed two closely related genes (Fig. 20.8). In Southern blotting, genomic DNA fragments produced by restriction endonuclease digestion were screened with labelled probes for specific genes (Sambrook *et al.*, 1989). In this case, the

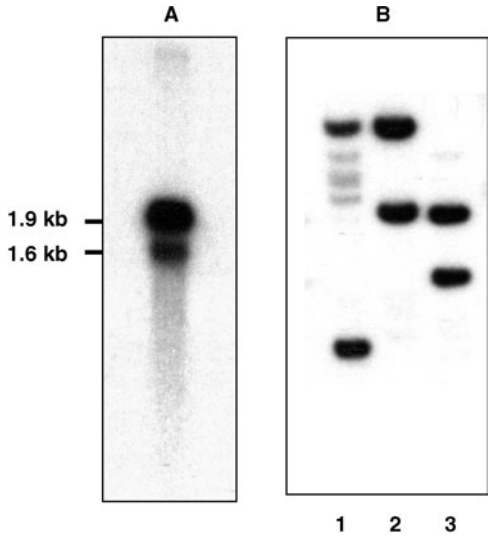


Fig. 20.8. Northern and Southern blotting analysis. In the Northern blot in panel (A), poly-A$^+$ RNA from *I. multifiliis* was size-fractionated on a 1.2% agarose gel, blotted onto nylon and probed with a ^{32}P-labelled cDNA for the 48 kDa i-antigen of parasite isolate G1 (serotype A). The probe recognized two distinct RNA transcripts of 1.6 and 1.9 kb. In the Southern blot in panel (B), genomic DNA from *Ichthyophthirius* strain G1 was digested with *Eco*RI (lane 1), *Hind* III (lane 2) or *Sma*I (lane 3), then fractionated on a 0.8% agarose gel and blotted as above for Northern analysis. When screened with the same cDNA probe used in (A), two major bands were seen in each of the restriction digests. Consistent with the presence of two transcripts in (A), the Southern blot suggests that two i-antigen genes with strong homology to the probes are present in the G1 parasite strain.

1.2 kb cDNA revealed two prominent bands in *I. multifiliis* DNA cut with different restriction enzymes, indicating the presence of two, closely related, i-antigen genes in the G1 parasite strain (Clark *et al.*, 1992).

With the 1.2 kb cDNA in hand, it became possible to isolate the gene for the 48 kDa i-antigen (Clark *et al.*, 1999). The gene, designated *IAG48[G1]*, was obtained from a lambda phage library prepared from genomic DNA fragments cut with the restriction enzymes *Swa*I and probed with the 1.2 kb cDNA. The gene was found to contain a single, uninterrupted, reading frame that predicts a full-length (that is,

preprocessed) protein of 442 amino acids. Interestingly, the coding sequence of the gene differed from the cDNA at its 3′ end. The gene (in contrast to the cDNA) predicted a protein with a hydrophobic C-terminus having all the hallmarks of a signal peptide for a GPI-anchored protein. Since the i-antigens are known to be GPI-anchored (Clark *et al.*, 2001), the 1.2 kb cDNA represented an alternatively spliced transcript or was the product of a cloning artefact. The latter seems to be the case since: (i) primers flanking the coding region of the gene amplify a fragment identical to the gene itself when RNA is used as a template (i.e. by reverse-transcriptase PCR (RT-PCR)) (Clark *et al.*, 1999); (ii) the 3′ sequence of the 1.2 kb cDNA recognizes an entirely different transcript on Northern blots than does a probe from the 5′ end of the gene (Clark *et al.*, 1999); and (iii) no other i-antigen-specific cDNA clones encoding the anomalous 3′ end have been identified (in > 100 independent clones) (T.G. Clark, unpublished).

Analysis of the coding region of the gene for the 48 kDa antigen made it clear that *Ichthyophthirius*, like other hymenostome ciliates, utilizes a non-standard genetic code, in which the normal UAA and UAG stop codons specify glutamine instead (Clark *et al.*, 1992, 1999). This has important implications in respect of vaccine development, since the native gene sequence would yield truncated proteins when produced in conventional heterologous protein expression systems, such as *E. coli* or yeast. The gene for the 48 kDa antigen contains 18 such 'stop' codons, including several near the N-terminus itself (Clark *et al.*, 1992, 1999). To overcome this problem, He *et al.* (1997) used overlapping oligonucleotides (primers) to synthesize a 315 nucleotide fragment (corresponding to roughly one tandem repeat near the N-terminus of the 48 kDa protein) lacking seven potential UAA glutamine codons. The synthetic gene fragment was then expressed as a GST fusion protein in *E. coli* and tested as a subunit vaccine in goldfish. In a single vaccine trial, 15 μg of the fusion protein administered intraperitoneally in Freund's complete adjuvant was reported to provide significant protection in terms of parasite load and overall mortality (although the challenge dose itself killed fewer than half the fish) (He *et al.*, 1997). This result is clearly interesting and deserves further investigation, particularly in light of studies indicating that protection afforded by purified subunit antigens is serotoype-specific. As shown by Wang *et al.* (2002), channel catfish injected with i-antigens purified directly from the parasite confer protection against only those strains from which the antigens are obtained. Similarly, passive protection using i-antigen-specific mouse monoclonal antibodies is serotype-specific, with the conformational epitopes recognized by these antibodies being unique to a given serological strain (Clark and Dickerson, 1997). In the studies of He *et al.* (1997), the parasite strain used for challenge was undefined, as was the nature of the epitope(s) associated with the recombinant fusion protein.

Recently, paralogous surface antigen genes have been isolated from a different *I. multifiliis* strain (G5), representing a distinct i-antigen serotype (namely, D) (Lin *et al.*, 2002b). These genes, designated *IAG52A*[*G5*] and *IAG52B*[*G5*], encode surface antigens of 468 and 460 amino acids, respectively. As with the 48 kDa protein from the G1 isolate, the serotype D proteins are predicted to contain hydrophobic signal peptides at their N- and C-termini and a series of imperfect tandem repeats of ~ 80 amino acids each spanning their length. Antibodies against affinity-purified i-antigens of serotype D recognize a broad band of 52/55 kDa on Western blots, which resolves into four or more spots on two dimensional gels. Along with *IAG48*, the *IAG52A* and *IAG52B* genes have recently been cloned and expressed in *Tetrahymena thermophila*, a non-pathogenic, freshwater ciliate that is taxonomically related to *Ichthyophthirius* (Shang *et al.*, 2002; T.G. Clark, Y. Bisharyan and D.M. Cassidy-Hanley, unpublished). Following their expression, *T. thermophila* cell lines are rapidly immobilized by specific antibodies against the parasite proteins in a manner almost identical to that seen with *I. multifiliis* (T.G. Clark, Y. Bisharyan

and D.M. Cassidy-Hanley, unpublished). Expression of the native *I. multifiliis* genes in *Tetrahymena* was possible because *T. thermophila* and *Ichthyophthirius* share identical codon usage with respect to UAA and UAG triplets. Synthetic genes in which the native sequences of the 48 and 52/55 kDa antigens were replaced with the preferred codon usage of channel catfish have also been constructed. However, unlike the recombinant antigens expressed in *Tetrahymena*, the synthetic gene products expressed in *E. coli* and mammalian tissue-culture cells do not bind to protective monoclonal antibodies, indicating that the proteins did not fold correctly in these heterologous (non-ciliate) systems (Lin *et al.*, 2002a). Consistent with this observation, plasmid constructs encoding these proteins elicit only weak protection against parasite challenge when administered as DNA vaccines (Lin, 2002).

Although to date, DNA vaccines (also known as genetic immunization) have proved less than successful with *Ichthyophthirius*, this general approach has shown that it has enormous potential against a variety of microbial agents and may serve an important role in the prevention of parasitic diseases of fish. A DNA vaccine is constructed by introducing the gene for a protective antigen into a plasmid expression vector that contains a promoter element, which can drive transcription of the cloned sequence in host cells (Donnelly *et al.*, 1997; Heppell and Davis, 2000). Promoters from viruses normally associated with mammalian infection (for example, cytomegalovirus) have been shown to be highly efficient even in fish. The vaccine, in the form of naked DNA, is introduced into an animal by parenteral injection, where it is taken up by host cells. Once expressed, the vector-encoded antigen is recognized by the immune system as a foreign substance, leading to the production of humoral and cell-mediated immune responses.

Apart from genetic immunization, recombinant subunit antigens are also effective as vaccines, and pilot studies with i-antigens expressed in *Tetrahymena* have been quite promising (X. Wang, T.G. Clark and H. Dickerson unpublished). Needless to say, similar methods are being used against other fish parasites, particularly those of economic importance within the aquaculture industry. In this regard, a large-scale effort to identify potential targets for vaccine development against sea lice and *Neoparamoeba pemaquidensis* (the causative agent of amoebic gill disease in Atlantic salmon) are now well under way (Raynard *et al.*, 2002; B. Nowak, personal communication; International Association of Biologicals, 3rd International Symposium on Fish Vaccinology, Bergen, Norway, 2003).

Conclusions

Over the past 15 years, molecular genetics techniques have proved exceedingly useful in studies of fish parasites and have revealed novel aspects of the biology, evolution and life styles of these organisms. In many instances, such studies have gone beyond fish parasites and shed important light on evolutionary biology as a whole. In more practical terms, molecular cloning techniques provide sensitive and specific methods for diagnosing parasite infections of fish. They have contributed significantly towards our understanding of the epidemiology of parasite disease, and are beginning to play a role in the development of efficacious vaccines for the prevention of parasitic infections in farm-raised fish. Despite this explosion of new information, continued studies at the molecular level will be needed to unravel species differences and the precise evolutionary relationships among and between taxa for a variety of parasite lineages. One would imagine that molecular approaches will continue to be applied in the arena of vaccine development, since these offer the best hope for protecting farm-raised fish against a number of the most important disease-causing agents of commercial aquaculture. Finally, the bioinformatics revolution spawned by genomic and proteomics-based approaches to biology have begun to find their way into the study of fish parasites and will almost certainly provide the next wave of understanding of these complex and fascinating organisms.

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Glossary

Abdomen: The part of the crustacean body that lies behind the genital segments; the last genital segment normally corresponds to the last thoracic segment.

Abiotic factors: Physical factors which affect the development/survival of an organism.

Abscess: A localized collection of pus in a cavity formed by disintegration of issues.

Acanthella: An acanthocephalan larva that lacks a proboscis and develops from an acanthor in the intermediate host.

Acanthor: An acanthocephalan larva that develops from a zygote and is infective to the intermediate host.

Accessory cell: A cell required for, but not actually mediating, a specific immune response; often used to describe antigen-presenting cells (APC; see below).

Accessory filaments: Fibrillar structures, such as striated lamella, that may form part of the support in cells; accessory filaments are distinct from microtubules.

Acetabulum: The ventral sucker in trematodes.

Aclid organ: A structure with large, tissue-cutting spines which is located anteriorly on the acanthor.

Acquired (adaptive) immunity: A series of host defences against a parasite which are characterized by extreme specificity and immunological memory; the defences are mediated by antibody and/or T-cells.

Adenoma: A tumour which consists of glandular tissues.

Adhesion: A pathological condition which results in connective tissue proliferation within and around an organ.

Affinity: The strength of interaction or binding between an antibody binding site and an antigenic determinant.

Agarose gel electrophoresis: A method for separating DNA or RNA fragments (in an electric field) of different lengths, based on their differential rates of migration in an agarose gel.

Agglutinate: The aggregation of organisms or particles into clumps; this may be due to the reaction of specific antibodies against surface antigenic determinants on the organism.

Agglutinin: Any substance, not necessarily antibody, which is capable of forming bridges between antigenic determinants on contiguous cells to form visible clumps.

Alae: Lateral cuticular expansions along the body of some nematodes.

Allograft: A tissue transplant (graft) between two genetically non-identical members of a species.

Alternative complement pathway: The activation of complement through involvement of properdin factor D, properdin factor B, C3b, C3 and then progressing as in the classical pathway.

Amastigote: The ovoid developmental stage (usually intracellular) of a trypanosome in which there is no emergent flagellum.
Amplicon: A fragment of DNA synthesized using the polymerase chain reaction.
Anadromous: Fishes that migrate from the sea to fresh water to spawn.
Anaemia: A deficiency in blood or red blood cells.
Anchor: An outgrowth from the head of some female copepods; it is used for attachment to the host.
Anchoring disc: A laminar structure at the anterior end of a microsporidia spore.
Anoneme: The core of a cilium or flagellum which comprises the microtubule skeleton.
Anorexia: The loss of appetite for food.
Anoxia: The absence of oxygen in tissues.
Antibody (Ab): A protein with the molecular properties of an immunoglobulin and capable of combination with an antigen; it is produced by lymphoid cells, especially plasma cells, in response to antigenic stimulation.
Antibody titre: A measure of the amount of antibody, usually in the blood of an animal.
Antigen: A substance or cell that elicits a specific immune response when introduced into an animal.
Antigen-binding site: The part of an immunoglobulin that specifically binds to antigen(s).
Antigenic determinant: A single antigenic site or epitope on a complex antigenic molecule or particle.
Antigen-presenting cell (APC): A specialized type of cell (e.g. macrophage, dendritic cells), which bears cell surface class II MHC (major histocompatibility complex) molecules and is involved in processing and presentation of antigen to T-cells.
Antigen processing: Large molecules are broken down into peptides in macrophages and are presented within the groove of MHC molecules.
Antigen receptor: The specific antigen-binding receptor on T- or B-lymphocytes; these receptors are transcribed and translated from rearrangements of V genes.
Antiglobulin test (Coombs test): A technique to detect cell-bound immunoglobulin.
Antisera: Sera containing antibodies induced in animals against known antigens.
Aplanospore: The trophozoite or vegetative stage of the protozoa *Perkinsus* sp. and Labyrinthomorpha.
Appendix: A finger-like posterior projection of the ventiruculus, common in some ascaridoid nematodes.
Aqueous humour: Anterior chamber of the eyeball.
Ascites: The presence of fluid in the abdominal cavity.
Atrophy: A decrease in the amount of tissue or the size of an organ after normal growth has been achieved.
Attachment plate: A region of the trophont of a dinoflagellate which is modified for attachment to the host.
Autogamy: A kind of sexual reproduction in which the zygote is formed by fusion of two haploid nuclei from one individual.
Avidity: The summation of multiple affinities, for example, when a polyvalent antibody binds to a polyvalent antigen.
Axiel structure: A supporting structure aligned along the long axis of a cell.
Axostyle: A rod-like supporting organelle composed of microtubules arranged in sheets or ribbons, e.g. in the Trichomonadida.

Basement membrane: The extracellular supporting layer of mucopolysaccharides and proteins underlying the epithelium.
Basic local alignment search tool (BLAST): A computational algorithm that allows one to search for sequence homologues (similar or identical genes, proteins, etc.) within an electronic database of stored sequences.

Benthos: The bottom of the sea.
Binary fission: Asexual reproduction by an organism which results in two progenies.
B-lymphocytes (B-cells): These are precursors of antibody-forming plasma cells; these cells carry immunoglobulin and class II MHC (major histocompatibility complex) antigens on their surfaces.
Biomass: Mass of biological tissue commonly expressed in units of weight.
Borborygmus: A rumbling noise caused by gas in the intestine.
Boring tooth: A cuticular tooth-like projection at the cephalic end of a larval nematode.
Branchiae: Leaf-like or finger-like lateral appendages on the urosome of some leeches.
Buccal: Pertaining to the oral cavity.
Buccal cavity: A pouch in ciliates equipped with compound ciliary organelles (*see* Cilium) and which precedes the cytostome.
Buccal ciliary apparatus: The specialized ciliature inside and around the buccal cavity in ciliates for food uptake.

Cachexia: The wasting of body tissues due to extreme malnutrition.
Caecum: An anterior projection of the intestine from the region of the oesophageal–intestine junction, common in some ascaridoids and spirurids.
Calcification: The deposition of calcium compounds occasionally associated with capsule formation.
Carcinoma: An epithelial tumour which tends to invade the lymph spaces of surrounding connective tissues.
Carrier: A large immunogenic molecule or particle to which an antigenic determinant is attached, thus allowing the determinant to become immunogenic.
Catadromous: Fishes that migrate from inland waters to the sea to spawn.
Cataract: The loss of transparency in the eye lens.
Catarrhal: The discharge of fluid due to inflammation of the mucus membrane.
Caudal cilium: An unusually long somatic cilium at the posterior pole of a ciliate.
cDNA: copy DNA, made from isolated messenger RNA.
C domain: The constant homology region of an immunoglobulin molecule.
Cell-mediated immunity (also **cellular immunity**): Specific immune response which is dependent on T-lymphocytes.
Cercariae: Free-swimming larvae of digeneans; these stages are usually released from the gastropod intermediate host.
Chalimus: The modified copepodid stage of a copepod; the parasite attaches to its host by a filament.
Chemotherapy: The use of chemicals to control an infection.
Cholangitis: The inflammation of the bile duct.
Cholangiocarcinoma: Cancer of the bile duct.
Chondroblasts: Cartilage-forming cells.
Chorioallantois: The vascular membrane which encloses the chick embryo.
Chromophores: Dermal cells which contribute to skin colour.
Cilium: A locomotory organelle rooted in a kinetosome, composed of a microtubular axoneme and covered by the outer cell membrane. The axoneme consists of nine peripheral doublets and a central paid of microtubules. Cilia may be grouped together in functionally specialized compound ciliary organelles (e.g. membranelles).
Cirrus: Coarse cilia which are longer than normal.
Classical complement pathway: The activation of complement components (in a sequential enzymatic cascade; C1, 4, 2, 3, 5, 6, 7, 8, 9) as a result of an antigen-antibody reaction which ultimately leads to cell lysis.
Class I and II MHC molecules: Proteins encoded by genes in the major histocompatibility complex.

Class switch: *See* Isotype switch.
Clitellum: The portion of a leech which is located betweeen the trachelosome and urosome; it secretes the cocoon.
Clonal deletion: A concept related to the clonal selection theory, which suggests that tolerance to 'self' antigens results from the deletion of autoreactive lymphocyte clones during embryonic development.
Clone: A population which is derived from a single individual.
Club cells: Large, roundish or club-shaped cells in the epidermis of fishes. In carps and crucian carps they are the Leydig cells, in channel catfish they are the alarm substance cells and in eel and loach they are the clavate cells.
Coccoid: A globular shape that resembles a coccus bacterium.
Cocoon: A brooding capsule for leech eggs.
Ceolozoic parasites: Parasites which occur in organ cavities.
Collagen: The principal proteinaceous substance in connective fibres.
Complement: A group of serum proteins that is the primary humoral mediator of antigen-antibody reactions and as opsonins in cellular immunity to enable leucocytes to phagocytose foreign particles.
Complement fixation: A serological assay used for the detection of an antigen–antibody reaction in which complement is fixed as a result of the formation of an immune complex.
Conchiolin: A nitrogenous albuminoid substance which is usually dark brown in colour; the organic base of many molluscan shells.
Congested: An excessive or abnormal accumulation of blood in an organ or tissues.
Conjugation: A sexual process in ciliates, in which two individuals fuse; their micronuclei undergo meiotic division followed by exchange and fusion of the haploid nuclei in the conjugants.
Constant region (C region): The invariant carboxyl-terminal portion of an antibody molecule, as distinct from the variable region, which is at the amino-terminal of the chain.
Contractile vacuole (or water expulsion vesicle): The pulsating vacuole in a protozoan that has osmoregulatory and excretory functions.
Coombs test: *See* Antiglobulin test.
Copepods: Small planktonic or bottom-dwelling crustaceans that are important components of the food chain of aquatic animals.
Copepodid: The larval stage of a copepod; between the nauplius and the pre-adult stages.
Cornified: Containing fleshy fibrous tissue.
Corticosteroid: Any of the adrenal-cortex steroids.
Cortisol: The major natural glucocorticoid synthesized by the adrenal gland.
Cytokine: A soluble substance (e.g. interleukin 1) secreted by cells that has a number of effects on other cells.
C-reactive protein (also **acute phase protein**): A serum protein which increases during acute inflammation; it has calcium-dependent phosphorylcholine binding properties.
C3 receptor: The cell surface receptor which is capable of selectively combining with the third component of complement.
Cyst: A non-motile, resistant, dormant stage.
Cystacanth: A juvenile acanthocephalan that develops from an acanthella; it possesses a proboscis and is infective to the definitive host.
Cystogenous glands: Glands which produce encystment substances.
Cytopharynx: A tube (**cytopharyngeal tube**) which leads from the cytostome into the endoplasm of a protozoan.
Cytostome: An oral opening or aperture for ingestion of food particles.
Cytotoxic T-cell: A cell that kills target cells bearing appropriate antigen within the groove of an MHC class I molecule that is identical to that of the T-cell.

Definitive host: The host in which the parasite usually undergoes sexual reproduction.
Degenerate primer: A set of oligonucleotides, each coding for the same region of a protein, but differing at the third position of codons. Degenerate primers are used to amplify genes from related organisms based on regions of conserved sequence within proteins.
Delayed-type hypersensitivity (DTH): A T-cell-mediated reaction to an antigen, which takes 24–48 h to develop fully in an animal, and which involves the release of lymphokines and the recruitment of monocytes and macrophages. Also called cell-mediated immunity.
Dermatitis: The inflammation of the skin.
Determinant: Part of the antigen molecule that binds to an antibody-combining site or to a receptor on T-cells.
Diadromous: The life history of fish with both freshwater and marine phases.
Diapedesis: The movement of haemocytes through intact epithelium (i.e. through the gut or mantle) and thus, are voided from the body.
Digestive gland: Part of the digestive tract of molluscs that extends from the midgut and is composed of numerous ducts and blind tubules. Forms a discrete organ that functions in nutrient acquisition.
Digitiform: Finger-like.
Dimorphic: Existing in two morphologically different forms.
Dinospore: A unicellular, haploid, motile, parasitic stage that resembles a free-living dinoflagellate. The dinospore is covered by thecal armour and may contain pigment, starch, lipid and a stigma.
Diplokaryon: Two closely apposed nuclei with their membranes adhering to each other in a binucleated cell.
Diplomond: A flagellate with one or two karyomastigonts, each with one to four flagella and accessary organelles; duplozoic individuals have bilateral symmetry.
Diplozoic: Having two karyomastigonts.
Disporic: A small sporogonic plasmodium or pseudoplasmodium which produces only one spore.
Disporoblastic sporogony: A process of spore production in which two sporoblasts arise from one sporont.
DNA polymerase: An enzyme that catalyses the synthesis of DNA strands. PCR (polymerase chain reaction) reactions utilize DNA polymerase enzymes from thermophilic bacteria, which can withstand denaturation at the high temperatures required for DNA strand separation.
DNA probes: Segments of DNA from an organism which are used to identify homologous segments of DNA from another organism. These probes are usually used for rapid identification of parasites.
DNA sequencing: A method of determining the primary nucleotide sequence of a DNA molecule.
Domain: A compact segment of an immunoglobulin molecule, made up of about 110 amino acids around an S–S bond, and encoded by a unique segment of DNA surrounded by non-translated sequences.
Dropsy: The abnormal accumulation of serous fluid in cellular tissue or the body cavity.
Ductus ejaculatorus: The functional penis in flatworms.
Dyplasia: The regressive change in adult cells.
Dysmea: Difficulty in breathing.

Ecchymosis: A small haemorrhagic spot, larger than a petechia, in the skin or mucus membrane.
Ecdysis: The shedding of the nematode cuticle following moulting.

Electrophoresis: The separation of proteins across an electrical gradient according to their mass and charge.
ELISA (Enzyme-linked immunosorbent assay): An immunological test to detect minute quantities of an antigen (antigen capture ELISA) or an antibody (antibody ELISA).
Emaciation: The deficiency of muscle and fat in an organism due to undernourishment.
Encapsulation: The covering of a parasite by the host with materials, mostly, if not entirely, of host origin.
Encephalomyelitis: The inflammation of the brain and the spinal cord.
Encystment: The covering of a parasite with materials of parasite origin.
Endocytosis: The uptake by a cell of material from the environment by invagination of its plasma membrane.
Endoplasmic reticulum: The network of fine tubules in the cytoplasm of cells for structural framework and circulating pathway.
Endosome: The central dark staining body in the nucleus of an amoeba that probably corresponds to the nucleolus.
Endospore: The thick inner chitinous layer of the wall in a microsporidia spore.
Endothelium: The innermost lining of the blood vessels.
Enteritis: The inflammation of the intestine.
Enzyme: An organic compound, frequently a protein, capable of accelerating or producing by catalytic action with some change in a specific substrate.
Eosinophilia: An increase in the number of eosinophils in circulation or in tissues.
Eosinophilic granulocytes: Leucocytes which contain acid staining granules.
Eosiniphilic meningoencephalitis: Meningitis characterized by the presence of numerous eosinophils.
Epimastogote: The elongated development stage of a trypanosome in which the flagellum arises anterior to the nucleus and emerges laterally to form a short undulating membrane along the body to the anterior end.
Epistomial disc: The vaulted apical cell surface in peririchous ciliates, it is encircled by haplo and polykinety and these two rows together form the **adoral zone**.
Epithelioid cells: Mononuclear cells which are involved in the inflammatory reaction and are common in granulomatous lesions. They are formed from the transformation of macrophages during encapsulation.
Epitope: An alternative term for antigenic determinant.
Epizootic: An outbreak of a disease or an unusually large increase in prevalence and/or intensity of a parasite in an animal population.
Epizootiology: The study of disease in animal populations.
Esterase: An enzyme that catalyses the hydrolysis of an ester into its alcohol and acid.
Estuarine: The transitory aquatic habitat between river and sea.
Ethidium bromide: A fluorescent dye that intercalates into the double-stranded DNA and emits a red-orange light (590 nm) in response to ultraviolet (UV) radiation.
Eukaryote: An organism whose cell(s) has a double nuclear envelope.
Eutropic: An aquatic habitat with abundant organic matter.
Exfoliation: The sloughing of cells from the tissue surface.
Exocytosis: The discharge from a cell of particles that are too large to diffuse through the cell membrane.
Exogenous budding: Reproduction by outgrowth of a protrusion (one or more buds) that is smaller than the parental cell.
Exophthalmus: Protrusion of the eye.
Exospore: The proteinaceous outer layer of the wall in a microsporidia spore.
Expressed sequence tag (EST): A single-pass sequence of a cDNA insert coding for a given protein.

Expression library: A DNA library in which cloned gene fragments (inserts) are expressed as proteins in a bacterial cell host. Expression of the DNA is driven by a promoter element in the cloning vector which is either constitutively active or induced by the conditions of cell culture.
Extracellular: Situated or occurring outside a cell.
Extrasporogonic: A phase of the development cycle which occurs parallel to the sporogonic phase.
Extravascular: Situated or occurring outside a vessel.
Extrusion apparatus: An elaborate apparatus that serves for injection of the sporoplasm of a microsporidia into the host cell.

Fab: A fragment of an antibody containing the antigen-binding site generated by cleavage of the antibody with the enzyme papain.
Fc: A fragment of an antibody without antigen-binding sites generated by cleavage with papain; the Fc fragment contains the C-terminal domains of the heavy immunoglobulin chain.
Fibroblastic response: The response characterized by intensive hyperplasia of fibroblasts.
Fibroblasts: A type of connective tissue cell which is responsible for synthesis of collagen.
Fibrosis: The proliferation of connective tissues which consists of a high proportion of fibroblasts.
Fingerling: A young salmonid fish which is older than a fry, and is approximately finger size.
Flagllar pocket: An elongate tubular in-tucking of the body surface from which emerges a flagellum.
Flame cells: Cilia bearing cells in flatworms that have excretory and/or osmoregulatory functions.
Flashing: An erratic swimming behaviour in fishes.
Formalin: A 37% solution of formaldehyde.
Freund's complete adjuvant: A water-in-oil emulsion with killed mycobacteria; addition of an antigen to the emulsion significantly increases the production of antibody against the antigen.
Friable: Easily pulverized.
Funis: One of three bands of microtubular ribbons in *Hexamita* and *Spironucleus*; the ribbon (M_3) originates at kinetosome R, and passes from a nucleus posteriorly along the flagellar pocket.

Gamogony: Part of sexual reproduction; it generally precedes fertilization of macrogametes by microgametes.
Gastritis: The inflammation of the stomach.
Gastroderm: The epithelium lining the gastric lumen.
Genome: The complete set of genetic material of a living organism. In eukaryotes, the genome can be further subdivided into the DNA within individual cellular compartments such as the nucleus, mitochondria and other plastid organelles.
Genomic DNA library: A collection of individually cloned DNA fragments, typically in a bacterial plasmid or phage vector. Genomic DNA libraries represent the sum of all DNA present within the genome, while cDNA libraries represent the mRNA transcripts being expressed by a cell at the time RNA was extracted.
Genomics: The study of the genome of a particular organism, especially the sequence and organization of its genes.
Germinal cells: Non-differentiated cells in a larval trematode, which serve as a source of cells for the next larval generation.

Germ line: Refers to genes in germ cells as opposed to those in somatic cells; that is, genes in their unrearranged state rather than those rearranged for production of proteins.
Gliosis: The disease condition associated with the presence of glioma (neuroglial tumour) or with the development of neuroglia tissue.
Glomerulitis: The inflammation of the glomeruli of the kidney, with proliferative or necrotizing changes of the endothelial or epithelial cells or thickening of the basement membrane.
Glycocalyx: The glycoprotein and polysaccharide covering that surrounds many cells.
Golgi complex: The internal reticular apparatus in the cell cytoplasm which is involved in the secretory process.
Gonopore: The external opening of the reproductive system of a crustacean.
Graft: A piece of transplanted tissue.
Granulation tissues: Newly formed tissues (proliferating fibroblasts, endothelial cells, histiocytes and macrophages) involved in the healing of various types of lesions and in the reparative stage of inflammation.
Granulocytes: Granular leucocytes (eosinophils, basophils and neutrophils).
Granuloma: A lesion resembling a tumour which results from chronic inflammation and consisting primarily of aggregation of macrophages, epithelioid cells and some connective tissue elements.
Granulomatous inflammation: The type of inflammation characterized by the exudate composed of macrophages, epithelioid and giant cells and by connective tissue formation.

Haematophagous: Feeding on blood or lymph.
Haematuria: The presence of blood in the urine.
Haemodilution: The increase of fluid content in the blood with resulting decrease in concentration of its red blood cells.
Haemorrhage: Internal bleeding and subsequent clotting caused by the rupture of blood vessels.
Hamuli: Large sclerites that occur in pairs which are located centrally in the haptor of monogeneans.
Haplokinety: Paroral membrane of exceptional length which is around the epistomial disc in peritrichous ciliates.
Haplotype: A particular combination of closely linked genes on a chromosome inherited from one individual.
Hapten: A substance that cannot initiate an immune response in an animal unless it is bound to a carrier because of its small size.
Haptor: The posterior and principal organ of attachment used by monogeneans.
Heavy chain (H chain): The larger of the two types of chains that comprise a normal immunoglobulin molecule.
Helper T-cells: A class of T-cells that helps to trigger B-cells to produce antibodies against thymus-dependent antigens. Helper T-cells also help to generate cytotoxic T-cells.
Hepatomegaly: The enlargement of the liver.
Hepatopancreas: A part of the digestive tract in crustacea that extends from the midgut and is composed of numerous ducts and blind tubules.
Hermaphrodites: Organisms with both male and female genital systems.
Herniation: Protrusion of part of a membrane-covered organ through a defect or opening.
Heteroxenous parasite: A parasite that requires more than one host to complete its cycle.
Histocompatibility: The ability of transplanted tissues to get along with host tissues; these antigens are collectively referred to as histocompatibility antigens.
Histiocyte: A type of cell with phagocytic ‘macrophage’-like properties; found in connective tissues.

Histolysis: The breakdown of host tissues.
Histophagous: Feeding on tissues of animals.
Holarctic: Of the entire Arctic region.
Holdfast: The attachment organ of some parasites (e.g. copepods, cestodes).
Holotrichous: Ciliates with cilia which cover the entire body surface.
Homologous recombination: Site-specific integration of foreign DNA into the genome of a host organism as a result of recombination between sequences that flank the DNA insert and homologous sequences within the genome.
Humoral immunity: Specific immunity mediated by antibodies.
Hyalinization: Describes an abnormally smooth and glassy appearance in tissues under the light microscopic.
Hyaluronidase: An enzyme that catalyses the hydrolysis of hyaluronic acid, the cement substance.
Hybridization: Annealing between complementary strands of DNA.
Hydropic: Pertaining to or affected with dropsy.
Hyperaemia: The increase in blood flow during acute inflammation.
Hyperbiotic: Pathological processes which have in common an increase in cell metabolism resulting in excessive growth.
Hyperosmotic: The increase in concentration of osmotically active components in a solution.
Hyperplasia: The increase in size of a tissue or an organ by the formation and growth of new cells.
Hyperplastic: Pertaining to abnormal multiplication or increase in the number of normal cells in normal arrangement in a tissue.
Hypertrophic: Pertaining to enlargement or overgrowth of an organ.
Hypertrophy: An increase in size of a tissue or an organ due to an increase in size of individual cells.
Hypnospores: A term used by phycologists for resting cells. This term has also been applied to the pre-zoosporangium of *Perkinsus* spp., which is produced by incubating the parasite from infected hosts in fluid thioglycollate medium for several days.
Hypobiotic: Pathological processes which have in common a decrease in cell metabolism resulting in deficient growth or abnormal patterns of growth.
Hypochronic: A condition in which the amount of haemoglobin in red blood cells is lower than normal.
Hypoesthesia: Abnormal decreased sensitivity to stimulation.
Hypoxia: The reduction of oxygen supply to tissues below physiological levels despite adequate perfusion of the tissue by blood.

Immune complex: An antigen bound to an antibody.
Immunization: The protection of an organism against disease by exposing the animal to parasite antigens so that its immune system recognizes and produces a rapid response against the same antigens on subsequent exposures.
Immunodepression: The decrease in response of the immune system to antigenic materials because of an established infection or exposure to some chemical agents.
Immunoglobulin (Ig): A family of proteins which is made up of light and heavy chains linked together by disulphide bonds; usually produced in response to an antigenic stimulation.
Indirect fluorescent antibody technique (IFAT): A technique in which unlabelled antibody is incubated with the antigen and then overlaid with a fluorescent conjugated anti-immunoglobulin to form a sandwich.
Indirect (passive) agglutination test: The agglutination by specific antibodies of particles or red blood cells to which antigens have been chemically absorbed on their surfaces.

Inflammation: The initial response in vertebrates to tissue injury; this is characterized by the release of amines which case vasodilation, infiltration of blood cells and proteins, and redness.

Infranuclear microtubules: One of three bands of microtubular ribbons in *Hexamita* and *Spironucleus*; the ribbon (M_2) originates at kinetosome R_1, passes below one nucleus, crosses to the other, and then passes posteriorly along the flagellar pocket.

Infundibulum: The funnel-shaped buccal cavity of peritrichous ciliates; it is equipped with buccal ciliary apparatus.

Innate immunity: Various host defences against a parasite which are not dependent on prior exposure to the parasite nor on immunological memory.

Insert: A DNA fragment that has been cloned into a plasmid or bacteriophage vector.

Integument: The tissue which covers an organism.

Intensity: The number of parasites per infected host.

Intercellular: Situated or occurring between cells in a structure.

Interferon: A group of low molecular weight proteins that have antiviral activities, and are capable of enhancing and modifying the immune response.

Interleukin (IL): Glycoproteins derived from macrophages or T-cells which exert regulatory effects on other cells.

Interleukin-1: A macrophage-derived factor that promotes short-term proliferation of T-cells.

Interleukin-2: A lymphocyte-derived factor that promotes long-term T-cell proliferation in cultures.

Interleukin-8: A factor produced by macrophages that chemoattracts T-cells and neutrophils.

Intermediate host: A host in which there is development of the asexual or immature stages of a parasite.

Internal transcribed spacer (ITS): The spacer between the ribosomal subunits coding.

Intercellular: Situated or occurring inside a cell.

Interspecific: Between two different species.

Intraspecific: Among individuals of a species.

Introperitoneal: Within a peritoneal cavity.

Iridoclitis: Inflammation of the iris and the ciliary body.

Isoelectric focusing (IEF): A method that separates isozymes based on their PI (isoelectric point) on a gel.

Isotypes: Classes of antibody that differ in the constant region of their heavy chain (Fc portion); distinguishable also on the basis of reaction with antisera raised in another species. These differences also result in different biological activities of the antibodies.

Isotype switch: The process of changing synthesis of heavy-chain isotypes, e.g. from μ to γ in B-cells.

Isozymes: Different molecular forms of an enzyme with different physical properties.

Karyomastigont: The unit of mastigonts and associated nucleus.

Kilodaltons: The expression of the molecular weight (size) of a compound, expressed as thousand atomic mass.

Kinetoplast: The part of the mitochondrion which contains DNA and is conspicuous after staining; it is located near the base of the flagellum.

Kinetosomal complexes: Clusters of kinetosomes, in *Hexamita* and *Spironucleus*, each complex comprises one anterior pair and one posterior pair of kinetosomes.

Kinetosomal pocket: A depression in the nucleus that accommodates the kinetosome.

Kinetostome (or basal body): A subpellicular cylinder of nine skewed, peripheral structures each composed of three microtubules. It produces a cilium or flagellum at its distal end.

Kinety: Rows of cilia; structurally and functionally integrated entity of kinetosomes and their cilia but not all kinetosomes are necessarily ciliferous.

Lacunar system: A system of fluid-filled sinuses in the tegument of acanthocephalans that may act to distribute nutrients.
Lacustrine: Of the lake habitat.
Lanceolate: Tapering at both ends.
Lemniscus: One of a pair of tegumental projections into the anterior portion of the acanthocephalan pseudocoelom.
Lesions: Abnormal changes in tissues or body functions.
Leucocyte (or **Leukocyte):** A white blood cell.
Leucocytosis: An increase in the number of white blood cells.
Leucorrhoea: A whitish discharge from the vagina.
Library: *See* Genomic DNA library.
Light chain (L chain): A polypeptide chain common in all immunoglobulin molecules; two forms (kappa and lambda) exist in most animals.
Linguiform lobopodium: A tongue-shaped lobular pseudopodium of an amoeba, which often has a hyaline cap at the advancing tip; it is used in both locomotion and feeding.
Lipopolysaccharide (endotoxin): A compound from Gram-negative enteric bacteria; it has a variety of biological functions, including mitogenic activity for B-lymphocytes.
Liquefaction: The result of necrobiosis; conversion of tissue into a semisolid or fluid mass.
Littoral: The coastal zone.
Lymphocyte: A mononuclear, nongranular leucocyte with a dark staining nucleus and pale blue cytoplasm; this class of leucocyte includes cells responsible for producing antibodies and is in circulation and in organs such as lymph nodes, spleen, thymus, tonsils.
Lymphokines: Soluble substances secreted by lymphocytes, which have a variety of effects on lymphocytes and other cell types.
Lysin: An antibody which cases cell lysis (e.g. haemolysin) or a bacterial toxin which lyses cells.

Macrogamogenesis: The formation of macrogamonts during sexual reproduction.
Macronucleus: A large, usually highly polyploid vegetative nucleus in ciliates which controls tropic functions.
Macrophages: Leucocytes which are important in cellular defence and have phagocytic activity.
Macrospores: Large spores in microsporidia characterized by spore di- or polymorphism.
Major histocompatibility complex (MHC): A cluster of genes encoding cell surface molecules that are polymorphic and code for antigens that lead to rapid graft rejection. Several classes of protein, such as MHC class I and II proteins, are encoded in this region, and MHC molecules also play an important role in antigen presentation.
Manca: The larval stage of a parasitic isopod; it has six pairs of legs and usually has good swimming ability.
Manubrium: The stalk of the bulla in a lernaeopodid copepod.
Marsupium: The chamber in which eggs are brooded in an isopod. It is formed by oostegites.
Mastigonemes: Fine filamentous structures arranged in one or more rows or bands and typically appearing at right angles to the flagellar shaft of some protozoa that have flagellated stages.
Mastigont: A complex of flagella-associated organelles in flagellated protozoa, including basal bodies (also called kinetosomes) and projecting and trailing flagella.
Mehlis gland: A gland which evacuates into the ootype in the digenean female system.
Melano-macrophage centres: Localized accumulations of macrophages in organs, which contain haemosiderin, lipofuschin, ceroid and melanin-staining pigments.
Melanomacrophages: Pigment bearing macrophages which aggregate in the haeopoietic tissues to form melanomacrophage centres. They contain melanin, maemosiderin and lipid pigments.

Melanophores: Dermal cells containing melanin.
Membranelle: An organelle in the buccal cavity or ciliates, it is composed of three short rows of densely set kinetosomes.
Memory (immunological): The ability of the immune system to recall a previous exposure to an antigen and to respond with an earlier and/or higher response.
Meningitis: Inflammation of the meninges, which are membranes that cover the brain and spinal cord.
Merogony: The sequence of asexual multiplication (syn. schizogony) to produce a great number of vegetative parasite stages.
Meront: An asexual development stage to produce merozoites.
Merozoite: A crescent stage formed by a meront during asexual reproduction; a stage which may initiate another merogony or gamogony.
Mesangial cells: Irregularly shaped, phagocytic cells which support capilliary loops in the renal corpuscles.
Mesotrophic: Aquatic habitat with intermediate organic load.
Metacercariae: Encysted cercariae of digeneans.
Metaplasia: A change in tissue type.
Metatrypanosome: The trypomastigote stage of a trypanosome that is formed in the vector after cyclopropagative development.
Microarray: A large number of specific sequences (genes, gene fragments or oligonucleotides) spotted on an inert support (e.g. a glass microscope slide) in a pattern or grid. Such arrays are used to evaluate alterations in gene expression within a given tissue or organism under different sets of conditions, by hybridization with RNA obtained from cells subjected to those conditions.
Microcytic: A condition in which red blood cells are smaller than normal.
Microfilaments: Very fine filaments, such as those occurring in longitudinal rows of tufts on the surface of *Spironucleus elegans.*
Microgamete: A male gamete which is usually motile.
Microgamogenesis: The formation of microgamonts and microgametes.
Microgamont: A cell which produces microgametes.
Micronucleus: An usually very small, diploid generative nucleus in ciliates which carries the genetic information.
Micropyle: An opening of the oocyst wall.
Microspores: Small spores in microsporidia which are di- or polymorphic.
Microtubular ribbons: Multiple linked microtubules in a flat, ribbon-like arrangement; they are also called microtubular bands or fibres.
Microtubule: A hollow rigid cylindrical structure of 20–25 nm in diameter; microtubules may be linked to others to form a ribbon or band; they comprise the '9 + 2' structure in flagella.
Microvilli: Extensions from the surface of epithelial cells or tegument (in Platyhelminthes).
Mictosporic: A Myxosporean species whose plasmodia forms various types of spores.
Miracidia: The larval stage that hatches from a digenean egg.
Mitochondrion: A filamentous or granular organelle in the cytoplasm; it is the principal site of oxidative reactions by which energy is made available for endergonic processes.
Mitogen: Any agent which induces mitosis in cells.
Mixed lymphocyte reaction (MLR): When lymphocytes from two individuals are cultured together, a proliferative response is generally observed, as the result of reactions of T-cells of one individual to MHC antigens on the other individual's cells.
Molluscicide: An agent (usually a chemical) that is used to control molluscs.
Monoclonal antibody: Identical antibody (immunoglobulin) molecules produced by a cloned antibody producing cell.
Monocytes: Non-granular, usually phagocytic leucocytes.

Monopisthocotyleans: Tissue feeding monogeans which have sclerotized hamuli in the haptor at some point in their life cycle.
Monosporic: A plasmodium or pseudoplasmodium which produces a single spore.
Monoxenous: A life cycle which involves one host.
Monozoic: Flagellates that have a single karyomastigont or cestodes that have a single set of reproductive organs.
Moulting: The shedding of an old cuticle during the development of a nematode.
Monoclonal: In immunology, monoclonal generally describes a preparation of antibody that is monogenous or cells of a single specificity.
Monokine: A substance released by a macrophage or a monocyte that affects the function of another cell.
mRNA: Messenger RNA.
Mucocyst: A membranous and/or fibrous structure located beneath the surface membrane of a ciliate or a flagellate; it is discharged through an opening in the pellicle as a mucus-like mass.
Mucopolysaccarides: Mucin substances which are composed of protein-sugar complexes.
Mucron: A cuticular spine at the posterior end in a nematode.
Mucus: A secretion that is usually rich in mucin and is produced by epithelial goblet cells.
Multiple fission: Cell division in which the parent cell undergoes a series of nuclear divisions before the cytoplasm divides to produce daughter cells.
Muscularis externa: The external muscular layer of the vertebrate gut.
Myelitis: The inflammation of the spinal cord.
Myofibrils: Microfibrillar strands or bundles with contractile function in protozoan cells.

Nacre: The iridescent inner layer (mother-of-pearl) of many mollusc shells.
Natural killer cells (NK cells): Large granular lymphocyte-like killer cells that kill various tumour cells, do not exhibit antigenic specificity, and their number does not increase by immunization.
Nauplius: The early larval stage of copepods and barnacles; it has three pairs of swimming appendages.
Necrosis: The alteration of tissue which results in cell death and formation of exudate.
Necrotic: Pertaining to cell death and enzymatic degradation in a limited area in an organism.
Nematocyst: A specialized organelle in coelenterates which contains a capsule with extrusible polar filament. Serves mostly for capture of prey.
Nematodesma: A bundle of microtubules in paracrystalline arrangement, associated with kinetosomes, reinforcing the walls of the cytopharyngeal tube in ciliates.
Neutralization: The process by which an antibody neutralizes the infectivity of a parasite.
Neutrophils: Leucocytes with polymorphic nuclei, and neutral staining granules; they have phagocytic properties and are present during inflammation.
Northern blot: A technique in which RNA is transferred from an agarose gel to a membrane and hybridized with the complementary DNA.

Obligate parasite: An organism that can only survive as a parasite on or in another animal.
Ocelli: Punctiform or crescentiform pigment markings, usually arranged segmentally on the urosome of leeches or radially around the caudal sucker.
Oedema: The accumulation of fluid in tissues.
Oesophagus: Part of the gut between the pharynx and the stomach.
Oligohaline: Refers to water (brackish) in the area where fresh water and sea water mix (e.g. in an estuary, bay or areas adjacent to the shoreline) and the salinity of the water tends to vary widely, depending on the kinetics of the mix of marine and fresh water.

Oligonucleotide primers: Short single strands of DNA used to initiate the DNA polymerase chain reaction on the template for the synthesis of the complementary strand.
Omnivorous: An animal with diverse diet.
Oncomiracidium: The free-swimming or crawling infection stage of monogeneans.
Oocyst: A one-to-three-layered wall or membrane-bound sack surrounding a sporont or sporocysts.
Oostegite: Thoracid plates that extend under a female isopod to form a marsupium in which the eggs are carried.
Ootype: A central chamber in the digenean female system which connects the oviduct, the seminal receptacle, the vaginal and the vitelline ducts.
Operculum: A cup-like opening of the egg of flatworms.
Opsonin: A factor which binds to particles or parasites and increases their susceptibility to phagocytosis.
Opsonization: The coating of bacteria (or protozoans) with antibody and/or complement, which leads to enhanced phagocytosis of the microorganisms by phagocytic cells.
Oral kinety: Any kinety associated with the oral region in ciliates.
Oral vaccination: The immunization of an animal in which the vaccine is mixed with food.
Osmolarity: The concentration of osmotically active particles in solution.
Osmoregulation: The adjustment of internal osmotic pressure in an organism in relation to changes in the external osmotic environment.
Oviparous: An organism that lays eggs.

Palintomy: Rapid binary fissions typically within a cyst and essentially without intervening growth.
Pansporoblast: A spore-producing formation within a polysporic plasmodium. It originates by the union of two generative cells (the pericyte and the sporogonic cell). The pericyte gives rise to the pansporoblast envelope while the sporogonic cell divides to produce the sporoblast cells.
Papillae: Small conical projections on the body surface.
Paraoral membrane: A ciliary organelle at the right border of the buccal cavity in ciliates. It consists of two rows of kinetosomes, of which only the outer one is ciliated.
Parasome: An organelle-like structure beside the nucleus of *Paramoeba* sp., which may be a discrete organism of unknown taxonomic affinities and is not an organelle of the amoeba.
Paratenic host: A transport host in which the larval stage of a parasite undergoes no development and its only function is to transfer the parasite to the next host.
Paraxial rod: An electron-dense ribbon or rod along the axoneme in the flagellar membrane.
Paresthesia: Morbid or abnormal sensation such as burning, prickling.
PAS reaction: The Periodic-Acid-Schiff (PAS) reaction used for specific staining of glycogen, neutral mucin, basement membranes and fungi.
Pathognomonic: Specifically distinctive or characteristic of a disease or pathological condition; a sign or indicant on which a diagnosis can be made.
Pectinelle: One of an equatorial band of short oblique rows of up to about six closely apposed cilia. It is used for locomotion in telotrochs of peritrichous ciliates; in adult ciliates the cilia are usually resorbed.
Pellicle: The cortex of the ciliate cell; it is composed of an outer cell membrane subtended with flat pellicular alveoli with underlying fibrous layer, the epiplasm.
Penmiculus: A modified membranelle, with kinetosomes slightly more apart, forming sometimes more than three rows (up to seven) across.
Pentamer: A molecule consisting of five (sub)units.
Performatorium: A cortical structure at the anterior of theronts and trophonts, used for penetration of epithelium and feeding.

Perichondrium: Cartilage-forming tissue in an animal.
Pericyte: *See* Pansporoblast.
Periopod: A walking leg of a crustacean.
Peristomial lip: A pellicular fold in peritrichous ciliates, it skirts the groove in which the cilia encircling the epistornial disc are inserted.
Petechial haemorrhage: A tiny haemorrhage about the size of a pin head.
Phagocytosis: The engulfment of microorganisms or other particulate material by cells.
Phagosome: A closed membrane-bound intracellular vesicle in a phagocyte formed by invagination of the cell membrane and the phagocytized material.
Pharynx: The muscular bulb at the anterior end of the gut in flatworms.
Phosphatase: An enzyme that splits phosphate from its organic compounds (esters).
Plankton: Small organisms which float in water.
Plasma cell: End-stage differentiation of a B-cell to an antibody-producing cell.
Plasma membrane: The trilaminated cell membrane.
Plasmid: A circular DNA molecule that can act as a replicating unit of DNA. By inserting a foreign DNA element into a plasmid, that element can be maintained following introduction of the plasmid into a host cell.
Plasmodium: A multinucleate mass of protoplasm which is generally produced from a uninucleate stage.
Plasmotomy: The division of a multinucleate cell by cleavage in two or more cells.
Pleopod: An abdominal appendage of a crustacean.
Pleocercoid: The larval stage of cestodes which is adapted to aquatic transmission; the larval stage is usually in the fish intermediate host.
Poikilotherms: Vertebrates (fish, amphibians and reptiles) whose body temperature fluctuates with the ambient temperature.
Polar cap: A body stained by PAS (Periodic-acid-Schiff) in the apex of a microsporidian spore; it is essentially identical to the anchoring disc.
Polar capsule: A thick-walled vesicle in myxozoan spores with an inverted polar filament; it forms a coil and on stimulation everts inside out.
Polar filament: *See* Polar capsule.
Polarplast: An organelle in the anterior part of a microsporidian spore which consists of an anterior membraneous and posterior vesicular part; it is instrumental in the extrusion of the polar tube.
Polar tube: A tubular coiled organelle inside a microsporidian spore; it is used to inject the sporoplasm into a host cell.
Poly-A$^+$ RNA: Synonymous with messenger RNA from eukaryotic cells. These RNAs are post-transcriptionally modifed to contain a homopolymeric A tail (in the range of 50–200 nucleotides) at their 3′ end.
Polykinety: An extremely elongated membranelle in peritrichous ciliates which encircles the epistomial disc.
Polymerase chain reaction (PCR): A method that allows amplification of specific DNA sequences, utilizing oppositely oriented (forward and reverse) oligonucleotide primers that flank a given region of DNA. Template DNA is denatured (melted) at high temperatures and allowed to reanneal in the presence of a large molar excess of flanking primers. The region between primers is then synthesized in the presence of a thermostabile DNA polymerase. Successive rounds of melting, reannealing and synthesis are carried out leading to a geometric increase in the amount of the specific sequence with each round of synthesis.
Polymorphic: The ability of biomolecules, such as some enzymes, to exist in more than one molecular form.
Polyopisthocotyleans: These are blood-feeding mongeneans, which attach to fish gills by means of sclerotized clamps.

Polypeptide: A peptide which on hydrolysis yields more than two amino acids.
Polysporic: A plasmodium which produces several spores.
Postoral kinety: A kinety which runs from the buccal cavity on the central side of a ciliate.
Pranzia: The larval stage of a gnathid isopod.
Precipitin: An antibody or other substance which reacts with a soluble antigen to form a precipitate.
Precocious: An animal attaining maturity at early age (larval age).
Premunition: The resistance to infection against the same or closely related pathogen after an acute infection has become chronic. The protection lasts as long as the infection persists.
Prepatent period: The period after infection but before the causative agent can be found using the usual diagnostic techniques.
Presporogonic: A sequence of development which precedes sporogony.
Prevalence: The percentage of animals in a population which are infected at any one time by a particular organism.
Primary lymphoid organs: Organs in which the maturation of T- and B-lymphocytes takes place and antigen-specific receptors are first acquired.
Primary responses: The immune response to a first encounter with an antigen. The primary response is generally small, has a long induction phase or lag period, consists primarily of IgM antibodies and generates immunological memory.
Primer: An oligodeoxynucleotide used to prime DNA synthesis reactions in PCR or DNA sequencing protocols. The free 3′ hydroxyl group of the terminal deoxyribose sugar initiates DNA strand synthesis.
Primordia: The earliest discernible indication of an emerging organ or part of it.
Proboscis: A muscular, protrusible feeding organ in rhynchobdellid leeches.
Proboscis sheath: The space surrounding the proboscis when it is retracted.
Proceroid: The first larval stage of many cestodes which develop inside the body cavity of the invertebrate (first) intermediate host.
Productive inflammation: *See* Proliferative inflammation.
Progenetic: Larval stage that attains sexual maturity and produces eggs.
Prohaptor: A non-sclerotized anterior organ of attachment used by monogeneans.
Proliferative inflammation: An inflammation which is characterized by a pronounced multiplication of fibroblasts: histiocytes and endothelial cells.
Pronephros: The primary kidney which is incorporated into the head kidney in adult fish.
Prostate gland: A gland which provides the supporting medium for male gametes.
Protandry: A type of hermaphroditism in which the individual first functions as a male and then as a female.
Protease: A general term for proteolytic enzymes.
Protista: Single-cell (or very few-celled and, therefore, still microscopic) eukaryotic microorganisms which diversified to give rise to animal, plant and fungal evolutionary lines.
Protonephridium: An organ for osmoregulation and excretion; it consists of flame cells connected to a tubule complex that opens to the exterior.
Pseudocyst: A cyst-like structure surrounded by a dense fibrous capsule. It does not possess an inner lining and the formation of a pseudocyst follows necrotic changes.
Pseudopodium: A temporary cytoplasmic projection of an organism which is used for feeding and/or for locomotion.
Pulsatile vesicles: Blister-like lateral appendages on the urosome of some piscicolid leeches; they are part of the coelomic system, and contract/expand to circulate coelomic fluid.
Pyknotic: The degeneration of a cell in which the nucleus shrinks and the chromalin condenses to a solid amorphous mass.
Pyriform: Pear-shaped.

Raceway: An elongated earth pond or cement lined container with constant water flow.
Rapid amplification of cDNA ends (RACE): A method to obtain sequence information from the 5′ and 3′ ends of cDNA molecules. Starting with the sequence of a partial cDNA, this method allows one to obtain the full-length sequence of an RNA transcript by PCR.
Random amplified polymorphic DNA (RAPD): DNA fragments generated by PCR using pairs of random oligodeoxynucleotide primers. Patterns of bands generated with given sets of primers can be diagnostic for a given organism, strain or species, and can be used as a tool in genotype analysis.
Receptaculum seminis: A sac for storing received spermatozoans in the female genital system of trematodes.
Recurrent: Directed towards the posterior end.
Redia: The third larval stage of digeneans.
Regressive changes: A pathological process which is related to metabolic disorders and includes dystrophic (degenerative) changes and necrosis.
Repair: A process to reestablish anatomical and functional integrity of tissues after an injury or inflammation.
Respiratory burst: Oxygen-dependent increase in metabolic activity within phagocytic cells usually stimulated by bacteria or parasites.
Restriction endonuclease: An enzyme that recognizes specific sequences within duplex DNA and cleaves (digests) the double strand, leaving either blunt ends or overhangs, which can act as sticky ends for DNA cloning. Digestion of genomic DNA with these enzymes creates a unique pattern of fragments for any given endonuclease.
Restriction fragment length polymorphism (RFLP): DNA fragments of different lengths after the DNA has been cleaved by restriction endonucleases at specific sites.
Rete system: A series of canals associated with the musculature in the body wall in an acanthocephala.
Reticuloendothelial system (or mononuclear phagocyte system): A network of phagocytic cells primarily in the reticular connective tissues of lymphoid and other organs.
Retina: The sensory lining on the back of an eye.
Rhizocyst: A nail-like organelle which projects from the attachment disc of some parasitic dinoflagellates, e.g. *Piscinoodinium*; the organelle penetrates the host cells to provide anchorage for the parasite.
Rhizoids: Modified cytoplasmic projections which arise from the basal end of some parasitic dinoflagellates, e.g. *Amyloodinium, Crepidoodinium*; these projections attach to or penetrate host cells to provide anchorage.
Rhynchi: Anterior tentacles or fimbriae in bucephalid digeneans.
Ribosomal DNA (rDNA): A region of the genome that contains the genes for ribosomal RNA.
Ribosomal RNA (rRNA): A structural unit of the ribosome. Ribosomal RNA is expressed in the nucleus as a large primary transcript that is processed to form 18S and 28S RNAs that associate with the small (40S) and large (60S) subunits of eukaryotic ribosomes, respectively.
Ribosomes: Intracytoplasmic granules which are rich in RNA and function in protein synthesis.
RNA interference (RNAi): A method for suppressing the expression of specific genes in eukaryotic cells using double-stranded RNA.
Rootlet fibril: *See* Striated lamella.

Sagenogenetosomes: Complex pit-like structures in the cell surface of the Labyrinthomorpha; they are presumed responsible for production of the ectoplasmic net which is involved in movement and nutrient acquisition.
Sanguiniferous: Blood feeders.
Second set rejection: Accelerated rejection of an allograft in an already immune recipient.

Schizogony: *See* Merogony.
Schizont: *See* Meront.
Scolex: *See* Holdfast.
***Scolex pleuronectis*:** A scientific name which is used widely to describe tetraphyllidean plerocercoids whose adult stages are unknown.
Scopula: A small area of the pellicle at the aboral body end of sessiline peritrich ciliates. It consists of very short, imperfect and immobile cilia and is used for attachment to the substrate, either directly or by means of a secreted stalk.
Secondary lymphoid organ: An organ which contains the effector cells (activated lymphocytes and plasma cells) after an antigenic stimulation.
SEM (scanning electron microscopy): A technique which utilizes electronics to study the surface structures of an organism; this method provides increased resolutions at high magnifications compared with what is achieved with light microscopy.
Senescent: The depression of cellular functions in an animal as part of the ageing process.
Serosanguineous: Pertaining to or containing both serum and blood.
Shell valve: one of the parts of the myxosporean spore wall.
Shucking: A procedure in which a mollusc is removed from its shell.
Smolt: A young salmon or sea trout that is about two years old.
Somatic: Pertaining to the body.
Somatic cilia: All cilia on the body surface which are outside the oral region.
Sonicated: Disruption of an organism by ultrasonic waves.
Southern blot: A method for detecting specific genes or DNA fragments. Typically, genomic DNA is digested with restriction endonucleases, and the resulting digests are fractionated by agarose gel electrophoresis. DNA is then transferred (blotted) on to a filter, and screened with a labelled DNA or RNA probe for a gene of interest. Southern blots reveal the presence or absence of a particular DNA element and its organization within the genome.
Spermatophore: A package of sperm, usually deposited by a male crustacean on to a female.
Sphaeromastigote: The round developmental stage of a trypanosome in which the flagellum is visible but the anterior end of the organism cannot be identified.
Splenomegaly: The enlargement of the spleen.
Spongiosis: Intercellular oedema of the spongy layer (malpighian layer) of the skin.
Sporangium: The developmental stage of some protozoa that is sac-shaped and is destined to produce multiple spores or trophozoites as in *Perkinsus* sp.
Spore: The infective stage of an organism that is usually protected from the environment by one or more protective membranes.
Sporoblast: A cell which developed into a spore; it is produced generally by the division of a sporant.
Sporocyst: A membrane bound sac which contains sporozoites of Apicomplexa or the second larval stage of digeneans in the gastropod intermediate host.
Sporogonial plasmodium: A multinuclear cell with many generative cells engaged in sporogony.
Sporogony: A phase in the development of an Apicomplex in which the zygote initiates asexual reproduction and results in production of infective sporozoites.
Sporont: A developmental stage that gives rise to one or many sporoblasts.
Sporophorocyst: A thick envelope which is produced by early stages of heterosporis, and later is enlarged to contain all ensuing stages of merogony and sporogony.
Sporophorous vesicle: A solid envelope of parasite origin which encloses sporogonic stages and mature spores when they are formed.
Sporoplasm: The infectious component in spores.
Sporoplasmosome: An electron-dense inclusion in the sporoplasm.
Sporozoite: A nucleated infective stage formed by division of the sporont.

Sporulation: The formation of sporocysts and sporozoites within an oocyst.
Stigma: A pigmented red spot in diniflagellates, it may also be present in the dinospore and other stages.
Stomopode: A membrane-bound tubular projection which arises proximal to the sulcus in the trophont stage of *Amyloodinium.*
Stress: An adverse stimulus which results in disruption of homeostasis in an organism.
Striated lamella: The fibrillar structure which extends from a kinetosome (R in Hexamitinae), into the deeper cytoplasm of the cell; typically it is cross-banded (striated).
Sublabia: Lip-like structures which are located below true lips, and they open into the oral cavity in some nematodes.
Suctorial tentacle: A rod-like organelle which extends from the body of suctorian ciliates. It is retractile and contains a microtubular cylinder, and has a capitate end. This end has a battery of haptocysts whose extrusomes contain toxic and lytic substances to capture prey and the prey cytoplasm is ingested through the tentacle.
Supranuclear microtubules: One of three bands of microtubular ribbons in *Hexamita* and *Spironucleus*; the ribbon (M_1) originates at kinetosome K_1, and passes from the top of one nucleus across to the top of the other nucleus.
Surfactant: A surface-active agent such as a soap or a detergent.
Suture line: A line of convergence of somatic kineties from different areas of the surface of a ciliate. There may be preoral sutures (in front of) and postoral sutures (behind the oral area).
Symmetrogenic division: A type of binary fission (generally longitudinal) in which the progenies are mirror images of each other with respect to principal structures.
Syncytium: A multinucleated mass of cytoplasm.
Syrian–African rift: Formed between Syria and central Africa by sinking of land between two roughly parallel faults.
Syzygy: Side-by-side or end-to-end association of gamonts after the formation of gametocysts and gametes.

T-cell: A rhymus derived lymphpcyte (*see* T-lymphocyte).
Tegument: The nonciliated body wall of platyhelminths.
Tetramer: A molecule which consists of four (sub)units.
T-dependent antigen: An immunogen that is able to induce antibody synthesis only in the presence of lymphokines released by helper T-cells.
Theca/rhizotheca/amphiesma: A multilayered membranous sheath that covers most of the surface of a dinoflagellate.
Theront: The free-swimming infective form of *Icthyophthirius* and *Cryptocaryon.*
Thigmotactic ciliature: Specialized somatic cilia which are presumed to have sensory-tactile or adhering function.
Thrombocytes (platelets): Involved in clotting and in phagocytosis; they are presumed to be produced in the spleen
Thrombus: (pl. thrombi): Blood clot.
Thyroxine: A hormone secreted by the thyroid; its main function is to increase the rate of cell metabolism.
T-independent antigen: An immunogen that induces antibody synthesis in the absence of lymphokines released by T-cells; the antibodies are generally only IgM isotype.
Titre: The reciprocal of the last dilution of an antiserum titration giving a measurable effect; e.g. if the last dilution giving significant agglutination is 1 : 128, then the titre is 128.
T-lymphocyte: A subpopulation of lymphocytes whose development occurs in the thymus; these are effector cells in cell-mediated immune response and regulatory cells of the immune system.

Tolerance: Diminished or absent capacity to respond to a specific antigen; it is usually produced as a result of contact with that antigen under non-immunizing conditions.
Tomites: Cells within the tomont which result from serial binary division.
Tomont: A cyst-like structure formed by the trophont following detachment from the host; it supports binary division of the tomites and their maturation.
Torus (pl. tori)**:** Rounded swelling.
Trachelosome: The neck region of a piscicolid leech that includes clitellar and preclitellar segments.
Transcript: An RNA molecule copied from genomic DNA (often synonymous with messenger RNA).
Trophont: The feeding and growing stage of a dinoflagellate or a ciliate which differentiates into the reproductive tomont following detachment.
Trophozoite: A vegetative (feeding) stage in the life cycle of a parasitic protist that may undergo asexual multiplication (fission).
Trunk: The 'body' of many parasitic copepods; formed from fused and enlarged genital segments and may also incorporate other thoracic segments.
Trypomastigote: The elongated developmental stage of a trypanosome in which the flagellum arises posterior to the nucleus and emerges laterally to form a long undulating membrane along the body to the anterior end.
Tumour necrosis factor (TNF-α): Secreted by macrophages in response to inflammation and infection; cytotoxic to tumour cells but not to normal cells.

Ulcer: The excavation of the surface of an organ or tissue produced by sloughing of necrotic inflammatory tissue.
Ulceration: A pathological condition which is usually associated with the skin or mucosal surfaces; it involves lesions with erosion of surface epithelia, and inflammation with infiltration of leucocytes.
Urosome: The main body region of a piscicolid leech between the clitellum and caudal sucker.
Urticaria: An allergic condition marked by red wheals.
Uveitis: Inflammation of the uvea – the iris, ciliary body and choroid considered together.

Vaccination: Originally referred to immunization against smallpox with the less virulent cowpox (*Vaccinia*) virus; it is now used for any immunization against a pathogen.
Vaccine: An antigen preparation which consists of either whole cells or extracts of cells and is used to immunize animals.
Vacuolated: Containing spaces or cavities in the cytoplasm of a cell.
Valvogenic cell: One of the cells in a sporoblast in *Myxosporea* which differentiates into the shell valve.
Vascularization: The increase in supply of blood to a tissue; this is either by increasing blood volume (dilation of blood vessels) or the development of new blood vessels.
Vas efferens: Proximal sperm-carrying duct.
Vector (molecular biology)**:** A vehicle for cloning genes, most often consisting of plasmid or bacteriophage DNA that has been modified to accept foreign DNA inserts.
Ventriculus: The posterior glandular part of the oesophagus, this structure is characteristic of many ascaridoid mematodes.
Vesicle: A small circumscribed elevation on the epidermis which contains serous fluid.
Vestibulum: A depression of the body surface which leads to the cytostome and is equipped with ciliature of somatic origin; if it is in the shape of a groove, it is called a vesticular groove.
VH genes: Genes which code for the variable region of a heavy chain in immunoglobulins.

Virulence: The capacity of a parasite to cause disease in an animal; the damage may be modified by the defence mechanism of the host.
Vitellaria: Yolk glands.
Vitreous humour: The posterior chamber of the eye.
Viviparous: The bearing of live young.

Western immunoblotting: A technique to identify specific antigens in a mixture by separation on polyacrylamide gels, blotting onto mitrocellulose, and labelling with radiolabelled or enzyme-labelled antibodies.

Xanthochromic: Yellow in colour.
Xenoma: A symbiotic complex formed by a hypertrophic host cell and an intracellular parasite which proliferates in its cytoplasm.

Yearling: A one-year-old salmonid fish.

Zooplankton: Animal-like organisms which float or drift almost passively at sea or in other large water bodies.

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