Fish Diseases and Disorders, Volume 2: Non-infectious Disorders, Second Edition

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Fish Diseases and Disorders, Volume 2: Non-infectious Disorders, Second Edition

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Preface

As for the first edition of this volume, the chapters comprise comprehensive discussions of the some of the major non-infectious disorders of finfish. It is the second volume of a three-volume series on fish diseases and disorders; Volume 1 deals with parasitic diseases and Volume 3 with microbial diseases. Reviews in the three volumes are written by leading international authorities who are actively working in the area or who have contributed greatly to our understanding of specific diseases or disorders.

The present book includes non-infectious disorders of development and growth and various aspects of the physiology of wild and captive species, including nutritional physiology, feeding activity, cardiovascular physiology, ionic and osmotic regulation, stress physiology, reproduction and endocrine physiology. In addition, chapters dealing with issues related to the diagnosis of non-infectious disorders, tumourigenesis and problems related to supersaturated gas issues in aquaculture practice are included. Because of the increasing concern of the effects of 'anthropogenic' chemicals on aquatic organisms, particularly, but not exclusively, those that act as hormone mimics or hormone-disrupting chemicals, several chapters address this issue from different perspectives. These chapters review the known effects of such chemicals on the endocrine, reproductive and immune systems, and explore the use of fish as sentinel organisms for the detection of such chemicals and monitoring of 'ecosystem health'. In addition, because of the increasing interest in animal welfare issues in aquaculture practice, a chapter dealing with this topic is included in this volume.

The second edition attempts to address emerging areas of interest and concern in fisheries health in both wild populations and captive stock, and to reflect changing attitudes toward the interpretation of fish health issues and the affects of non-infectious disorders on production issues in the wild and captive fish stocks. Several chapters are included that were not present in the first edition; new authors have contributed to some of the chapters that were present in the first edition, and some chapters have been updated from the first edition.

The principal audience of this volume, as for Volumes 1 and 3, is the fish and fisheries research community, in aquaculture and government fisheries management and researchers in academe; the community comprises environmental toxicologists, pure and applied fish physiologists, fish health specialists, and fish health consultants in government

laboratories, universities or the private sector. The volume is also relevant to graduate students and senior undergraduate students who are involved in studies related to the health of aquatic organisms.

J.F. Leatherland and P.T.K Woo

1 Introduction: Diagnostic Assessment of Non-infectious Disorders

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Introduction

The term diagnosis is generally used to describe the recognition of a disease or condition by its clinical signs and symptoms; however, the definition is commonly extended to include the second stage of the identification process, namely the determination of the underlying physiological, biochemical or molecular factors that are related to or responsible for the disease or condition. In human and veterinary medicine, even when a specific aetiological agent is known, a cluster of specific clinical signs (together with symptoms communicated by human patients) is used to formulate preliminary diagnoses. Based on the clinical signs, clinical tests are then used to confirm or refute the preliminary diagnosis, and, where possible, treatments and disease management strategies are developed to deal with the condition. This general approach is used extensively in veterinary practice related to the management of captive fish stocks and, to a lesser extent, to diagnose infectious conditions of wild fish populations; however, diagnosing non-infectious disorders in fish has tended to be much more problematic, and it has been particularly difficult to link the non-infectious conditions to a specific aetiological factor. Moreover, the follow-up evaluation of the physiological and biochemical responses of the organism rarely provides specific information about the root cause(s) of the dysfunctional condition.

This volume of the second edition of the fish diseases series comprises chapters that focus on the description of known and generally well-documented non-infectious disorders. The chapters examine the nature of the disorders, the biological implications of those disorders and the aetiologies of the disorders, as far as these are known. Some chapters survey the diseases and disorders associated with a specific organ system, such as the cardiovascular system; in other chapters the focus is on a particular aspect of fish disorders related to a specific theme, such as disorders associated with nutritional factors or with tumour genesis. Regardless of the scope of the interest, a primary challenge for investigators in this particular field is to identify when a specific animal, a captive stock or a wild population is exhibiting signs of a non-infectious disease or disorder. As will be explored in this chapter, most of our knowledge pertaining to non-infectious conditions is based on follow-up studies that have been prompted by observations of poor growth, reproductive problems or grossly evident lesions within a particular population or stock. As will be discussed in the following pages, for several reasons, an a priori diagnosis (or even a

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posteriori diagnosis) of a specific problem is often not possible.

Issues Related to the Diagnosis of Non-infectious Disorders

Infectious diseases are diagnosed by symptomatology (the study of symptoms) and the identification of the infectious agent or the product of that agent. For non-infectious disorders, because there is no infectious agent or the product of that infectious agent, the identification of a problem is limited to the recognition of clinical signs and symptoms. Moreover, non-infectious diseases may not be associated with a primary response of the innate or acquired immune system; hence, even immunological assessment tools may not be applicable. Consequently, many of the non-infectious conditions that have been recognized and studied in fish to date have been documented without the application of specific diagnostic methods. In fact, many of these cases were discovered serendipitously and the follow-up physiological or biochemical studies were made a posteriori, and it remains to be determined if these largely non-specific responses can be used as meaningful diagnostic tools. In fact, for the most part, these compensatory physiological and biochemical responses, albeit of value and interest to the investigator, are of limited diagnostic value. In contrast, in the short term, it is commonly the 'global' responses of a population, such as changes in the structure of a population or changes in the reproductive success of a population, that are the primary indicators of the existence of a health issue in that population. There are exceptions to the rule, such as changes in the cardiovascular physiology and xenobiotic-induced changes in the reproductive system of some fishes, which are explored in later chapters.

Figure 1.1 summarizes the several levels of biological organisation at which responses to non-infectious disorders can, in theory, be detected; however, it must be emphasized that non-infectious disorders and diseases that have very different root causes may elicit similar responses (such as poor growth) when measured at the population or stock level. The diagnostic and analytic problems are far more challenging for studies of disorders in wild fish populations, compared with studies of issues in captive stocks. In captive fish stocks, high mortality rates, reduced feeding and reduced reproductive success of the stock can be readily identified by facility managers; the cause(s) may not be directly evident but the outcomes are. In contrast, for wild populations, the reduction in fish numbers could be associated with increased mortality or reduced reproduction or both. Increased mortalities in wild populations may not be recognized unless there is an acute episode and then only if the dead fish are found, which is not likely to occur, for example, with benthic species. More commonly, increased mortality in a wild population is suspected when the numbers of fish in a population declines; however, a reduction in the size of the population may not necessarily be related to an increase in mortality rates, although this may be one component; several direct and indirect factors, including ecological factors may contribute to a decrease in population size, as summarized in Box 1.1.

All of the factors noted in Box 1.1 have been linked to reductions in the size of wild populations of diverse fish species, and they will be elaborated on later in this chapter. Because the reduction in the size of a population is the end product of the impact of these factors, other population indicators need to be used to examine the dynamics of the dysfunctional state in progress and these may be more useful indicators. For example, the absence of an age class in a population may be indicative of a reproductive problem, and skewed age/size distributions might indicate impaired growth and associated metabolic dysfunctions, which could possibly be attributed to several factors (Fig. 1.1). Information related to feeding activity source and quality of diet might provide an insight into changes in the structure of the population. Measurements of the relative concentration of stable isotopes in body tissues are currently being used by a

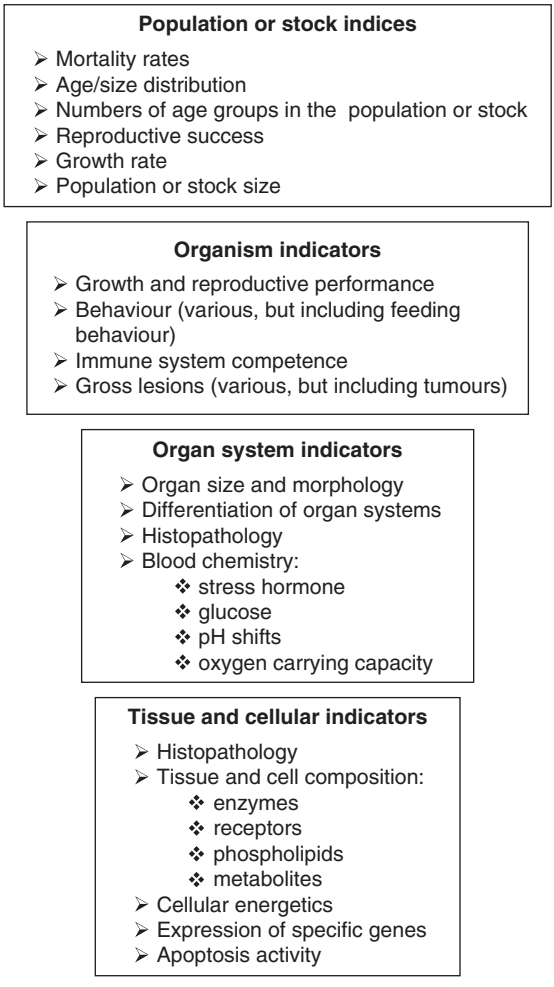


Fig. 1.1. Schematic summary of the levels of biological organization at which indicators of non-infectious diseases or disorders can be detected; at each level examples of key investigational methods are shown. The population or stock indicators are most commonly the first indicators of a non-infectious disease or disorder, although some organism indicators (for example, prevalence of lesions, including tumours) have also been the first indicators of a possible problem. For the most part, the organ system indicators and tissue and cellular indicators have not been primary indicators of a possible problem, but have been used for follow-up diagnostic purposes.

Box 1.1. Summary of factors that may contribute directly or indirectly to a decrease in the size of a wild population of fish.

Mortalities or impaired reproduction associated with contaminated environments.
Mortalities or impaired reproduction associated with hypoxic environments.
Mortalities associated with suppressed immune system function, leading to increased susceptibility to infectious disease.
Increased predation (including increased harvesting of natural resources by recreational and commercial fishing).
Reductions in the availability of suitable food resources.

number of investigators (Satterfield and Finney, 2002; Høie *et al.*, 2003; Schlechtriem *et al.*, 2004; Dubé *et al.*, 2006; Hutchinson and Trueman, 2006; Rojas *et al.*, 2006; Williamson *et al.*, 2009, among others) to determine the changing history of dietary sources of individual fish and populations. This approach offers a means of determining

dynamic aspects of population stability and could be a valuable tool in documenting trophic-related factors involved in population change.

Another compounding factor is the as yet poorly understood association between depressed immune system function and impaired growth and reproductive success. It is not clear whether the growth and reproductive condition bring about the depressed immune response or vice versa, or whether these are independently part of the relatively non-specific 'stress response' in fish. However, stress responses are an important consideration in the diagnosis of all non-infectious conditions in fish.

Table 1.1 summarizes some of the major stress responses in vertebrates. The general non-specific stress response in fish includes the rapid release of stress hormones, such as adrenal catecholamines (epinephrine and norepinephrine), within seconds of the onset of the stressor (the so-called 'primary stress response'). This is followed within minutes by an increase in the release of the glucocorticoid hormone cortisol from the steroidogenic cells of the interrenal gland, leading to an increase in circulating levels of the hormone, which lasts for several hours. In some literary sources this increase in plasma cortisol concentrations is considered to be a component of the 'primary stress response', but the temporal differences in the stressor-linked profiles of plasma hormone levels of catecholamine and glucocorticoid hormones argues for the cortisol release and its activation of glucocorticoid receptors to be considered as the 'secondary stress response'. The increase in circulating levels of the catecholamine and glucocorticoid hormones stimulates changes in blood metabolites, such as glucose; the catecholamines stimulate the release of glucose from glycogen by several tissues, but mostly by hepatocytes; cortisol stimulates the mobilization of lipid reserves and the production of *de novo* glucose by hepatic gluconeogenesis using non-carbohydrate substrates. In addition, the increased skeletal muscle activity that commonly accompanies the stress response gives rise to an increase in plasma lactic acid and changes in plasma pH, and there may also be changes in plasma electrolytes caused by increased blood flow through the gills and increased ion exchange across the gill epithelium.

The release of tissue carbohydrate reserves by catecholamines and the production of new glucose by hepatic gluconeogenesis supplies the increased metabolic needs of cells involved in the stress response, such as increased muscle and central nervous system activities; these metabolic responses represent the 'tertiary stress response', which is highly beneficial to the organism. However, the increased chronic secretion of cortisol has a depressive action on the immune system (see Chapter 6, this volume), which may increase the susceptibility of the organism to pathogens. Cortisol-induced immunosuppression may be considered as an example of the 'quaternary stress response', as could the suppression of growth and impaired reproduction. The reduction in growth may be caused by a decrease in feeding or increased activity of the fish, leading to energy sources being diverted from the support of somatic growth. Reduced reproductive success may also be caused by a decrease in availability of nutrients if the animal ceases to feed. However, stressor-induced changes in the activity of the hypothalamus–pituitary gland–gonad axis may lead to impaired gamete production, and direct inhibitory actions of cortisol on gonadal steroidogenesis have also been reported for some species (Reddy *et al.*, 1999; Leatherland *et al.*, 2010). These various levels of the stress response are discussed at more length in Chapter 6, this volume.

Whilst these global responses by a population (or stock) are important first signs, they usually provide little immediate information about the cause of a specific disorder; whole organism and organ indices may provide a second level of investigation. These might include measurement of the mass of specific organs, histopathological examination of tissues and organs to explore for lesions, assessments of immune response, monitoring of blood chemistry, measurement of the levels of energy reserves in key organs and assessment of the activities of key enzymes in intermediary metabolic

Table 1.1. Stages of the response of fish to a range of stressors.

Stage of response to stressors	Biochemical and physiological changes	Period of response
Primary	Rapid upregulation of the autonomic nervous system, increasing the adrenergic stimulation of the heart pacemaker Rapid release of catecholamines from the interrenal chromaffin cells; increased plasma catecholamine concentration Increased heart rate Mobilization of carbohydrate reserves Neural stimulation of hypothalamic corticotropin-releasing-hormone (CRH)-secreting cells to override the negative feedback control of plasma cortisol concentration	Within seconds
Secondary	Suppression of the negative feedback regulation of pituitary adrenocorticotropic cells to allow increased adrenocorticotropin (ACTH) secretion Increased plasma cortisol concentrations, beginning within minutes and progressing for several hours	Minutes to hours
Tertiary	Increased plasma glucose concentration in response to catecholamine stimulation of hepatic glycogenolysis Increased hepatic gluconeogenesis in response to glucocorticoid (cortisol) stimulation, leading to increased plasma glucose concentration Possible increased plasma lactic acid concentrations resulting from increased skeletal muscle activity	Hours
Quaternary	Physiological responses to chronic hypercortisolism; these may include: immunosuppression by glucocorticoids and increased susceptibility to pathogens, impairment of growth and impairment of reproduction	Days to months

pathways. The specificity of some of these diagnostic tests is still not well established, but they do provide valuable information about the nature of the animal's physiological condition. The third order of diagnostic examination, which explores the organ- and tissue-specific cellular and subcellular sites of the malfunction (Fig. 1.1), has similar limitations as regards the specificity of response.

This chapter provides an overview of this stepwise 'diagnostic approach'; it also outlines the strengths and weaknesses of some of these methodologies and emphasizes that there is no single template that can be applied to determine the causes of all known or suspected environmentally related conditions. Each outbreak of a problem needs to be investigated using first principles and the application of the most appropriate investigational tools.

This chapter also briefly explores how fish disorders can themselves be used as biological indicators of environmental problems and as a measure (bioassay) of the extent of the environmental problem. This use of so-called sentinel organisms in the wild as the 'miner's canary' to monitor the quality of the environment has provided an invaluable first step towards the recognition and subsequent understanding of sometimes broad-based problems. An excellent example of this approach is Sonstegard's (1977) documentation of regional differences in tumour prevalence in fish in the Great Lakes of North America. Sonstegard used tumour prevalence as an indicator of the extent of contamination of different regions of the lakes with chemicals that directly or indirectly induced tumourigenesis; follow-up studies were then used to determine the specific factors involved. Sonstegard's extensive

series of studies of the epizootiology of tumours in Great Lakes fish species set the stage for later work that used sentinel aquatic species as markers of contaminants in various lakes, coastal aquatic systems and rivers. Such sentinels have been used not only to monitor the presence of xenobiotics but also to determine seasonal and year-to-year changes in the level of contamination. Of particular note is the use of sentinel species to detect and monitor changing levels of endocrine-modulating toxicants in the effluents of pulp mills and sewage treatment plants; these are discussed at greater length later in this chapter and also in Chapters 3 and 4, this volume.

During the last few decades, there has been considerable interest in documenting the effects of human activities on the degradation and destabilization of ecosystems. Metaphors drawn from the human health sciences have been applied increasingly to describe changes in ecological systems, and terms such as 'ecosystem health' and 'stressed ecosystems' have become commonplace in the literature; indeed, university programmes of similar names have been developed during the same period. The application of the diagnostic methods and approaches that are currently used in human and veterinary medicine to the diagnosis of ecological problems was proposed by Fazey *et al.* (2004), and these approaches have been used to diagnose degradation of ecosystems that are very obviously impacted by human activities (e.g. removal of forests, draining of wetlands, pollution of terrestrial and aquatic systems, global climate change, etc.). However, our level of understanding of ecosystem interactions is still very limited, and indicators have not yet been developed that can distinguish between less severe human impact and the 'natural' changes that are characteristics of all ecosystems. Ecosystems are very diverse and are also not static entities; their character changes with season and with time, and each particular ecosystem exhibits its own characteristic responses to change. Ever since the emergence of life on this planet, both short-term and long-term climatic fluctuations have acted as stressors on living organisms and thus on the interactions of those organisms within a particular ecosystem. A change in the dynamics of an ecosystem does not necessarily mean that the system is unstable or unhealthy. However, changes in the physiological or clinical status of key sentinel organisms that comprise the biotic components of a particular ecosystem over time can be invaluable and sensitive monitors of ecosystem change and signal the occurrence of change long before there is a marked deterioration in the 'health' of an ecosystem.

Human activities have had major (and rapid) effects on the stability of ecosystems. These include the excessive harvesting of selected animal and plant species resulting in reduction in species diversity, the introduction of exotic organisms, the physical disturbance of key aspects of an organism (e.g. draining of wetlands that comprise the breeding areas for many aquatic ecosystems), changes in the availability of nutrients (e.g. fertilizer or pesticide runoff from cultivated land, the drainage of municipal sewage into aquatic systems or the depletion of nutrients following the introduction of exotic species), the contamination of ecosystems by toxic chemicals, and the potential effects of climate change and associated meteorological changes. All aquatic ecosystems have been impacted to some extent by one or more of these activities, and although attempts have been made to artificially 'stabilize' ecosystems, once the signs of change are evident, attempts to reverse the change have been largely ineffective. The human-associated escalation in the rate of environmental change has accompanied the spread of human populations. In particular the spread of industrial activities has led some evolutionary ecologists to conclude that the planet is well on its way toward a third major extinction, comparable in many ways to the mass extinctions that categorized the end of the Palaeozoic and Mesozoic eras (Ward, 1994). Therefore, although sentinel or indicator organisms have played a central role in monitoring both changes in environmental conditions and the rate of environmental change, reversing these changes has proved to be a challenge that is currently beyond the limits of our ability.

Fish as Sentinel Organisms

Non-infectious disorders of particular wild species have been used effectively to signal detrimental changes at a particular site or within an ecosystem. In some cases, fish that are susceptible to particular contaminants have been placed in cages in aquatic systems that are thought to be contaminated. Two examples of the use of sentinel fish species illustrate their value. One series of studies (summarized in Chapter 3, this volume) examined the effects of sewage treatment effluent on vitellogenin synthesis in fish held downstream of the effluent. Vitellogenin is a phospholipoprotein that is transferred to the oocytes during gonadal growth and maturation, a process referred to as vitellogenesis. Vitellogenin is synthesized by the liver under the influence of oestrogen, and therefore it is normally only synthesized by sexually mature females. The presence of vitellogenin in juvenile fish and adult males is indicative of the presence of environmental oestrogens (xeno-oestrogens). Sentinel fish held in cages downstream of sewage treatment plants in several countries were found to have elevated plasma vitellogenin levels, suggesting that the sewage treatment microflora were not able to fully metabolize the oestrogens (including contraceptive oestrogens) excreted by the human population from which the effluent is received. A second example of the application of sentinel fish species has been the examination of the effects of bleach kraft mill effluent (BKME) on the reproductive biology of fish in river and lake systems and of the dispersal of the effluent within the ecosystem (summarized in Chapter 3, this volume). The physiological responses of the sentinel animals have provided evidence of the presence of a contaminant or mixture of contaminants and, to some extent, the level of the contaminant.

For both freshwater and marine aquatic systems, teleost fishes have proved to be particularly valuable as sentinels as they occupy various trophic levels in an ecosystem; they accumulate xenobiotic chemicals both via the food chain and directly from the water column via the gills; and they 'biomagnify' many xenobiotic factors in specific tissues to a level that can be measured using currently available chemical analysis.

The value of such sentinels as bioassay systems is that they can be used as indicators without necessarily having a priori knowledge of the nature of the environmental insult (physical or chemical). This is particularly important in assessing the effects of man-made chemicals on the environment, because the total number of newly synthesized chemicals continues to increase at a rate that exceeds our capacity to undertake meaningful toxicology screening, and our knowledge of the interactions of chemicals in biological systems is still rudimentary. Moreover, the method is especially valuable in situations in which there is a mixture of chemicals being introduced into the environment, as is the case for BKME.

An additional value of the sentinel approach over the direct chemical measurement approach is the high level of sensitivity of the former for some classes of toxicants. Many environmental chemicals exert their effect by interacting with receptor proteins on the plasma membrane of cells. A low level of receptor–ligand (toxicant) interaction brings about changes in cellular activity, and the cellular response is biomagnified to the point that the physiology of the sentinel organism is changed to a degree that can be measured.

Each category of toxicant in a mixture of toxicants in a given ecosystem will have its own unique mode of action at the cellular or subcellular level; therefore, there is no single protocol to test for all toxicants, or even for all toxicants in a particular class of chemicals. For example, heavy metals exert their effects via different pathways. Some factors, such as organic phosphate, exert effects directly on an organ system; for example, the organic phosphates act on the central nervous system (Katzung, 2001). Members of the aromatic halogenated hydrocarbon group of chemicals, which includes the dioxins and polychlorinated biphenyl (PCB) families, exert a range of biological effects (Bruckner-Davis, 1998; Rolland, 2000a,b). In the case of the PCB family, the toxicity of different PCB congeners is

dependent on the structure of the congener. Some congeners act on the nucleus of cells, where they interact with the aryl hydrocarbon receptor (AhR). This leads to the increased expression of some genes, including those that code for the synthesis of cytochrome P450 (CYP) enzymes, which are mixed-function oxidases involved in detoxifying an animal of a range of compounds. The xenobiotic is a ligand for the AhR protein; ligand activation of the AhR causes it to form a heterodimer with a nuclear translocator protein, such as ARNT; the heterodimer acts as a transcription factor for the genes that encode for specific CYP enzymes. Other PCB congeners do not elicit a CYP response but can affect thyroid hormone metabolism (Brouwer *et al.*, 1998; Porterfield and Hendry, 1998; Naz, 2004). Other cellular sites of action of xenobiotics include actions on metabolic events, either by affecting cellular enzyme gene expression or by acting directly on the interaction of an enzyme with its substrate via multiple routes of action, membrane transport processes, and hormone and growth factor receptors in the plasma membrane or nucleus of target cells (Naz, 2004). Toxicants that act as ligands for several families of hormone or growth factor receptors may either activate the receptor (i.e. act as an agonist) or prevent the receptor binding to its native ligand (i.e. act as antagonists). These xenobiotic–receptor relationships may be transient or persistent. Persistent toxicants have a relatively long biological half-life, usually because the toxicants cannot be readily metabolized. Persistent agonistic compounds may have a relatively low affinity for a specific receptor relative to the native ligand, but their long half-life gives them an increased biological potency; this is the case for weak xeno-oestrogenic chemicals such as bisphenol A, which have a long biological half-life (Bjerregaard *et al.*, 2007; Crain *et al.*, 2007). This is particularly evident in fish because these compounds induce the synthesis of vitellogenin by the livers of fish exposed to environmental compounds that are weak oestrogens (Harries *et al.*, 1996); vitellogenin is a phospholipoprotein that is normally only found in female fish that are undergoing gonadal maturation; the presence of vitellogenin in immature female fish and male fish is commonly used as an indicator for the presence of environmental xeno-oestrogens (Crain *et al.*, 2007). Alternatively, persistent antagonistic toxicants bind to receptors without activating the receptors; the occupation of the binding site on the receptor may prevent the normal interaction between the receptor and its natural ligand, a hormone or other form of cytokine or growth factor; an example is the anti-androgenic action of some organochlorine compounds such as the DDT metabolite DDE (Kime, 1998; Rolland, 2000b; Norris and Carr, 2006). Yet other xenobiotics interact with proteins that are not receptors; for example, nonylphenol impairs gonadal steroidogenesis by inhibiting the movement of cholesterol into the mitochondria of steroidogenic cells, thus reducing the synthesis of the precursor steroid, pregnenolone (Kortner and Arukwe, 2006). Cholesterol flux into the mitochondria requires the presence of activated steroidogenic acute regulatory (StAR) protein; nonylphenol may prevent the activation of StAR or prevent its insertion into the outer mitochondrial membrane.

Epizootiological Measures of Disorders

Widespread disruptions of population stability caused by a disease outbreak, habitat destruction, depletion of food sources or the application of other environmental stressors may be accompanied by gross epizootic indications of distress. This is the case for both captive and wild fish, and the most common 'population indicators' include high mortality, skewed age/size distributions, impaired growth performance, low body metabolite reserves and impaired reproductive success (Fig. 1.1). In addition, as indicated earlier in the chapter, epizootics of gross lesions, particularly neoplasms, have been used as population indices, usually as indicators of the presence of contaminants (e.g. Sonstegard, 1977). The major limitation in the use of population indices as a diagnostic tool is their lack of specificity; few population indices are disease-, disorder- or condition-specific.

Mortality or reduction in population size

Each species of fish can tolerate environmental changes to which they are continually exposed; these may include temperature, pH and salinity of its aquatic environment; the availability of oxygen (and presence of carbon dioxide); and the availability of food (Fig. 1.2). The major organ systems undergo adaptive responses that adjust the homeostatic processes within this 'tolerance range'. At the upper and lower ends of the tolerance range, the fish will physiologically resist further physiological changes, but these so-called 'resistance ranges' are small and homeostatic balance is disturbed. If the homeostatic balance is not recovered rapidly, the animal reaches the extreme upper or lower end of the resistance range, at which point it dies; these are the upper and lower lethal points for a particular variable (Fig. 1.3). Death occurs as the end result of the breakdown of homeostatic processes, which can result from a myriad of events, including the presence of infectious agents or changes in the abiotic environment that exceed the upper or lower limits of the animal's tolerance range, as well as metabolic disorders and contamination of the environment by natural or man-made toxicants or infectious disease (Fig. 1.3). As such, although it is the most dramatic indicator of acute or chronic problems, the death of a significant percentage of a population (or captive stock), unless there is a diagnosable infectious aetiology, provides little direct information about the nature of the problem.

As indicated in an earlier section of this chapter, the disappearance of wild fish stocks cannot, per se, be directly attributed to increased mortality. Mortality caused by contaminated environments or infectious disease could be part of the problem, but, equally, changes in predator–prey relationships,

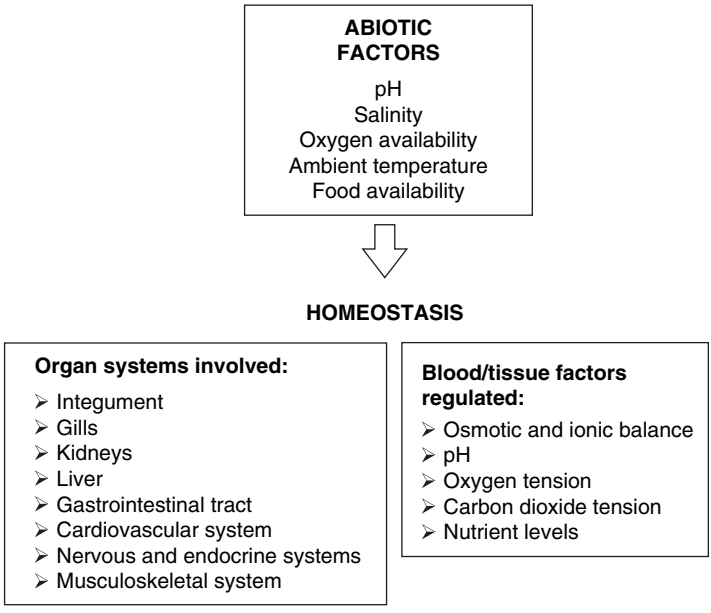


Fig. 1.2. Schematic summary of the relationship between abiotic factors and homeostasis, the physiological factors that are regulated and the main organ systems involved in homeostatic regulation. Abiotic factors impose a persistent adaptive stress on the organism, which can be accommodated within the normal homeostatic (physiological) range. The various organ systems that are involved are shown – it should be noted that these encompass virtually all of the body organ systems; only the reproductive system is not included. Some, but not all, of the blood and tissue factors that are regulated are also shown.

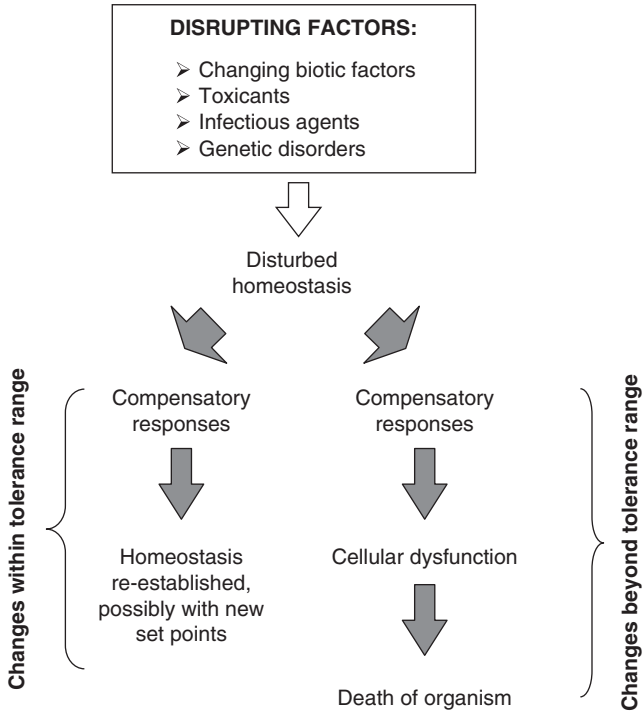


Fig. 1.3. Schematic representation of the processes which cause the organism's normal physiological range to be pushed beyond the tolerance range; physiological variations within the tolerance range can be accommodated, possibly with some adjustment to the homeostasis set points. Variations beyond the tolerance range cause the animal to resist further physiological change for short periods of time, but the process cannot be reversed; the animal will succumb when it reaches the upper or lower limits of the range – the upper and lower lethal points.

excess harvesting of fish stocks (or of the primary prey species of a particular fish stock), and factors such as contaminants, loss of spawning habitats or changes in water condition, such as hypoxia, resulting in reduced reproductive success, could be, and probably are, also involved.

Examples of the effects of such cumulative events on fish populations abound, but the catastrophic declines in the Atlantic cod (*Gadus morhua*), lake trout (*Salvelinus namaycush*) in the Great Lakes of North America, and sockeye salmon (*Oncorhynchus nerka*) stocks along the Pacific coast of North America bear testimony to the problem faced by a particular species, as does the drastic decline of the commercial fishing base in the Mediterranean Sea. It should be emphasized that although these examples represent recent events (most within the last 60 years), archaeological evidence attests to the long-term effect of human activities on animal and plant populations. Even in the absence of human activity, the fossil record provides similar evidence of the 'constancy of change' in population and community structures.

Thus, in captive or wild populations, high mortalities may provide an immediate indication of an acute or chronic problem (including infectious diseases) that exceeds the animal's tolerance and resistance ranges, but the mortalities may also be indicative of environmental issues related to the availability of reproductive resources. Even if the mortalities are related to factors exceeding the resistance limits of the fish, the specific cause of death can only be

established by the application of other diagnostic methods.

Changes in age/size distributions

Changes in the age/size distribution may be useful indicators, particularly of problems faced at specific stages in the life cycle. For example, the loss of early year classes may be indicative of an impaired recruitment of the population into brood stock or, equally, this may be caused by reproductive problems. Further, if a specific age group within a population is small, this may be an indication of impaired growth efficiency or increased size-specific mortality. A major limitation of this approach is that it requires a long-term study and necessitates the removal of a significant number of a resident population. Random sampling methods usually use lethal techniques, and the most accurate ageing techniques rely on the examination of the annual growth rings of the otoliths of the inner ear and are therefore only possible post-mortem. Furthermore, all of the limitations as regards the interpretation of the results of such studies that applied to the use of mortality rates as indicators of problems within a population are equally true in the evaluations of age/size data.

Impaired growth performance

In its simplest terms, growth is a measure of the change in the total energy content of an animal over time (Brett and Groves, 1979). It is the net difference between the acquisition and assimilation of nutrients and the metabolism of those nutrients to generate metabolic energy and heat (Fig. 1.4). Growth performance is affected by the quantity, quality, palatability and digestibility of the available nutrients, the rate of metabolism and activity, and factors that alter energy partitioning needs (e.g. gonadal development). Consequently, in real terms, growth of fish, as with that of all animals, is an extremely complex process and still surprisingly poorly understood. Recent excellent reviews by Katsanevakis and Maravelias (2008) and Kuparinen *et al.* (2008) illustrate the complex nature of modelling and understanding fish growth at a population level. In part, the limitations of our understanding of growth physiology are related to the imperfect methods currently available for measuring growth rates and growth performance of fish, particularly animals in the wild. Of these, changes in body length and mass (and condition factor) with time are widely used and have limited value for measures of wild populations, unless used in combination with valid age data (see above). More recently, measurement of the RNA:DNA ratios or of ornithine decarboxylase activity (the rate-limiting enzyme for nucleic acid production) in specific tissues have been used as indirect measures (Houlihan *et al.*, 1993; Arndt *et al.*, 1994; Mercaldo-Allen *et al.*, 2008), as have measurement of the isotope signature or stable isotope composition of otolyth and scale rings (Satterfield and Finney, 2002; Høie *et al.*, 2003; Gao *et al.*, 2004; Hutchinson and Trueman, 2006) and amino acid uptake by scales *in vitro* (Goolish and Adelman, 1983; Farbridge and Leatherland, 1987). In addition, changes in the activity of key metabolic enzymes in specific tissues have been used as measures of growth by some authors (Mathers *et al.*, 1992, 1993; Pelletier *et al.*, 1993, 1994; Guderley *et al.*, 1994). All of these approaches have strengths and weaknesses, and, with some exceptions, they are all a posteriori measures of growth. The problem of measuring growth in the long term is further compounded by the uneven nature of growth in fish. Fish inhabiting temperate regions do not exhibit a constant rate of growth; there are daily variations in growth rate, which overlay seasonal differences that are correlated with annual and semilunar rhythms (Leatherland *et al.*, 1992). Moreover, depending on the gender and phase of the life cycle (early ontogeny, sexually immature, sexually maturing, etc.), growth rate stanzas (Brett, 1979), expressed as changes in body weight over time, vary markedly (Ricker, 1979).

For any given set of conditions, the daily rate at which food is consumed is the

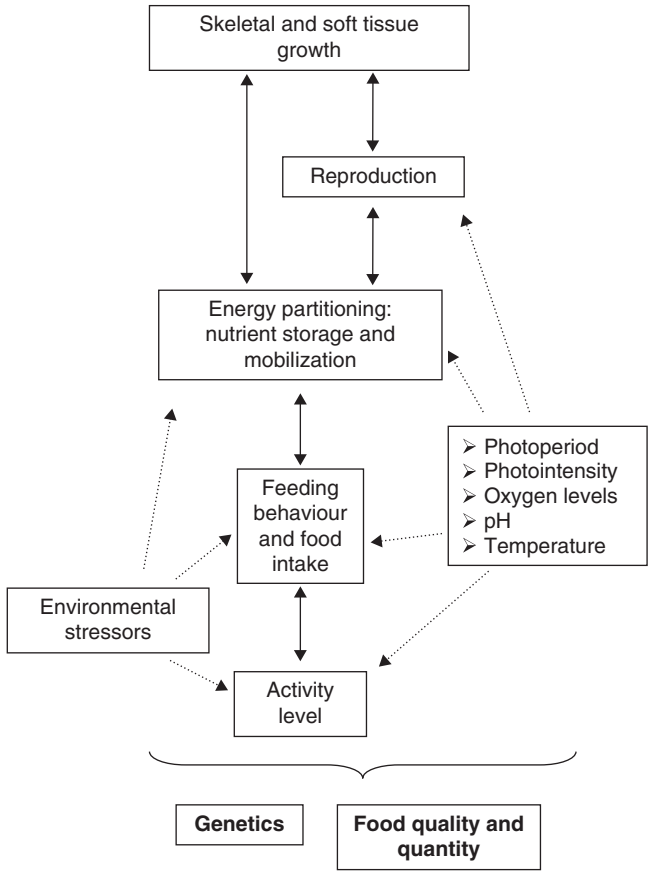


Fig. 1.4. Schematic representation of the interactive nature of metabolism and energy partitioning processes in fishes. The bold arrows indicate sites of action of environmental factors, such as photoperiod and temperature and environmental stressors ([e.g. toxicants, high population density, food deprivation, etc.) on the interactive net. The dashed arrows represent the energy partitioning interactions that occur as a result of life history events and activities.

prime determinant of growth rate in fish (Brett, 1979). However, annual seasonal cycles exert a major influence on the growth performance of wild ectothermic animals such as fishes, particularly for species that inhabit temperate climates. Annual rhythms of photoperiod, light intensity and water temperature often determine the amount of available food, the length of time that an animal can feed and the metabolic rate (Brett and Groves, 1979). Although the influence of these abiotic factors on growth performance of fishes is well established, there is no comprehensive understanding of how they exert their influence. Furthermore, the multiple interactions between abiotic and biotic factors in a complex ecosystem (and particularly disturbed ecosystems) are poorly understood. Consequently, the use of growth performance of wild fish species as a measure of environmental impact has limited value, unless it is combined with other investigational approaches; growth rates of individuals in a population are difficult to determine, and even if growth rates can be determined, the association of altered growth rate with a particular cause is usually very difficult to discern.

The established growth performance measures outlined above are considerably

easier to apply to evaluate captive stocks. 'Optimal' growth performance for a given species reared under established conditions on a particular diet is easy to measure, and thus any reduction in growth rate can be readily identified. However, even for these well-controlled situations, the value of impaired growth as a diagnostic tool is limited because it is only a preliminary indicator of a problem. Under controlled conditions, such as those found in many fish-farming situations, the quality and quantity of dietary sources probably exert the most significant influence on growth performance. A reduction in growth rate, under these conditions, is indicative of reduced food intake, impaired digestion and/or assimilation, or altered metabolism resulting in a reduced efficiency of nutrient assimilation. Specific identification of the cause is not possible and other diagnostic methodologies are required to determine the aetiology.

Impaired reproductive success and early ontogeny defects

This topic area is explored extensively in Chapters 3 and 4 of this book. In brief, reproductive problems and embryo development problems related to environmental contaminants have been reported in many wild fish populations (Kime, 1995, 1998; Monosson, 1997; Rolland 2000b; Norris and Carr, 2006), and there are likely to be issues in many species that have not yet been identified. These studies have shown that virtually all aspects of reproduction and early ontogeny may be affected, but the firm evidence of cause–effect linkages between exposure of the organism to contaminants and the observed reproductive and developmental effects has proved to be difficult. Moreover, in some instances, reproductive or development issues were attributed incorrectly to a contaminant aetiology. For example, M74 Syndrome in Baltic Sea Atlantic salmon (called Early Mortality Syndrome in the Great Lakes) is characterized by the sudden mortality of late yolk-sac-stage embryos. The condition was subsequently shown to be a vitamin B deficiency caused by overfishing of the primary prey species of the juvenile and adult fish (Börjeson and Norrgren, 1997). Smelt (*Osmerus* sp.) are the preferred prey species, but overfishing of smelt in the Baltic Sea and Great Lakes led to significant reductions in the availability of that species, and the Atlantic salmon increased predation of their secondary prey species, the alewife (*Alosa pseudoharengus*); alewife contain a vitamin B inhibitor, which reduced the ability of the adult salmon to acquire vitamin B. As a consequence, delivery of vitamin B from the maternal circulation into the developing oocytes was reduced, leading to vitamin B deficiency in the late-stage embryos when the yolk sac reserves were close to their final stages of absorption. The condition can be prevented by a single immersion of the embryos in a solution of vitamin B.

A second example of a reproductive problem that is brought about by 'natural' causes is the reproductive neuroendocrine functional changes in esturarine fish brought about by seasonal hypoxia (Thomas *et al.*, 2007). Hypoxia has been of increasing focus and has been related to specific gene expression (Rahman and Thomas, 2007) and compromised immunoresponse (Choi *et al.*, 2007), in addition to oxidative stress (Lushchak and Bagnyukova, 2007); this may be a factor that needs to be considered more prominently in future studies of non-infectious disorders in fish.

Laboratory studies, largely based on studies of exposure of fish to a single chemical, have provided some information about the mechanistic basis of reported reproductive problems. The list of suspect chemicals is long and includes polycyclic aromatic hydrocarbons (PAHs), PCBs, dioxins, organochlorine insecticides, metals (including cadmium, lead and selenium), phyto-oestrogens and synthetic oestrogens (Kavlock *et al.*, 1996; Rolland, 2000b). However, in the cases where effects have been seen over wide geographic regions or due to complex industrial effluents from pulp mill or sewage treatment facilities, the causative chemicals have often not been fully identified; this makes replication in the laboratory setting

difficult. Furthermore, the broad range of chemicals on this list illustrates that reproductive and development effects are influenced by multiple mechanistic pathways. Broad generalizations of how these will affect different species of fish should be viewed with caution, given the diversity of reproductive strategies, reproductive life histories and spawning strategies.

Also, the processes that are sensitive to the impact of environmental chemicals are diverse; thus, it should come as no surprise that there is no simple prescription for evaluating reproductive and developmental fitness in fish. Although standardized whole animal tests have been developed for examining the effects of anthropogenic chemicals on reproductive processes in fish (summarized by Leatherland *et al.*, 1998), these tests have been developed primarily for toxicity testing rather than a means of diagnosing *de novo* dysfunctional conditions; the tests were not intended to be diagnostic methods, and for the most part they are not suited to the diagnosis of emerging conditions that are of unknown aetiology. One possible exception is the prevalence of the yolk phospholipoprotein vitellogenin in sexually immature fish of both sexes or in males of all developmental stages; elevated plasma vitellogenin levels in male fish is a reasonably well-established diagnostic indicator of exposure of the fish to a xeno-oestrogen.

Organ, tissue and molecular indicators

Measures of tissue, organ or organism content of metabolites and calories have been used, together with growth per se, to assess the efficacy of specific diets or feeding protocols; the most common form of proximate analysis includes total carbohydrate, lipid and protein levels, as well as total caloric content. These are valuable indicators in the confirmation of pathologic emaciation that is linked to infectious disease, reduced food availability, diets that cannot be digested and absorbed, or diets that cause intestinal lesions that prevent the absorption of digesta. But, as with so many of the other indicators considered in the above sections of this chapter, the values are not diagnostic of a specific condition but merely indicative of impaired assimilation and partitioning of energy. In other words, they are gross estimates of the overall 'condition' of the fish. Most blood parameters, whether it be haematocrit, plasma metabolite levels, plasma enzyme activities or blood hormone levels (summarized in Leatherland *et al.*, 1998), are a posteriori indicators and not cause-specific; this is also true for most cellular or tissue indicators. There are some possible exceptions to this general statement. One example is the group of genes that is expressed in response to specific environmental changes, such as temperature changes and episodes of hypoxia (Lushchak and Bagnyukova, 2007); however, even these may be of limited value given daily and seasonal changes in environmental parameters. A second example is the group of enzymes that is associated with detoxification processes. The increased synthesis of these enzymes or the increased expression of the genes that encode for these enzymes is used as an indicator of the response of the animal to the presence of contaminants in its environment. A list of the key enzymes in this group is given in Leatherland *et al.* (1998). Of these, induction in the hepatic activity of mixed-function oxidases, including cytochrome P4501A activity, ethoxyresorufin-*O*-deethylase (EROD) and benzo(a)pyrene monooxygenase (B(a)PMO) (Addison *et al.*, 1979; Focardi *et al.*, 1992; Arinc *et al.*, 2000; Corsi *et al.*, 2004), has been used as an indicator of hepatotoxic responses to environmental chemicals. In addition, the induction of the glutathione-S-transferase (GST) family of enzymes has been used in some fish species as a marker of the level of toxic challenges faced by a population or stock of animals. The GST family of enzymes in fish closely resembles similar enzymes in mammals (Dominey *et al.*, 1991; Henson *et al.*, 2000); they contribute to the biotransformation of a wide range of compounds, including xenobiotics and endogenous compounds. GST enzyme levels based on functional activity or immunohistochemical evaluation in blood, gill, liver, kidney and intestine

have been correlated with toxicant levels in several fish species (Van Veld and Lee, 1988; Al-Ghais and Ali, 1995; Al-Ghais, 1997; Henson and Gallagher, 2004; Skuratovskaia, 2005). However, it must be remembered that these are not specific to a particular contaminant and variations in enzyme levels may not necessarily be related to xenobiotics; dietary changes that are not necessarily health threatening may also induce changes in GST activity, particularly in hepatocytes.

Notwithstanding these limitations, measurement of the induction of the detoxification enzymes or changes in the expression of genes that encode for these enzymes offers a valuable assessment tool in the identification of possible biochemical stress. The tremendous advancements in genomic and proteomic technologies over the last decade have provided fish pathologists with some of the diagnostic tools that are routinely applied to human and veterinary medicine, and these are most likely to be the best hope for diagnostic advances, if not at the individual animal level at least at the population or stock level.

Conclusions

The assessment of the effects of a detrimental environmental impact on a population or stock of aquatic animals is a complex task, and there is no easy formula with which to develop an appropriate approach to deal with a specific problem. Disorders that bring about reduced growth, reduced fecundity or high mortalities (the gross population indicators of a problem) may have a range of possible causes. There may be a single aetiological agent (e.g. a particular toxicant), although in field situations, this is atypical. More commonly, the cause of the disorder is the result of several factors acting in combination (e.g. dietary problems, inappropriate temperature regimes, single or multiple toxicants), often in association with human activities, such as the physical destruction of habitats. The Great Lakes of North America and the Mediterranean Sea are 'classical' examples of interactions of multiple events, culminating in irreversible devastation of once diverse and complex aquatic ecosystems. Understanding the root causes of such catastrophes is important, even though full restoration may be impossible. By comprehending the nature of the problem, there are lessons to be learned in terms of diagnosing the causes of present and future disorders of wild and captive populations.

The gross population indicators can form the basis of further investigations, which, depending on the particular situation, might involve sampling from the afflicted stock, testing of hypotheses using controlled experimental trials, hypothesis testing in the field, comparing situations of afflicted and non-afflicted populations of the same species, etc. Ultimately, if the mechanistic questions need to be addressed, studies at the organelle level, including the application of molecular genomic and proteomic investigative techniques currently not available, will be required.

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2 Neoplasms and Related Disorders

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Introduction

Fish oncology is important not only because of the effects of neoplasms on individual fish and fish populations but also because fish can be models for furthering our understanding of neoplasia in general (Ostrander and Rotchell, 2005). Fish are especially useful in the evaluation of carcinogenicity of chemicals (Hoover, 1984a; Hawkins *et al.*, 1995; Bailey *et al.*, 1996) and the study of factors affecting carcinogenicity (Pratt *et al.*, 2007), including the determination of genetic factors regulating oncogenesis (Walter and Kazianis, 2001; Stern and Zon, 2003; Berghmans *et al.*, 2005a; Tilton *et al.*, 2005; Lam *et al.*, 2006; Lee *et al.*, 2008). Fish neoplasms can also serve as indicators for the presence of environmental carcinogens (Dawe and Harshbarger, 1975; Sonstegard and Leatherland, 1980; Grizzle, 1985, 1990; Harshbarger *et al.*, 1993; Hinton *et al.*, 2005).

In this chapter, we review the neoplastic diseases of fish, with an emphasis on aetiology. Selected non-neoplastic lesions that could be confused with neoplasia are included, and differences and similarities between these lesions are discussed. Laboratory experiments have demonstrated that certain viruses, chemicals, inherited characteristics and radiation can cause neoplasms in fish. Although causes of neoplasms in wild fish are more difficult to ascertain, there is strong evidence that chemical pollutants (Baumann, 1998; Myers *et al.*, 2003) and oncogenic viruses (Davidov *et al.*, 2002) are important in certain fish populations. In other instances, neoplasms occur sporadically and at very low prevalence, so epizootiology may not be useful for determining the nature of the aetiological agent.

Neoplasia in fish has been a popular topic for reviews. Some reviews have provided a broad coverage of this topic (Martineau and Ferguson, 2006), and most general reviews of fish neoplasms have been organized phylogenetically or by tissue, organ or organ system (Schlumberger and Lucké, 1948; Nigrelli, 1954; Wellings, 1969; Mawdesley-Thomas, 1975; Peters, 1984; Sindermann, 1990; Roberts, 2001). These references can be consulted for an overview of the types of neoplasms that occur in fish. Fish have been included in discussions of comparative oncology (Squire *et al.*, 1978; Dawe, 1982), and several symposia have provided overviews of fish oncology (Dawe and Harshbarger, 1969; Dawe *et al.*, 1976, 1981; Kraybill *et al.*, 1977; Hoover, 1984a; Malins, 1988; Woodhead and Chen, 2001). Reviews related to molecular oncogenesis include Wellbrock *et al.* (2002) and Berghmans *et al.* (2005a). Previous reviews of aetiological factors associated with fish neoplasia have

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focused on viruses (Essbauer and Ahne, 2001; Smail and Munro, 2001), genetics (Walter and Kazianis, 2001; Meierjohann and Schartl, 2006), pollutants (Grizzle, 1990; Harshbarger and Clark, 1990; Bucke, 1993; Harshbarger *et al.*, 1993; Baumann, 1998) or chemical carcinogens generally (Moore and Myers, 1994; Hawkins *et al.*, 1995; Bunton, 1996).

General Characteristics of Neoplasia

Definition

Neoplasia is a disease in which genetically altered cells escape from normal growth regulation. Important concepts in the definition of neoplasia include: (i) the presence of an abnormal cell population, often forming a mass, with growth that is uncoordinated with normal tissues; and (ii) persistence of excessive growth after cessation of the stimulus evoking the lesion. The abnormal growth is to some extent structurally and functionally independent of the host because neoplastic cells are partially free of the controls that act to regulate and limit growth of normal cells (Kumar *et al.*, 2005). Persistence of growth after removal of the factor evoking the neoplasm indicates that there has been a change in the structure or expression of DNA, which is inherited by succeeding generations of neoplastic cells.

Several morphological features distinguish neoplasms from normal tissues and from other types of lesions. The loss of constraints that limit the replication of normal cells results in a persistent, expanding or infiltrating growth without the architecture of normal tissue. Neoplasms commonly form grossly visible masses, but this is not an essential part of the concept of neoplasia; for example, some types of lymphomas consist of invasive cells that do not form macroscopically visible tumours (Kieser *et al.*, 1991; Langenau *et al.*, 2005). Neoplasms have varying degrees of abnormality in cellular appearance and growth rates, and there are often functional differences between neoplasms and related normal cells.

The molecular and morphological aspects of neoplasia in fish are generally similar to those of mammals. Similarities are seen in mutations or altered expression of oncogenes and tumour suppressor genes (Goodwin and Grizzle, 1994; Van Beneden and Ostrander, 1994; Du Corbier *et al.*, 2005; Lam *et al.*, 2006), as well as in protein markers (Thiyagarajah *et al.*, 1995; Bunton, 2000). There is also similarity in morphological progression for some types of neoplasms (Boorman *et al.*, 1997). The genetic information available for zebrafish (*Danio rerio*) has been useful for exploring the molecular similarities between fish and mammalian neoplasms (Lam and Gong, 2006; Feitsma and Cuppen, 2008; Stoletov and Klemke, 2008).

Hyperplasia can be difficult to distinguish from neoplasia in some cases. Hyperplastic growth can form a mass, but cessation of the stimulus causing the lesion results in regression of the growth. Usually the cellular appearance and tissue architecture of hyperplastic masses more closely resemble normal tissue than neoplasms. Examples of lesions that resemble neoplasia or have been confused with neoplasia are presented later in this chapter under the heading of Pseudoneoplasms. The term 'hyperplasia' has been used by some authors to include proliferation of cells in neoplasia, but in this chapter, hyperplasia will only be used to describe non-neoplastic lesions.

Terms used for neoplasms

The term 'tumour' is usually a synonym for neoplasm (Kumar *et al.*, 2005), but it has also been used in a broader context to indicate any tissue swelling or mass, including those that are not neoplastic. Non-neoplastic diseases such as lymphocystis and *Mycobacterium* infection have sometimes been referred to as tumours (Weissenberg, 1965; Post, 1987; Berthiaume *et al.*, 1993; Anders and Yoshimizu, 1994). Campana (1983) stated that he used tumour 'in a loose sense' because of uncertainty about whether skin

lesions of starry flounders (*Platichthys stellatus*) were neoplastic. Because the term 'tumour' can be ambiguous, the terms neoplasia (for the disease) and neoplasm (for the lesion) are preferred when the objective is to clearly state the diagnosis.

The names used for fish neoplasms are similar to those used for mammalian neoplasms. Typically the name includes an indication of the tissue or cell type of origin and whether the disease is benign or malignant. However, the names of some neoplasms vary from this pattern. Papillomas, for example, are named for the papillary appearance of the mass rather than for the cell type. The term 'papilloma' has also been used for some growths that are probably hyperplastic rather than neoplastic (Sano *et al.*, 1991; Kortet *et al.*, 2002; Korkea-aho *et al.*, 2006).

Malignant neoplasia, commonly known as cancer, is usually indicated by the terms carcinoma or sarcoma. Exceptions are certain invariably malignant neoplasms, e.g. lymphoma, melanoma and various 'blastomas' (such as nephroblastoma). There have also been changes over time in the names used for some types of neoplasms; e.g. hepatocellular carcinoma was usually termed 'hepatoma' in older literature.

Indications that a fish neoplasm is malignant include the cellular appearance and behaviour of the lesion. These criteria are similar to those used for mammalian neoplasms, but there is considerably less documentation (and for many lesion types, no documentation) about recurrence after surgery or the clinicopathological outcome. For most fish neoplasms, invasiveness is perhaps the most important criterion used to determine malignancy.

The categories of benign and malignant for neoplasms of fish have been questioned because of the prognostication implied with the term 'malignant' (i.e. potentially life threatening) and because fish neoplasms are less aggressive than their mammalian counterparts (Martineau and Ferguson, 2006). As previously mentioned, clinical experience with most types of neoplasms in fish is limited, so the eventual outcome is unknown. A conclusion that a fish neoplasm is malignant implies that some of the morphological features associated with malignant neoplasms of mammals are present and generally is descriptive of its histological characteristics rather than a clinical assessment.

Metastasis

Metastasis has been reported for certain types of fish neoplasms, including nephroblastomas (Masahito *et al.*, 1992), pigment cell neoplasms (Okihiro *et al.*, 1993), hepatic neoplasms (Okihiro and Hinton, 1999) and lymphomas (Nigrelli, 1947). Melanomas commonly metastasize in some fish (Fig. 2.1), although this may not occur in all species. There are several reports of metastasis of hepatic neoplasms; these and other metastatic neoplasms of fish were reviewed by Machotka *et al.* (1989). Overall, metastasis in fish may be less common than in mammals because several common metastatic primary tumours in mammals (lung, breast, cervix, prostate and uterus) and some of the most frequent sites of metastases (lungs, lymph nodes and bone marrow) are not present in fish. Many common neoplasms of fish are relatively well differentiated, and this could also be related to their weakly malignant behaviour. Other reasons for the less frequent occurrence of metastasis in fish compared with mammals have been proposed, including differences in the 'lymphatic system' (Haddow and Blake, 1933; Machotka *et al.*, 1989) and lower body temperature of fish (Hendricks *et al.*, 1984b). The 'lymphatic system' of fish is better described as a secondary vascular system, which differs from the lymphatic system of tetrapods by receiving fluid from arteries (Steffensen and Lomholt, 1992). Further study is needed to determine how the lack of a lymphatic system in fish affects metastasis of neoplasms. Protocols used for experimental exposure of fish to carcinogens typically involve necropsy of the fish soon after neoplasms are likely to be present; if these fish were allowed to live longer, metastasis of experimentally induced neoplasms might be more common (Hendricks *et al.*, 1984b).

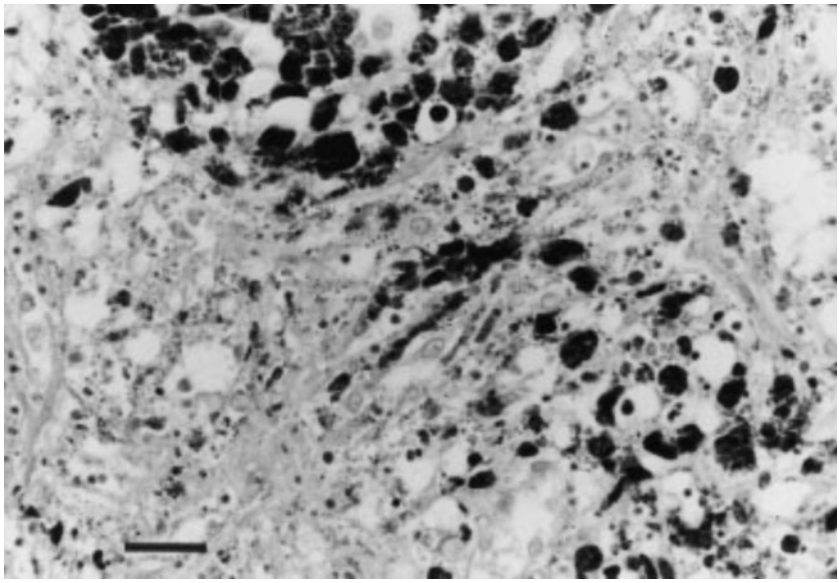


Fig. 2.1. Melanoma in the skin of a channel catfish (*Ictalurus punctatus*). This fish had multiple, black, slightly raised lesions scattered over the body. Bar = 25 μm. Registry of Tumors in Lower Animals (RTLA) Accession No. 5202; specimen contributed by Rodney W. Horner and L. Durham.

Effects of Neoplasms on Captive and Wild Fish

The life-threatening aspects of neoplasia are not always obvious. Effects of external neoplasms can include mechanical impediments to locomotion, interference with protective coloration and increased susceptibility to predation. Some species of wild fish would be more susceptible to capture by gill nets. For both cultured and wild fish, neoplasia can also result in the fish being affected by secondary infections or osmotic imbalance, and neoplasms on the jaws or lips can physically interfere with feeding. Plasmacytoid leukaemia of chinook salmon (*Oncorhynchus tshawytscha*) grown in netpens can directly cause a high rate of mortality (Kent *et al.*, 1990).

Other examples of decreased longevity related to neoplasia involve the loss of older age groups from affected wild fish populations. Brown bullheads (*Ameiurus nebulosus*) older than 4 years were scarce in the polluted Black River, Ohio, compared with populations at a reference site and in previous studies (Baumann *et al.*, 1990). Similarly, in the Hudson River estuary there was an abnormal age distribution of Atlantic tomcod (*Microgadus tomcod*), which probably resulted from the early death of 3-year-old fish that had carcinomas and other hepatic lesions (Dey *et al.*, 1993). However, in wild populations the role of neoplasia in changing age structure is uncertain because the incidence of diseases other than neoplasia could have increased.

Because of concern about adverse effects on humans and ecosystems, considerable emphasis has been placed on the use of fish neoplasms as sentinels for the presence of chemical carcinogens (Sonstegard and Leatherland, 1980; Grizzle, 1990; Feist *et al.*, 2004; Hinton *et al.*, 2005; Blazer *et al.*, 2006). However, a fish population exposed to chemical carcinogens could also be adversely affected by the toxicity of environmental pollutants; therefore, neoplasms can also be considered as sentinels for less conspicuous impacts of pollutants on the fish themselves. The non-neoplastic effects of chemical carcinogens include changes in behaviour (Ostrander

et al., 1988) and the immune system (Faisal *et al.*, 1991; Seeley and Weeks-Perkins, 1991; Weeks *et al.*, 1992). Because of complex effects of pollutants on food chains, growth rates of fish in polluted environments can increase or may not change, but reduced growth rates of fish have occurred in some polluted environments (Grizzle *et al.*, 1988a). Lack of successful reproduction can be caused by several mechanisms, including toxicity to fish larvae (Weis and Weis, 1987; Walker *et al.*, 1991) and decreased serum levels of vitellogenin (Chen *et al.*, 1986; Sherry *et al.*, 2006). Genotoxic carcinogens could also cause germ-cell mutations, which would be of greater concern than somatic changes in populations with surplus reproduction (Würgler and Kramers, 1992).

Pseudoneoplasms

Non-neoplastic lesions that resemble neoplasms have been called pseudoneoplasms (Harshbarger, 1984). These are typically hyperplastic or chronically inflamed lesions and can be caused by a variety of stimuli. Often the resemblance between neoplasms and pseudoneoplasms is superficial, and they can be easily distinguished by histopathology. However, there is a lack of consensus about the neoplastic nature of some types of lesions.

Virally induced hyperplasia or hypertrophy

Several viral diseases are characterized by cutaneous growths. Some of these lesions are neoplasms, but others such as 'carp pox' are epidermal hyperplasia of well-differentiated cells with little or no involvement of the dermis (Schlumberger and Lucké, 1948; Nigrelli, 1954). Other virally induced masses, most notably lymphocystis disease, are characterized by hypertrophied cells and are easily distinguished from neoplasia. Non-neoplastic diseases that have been associated with viruses are discussed further in the virology section of this chapter.

Parasitic diseases

Some parasitic diseases closely mimic neoplasia (Ferguson and Roberts, 1976), but more often the resemblance to neoplasia is superficial. Examples of lesions that are readily recognized histologically as non-neoplastic include cutaneous melanosis and inflammation, which are caused by a variety of parasites (Fig. 2.2). Certain Myxosporea and Microsporea can form large cysts filled with spores (El–Matbouli *et al.*, 1992; Lom and Dyková, 1992). Grossly, these masses could be confused with neoplasms, but after microscopic examination the cause of the cysts is apparent because of the distinctive appearance of the spores.

Growths consisting of 'X-cells' commonly occur in the skin, gills or pseudobranchs of certain species in the families Pleuronectidae and Gadidae (Alpers *et al.*, 1977; Eaton *et al.*, 1991a; Watermann *et al.*, 1993) and less commonly in other families of marine fish (Diamant *et al.*, 1994). X-cells are protists with some characteristics reminiscent of amoebas (Dawe, 1981; Harshbarger, 1984; Waterman *et al.*, 1993) but do not appear to be closely related to other protist groups (Miwa *et al.*, 2004). Virus-like particles have been observed in some X-cell lesions (Wellings and Chuinard, 1964; McArn *et al.*, 1968), but the role of viruses in this disease is uncertain (Watermann *et al.*, 1993). X-cells have cytoplasmic granules, unusually large mitochondria, prominent nucleoli, an extracellular envelope and a larger size than stromal cells (Brooks *et al.*, 1969). Although the masses formed by X-cells have been called 'papillomas' by some authors, this disease is not neoplastic.

Inflammation

Regardless of the cause of the inflammatory response, granulomatous inflammation and granulation tissue can resemble neoplasms, and the suffix of the term granuloma adds to the potential confusion. A common cause of granulomas in fish is mycobacteria (Nigrelli

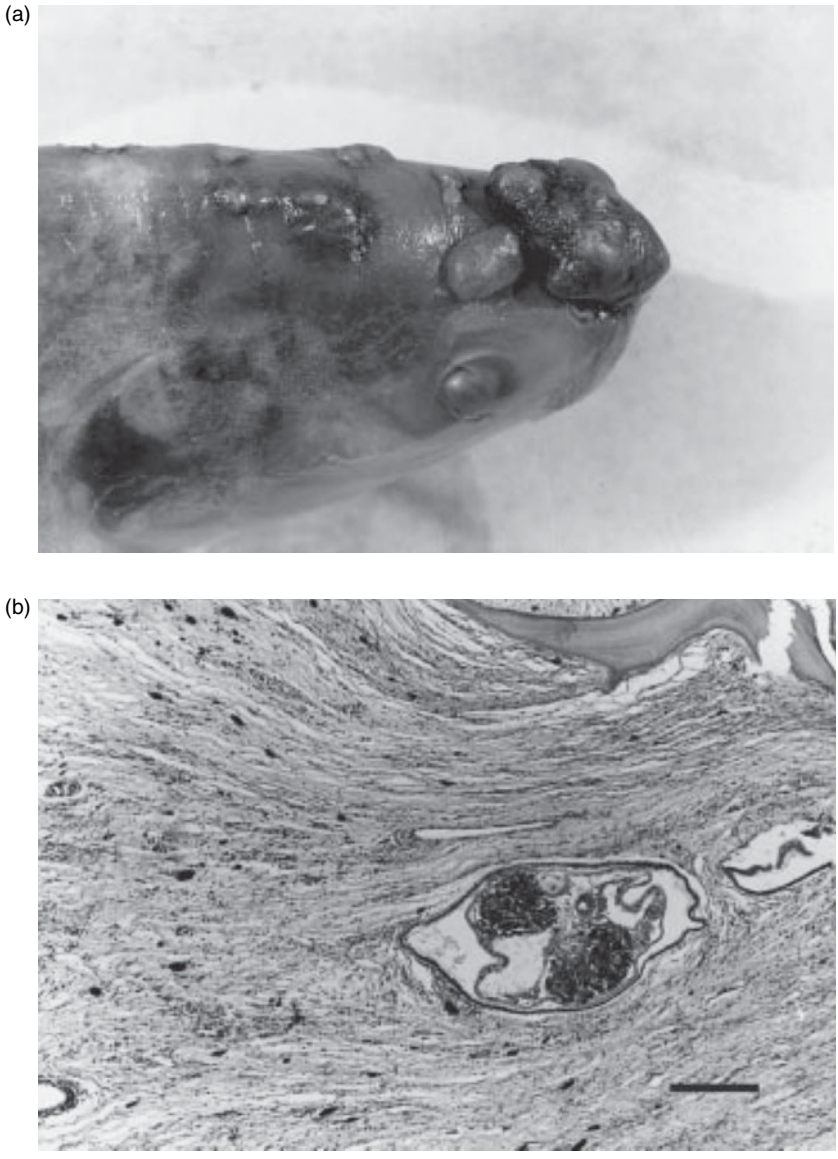


Fig. 2.2. (a) A black growth on the snout of a gizzard shad (*Dorosoma cepedianum*). This non-neoplastic, inflammatory lesion was caused by digenetic trematodes, *Bucephalopsis labiatus*. (b) Histologically, the mass consisted of granulation tissue with large numbers of well-differentiated melanocytes. Bar = 150 µm.

and Vogel, 1963; Beckwith and Malsberger, 1980; Gómez, 2008; Davis and Ramakrishnan, 2009), but similar lesions are caused by other pathogens (Majeed *et al.*, 1981; McVicar and McLay, 1985) or egg-associated inflammation (Whipps *et al.*, 2008) or they are idiopathic (Munkittrick *et al.*, 1985). In some cases, granulomatous exudate can occur in multiple sites, displace normal tissue and cause a distention of the body (Fig. 2.3). Identification of the infiltrating cells as macrophages is difficult in routinely stained sections, and these lesions could be mistaken for neoplasia, especially when the cause of

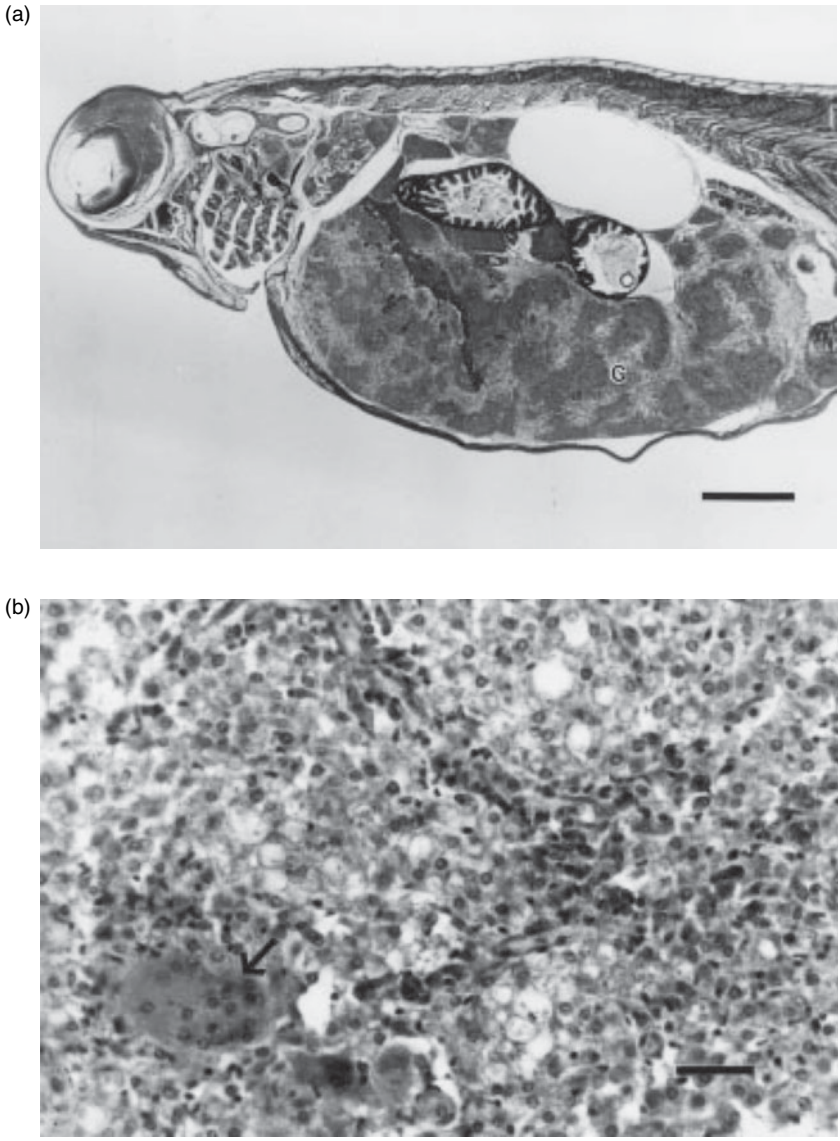


Fig. 2.3. A non-neoplastic, inflammatory disease in mangrove rivulus; the aetiological agent is unknown. (a) Granulomatous exudate (G) causing distention of the peritoneal cavity. Bar = 500 µm. (b) Higher magnification of (a). Macrophages are the most prominent component of the exudate. Giant cells (arrow) are present. Bar = 25 µm.

the lesion is not apparent. Granulation tissue and granulomas have been the cause of erroneous reports of neoplasms in experimental studies (Beckwith and Malsberger, 1980; Raiten and Titus, 1994).

Thyroid hyperplasia

Although thyroid enlargement has been commonly reported in fish, most of these thyroid masses were probably hyperplastic

rather than neoplastic (Leatherland and Down, 2001; Fournie *et al.*, 2005). Thyroid hyperplasia occurs most often in captive fish (Hoover, 1984b; Crow *et al.*, 2001) or in wild fish from certain geographical areas, such as the Great Lakes. Prevalence of these lesions can be high, up to 93.5% in Lake Erie coho salmon (*Oncorhynchus kisutch*), and the lesions can occur seasonally (Leatherland and Sonstegard, 1980). Causes of goiter in fish are not always evident but can include endocrine stimulation of the thyroid, problems with iodine metabolism or direct stimulation of the thyroid (Leatherland, 1994). Exposure to goitrogens can reduce or eliminate thyroxine (T_4) synthesis or release from the thyroid; without the normal negative feedback of T_4 on the pituitary, thyrotropin secretion rates increase. The higher concentration of circulating thyrotropin stimulates the thyroid, resulting in hyperplasia and depletion of colloid reserve.

Invasiveness and apparent metastasis are common features of hyperplastic thyroid in fish. The thyroid in many teleosts is a diffuse organ located in the hypobranchial area near the ventral aorta and afferent branchial arteries; although some fish families, such as parrotfish (Scaridae) have a compact, circumscribed thyroid (Grau *et al.*, 1986). The commonly observed invasiveness of goiter in teleosts is probably related to the unencapsulated and diffuse nature of the thyroid. Ectopic follicles are often in the spleen, kidney and other organs of fish without thyroid hyperplasia, especially when iodine is limiting (Baker, 1959); therefore, invasive or apparently 'metastatic' lesions in fish with thyroid hyperplasia do not indicate that the lesion is neoplastic.

Histological criteria have been established for fish thyroid lesions to distinguish between hyperplasia and neoplasia (Fournie *et al.*, 2005). In addition to histological appearance, iodine supplementation and transplantation experiments are two approaches for aiding in the distinction between thyroid hyperplasia and carcinoma. Both of these techniques were used in an experiment in which thyroid masses were apparent 2 months after 7-day-old mangrove rivulus (*Kryptolebias* (= *Rivulus*) *marmoratus*) were exposed for 2 h to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Park *et al.*, 1993). Throughout the experiment, 50 µg iodine/l was added to the water to achieve a total iodine concentration of 150–200 µg/l. While no thyroid lesions were found in controls, thyroid masses were present in almost all fish exposed to the highest dose of MNNG (25 mg/l) for 4 months, and most lesions were diagnosed as papillary carcinomas. The thyroid carcinomas were successfully transplanted to the anterior chamber of the eye of other mangrove rivulus. Control thyroid transplants degenerated, even though the recipients were probably isogenic.

Nutrition

Largemouth bass (*Micropterus salmoides*) fed diets that were higher in carbohydrates than their normal diet (insects and vertebrates) accumulated large amounts of glycogen in their hepatocytes (Goodwin *et al.*, 2002). This accumulation led to a catastrophic necrosis of hepatocytes. In fish that survived this acute phase, the liver regenerated as nodules. These livers had the gross appearance of hepatocellular carcinomas (Fig. 2.4), but histology revealed nodules of hepatocytes with a normal cellular appearance but little glycogen storage. The nodules were initially surrounded by inflammation that included residual hepatic stroma and numerous eosinophils. As the lesion progressed, the nodules grew together and produced an atypically shaped liver with a somewhat disorganized structure.

Factors Influencing Oncogenesis

Age

Neoplasms typically become more common in older fish (Ozato and Wakamatsu, 1981; Etoh *et al.*, 1983). This relationship between age of fish and tumour frequency also occurs

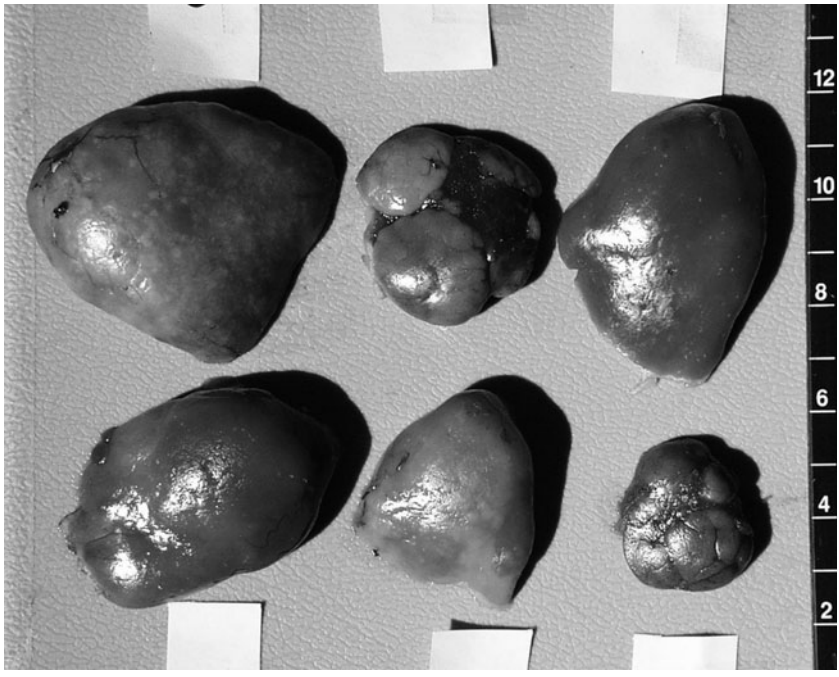


Fig. 2.4. Non-neoplastic nodular regeneration following necrosis in livers from 0.75-kg largemouth bass fed a diet high in available carbohydrates. Scale bar is in centimetres.

in wild fish exposed to chemical carcinogens (Baumann *et al.*, 1987, 1990; Becker *et al.*, 1987; Rhodes *et al.*, 1987; Mikaelian *et al.*, 2002). However, the relationship between fish age and neoplasms caused by viruses may be more complex. The percentage of walleye (*Sander vitreus*) developing dermal sarcomas caused by a retrovirus increased for fish from 3 to 6 years old but decreased in older fish (Getchell *et al.*, 2000b, 2004).

The stage of development at which fish are exposed to carcinogens can also affect carcinogenicity. The percentage of rainbow trout (*Oncorhynchus mykiss*) with neoplasms 10–12 months after a pre-hatching exposure to aflatoxin B_1 (AFB_1) was higher if embryos were exposed after, rather than before, they reached the stage when the liver is present as a discrete organ (Wales *et al.*, 1978). Compared with optimal embryo exposure, carcinogenicity of AFB_1 was similar or even greater if recently hatched rainbow trout were exposed (Hendricks *et al.*, 1980d). For *Xiphophorus*, exposure to methylnitrosourea (nitrosomethylurea, MNU) or X-rays at 6 weeks of age resulted in a higher frequency of neoplasia than for fish exposed at 6 months of age (Schwab *et al.*, 1978). A similar tendency for younger fish to be more sensitive to carcinogens has been found in several studies (Thiyagarajah and Grizzle, 1986; Grizzle and Thiyagarajah, 1988; Boorman *et al.*, 1997).

Gender

In some cases the gender of the fish affects oncogenesis. Male F_1 hybrids of southern platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus helleri*) had a higher prevalence of hereditary melanomas than did female F_1 hybrids (85.2% compared with 55.9%), although almost all fish of both sexes developed melanosis (Siciliano *et al.*, 1971). After exposure to MNNG, only male medaka (*Oryzias latipes*) developed thyroid neoplasms (Bunton and Wolfe,

1996), and male zebrafish had an increased risk of neoplasia following an embryonic exposure (Spitsbergen *et al.*, 2000b). Neoplasms were more common in male than in female guppies (*Poecilia reticulata*) and medaka exposed to 2,2-bis(bromomethyl)-1,3-propanediol (BMP) in water (Kissling *et al.*, 2006). There was also a higher incidence of gastric papillomas in male than in female rainbow trout fed 1,2-dibromoethane (DBE) (Hendricks *et al.*, 1995).

In contrast to the above studies, in which male fish were more susceptible than females to chemical carcinogens, hepatic neoplasms were more common in female salmonids than in males, and neoplasms did not occur until fish were sexually mature in Japanese hatcheries, (Takashima, 1976). Spontaneous tumours were also more common in the liver of female medaka than in males, but only for fish older than 3 years (Masahito *et al.*, 1989). After exposure to diethylnitrosamine (*N*-nitrosodiethylamine, DEN), hepatic neoplasia was two to three times more common in female medaka than in males (Teh and Hinton, 1998). Hepatocellular carcinomas, but not cholangiocarcinomas, were more common in female than in male lake whitefish (*Coregonus clupeaformis*) from the St Lawrence River in Quebec (Mikaelian *et al.*, 2002) and in brown bullheads from the Black River, Ohio (Baumann *et al.*, 1990). Liver neoplasms were also about four times more common in female than in male brown bullheads in the Anacostia River, Washington, DC (Pinkney *et al.*, 2004b). In Green Bay, Wisconsin, 17% of the female walleye between 5 and 8 years old had hepatic tumours, while no tumours were found in a sample of 23 males (Barron *et al.*, 2000).

Higher rates of certain types of neoplasms in females could be related to oestradiol, which can act as a promoter (Núñez *et al.*, 1989; Cooke and Hinton, 1999). Predisposition to neoplasia can also result from sex-linked, inherited characteristics; the melanoma locus in *Xiphophorus* spp. is a well-studied example (Walter and Kazianis, 2001; Meierjohann and Schartl, 2006). For European flounder (*Platichthys flesus*) collected from polluted areas of the German Wadden Sea coast, where hepatic neoplasms were found in female but not in male flounder (Koehler, 2004), the preferential use of NADPH for the production of vitellogenin in female fish, rather than for CYP1A biotransformations or other detoxification processes, may increase susceptibility to carcinogens (Koehler and Van Noorden, 2003). Studies that do not show a correlation between tumour development and gender are often those that were terminated before or soon after sexual maturity (Hendricks *et al.*, 1995).

Temperature

Environmental temperature is an important factor in any aspect of fish pathology because the temperature of most fish is essentially the same as that of the surrounding water. Low temperatures usually reduce the occurrence, or at least increase the duration of latency, of neoplasms in fish exposed to chemical carcinogens (Egami *et al.*, 1981; Hendricks *et al.*, 1984b; Kyono-Hamaguchi, 1984; Curtis *et al.*, 1995; El-Zahr *et al.*, 2002). However, the melanosis and melanomas that develop in hybrid *Xiphophorus* kept at 26.0–27.5 °C do not develop at 31.0–32.0 °C (Perlmutter and Potter, 1988).

Genetic predisposition

Genetic predisposition is an important factor affecting the occurrence of most neoplasms. The tendency of certain species to develop particular types of tumours is a well-known aspect of oncology and is also a characteristic of neoplasia in fish (Schlumberger, 1957). The frequency of neoplasia varies in different fish species, but there are no taxa known to be completely refractory (Harshbarger *et al.*, 1981). The frequency of reports about neoplasms in various species is undoubtedly affected by several factors other than disease prevalence. For example, although neoplasms occur in sharks (Fig. 2.5) and rays, there are relatively few published reports of neoplasms in these groups (Ostrander *et al.*, 2004; Borucinska *et al.*, 2008). This could be related to the small number of chondrichthyans kept in captivity and the infrequency of

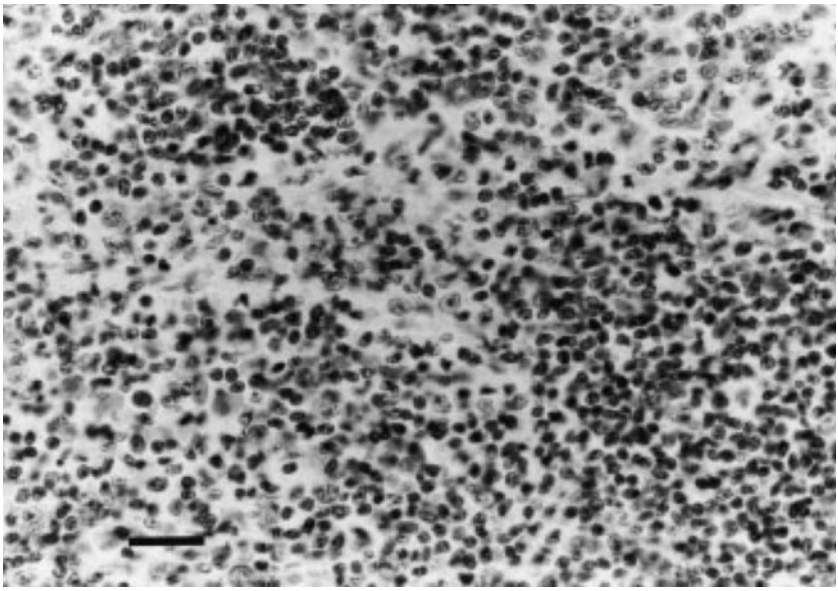


Fig. 2.5. Reticulum cell sarcoma in the spleen of a sandbar shark (*Carcharhinus plumbeus*). Bar = 25 µm. RTLA Accession No. 523; submitted by R. O'Gara and V.T. Oliverio.

experimental oncology with these animals. Sharks with tumours could also be at an extreme disadvantage for capturing prey and for avoiding becoming prey.

The relative importance of genetic predisposition in comparison with species-dependent factors, such as types of food eaten and contact with sediment, is difficult to determine in studies of wild fish. Species differences in metabolism, however, indicate that biochemical differences, rather than differences in exposure, are sometimes related to differences in susceptibility to neoplasia (Willett *et al.*, 2000). Variation in DNA-repair capability is also likely to be an important reason for differences in susceptibility of different species and different organs (David *et al.*, 2004).

Laboratory experiments have confirmed that there can be differences in sensitivity to carcinogens both between species (Ashley, 1970; Hawkins *et al.*, 1988a) and within a species (Sinnhuber *et al.*, 1977; Hyodo-Taguchi and Matsudaira, 1984; Schultz and Schultz, 1988; Bailey *et al.*, 1989). Inbreeding (Etoh *et al.*, 1983) and hybridization can also result in predisposition to the occurrence of neoplasms. For example, the various species of *Xiphophorus* are relatively insensitive to chemical carcinogens and radiation, but certain hybrid *Xiphophorus* are highly sensitive (Schwab *et al.*, 1978; A. Anders *et al.*, 1991). Several mutant or clonal lines of zebrafish also have an increased risk of induced and spontaneous neoplasms (Amsterdam *et al.*, 2004; Berghmans *et al.*, 2005b; Shepard *et al.*, 2005, 2007; Haramis *et al.*, 2006; Moore *et al.*, 2006). Transgenic modification resulting in altered expression of oncogenes has been used to induce several types of neoplasms (Yang *et al.*, 2004; Langenau *et al.*, 2005, 2007; Patton *et al.*, 2005; Feng *et al.*, 2007; Le *et al.*, 2007; Park *et al.*, 2008).

Triploid rainbow trout were less susceptible than diploids to neoplasia induced by exposure to chemical carcinogens (Thorgaard *et al.*, 1999). A lower probability that the carcinogen would alter all copies of tumour suppressor genes was suggested as a potential mechanism. Mizgireuv *et al.* (2004) concluded that triploid zebrafish also have an increased resistance to the chemical carcinogen dimethylnitrosamine (*N*-nitrosodimethylamine, DMN);

this conclusion was based on the longer latency compared with diploid zebrafish. However, the percentage of triploid zebrafish developing hepatocellular neoplasms, though not other types of neoplasms, was actually greater than for diploids.

Promoters and inhibitors

Several chemicals increase or decrease the development of neoplasia initiated by other factors (see additional information about this topic in the Chemical Carcinogenesis section of this chapter). In addition, pathogens can sometimes alter carcinogenesis. For example, neoplasms were more common in zebrafish with the nematode *Pseudocapillaria tomentosa* and exposed to 7,12-dimethylbenz[*a*] anthracene (DMBA) than in zebrafish exposed to the same chemical carcinogen but without the nematode (Kent *et al.*, 2002). This nematode was often physically associated with the neoplasms and appeared to serve as a promoter of carcinogenicity.

Hereditary Neoplasms

Most research about hereditary neoplasms of fish has been conducted with melanomas of hybrid *Xiphophorus*. An inherited neoplasm of pigment cells has also been documented in Amazon mollies (*Poecilia formosa*). Although the inheritance of other neoplasms is not well established, gonadal tumours in hybrids of goldfish (*Carassius auratus*) × common carp (*Cyprinus carpio*) are discussed in this section as another example of a genetically related neoplasm.

Genetically modified fish have been developed that are predisposed to neoplasia, and these fish provide models for the study of molecular mechanisms of oncogenesis. Examples of neoplasms that occur in zebrafish models include leukaemia (Langenau *et al.*, 2005; Chen *et al.*, 2007), rhabdomyosarcoma (Langenau *et al.*, 2007), exocrine pancreatic carcinoma (Park *et al.*, 2008), peripheral nerve sheath tumours (PNST) (Amsterdam *et al.*, 2004; Berghmans *et al.*, 2005b) and pancreatic neuroendocrine carcinoma (Yang *et al.*, 2004). Medaka with a non-functional *p53* gene, obtained by ethylnitrosourea (ENU) mutagenesis, developed several types of neoplasms (Taniguchi *et al.*, 2006).

Melanoma in *Xiphophorus* hybrids

Melanomas can result from matings between southern platyfish from different populations (Gordon, 1948; Kallman, 1975) or between *Xiphophorus* of different species (Figs 2.6 and 2.7). The most frequently studied *Xiphophorus* hybrids are inbred strains of southern platyfish × swordtail, but other *Xiphophorus* species have also been used (Walter and Kazianis, 2001; Wellbrock *et al.*, 2002). Similar melanomas sometimes occur in certain strains of purebred *Xiphophorus* spp. (Kazianis and Borowsky, 1995; Schartl *et al.*, 1995). Melanomas in hybrids of *Xiphophorus* were reported in 1912–1913, and early studies on genetics of these hybrids were published in 1927–1928 (Schwab, 1986; Anders, 1991). Classification of *Xiphophorus* melanomas was described by Gimenez-Conti *et al.* (2001).

A key feature of the *Xiphophorus* melanoma model is the macromelanophore, a distinctive type of pigment cell. Macromelanophores are up to 500 μm in diameter compared with normal melanophores, which are about 100 μm in diameter (Gordon, 1959). Macromelanophores form conspicuous clusters or spots because they are closely spaced; these cells do not seem to be subject to distance-dependent regulation affecting spacing between normal melanophores (Anders *et al.*, 1984). The presence of macromelanophores is sex-linked and causes various pigmentation patterns that are determined by Mendelian dominant genes (Gordon, 1931; Kallman, 1975). The presence of macromelanophores identifies broodfish carrying the oncogene for melanoma. Although this oncogene is closely linked to the macromelanophore-determining locus, they are separate genetic entities (Weis and Schartl, 1998).

X*mrk* (*Xiphophorus* melanoma-inducing receptor kinase) is the melanoma-inducing

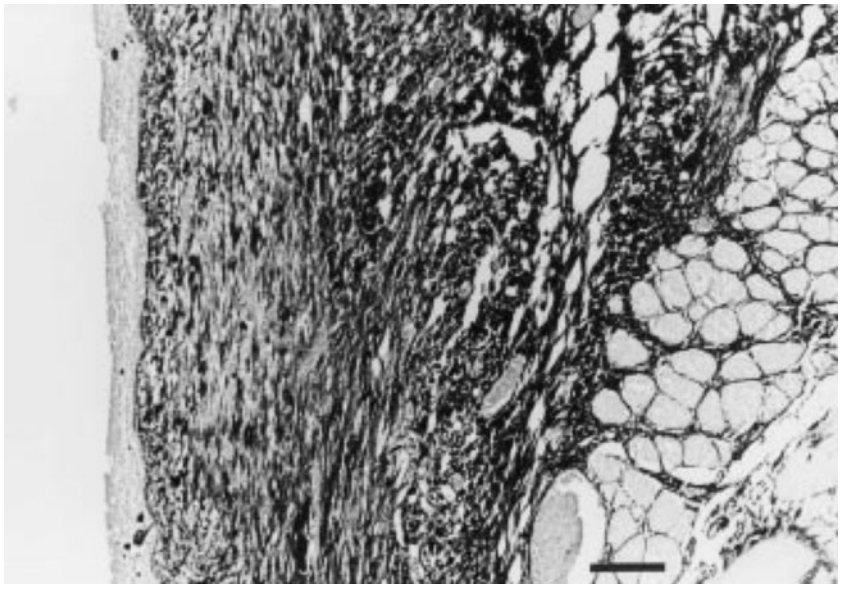


Fig. 2.6. A melanoma from a *Xiphophorus* hybrid. The densely pigmented melanoma has invaded the dermis and underlying musculature. Bar = 100 μm. RTLA Accession No. 230, specimen contributed by I.L. Gorman.

oncogene in *Xiphophorus* (Fig. 2.7) and is a mutated copy of an epidermal growth factor receptor (Volff *et al.*, 2003; Meierjohann *et al.*, 2004). This oncogene is overexpressed in melanomas (Mäueler *et al.*, 1988; Adam *et al.*, 1991; Wittbrodt *et al.*, 1992; Dimitrijevic *et al.*, 1998) and the mutations in X*mrk* are sufficient to induce neoplasia (Winnemoeller *et al.*, 2005).

In most purebred fish, the oncogenic action of the X*mrk* oncogene is inhibited by the 'differentiation gene' (*Diff*), a non-sex-linked locus (Fig. 2.7) that represses melanoma formation by inducing differentiation of macromelanophores (Vielkind, 1976; Anders and Anders, 1978). In wild fish, macromelanophores are completely differentiated and do not become neoplastic; the development of neoplasms requires that differentiation does not occur. A cyclin-dependent kinase inhibitor gene (*CDKN2*) is a candidate for the tumour-suppressor locus *Diff* (Kazianis *et al.*, 2004).

Hybrids that are heterozygous for both the X*mrk* oncogene and the melanoma suppressor locus *Diff* (F_1 hybrid in Fig. 2.7) develop melanosis soon after birth (Gordon, 1958; Atz, 1962; Ozato and Wakamatsu, 1981). Melanotic areas have melanophores that are less differentiated than normal macromelanophores (Vielkind, 1976), and the location of melanosis is related to the location of the pigment pattern on the parent (Gordon, 1931). These melanotic areas often develop into melanomas in adult fish (Anders, 1967; Wakamatsu, 1980; Ozato and Wakamatsu, 1981). These melanomas have invasive, sparsely pigmented neoplastic cells; the neoplastic mass grows to a large size; and the fish usually dies within 2 months (Wakamatsu, 1980). The neoplastic cells are less differentiated than in melanomas that develop earlier in life in certain backcross hybrids of *Xiphophorus* (Esaka *et al.*, 1981).

Backcross hybrids (Fig. 2.7) that carry the X*mrk* oncogene and are homozygous for the absence of the repressor gene *Diff* develop melanomas before birth or soon after birth (Gordon, 1937; Gordon and Smith, 1938; Wakamatsu, 1980). Initially located in the dermis, neoplastic cells infiltrate the adjacent muscle and spread through most outer portions of the body, causing destruction of fin rays and muscle (Gordon and Smith, 1938;

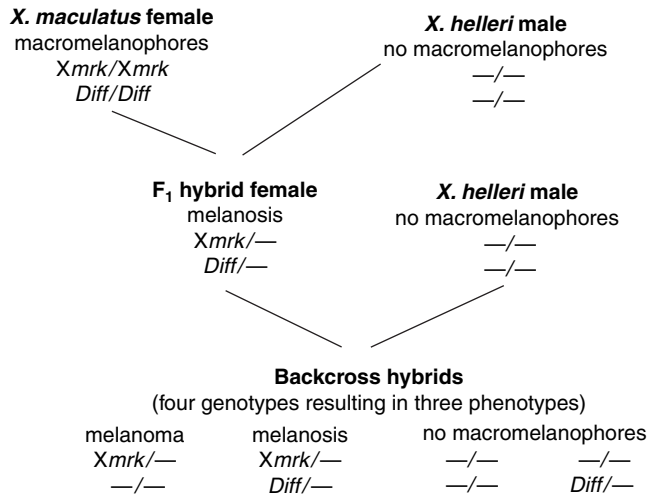


Fig. 2.7. Inheritance of melanoma in hybrids of southern platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus helleri*). The X*mrk* oncogene results in melanoma unless repressed by the melanoma suppressor locus *Diff*. Macromelanophores are present in fish with the X*mrk* gene and homozygous for *Diff*. Hybrids (F_1 hybrids and some backcross hybrids) that carry X*mrk* and are heterozygous for *Diff* have melanosis and sometime develop melanoma when they are adults. Melanomas occur in very young backcross hybrids carrying the X*mrk* oncogene but lacking *Diff*. Based on Vielkind (1976), Anders and Anders (1978), Walter and Kazianis (2001), Meierjohann *et al.* (2004), and Winnemoeller *et al.* (2005).

Esaka *et al.*, 1981). Invasion of myomeres extends inward to the vertebrae; however, mitotic figures are infrequent and metastasis has not been reported. In addition, melanomas similar to the type that occurs in F_1 hybrids also develop in some adult backcross hybrids that already have early-onset melanoma.

Amelanotic melanomas occur if an albino swordtail is mated with an appropriate F_1 hybrid (Fig. 2.7). Compared with pigmented melanomas, amelanotic melanomas grow more rapidly, have more DNA and contain less-differentiated melanocytes (Vielkind *et al.*, 1971; Esaka *et al.*, 1981).

Pigment cell neoplasms in Amazon mollies

Approximately 5% of the Amazon mollies in a certain clone (M-clone) develop cutaneous pigment cell neoplasms (Schartl *et al.*, 1997). Clones occur in this gynogenetic species because descendants from a given female usually contain only maternal DNA. Embryogenesis of diploid eggs occurs after insemination by males of related species, but paternal DNA is not usually contributed to offspring. In rare matings, however, paternal microchromosomes enter the egg, resulting in a new clone. Fish of the M-clone have macromelanophores, the cell type giving rise to melanoma in the related genus *Xiphophorus*, but the oncogene involved in melanoma of *Xiphophorus* does not appear to be involved with the pigment cell tumours of Amazon mollies.

Although M-clone Amazon mollies are genetically uniform, there is considerable variation in the pigment cell neoplasms of these fish (Schartl *et al.*, 1997). There is variation in the growth, invasiveness and age of onset, and yellow pigment occurs in addition to the more common melanin. Schartl *et al.* (1997) consider these neoplasms to be chromatoblastomas.

Gonadal tumours in goldfish × carp hybrids

A high prevalence of gonadal neoplasms occurs in hybrids of goldfish × common

carp in the Great Lakes (Sonstegard, 1977; Down and Leatherland, 1989). Onset of tumour formation coincides with the age of first sexual maturity, and prevalence increases with age. Overall prevalence was 0.57% in carp, 4.1% in goldfish and 68% in hybrids, and prevalence was 100% in some samples of hybrids. Sonstegard (1977) hypothesized that this condition was caused by polychlorinated biphenyls (PCB) or DDT, but Down and Leatherland (1989) found that these neoplasms were as common in areas relatively free of industrial or heavy domestic discharge as they were in polluted locations. Although the cause of these lesions is uncertain, they are undoubtedly related to genetic factors. Ornamental carp (*C. carpio*), with complex genetic histories, also develop ovarian neoplasms that may be hereditary (Ishikawa and Takayama, 1977).

Goldfish × common carp hybrids with neoplasms had pronounced hyperplasia of gonadotropic cells of the pituitary, resulting in large amounts of gonadotropin in the pituitary and serum (Down *et al.*, 1990). Serum levels of testosterone and 11-ketotestosterone were also elevated in hybrids with neoplasms consisting of poorly differentiated cells that were probably of Sertoli cell origin. This hormonal imbalance could be related to oncogenesis directly or could result in promotion of pre-neoplastic changes induced by environmental factors (Down, 1984).

Radiation

Most studies of radiation as a cause of neoplasia in fish have used *Xiphophorus* hybrids that are unusually sensitive to oncogenic stimuli. Therefore, the susceptibility of fish in general should not be inferred from these studies.

Ultraviolet light

Four months after exposure to ultraviolet (UV) light, *Xiphophorus* hybrids had a melanoma prevalence of 20–40% compared with 2–12% in similar hybrids not exposed to UV light (Setlow *et al.*, 1989). Wavelengths from 302 to at least 405 nm induced melanomas in *Xiphophorus* hybrids, even though the longer wavelengths are not absorbed directly by DNA (Setlow *et al.*, 1993). The production of reactive melanin radicals by the longer wavelength UV is a potential cause of these melanomas (Wood *et al.*, 2006). The *Xiphophorus* homologue of the mammalian *CDKN2* gene has been implicated in enhancing the susceptibility of certain backcross hybrids to UV-induced melanoma (Nairn *et al.*, 2001).

In Amazon mollies, thyroid tumours developed after thyroid cells were irradiated *in vitro* with UV radiation (254 nm) and then injected into isogenic recipients (Hart *et al.*, 1977). Thyroid growths were found in most fish injected with cells exposed to an average incident dose of 10–20 J/m^2. Lower incidence of thyroid growths occurred in fish injected with cells having lesser or greater exposures to UV radiation. *In vitro* exposure of thyroid cells to photoreactivation light (360 nm) after UV irradiation prevented formation of tumours in recipient fish. Hart *et al.* (1977) presented several types of evidence, including transplantation of thyroid growths to Amazon mollies that were not isogenic, that these thyroid masses were neoplasms rather than goiters. However, the cells and follicles in the affected fish were well differentiated, with no indication of cellular atypia.

X-ray

X-rays caused a wide spectrum of neoplasms in hybrid *Xiphophorus* after three whole-body exposures to 1000 R for 45 min at 6-week intervals (Schwab *et al.*, 1978). The more common types of neoplasms included melanoma, fibrosarcoma and neuroblastoma. The age of the fish when exposed and the genotype were both highly related to the occurrence of neoplasia. The only fish to develop neuroblastomas were those carrying the 'lineatus' locus; however, the parent species carrying this trait (*Xiphophorus variatus*) and the hybrid used to produce the susceptible backcross did not develop this

type of neoplasm. The increased susceptibility of backcross fish is presumably related to the absence of repressor genes, as discussed for genetically related melanomas.

Oncogenic Viruses of Fish

Indications that a virus is associated with a neoplasm include isolation of virus in cell culture, detection of viral nucleic acid, experimental transmission of the tumour by cell-free filtrate, visualization of virus-like particles with electron microscopy and epizootiologic evidence. Previous reviews that consider oncogenic viruses of fish include Pilcher and Fryer (1980), Gross (1983), Wolf (1988), Anders and Yoshimizu (1994), Essbauer and Ahne (2001), Smail and Munro (2001), and Davidov *et al.* (2002). In addition, Getchell *et al.* (1998) reviewed the seasonal occurrence of virally induced cutaneous tumours.

Most of the conclusive research on viruses as a cause of fish neoplasia has involved two viral families: an RNA family, *Retroviridae*, and a DNA family, *Alloherpesviridae* (order *Herpes-virales*). Both of these families include not only oncogenic species but also species that cause non-neoplastic diseases. This section is organized by viral families and includes some of the neoplasms caused, or suspected to be caused, by a virus. In addition, we review selected neoplasms that have historically been considered viral, but may be caused by other factors, and virally induced non-neoplastic diseases resembling neoplasms either macroscopically or microscopically. As discussed below, the category in which a particular disease fits is uncertain for several diseases.

Retroviridae

Neoplasms

The neoplasms caused by retroviruses are diverse and include lymphosarcoma or leukaemia, dermal sarcoma, fibroma, leiomyosarcoma, papilloma and neural neoplasms (Bowser and Casey 1993; Quackenbush *et al.*, 2001). Viruses causing these diseases are difficult to isolate in cell culture, but transmission of the disease by cell-free inoculum, the presence of reverse transcriptase activity and identification of retroviral sequences provide evidence that retroviruses are the aetiological agents causing certain neoplasms of fish. Virus-like particles, typically C-type particles, have been seen in some lesions thought to be caused by retroviruses, but this evidence must be interpreted cautiously because of the similar-appearing neurosecretory granules in some cells (Harada *et al.*, 1990).

Lymphosarcoma in northern pike (*Esox lucius*) and muskellunge (*Esox masquinongy*) is probably caused by a retrovirus. This neoplasm also occurs in tiger muskellunge, a hybrid of northern pike and muskellunge (Bowser *et al.*, 2002a). The neoplastic cells contain C-type particles and reverse transcriptase (Papas *et al.*, 1976, 1977; Sonstegard, 1976), and neoplasms were transmitted by cell-free tumour homogenate (Mulcahy and O'Leary, 1970; Brown *et al.*, 1975; Sonstegard, 1976). The most common lesions in this disease are large, infiltrating masses in skin and underlying muscle. Neoplastic cells resemble haemocytoblasts (Mulcahy *et al.*, 1970) or lymphoblasts (Sonstegard, 1975), and they are present in blood. Metastases occur in kidney, spleen and liver (Sonstegard, 1975). Increased prevalence of this disease was reported in polluted waters (Brown *et al.*, 1973, 1977), but studies in Ireland discounted the role of pollution (Mulcahy, 1976).

A plasmacytoid leukaemia of chinook salmon was transmitted with a cell-free filtrate (Kent and Dawe, 1993), and reverse transcriptase activity and virus-like particles were demonstrated (Eaton and Kent, 1992). In this neoplasm, proliferating cells, which appeared to be plasmablasts, infiltrated most organs. Anaemia and high mortality rate of chinook salmon in netpens were caused by this leukaemia (Kent *et al.*, 1990), which also occurs in wild chinook salmon (Eaton *et al.*, 1994).

Lymphosarcoma in medaka consisted of dermal masses of homogeneous blast

cells infiltrating through muscle (Harada *et al.*, 1990). The neoplasms spread directly to adjacent sites, and also reached the thymus, spleen and kidney. C-type particles were in the neoplastic cells, but the similarity in appearance of these particles and neurosecretory granules complicates the conclusion that these particles are retroviruses.

Sarcomas in fish can also be caused by retroviruses. The best studied of these is walleye dermal sarcoma (Holzschu *et al.*, 2003), which is common in some wild populations of walleye and can affect experimentally infected or captive yellow perch (*Perca flavescens*) (Bowser *et al.*, 2001, 2005) and sauger (*Sander canadensis*) (Holzschu *et al.*, 1998). This neoplasm is caused by Walleye dermal sarcoma virus (WDSV), which is the type species of the genus *Epsilonretrovirus*. Experimentally, WDSV has been transmitted by intramuscular injection (Bowser *et al.*, 1990, 1996; Martineau *et al.*, 1990a) or topical application (Bowser *et al.*, 2001; Getchell *et al.*, 2002) of cell-free filtrate of tumour homogenate and by waterborne exposure (Bowser *et al.*, 1999). Viral RNA and DNA were detected in both tumour-bearing and tumour-free walleye from an infected population (Poulet *et al.*, 1996). These neoplasms are typically composed of fibroblast-like cells, but the tumours sometimes contain osteoid material and resemble osteosarcomas (Martineau *et al.*, 1990b; Earnest-Koons *et al.*, 1996). Cells are anaplastic and in most cases are limited to the dermis with no indication of invasion or metastasis, although locally invasive lesions occur (Earnest-Koons *et al.*, 1996; Bowser *et al.*, 2002b, 2005). Viral particles are visible in some tumours (Walker, 1969) but are not seen in others (Martineau *et al.*, 1990b). There are seasonal changes in prevalence of this disease, with lowest prevalence in summer (Bowser and Wooster, 1991), and infiltration by lymphocytes was associated with degeneration and necrosis in some neoplasms (Martineau *et al.*, 1990b). Although the density of lymphocytes was not significantly related to season, immunologic functions of these cells could be affected by temperature. Experimentally, the regression of tumours was more common at higher temperatures (Getchell *et al.*, 2000a). Acquired immunity against WDSV was also indicated by an experiment that demonstrated that most walleyes were resistant to a second exposure to the virus (Getchell *et al.*, 2001).

Swimbladder sarcoma virus is a retrovirus associated with swimbladder leiomyosarcomas of Atlantic salmon (*Salmo salar*). The neoplasms consist of well-differentiated, spindle-shaped cells with elongated cytoplasmic processes, minimal collagen and a high mitotic index (McKnight, 1978). Retrovirus-like particles were observed in swimbladder leiomyosarcomas of Atlantic salmon reared in cages in Scotland (Duncan, 1978). Another outbreak of swimbladder leiomyosarcoma occurred in a hatchery in Maine, USA, and provided samples that were used to obtain the genetic sequence of the virus (Paul *et al.*, 2006).

Retrovirus-like particles were also observed in fibromas on the lips of freshwater angelfish (*Pterophyllum scalare*) from several sources (Francis-Floyd *et al.*, 1993). These lesions were surgically removed and did not recur in 12 months. These viruses were not isolated or experimentally transmitted, and their contribution to development of these neoplasms is uncertain.

Fibromas or fibrosarcomas were found by K. Anders *et al.* (1991) in the skin of 11 (N = 1653) hooknose (*Agonus cataphractus*), a benthic marine fish found in European coastal waters. Of the seven tumours examined histologically, one appeared to be invasive but the others were benign. Electron microscopy revealed virus-like particles in cytoplasmic vacuoles of cells within the neoplasms. These particles were spherical and averaged 99 nm in diameter (range 86–132 nm). K. Anders *et al.* (1991) concluded that these virus-like particles morphologically resembled viruses in the genus *Lentivirus*, which are retroviruses. All of the well-characterized lentiviruses infect mammals and are not oncogenic.

White suckers (*Catostomus commersonii*) from Burlington Harbour and Oakville Creek in western Lake Ontario had oral papilloma prevalences of 35.1% and 50.8%, respectively (Sonstegard, 1977). Electron

microscopy revealed C-type particles in the papillomas, and reverse transcriptase activity was associated with particulate fractions separated on sucrose gradients. These papillomas were less common on fish from less polluted areas. Similar tumours were transmitted by injection of cell-free filtrate of papillomas (Premdas and Metcalfe, 1996), but virus-like particles were not seen in later studies (Smith *et al.*, 1989; Premdas and Metcalfe, 1996). Determining the role of viruses in these neoplasms is complicated by the presence of chemical carcinogens, but for some types of neoplasms, factors other than exposure to chemical carcinogens seem to be involved (Hayes *et al.*, 1990).

Neoplasms of hybrid *Xiphophorus* contain virus-like particles, but the relation between viruses and these neoplasms is unknown. Particles resembling retroviruses were seen in MNU-induced neuroblastomas of fish injected with 5-bromodeoxyuridine but not in similar tumours of fish that had not been injected with 5-bromodeoxyuridine (Kollinger *et al.*, 1979). A retrovirus was also found in a cell line established from melanomas of southern platyfish (Petry *et al.*, 1992). Other virus-like particles that were not retroviruses were also seen in melanomas of *Xiphophorus* (Kollinger *et al.*, 1979; Esaka *et al.*, 1981).

Non-neoplastic retroviral lesions

Northern pike and walleye have discrete hyperplastic epidermal lesions that are probably caused by retroviruses (Yamamoto *et al.*, 1983, 1985a,b; Bowser *et al.*, 1998). There are two closely related epsilonretroviruses associated with walleye discrete epidermal hyperplasia (LaPierre *et al.*, 1998), and the disease is more common in older fish (Getchell *et al.*, 2004). The lesions are smooth, translucent masses with thickness up to 2 mm and variable diameter up to 20 mm. Within the masses are occasional pegs of dermis, and there is generally a lack of goblet cell differentiation over most of the mass. Walleye epidermal hyperplastic lesions containing retrovirus tend to be more discrete and well demarcated (LaPierre *et al.*, 1998) than the hyperplastic lesions caused by walleye herpesvirus (Kelly *et al.*, 1983). The cellular differentiation and minimal change in the relationship between the dermis and epidermis distinguishes these lesions from papillomas and other neoplasms. However, this disease has been considered as neoplastic by some authors (Wolf, 1988; Eaton and Kent, 1992).

Alloherpesviridae

Neoplasms

Salmonid herpesvirus 2 (SalHV-2) can cause cutaneous carcinoma (Fig. 2.8). In addition to neoplasms, SalHV-2 also causes a lethal, acute disease in young salmonids (Kimura *et al.*, 1983; Furihata *et al.*, 2005). This viral species includes isolates known as *Oncorhynchus masou* virus (OMV), Yamame tumour virus (YTV), nerka virus in Towada Lake, Akita and Aomori Prefecture (NeVTA) and coho salmon tumour virus (CSTV). These viruses have been isolated from coho salmon, chum salmon (*Oncorhynchus keta*), cherry salmon (*Oncorhynchus masou*), sockeye salmon and rainbow trout (Sano, 1976; Kimura *et al.*, 1981; Sano *et al.*, 1983; Yoshimizu *et al.*, 1987, 1988). The relatedness of SalHV-2 isolates has been demonstrated serologically (Hedrick *et al.*, 1987; Yoshimizu *et al.*, 1995) and genetically (Eaton *et al.*, 1991b). However, the carcinogenicity of various isolates of SalHV-2 may vary. There is evidence that some isolates of both OMV (Yoshimizu *et al.*, 1987) and YTV (Sano *et al.*, 1983) are oncogenic; virus was reisolated from neoplasms of experimentally infected fish. However in another experiment, rainbow trout infected with OMV did not develop tumours during 530 days of observation (Furihata *et al.*, 2005).

Neoplasms caused by SalHV-2 developed 120–270 days (depending on fish species) after experimental exposure and occurred most commonly on the jaws but also on fins, cornea and operculum (Sano *et al.*, 1983; Yoshimizu *et al.*, 1987). These neoplasms were composed of epithelial cells with enlarged nuclei, and there was invasion of adjacent connective tissue (Sano *et al.*, 1983;

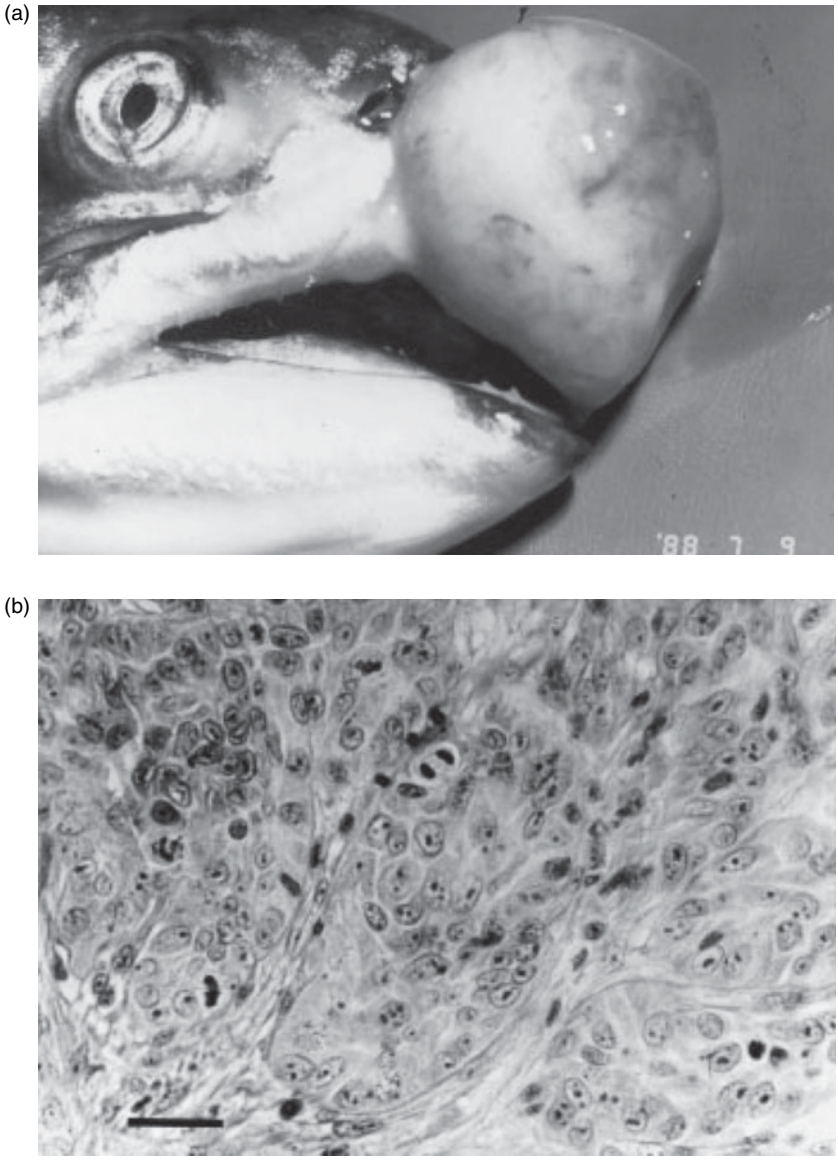


Fig. 2.8. (a) Coho salmon with a carcinoma on the upper jaw. *Oncorhynchus masou* virus (OMV) was isolated from this tumour. (b) Histological section of a carcinoma caused by OMV. Bar = 20 µm. Photographs provided by Takahisa Kimura.

Yoshimizu *et al.*, 1988). Two types of neoplasms developed in the kidney; one resembled the cutaneous lesions, and the second type contained hyperplastic renal tubules and cells resembling smooth muscle. The similarity between the cutaneous and renal neoplasms suggests the possibility of metastasis, but further study is needed to confirm this. Other malignant characteristics of these lesions were invasion of connective tissue and rapid growth.

Morrison *et al.* (1996) observed virions with the appearance of herpesvirus in papillomas and squamous cell carcinomas of

rainbow smelt (*Osmerus mordax*). An earlier attempt to find virus in similar carcinomas from this species was unsuccessful (Herman, 1988). Although similar in gross appearance, these lesions of rainbow smelt had malignant features that distinguished them from hyperplastic lesions common on European smelt (*Osmerus eperlanus*). However, particles resembling herpesvirus have also been observed in hyperplastic lesions of both rainbow smelt (Herman *et al.*, 1997) and European smelt (Anders and Möller, 1985).

Non-neoplastic herpesviral lesions

Although some authors have considered the epidermal masses described below to be neoplasms, these lesions are characterized by well-differentiated cells and have little or no change from the normal tissue arrangement. The interdigitation between epithelial and supportive stromal tissues, which is characteristic of papillomas, is not typically present or is not distinctly different from in normal skin. Note that lesions associated with pike herpesvirus (discussed below) are characterized by epidermal hypertrophy and are therefore quite different from other fish diseases caused by herpesviruses.

A disease known as 'carp pox' is one of the oldest recognized diseases of fish (Wolf, 1988). The virus that causes carp pox, cyprinid herpesvirus-1 (CyHV-1), has been isolated from ornamental carp (Sano *et al.*, 1985a,b, 1991). Thickened areas of epidermis developed 5–6 months after immersion exposure of carp. The lesions sloughed spontaneously and then recurred 7.5 months later (Sano *et al.*, 1991). The virus was re-isolated from the hyperplastic lesions, fulfilling Rivers' postulates. *In situ* hybridization was used to detect the viral genome in lesions and other locations of fish with active infections, and after lesions had regressed the viral genome could still be detected in gills, cranial nerve ganglia and spinal nerves (Sano *et al.*, 1993).

The historically entrenched name 'carp pox' is a misnomer because the causative agent is not a poxvirus. Several other names have been used for this condition, including fish pox, cutaneous warts, epithelioma papulosum, hyperplastic epidermal disease, papillosum cyprini, plaque warty hyperplasia and variola (Wolf, 1988). Cyprinids other than carp are affected, and some reports indicate that non-cyprinids, including zander (*Sander lucioperca*) and European smelt, are also susceptible (van Duijn, 1973). Epidermal growths on wels (*Silurus glanis*) (Wolf, 1988) and spawning European smelt (Anders and Möller, 1985; Lee and Whitfield, 1992) are similar to carp pox. Virus-like particles that resemble herpesvirus are visible in hyperplastic lesions of wels and European smelt, but viruses have not been isolated.

In addition to nomenclatural problems posed by carp pox, the neoplastic nature of the lesions also needs additional consideration. Lesions associated with this disease have been considered non-neoplastic by some authors (Schlumberger and Lucké, 1948; Nigrelli, 1954), while other authors describe the lesions as papillomas (Sano *et al.*, 1991). There may be a progression from early non-neoplastic lesions to papillomas, but this has not been adequately described. The use of the term papilloma for these non-neoplastic lesions has unfortunately led some authors to make inappropriate comparisons between hyperplastic diseases and neoplasms (e.g. Korkea-aho *et al.*, 2006).

Carp pox lesions are white plaques composed of hyperplastic epithelial cells, and the lesions tend to harden with age (Schäperclaus, 1991). There is typically minimal involvement by the dermis (McAllister *et al.*, 1985; Sano *et al.*, 1991). Epidermal cells generally appear differentiated, and some goblet cells are present. As with many viral diseases of fish skin, the masses are transient and often regress as water temperature increases (McAllister *et al.*, 1985) or during other critical phases of the fish's life cycle (Anders, 1989). Sano *et al.* (1991) speculated that replication of the virus in the hyperplastic tissue was suppressed or enhanced depending on water temperature. Lymphocytes are probably an important factor related to regression of the lesions (Morita and Sano, 1990).

Walleye have four types of cutaneous masses that are difficult to distinguish based on macroscopic examination (Yamamoto

et al., 1985b). One of these diseases resembles carp pox and is probably caused by a walleye herpesvirus (Kelly *et al.*, 1983). This virus, known as percid herpesvirus 1, was isolated from hyperplastic epidermis that typically occurs during the spawning season and then regresses. The lesions are flat, translucent masses with diameters up to several centimetres. Superficially these lesions resembled areas of thickened mucus without well-demarcated margins.

One type of cutaneous mass found on northern pike is caused by northern pike herpesvirus (esocid herpesvirus 1), and the lesion consists of hypertrophied epithelial cells (Yamamoto *et al.*, 1983; Graham *et al.*, 2004). Enlarged cells are up to 150 μm in diameter and are interspersed with normal-sized epidermal cells. Lesions appear as flattened, bluish-white masses with a granular texture. Enlarged nuclei of the hypertrophied cells contain herpesvirus capsids measuring 100 nm in diameter. Northern pike can also have lymphocystis, another disease characterized by hypertrophied cells, but lesions caused by pike herpesvirus lack a hyaline capsule and have an epidermal location (Yamamoto *et al.*, 1983).

Iridoviridae

Lymphocystis is a common non-neoplastic disease of fish and is caused by an iridovirus (Flügel, 1985). The cutaneous masses typical of this disease are formed by massive hypertrophy of infected cells (Weissenberg, 1965). These lesions might be confused with neoplasia grossly but are clearly and easily distinguished from neoplasia by histopathology. Infected cells increase in size, commonly to 100–500 μm, with the maximum size varying depending on the fish species (Nigrelli and Ruggieri, 1965). Cells have a hyaline capsule, a centrally located and enlarged nucleus, and prominent basophilic cytoplasmic inclusions. Rivers' postulates were fulfilled by Wolf *et al.* (1966). This disease is widespread geographically and taxonomically (Lawler *et al.*, 1977). It occurs in both freshwater and marine species, but is more common in higher phylogenetic groups (Nigrelli and Ruggieri, 1965; Wolf, 1988).

Unclassified viruses associated with neoplasms

Neurofibromatosis of bicolour damselfish (*Stegastes partitus*) can be transmitted by injection of filtered (0.2 μm) tumour homogenate (Schmale, 1995), and epizootiological evidence suggests that an infectious agent is transmitted horizontally to spread this disease (Schmale, 1991). Damselfish with neurofibromastosis have PNST (neurofibromas and neurofibromosarcomas) and chromatophoromas. A retrovirus was found in tumourigenic cell lines derived from fish with spontaneous or experimentally induced neurofibromatosis (Schmale *et al.*, 1996); however, retroviral genomes were not detected consistently and are not considered to be the cause of this disease (Schmale *et al.*, 2002). A damselfish virus-like agent detected by molecular techniques is the most likely cause of neurofibromatosis in this fish species (Schmale *et al.*, 2002; Rahn *et al.*, 2004).

Papillomas of brown bullheads have been reported to contain virus-like particles measuring 50 nm in diameter (Edwards *et al.*, 1977). However, other studies failed to confirm this observation (Harshbarger *et al.*, 1993; Poulet and Spitsbergen, 1996). An RNA-dependent DNA polymerase activity, presumably reverse transcriptase, was present in brown bullhead papillomas, but no other indication of a viral agent was found by Poulet *et al.* (1993). Chemical carcinogens have also been suggested as causes of papillomas in some brown bullhead populations (Black *et al.*, 1985a). Papillomas were present on 60% of brown bullheads in samples from Silver Stream Reservoir, New York, during October 1986 (Bowser *et al.*, 1991). This reservoir had relatively low concentrations of contaminants; polycyclic aromatic hydrocarbon (PAH) levels in sediment were similar to those at reference sites used during studies of neoplasms in fish from Puget Sound. However, in a sample from Silver

Stream Reservoir the following July, no brown bullheads with papillomas were found, suggesting that there is a pronounced seasonal fluctuation in prevalence of papillomas in some brown bullhead populations (Bowser *et al.*, 1991). The widespread occurrence of papillomas and carcinomas on brown bullheads from both polluted and unpolluted sites suggests that the causes of these lesions are complex or variable (Poulet *et al.*, 1994).

Papillomas occur on Atlantic pleuronectids (Sindermann, 1990). Small (30 nm) cytoplasmic virus-like particles that apparently contained DNA were found in cutaneous growths on winter flounder (*Pseudopleuronectes americanus*) from the north-western Atlantic (Emerson *et al.*, 1985). Particles resembling adenovirus were observed in hyperplastic epithelial cells and papillomas of dab (*Limanda limanda*) from the North Sea (Bloch *et al.*, 1986). These papillomas were distinguished from hyperplastic lesions by the epithelial folding and dermal extensions characteristic of papillomas. The adenovirus-like particles measured about 80 nm in diameter and were present in nuclei of epithelial cells near the surface of the lesions.

Papillomas of European eel (*Anguilla anguilla*) have often been considered to be caused by viruses (Pilcher and Fryer, 1980). These lesions are typically located on the jaws and other parts of the head, and the disease is sometimes termed stomatopapilloma or 'cauliflower disease'. Although viruses have been isolated from eels with papillomas, they can also be isolated from eels without papillomas. The role of these viruses in the pathogenesis of papillomas is questionable (Wolf, 1988), although an interaction between a virus and unidentified environmental factor(s) could be involved with tumour formation (Peters, 1984; Roberts, 2001).

Chemical Carcinogenesis

Various aspects of chemical carcinogenicity in fish have been reviewed by Hendricks (1982), Couch and Harshbarger (1985), Calabrese *et al.* (1992), Moore and Myers (1994), Hawkins *et al.* (1995), Bailey *et al.* (1996), Baumann (1998) and Chen and White (2004). Our review includes selected groups of chemicals that have been clearly associated with neoplasms of wild or hatchery fish or that have been used extensively in laboratory experiments.

High prevalences of neoplasia have been discovered in some waters polluted with mixtures of chemicals. In many of these locations, it is likely that the tumours result from the chemical mixture, which could include not only carcinogens but promoters as well. Some of these cases have been included in this review under a particular category of chemical carcinogen because of evidence implicating that agent as most responsible for initiation of the neoplasms. Other locations have highly complex mixtures, and association of the neoplasia with a single category of chemical seems unwarranted without further study. Examples of epizootics of neoplasia, either papillomas or hepatic tumours, associated with complex mixtures of pollutants include dab and European flounder in German and Dutch coastal areas (Vethaak *et al.*, 1992; Vethaak and Jol, 1996; Vethaak and Wester, 1996; Koehler 2004); Atlantic tomcod in the Hudson River, New York, estuary (Dey *et al.*, 1993); walleye in Green Bay, Wisconsin (Barron *et al.*, 2000); and lake whitefish and white suckers in the St Lawrence River (Mikaelian *et al.*, 2000, 2002).

Chemical enhancers and inhibitors of carcinogenesis

A variety of chemicals can alter the course of oncogenesis in fish by acting as co-carcinogens, promoters or anti-carcinogens (Bailey *et al.*, 1987; Tilton *et al.*, 2006). Induction of cytochrome P450 is also an important aspect of chemical carcinogenesis (Williams *et al.*, 1998). Certain pollutants seem to be involved in increasing the prevalence of neoplasms in fish, but in many cases it is not known whether these chemicals act as carcinogens, promoters or co-carcinogens, or as activators of oncogenic viruses. Some chemicals are probably both carcinogens and promoters; an initial exposure causes genetic change and continuing exposure

stimulates development and growth of the neoplasm. Whether a particular compound enhances or inhibits carcinogenicity can depend on several factors, including the initiating chemical. For example, Aroclor inhibited the effect of AFB_1 but enhanced the effect of DEN (Shelton *et al.*, 1983, 1984).

Metcalfe and Sonstegard (1985) demonstrated that pollutants can act as co-carcinogens. They injected rainbow trout embryos simultaneously with AFB_1 and an extract of oil refinery effluent; after a year the frequency of neoplasms was higher in fish from this treatment than for fish that received only AFB_1. Co-carcinogenic activity of the extract did not increase the carcinogenicity of MNNG, a direct-acting carcinogen.

Gardner *et al.* (1998) studied another complex mixture of chemicals that enhanced the carcinogenicity of DEN. Medaka were exposed to DEN for 48 h and then exposed for 6 months to various dilutions of groundwater containing an average of 0.125 mg/l trichloroethylene. The groundwater also contained smaller amounts of unidentified contaminants. The fish exposed to the contaminated groundwater, but not previously exposed to DEN, did not develop neoplasms; however, fish exposed to both DEN and contaminated groundwater had more neoplasms than those exposed only to DEN. However, similar exposures of fish to trichloroethylene, rather than the contaminated groundwater, did not produce tumours in excess of DEN exposure alone. These results suggest that the promotional effect of the contaminated groundwater was the result of the mixture of trichloroethylene plus the unidentified pollutants.

Several chemicals have been found to modulate the effects of chemical carcinogens in rainbow trout. Dietary tomatine (Friedman *et al.*, 2007), chlorophyllin (Reddy *et al.*, 1999; Pratt *et al.*, 2007) or chlorophyll (Simonich *et al.*, 2008) inhibited the development of hepatic and gastric tumours in rainbow trout fed dibenzo[*a,l*]pyrene (DBP). Dietary treatment of rainbow trout with indole-3-carbinol, β-naphthoflavone or chlorophyllin before or during exposure to AFB_1 reduced the occurrence of hepatocellular carcinomas compared with fish receiving only AFB_1 (Nixon *et al.*, 1984; Goeger *et al.*, 1988; Dashwood *et al.*, 1998). In contrast, when indole-3-carbinol, 3,3'-diindolylmethane or β-naphthoflavone was given after exposure to AFB_1, the percentage of fish with carcinomas increased (Goeger *et al.*, 1988; Dashwood *et al.*, 1991; Oganesian *et al.*, 1999; Tilton *et al.*, 2007). Carcinogenicity was also enhanced when 17β-estradiol, indole-3-carbinol, β-naphthoflavone, DDT or dehydroepiandrosterone was fed to fish after a single exposure to AFB_1 or MNNG (Núñez *et al.*, 1988, 1989; Orner *et al.*, 1995). Dietary exposure to perfluorooctanoic acid (PFOA) promoted hepatocarcinogenicity in rainbow trout previously exposed to AFB_1, and this effect was related to an oestrogenic action of PFOA rather than peroxisome proliferation as in rodent models (Tilton *et al.*, 2008).

Premdas *et al.* (2001) also demonstrated the potential of 17β-estradiol to serve as a tumour promoter. Injections of either 17β-estradiol or testosterone stimulated the development of papillomas on white suckers from locations polluted with several organic chemicals. As further evidence, injection of tamoxifen, an oestrogen-receptor blocker, caused regression and inhibited development of papillomas on these fish.

Maternal transfer of pollutants to offspring can also affect carcinogenesis. Aroclor 1254 was present in embryos after this PCB was fed to female rainbow trout for 2 months before spawning (Hendricks *et al.*, 1981). After embryo exposure to AFB_1, incidence of hepatocellular carcinoma was enhanced by maternally derived PCB.

The promotional activity of 41 agents was tested with a strain of hybrid *Xiphophorus* that was genetically predisposed to melanoma (A. Anders *et al.*, 1991). Thirty of these agents were positive, including the carcinogens MNU and ENU, and 11 were negative. Chemicals that were negative for promoting activity in this test included DEN.

Carbon tetrachloride enhances hepatocarcinogenesis in rainbow trout given a single injection of AFB_1 (Kotsanis and Metcalfe, 1991). The CCl_4 was administered intraperitoneally at 21-day intervals starting 25 days after yolk-sac larvae were injected with AFB_1. After 3 months, incidence of carcinomas in fish receiving both CCl_4 and AFB_1

was double the rate for fish injected with only AFB_1. However, after 6 months there was no significant difference between these treatments.

Hydrogen peroxide in the diet enhanced carcinogenicity in MNNG-initiated rainbow trout (Kelly *et al.*, 1992). Fish fed hydrogen peroxide had increased levels of the mutagenic DNA adduct 8-hydroxy-2′-deoxyguanosine, which is an indication of oxidative DNA damage. Vitamin E, an antioxidant, did not have a significant effect in this study.

Mycotoxins

Aflatoxin

Hepatic carcinomas in rainbow trout grown in hatcheries have been linked to feed contaminated with aflatoxin. Although there is some continuing interest in the effects of aflatoxin-contaminated feeds in aquaculture (Arana *et al.*, 2002; Tuan *et al.*, 2002; Manning, 2005), most recent research with fish has been related to experimental carcinogenesis. Reviews of aflatoxin carcinogenicity include Hendricks (1994) and Santacroce *et al.* (2008).

Epizootics of hepatic carcinomas were discovered after dry feeds for trout came into wide use during the 1950s (Hueper and Payne, 1961; Rucker *et al.*, 1961; Wood and Larson, 1961; Scarpelli *et al.*, 1963), although earlier problems with hepatic neoplasms had occurred in hatchery-reared salmonids (Haddow and Blake, 1933; Nigrelli, 1954; Wales and Sinnhuber, 1966). Aflatoxin in cottonseed meal was the primary cause of these epizootics (Wolf and Jackson, 1963; Ashley *et al.*, 1964; Sinnhuber, 1967; Halver, 1967); however, carcinogenicity of aflatoxin was enhanced by cyclopropenoid fatty acids (malvalic and sterculic acids) occurring naturally in cottonseeds (Lee *et al.*, 1968, 1971; Sinnhuber *et al.*, 1968, 1974; Hendricks *et al.*, 1980a). Epizootics of hepatic carcinomas have occurred more recently (Majeed *et al.*, 1984; Rasmussen *et al.*, 1986), but problems in aquaculture have been reduced by avoiding feed ingredients with high concentrations of aflatoxin. Feed ingredients most likely to be contaminated with aflatoxin are maize, cottonseed and groundnuts (CAST, 2002).

Aflatoxins can be produced by four species of *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. nomius* and *A. pseudotamarii* (CAST, 2002). Several types of aflatoxin are produced by these fungi, but AFB_1 is a major component and is also the form most often used in experimental exposures of fish. Aflatoxin B_1 is not carcinogenic until conversion to the electrophilic 8,9-epoxide, which can form adducts with DNA (Swenson *et al.*, 1977; Baertschi *et al.*, 1988). This metabolic activation is mediated by cytochrome P450, and the extreme carcinogenicity of AFB_1 in rainbow trout is related to the preferential formation of the ultimate carcinogen rather than the formation of less carcinogenic metabolites (Williams and Buhler, 1983; Bailey *et al.*, 1988, 1998). Aflatoxin B_1 is also metabolized to compounds that can be conjugated and excreted; however, in rainbow trout some of these metabolites are carcinogenic, including aflatoxin M_1 (Sinnhuber *et al.*, 1974), aflatoxin Q_1 (Hendricks *et al.*, 1980a) and aflatoxicol (Schoenhard *et al.*, 1981). Aflatoxicol is a major metabolite of AFB_1 in rainbow trout, and the tendency to form aflatoxicol, rather then less carcinogenic metabolites, during metabolism of AFB_1 could contribute to the sensitivity of rainbow trout to AFB_1 (Schoenhard *et al.*, 1981).

Types of neoplasms in rainbow trout exposed to aflatoxin are hepatocellular adenomas, hepatocellular carcinomas and mixed carcinomas containing both hepatocellular and cholangiolar components (Núñez *et al.*, 1989, 1991). Hepatocellular adenomas consist of basophilic cells with less glycogen than normal hepatocytes. Hepatocytes within these adenomas are usually organized in tubules having the normal two-cell thickness. Compression and invasion of adjacent sites are absent. Hepatocellular adenomas are uncommon and appear to be a transitional stage between pre-neoplastic basophilic foci and hepatocellular carcinoma (Hendricks *et al.*, 1984b; Núñez *et al.*, 1991). A tubular pattern with well-differentiated

hepatocytes is the most common form of hepatocellular carcinoma (Hendricks *et al.*, 1984b). These carcinomas are distinguished from hepatocellular adenoma by their invasiveness and expansion of tubules to five or more cells thick (Núñez *et al.*, 1991). Metastases and emboli of carcinoma cells occur (Hueper and Payne, 1961; Wood and Larson, 1961; Ashley and Halver, 1963; Yasutake and Rucker, 1967; Núñez *et al.*, 1989), but experimental studies are usually terminated before metastasis is observed.

Although mixed carcinomas are usually the most common neoplasm in rainbow trout exposed to aflatoxin, experimental exposures sometimes result in only hepatocellular carcinomas (Núñez *et al.*, 1991). Hepatocytes within neoplasms caused by aflatoxin can function normally, so affected fish survive even after the liver has been almost totally replaced by neoplastic tissue (Hendricks, 1982).

Triploid and diploid rainbow trout exposed to aflatoxin by a single immersion in 0.25 mg/l for 30 min when they were 4 months old developed only hepatic neoplasms (Thorgaard *et al.*, 1999). There were 50% of the diploid fish and 16% of the triploid fish with hepatic tumours. The kidney, stomach and swimbladder, which had neoplasms in fish exposed to MNNG or DMBA in this study, did not have neoplasms after exposure to AFB_1.

An unusual lesion in rainbow trout fed aflatoxin is pancreatic acinar cell metaplasia within hepatocellular carcinomas (Hendricks *et al.*, 1984b). Unlike many other teleosts, salmonids do not normally have pancreatic acini associated with the hepatic portal veins within the liver (Yasutake and Wales, 1983). Therefore, occurrence of exocrine pancreatic cells within the liver of aflatoxin-exposed rainbow trout is probably related to the origin of both tissues from a single pluripotent stem cell.

Fish species and strains vary dramatically regarding their sensitivity to aflatoxin. Rainbow trout are more sensitive to the carcinogenic action of dietary aflatoxin than are other animals studied (Hendricks, 1994); 14% of the rainbow trout fed 0.4 μg AFB_1/kg of feed developed liver neoplasms after 15 months (Lee *et al.*, 1968). Shasta strain rainbow trout are the most sensitive strain of rainbow trout (Sinnhuber *et al.*, 1977; Bailey *et al.*, 1989) and are the most commonly used fish in studies involving aflatoxin-induced carcinogenicity. However, this sensitivity is not a universal feature of fish or even of salmonids. Rats of the Fischer strain are more sensitive than coho salmon (Halver *et al.*, 1969; Wogan *et al.*, 1974; Bailey *et al.*, 1988) or guppies (Sato *et al.*, 1973). Sockeye salmon (*Oncorhynchus nerka*) fed aflatoxin develop carcinomas only if synergists, such as cyclopropenoid fatty acids, are included in the diet (Wales and Sinnhuber, 1972). Not only is a high dose of AFB_1 required for coho salmon to develop neoplasms but also the neoplasms that develop in coho salmon are adenomas rather than carcinomas. Compared with salmonids, channel catfish (*Ictalurus punctatus*) are much less sensitive to the acute and oncogenic properties of AFB_1 (Ashley, 1970; Jantrarotai and Lovell, 1990; Jantrarotai *et al.*, 1990). The low sensitivity of channel catfish could be related to incomplete absorption and rapid elimination of AFB_1 (Plakas *et al.*, 1991). Similarly, wild-type zebrafish exposed at any life stage are remarkably resistant to the carcinogenic effects of AFB_1 (Spitsbergen and Kent, 2003).

Aflatoxin can also be used to initiate carcinogenesis before fish hatch. Rainbow trout embryos immersed in a solution of AFB_1 for 30 min will develop hepatic neoplasms 9–12 months later (Sinnhuber and Wales, 1974; Wales *et al.*, 1978; Hendricks *et al.*, 1980d). Age of the exposed embryo is important because exposure after liver development increases sensitivity to AFB_1 (Wales *et al.*, 1978). An AFB_1 concentration of 0.125 mg/l and a duration of exposure of 30 min resulted in an incidence of hepatic neoplasms of 5% for 9 months (Núñez *et al.*, 1989).

Exposure of fish embryos or yolk-sac larvae can also be accomplished by microinjection of carcinogen, which offers the advantages of reducing the amount of carcinogen needed and ensuring exposure to water-insoluble compounds (Metcalfe and Sonstegard, 1984; Black *et al.*, 1985b; Metcalfe *et al.*, 1988). Both rainbow trout and

coho salmon have been used successfully for embryo injection of AFB_1 (Black *et al.*, 1988), and coho salmon offer the advantage of relatively large eggs (200 mg).

Other mycotoxins

Versicolorin A and sterigmatocystin are synthesized by *Aspergillus* spp. and are precursors in the synthesis of AFB_1. Both of these mycotoxins caused hepatic carcinomas in the rainbow trout embryo exposure assay (Hendricks *et al.*, 1980b).

Fumonisins are mycotoxins commonly found on maize. Laboratory exposures of rainbow trout indicated that fumonisin B_1 was not a complete carcinogen in this model. However, fumonisin B_1 did promote the carcinogenicity of other chemical carcinogens in some organs, including liver neoplasia initiated by aflatoxin B_1 (Carlson *et al.*, 2001).

Cyclopropenoid fatty acids

Cyclopropenoid fatty acids (malvalic and sterculic acids) are natural components of cottonseed meal. These compounds are co-carcinogens of AFB_1 and its metabolites (Lee *et al.*, 1968, 1971; Sinnhuber *et al.*, 1968, 1974; Hendricks *et al.*, 1980a; Schoenhard *et al.*, 1981), but they are also primary carcinogens in rainbow trout (Hendricks *et al.*, 1980c).

Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is a major circulating steroid and is used for treatment of diseases in mammals. Rainbow trout fed a diet containing DHEA for 30 weeks developed hepatic neoplasms, and there was also an enhancement of MNNG- (Orner *et al.*, 1996) and AFB_1-initiated carcinogenicity (Orner *et al.*, 1995). Daily doses lower than used in human clinical trials were carcinogenic in rainbow trout. The latency of tumour formation in rainbow trout initiation with AFB_1 was shortened when DHEA was fed to the fish after initiation, compared with administration of DHEA before or during initiation (Orner *et al.*, 1998). The fish fed DHEA also had decreased levels of the proteins p53 and p34cdc2, which are involved in regulation of the cell cycle.

In contrast to the above results, there was no evidence that DHEA caused neoplasms in zebrafish fed DHEA for 6 months (Tsai, 1996). There was also no statistically significant promotion of neoplasia in zebrafish previously exposed to AFB_1. The lack of positive results in zebrafish is probably related to the resistance of wild-type zebrafish to chemical carcinogenicity.

Halogenated compounds

Halogenated chemicals have numerous industrial and agricultural uses. In addition, chlorine used for treatment of drinking water and wastewater combines with organic chemicals to form chlorinated compounds such as chloroform. Some processes used to manufacture paper also use chlorine and can form chlorinated compounds. Several halogenated compounds are known or suspected mammalian carcinogens.

Oral papillomas (Fig. 2.9) occurred on 73% of black bullheads (*Ameiurus melas*) living in a pond filled with chlorinated wastewater of domestic origin (Grizzle *et al.*, 1981). There was no evidence that viruses were present in the oral papillomas (Grizzle *et al.*, 1984). After neoplasms were discovered, less chlorine was used for effluent disinfection, and the total residual chlorine concentration entering the pond decreased from 1.0–3.1 mg/l to 0.25–1.2 mg/l (monthly averages). Three years after the chlorination rate was reduced, prevalence of neoplasms had decreased to 23% (Grizzle *et al.*, 1984). This population of fish has since been extirpated, presumably because reproduction was not successful in the contaminated water. Except for low concentrations of chloroform (9.0–13.5 µg/l) and bromodichloromethane (0.7 µg/l) present in the water, chemicals suspected to be carcinogens were not detected in water or sediment of the pond. Some organic extracts of the wastewater tested positive for mutagenicity in Ames tests; extracts were

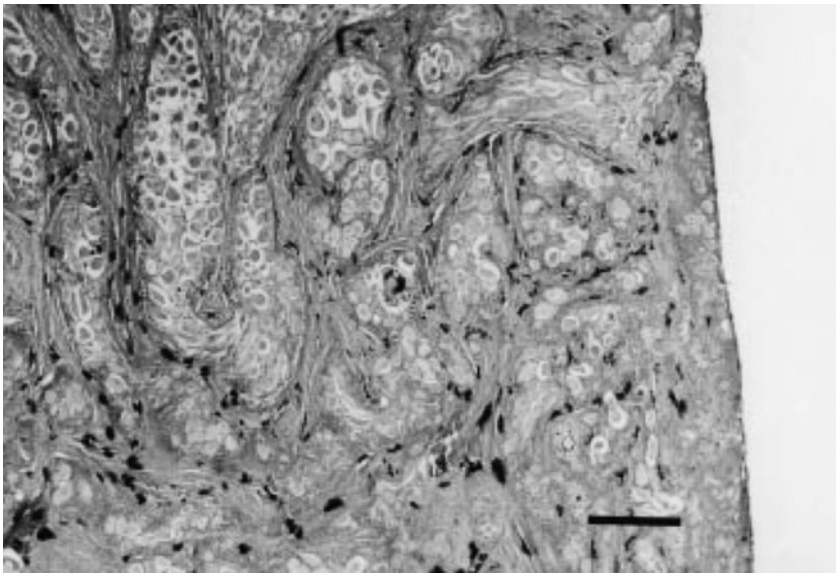


Fig. 2.9. Papilloma from the head of a black bullhead. The fish was from a pond receiving chlorinated wastewater effluent. Bar = 300 μm.

most mutagenic during the summer (Grizzle *et al.*, 1984). Tan *et al.* (1981) presented evidence for induction of mixed-function oxidase systems and for hepatic dysfunction in black bullheads exposed to this chlorinated wastewater.

Black bullheads confined to cages in this pond receiving chlorinated wastewater developed oral papillomas after 2–18 months (Grizzle *et al.*, 1984). Papillomas did not develop in control fish or in exposed brown bullheads, yellow bullheads (*Ameiurus natalis*) and channel catfish. Compared with exposed black bullheads and control channel catfish, exposed channel catfish had increased levels of hepatic glucuronosyltransferase, which could conjugate active metabolites and thereby reduce the effects of carcinogens.

Neuroblastomas in coho salmon were attributed to halogenated compounds in water that had been chlorinated and then dechlorinated (Meyers and Hendricks, 1984). However, similar neoplasms, diagnosed as malignant schwannomas and ependymoblastomas, also occurred in coho salmon reared in well water that had not been chlorinated (Masahito *et al.*, 1985).

Nibe croaker (*Nibea mitsukurii*) collected from several locations along the Pacific coast of Japan had chromatophoromas, but prevalence was especially high at a location polluted by effluent from a pulp mill (Kinae *et al.*, 1990). An ether extract of effluent from the pulp mill was mutagenic, and several chlorinated compounds were identified by gas chromatography/mass spectrometry. During surveys from 1973 to 1981, frequency of chromatophoromas on Nibe croaker collected near the pulp mill averaged 47.3%, compared with 0–8.5% at other locations. Between 1977 and 1979, treatment of the wastewater was improved and contaminated sediment was removed; prevalence of chromatophoromas decreased to 20% for 1984–1987. Neoplasms were noted on other fish species collected from the area polluted by the pulp mill, but the number of fish sampled was insufficient for analysis. Striped eel-catfish (*Plotosus lineatus* [= *anguillaris*]) from this location had a 13.5% prevalence of cutaneous melanosis. A chromatophoroma developed on one of the 100 Nibe croakers exposed for 13 months to seawater containing 10% effluent. Melanosis developed on 70% of the experimentally exposed

striped eel-catfish, compared with 10% of the control fish.

Guppies and medaka exposed to 1,2,3-trichloropropane (4.5–18 mg/l) developed hepatic neoplasms, and medaka also developed adenomas in the gallbladder (Kissling *et al.*, 2006). This chlorinated solvent is carcinogenic in rodents exposed by gavage, and several organs are affected.

Rainbow trout fed 1,2-dibromoethane (2 g/kg dry weight in diet) developed gastric papillomas and a low incidence of hepatocellular carcinomas (Hendricks *et al.*, 1995). After 18 months, frequency of these gastric papillomas was higher in males (41%) than in females (21%).

Medaka exposed to 1,2-dibromoethane in the water developed hepatic and gallbladder neoplasms (Hawkins *et al.*, 1998). Exposures began when fish were 7 days old and continued for 73–97 days. This compound was clearly carcinogenic at concentrations of 6.2 mg/l and higher. A concentration of 1.0 mg/l induced hepatic glutathione S-transferase, which is part of the enzyme pathway forming the reactive metabolite of 1,2-dibromoethane.

Another brominated compound, 2,2-bis(bromomethyl)-1,3-propanediol (BMP), which is used as a fire retardant, caused hepatocellular neoplasms in male guppies and medaka (Kissling *et al.*, 2006). Neoplasms were not found in female fish or in organs other than liver. This compound is carcinogenic in both male and female rodents, with neoplasms occurring in several organs. In the test by Kissling *et al.* (2006), fish were exposed to 24–150 mg BMP/l in the water, rather than the higher concentrations fed to rodents.

N-nitroso compounds

N-nitroso compounds are produced by reactions of amines with nitrites. These reactions occur in foods, cosmetics, tobacco products, cutting oils and in rubber manufacture (Lijinsky, 1992). Several *N*-nitroso compounds have been shown to form spontaneously in sewage and lake water containing simultaneously high levels of nitrates or nitrites and dimethyl- or trimethylamines (Ayanaba and Alexander, 1974). It has also been reported that mutagenic *N*-nitroso compounds can be formed in the muscle of fish exposed to high levels of environmental nitrate (De Flora and Arillo, 1983). There have been no reports of neoplasia in wild fish exposed to nitrosamines; however, the *N*-nitroso compounds have been widely used as carcinogens in experimental exposures.

Diethylnitrosamine

Diethylnitrosamine and the related *N*-nitroso compound DMN are metabolized by vertebrates to form carcinogenic metabolites. In fish (Kaplan *et al.*, 1991) as well as in mammals (Lijinsky, 1992), the primary site of DEN and DMN metabolism is the liver; therefore, most neoplasms resulting from experimental exposure are associated with the liver (Fig. 2.10). Since Stanton (1965) reported neoplasms in zebrafish exposed to DEN, this carcinogen has been commonly used for experimental carcinogenesis in fish. Examples of studies related to DEN carcinogenesis in fish include Shelton *et al.* (1984), Thiyagarajah and Grizzle (1985), Bunton (1990, 1991, 1995), Couch (1990, 1991, 1993), Braunbeck *et al.* (1992), Hinton *et al.* (1992), Teh and Hinton (1993, 1998), Hendricks *et al.* (1994), Goodwin and Grizzle (1994), Boorman *et al.* (1997), Brown-Peterson *et al.* (1999), Okihiro and Hinton (1999), and Mizgireuv and Revskoy (2006).

In addition to hepatocytes, other cell types in livers of fish exposed to DEN are also transformed, presumably due to *N*-nitroso metabolites released by hepatocytes. In DEN-exposed *Poeciliopsis* (Schultz and Schultz, 1985), mangrove rivulus (Grizzle and Thiyagarajah, 1988), medaka (Bunton, 1990, 1991) and sheepshead minnows (*Cyprinodon variegatus*) (Couch and Courtney, 1987; Couch, 1990), neoplastic pericytes form haemangiopericytomas consisting of spindle-shaped cells arranged in whorls around small blood vessels (Fig. 2.10e). Pericytomas that are distinct from haemangiopericytomas have been reported in sheepshead minnows

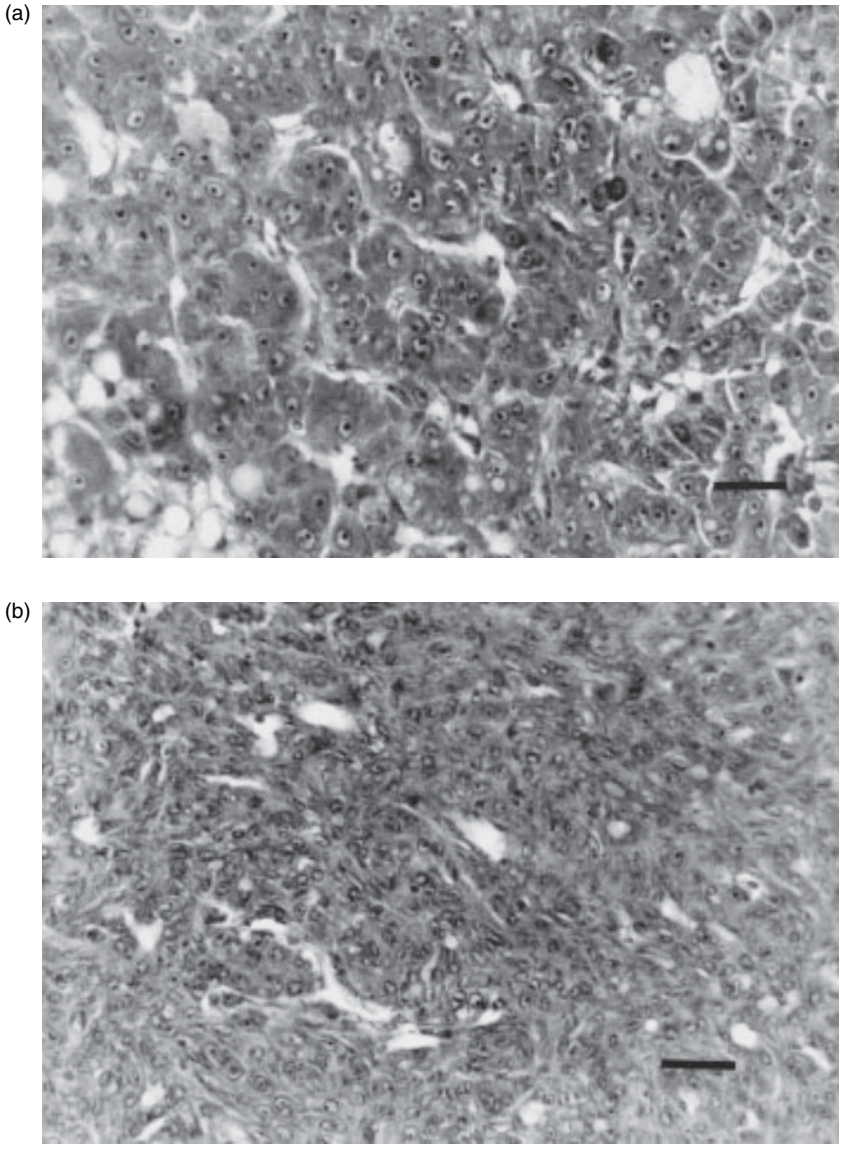


Fig. 2.10. Hepatic lesions in mangrove rivulus exposed to diethylnitrosamine. (a) Trabecular hepatocellular carcinoma. Bar = 25 µm. (b) Anaplastic hepatocellular carcinoma. Bar = 25 µm. (c) Cholangiocarcinoma invading adjacent hepatic parenchyma. Bar = 100 µm. (d) Spongiosis hepatis. Bar = 50 µm. (e) Haemagiopericytoma. Bar = 25 µm. (f) Haemangioma. Bar = 50 µm.

Continued

(Couch and Courtney, 1987). Endothelial cells are also subject to neoplastic transformation by DEN and form haemangiomas (Fig. 2.10f) (Thiyagarajah and Grizzle, 1985; Grizzle and Thiyagarajah, 1988) or haemangioendotheliomas (Bunton, 1990).

Medaka, mangrove rivulus and sheepshead minnows exposed to DEN develop spongiosis hepatis (Fig. 2.10d), a hepatic lesion consisting of multilocular hepatic foci filled with weakly eosinophilic fluid (Hinton *et al.*, 1984, 1988; Grizzle and Thiyagarajah,

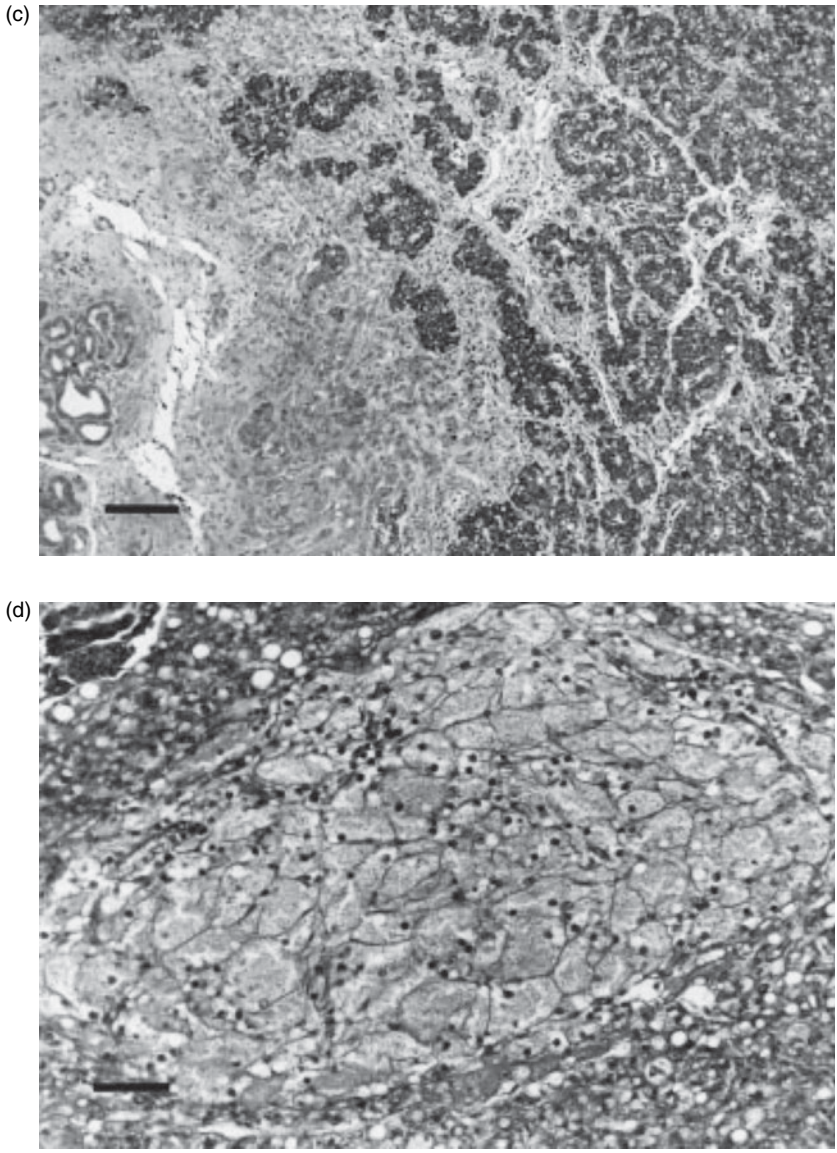


Fig. 2.10. *Continued*.

1988; Couch, 1991; Braunbeck *et al*., 1992). This lesion has also been reported in control medaka (Bunton, 1990; Boorman *et al*., 1997; Brown-Peterson *et al*., 1999). Spongiosis hepatis is formed by a meshwork of interconnected cytoplasmic extensions of perisinusoidal stellate cells, sometimes accompanied by leucocytes (Couch, 1991). In sheepshead minnows, polymorphic cell neoplasms apparently arise within areas of spongiosis hepatis. These neoplasms consist of an avascular population of belt-like, stellate or spindle-shaped eosinophilic cells with tenuous cell-to-cell contacts and frequent mitotic figures (Couch, 1991). Even after similar exposure protocols, spongiosis

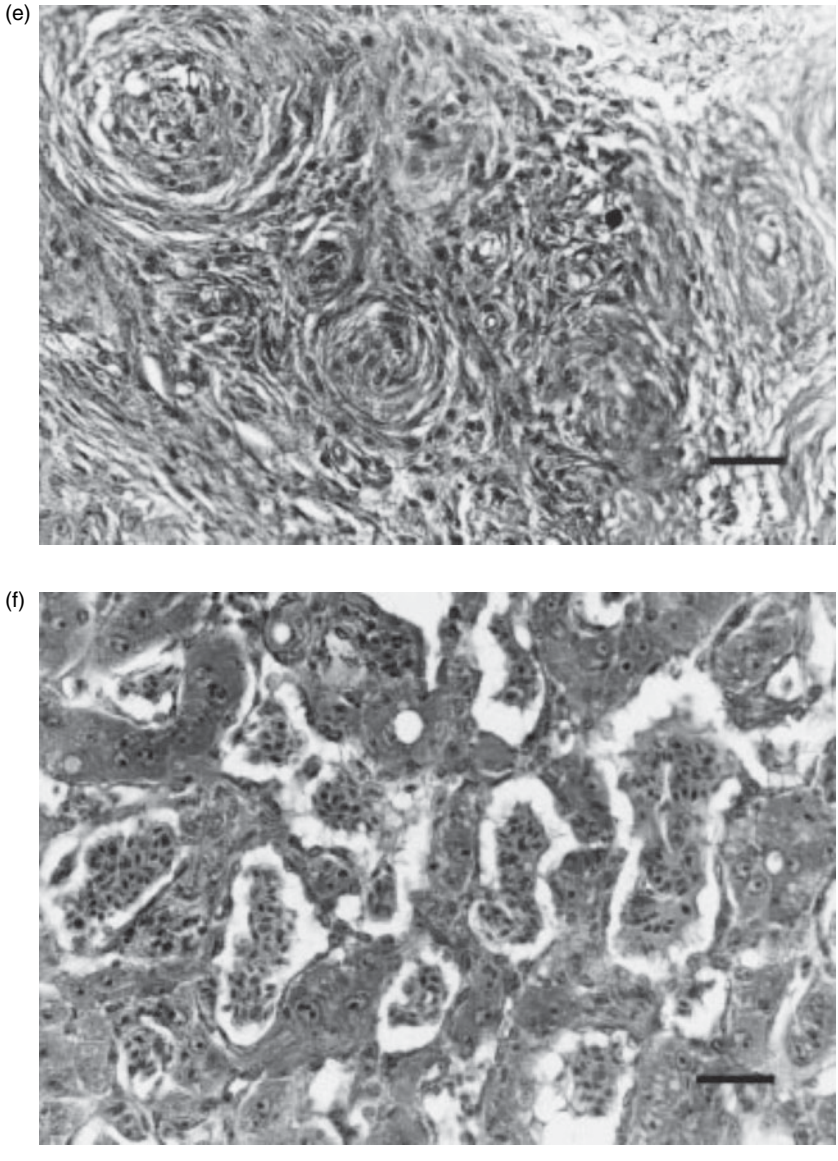


Fig. 2.10. *Continued*.

hepatis has not been experimentally induced in fish species that are not in the order Cyprinodontiformes.

Exocrine pancreas, which is located within or adjacent to the liver in some fish, is also affected by DEN metabolites. Exposure of larval or juvenile mangrove rivulus for 1 week or continuously to DEN produced pancreatic adenomas composed of duct-like arrangements of cuboidal or flattened exocrine pancreatic cells (Thiyagarajah and Grizzle, 1986). Mangrove rivulus that were first exposed while larvae, but not those first exposed as juveniles, developed cystadenomas and adenocarcinomas after continuous exposure to DEN for 20 weeks. Cystadenomas

consisted of cystic pancreatic ducts that were occasionally folded and were surrounded by moderate amounts of periductal collagen. Adenocarcinomas were characterized by extensive duct-like structures infiltrating mesenteries and adipose tissue. Rainbow trout exposed to DEN had metaplastic pancreatic acinar cells in the liver (Lee *et al.*, 1989a). These pancreatic cells apparently developed from hepatocytes, and this change was most common near cholangiocarcinomas.

Zebrafish from clonal line CB1 were immersed in 100 mg DEN/l for 8 weeks, beginning when the fish were 2.5 months old (Mizgireuv and Revskoy, 2006). In the 65 fish exposed to DEN, 35 tumours were found and all were derived from the liver, except for one pancreatic acinar cell carcinoma. In addition, there were spontaneous carcinomas of the pancreas in control fish. Mizgireuv and Revskoy (2006) suggested that the CB1 clonal line of zebrafish might have a predisposition to development of pancreatic neoplasms because of the possible loss of heterozygosity of tumour suppressor genes. Grossly visible tumours were selected for transplantation to homozygous fish, and both the hepatic and pancreatic neoplasms were successfully transplanted to syngeneic and isogeneic zebrafish but not to wild-type zebrafish.

In contrast with some clonal lines of zebrafish, wild-type zebrafish are relatively resistant to DEN carcinogenesis (Tsai, 1996). No neoplasms were found in zebrafish fed up to 2000 mg DEN/kg of feed for 3 months and then examined 6 months after the beginning of exposure. A year after a 24-hour immersion of 2-week-old zebrafish in DEN concentrations up to 2000 mg/l, only hepatocellular and biliary neoplasms were found. Extrahepatic neoplasms developed only after DEN exposure of embryos, and even then they were rare.

Dimethylnitrosamine

Hepatic neoplasms were common in rainbow trout fed DMN (Ashley, 1970). There was also an infrequent occurrence of nephroblastomas, which were composed of abortive nephrons and neoplastic epithelioid cells. Haematopoietic tissue and melanin, which are found in normal kidney of rainbow trout, were not present within the nephroblastomas. A 24-hour bath of rainbow trout embryos (21 days post-fertilization) in DMN also caused hepatocellular carcinoma (Hendricks *et al.*, 1980d).

Zebrafish immersed in DMN for 24 h when 2 weeks old had neoplasms in the liver and less commonly in the intestine when examined 1 year after exposure (Tsai, 1996). The intestinal neoplasms were leiomyosarcomas. In contrast, feeding DMN for 3 months did not cause neoplasms in wild-type zebrafish examined 6 months after the beginning of exposure.

Mizgireuv *et al.* (2004) exposed diploid and triploid zebrafish to DMN. Immersion exposure to 50 mg DMN/l for 8 weeks began when the fish were 5–6 weeks old. For fish examined 24 weeks after the beginning of exposure, hepatocellular neoplasms occurred at similar rates in the diploid and triploid zebrafish, but biliary neoplasms occurred only in diploid fish. However, after 36 weeks, hepatocellular neoplasms were less common in diploid fish than in triploids, and the prevalence of biliary neoplasms was similar for diploid and triploid fish.

N-*methyl*-N′-*nitro*-N-*nitrosoguanidine* (*MNNG*)

Because MNNG does not require activation by tissue-specific enzymes, it causes neoplasms not only in the liver of fish (Hendricks *et al.*, 1980e; Black *et al.*, 1985b; Núñez *et al.*, 1988) but also in many other locations (Bunton and Wolfe, 1996; Chen *et al.*, 1996; Spitsbergen *et al.*, 2000b). There are also variations in response of different species (Chen *et al.*, 1996) and sexes of fish (Bunton and Wolfe, 1996; Spitsbergen *et al.*, 2000b).

Branchial blastomas occur in medaka and channel catfish exposed to MNNG as a bath (Brittelli *et al.*, 1985; Chen *et al.*, 1996). These tumours are characterized by poorly differentiated anaplastic cells in nodules or cords that are highly proliferative and invade adjacent tissues. Papillomas also occur on gills of MNNG-exposed channel catfish (Chen *et al.*, 1996).

Nephroblastomas, gastric adenomas and pancreatic metaplasia develop in rainbow trout exposed as larvae or embryos to an aqueous solution of MNNG (Hendricks *et al.*, 1980e; Núñez *et al.*, 1988; Lee *et al.*, 1989b). The gastric adenomas were polyp-shaped growths of tall, mucinous epithelial cells that formed both surface epithelium and subsurface glands. These tumours were well differentiated and non-invasive. The most common renal neoplasms were unencapsulated, invasive nephroblastomas.

Rainbow trout exposed to MNNG by immersion when 4 months old developed neoplasms in the liver, kidney, stomach and swimbladder (Thorgaard *et al.*, 1999). Most common were stomach tumours, which were found in 81% of diploid fish and 11% of triploid fish. Only 7% of the diploid and 6% of the triploid fish had hepatic neoplasms.

All rainbow trout fed MNNG for 18 months developed papillary adenomas in the glandular region of the stomach (Hendricks *et al.*, 1995). Neoplasms did not develop in other organs, in contrast to the widespread effects of MNNG after immersion exposure of rainbow trout.

A pancreatic adenocarcinoma developed after injection of gulf killifish (*Fundulus grandis*) embryos with MNNG (Grizzle *et al.*, 1988b). There are relatively few reports of experimentally induced pancreatic neoplasms in fish, and most of these studies involved species in the order Cyprinodontiformes and exposure of embryos or recently hatched fish (Thiyagarajah and Grizzle, 1986; Fournie *et al.*, 1987; Grizzle *et al.*, 1988b; Fabacher *et al.*, 1991; Bunton and Wolfe, 1996). An exception to this trend is the occurrence of pancreatic neoplasms in zebrafish, either spontaneously (Mizgireuv and Revskoy, 2006) or after exposure to chemical carcinogens (Spitsbergen 2000a,b; Haramis *et al.*, 2006).

Thyroid carcinomas developed 2–4 months after mangrove rivulus were exposed to MNNG (Park *et al.*, 1993). Histological distinctions between thyroid hyperplasia and neoplasia are difficult, and iodine supplementation and transplantation experiments were used to support the diagnosis. Most of the lesions were papillary carcinomas with enlarged, highly folded follicles. Less common were invasive follicular carcinomas of variably sized and rudimentary follicles composed of anaplastic cells. There were also adenomas where folds of follicular epithelium formed papillary structures within a cystic lumen. Medaka exposed to MNNG also developed thyroid neoplasms, but only in males (Bunton and Wolfe, 1996).

Lipomas were one of several types of neoplasms that occurred in channel catfish exposed to MNNG (Chen *et al.*, 1996). Other neoplasms observed in this study were lymphosarcoma, papilloma, squamous cell carcinoma, fibroma, osteosarcoma, branchioblastoma and epithelial thymoma, and incidence of all types of tumours was low. Three fish (of 172 examined) developed lipomas, which have seldom been investigated experimentally.

Melanomas occurred in two inbred strains of medaka exposed to MNNG (Hyodo-Taguchi and Matsudiara, 1984). The strain that was less sensitive to the acute toxicity of MNNG had a higher incidence of amelanotic melanomas. These tumours were successfully transplanted to the anterior chamber of eyes of syngeneic and allogeneic fish. Hybrids of these inbred strains (F_1) exposed to MNNG developed a wider variety of neoplasms, including melanoma, papilloma, ovarian tumours, olfactory epithelioma, branchioblastoma and fibroma (Hyodo-Taguchi and Matsudiara, 1987). The cumulative incidence of melanoma was higher in F_1 hybrids compared with the parental strains. Another type of pigment cell tumour, chromatophoroma, developed in Nibe croaker exposed to MNNG (Kimura *et al.*, 1984).

Spitsbergen *et al.* (2000b) immersed zebrafish embryos (83 h post-fertilization) in MNNG (1, 5 or 10 mg/l) for 1 h. Embryos (72 h post-fertilization) were also injected with 96 ng of MNNG per embryo. Zebrafish larvae (3 weeks post-hatch) were immersed in MNNG (0.5, 1, 1.5 or 2 mg/l) for 24 h. For both age groups and exposure methods, the liver was the most common location of neoplasms, including both hepatocellular and biliary tumours. Seminomas were also common, and other locations with neoplasms

were blood vessels, gill, intestine, swimbladder, exocrine pancreas, kidney and ultimobranchial organ. In contrast, no neoplasms were found in zebrafish fed diets containing MNNG (500, 1000 or 2000 mg/kg) for 3 months beginning 2 months after hatching. The zebrafish that had been fed MNNG were periodically examined histologically, with the final sample 6 months after the beginning of exposure.

Immersion exposure to MNNG has been used to determine that zebrafish with certain mutations can have an increased susceptibility to neoplasia. Zebrafish that were heterozygous for the deficient function of the transcriptional regulator gene *bmyb* (Shepard *et al.*, 2005) or a gene involved with separation of sister chromatids during mitosis (Shepard *et al.*, 2007) were about twofold more susceptible to MNNG-induced neoplasia than were wild-type zebrafish.

Other N-*Nitroso compounds*

The direct-acting *N*-nitroso compound MNU was used in experiments with several *Xiphophorus* species, their hybrids and backcrosses (Schwab *et al.*, 1978). The most common types of neoplasms were melanomas, neuroblastomas, fibrosarcomas, rhabdomyosarcoma and papillomas, and the occurrence of neoplasms varied depending on the genotype.

Backcross hybrid *Xiphophorus* (produced by mating a male Monterrey platyfish (*Xiphophorus couchianus*) with an F_1 Monterrey platyfish × southern platyfish (strain Jp 163 A)) were exposed to 103 mg MNU/l (Kazianis *et al.*, 2001a). Exposed fish developed schwannomas (2.8%), fibrosarcomas (6.6%) and retinoblastomas (3.8%); these neoplasms were not found in control fish.

In another study, backcross hybrid *Xiphophorus* (F_1 southern platyfish × swordtails mated to a male swordtail) were exposed to 103 mg MNU/l (Kazianis *et al.*, 2001b). The southern platyfish used for hybridization were homozygous for a spot-sided pigment pattern (Sp). When the fish were 6 months old, 36.8% (25 of 68) of the MNU-exposed backcross hybrids having the Sp characteristic had melanoma, compared with 7.2% of the control fish. Melanomas did not occur in any fish without the Sp trait, presumably because the X*mrk* oncogene was not present in these fish. When the MNU-exposed fish were 1 year old, 57.4% had melanoma, but apparently control fish were not examined after 6 months of age. Low numbers of exposed fish had renal adenocarcinoma (one fish), papilloma (one fish) and fibrosarcoma (two fish).

Mangrove rivulus were exposed to 50 mg MNU/l for 2 h, and 4 months later 95% of the exposed fish had thyroid neoplasms (Lee *et al.*, 2000). These tumours resembled those induced by MNNG in this species. Other types of neoplasms were not mentioned in this report.

Ethylnitrosourea is commonly used as a mutagen in studies of zebrafish genetics (Berghman *et al.*, 2005a; Feitsma and Cuppen, 2008), but there are few studies related to its carcinogenicity. Beckwith *et al.* (2000) exposed adult (7–9 months old) male zebrafish by immersing the fish in ENU solutions for 1 h every 3 days for a total of three exposures. By 10–12 months after exposure, all 18 of the ENU-exposed zebrafish had epidermal papillomas, and two fish had additional neoplasms. None of the five controls developed tumours. Fish exposed to 293 mg ENU/l had 1 to 7 papillomas per fish, and the fish exposed to 351 mg ENU/l had 1 to 22 papillomas per fish. The other neoplasms found in these exposed fish were malignant PNST and cavernous haemangioma. It is noteworthy that during mutagenesis experiments in other laboratories, zebrafish are exposed to ENU in a manner similar to the protocol used by Beckwith *et al.* (2000), but cutaneous papillomas have not been reported.

The papillomas observed by Beckwith *et al.* (2000) did not develop during a later study of the carcinogenicity of ENU to zebrafish. Spitsbergen and Kent (2003) exposed *long fin leopard* mutant line zebrafish (3 weeks old) to 293 mg ENU/l in a 1-h bath. In addition, wild-type zebrafish were exposed by immersion in 293 mg/l ENU three times when they were 3, 5 and 7 weeks of age. One year after exposure of the mutant zebrafish and after 1 and 2 years

for the wild-type fish, no papillomas were observed. However, there were several other types of neoplasms in the ENU-exposed fish, including haemangiomas and hepatic and neural neoplasms.

Nitrosomorpholine causes hepatocellular carcinoma, cholangiocarcinoma, intestinal adenocarcinoma and multiple esophageal papillomas in guppies and zebrafish (Khudoley, 1984). The intestinal adenocarcinomas were invasive and were composed of desmoplastic growths of pleomorphic, mucin-laden epithelium that invaded the intestinal wall. The esophageal papillomas were composed of basophilic epithelial cells with large nuclei.

Simon and Lapis (1984) tested DEN, *N-N′*-dinitrosopiperazine and several chemicals of unknown carcinogenicity. Both *N-N′*-dinitrosopiperazine and DEN produced hepatocellular carcinomas, esophageal papillomas and intestinal polyps in guppies, but incidence was higher and latency was shorter in fish exposed to DEN.

Liver carcinomas developed after exposure of rainbow trout embryos to 2,6-dimethylnitrosomorpholine, nitrosopyrrolidine and nitrosomorpholine but not after exposure to either dibutylnitrosamine or ENU (Hendricks *et al.*, 1984a). A dietary exposure to 2,6-dimethylnitrosomorpholine also caused hepatocellular neoplasms, papillary adenomas of the glandular stomach and a low incidence of swimbladder papillomas in rainbow trout (Hendricks *et al.*, 1995).

Methylazoxymethanol

Methylazoxymethanol (MAM) is a potent carcinogen present in the nuts of cycad trees as methylazoxymethanol β-D-glucoside and is commonly used in a synthetic form, methylazoxymethanol acetate (MAM-Ac), to experimentally produce neoplasms in fish (Hawkins *et al.*, 1988a) and mammals (Sohn *et al.*, 1991). Methylazoxymethanol is not important as an environmental pollutant, but 1,1-dimethylhydrazine, a metabolic precursor of MAM, is manufactured as a rocket fuel (NTP, 2005). Methylazoxymethanol is metabolized to carbonium ions that alkylate DNA in the same manner as nitrosamines. Enzymes necessary to metabolize MAM compounds to ultimate carcinogens are species, tissue and age specific, leading to considerable variability in tumour incidence and type between fish of different species and ages.

In an experiment in which seven species of fish were exposed to MAM-Ac when they were 6–10 days old, frequency of hepatic neoplasms ranged from 7 to 67% (Hawkins *et al.*, 1988a). The highest incidence of neoplasms occurred in guppies, with a latent period of about 1 month. In contrast, the lowest incidence occurred in fathead minnows (*Pimephales promelas*), which had a latent period of 6 months. Medaka, guppy and sheepshead minnow had the greatest diversity of tumour types; neoplasms were found in six tissues of medaka exposed to MAM-Ac. In addition, a single medaka was found with an exocrine pancreatic carcinoma, but the low incidence of this lesion prevents a conclusive link to MAM-Ac exposure (Hawkins *et al.*, 1991).

In a similar study, western mosquitofish (*Gambusia affinis*) were exposed to 10 mg MAM-Ac/l for 2 h and developed hepatocellular and cholangiocellular neoplasms within 25 weeks (Law *et al.*, 1994). By 40 weeks, 52% of these fish had hepatic neoplasms, but lesions were found only in the liver.

For zebrafish exposed to MAM-Ac by diet or by short-term immersion of larvae or embryos, the liver was the most common site of neoplasia, but there was a wide spectrum of extrahepatic neoplasms (Tsai, 1996). The greatest variety of neoplasms developed after exposure of embryos, but each type of extrahepatic neoplasm was found at low frequency. Tsai (1996) also fed MAM-Ac to medaka and found that the percentage of fish with neoplasia was similar for medaka and zebrafish. However, neoplasms in medaka fed MAM-Ac were found only in the liver.

The types of neoplasms that develop in medaka after MAM-Ac exposure depend on the age of fish exposed. One-year-old fish primarily develop hepatic neoplasms, including

hepatocellular carcinomas (trabecular and spindle shaped), cholangiomas and cholangiocarcinomas (Harada *et al.*, 1988). Medaka exposed to MAM-Ac when only 6–10 days old develop not only hepatic neoplasms but also rhabdomyosarcoma, fibrosarcoma, nephroblastoma, undifferentiated mesenchymal sarcoma, and medulloepithelioma (Hawkins *et al.*, 1988a). Additional neoplasms found in medaka exposed when 1 month old were leiomyosarcoma and haemagiopericytoma (Fabacher *et al.*, 1991).

Retinal medulloepitheliomas arise from the primitive medullary epithelium and form three cellular patterns in medaka exposed to MAM-Ac (Hawkins *et al.*, 1986). Cells differentiating along the photoreceptor cell pathway form neoplasms that contain photoreceptor cells that are frequently in ductular or rosette patterns. Those with rosette patterns are especially interesting because of their resemblance to human retinoblastomas. Medulloepithelioma cells differentiating towards cells other than photoreceptors form pigmented neoplasms of cuboidal or columnar cells in a glandular pattern. A third type of eye tumour found in medaka exposed to MAM-Ac is an invasive teratoid neoplasm that differentiates into striated muscle, mesenchymal tissues and hyalin cartilage.

Guppies exposed to low doses (10 mg/l or less) of MAM-Ac for 2 h develop adenomas or carcinomas of the exocrine pancreas (Fournie *et al.*, 1987; Fournie and Hawkins, 2002). Interestingly, guppies exposed to higher concentrations of MAM-Ac did not develop pancreatic neoplasms, and the highest prevalence of pancreatic neoplasms (28%) was for the guppies exposed to 4 mg/l. This inverse dose response could be related to higher mortality of guppies treated with 50–100 mg MAM-Ac/l, but an inverse relationship between dose of carcinogen and incidence of pancreatic carcinomas was also found by Thiyagarajah and Grizzle (1986). The exocrine pancreatic neoplasms in guppies fall into three categories: (i) adenomas consisting of large masses of well-differentiated pancreatic cells in a pattern similar to that of normal pancreas and containing zymogen granules; (ii) acinar cell carcinomas that are invasive and vary from well-differentiated to poorly differentiated; and (iii) adenocarcinomas of ductal elements containing eosinophilic material. The similarity in appearance and location between the poorly differentiated acinar cells described by Fournie *et al.* (1987) and the hepatocytes in some forms of hepatocellular carcinoma is probably an impediment to the diagnosis of exocrine pancreatic neoplasms.

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are widely distributed in the environment and probably cause neoplasms in wild fish (Baumann, 1998; Myers *et al.*, 2003; Vogelbein and Unger, 2006). The PAH carcinogens consist of two to six fused benzene rings with or without alkyl substitutions, and typically occur as mixtures of different compounds. Examples of PAH that have been used in experiments with fish include DMBA, benzo[*a*]pyrene and DBP.

Sources of PAH are diverse and include crude oil and products produced during burning of fossil fuels or organic matter (Douben, 2003). Most PAH are delivered to aquatic environments by atmospheric deposition or through runoff, but there are examples of locally high levels of PAH related to industrial sources such as creosote plants.

Although PAH are degraded by some fungi and bacteria under aerobic conditions (Cerniglia and Heitkamp, 1989), PAH tend to accumulate in sediments and in some aquatic animals (Chen and White, 2004). Fish and shrimp can efficiently metabolize and excrete PAH; therefore, less accumulation of PAH occurs than in bivalves and gastropods, which metabolize PAH slowly and so are subject to PAH accumulation (Neff *et al.*, 1976; Roesijadi *et al.*, 1978; Varanasi *et al.*, 1985). In a Puget Sound study, English sole (*Parophrys vetulus*) were found to have liver concentrations of benzo[*a*]pyrene that were below detection limits (<25 ng/g dry weight), while their stomach contents (annelids, mollusks, crustaceans and echinoderms) had 570 ng/g dry weight, and

sediments in the collection area had from 170 to 550 ng benzo[*a*]pyrene/g dry sediment. None of the 25 hydrocarbons quantified were present in fish liver in higher concentrations than levels in stomach contents or sediment (Malins *et al.*, 1985).

Metabolism of PAH by fish and other animals has been extensively studied (Douben, 2003; Luch, 2005). Fish metabolize PAH to form unstable intermediates that can form DNA adducts and lead to mutations. Common carp have a much lower neoplasm frequency than brown bullheads in environments with high PAH levels (Brown *et al.*, 1973), but contrary to expectations, common carp make more PAH-related DNA adducts than do brown bullheads (Steward *et al.*, 1989; Sikka *et al.*, 1990). Channel catfish also do not appear prone to develop neoplasms when exposed to chemical carcinogens requiring metabolic activation. Comparison of benzo[*a*]pyrene metabolism by channel catfish and brown bullheads revealed that preferential formation of DNA-reactive metabolites (Willett *et al.*, 2000) and a higher level of DNA adducts (Ploch *et al.*, 1998) in brown bullheads could explain the difference in susceptibility to chemical carcinogens.

Field studies

Several epizootics of neoplasia in fish appear to be related to PAH contamination. However, most of these cases involve complex mixtures of chemicals, and the contribution of a single carcinogen to the overall incidence of neoplasia is difficult to discern. The following studies implicate PAH as a cause of neoplasia in certain populations of wild fish.

The Elizabeth River runs through a heavily industrialized area of Virginia and is highly contaminated with PAH (Bieri *et al.*, 1986). Mummichogs (*Fundulus heteroclitus*) from a portion of the river that had up to 3900 mg PAH/kg of sediment (adjacent to an abandoned creosote plant and an active oil transfer and storage site) had papilloma, schwannoma and haemangioendothelioma (Hargis *et al.*, 1989). The overall prevalence of neoplasms was 2%. Mummichogs in later collections in the Elizabeth River at a site with 2200 mg PAH/kg of sediment had a 73.3% prevalence of hepatic foci of alteration and a 35% prevalence of hepatocellular neoplasms (Vogelbein *et al.*, 1990). Only 600 m away and across the river, PAH concentration was 61 mg/kg sediment, and mummichogs from this area had no hepatic lesions. Mummichogs from the contaminated site in the Elizabeth River also had neoplasms of the exocrine pancreas (Fournie and Vogelbein, 1994). Other locations contaminated with creosote also have mummichog populations with neoplasia (Pinkney and Harshbarger, 2006).

The Niagara River area near Buffalo, New York, has several sites that contain high concentrations of PAH (Black, 1983). Neoplasms of fish from this area included dermal neoplasms in freshwater drum (*Aplodinotus grunniens*) and oral papillomas in white suckers. Freshwater drum had dermal neoplasms with frequencies as high as 16.7% in Lake Erie near Wanakah, New York, and 13.3% at the confluence of Frenchmans Creek and the Niagara River. The neoplasms of freshwater drum were more common in larger fish. White suckers over 30 cm long had oral papillomas with an overall frequency of 8.5%. Although a high prevalence of neoplasms was observed at some locations with relatively low concentrations of PAH in sediment, freshwater drum and white suckers can move freely from areas of high sediment concentration of PAH to nearby areas with low concentration. Various types of neoplasms were found in five additional species of fish, including a 17% prevalence of grossly visible skin or liver neoplasms in large adult brown bullheads in the Buffalo River, New York (Black, 1983; Black *et al.*, 1985a).

The Black River in northern Ohio was contaminated with high concentrations of PAH, but contaminant levels decreased after the 1983 closure of the principal source of PAH and were further reduced by dredging of the most contaminated sediments in 1990 (Baumann and Harshbarger, 1998). Concentrations of total PAH in sediment decreased from 1096 mg/kg in 1980 to

9.8 mg/kg in 1994. While the PAH concentrations were high, ten types of PAH were identified in brown bullheads from the Black River, and concentrations of these PAH were much higher than in reference fish (Baumann *et al.*, 1987). Brown bullhead from this area contained 3.1 mg/kg of phenanthrene plus lower levels of other PAH. There was also 1.3 mg PCB/kg wet weight in Black River fish, compared with 0.050 mg/kg in reference fish.

During the 1980s, brown bullheads collected from the Black River had a high prevalence of liver, skin and lip neoplasms (Baumann *et al.*, 1987, 1990). Most liver neoplasms in brown bullheads were cholangiocarcinomas; approximately 60% of the skin and lip neoplasms were papillomas; and the remaining skin and lip tumours were squamous cell carcinomas. No evidence of viruses in the lesions was found with electron microscopy. Prevalence of neoplasms in these fish was age dependent. Skin and lip neoplasms occurred in less than 1% of 2 year olds, but frequencies in age-4 fish were as high as 32% for lip neoplasms and 18% for skin neoplasms. Prevalence of liver tumours was less than 2% in 2 year olds, exceeded 11% in 3 year olds, and was 28–44% in age-4 fish. Prevalence was even higher in 4 year olds sampled in September (54%) and in 5-year-old fish (60%), but few fish survived to this age. Brown bullheads collected from two reference sites had no liver neoplasms, but there was a 1.5% frequency of lip tumours in 3 year olds at one site.

The cause-and-effect relationship between PAH exposure and hepatic neoplasms in the Black River was strengthened by the decline in prevalence of hepatic neoplasms in brown bullheads after PAH levels decreased following closure of a coking facility in 1983 and removal of the most contaminated sediments in 1990 (Baumann and Harshbarger, 1998). In 1994, hepatic neoplasms were not found in age-3 brown bullheads, which were hatched after the removal of contaminated sediment.

Balch *et al.* (1995) investigated brown bullheads in Hamilton Harbour, Ontario, another PAH-contaminated location in the Great Lakes region. Brown bullheads in this harbour had hepatic and cutaneous neoplasms. Hepatic enzyme induction, aromatic metabolites in bile and DNA adducts provided evidence of a link between PAH and neoplasia in brown bullheads.

Pinkney *et al.* (2001, 2004a,b) examined brown bullheads from rivers draining into the Chesapeake Bay and found an increased prevalence of skin and liver neoplasms in fish from polluted areas. In the Anacostia River, Washington, DC, 50–68% of the brown bullheads that were at least 3 years old had hepatic neoplasms and 13–23% had cutaneous neoplasms (Pinkney *et al.*, 2004b). The sediment of this river had high levels of PAH, and regardless of age, the brown bullheads had high concentrations of biliary PAH metabolites and DNA adducts. Other pollutants, including PCB, were also present and could have contributed to the carcinogenicity. Less pronounced increases in tumour prevalence were found in brown bullheads from other rivers in this area.

Puget Sound is perhaps the best-characterized site of a PAH-associated epizootic of fish neoplasms (Myers *et al.*, 1990, 1991, 2003; Stein *et al.*, 1990). Although a few areas in Puget Sound have sediments with high concentrations of anthropogenic chemicals, most of Puget Sound is less polluted, allowing comparisons of fish collected from locations with different levels of sediment contamination. Over 900 different organic compounds were identified in sediments of Commencement Bay (Malins *et al.*, 1984a). Aromatic hydrocarbons were also found in invertebrate animals recovered from the stomach of English sole from Puget Sound (Malins *et al.*, 1985), indicating that organic chemicals present in sediment are available through the diet. There were positive correlations between prevalence of hepatic neoplasms in English sole from several locations and sediment concentrations of PAH and metals (Malins *et al.*, 1984b). However, there was a higher correlation between concentration of bile metabolites of aromatic compounds and prevalence of hepatic neoplasms (Krahn *et al.*, 1986).

Prevalences of hepatic neoplasms in English sole from polluted areas of Puget

Sound varied from 2.6 to 32% depending on collection site during the 1970s and 1980s (Pierce *et al.*, 1978; Malins *et al.*, 1984b, 1985; Becker *et al.*, 1987; Myers *et al.*, 1987), while no fish with neoplasms were found in several minimally polluted locations. Site of capture and fish age were the most important risk factors for neoplasms as well as for other hepatic lesions (Rhodes *et al.*, 1987). Myers *et al.* (1987, 1998) found that certain types of non-neoplastic lesions in the liver of English sole had high frequencies of co-occurrence with hepatic neoplasms and were useful indicators of exposure to carcinogens.

The starry flounder inhabits the same areas of Puget Sound as the English sole and has similar feeding habits but a much lower prevalence of neoplasia (Pierce *et al.*, 1980). This difference is caused by quicker conversion of PAH to proximate carcinogens and slower detoxification of reactive intermediates by English sole (Collier *et al.*, 1992).

Hepatic neoplasms were found in 8% of the winter flounder examined from Boston Harbor in 1984 (Murchelano and Wolke, 1985). The tumours were cholangiocarinomas and hepatocellular carcinomas. At that time, Boston Harbor received untreated sewage containing a complex mixture of pollutants, including PAH, PCB, hexachlorobenzene, DDT, chlordane and several metals. During the 1990s, the concentrations of pollutants entering Boston Harbor decreased because of source reduction of toxicants, better wastewater treatment and relocation of discharges, and no neoplasms have been found in winter flounder since 1998 (Moore *et al.*, 2005). Although the complexity of the pollutant mixture in Boston Harbor prevents a simple link between any single contaminant and neoplasia, the reduction in tumour prevalence in this case strengthens the link between the presence of chemical carcinogens, e.g. PAH, and the occurrence of neoplasia in wild fish.

Hepatic neoplasms were also found in winter flounder collected from six additional sites in the north-eastern USA between Long Island Sound and Boston Harbor (Gardner *et al.*, 1989). The highest prevalence of neoplasia was 26% in New Bedford Harbor, and for all of the sites there was a trend of increasing prevalence of neoplasia with higher concentration of PAH in the sediment. These sites were also contaminated with PCB and metals.

Stentiford *et al.* (2003) examined European flounder, sand goby (*Pomatoschistus minutus*) and viviparous blenny (*Zoarces viviparus*) from three British estuaries contaminated with PAH: Tyne, Tees and Mersey. Fish from Alde estuary were used for reference. For a collection of 30 European flounder from the Mersey, the prevalence of hepatocellular adenoma was 10%. These were the only neoplasms found, except for one cholangioma in a viviparous blenny from the Tyne estuary. Sediment concentrations of PAH in the Mersey estuary were up to 6 mg/kg, which was lower than for the other polluted sites sampled in this study (Woodhead *et al.*, 1999).

With some of the characteristics of both a field and laboratory study, hepatocellular adenoma developed in European flounder that were directly or indirectly exposed in mesocosms to sediment removed from Rotterdam Harbour (Vethaak *et al.*, 1996). The first neoplasm occurred after 2.5 years of exposure. The sediment contained PAH, but the complex mixture of chemicals in the tested sediment prevents a firm conclusion that PAH caused the tumours.

Laboratory studies

Several types of PAH, both single compounds and mixtures from contaminated sediment, have been used to induce neoplasia in controlled experiments with fish. Immersion, feeding, injection and application to skin have been used successfully to induce neoplasia in several species. Results of laboratory experiments with PAH, along with evidence from field studies, provide convincing evidence that PAH are a likely cause of neoplasia in some wild fish populations living in environments contaminated with PAH.

Guppies and medaka were exposed to benzo[*a*]pyrene by immersion (Hawkins *et al.*, 1988b). Both species developed invasive, polymorphic, trabecular hepatocellular

carcinomas with high mitotic rates, but there was a higher frequency of neoplasms in medaka than in guppies at all sampling intervals. Hepatic neoplasms were the most common tumours in guppies exposed for 6 h once weekly for 4 weeks to DMBA, but low numbers of rhabdomyosarcoma, renal adenocarcinoma, neurilemmoma, and undifferentiated sarcoma also occurred (Hawkins *et al.*, 1989).

Immersion exposure to DMBA has been successfully used to induce neoplasia in several fish species. Multiple exposures of *Poeciliopsis* spp. to a 5 mg/l aqueous suspension of DMBA at weekly intervals produced hepatic neoplasms with a frequency of nearly 50% at 7–8 months (Schultz and Schultz, 1982). These hepatocellular carcinomas ranged from well-differentiated trabecular forms to anaplastic types with deeply basophilic, pleomorphic, spindle-shaped cells. This experiment also produced several highly invasive lymphosarcomas.

Nine months after triploid and diploid rainbow trout (4 months old) were given a single 20-h bath in 5 mg DMBA/l, neoplasms were found in the liver, kidney, stomach and swimbladder (Thorgaard *et al.*, 1999). The stomach was the most commonly affected organ; frequency of stomach tumours was 98% in diploid fish and 16% in triploid fish. The kidney was the least common location of neoplasms, with 2% of diploid and none of the triploids affected. In another experiment with rainbow trout, neoplasms were found in the liver, stomach and swimbladder after a 20-h immersion in 1 mg DMBA/l (El-Zahr *et al.*, 2002).

Immersion of fish embryos in DMBA has also been used to induce neoplasia. Nine months after rainbow trout embryos were bathed in DMBA, there was a high frequency of hepatic neoplasms; gastric adenomas and nephroblastomas were also present (Fong *et al.*, 1993).

Mutant zebrafish that were heterozygous for truncating of a tumour suppressor gene (adenomatous polyposis coli) were predisposed to spontaneous neoplasms in the liver and intestine (Haramis *et al.*, 2006). The occurrence of these neoplasms increased and acinar cell neoplasms also developed after a single, overnight immersion exposure of 3-week-old fish in 5 mg DMBA/l.

Spitsbergen *et al.* (2000a) exposed three different ages of wild-type zebrafish to DMBA. Embryos (60-h post-fertilization) were exposed by immersion for 24 h in concentrations of DMBA from 0.25 to 1.0 mg/l. When examined 1 year after exposure, 5% of these fish had neoplasms, and most of these neoplasms were hepatocellular or biliary. The only other neoplasm found in the zebrafish exposed to DMBA while embryos was PNST. Larvae (21 days after hatching) were immersed for 24 h in 1.25–5.0 mg DMBA/l. Nine months after exposure, the prevalence of neoplasia was 45–66% for zebrafish exposed to DMBA, and the most commonly affected organ was the liver. There were 14 types of neoplasms in the zebrafish exposed as larvae, including tumours in gills, blood vessels, intestine, pancreas, thyroid and nervous system. The third age exposed was juveniles (2 months old at the beginning of exposure), which were fed a diet containing 100–1000 mg DMBA/kg of feed for 4 months. When these fish were examined 7 months after the beginning of exposure, neoplasms were found only in fish fed 500 or 1000 mg/kg, and the prevalence of neoplasia in fish fed the highest concentration was 18%. The intestine was the most common organ affected, and the neoplasms in the intestine were histologically diverse.

The oral route of exposure to PAH has also been evaluated in rainbow trout. Feeding DMBA to Shasta strain rainbow trout for 8 weeks resulted in neoplasms in 4% of livers, 92% of stomachs and 46% of swimbladders 7 months after the end of DMBA exposure (Weimer *et al.*, 2000). Feeding β-naphthoflavone (500 mg/kg of feed) for 10 weeks, starting 1 week before the DMBA exposure, significantly reduced the percentages of fish with stomach and swimbladder tumours. Hendricks *et al.* (1985) had previously shown that a dietary exposure of rainbow trout to benzo[*a*]pyrene caused hepatocellular carcinoma.

Rainbow trout have also been fed DBP; this PAH caused neoplasms in the liver, stomach and swimbladder (Reddy *et al.*,

1999). Feeding chlorophyllin along with the DBP reduced the number of tumours. In a similar study, Pratt *et al.* (2007) found that the frequency of hepatic neoplasms reached a plateau of about 60% when rainbow trout were fed DBP and that the effect of a given chlorophyllin dose depended on the dose of DBP. For DBP doses ≤80 mg/kg of feed, there was a dose-dependent increase in tumours with increasing amount of DBP, and the addition of chlorophyllin to the diet resulted in a dose-dependent reduction in tumour formation. The hepatic neoplasms caused by DBP were usually hepatocellular carcinomas or adenomas, and all tumours of the stomach and swimbladder were papillary adenomas.

Injection has also been used for experimental exposures of fish to PAH. Intraperitoneal injection of rainbow trout (10 months old at first dose) with benzo[*a*]pyrene monthly for 12 months resulted in 50% of the fish developing hepatocellular carcinomas (Hendricks *et al.*, 1985). In addition, one fish developed hepatic fibrosarcoma and one fish developed a papillary adenoma of the swimbladder. Rainbow trout also develop hepatic neoplasms after a single injection of benzo[a]pyrene into the yolk of embryos (Black *et al.*, 1985b).

Sediment extracts from locations polluted with PAH have been used in laboratory exposures of fish. Brown bullheads developed papillomas after topical application of an extract of sediment from the Buffalo River, New York (Black *et al.*, 1985a). The sediment extract was applied to the skin weekly, and papillomas were first evident between 14 and 18 months. Papillomas and carcinomas also developed on the skin of mice after topical application of this extract.

Neoplasms developed in medaka exposed by immersion in sediment extracts from two of four locations contaminated with PAH (Fabacher *et al.*, 1991). Neoplasms in fish exposed to extracts from the Black River, Ohio, or the Fox River, Wisconsin, were hepatocellular adenoma, hepatocellular carcinoma, cholangioma and pancreatic ductular adenoma. In another study, medaka embryos were exposed for 10 days to sediment spiked with benzo[*a*]pyrene or extracts from PAH- and PCB-contaminated sediment from the Seine estuary (Cachot *et al.*, 2007). Eight months after exposure, hepatocellular carcinoma was found in 1 of 25 fish exposed to benzo[*a*]pyrene, and a dysgerminoma was found in 1 of 24 fish exposed to Seine estuary sediment extract.

Extract of sediment from another Great Lakes location, Hamilton Harbour, Ontario, contained high levels of PAH and PCB, and 12 months after rainbow trout yolk-sac larvae were given a single injection of this sediment extract they had hepatic neoplasms (Metcalfe *et al.*, 1990).

Idiopathic Neoplasms

There are numerous reports of idiopathic neoplasms in fish, but in most cases few fish in a population are affected. However, there are relatively rare reports of idiopathic neoplasms occurring at easily detectable levels in a fish population for an extended time. Some examples of idiopathic neoplasms that are common in certain species or populations are presented.

Peripheral nerve sheath tumours of goldfish

Peripheral nerve sheath tumours (including neurofibromas, neurofibrosarcomas, neurilemmomas and schwannomas) of goldfish (Schlumberger, 1952; Grizzle *et al.*, 1995) have occurred in high prevalence in some populations. Histologically these tumours resemble bicolour damselfish PNST, which are probably caused by an unclassified virus (Schmale *et al.*, 2002; Rahn *et al.*, 2004). However, transmissible agents have not been detected in PNST of species other than bicolour damselfish. Peripheral nerve sheath tumours also appear to be common in some populations of snappers, *Lutjanus* spp. (Lucké, 1942; Overstreet, 1988). Marino *et al.* (2007) used calretinin immunostaining to aid diagnosis of goldfish schwannomas.

Lipomas

Lipomas occur sporadically and at low prevalence, and there is no indication of the cause of these benign neoplasms in wild or aquaculture fish. Lipomas have been reported in diverse species including dab (Bruno *et al.*, 1991), European eel (Easa *et al.*, 1989a), striped mullet, *Mugil cephalus* (Easa *et al.*, 1989b), bluefin tuna, *Thunnus thynnus* (Marino *et al.*, 2006) and southern bluefin tuna, *Thunnus maccoyii* (Johnston *et al.*, 2008). Channel catfish with lipomas were found in two commercial fish ponds and from a research pond; none of these sites had any known carcinogenic contaminants (McCoy *et al.*, 1985). In surveys of fish from polluted areas, no increase in prevalence of lipomas was found, but these neoplasms were more common in mature females and in certain geographic locations (Bruno *et al.*, 1991).

Nephroblastomas in Japanese eel

Nephroblastomas were observed in 50 Japanese eels (*Anguilla japonica*) in indoor tanks with water temperature controlled at about 26 °C (Masahito *et al.*, 1992). Potential causes for these nephroblastomas included chemical carcinogens or promoters in the water, perhaps related to the high levels of nitrous acid resulting from the dense population of eels in culture tanks. Elevated water temperature and genetic influences were also potential factors. The potential role of the Japanese eel counterpart of the Wilm's tumour 1 gene in genesis of these nephroblastomas was considered by Nakatsuru *et al.* (2000).

Pigmented neoplasms of the skin

Chromatophoromas were observed over an 11-year period in two species of butterflyfish (*Chaetodon multicinctus* and *Chaetodon miliaris*) on Hawaiian reefs (Okihiro, 1988). In 1987, 50% of the *C. multicinctus* sampled had chromatophoromas, about double the percentage in 1976. The chromatophoromas were variable and included melanophoromas, iridophoromas and mixed chromatophoromas, and there was a tendency for the tumours to become invasive. Fish with neoplasia were restricted to certain locations of the reefs off the islands of Maui, Lanai and Molokini, and the prevalence decreased as water depth and distance from shore increased. Agricultural lands were near these reefs, but the presence of chemical carcinogens was not documented.

Chromatophoromas were also found in five species of Pacific rockfish (*Sebastes* spp.) collected from the Cordell Bank off the California coast over a 6-year period (Okihiro *et al.*, 1993). Neoplasms included melanophoromas, xanthophoromas, erythrophoromas and mixed chromatophoromas. Lesions consisting of hyperplastic chromatophores were also present and were not reliably distinguishable by gross appearance from neoplastic lesions. Prevalence of affected fish (including melanosis) was over 30% in some samples. Although the cause of these lesions was not determined, a waste dump was located 30 km from the collecting site.

Dermal neoplasms composed of fusiform cells and containing melanin were found in adult gizzard shad (*Dorosoma cepedianum*) from four reservoirs located in southern and north-western Oklahoma (Ostrander *et al.*, 1995, 1999; Jacobs and Ostrander, 1995; Geter *et al.*, 1998). The cell of origin of these tumours is unknown but could be melanocytes or peripheral nerve sheath cells. Mean prevalence in adults (2–5 years old) was 13–22% in several samples collected over several years; grossly visible neoplasms were not found in juveniles. Prevalence of these neoplasms did not appear to be seasonal. There was no evidence from electron microscopy and reverse transcriptase assays that a virus was involved, and environmental radiation and metals were not elevated. Although genetic markers did not distinguish between individual fish with and without tumours, this neoplasm was not found on gizzard shad from a reservoir in a different area in Oklahoma (Geter *et al.*, 1998; Ostrander *et al.*, 1999). However, similar neoplasms occur in a population of

gizzard shad in Alabama (J.M. Grizzle, unpublished observations).

Endothelial cardiac neoplasms in mangrove rivulus

Mangrove rivulus fed freeze-dried chicken liver developed endothelial neoplasms in the ventricle and bulbus arteriosus (Couch, 1995). Prevalence of these cardiac neoplasms was 25% in 204 fish, and 9 of the affected fish had possible metastatic neoplasms in the gills. An avian virus in the chicken liver, activation of an endogenous virus or unintended exposure to a chemical carcinogen are possible causes of these neoplasms, but additional study is required to determine the cause of the neoplasms.

Conclusions

Progress has been made concerning the causes of neoplasms in fish, but many questions remain. The following conclusions and suggestions for additional study are based on literature reviewed in this chapter.

1. Both oncogenic viruses and chemical carcinogens appear to be common causes of fish neoplasia. However, interactions between viruses of fish and environmental factors, especially chemical pollutants, have not been adequately considered.

2. Differentiation between neoplasia and various non-neoplastic lesions continues to be a problem. Historically, neoplasia in fish has been defined almost exclusively by histological appearance. Molecular markers provide an additional approach for recognition of neoplasms, but more research is needed to better define the molecular characteristics of various neoplasms.

3. Regression of fish neoplasms, including some considered malignant, needs additional study. Frequent or rapid regression suggests that there were changes in the immune system or that the growth advantage of neoplastic cells was altered. Another possibility is that some of these lesions are not neoplasms, or at least are not malignant.

4. Temperature has important effects on development and regression of fish neoplasms. Although temperature is a major factor in all aspects of poikilothermic physiology, specific mechanisms involved in temperature-related changes in the behaviour of fish neoplasms have not been adequately considered.

5. The importance and usefulness of transplantation of neoplastic tissue from one fish to another need to be clarified. Successful transplantation of tumours between inbred or syngeneic fish has occasionally been used as evidence for the neoplastic nature of the lesion. Basic information is needed about transplant rejection in fish, and factors that affect growth of normal tissue when transplanted to syngeneic fish need to be determined.

6. Fish neoplasms metastasize less often and less aggressively than do similar tumours in mammals. Although several hypotheses for this difference have been proposed, additional research is required to test these possibilities.

7. Most neoplasms of wild fish do not appear to affect the size of fish populations; however, shifts in age distribution have been reported. Additional consideration should be given to potential long-term effects if high frequencies of neoplasms occur for several generations.

8. A common theme in many studies of neoplasms occurring in wild fish is the usefulness of certain types of tumours as sentinels for the presence of chemical carcinogens that could have human health implications. These neoplasms can also be useful as indicators of environmental degradation that has serious direct effects on aquatic ecosystems, including fish populations.

9. Fish may offer some advantages over other animals in screening for carcinogenicity; however, the sensitivity of fish in some protocols was lower than for rodents (Kissling *et al.*, 2006). Evaluations of chemicals for carcinogenicity should consider the most

appropriate fish species and routes for administering the test chemical.

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3 Endocrine and Reproductive Systems, Including Their Interaction with the Immune System

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Introduction

This chapter examines a range of disorders that have been reported in the endocrine and reproductive systems of fishes. To understand the development of these dysfunctional states, or how the endocrine system is involved, a basic understanding of the structure and function of the endocrine system of fish is essential. The following section briefly outlines the organization of the endocrine system, the manner in which hormones are secreted, the process by which hormones are transported from the glandular cells that secrete them to the target cells and the events that trigger a response of the target cells. Similarly, an understanding of the dysfunctional states of the reproductive system requires some knowledge of the normal anatomy and physiology of that system, and that is provided in a later section of this chapter.

The immune system in vertebrates is a collection of biological barriers and processes that protects the animal from a broad spectrum of pathogens, from viruses to parasitic worms; the processes also act to destroy tumour cells. Although this book deals primarily with non-infectious disorders, there are many physiological interactions between the endocrine and immune systems (e.g. Harris and Bird, 2000); the immune system is affected by environmental factors (Bowden, 2008), and some environmental contaminants appear to have direct effects on components of the immune system in fish (explored at more length in Chapter 9, this volume). Thus, this chapter will briefly consider non-infectious factors affecting the immune system, particularly those related to the interaction of endocrine and immune system roles.

Introduction to the Endocrine System

Hormones and how they work

Hormones and other chemical signalling molecules

All multicellular animals use chemical signalling for most cell-to-cell communication. The hormones of the endocrine 'system' represent just one category of these signalling chemicals. The concept of an endocrine 'system', per se, has been largely replaced by the recognition of a complex chemical communication network that regulates many facets of cell function, and thus, by extension, regulates tissue and organ system activity. The main categories of chemical cellular regulating factors include cytokines of the

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immune system, cellular and tissue growth factors, cell-to-cell adhesion molecules produced in many types of cells, angiogenesis-regulating growth factors of the vascular system, neurotransmitter substances of the nervous system and hormones. All of these factors play key roles in regulating cellular activity and allow the animal to respond to changes in its environment by regulating the adjustment of cellular activity.

The lines that formally separated the different types of regulatory chemicals into discreet categories are now blurred. Indeed, some factors (e.g. epinephrine) that are classed as hormones under some circumstances are also in another category of signalling chemical (in the case of epinephrine, a neurotransmitter).

For the most part, hormones are involved in the regulation of processes that occur over long periods of time. These include the constant regulation of metabolic rate, growth and reproductive activity; however, some hormones, such as epinephrine, elicit rapid physiological response. Hormones differ from other chemical signalling factors in that they are synthesized and released from glandular tissues that are independent of the target tissue that responds to them; hormones are generally transported by the circulatory system from the cells that secrete them to their target tissues, although some hormones, such as the steroid hormones secreted by steroidogenic cells within the gonads, in addition to being released into the blood, also play regulatory roles within the testis and ovary.

Hormones are synthesized by secretory cells and are released into the extracellular fluid, from where they may find their way by diffusion, or by transport across membranes by transport proteins, into the circulatory system. They are then carried in the blood, and after moving out of the circulatory system into the extracellular fluid, exert effects on peripheral 'target cells' (Fig. 3.1). Some hormones may have local rather than peripheral actions; these may act on the same cell that secreted them ('autocrine') or on adjacent cells ('paracrine') (Fig. 3.1). All hormones (and most of the other classes of chemical signalling factors) exert their effects on target cells by activating receptor proteins that are synthesized within the target cells. The specificity and the intensity of the response of a target cell to a particular hormone is determined by several factors: (i) whether or not the cell produces the receptor that is specific for a particular hormone; (ii) by the number of the receptor protein units synthesized locally by the target cell; and (iii) by the concentration of hormone present in the extracellular fluid that is in contact with the target cell.

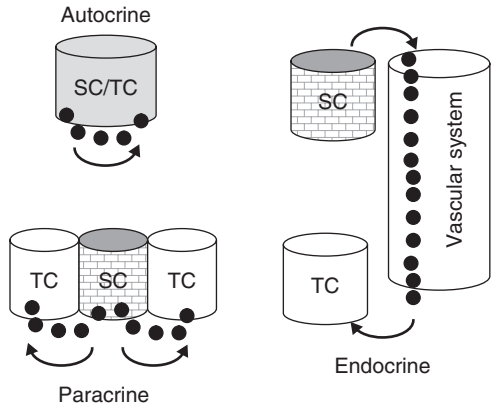


Fig. 3.1. Autocrine, paracrine and endocrine relationships. The diagram shows the autocrine, paracrine and endocrine relationship between secretory cells (SC) and target cells (TC). The black circles represent molecules of either a hormone or other form of regulatory chemical, such as a growth factor. In the case of the autocrine relationship, the secretory and target cell are one and the same. The hormone or other regulatory chemical is released from the cell into the extracellular space and it acts on receptors that are present on the plasma membrane of the same cell. In a paracrine relationship, the regulatory factor is released from secretory cells and it diffuses through the extracellular fluid and activates receptors in adjacent cells. For both of these relationships, the regulatory chemical is acting locally. In the case of the endocrine relationship, the hormone moves from the extracellular fluid into the vascular system and is carried away from the site of hormone production to act on distant target cells.

Nucleus-associated or genomic hormone receptors

Two families of hormones, the thyroid hormones and the steroid hormones, exert at

least some of their actions by interacting with receptors that are members of the same superfamily of DNA-binding receptors. These *nuclear receptors* attach to specific sequences of nucleotide bases (called hormone response elements) that are present in the gene promoter region, which is located 'upstream' of the coded gene sequence of the genes that respond to a particular hormone. The activation of these receptors by binding to their ligand (the hormone) brings about changes in the rate of expression of specific genes (Fig. 3.2).

These hormone–receptor–intracellular response relationships are extremely complex and are only partly understood, particularly in non-mammalian taxa. It is beyond the scope of this chapter to deal with this very interesting area of regulatory biology, and the reader is referred to sources that will provide a more detailed background (Griffin and Ojeda, 2000; Kacsoh, 2000). Increasingly, we are discovering that many of the known endocrine disorders are the result of dysfunctional hormone receptors caused by mutation of the genes that encode

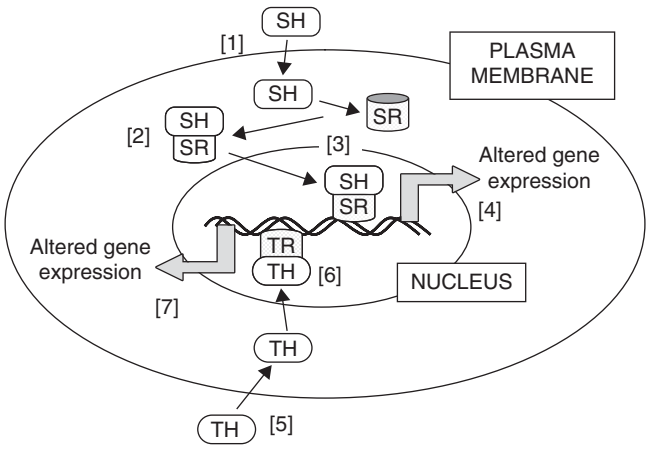


Fig. 3.2. Steroid and thyroid hormone receptors that act to alter gene expression by target cells. The diagram is a very simple schematic to illustrate how steroid hormones (SH) and thyroid hormones (TH) interact with their specific steroid and thyroid hormone receptors (SR and TR, respectively). For SH, the hormone moves into the cytoplasm of the target cell [1] and interacts with a specific steroid receptor protein (SR) that is present in the cytoplasm [2]. The SR–SH complex then moves by diffusion through the nuclear pores and attaches at specific sites, the steroid hormone response elements (SRE) (not shown in the diagram), on the nuclear DNA [3]. Small differences in the sequence of the SRE determine which SR can attach to the DNA at that point and thus determine the specificity of the target cell response to a particular hormone. The SR–SH complex acts as a transcription factor for specific genes and determines the rate of transcription of those particular genes [4]. Genes may have several transcriptional factors that are involved in regulating their expression. There are variations in the pattern of events, depending on the steroid hormone. For some steroid hormones, the SR is located in the nucleus but not attached to the DNA.

For TH, the hormone involved is triiodothyronine (T_3). The hormone enters the target cell by means of carrier proteins that are constituent proteins of the target cell plasma membrane [5]. The TR is present in the nucleus of the target cell, where it is probably attached to the thyroid hormone response element (TRE), even when the hormone is not present [6]. In the absence of the TH, the attachment of the TR protein to its response element exerts a 'gene silencing action', suppressing or preventing the expression of particular genes. In the presence of T_3, however, the TR is activated, and the TR–TH complex acts as a transcription factor, affecting gene expression [7]. The TREs differ in their nucleotide base sequence. Some TRE sequences are associated with enhancing gene expression and some with suppressing gene expression. Thus, T_3 may enhance the expression of some genes in some type of cells and inhibit the expression of other genes in the same or different cell types.

for the receptors; some disorders may also be caused by the receptors interacting with environmental contaminants that act as hormone mimics. Where such interactions occur, the receptors may be activated, in which case the environmental factor is termed a 'hormone agonist', or they may be rendered non-responsive to the native hormone, in which case the environmental factor is termed a 'hormone antagonist'.

Plasma membrane-associated hormone receptors

The receptor proteins for most hormones are located on the plasma membrane of target cells (Fig. 3.3) and are therefore termed *membrane receptors*. There is a constant turnover of receptor proteins, with new proteins being inserted into the membrane as older receptor proteins (sometimes with the hormone attached) being internalized by the target cells and metabolized. This constant turnover of the receptors is critical to maintaining the sensitivity of the target cells to the hormonal stimulus.

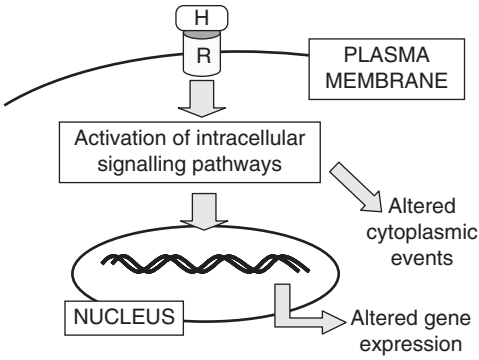


Fig. 3.3. Hormone receptors in the plasma membrane of target cells. The diagram is a very simple schematic to illustrate the manner in which a hormone (H) interacts with its receptor (R), which is located in the plasma membrane of the target cell. Binding of the hormone to its receptor causes conformational changes in the receptor, which causes the activation of intracellular chemical signalling cascades. Some of these cascades regulate cytoplasmic events; some may effect changes in the membrane characteristics of the target cell; and some result in the production of proteins that can affect gene expression in the nucleus of the target cell. Most cells are target cells for several hormones and other regulatory factors. The sensitivity of a particular cell to a particular hormone is partly determined by the number of receptors present in its plasma membrane and the number of hormone molecules (i.e. the hormone concentration in the extracellular fluid). The number of receptors is not fixed; the receptor population is constantly turned over, with new receptors replacing ones that are internalized by the cell and destroyed.

The binding of a hormone to its membrane receptor activates or suppresses intracellular signalling pathways within the target cell. These intracellular pathways regulate cellular activities; some of these signalling pathways are largely cytoplasmic events and some result in the production of proteins that alter the expression of specific genes (transcription factors) in the nucleus of the target cells.

As indicated above, thyroid and steroid hormones have genomic receptors, which act as transcription factors. In addition, membrane receptors are known for both groups of hormones in vertebrates, including fish (Borski, 2000; Davis *et al.*, 2005; Tasker *et al.*, 2006; Hanna and Zhu, 2009; Pang and Thomas, 2009). Ligand activation of these receptors has been involved in rapid intracellular signalling events, some of which will be explored at more length in the following sections of this chapter.

The organization of the endocrine system in fish

The main hormone 'systems' and their primary hormones considered in this chapter are listed in Tables 3.1 to 3.4. In general, the 'systems' fall into one of two primary categories, defined by their organization. One category comprises axes (Figs 3.4 and 3.5) that involve the hypothalamus and pituitary gland, while the second type of endocrine system is independent of hypothalamus–pituitary gland regulation. Hormone-secreting cells may be gathered together in the form of glandular tissues (e.g. Brockman bodies found in some fish species, containing largely insulin-secreting cells) or may be present dispersed among non-endocrine tissues (e.g. the

Table 3.1. Summary of the major fish hormone systems, the hormones produced and the major proposed physiological roles of the hormones associated with the central nervous system.[a]

Gland/tissue	Hormones	Chemical nature of the hormones	Primary role(s) of the hormones
Pineal gland	Melatonin	Amino acid derivative	Regulation of circadian and seasonal rhythms, reproductive cycles, seasonal migratory behaviour
Hypophyseotropic hormones[b]	Various (discussed in text)	Peptides, amino acid derivatives	Direct and indirect control of the secretion of hormones from the anterior pituitary gland
Hypothalamus (*pars nervosa* hormones)[c]	AVT	Peptide	Possible regulation of some aspects of ionic homeostasis
	Ichthyotocin (teleost fishes)	Peptide	Role not known
	Glumitocin (elasmobranch fishes)	Peptide	Role not known
Urophysis	Urotensin (several isoforms)	Peptide	Local control of some aspects of cardiovascular physiology
Chromaffin cells of the interrenal tissue	Catecholamines (epinephrine and norepinephrine)	Amino acid derivatives	Regulation of blood glucose homeostasis, including elevating blood glucose levels in response to stressors

[a]Except where indicated to the contrary, much of the information is derived from teleostean fishes; [b]Hypophyseotropic hormones are synthesized in the cell body of specialized hypothalamic neurones, and the hormones are released from synapses associated with the anterior pituitary gland; [c]AVT and the other posterior pituitary hormones are synthesized in the hypothalamus and released from the posterior pituitary gland.

Table 3.2. Summary of the major fish hormones synthesized in and released from the *pars distalis* and *pars intermedia* of the anterior pituitary gland (synonym adenohypophysis) and the major proposed physiological roles of the hormones.[a]

Gland/tissue	Hormones	Chemical nature of the hormones	Primary role(s) of the hormones
Anterior pituitary gland (*pars distalis*)	Prolactin (PRL)	Protein	Regulation of some aspects of ionic and osmotic homeostasis Regulation of some aspects of metabolism
	Growth hormone (somatotropin) (GH)	Protein	Regulation of some aspects of metabolism Enhancing skeletal and somatic growth, possibly via its metabolic regulating actions
	Adrenocorticotropin (ACTH)	Peptide	Regulation of interrenal steroid hormone secretion
	Thyrotropin (TSH)	Glycoprotein	Regulation of thyroid hormone secretion
	Gonadotropin (GtH)	Glycoprotein	Regulation of gonadal steroid hormone secretion
Anterior pituitary gland (*pars intermedia*)	Melanocyte-stimulating hormone (MSH)	Peptide	Role not known
	Melanin-concentrating hormone (MCH)	Peptide	Role not known
	Somatolactin (SL)	Protein	Possible involvement in calcium regulation in some species

[a]Except where indicated to the contrary, much of the information is derived from teleostean fishes.

Table 3.3. Summary of the major fish hormones synthesized in and released from various organ systems other than the nervous system and the anterior pituitary gland, and the major proposed physiological roles of the hormones.[a]

Gland/tissue	Hormones	Chemical nature of the hormones	Primary role(s) of the hormones
Interrenal tissue (steroidogenic adrenocortical tissue)	Cortisol (and small amounts of corticosterone and cortisone)	Steroid	Regulation of some aspects of metabolism, and facilitating adaptive responses to stressors
Corpuscles of Stannius	Stanniocalcin	Glycoprotein	Regulation of blood calcium ion homeostasis
Ultimobranchial gland	Calcitonin	Peptide	Regulation of blood calcium ion homeostasis
Thyroid tissue	Thyroxine (T_4) and triiodothyronine (T_3)[b]	Iodinated amino acid	T_4 is a prohormone, from which triiodothyronine (T_3) is synthesized; T_3 is involved in the regulation of some aspects of early embryo development and some aspects of metabolism
Testis (Leydig and Sertoli cells)	Testosterone and 11-ketotestosterone	Steroid	Regulation of spermatogenesis, and promotion of male secondary sexual characteristics
Ovary (theca and granulosa cells)	17β-Oestradiol and progestogens	Steroid	Regulation of oogenesis and promotion of female secondary sexual characteristics (oestrogen). Stimulation of ovulation (progestogen) Stimulation of vitellogenin synthesis by liver Mobilization of lipid from liver, muscle and adipocytes
Kidney	Erythropoietin	Glycoprotein	Regulation of the production of red blood cells
Heart	Natriuretin	Peptide	Possible regulation of some aspects of ionoregulation
Vascular system	Various (discussed in text)	Peptides	Local vasoconstriction of blood vessels

[a]Except where indicated to the contrary, much of the information is derived from teleostean fishes; [b]Some T_3 is released from the thyroid tissue, but most circulating hormone is produced by the monodeiodination of T_4 by peripheral tissues (liver, kidney, skin, brain, pituitary gland, intestinal tract).

Table 3.4. Summary of the major fish hormones synthesized in and released from various organ systems other than the nervous system and the anterior pituitary gland, and the major proposed physiological roles of the hormones.[a]

Gland/tissue	Hormones	Chemical nature of the hormones	Primary role(s) of the hormones
Liver	Insulin-like growth factor-1 (IGF-1)	Peptide	Regulation of some aspects of metabolism Enhancing skeletal and somatic growth, possibly via its metabolic regulating actions[b]
Pancreatic islet[c] and gastrointestinal (GI) tract	Insulin	Large peptide	Regulation of some aspects of protein metabolism
	Glucagon-like peptide	Peptide	Regulation of carbohydrate metabolism
	SRIF-22 and SRIF-24	Peptides	Regulation of some aspects of protein metabolism
	Pancreastatin	Peptide	Regulation of insulin secretion
	Guanylins	Peptides	Regulation of water and electrolyte transport across gastrointestinal tract epithelium
	Ghrelin	Peptide	Multifunctional, includes regulation of GH secretion
	Gastrin	Peptide	Regulation of GI tract function
	Secretin	Peptide	Regulation of GI tract function
Adipose tissue	Adiponectin	Large peptide	Regulation of some aspects of carbohydrate and fatty acid metabolism
	Leptin	Protein	Regulation of lipid metabolism and processing

[a]Except where indicated to the contrary, much of the information is derived from teleostean fishes; [b]IGF-1 secretion by hepatocytes is regulated by GH; IGF, in turn, exerts a negative feedback control over the release of GH from the anterior pituitary gland; [c]In some species the islet cells are scattered through the mucosa of the gastrointestinal tract; in others species the cells are present as a distinct glandular organ (Brockman body).

endocrine cells present in the mucosa of the gastrointestinal tract).

The term 'axes' (Figs 3.4 and 3.5) refers to the functional association between hormone-secreting neurons in the hypothalamus, the secretory cells of the anterior pituitary gland and the target cells that are regulated by the anterior pituitary hormones. The neurosecretory neurons of the hypothalamus synthesize and release hypophyseotropic hormones, which regulate the activity of the hormone-secreting cells of the anterior pituitary gland; the activity of these neurosecretory neurons is influenced by multiple

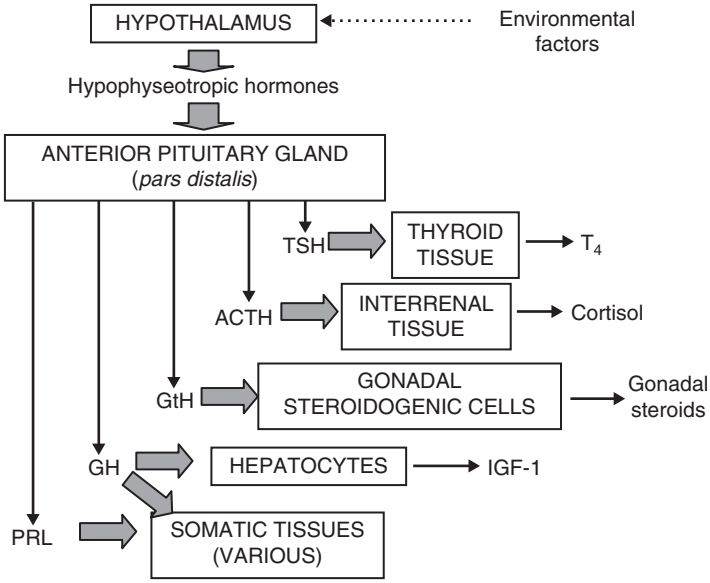


Fig. 3.4. The 'axes' hormones acting via the *pars distalis* of the pituitary gland. The figure illustrates the major hormones of the hypothalamus–*pars distalis*–peripheral tissue axes. Specialized neurons in the hypothalamus synthesize and secrete a range of amine and peptide hormones: the hypophyseotropic hormones, which play major roles in the regulation of the activity of the cells of the region of the anterior pituitary gland, termed the *pars distalis*. For fish, the nature of some of the hypophyseotropic hormones is still not fully known. Undoubtedly, there are factors that have not yet been chemically or biologically identified. The activity of the hypophyseotropic hypothalamic neurons is determined by higher brain centres, which in turn are influenced by environmental cues. Thus, the rate of activity of the axes may be affected by photoperiod, temperature, availability of food, stressors and many other abiotic factors. The main hormones of the *pars distalis* are shown. Thyrotropin (or thyroid-stimulating hormone) (TSH) is a glycoprotein that regulates much of the activity of the thyrocytes (the cells of the thyroid tissue), leading to the synthesis and release of the iodinated thyronine hormone, thyroxine (T_4). Adrenocorticotropin (ACTH) is a peptide that regulates the activity of the adrenocortical cells of the interrenal gland (the equivalent of the adrenal cortex in mammals). ACTH is the main factor regulating the synthesis of adrenocorticosteroids, the main one, in most fish species, being cortisol. Gonadotropin (GtH) is in the form of GtH-1 and GtH-2 (the homologues of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in mammals), and these are responsible for regulating steroid hormone production in the testis and ovary. Growth hormone (GH) activates receptors present in the plasma membrane of many cells and plays a role throughout the life of the animal in regulating aspects of cell metabolism. Some of this metabolic activity is linked to the regulation of growth, from which the hormone gets its name. GH also has a specific action on hepatocytes, stimulating the synthesis of insulin-like growth factor-1 (IGF-1), a hormone in the same family as insulin, which exerts effects that are complementary to the actions of GH. Prolactin (PRL) appears to play many roles in fish, but its best known is that as facilitating ionic and osmotic regulation in fresh water. It appears to exert its actions by acting directly on specific somatic cell types.

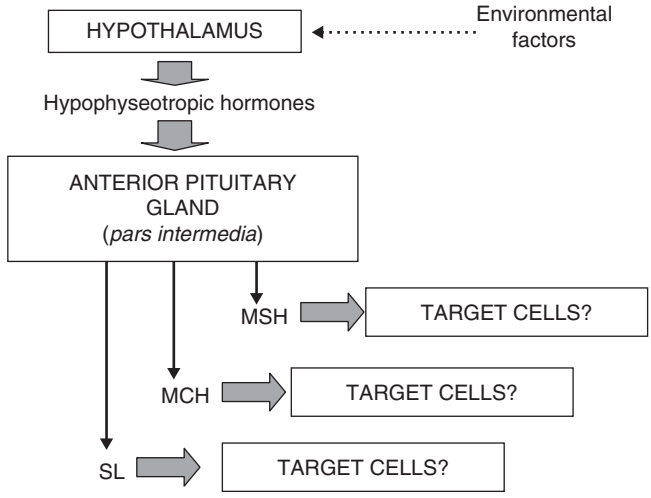


Fig. 3.5. The 'axes' hormones acting via the *pars intermedia* of the anterior pituitary gland. The figure illustrates the known hormones of the hypothalamus–*pars intermedia* axes. The relationships are similar to those described in Fig. 3.4. The *pars intermedia* comprises cells that synthesize and secrete several hormones, including melanocyte-stimulating hormone (MSH), melanocyte-concentrating hormone (MCH) and somatolactin (SL). Among fishes, there is considerable species variability as to which hormones are produced by the *pars intermedia*, and very little is known about their specific roles. Their names may be misleading, since MSH and MCH may not act on melanocytes in fish, and SL appears to have a calcium-regulating role.

environmental factors, which include photoperiod, ambient temperature and nutrient availability, and they are also indirectly influenced by a broad range of stressors. Unlike mammals, in which the hypophyseotropic hormones reach the anterior pituitary gland via a portal capillary system, in fish the axons of the hypophyseotropic neurons extend into the pituitary gland (Fig. 3.6). The neurohormones are released at synaptic terminals and enter the extracellular fluid surrounding the pituitary cells and activate specific receptors in the membrane of their pituitary target cells.

The hormones produced by the anterior pituitary cells under the influence of the hypophyseotropic hormones exert their effects on peripheral target cells, most of which are themselves hormone-producing cells; these include the cells of thyroid and interrenal tissue, the Leydig cells of the testis, the theca and granulosa cells of the ovary, and the IGF-secreting hepatocytes (Fig. 3.4). Some pituitary hormones, such as PRL, GH, SL, MSH and MCH, exert effects on non-endocrine peripheral target cells (Figs 3.4 and 3.5).

The brief description of the vertebrate endocrine system given above represents the classical overview of its organization (Griffin and Ojeda, 2000; Kacsoh, 2000), and apart from the differences in the relationship of the hypothalamus with the anterior pituitary gland in fish and mammals (Takei and Loretz, 2006) and the detailed morphology of some endocrine tissues in the two taxa, there is broad conservation of the endocrine 'systems'. However, there is considerable variation among taxa as regards the roles that a specific hormone plays.

It must be emphasized that the same hormone may be synthesized in, and secreted by, several different tissues. For example, many of the hypophyseotropic hormones that are synthesized in the hypothalamus and involved in the regulation of anterior pituitary cell activity are also produced by other organ systems, such as the gastrointestinal tract. These are sometimes referred to as the 'brain–gut' hormones. One specific example

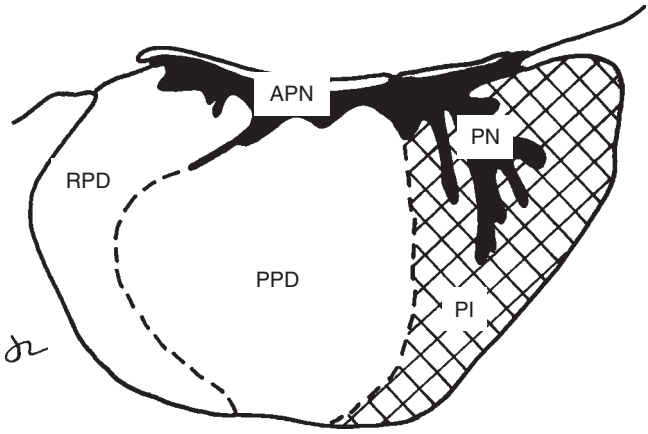


Fig. 3.6. Diagram showing a transverse section through the pituitary gland of a three-spine stickleback (*Gasterosteus aculeatus* form *trachurus*). The diagram shows the *rostral* and *proximal pars distalis* (RPD and PPD, respectively) of the anterior pituitary gland, the *pars intermedia* (PI) of the anterior pituitary gland, which is closely associated with axons of the *pars nervosa* (PN). The diagram also shows the penetration of the RPD and PPD by the axons of hypothalamic neurons (APN).

is the peptide hormone somatostatin (SRIF), which is synthesized by specific neurons of the hypothalamus and acts on the GH-secreting cells of the anterior pituitary gland to suppress GH synthesis; however, SRIF is also synthesized by multiple non-hypothalamic tissues, which release the hormone into the extracellular fluid, and it appears to play multiple autocrine and paracrine roles. Insulin-like growth factor-1 (IGF-1) is another example of a hormone that is synthesized in multiple sites and exerts many different actions depending on the source. IGF-1 is best known as a factor produced by liver cells under the influence of GH (Fig. 3.4), the so-called somatotropic axis; however, mRNA transcripts encoding for IGF-1 are found in many non-hepatic cell types in fish (e.g. Li *et al*., 2006, 2007; Li and Leatherland, 2008), and the hormone probably acts as an autocrine or paracrine growth factor. The functions of these local sources of IGF-1 and other hormones in fish are still not well understood.

Direct and permissive actions of hormones

Only rarely do hormones act in isolation from other hormones or growth factors. Some hormones work in opposition to one another in the regulation of a particular physiological process. Several hormones may be involved in regulating the same process and exert additive effects, where each hormone contributes its own level of stimulation. Yet other hormone interactions are synergistic, with the overall response being greater than can be accounted for by the simple sum of the actions of the individual hormones. Many hormones also act together 'permissively'. Permissive interactions may mean that both hormones are needed for a particularly event to occur. For example, the maximum expression of many genes relies on the activation of transcription factors in the promoter region of the gene. The receptors of several hormones, or proteins produced by hormone action on a target cell, may be transcription factors for a given gene; several hormones may be needed in order to obtain optimal gene expression (Griffin and Ojeda, 2000; Kacsoh, 2000). Yet another form of permissive action is found, in which one hormone regulates either the synthesis of a second hormone (e.g. thyroid hormone is needed for the synthesis of GH) or the synthesis of the receptor for a second hormone (Griffin and Ojeda, 2000; Kacsoh, 2000).

Hormone release from hormone secretory cells, hormone delivery to target cells and the role of hormone transport proteins

Hormones are released from the secretory cells into the extracellular fluid that surrounds these cells and from there they enter the vascular system. Some hormones (e.g. the hormones of the anterior pituitary gland) are stored as granules contained in cytoplasmic vesicles. Hormone release to the extracellular fluid entails the movement of the granules across the plasma membrane of the secretory cell by exocytosis. Steroid and thyroid hormones are not stored intracellularly in the glands that synthesize them. As hormones are synthesized by steroid-producing (steroidogenic) cells, the hormones diffuse across the plasma membranes of the secretory cells. Conversely, thyroid hormones are stored extracellularly as part of the molecular structure of the protein thyroglobulin, which is found in the lumen of the thyroid follicles. Thyroid hormone release involves the endocytosis of thyroglobulin by the thyroid follicle cells (thyrocytes) and the proteolysis of the thyroglobulin within the cytoplasm of the thyrocytes; the thyroid hormones then leave the thyrocytes and enter the extracellular fluid compartment. The movement of thyroid hormones from the thyrocytes to the extracellular fluid probably requires the presence of membrane transport proteins in the basal plasma membrane of the thyrocytes (Abe *et al*., 2002; Bernal, 2006).

Many hormones are bound non-covalently with specific proteins in the blood. For small molecules, such as the thyroid hormones, steroid hormones and small peptide hormones, the vast majority (>99%) of the total plasma hormone may be present in this protein-bound form. The association of small hormone molecules with the larger plasma transport proteins provides some protection from the passive loss of the small molecules via gills and kidney and ensures a ready supply of hormone for transfer to target cells. The ratio of bound to unbound ('free') hormone is determined by mass-action equations, and as the 'free' hormone enters the target cells, some of the bound hormone become dissociated from the transport protein to maintain a relatively constant bound to 'free' ratio. Even relatively large hormone molecules, such as GH, are transported in the blood in association with transport proteins; in these cases, the transport proteins appear to play an integral role in regulating the access of these hormones to their receptor proteins on the target cell membrane (Griffin and Ojeda, 2000; Kacsoh, 2000).

Biotransformation of hormones in peripheral tissues

Many cells take up hormones from the extracellular fluid and biotransform them into other hormones. In part, the biotransformation is a key process leading to the excretion of these hormones, but it may also be a vital step in the production of biologically active hormones. These *de novo* hormones may be active in the same cell in which they are produced, or they may pass into the general circulation and act on other target cells. One well-established example is the transformation of androgens into oestrogens that occurs in several non-gonadal sites. Specific isoforms of the cytochrome P450 aromatase enzyme (CYP 19 or P450arom) are involved in the conversion of androgens to oestrogens, and these enzymes are expressed in brain and fat tissue, and other organ systems. Thyroid hormone biotransformation also occurs in peripheral tissues, involving the enzymatic removal of iodide from T_4 (which has four iodides) to form biologically active T_3 or biologically inactive reverse T_3 (rT_3) (both of which have three iodides). The physiological value of the production of a biologically inactive product is that it allows the target cells to regulate the intracellular levels of the biologically active form of the hormone, and thus allows local regulation of the response of target cells to the hormone signal (Griffin and Ojeda, 2000; Kacsoh, 2000).

Systematic Survey of Endocrine Systems in Fish

Neuroendocrine tissues and their hormones

The neuroendocrine tissues include: (i) the neurohypophyseal neurons of the

hypothalamus; (ii) the pineal gland of the roof of the diencephalon; (iii) the caudal neurosecretory system; and (iv) the chromaffin cells of the interrenal gland (the homologue of the adrenal medulla of mammals) (Table 3.1).

The neurohypophyseal neurons are of two types, depending on the nature of their hormones and where the neurohormones are released. One group of these neurons synthesizes specific amine or peptide hypophyseotropic hormones that regulate the activity of the anterior pituitary gland; these hypophyseotropic hormones are released at axonal endings on the dorsal surface of the *rostral* and *proximal pars distalis* of the anterior pituitary gland (Fig. 3.6). Some of these hormones have yet to be characterized in fish, but some, such as CRH, GnRH, L-dopamine and SRIF-14, have been identified as factors that regulate anterior pituitary gland function (Sherwood and Parker, 1990; Holloway *et al.*, 1997; Fryer, 1989; Holloway and Leatherland, 1998; Lovejoy and Balment, 1999; Chen and Fernald, 2008). Some of the names that are currently used for these hormones reflect their possible functions in mammalian species and may not necessarily reflect the function of these hormones in fish. An example is the neurohormone gonadotropin-releasing hormone (GnRH). GnRH gets its name from its stimulatory action on the pituitary cells that produce the gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in mammals. In several species of teleostean fishes studied to date, GnRH has been found to be a potent stimulator of GH release from pituitary somatotropic cells (Holloway and Leatherland, 1998). Similarly, although another of the hypothalamic neuropeptides, CRH, does, as its name suggests, play a major role in regulating the activity of the ACTH-secreting cells of the anterior pituitary gland, it is also known to have other actions on pituitary gland function (e.g. regulating TSH secretion: De Groef *et al.*, 2006) and to act at many other sites, including the gonads.

A second type of neurohypophyseal neuron synthesizes the octapeptide hormone AVT. AVT is released from synapses in the neurohypophysis (synonym, *pars nervosa*); the *pars nervosa* is shown diagrammatically in Fig. 3.6 and as part of the neurointermediate lobe in Fig. 3.7. The neurointermediate lobe comprises the *partes nervosa* and *intermedia*. AVT may play ionoregulatory or osmoregulatory roles (Haruta *et al.*, 1991; Balment *et al.*, 1993), but few details of its physiological relevance in fish are known. Octapeptides, in addition to AVT, have been found in bony fishes (termed ichthyotocin) and cartilaginous fishes (termed glumitocin); they may have roles in aspects of reproductive physiology, but the specific nature of their actions is currently not clear.

The pineal gland secretes the amine hormone melatonin, which is released into the circulation during the scotophase (dark phase) of the photoperiod (Iigo *et al.*, 1991; Falcón *et al.*, 1992; Zachmann *et al.*, 1992). The square-wave circadian variations in plasma melatonin concentrations act as a signal that links changes in season to tissue and organ activity; thus, seasonal changes in the length of the scotophase, and a concomitant change in the daily period of elevated melatonin concentrations, is used as a signal that allows physiological adaptations to the changing seasons (e.g. growth, feeding activity, reproductive activity). Melatonin receptors are present in many tissues, suggesting that the hormone has multiple, but as yet poorly defined, physiological roles in fish and other vertebrate animals.

The neurons of the caudal neurosecretory system synthesize the peptides urotensin I (UI) and II (UII), which are structurally similar to two of the hypophyseotropic peptides, CRH and SRIF, respectively. In fish, UII has been shown to affect cortisol secretion, influence Na^+ transport and affect some aspects of metabolism (Affolter and Webb, 2001), and UI may play some roles in the stress response and appetite control of fish (Bernier and Peter, 2001; Craig *et al.*, 2005).

Chromaffin cells, so-called because of their staining properties in histological preparations, are interspersed among, and histologically distinct from, the steroidogenic interrenal cells that are associated with major blood vessels in the anterior (head) kidney (Fig. 3.8). The chromaffin cells represent the homologue of the adrenal medulla

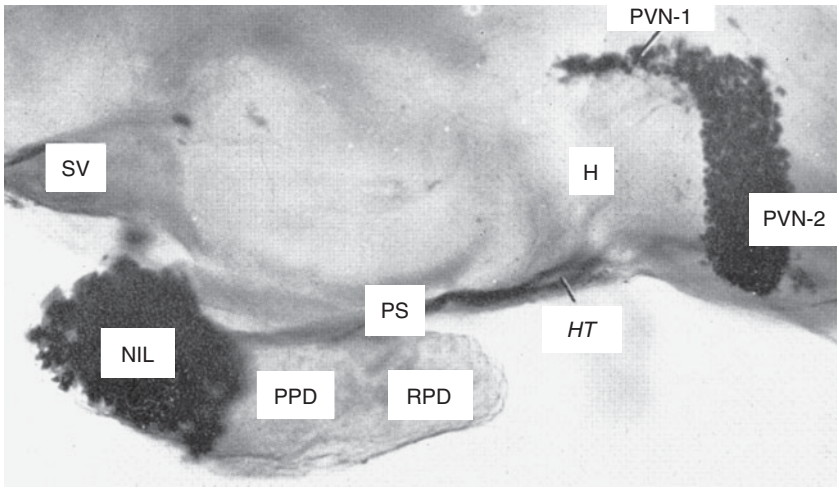


Fig. 3.7. Whole preparation of part of the brain of a European eel (*Anguilla anguilla*). The preparation has been stained to show the granules that contain the hormones that are released from the posterior pituitary gland, and the tissue has been cleared to make it transparent. The hormone granules appear black in this image. The cell bodies of these neurons are gathered into a pair of nuclei, called the paraventricular nuclei (PVN), each of which has a horizontal (PVN-1) and vertical (PVN-2) component. The axons of these cells pass through the hypothalamus (H) and gather together to pass along the pituitary stalk (PS), and terminate in the *pars nervosa* of the neurointermediate lobe (NIL). The *rostral* and *proximal pars distalis* of the anterior pituitary gland (RPD and PPD, respectively) and the *saccus vasculosus* (SV), a capillary cluster that lies just posterior to the pituitary gland, are also labelled.

of mammals. Catecholamine hormones, epinephrine and norepinephrine and other amino acid derivatives and small peptides have been identified as products of these cells in various fish species. The catecholamines are probably involved in the primary stress response, bringing about rapid changes in cardiovascular events and possibly also metabolic events leading to mobilization of metabolic reserves (Danulat and Mommsen, 1990; Fabbri *et al*., 1998).

Apart from the 'normal' changes in activity of the chromaffin cells associated with stress responses, there are no reported dysfunctional conditions of these neuroendocrine systems in fish. The stress response is considered in more detail in Chapter 7.

Anterior pituitary gland morphology and hormones

The anterior pituitary gland in fish comprises two morphologically distinct regions, the *pars distalis* and the *pars intermedia* (Fig. 3.6). The *pars distalis* is associated with hypophyseotropic neurons, whereas the *pars intermedia* is highly interdigitated with the posterior pituitary gland (*pars nervosa*) (Figs 3.6 and 3.7).

The anterior pituitary gland has its embryological origin as an up-pushing of the dorsal pharyngeal region to form a structure called Rathke's pouch. The pouch migrates dorsally to meet a down-pushing of the floor of the hypothalamus; the latter forms the *pars distalis*. Some dipnoan and teleostean species (e.g. alewife, *Alosa pseudoharengus*) retain a tubular connection of the anterior pituitary gland with the lumen of the gastrointestinal tract. There is some evidence to show that the hormone granules of the cells that synthesize PRL are released into the lumen of the gastrointestinal tract of these species.

Although the structure of the pituitary gland in teleostean fishes is highly conserved, there are species differences. One

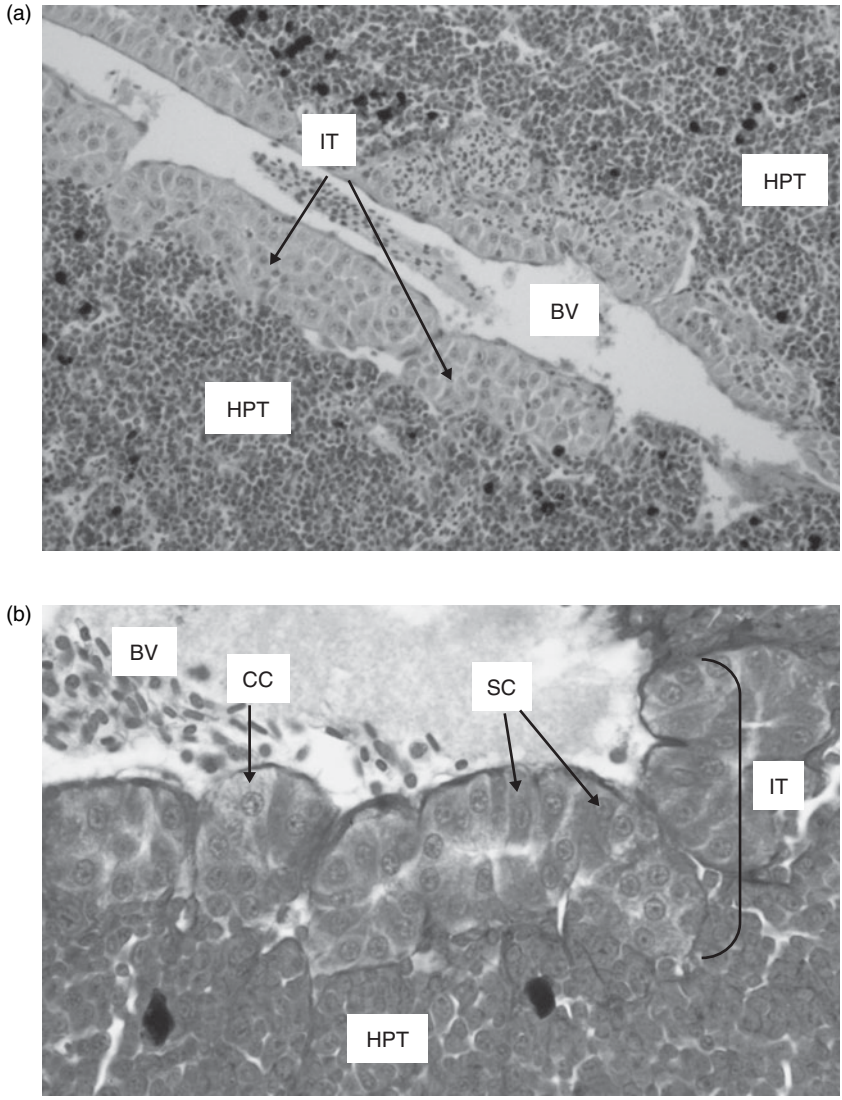


Fig. 3.8. Histological section through part of the anterior (head) kidney of a rainbow trout (*Oncorhynchus mykiss*) and a coho salmon (*Oncorhynchus kisutch*) (Figs 3.8a and 3.8b, respectively). The figures show interrenal tissue (IT) in juxtaposition to a blood vessel (BV). In both figures the IT is clearly differentiated from the haematopoeitic tissue (HPT) that makes up most of the head kidney. The dark cells among the HPT are melanocytes. The histological preparation shown in Fig. 3.8a is stained with haematoxylin and eosin and does not differentiate between the chromaffin cells (the adrenal medulla homologue) and the steroid-secreting cells (the adrenal cortex homologue). The histological preparation shown in Fig. 3.8b is stained with a trichrome stain that differentiates between the chromaffin cells (CC), which have clear cytoplasm in this preparation, and the steroid-secreting cells (the adrenal cortex homologue) (SC), in which the cytoplasm appears granular in this preparation.

notable difference among species is the organization of cells in the most anterior region of the *pars distalis* (the *rostral pars distalis*), which comprises largely ACTH- and PRL-secreting cells (Fig. 3.9). In anguillid and salmonid species, the PRL cells, together with non-granular stellate cells, are arranged in the form of follicles surrounding

a fluid-filled lumen (Figs 3.9a and 3.9b). In other teleostean fish taxa, the PRL cells are intermixed with non-granulated stellate cells (Fig. 3.10), but there is no follicular form (Fig. 3.9c); the functional significance (if any) of these morphological differences is not known. In some species, the thyrotropin (TSH)-secreting cells may be located in the same region as the PRL and ACTH cells (i.e. the *pars distalis*), but in others, the TSH cells may be gathered together in the dorsal region of the posterior part of the *pars distalis* (*proximal pars distalis*) (Ball and Baker, 1969; Farbridge and Leatherland, 1986).

The major hormones produced by the *pars distalis* and their major known roles are listed in Table 3.2. Briefly, PRL plays a role in osmotic and ionic regulation in freshwater fish, and probably also has metabolic roles and influences some immune system responses (Manzon, 2002). ACTH is the major regulator of adrenal steroidogenesis, although MSH may also play a similar role during some life history stages, particularly during the migration and sexual maturation phases (Lamers *et al.*, 1992). TSH is the main pituitary factor regulating thyroid tissue function. As the name suggests, the isoforms of GtH (GtH I and II) play essential roles in regulating gonadal development and maturation in fish. In fish, GH plays multiple roles, including the stimulation of IGF-1 synthesis by the liver, the regulation of ionic and osmotic homeostasis, the stimulation of cartilage growth and the regulation of several aspects of metabolism, most notably enhancing protein assimilation and lipid mobilization (Björnsson, 1997; Cameron *et al.*, 2002, 2005, 2007). The growth-regulating actions of GH probably operate via the metabolic-regulating actions of the hormone. SL, a member of the same family of hormones as GH and PRL, is synthesized by cells in the *pars intermedia*; it appears to play a role in calcium regulation in some species (Kaneko and Hirano, 1993; Kakizawa *et al.*, 1995); because of the similarity in the structure of GH, PRL and SL, the overlap in the apparent roles of the three hormones may be related to non-specific interaction with the several receptors. MSH is produced by the majority of the cells of the *pars intermedia* and some species produce MCH, but their physiological roles are not well understood; they may play roles in colour change in some fish species, but MSH may also have important roles in the regulation of the stress response, possibly operating via regulation of interrenal gland function (Baker *et al.*, 1986; Burton, 1993; Rotllant *et al.*, 2003).

The pituitary gland disorders that have been reported in fishes are described and discussed in a later section of this chapter.

Thyroid tissue morphology and thyroid hormone synthesis

Thyroid morphology

The thyroid tissue can be seen in histological sections of the lower jaw of most fishes apparently as follicles dispersed among the aereolar tissue of the lower jaw and lying in close association with the ventral aorta (Fig. 3.11). Because it is dispersed, the 'gland' is usually referred to as thyroid tissue. In a few teleostean species, notably the parrot fishes (*Scaras* spp.) and swordfish (*Xiphias gladius*), and in elasmobranch fishes generally, the thyroid has a glandular form. Ectopic thyroid tissue has been reported in the eye, anterior (head) kidney, spleen and heart of various fish species, usually in fish that have enlarged thyroid masses (goitres, which will be discussed in more detail later in this chapter). In cyprinid species, however, thyroid tissue is a normal component of both the pharyngeal region close to the ventral aorta, and the head kidney (Leatherland, 1994).

The traditional view of thyroid tissue structure in bony fishes is that the functional units are follicles, comprising a tight epithelium of thyroid folliculo-epithelial cells (synonym, thyrocytes). The follicle lumen contains colloidal thyroglobulin, a 660 kDa glycoprotein that has, as part of its chemical structure, the thyroid hormones T_4 and T_3 (Leatherland and Down, 2001). The thyroid tissue in vertebrates has its embryonic origin as a simple hollow ball of cells; in salmonid fishes, this primordium then elongates and tubular outgrowths form;

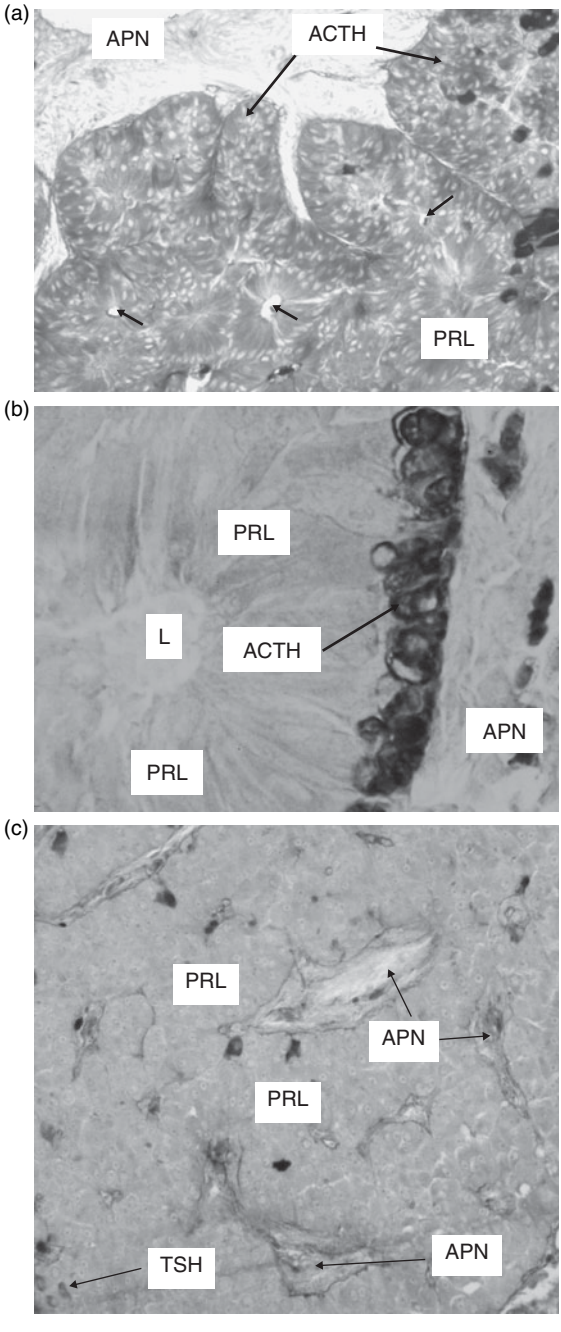


Fig. 3.9. Histological sections through part of the *rostral pars distalis* of the anterior pituitary gland of a coho salmon (*Oncorhynchus kisutch*), a European eel (*Anguilla anguilla*) and a carp (*Cyprinus carpio*) (Figs 3.9a, 3.9b and 3.9c respectively). Figure 3.9a shows a layer of adrenocorticotropic cells (ACTH) lining the interface of the *rostral pars distalis* with the anterior component of the *pars nervosa* (APN). The prolactin-secreting cells (PRL) lying below the ACTH layer are arranged in the form of follicles; the lumens of several follicles are marked by arrows. The follicle epithelium is made up of the granular PRL cells interspersed with non-granulated (clear) cells (not labeled). Figure 3.9b is stained to show the ACTH cells (darkly stained) at the interface between the APN; the PRL cells, arranged as follicles, and the follicle lumen (L) are also evident. Figure 3.9c shows a region of the *rostral pars distalis* that contains predominantly lightly stained PRL cells; note that these are not arranged in the form of follicles. The PRL cells are interspersed with non-granulated cells, but these can only be seen under the electron microscope (see Fig. 3.10). The small dark cells are probably thyroid-stimulating hormone (TSH)-secreting cells. Also seen are sections through finger-like projections of the anterior *pars nervosa* (APN); the projections contain axons of the hypothalamic neurons and blood vessels.

these tubular systems are still present well into early adult life, and possibly throughout the life of the animal (Fig. 3.12) (Raine and Leatherland, 2000; Raine *et al.*, 2005).

The published literature concerning fish thyroid morphology is replete with descriptions of 'large' and 'irregularly shaped' follicles that are most likely tubules, suggesting

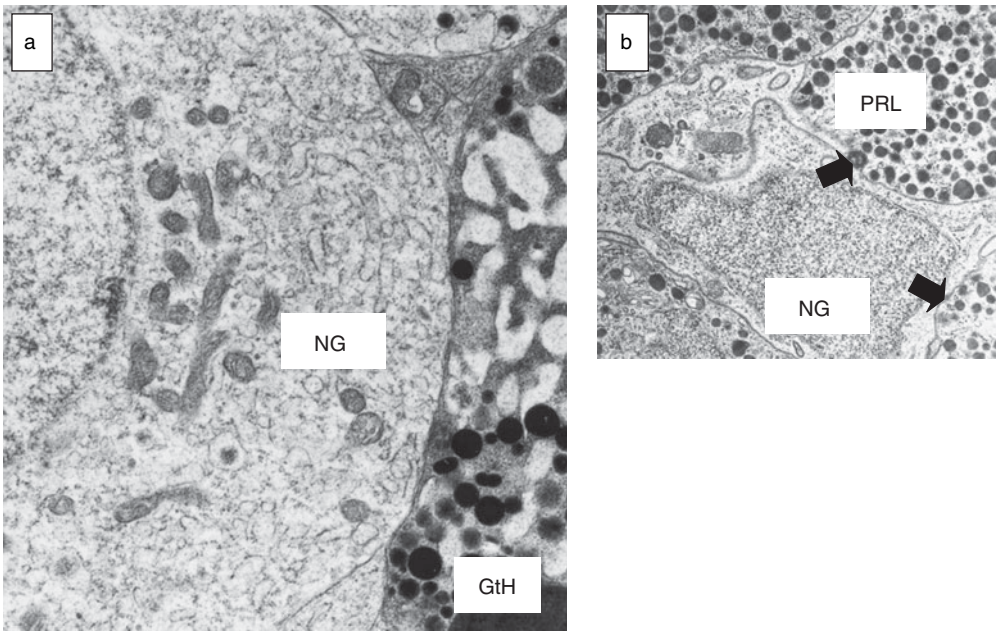


Fig. 3.10. Electron microscope images of the *proximal pars distalis* (a) and part of the *rostral pars distalis* (b) of a tilapia (*Oreochromis niloticus*). The two images show the non-granulated (NG) cells present in these two regions of the pituitary gland. In (a), the adjacent granulated cell is a gonadotropin-secreting cell (GtH); in (b), the adjacent granulated cells are prolactin-secreting cells (PRL).

that the thyroid tissue in many fish species may be tubular rather than follicular.

Thyroid hormone synthesis

The thyroid hormones, T_4 and T_3 (Table 3.3), are iodinated thyronine compounds, and their synthesis (shown diagrammatically in Fig. 3.13a) requires access to a source of iodide. The ion is actively taken up from food by the intestinal tract and from ambient water by the gills, by processes that probably involve some form of secondary active transport. The ion enters the blood, and thence the extracellular fluid, and is selectively extracted from the extracellular fluid by thyrocytes by means of secondary active transport, using a sodium ion (Na^+)–iodide symporter (NIS) protein. The NIS proteins are constitutive proteins of the basal cell pole (Fig. 3.13a) and belong to the solute-linked carrier (SLC) transporter family. NIS is a member of the SLC5A subfamily of Na^+-dependent anion transporters; the proteins transport complex anions such as perchlorates, which is why perchlorates are competitive inhibitors of iodide transport by NIS proteins (Wolff, 1998; Van Sande *et al.*, 2003). Iodide moves from the cytoplasm of the thyrocytes into the lumen of the follicles or tubules via specific iodide channels; on the luminal side of the apical membrane the iodide is converted to a free radical form, usually expressed as $I^{\bullet}$, by an oxidative enzyme (thyroid peroxidase (TPO)) reaction; the $I^{\bullet}$ becomes covalently attached to tyrosine elements in thyroglobulin. A subsequent oxidative reaction, also involving TPO, condenses some of the iodinated tyrosine elements to form the iodinated thyronine compounds T_4 and T_3, which at this point are still part of the molecular structure of the thyroglobulin protein.

In marine and brackish-water ecosystems, iodide is usually readily available to aquatic species, but in freshwater environments, iodide availability is limited, sometimes severely. Many of the known disorders of the thyroid relate to an inadequate supply

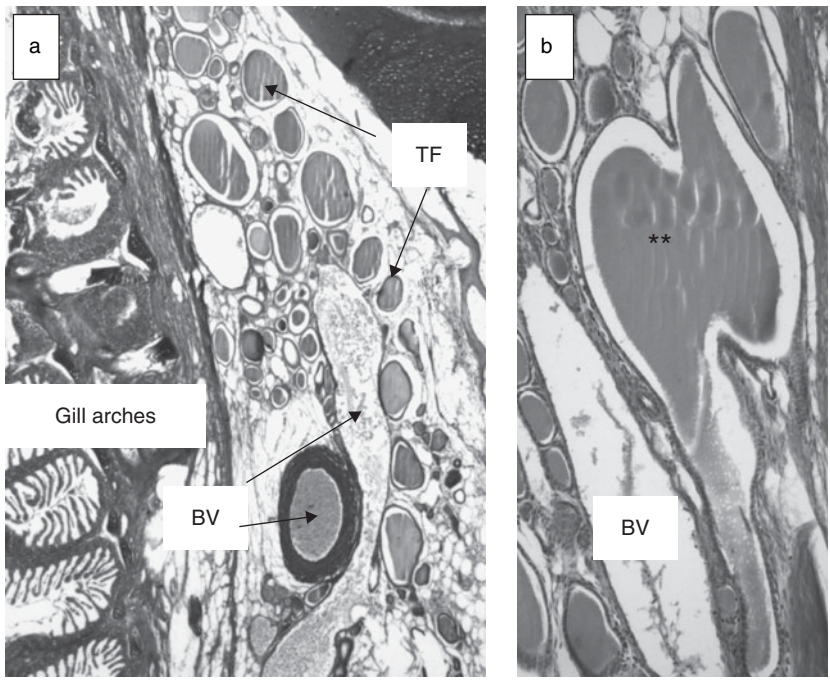


Fig. 3.11. Histological sections through part of the lower jaw of an adult sexually immature rainbow trout (*Oncorhynchus mykiss*). The section shows colloid-filled thyroid follicles that are closely associated with major blood vessels (BV) in the lower jaw. On the left of the section in (a) are bases of the gill arches. Figure (b) shows a 'follicle' (**) adjacent to a blood vessel (BV) that is distinctly tubular in appearance.

of iodide, to chemical impairment of the uptake of iodide from the environment or to chemical impairment of the oxidative iodination of tyrosine elements in the thyroglobulin. These factors all result in a reduced synthesis of thyroid hormone, a lowering of plasma thyroid hormone levels and a resultant increase in TSH release from the anterior pituitary gland. The increased TSH stimulation promotes growth of the thyroid tissue (a goitre) without a concomitant increase in thyroid hormone synthesis. Many of the reported disorders of the thyroid tissue in fishes are of this type, and they will be discussed in later sections of this chapter.

The thyroid hormones need to be released from the thyroglobulin molecule before they can enter the general circulation. The release of the hormones (shown diagrammatically in Fig. 3.13b) is under the influence of TSH, which stimulates the thyrocytes to take up, by endocytosis, droplets of thyroglobulin from the lumen. TSH also stimulates the thyrocytes to produce primary lysosome vesicles containing proteolytic enzymes; these vesicles fuse with the thyroglobulin droplets, and the thyroglobulin is digested to release T_4 and a smaller amount of T_3. Additional T_3 is produced within the thyrocytes by the enzymatic conversion (monodeiodination) of T_4 to T_3. Both hormones leave the thyrocytes via monocarboxylate transporter proteins in the basal cell membrane (Abe *et al.*, 2002) and enter the extracellular fluid. From the extracellular fluid the hormones enter the general circulation, where they become non-covalently bound to plasma transport proteins (Eales and Brown, 1993).

Monodeiodination of thyroxine by non-thyroidal cells

T_3 has a higher affinity than T_4 for the thyroid hormone receptor (TR), and thus T_3 is

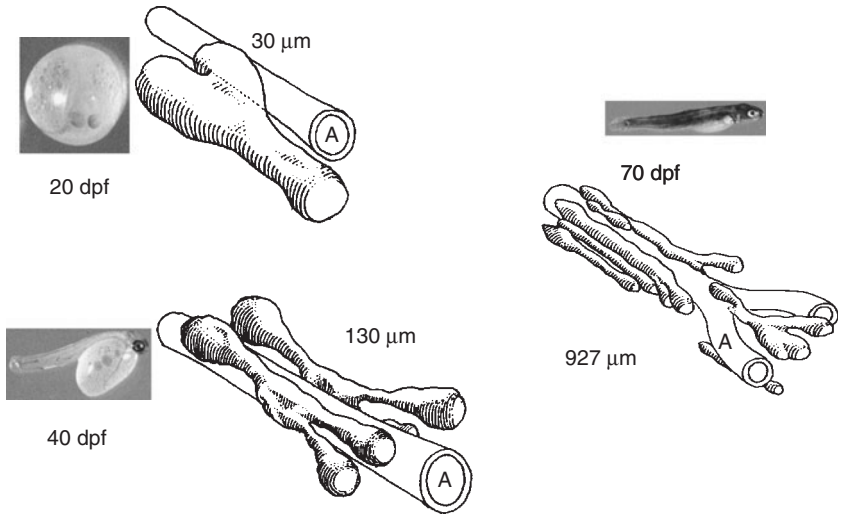


Fig. 3.12. Diagrams showing the formation of the thyroid primordium in a rainbow trout (*Oncorhynchus mykiss*). The drawings are based on serial sections of the lower jaw of embryos sampled 20 days post-fertilization (dpf) (before hatching), 40 dpf (after hatching) and 70 dpf (when the yolk in the yolk sac was almost completely absorbed). The numbers represent the total length of the thyroid tissue unit. At 20 dpf, the thyroid primordium lies just below the ventral aorta (A) in the lower jaw region; it takes the form of a simple tubular structure that is bifurcated posteriorly. By 40 dpf, the thyroid tissue is still in tubular form, but the tubular components are more elaborate and beginning to encase the ventral aorta. By 70 dpf, the tubular structure is still evident; it is more elaborate and branching has taken place, but there is still no evidence of the formation of follicles. In transverse section these tubules appear as follicles. (Modified from Raine *et al.*, 2005.)

the biologically active form of thyroid hormone. Most of the T_3 in the circulation is produced by enzymatic monodeiodination of T_4 by peripheral (non-thyroidal) organs, such as the liver and kidney; the T_3 thus synthesized is released back into the vascular system. In addition, some cells produce T_3, which acts on either TRs within the same cell or receptors that are contained in adjacent cells; one such example is the relationship between the astrocytes and neurones of the central nervous system. The astrocytes, which express the monodeiodinase necessary for the conversion of T_4 to T_3, produce T_3 to meet both their own needs and those of associated neurons (which do not express the monodeiodinase) (Griffin and Ojeda, 2000; Kacsoh, 2000).

In addition to the formation of T_3 from T_4, a second form of monodeiodinase acts to convert T_4 into an inactive form of T_3 called reverse T_3 (rT_3); rT_3 does not interact with the TR, and thus excess intracellular T_4 can be degraded without forming a product (T_3) that has high biological potency. The selective expression of genes that encode for the two forms of monodeiodinase and the selective translation of the gene products into proteins allows cells to regulate and moderate the level of their response to the thyroid hormones that enter the cell (Griffin and Ojeda, 2000; Kacsoh, 2000).

Thyroid hormone receptors in target cells

The TRs (and the steroid hormone receptors) belong to a superfamily of DNA-binding receptor proteins. TRs form dimers with the retinol receptor (RXR) and the dimers attach to specific sequences of DNA nucleotide bases called thyroid hormone response elements (TREs). The TREs are found in the promoter region of specific genes; the TR/RXR heterodimer complex acts as one of the

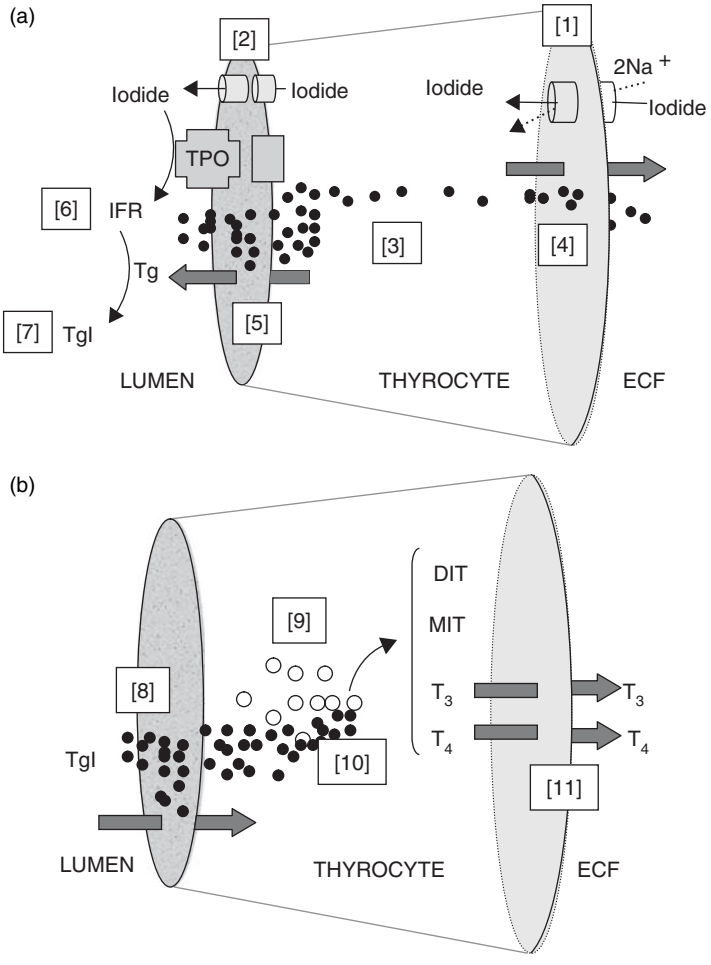


Fig. 3.13. Diagrams illustrating the basic components of the synthesis of thyroid hormones (thyroid hormonogenesis) (a) and thyroid hormone release (b). (a) is a diagram of a single thyroid epithelial cell (thyrocyte); the end of the cell towards the right (the basal cell pole) is in contact with the extracellular fluid (ECF) that surrounds the thyroid follicle (or tubule); the end of the cell to the left (the apical cell pole) is in contact with the lumen of the follicle (or tubule). Thyroid hormonogenesis requires two components: iodide and the protein thyroglobulin (Tg). Iodide is taken up from the ECF at the basal pole of the cell by a transport protein, the Na^+–iodide symporter (NIS) [1]; the NIS transporter uses the energy of the Na^+ influx to co-transport the iodide against a concentration gradient (secondary active transport). The iodide then diffuses through the thyrocyte and leaves the thyrocyte via iodide channels located in the apical cell membrane [2]. Tg is synthesized within the thyrocyte and packaged in the form of vesicles [3]; some of these vesicles leave the thyrocyte by exocytosis via the basal cell membrane [4], and enter the circulation. Most of the Tg vesicles pass by exocytosis through the apical cell membrane into the lumen [5]. In the lumen, the iodide is converted in the presence of hydrogen peroxide into a free radical form (IFR) [6] by the enzyme thyroid peroxidase (TPO); TPO is one of the apical membrane proteins; the enzyme domain faces the lumen. The IFR reacts with tyrosine elements of the Tg to form iodinated Tg (TgI) [7]. The oxidative iodination causes either the monoiodination of tyrosine components of the Tg to form monoiodotyrosine (MIT) or the diodination of the tyrosine elements to form diiodotyrosine (DIT). A further oxidative process, also involving TPO, causes the condensation of these iodinated tyrosine units to form the thyroid hormones tetraiodothyronine (thyroxine or T_4) (the condensation of two DIT units) and triiodothyronine (T_3) (the condensation of an MIT and a DIT unit); the thyroid hormones remain as components of the TgI molecule.

Continued

Fig. 3.13. *Continued*. (b) is a diagram showing the processes involved in the release of the thyroid hormones from the TgI. Droplets of Tg (probably both iodinated and non-iodinated) pass through the apical cell membrane by endocytosis [8]; the vesicles of Tg (shown as black circles) fuse with primary lysosomes (shown as open circles) [9] that contain proteolytic enzymes. The proteolytic enzymes digest the Tg, releasing the iodinated thyronine compounds (T_4 and T_3) together with uncondensed iodinated tyrosine compounds (DIT and MIT) [10]. DIT and MIT are enzymatically deiodinated by dehalogenases within the thyrocyte to release the iodide and tyrosine; the thyroid hormones leave the thyrocyte via the basal cell pole, probably by the action of a membrane transport protein [11].

several transcription factors that regulate the expression of specific genes. The TR/TXR dimers are present in the nucleus of target cells, and they appear to attach to the TREs in the absence of the TR hormone ligand T_3 and exert a 'gene silencing' action. The receptor is activated by T_3 binding to the TR, and the TR/RXR complex then becomes involved in the regulation of gene activity. There are different TRE sequences, and attachment of the activated RXR/TR dimer to some TRE sequences brings about an increase in gene expression (stimulatory TREs), but the association of RXR/TR dimers to other TRE sequences results in the inhibition of gene expression (Griffin and Ojeda, 2000; Kacsoh, 2000).

Although the RXR/TR heterodimer appears to be the most common form of the receptor complex, TR homodimers can also form, and some of these are known to be functional transcription factors. The picture is further complicated by the presence of two separate TR gene products, TRα and TRβ, and by post-translational subtypes of those major TR classes, which are synthesized at different stages of the life history of fish. Theoretically, heterodimers of these various isoforms could also form. Further, there are several isoforms of the RXR protein and thus multiple possible permutations and combinations of receptor protein associations (Griffin and Ojeda, 2000; Kacsoh, 2000). The physiological significance (if any) of these various forms of receptor protein associations is not known at this time for any of the vertebrate classes.

Further, some of the known effects of thyroid hormones are on genes that do not have a TRE, and therefore there are pathways of hormone–receptor interactions with these genes that do not involve the association of the TR with the DNA in the promoter region of the gene. It is beyond the scope of this chapter to deal in detail with this important aspect of thyroid hormone function; the excellent reviews by Yen (2001), Wu and Koenig (2000), and Flamant and Samarut (2003) provide additional information.

The nuclear TRs described above are the best known of the pathways by which the thyroid hormones exert their actions at the target cell level. Recently, however, an additional TR has been identified; it is found in the plasma membrane of target cells, has a high affinity for T_4 and when activated stimulates one of the intracellular signalling pathways of the target cells (Davis *et al.*, 2005). Some of the actions of thyroid hormones in fishes cannot be readily explained on the basis of the stimulation or inhibition of gene expression, thus it is highly likely that the T_4 receptor is not restricted to mammals.

Physiological actions of the thyroid hormones

Thyroid hormones have been proposed as regulatory agents in various aspects of metabolism, growth, ionoregulation, osmoregulation, reproduction and development in fish (Leatherland, 1994). Despite considerable research effort, surprisingly little is known about the specific details of the roles of these hormones in fishes. This is probably because many, if not most, of the actions of the thyroid hormones are 'permissive' in nature, i.e. they allow the full expression of the effects of other hormones or other growth factors. Experimental elevation of plasma thyroid hormone levels by administration of exogenous sources of hormones or reducing plasma hormone levels by administration of drugs that block thyroid hormone synthesis affect metabolism and rates of development of fish. However, the experimental conditions

do not lend themselves to careful study of the normal (and probably subtle) roles of the thyroid hormones, particularly those involving the interactions of these hormones with other regulatory factors. Administration of exogenous hormone by injection or immersion results in pharmacological levels of blood hormone, thus the responses can best be described as pathological. Similarly, the chemical agents used to reduce plasma hormone levels are all themselves toxic, and it is sometimes difficult to differentiate between the actions of these toxicants and the cellular responses to reduced levels of thyroid hormones.

A point that cannot be overemphasized is that the thyroid hormones do not, in ectothermic animals such as fish, exert the same level of control over metabolic rate (MR) as they do in endothermic animals such as mammals and birds. In endothermic animals, MR is generally very high and tightly controlled by several components of the endocrine system, including the hormones of the thyroid and adrenal medulla. Daily thyroid hormone production and turnover in mammals greatly exceeds that seen in the fish species studied to date, and this is directly related to the energetic demands imposed by homeothermy, which requires the generation of heat (thermogenesis). In contrast, the MR of ectothermic animals is determined to a considerable extent by ambient temperature; consequently, the possibility for endocrine regulation of MR independent of environmental temperature is very limited. This may account for why some types of environmentally related disorders of thyroid function reported in mammals and birds are not found in fish (these are discussed later in this chapter).

Pancreatic hormones and other gastrointestinal tract hormones

The pancreatic hormones (Table 3.4) identified to date in fish include insulin, glucagon-like peptide (GLP), SRIF-22, SRIF-24, pancreastatin, guanylins and ghrelin (Kaiya *et al.*, 2003; Yuge *et al.*, 2003; Reinecke *et al.*, 2006); however, only a few fish species have been studied and relatively little is known about the roles of these hormones. The cells that produce these hormones are found as islets throughout the tissue of the exocrine pancreas (Fig. 3.14); these islets are homologous to the islets of Langerhans of mammals. A few fish species exhibit a larger gathering of these cells in the form of Brockman bodies, sometimes termed 'principal islets'. Insulin and the isoforms of SRIF may regulate some aspects of protein metabolism and may be involved in the regulation of growth, whereas GLP may induce hyperglycemia by stimulating hepatic gluconeogenesis (Reinecke *et al.*, 2006).

Even less is known about the roles of the gastrointestinal tract hormones in fish. Several factors have been identified in the mucosa, including the homologues of mammalian gastrin and secretin, and several neuropeptides. Some of these may have regulatory roles similar to those found in mammals, but the details of the physiological function of many of these factors is still not known (Reinecke *et al.*, 2006). As discussed earlier in this chapter, several of the gastrointestinal neuropeptides are also synthesized in hypothalamic cells and are involved in the regulation of the secretion of anterior pituitary hormones.

IGF-1 is a member of the insulin family of peptide hormones; it is synthesized by hepatocytes under the influence of GH. In turn, IGF-1 exerts chronic and acute negative feedback control over the secretion of GH by the pituitary gland (Cameron *et al.*, 2005). Because of this close relationship between GH and IGF-1 physiology, it is difficult to differentiate between the actions attributed to GH and those of IGF-1, per se. IGF-1 and GH appear to play important roles in the regulation of metabolism in fish, particularly during fasting and recovery from fasting (Pierce *et al.*, 2005; Cameron *et al.*, 2007). IGF-2 is also synthesized by fish, and IGF-1 and IGF-2 appear to have functions in early developmental periods, but these are likely to be hormones produced at the local tissue level, and they probably play autocrine or paracrine roles (Li *et al.*, 2006, 2007; Li and Leatherland, 2008).

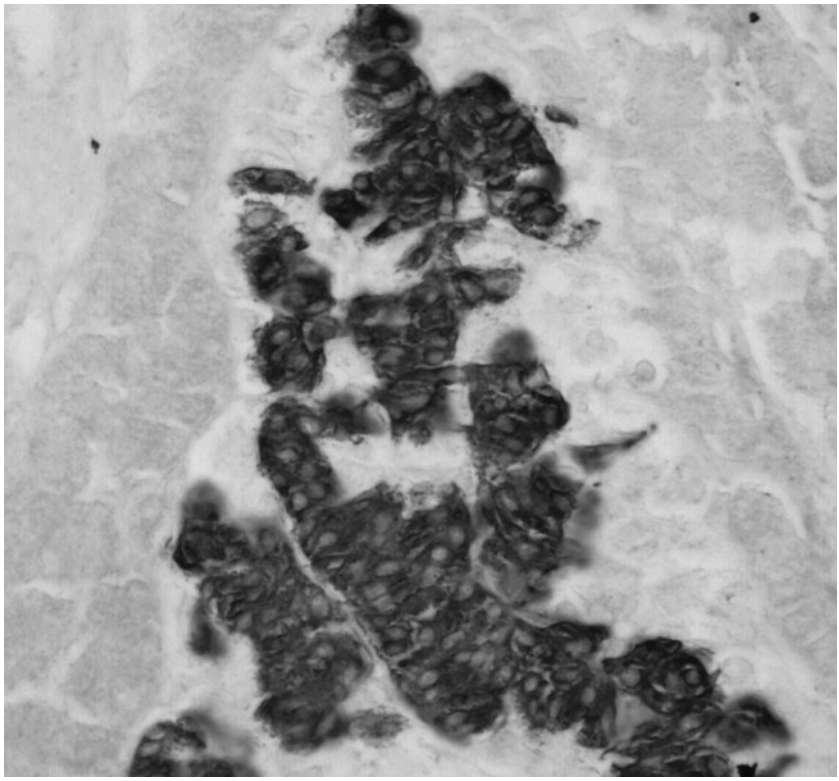


Fig. 3.14. Histological section through part of the pancreatic tissue of a rainbow trout (*Oncorhynchus mykiss*). The section is stained to show the insulin-containing cells in the tissue; these cells appear dark in this figure.

There are no known disorders that can be directly related to dysfunction of the pancreatic or gastrointestinal endocrine systems in fish.

Steroidogenic interrenal tissue

Steroidogenic cells, which are the homologue of the adrenal cortex in mammals, are found close to major blood vessels of the posterior cardinal veins in the anterior region of the kidney, commonly called the head kidney. Histologically, these cells are clearly distinguishable from the surrounding haematopoietic tissue (Fig. 3.8) and the catecholamine-secreting chromaffin cells, which are also part of the interrenal tissue (discussed earlier).

ACTH, acting through its G-protein-linked receptor, is the main regulator of steroidogenesis by the interrenal cells. Activation of the ACTH receptors in the plasma membrane of the steroidogenic cells promotes multiple intracellular signalling pathways, including the synthesis of cAMP, and changes in intracellular calcium ion levels. The details of the intracellular signalling pathways have yet to be elucidated, but they regulate the rate of movement of cholesterol into the inner compartment of the steroid-secreting cell mitochondria and affect the activity of some of the steroidogenic enzymes (see Chapter 6, this volume). The movement of cholesterol into the mitochondria appears to be the rate-limiting step in steroidogenesis and requires the involvement of two transport proteins: steroidogenic acute-regulatory

(StAR) protein (Aluru *et al.*, 2005; Hagen *et al.*, 2006; Miller, 2007), which associates with the outer mitochondrial membrane, and peripheral-type benzodiazepine receptor (PBR), which may control the import and processing of StAR protein in the outer mitochondrial membrane (Lacapère and Papadopoulos, 2003; Papadopoulos, 2004). In the inner mitochondrial compartment, cholesterol is biotransformed by specific isoforms of the cytochrome P450 side chain cleavage (CYP 11A or P450scc) enzyme into pregnenolone, the first steroid in the steroidogenic cascade. Pregnenolone then leaves the mitochondria, and steroidogenic enzymes associated with the smooth endoplasmic reticulum of the cytoplasm convert pregnenolone through a series of biotransformations that result in the formation of largely cortisol and smaller amounts of 11-deoxycortisol; the final enzymatic steps in the formation of these compounds occur in the mitochondria (Griffin and Ojeda, 2000; Kacsoh, 2000; see also Chapter 6, this volume).

Although ACTH is a major regulator of adrenal steroidogenesis, melanophore-stimulating hormone (α-MSH) acts to elevate plasma cortisol levels when administered experimentally (Baker *et al.*, 1986) and may stimulate steroidogenesis at some stages of the life history of some fishes, particularly during the migration and reproductive stages of salmonid fish, when glucocorticoid hormone levels are chronically elevated (Schreck *et al.*, 1989). In addition, thyroid hormones, catecholamines and possibly GH may also play important roles in regulating interrenal steroidogenesis.

Cortisol and 11-deoxycortisol (Table 3.3) are secreted in increasing amounts in response to a range of stressors, probably as a means of mobilizing nutrient reserves, enabling the fish to respond to the stressor. The review by Mommsen *et al.* (1999) provides a detailed discussion of the secretion and function of glucocorticoids in fish, including a review of the mechanisms of action of the hormones.

As was the case for thyroid hormones, only a small fraction of the total plasma glucocorticoids are present as free hormone; most are non-covalently bound to blood proteins. Free steroid hormone enters target cells and associates with glucocorticoid receptor (GR) protein located in the cell cytoplasm. Activation of the GR protein molecule by the hormone allows it to form a homodimer with other activated GR protein, and the homodimers move through the nuclear pores into the nucleus and attach to glucocorticoid response elements (GRE) in the promoter region of specific genes. The hormone–GR dimer complex acts as a transcription factor and regulates the rates of gene expression of target genes. In mammals, the GR may inhibit the expression of some genes by binding to other transcription factor proteins and inhibiting their actions; however, it is not known whether this is the case in fish. Many genes contain a GRE, and, as is the situation with the thyroid hormones, many of the actions of the glucocorticoids are permissive in nature (Mommsen *et al.*, 1999; see also Chapter 6, this volume).

Glucocorticoids play a central role in intermediary metabolism, affecting the expression of several key metabolic enzymes, particularly during food deprivation and stressful situations (Mommsen *et al.*, 1999). Glucocorticoid levels are elevated as part of the response to several stressors (see Chapter 6, this volume), and this elicits changes in metabolism that tend to increase glucose availability for cellular function, and simultaneously suppresses immune responses, making fish more susceptible to several diseases. To date, no disorders of interrenal tissue activity, other than those resulting from stress responses, have been reported.

Angiotensins, the renin–angiotensin system and other factors involved in cardiovascular function

The renin–angiotensin (RA) system, which is common to all vertebrate taxa, comprises several components, namely: (i) the juxtaglomerular apparatus (JGA) of the kidney; (ii) the enzyme renin, secreted by specific JGA cells; and (iii) two peptide factors, angiotensin I (AngI) and angiotensin II (AngII) (Arillo

et al., 1981; Bailey and Randall, 1981; Perrott and Balment, 1990; Takei *et al.*, 2004).

The active angiotensin factor is AngII, an octapeptide molecule produced by the catalytic action of angiotensin-converting enzyme (ACE) on the decapeptide molecule AngI. AngII production occurs in cells that contain ACE, largely endothelial cells of blood vessels and cardiomyocytes. AngI is produced by the action of the enzyme renin on the protein angiotensinogen, one of the blood proteins produced by the liver. Renin, in turn, is synthesized in, and released from, cells that are components of the JGA of the kidney. In addition to the renin-secretory cells, the JGA contains sensory cells that monitor the Na^+ concentration of the fluid in the kidney tubules (renal glomerular filtrate) and blood pressure in specific blood vessels in the kidney; changes in these parameters determine the rate of secretion of renin and thereby the amount of circulating AngI. The amount of AngII that is produced in peripheral tissues depends on the activity levels of ACE in specific cells, which change according to need.

In mammals, the RA system is best known for its role in regulating blood pressure, blood volume and blood Na^+ and K^+ levels. AngII plays an essential role in causing local vasoconstriction of peripheral blood vessels; AngII thus is important for regulating local blood flow and thus exerting an effect on systemic blood pressure (Nishimura, 1985; Kacsoh, 2000). AngII also directly stimulates the synthesis of the adrenal mineralocorticoid aldosterone, which has potent effects on the retention of Na^+ and the excretion of K^+. The degree of Na^+ retention also contributes to blood pressure and blood volume values. In fish, much less is known about the roles of the RA system, but there are similarities in function to the roles played in mammals (Nishimura, 1985). Since aldosterone has not yet been found in fish, the RA system may not exert an action via mineralocorticoid hormones; however, there is evidence to suggest a role of the RA system in some aspects of ionic or osmotic regulation via modulation of glomerular diuresis in some fish species (Wells *et al.*, 2003). In addition to vasoconstrictive actions of the angiotensins in fish (Opdyke and Holcombe, 1976; Platzack *et al.*, 1993), the peptides have also been postulated to play a direct role in the control of ovulation (Hsu and Goetz, 1992) and regulation of plasma Ca^{2+} concentrations (Pang *et al.*, 1981).

In addition to the roles of the RA system, other factors may also contribute to a network of biologically active chemicals that play essential roles in cardiovascular regulation, including the cardionatrin (natriuretin) peptides (Table 3.3), the kallekrein–kinin system and endothelins (Takei and Loretz, 2006). In addition, AVT, by virtue of its probable role in ionoregulation, is also a likely contributor to aspects of blood pressure regulation (Takei and Loretz, 2006).

No non-infectious disorders of the RA or kallekrein–kinin systems or of the cardionatrins have been reported in fish.

Corpuscles of Stannius and the ultimobranchial gland

The corpuscles of Stannius (CS) are glandular structures found associated with the kidneys of holostean and teleostean fishes. The secretory cells of the CS, the stanniocytes, secrete the glycoprotein hormone stanniocalcin, also called hypocalcin and teleocalcin (Table 3.3), which appears to play a role in regulating calcium homeostasis, specifically by preventing calcium uptake, thereby preventing hypocalcaemia (Pang, 1973; Wagner and Freisen, 1989; Pierson *et al.*, 2004).

The cells of the ultimobranchial gland (UB) are located in the transverse septum that separates the heart from the abdominal cavity; they secrete a 32 amino acid peptide hormone, calcitonin (Table 3.3), into capillaries that drain into the sinus venosus. Calcitonin has a potent hypocalcaemic role in some mammals and may play a similar role in fish (Pang, 1973; Wendelaar Bonga and Pang, 1991; Ishibashi and Imai, 2002; Mukherjee *et al.*, 2004; Suzuki, 2005).

Genes encoding for different isoforms of stanniocalcin and for calcitonin have been found in diverse tissues other than the corpuscles of Stannius and ultimobranchial

glands, respectively. There is increasing evidence to suggest that in addition to playing a role in calcium regulation, these hormones may play local autocrine or paracrine regulatory roles in several tissues (Clark *et al.*, 2002; Luo *et al.*, 2005).

No disorders associated with CS or UB gland function in fish have been reported.

Various other hormones

As briefly summarized in Table 3.3, the kidney, in addition to its role in the renin–angiotensin system, secretes the glycoprotein hormone erythropoietin, which plays a role in the production of red blood cells by haematopoietic tissue of the head kidney in fish. The heart produces the peptide natriuretin, an endocrine factor involved in aspects of ionoregulation in fish, and adipocytes secrete leptin and adiponectin into systemic blood. Leptin is involved in aspects of lipid metabolism, and adiponectin in aspects of glucose regulation and fatty acid metabolism.

Endocrine tissues of the testis and ovary

The primary endocrine tissues of the testis in fish are the Leydig cells of the interstitial tissues (synonym interstitial cells) (Figs 3.15–3.17), found associated with blood vessels in the matrix of the testis, which lies outside the seminiferous lobules or tubules (Cerdà *et al.*, 2008). The Sertoli cells, which make up the epithelium of the seminiferous lobules or tubules, may carry out steroid biotransformation of androgens to oestrogens in some fish species. In the fish ovary, the steroidogenic cells are the theca and granulosa cells, which form a one-cell-thick layer around each oocyte of the ovary, with the granulosa on the inside and the theca on the outside; the theca–granulosa cell layers

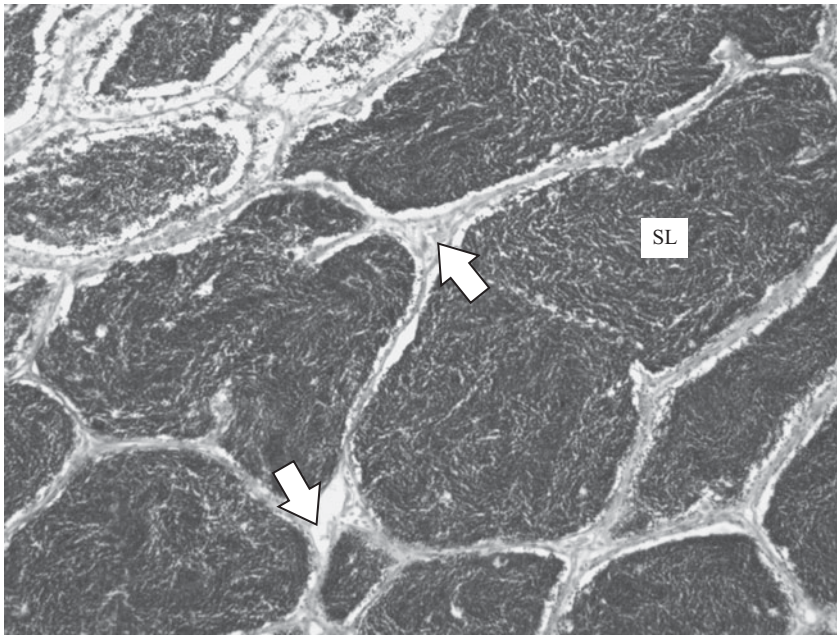


Fig. 3.15. Histological section of part of the testis of an adult, sexually mature rainbow trout (*Oncorhynchus mykiss*), which spawn once a year (total spawners). In this section the seminiferous lobules (SL) that make up the majority of the testis are filled with spermatozoa, and there are no other stages of gamete present; arrows indicate interstitial tissue.

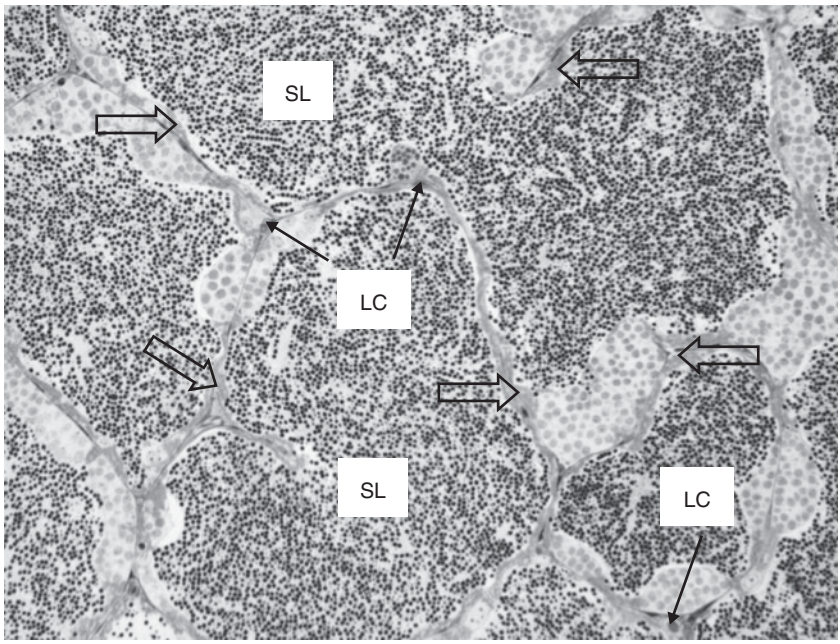


Fig. 3.16. Histological section of part of a testis of an adult, sexually mature goldfish (*Carassius auratus*). The testis comprises seminiferous lobules (SL) that are filled with spermatozoa; clusters of immature gamete cells can be seen within each of the lobules. The epithelium of the SL is formed from Sertoli cells, but these are very difficult to discern in light microscope preparations; the areas indicated by the open arrows are parts of the Sertoli cells; the cytoplasm is filled with lipid droplets, which have stained a dark colour. Between the lobules is interstitial tissue, which comprises connective tissue and blood vessels; within the interstitial tissue are the steroid hormone-secreting cells of the testis, the Leydig cells (LC) (also called interstitial cells).

overlay the proteinaceous acellular *zona pellucida* (synonym *zona radiata*), which envelops the oocyte (Figs 3.18 and 3.19). Further details of hormonogenesis by the gonadal endocrine tissues are provided in the section of this chapter that deals with reproductive function.

Interactions Between the Endocrine and Immune Systems

It is beyond the scope of this chapter to give a detailed overview of the immune system in fish. The reader is directed to recent reviews and pertinent articles for a more detailed description of the anatomy and histology of lymphoid organs and the function of the immune system components (Zhang *et al.*, 1999; Ewart *et al.*, 2001; Tort *et al.*, 2003; Russell and Lumsden, 2005; Boshra *et al.*, 2006; Fisher *et al.*, 2006; Magnadóttir, 2006; Noga, 2006; Reite and Evensen, 2006; Robertson, 2006; Zapata *et al.*, 2006; Hall *et al.*, 2008; Zapata and Cortés, 2008).

One component of the immune system is innate immunity, comprising surface barriers. In fish, the skin and the mucus that it produces contain antimicrobial factors that generally act non-specifically. Other non-specific humoral molecules of innate immunity in fish include complement, lectins, iron-binding proteins and lysozymes; non-specific cellular components include monocytes, tissue macrophages, neutrophils and cytotoxic cells. An additional humoral factor that has been shown to have antibacterial and haemagglutinating activities in fish is the yolk phospholipoprotein vitellogenin (Shi *et al.*, 2006). The second component of

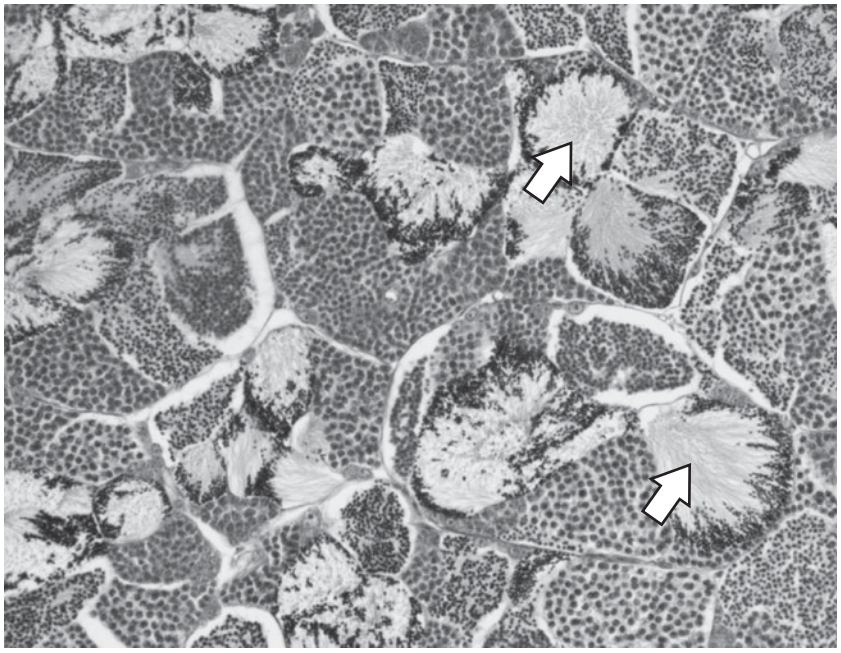


Fig. 3.17. Histological section of part of the testis of an adult, sexually mature Pacific wrasse (*Haliochoeres trimaculatus*), which exhibit a lunar periodicity in spawning and spawn several times during the breeding season (batch spawners). Note the markedly different appearance compared with sections of testis shown in Fig. 3.15. Seminiferous lobules with a range of stages of gamete maturation are evident, including lobules filled with spermatozoa (marked with arrows).

the immune system, adaptive or acquired immunity, includes humoral and cell-mediated responses that are similar to those found in mammals.

Cortisol is perhaps the best-known endocrine factor interacting with the immune system, and it has an immunosuppressive action. In fish, cortisol has been shown to reduce the number of circulating lymphocytes, decrease lymphocyte proliferation, decrease the number of B-lymphocytes, decrease antibody production, decrease phagocytosis and increase apoptosis (Harris and Bird, 2000; Cuesta *et al.*, 2006; see also Chapter 6, this volume). Cortisol has also been shown to enhance the local expression of genes that encode for IGF-1 and IGF-2 in tilapia gonads (Huang *et al.*, 2007). The role of cortisol in 'normal' immune system regulation is to prevent excessive positive feedback of cytokines, so that inflammatory reactions to pathogens or damaged tissue are controlled. However, under chronic stress situations, when blood cortisol levels are enhanced over a long period of time, the net effect of increased plasma cortisol levels is associated with a decreased resistance to pathogens (Cuesta *et al.*, 2006).

Some pituitary hormones have also been shown to affect some aspects of immune system function. For example, PRL administration to gilthead seabream has been shown to suppress circulating IgM levels, and administration of PRL or GH to that species suppresses complement activity levels (Cuesta *et al.*, 2006). In addition, GH, PRL and two hormones of the *pars intermedia*, α-MSH and MCH, and the POMC-derived hormone β-endorphin have all been shown to stimulate phagocytosis and/or mitogenesis of lymphoid tissues in fish (reviewed by Harris and Bird, 2000), and Carpio *et al.* (2008) recently showed that pituitary adenylate cyclase-activating polypeptide (PACAP), a

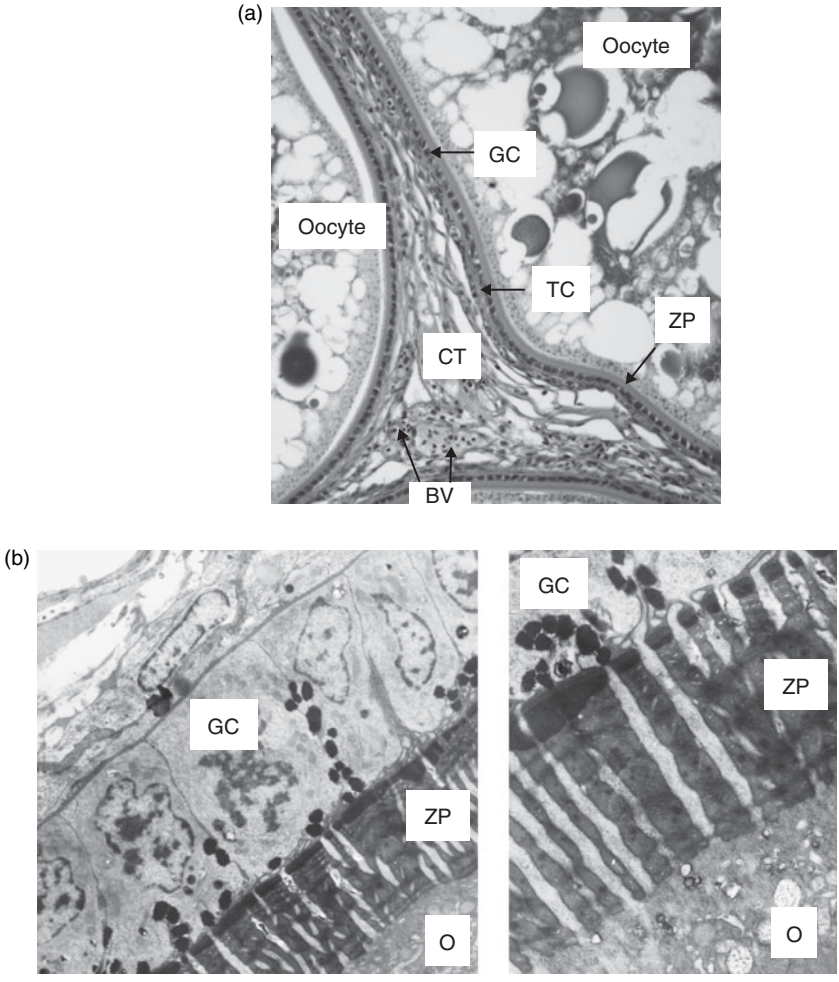


Fig. 3.18. Histological section of part of three ovarian follicles in an adult, sexually mature rainbow trout (*Oncorhynchus mykiss*) (a) and electron microscope images of the *zona pellucida* (ZP) (synonym *zona radiata*) of the hermaphroditic fish *Kryptolebias marmoratus* (formerly *Rivulus marmoratus*). In (a) two of the oocytes are labelled. The cytoplasm of the oocytes contains many apparently empty vesicles; these formerly contained lipid, which was washed out of the tissue during preparation for embedding and sectioning; the dark vesicles contain the yolk protein vitellogenin. Surrounding each oocyte is an acellular layer of protein, the *zona pellucida* (ZP) (also called the *zona radiata* because of its apparent striated appearance). Overlying the ZP is a layer of cuboidal cells, the granulosa cells (GC); extensions of the GC cytoplasm pass through the ZP (giving the layer its striated appearance) and contact the oocyte; similarly, there may be extensions of the oocyte cytoplasm that make contact with the GCs (b). Overlying the layer of GCs is a layer of small fusiform-shaped cells, the theca cells (TC). The oocyte surrounded by its ZP, GC and TC layers represents the ovarian follicle. The space in between the ovarian follicle comprises connective tissue (CT), which contains many blood vessels (BV); the cells in the BVs are nucleated red blood cells. The TC and GC layers represent the steroid-secreting cells of the ovary; the TCs synthesize progestogens and androgens, particularly testosterone, and the GCs convert the testosterone into the main steroid products of the ovary, namely oestrogens.

In the electron microscope images shown in (b), the multiple extensions of oocyte cytoplasm passing through the ZP are clearly evident. The dark granules associated with the GC layer comprise protein that is being deposited in the ZP; O, oocyte cytoplasm.

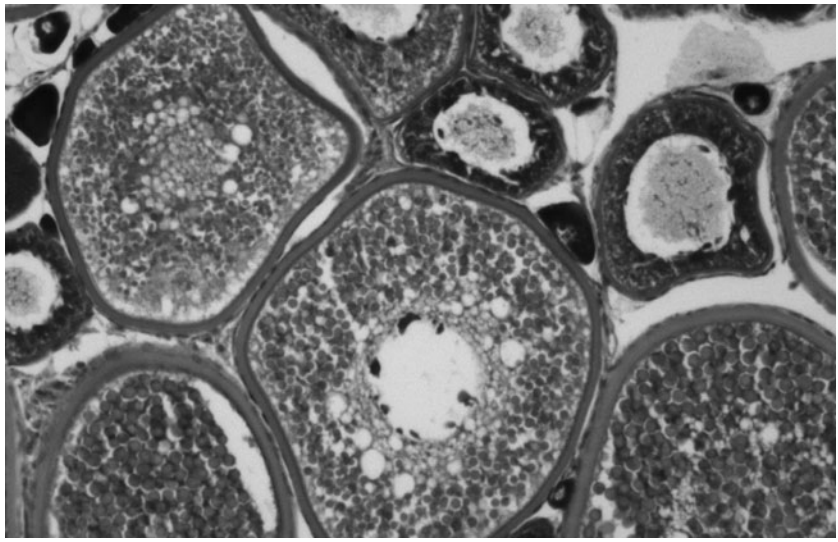


Fig. 3.19. Histological section of part of the ovary of a Pacific wrasse (*Haliochoeres trimaculatus*). The section shows ovarian follicles at several developmental stages of this species, which spawns multiple times during the reproductive period.

factor thought to be involved in GH secretion in fish, promoted growth of African catfish (*Clarius gariepinus*), but also stimulated lysozyme activity and NO synthase metabolites, and promoted antioxidant defenses, all of which are part of the innate immune response. In addition, Yada (2007) reported immunomodulatory effects of extrapituitary sources of GH and extrahepatic sources of IGF-1; the hormones are secreted in significant amounts by tilapia leucocytes and were found to enhance superoxide formation associated with phagocytosis by leucocytes; both IGF-1 and GH appear to play paracrine roles in immune cell function (Yada, 2007). Cortisol has also been shown to enhance the local expression of genes that encode for IGF-1 and IGF-2 in tilapia gonads (Huang *et al.*, 2007). These findings suggest a complex two-way interaction between these hormones (or paracrine factors) and the endocrine and immune systems.

There is also some evidence showing that 17β-oestradiol stimulates phagocytosis and/or mitogenesis of lymphoid tissues in fish (reviewed by Harris and Bird, 2000), but the biological value of this interaction is not well understood. However, endocrine mimics that exert effects on reproductive systems (discussed later in this chapter and in Chapter 9, this volume) are known to adversely affect immune system function, which suggests an important interactive relationship between gonadal function and immune system function.

Male and Female Reproductive Systems

Fish have evolved a broad array of reproductive strategies, including species such as the oncorhynchids, which spawn only once in their life (semelparous) and die thereafter, and species that reproduce several times in their life (iteroparous). Among the iteroparous species there may be total spawning at a single time or the release of batches of eggs over a period of time. In addition, there are differences in gender systems. Some fish species have at least two distinct sexes that are genetically determined (gonochoristic), whereas others are hermaphroditic (reviewed by Sardovy de Mitcheson and Liu, 2008) or parthenogenic; yet others require

sperm to activate the egg, but do not require the sperm to fertilize (gynogenic). Moreover, a large number of species are able to undergo sex reversal.

There is also a great range in the number of gametes produced at each spawning, from extremely large numbers in species that provide no parental care to a small number in species, such as sticklebacks, minnows and some tilapia, that provide brood care for their eggs or embryos. Most fishes use external fertilization of eggs, but some rely on internal fertilization, including self-fertilization in at least one species (*Kryptolebias marmoratus* (formerly *Rivulus marmoratus*) (Lee *et al.*, 2008)). For species that employ internal fertilization, the fertilized eggs are released and develop outside of the body cavity (oviparous), whereas for others the embryos develop within the body cavity of the female, hatch and are released as live young (ovoviparous) (Wootton, 1990; Murua and Saborido-Rey, 2003). The anatomy and reproductive endocrinology of each species has evolved to support these diverse reproductive strategies, and there are marked species differences in the structure of the gonads and associated reproductive organs, the gonadal steroid hormones that are produced and the nature of the control of gonadal steroidogenesis. It is not possible in this chapter to adequately review the diversity of reproductive adaptations found in fish taxa; the following is a general guide based largely on studies of gonochoristic species.

Morphology of the gonads

Testis

In fish, testes are commonly paired, but in some species they are fused as a single medial testis. The organ comprises largely tubules or lobules formed by a tight epithelium of Sertoli cells (Figs 3.15–3.17); these seminiferous tubules or lobules contain germ cells at various stages of maturation, depending on stage of development of the fish and season. In some species, the derivative germ cells, the spermatogonia, are found throughout the testis, and in others the spermatogonia are present at the distal end. In some species the gamete cells mature more or less synchronously, and at the end of testicular maturation the only gamete cells visible are spermatozoa (e.g. salmonid fishes) (Fig. 3.15). In other species, all stages of spermatogenesis are present most of the time during the reproductive season (e.g. goldfish (Fig. 3.16) and Pacific wrasse (Fig. 3.17)). For species in which the oocytes are fertilized internally (e.g. guppy, *Poecilia reticulata*), the testis may consist of spermatic cysts in which the spermatogenic cells mature synchronously. The steroidogenic Leydig cells (synonym interstitial cells) lie outside of the seminiferous tubule epithelium in between the tubular/lobular elements (Figs 3.15–3.17); primary spermatogonia are also outside of the seminiferous epithelium, in close contact with the basal pole of the Sertoli cells.

Ovary

In fish, ovaries may be paired or partially fused in the midline. In some species, an oviduct is present and eggs are moved directly from the ovary to the outside. In other species, such as salmonid fishes, the oviduct is not complete and, at ovulation, the eggs accumulate in the peritoneal cavity and are released through a 'vent' just posterior to the anus.

The ovary comprises lobular parenchymal tissue encompassing the germinal elements. The latter, depending on the species and stage of gonadal maturation, may range from primary oogonia, which will be attached to the parenchyma, to the fully formed follicular elements contained within the lumen of the ovary. Ovarian follicles comprise the oocyte, contained within the *zona pellucida*, and the layers of the steroid-secreting theca and granulosa cells that overlay the *zona pellucida* (Figs 3.18 and 3.19).

In synchronously spawning fish, the follicles of an individual are at a similar maturational stage, but in other species that are 'batch spawners' (i.e. they spawn repeatedly within a reproductive season), germinal cells of all stages of maturation will usually be present (Fig. 3.19); post-ovulatory

follicles and atretic follicles may also be present.

Hypothalamus–Pituitary Gland–Gonad Axis

The control of steroid hormone production by the gonads in vertebrates is highly conserved. Steroidogenesis by the steroid-secreting cells of the testis and ovary is regulated by the hormones of the hypothalamus–pituitary gland (HP) axis. Peptide hormones of the hypothalamus, acting via membrane receptors on the gonadotropic cells of the pituitary gland, regulate the synthesis and release of glycoprotein gonadotropic hormones (GtH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH, in turn, act on their membrane receptors on steroidogenic cells of the gonads to regulate steroid synthesis (Yaron and Mann, 2006). In sexually immature fish, and in mature fish that are not reproductively active, the overall level of activity of the HP axis is reduced, and there is very low production of hypothalamic hypophyseotropic hormones, little synthesis or release of the GtHs, and very low levels of steroid synthesis by the gonadal steroidogenic cells. Gonadal recrudescence is brought about by increasing the activity of the HP axis, leading to increased steroid hormone output and the implementation of a negative feedback control of the axis activity, largely based on steroid hormone feedback at the level of the hypothalamus and pituitary gland (Schultz *et al.*, 2001; Planas and Swanson, 2007). In addition to steroid hormones, peptide hormones (inhibins (synonym follicostatins) and activins) are synthesized and secreted by the gonad, and these act on the pituitary gland to modify the negative feedback control of steroid hormone production (Ge *et al.*, 2003).

Testicular and ovarian steroidogenesis is largely regulated via the actions of the GtHs on their G-protein-coupled receptors located in the plasma membrane of the steroid-secreting cells, giving rise to increased intracellular cAMP production and the activation of other intracellular signalling pathways, including ones that bring about changes in intracellular calcium levels. As for interrenal cell steroidogenesis discussed earlier, the transfer of cholesterol into the inner mitochondrial membrane by means of StAR protein and PBR protein transporters (Ings and Van Der Kraak, 2006; Yaron and Mann, 2006) appears to be the rate-limiting step in gonadal steroidogenesis; within the mitochondria the cholesterol is converted into pregnenolone by specific isoforms of the CYP 11A (side chain cleavage) enzyme (P450scc). Pregnenolone leaves the mitochondria, and a series of steroidogenic enzymes located on the smooth endoplasmic reticulum of the gonadal steroidogenic cells sequentially transform pregnenolone into the end-point testicular or ovarian steroids (Leatherland *et al.*, 2003).

Testicular steroidogenesis

In fish, the primary sites of testicular steroidogenesis are the Leydig cells of the interstitial tissue (synonym interstitial cells) (Figs 3.15–3.17), which lie in the matrix of the testis, outside the seminiferous lobules or tubules. The Sertoli cells, which make up the epithelium of the seminiferous lobules or tubules, may also carry out steroid biotransformation of androgens to oestrogens in some fish species. Testicular fragments incubated *in vitro* produce several steroids from the steroid precursor molecule, pregnenolone. These include progesterone, 17α-hydroxyprogesterone, 17,20β-dihydroxy-4-pregnen-3-one, androstenedione, 11β-hydroxyandrostenedione, testosterone, 11β-hydroxytestosterone, 11-ketotestosterone and 17β-oestradiol. *In vivo*, however, testosterone and 11–ketotestosterone appear to be the main androgenic steroids present in the plasma of most fish species examined to date (e.g. Leatherland *et al.*, 2003). These androgens are involved in the regulation of secondary sexual characteristics and reproductive behaviours (operating via the peripheral circulation), and they are necessary for normal spermatogenesis and spermeogenesis; the androgens enter the seminiferous tubules, bind to transport proteins and accumulate at high concentrations in the seminiferous fluid,

and the gametogenic cells are bathed in the androgen-rich medium.

Ovarian steroidogenesis

In the fish ovary, the steroidogenic cells are the theca and granulosa cells; these cells each form a one-cell-thick layer around each oocyte of the ovary, with the granulosa on the inside and the theca on the outside (Figs 3.18 and 3.19); they overlay the proteinaceous acellular *zona pellucida*, which envelops the oocyte. The *zona pellucida* is perforated by channels; cytoplasmic extensions of the granulosa cells through these channels allow contact of the steroid-secreting cells with the oocyte (Fig. 3.18b).

When incubated in *in vitro* culture, GtH-stimulated theca/granulosal cells produce a range of progestogens, androgens and oestrogens, but the major gonadal steroids in the circulation are 17β-oestradiol (the primary oestrogen), oestriol (in small amounts), testosterone and progestogens (e.g. Kime, 1993; Reddy *et al.*, 1999). In some species, such as salmonid fishes, testosterone levels in sexually mature females may exceed those of sexually mature males. This is because the major androgen in these species is 11-ketotestosterone not testosterone. The progestogens produced, the so-called maturation-inducing steroids, are preferentially secreted late in gonadal maturation to induce ovulation; metabolites of these progestogens excreted into the urine may also act as pheromonal agents. The oestrogens and progestogens play essential direct and indirect roles in the growth and maturation of the oocytes (Kime, 1993; Higashino *et al.*, 2003; Burnard *et al.*, 2008; Hoysak and Stacey, 2008). Gene expression in the oocyte, leading to final maturation, may be affected directly by oestrogen. In addition, the oestrogens stimulate the hepatocytes to synthesize the phospholipoprotein vitellogenin (VtG), the major yolk protein, and the proteins that will form polymers that make up the *zona pellucida* of the ovarian follicles (Arukwe and Goksøyr, 2003); oestrogens also stimulate several tissues to mobilize fat stores to release triglycerides. The VtG is taken up from the blood by the oocytes by receptor-mediated endocytosis, and *in ovo* processing of the VtG by serine proteases and cathpepsins gives rise to yolk protein and some of the yolk lipid (Babin *et al.*, 2007; Cerdà *et al.*, 2008). The *zona pellucida* proteins in the blood are monomers; these are polymerized to form the *zona pellucida* (Modig *et al.*, 2007). The released triglycerides are transferred by lipoprotein receptors into the oocytes and contribute to the total lipid content of the oocyte.

This brief outline of gonadal structure and function does not reflect the complexity of the process; this aspect of fish physiology is the subject of considerable ongoing research, and the application of new molecular methodologies is demonstrating new dimensions in the manner in which the gonads function and the control of gametogenesis and oogenesis (Bobe *et al.*, 2006; Goetz and MacKenzie, 2008; Mclean, 2008; Sundell and Power, 2008).

Disorders of the Endocrine System of Fish

Pathophysiological considerations and limitations

Apart from the exceptions discussed in this section, there are relatively few reported cases of spontaneous or environmentally induced epizootics of endocrine dysfunctional states in fish in the wild. In large measure this may reflect the difficulties of working in the field and the technical difficulties of identifying epizootics within populations. In general, relatively little attention is paid to hormone-producing tissues during pathological evaluation of fish stocks or populations. Moreover, even when endocrine tissues are of interest, unless the problem is evident by gross examination, measuring the prevalence of an epizootic of a particular endocrine disorder necessitates the sampling and processing of large numbers of fish (both afflicted and normal). This is time-consuming and costly, particularly because of the dispersed nature of the tissues that

synthesize hormones and other growth factors, and relatively few research laboratories are equipped to carry out such work.

Notable exceptions to this general rule are investigations that use a particular fish species as a 'sentinel' species to monitor the effects of 'point source' contaminants on an ecosystem; examples include the use of fish responses to monitor the effects of bleached kraft mill effluent (BKME) generated by paper-producing facilities or sewage effluent on the reproductive physiology of key fish species. Even these types of studies have confounding issues that affect interpretation of the data. The degree of a 'problem' in a contaminated site, say a lake, is usually determined by comparing the situation in the study lake with that of an uncontaminated 'control' site. Sites that are not contaminated by a specific contaminant or cocktail of contaminants will undoubtedly have a distinctly different ecosystem from those that do contain the chemicals of interest. As a consequence, the physiological challenges of fish in the two sites will differ markedly and this will likely have a major influence on the growth, metabolism and reproduction of fish that inhabit the two sites. Differentiating between endocrine (including reproductive) responses that are specific to the actions of an environmental chemical factor, as opposed to endocrine changes that are responses to impaired growth (possibly related to diet), metabolic responses (possibly related to diet or changes in liver function) or impaired reproduction (possibly related to diet and available stores of metabolites), is problematic. Establishing convincing cause–effect relationships between contaminant(s) and response(s) in wild fish without associated laboratory studies is sometimes not possible.

In addition, this 'sentinel' species approach is often compromised when contaminants have only a transient effect, as is the case for some of the chemicals in BKME that elicit reproductive endocrine responses when present but do not necessarily provoke chronic responses. Moreover, when using wild species, it is not always possible to determine whether the fish sampled have been exposed to the suspect toxicant, and even if they have, the duration of their exposure may be unknown. This is particularly problematic if the fish forage widely throughout a lake or river system and/or the origin of contamination is a point source.

Chemical endocrine disruptors and their modes of action

Disorders of the endocrine system in vertebrates have attracted considerable attention in the last two decades, with the discovery of environmental (anthropogenic) chemicals that act as oestrogen mimics (xeno-oestrogens), antagonize androgen function or act to interfere with thyroid hormone function. Some of these compounds are discussed later in this section. Collectively, these compounds are commonly referred to as 'endocrine disruptors' or 'endocrine-disrupting chemicals' (EDCs). Many of these chemicals find their way into surface water, and therefore fish are susceptible to any potential biological impact. Many of these chemicals are lipophilic and accumulate in lipid-rich tissues; they are transferred from maternal tissues into the yolk of the developing oocytes, and the very early-stage embryos may be exposed to a mixture of these factors. The rapid cell division and limited ability of embryos to metabolize and clear the chemicals makes them particularly vulnerable. Paradoxically, although the exposure to *in ovo* sources of the chemicals can be significant in the early (pre-hatch) embryo stages, these embryos are less susceptible to other sources of lipophilic xenobiotics, probably because the *zona pellucida* binds some forms of xenobiotic compounds and prevents their access to the embryos (Finn, 2007). After hatching, the embryos assimilate these chemicals by uptake via the gills (and possibly also via their yolk reserves). Juvenile (post-yolk sac absorption) and adult stages can potentially assimilate the chemicals from contaminated environments via both the diet and transfer across the gills.

Some of the suspected actions of EDCs will be touched upon in the last section, but

it is beyond the scope of this chapter to deal with the subject in great detail. The reader is referred to several recent publications that explore the topic in greater depth (Heath, 1995; Kime, 1998; Korach, 1998; Naz, 1999, 2004; Guillette and Crain, 2000; Norris and Carr, 2006).

These compounds may have oestrogenic, anti-oestrogenic, anti-androgenic or anti-thyroidal properties by interacting with the functioning of endocrine systems directly, interacting (as an agonist or antagonist) with hormone receptors or affecting hormone transport (Kime, 1998; Korach, 1998; Guillette and Crain, 2000; Rolland, 2000b; Naz, 2004). In addition, the xenobiotic compounds trigger a detoxification response that in some instances, such as blue sac disease (BSD), may itself have lethal consequences. The detoxification process involves the synthesis of cytochrome P450 (CYP) enzymes that can biotransform xenobiotic compounds, making them more water soluble and easier to excrete. The presence of a xenobiotic compound in hepatocytes causes the activation (by xenobiotic ligand) of transcription factors, such as aryl hydrocarbon receptor (AHR); the activated AHR forms a heterodimer with another protein, a nuclear translocator protein (ARNT), and the heterodimer transcription factor enters the nucleus of the hepatocytes and interacts with DNA in the promoter region of genes that encode for specific CYP enzymes that bring about the staged biotransformation of a range of xenobiotic classes. In addition to the AHR/ARNT-mediated CYP gene expression, some fish CYP family genes can also be controlled by nuclear pregnane X receptor, constitutive androstane receptor and retinoic acid X receptor. Further details of the processes can be found in Lindblom and Dodd (2006) and Finn (2007). Whilst these processes have the biological value of removing potentially toxic compounds from tissues, exposure of fish to complex mixtures of xenobiotic compounds elicits a complex, multifaceted, but interrelated, set of detoxifying responses to the various types of xenobiotic substances, which have significant consequences for the physiology of the animal.

Primary and secondary disorders associated with impaired hormone synthesis

The dysfunctional endocrine conditions that have been well studied in vertebrates are not only associated with the synthesis of hormones, they may also be related to hormone transport, mutant receptor proteins, hormone mimics that alter endogenous hormone production or activity, dysfunction of the normal control mechanisms, leading to the production of too little or too much hormone, and other factors. The term 'primary' is used when the disorder is related to the production of a hormone by the gland of origin. If the disorder is related to dysfunctional states of endocrine systems (such as the anterior pituitary gland) that control the end-point hormone production (such as the thyroid hormones), the term 'secondary' is applied (Katzung, 2001).

Examples of primary disorders include:

1. Mutation of genes that encode for specific peptide or protein hormones, such as insulin or PRL, respectively, which results in low plasma levels of functional hormone.
2. Mutation of genes that encode for enzymes, such as the steroidogenic enzymes, that are integral to the production of the end-point steroids; this may lead to an attenuation of the levels of the physiologically relevant hormones, but may also, paradoxically, result in an inappropriate increase in the production of precursor hormones. An example is the steroidogenic pathway leading to the synthesis of oestrogens in the ovary; impaired production of CYP 19 (P450arom), the enzyme that converts androgens to 17β-oestradiol, may lead to elevated androgen levels; innate or xenobiotic-induced impairment of hormone synthesis could bring about similar responses.
3. Toxicant exposure that enhances or suppresses the synthesis of hormones. The action of naturally occurring and anthropogenic goitrogens that impair thyroid hormone synthesis is one example. These so-called goitrogens, including the glucosinolates of canola seeds, thiocyanates and perchlorates, inhibit the iodination of thyroglobulin protein, and therefore of thyroid hormone

synthesis. Another example is the effects of several different organochlorine (OC) contaminants on the *in vitro* expression of genes encoding for some pituitary hormones (Elango *et al*., 2006), although whether this is translated into changes in hormone production is not yet known.

Examples of secondary disorders include impaired production of the hypothalamic or pituitary hormones (or of their receptors), which enhances or inhibits the secretion of end-point hormones; this might explain the sterility of some hybrid fishes, as will be discussed later.

Dysfunction of hormone receptors and intracellular signalling pathways

Mutant genes encoding for hormone receptors or dysfunctional translation of receptor protein from its mRNA are known to account for some endocrine dysfunctional states in which there are reduced physiological responses at the target cell level despite normal plasma hormone values. The target cells become insensitive to the hormone. It must be remembered that such conditions may not only affect the response to a particular hormone, but they will affect the overall effectiveness of other hormones with which it interacts in a permissive fashion. Thus, for example, reduced thyroid hormone production will affect the expression of genes that are co-regulated by thyroid hormone and steroid hormone receptors. Similarly, reduced thyroid hormone production may impair GH synthesis, because thyroid hormone receptor activation is needed for the expression of the gene that encodes for GH.

Most hormone receptors have a high affinity for a particular hormone, which gives them their ligand specificity. However, most receptors also have a lower affinity for other chemicals, which may elicit a certain level of 'non-specific' response. These factors other than the primary ligand may activate the receptor (i.e. they are receptor agonists) or they may reduce the availability of the receptor to the principal ligand and therefore inhibit the cell response to that factor (i.e. they are receptor antagonists). Some xenobiotic compounds are known to interact in either an agonistic or antagonistic manner with hormone receptors. Perhaps the best known of these in fish are the xenooestrogens (discussed later in this chapter and in Chapter 4), which are oestrogen receptor agonists.

As discussed earlier in the chapter, the activation of membrane hormone receptors triggers complex intracellular signalling events (Fig. 3.2), which commonly involve protein phosphorylation and activation, the activation of specific enzymes and changes in the flux of ions across the cell membrane. There is considerable 'cross-talk' between the pathways induced by different hormones, and some hormone–receptor interactions may activate several pathways. The details of these interactions are poorly understood, but some xenobiotics appear to exert their effects at 'post-receptor' levels (reviewed by Thomas, 1999), probably by disrupting some aspect of the intracellular signalling cascade. One specific example is found in ovarian steroidogenic cells, in which PCBs affect steroidogenesis by altering Ca^{++} flux from intracellular and extracellular stores (Benninghoff and Thomas, 2005).

Impaired hormone transport

For many hormones, the plasma total hormone concentration is directly linked to the concentration of specific transport proteins in the blood. Factors that affect the concentration of the transport protein in the blood or factors that compete with the native hormones for binding sites on the transport protein will affect the blood total hormone concentration. The synthesis of some of the transport proteins (e.g. those involved in thyroid hormone transport) is influenced by hormones of other endocrine systems (e.g. oestrogen), and thus blood total hormone concentration may change with variations in the animal's physiological state. This may not necessarily affect the blood 'free' hormone concentration, and thus the target cells may be in a normal physiological state.

There are, however, endocrine disorders associated with reductions in the hormone transport capacity of blood proteins. One example is the competition of some xenobiotic compounds for binding sites on the proteins that are involved in the transport of thyroid hormones (discussed in greater detail later in this chapter); this leads to an increased loss of the unbound ('free') hormone via gills and kidneys, which increases the activity of the hypothalamus–pituitary gland–thyroid tissue axis, resulting in a benign hypertrophic and hyperplastic enlargement of the thyroid tissue (the formation of a goitre).

Impaired clearance of steroid and thyroid hormones

Genetic conditions associated with mutant genes for steroidogenic enzymes account for some of the known adrenal and gonadal conditions in mammals, but there is no record of such conditions in fish. Xenobiotic-induced impaired expression of the genes encoding for the enzymes involved in the steroid biotransformation in steroidogenesis and steroid metabolism, and in thyroid hormones' metabolism may impair the clearance of biologically active forms of the hormones. However, there is considerable redundancy in the intracellular pathways that regulate cellular responses to physiological change, and thus compensatory responses may ameliorate the effects of reduced enzyme production. This possibility of impaired hormone clearance as a potentially important site of toxicant action has been proposed for fish embryos; the expression of key genes and related developmental events appear to be closely linked to the embryo's hormonal environment. If the embryo is exposed to hormone mimics that cannot be metabolized and cleared from the animal's tissues, there is the potential for disruption of the normal pattern of gene expression and altered phenotypic outcomes. One example of this is the finding of sustained changes in immunocompentency of salmonid fishes following a single, *in ovo* exposure to one of the metabolites of DDT, o,p'-DDE (Milston *et al.*, 2003).

Disorders of the pituitary gland

Only few reports of pituitary gland disorders in fish appear in the literature, and most of these pertain to highly inbred individuals or hybrid forms. Histopathological pituitary lesions, comprising largely GtH-secreting cells (basophilic adenomas), have been reported in specimen cases of guppy (*P. reticulata*), molly (*Molliensia velifera*), Indian catfish (*Mystus seenghala*), and in a large sampling of wild carp–goldfish (*Cyprinus carpio–Carassius auratus*) hybrids taken from one region of Lake Ontario, Canada (reviewed by Leatherland and Down, 2001). In the case of the carp–goldfish hybrids, the lesions (Fig. 3.20) were associated with high pituitary and plasma GtH content but normal cytology of the GtH secreting cells. The fish also exhibited gonadal lesions of various types (Down *et al.*, 1988, 1990; Down and Leatherland, 1989), and are probably symptomatic of impaired gonadal steroidogenesis. The basophilic adenomas reported in the other species may also be linked to species hybridization and related gonadal dysfunction, but no data were collected to test that hypothesis.

Hypertrophy of pituitary TSH-secreting cells has also been reported in several species of salmonid fish collected from several of the North American Great Lakes (Leatherland and Sonstegard, 1980). These are related to the goitres of salmonid and other species (discussed later in this chapter). Reports of similar histological changes have been reported in case studies of other species; these may also be responses to reduced plasma thyroid hormone concentrations or to other factors that influence thyroid hormone homeostasis (Leatherland, 1982), but the data are not available to assess that possibility.

A single study of the effect of the herbicide 2,6 nitro-N, N-dipropyl-4-(trifluoromethyl) benzamine on sheepshead minnows (*Cyprinodon variegates*) reported fluid-filled pseudocysts in the anterior and posterior pituitary glands of over 50% of the fish exposed to the herbicide for 19 months (Couch, 1984); the cells types involved were not identified.

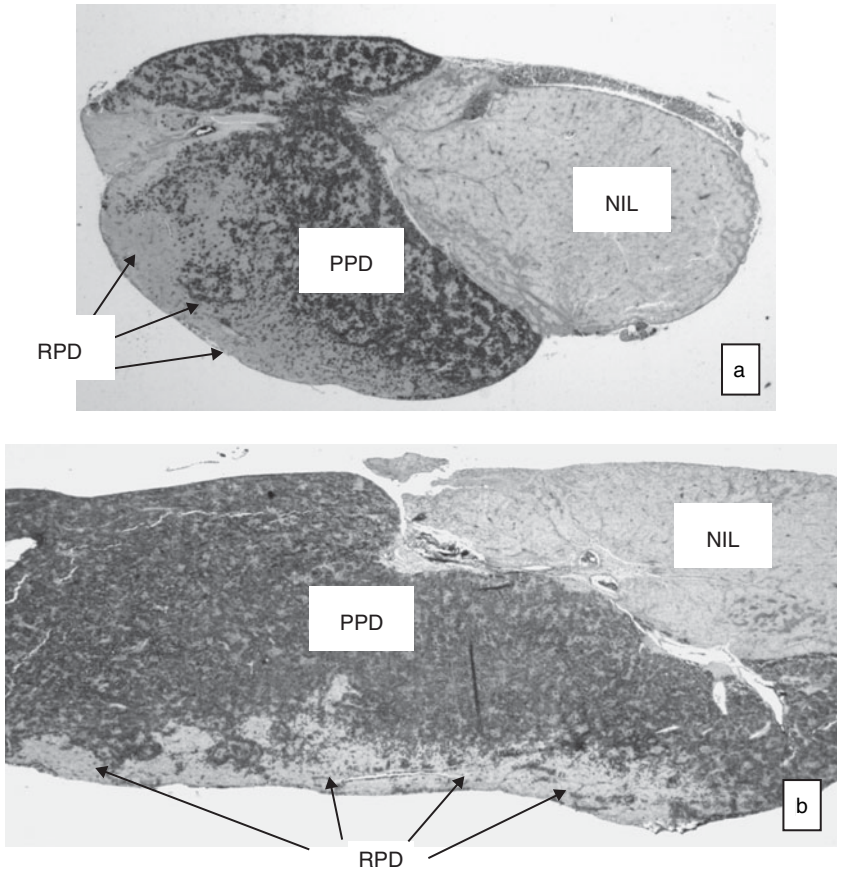


Fig. 3.20. Histological sections of the pituitary gland of a carp (*Cyprinus carpio*) (a) and a carp × goldfish (*Carassius auratus*) hybrid (b). The figure shows, in low magnification, sagittal or parasagittal sections of the pituitary gland; the images are of the same magnification. Although the pituitary gland of the hybrid is much larger than that of the carp, the two animals were of a similar age, and the carp was approximately three times larger than the hybrid. The section of the carp shows the *rostral* (marked with arrows) and *proximal pars distalis* (RPD and PPD, respectively) of the anterior pituitary gland, and the neurointermediate lobe (NIL), which comprises the axons of the *pars nervosa* interspersed with nodules of cells of the *pars intermedia*. Note the appearance of the PPD; the dark cells are basophilic-staining cells, predominantly gonadotropic hormone (GtH)-secreting cells, together with a smaller number of thyrotropin (TSH)-secreting cells; the pale cells in this region are growth hormone (GH)-secreting cells. Dark-staining cells (predominantly TSH-secreting cells) are also present in the RPD, but most of the RPD is made up of prolactin (PRL)-secreting cells. In the pituitary gland of the hybrid (b), the NIL and RPD are of similar size and cell composition is as found in the carp pituitary gland; however, the PPD is greatly enlarged. The increase in size is caused by hypertrophy and hyperplasia of the GtH-secreting cells. The tissue is partly fragmented because of compaction of the adenoma in the *sella tursica*, the cavity in the floor of the skull that normally encases the gland.

Disorders of the thyroid gland

Formation of goitres

For more details of this process, the reader is referred to earlier reviews (Leatherland, 1994; Leatherland and Down, 2001). As discussed previously, iodide is required for the synthesis of thyroid hormones. For mammals, the iodide is garnered from dietary sources, and dietary iodide insufficiency may lead to decreased plasma thyroid hormone

concentrations and clinical signs of hypothyroidism. The reduced plasma thyroid hormone levels trigger the increased synthesis and release of TSH, which stimulates the growth in size and number of the thyrocytes, leading to the formation of a simple goitre. Because of the iodide deficiency, the thyroid cannot increase thyroid hormone synthesis. In fish, iodide is obtained from both diet and ambient water, and experimentally inducing iodide deficiency to the point that gives rise to clinical hypothyroidism is very difficult to achieve in most fish species, even in extreme experimental situations.

Goitres associated with *hypothyroidism* may also form in situations where there is sufficient plasma iodide for potential synthesis of the thyroid hormones. Some naturally occurring and synthetic chemicals impair the incorporation of iodide into the thyroglobulin molecule. These chemicals act either to inhibit iodide uptake by the NIS protein or to inhibit the oxidative iodination of the thyroglobulin, thus impairing the animal's ability to synthesize thyroid hormones. Yet other goitres may be caused by impaired ability of the animal to take up iodide from environmental sources (dietary or waterborne). Exposure to nitrates, dietary or waterborne, has been associated with the formation of goitres in many vertebrates (Chaoui *et al.*, 2004; Eskiocak *et al.*, 2005), probably because of competition of nitrate and iodide for the same ion uptake system. Goitres of this potential aetiology have been found in fish (see below).

Goitres associate with *hyperthyroidism* are also known in mammals. These thyrotoxic goitres are associated with the secretion of excessive amounts of thyroid hormone caused by inappropriate stimulation of thyrocytes. The best known of these thyrotoxic goitres, Grave's disease, is caused by an autoimmune condition in which antibodies are produced to the subject's own TSH receptor (TSH-R). The TSH-R antibodies bind to the receptor close to the site of normal TSH attachment, and in so doing activate the receptor and promote thyroid hormone synthesis. There are no reports of goitres associated with hyperthyroidism in fish.

Some goitres are caused by factors that lead to inappropriate excretion of plasma thyroid hormones, usually via the kidney. Some anthropogenic chemicals, such as some congeners of polychlorinated biphenyls (PCBs), compete with thyroid hormones for the binding sites on the blood thyroid hormone transport proteins; some of these congeners have a higher affinity for the proteins than the thyroid hormones. This displacement of thyroid hormones from the transport protein leads to an increase in 'free' thyroid hormone, which is more vulnerable to loss via the kidney, and possibly also via the gills. The reduced plasma thyroid hormone levels induces a compensatory increase in the activity of the hypothalamus–pituitary gland axis, resulting in increased TSH production, which in turn stimulates an increase in growth of thyroid tissue and an accompanying increase in the production of thyroid hormone. For these conditions, clinical signs of hyper- or hypothyroidism are not usually evident. This type of goitre probably accounts for some of the reports of thyroid lesions in fish.

Goitres in fish: iodide deficiency or other aetiology?

Goitres (thyroid hyperplasia) (Fig. 3.21) represent the most commonly reported endocrine disorder in fishes. Such lesions have been reported in approximately 70 species from 28 Orders of bony fishes (Leatherland and Down, 2001) and approximately 20 species from 6 Orders of cartilaginous fishes (Crow *et al.*, 1998; Leatherland and Down, 2001). For the most part, these lesions (Fig. 3.22) have the appearance of simple hyperplasia (Leatherland and Down, 2001). Many of the case reports of bony fishes are of captive specimens; some held in seawater or brackish water, and usually fed commercial or natural diets that are iodide replete; iodide deficiency does not appear to explain the phenomenon. Most of the lesions found in cartilaginous fishes are also from captive specimens held in full seawater and fed diets that contain iodide; similarly, iodide deficiency would not appear to be an issue. However, Crow *et al.* (1998) report reductions

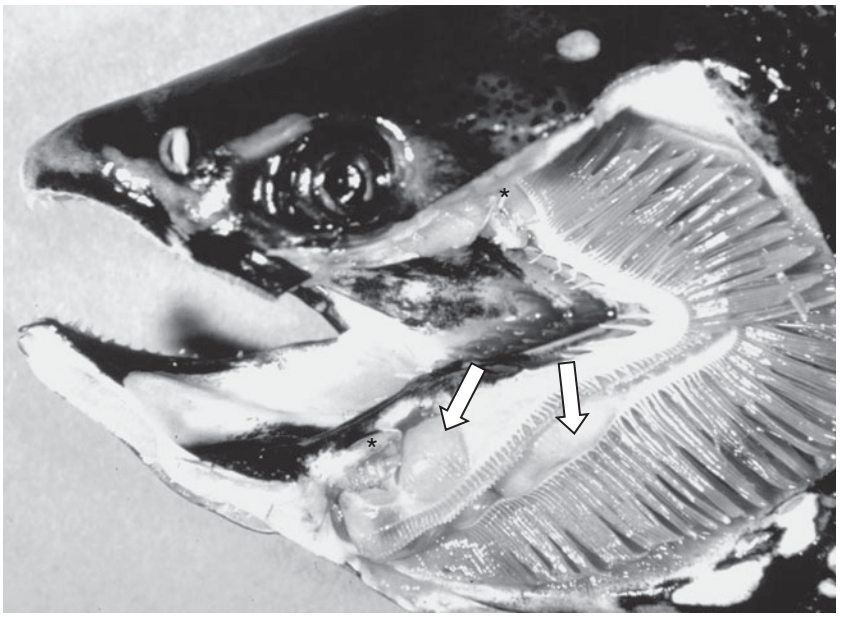


Fig. 3.21. Gross appearance of a goitre in an adult, sexually mature coho salmon (*Oncorhynchus kisutch*) collected from one of the Great Lakes of North America. The operculum has been removed to show the gill arches, and the first gill arch has been removed (the asterisks indicate the upper and lower insertion points of the gill arch) and the gill filaments of the second gill arch trimmed to show the lesions. Nodules (lesions) that contain thyroid tissue (marked with open arrows) can be seen at the base of the second and third gill arches.

in the size of the lesions in white-tip reef sharks (*Triaenodon obesus*) transferred from seawater that had low iodide and high nitrate to lagoon seawater containing high iodide and low nitrate. Although iodide availability might have been an issue in this study, nitrate toxicity might also be the cause. Nitrate and iodide uptake occurs via a common pathway, and high environmental nitrate is known to impair thyroid hormone synthesis (Chaoui *et al.*, 2004; Eskiocak *et al.*, 2005), resulting in goitre formation.

Naturally occurring goitrogenic chemicals, such as the glucosinolates found in some foods such as cassava, the cabbage family generally and canola meal, cause goitres in mammals, usually by interfering with iodide uptake or iodination of thyroglobulin, and thus reducing thyroid hormone synthesis. Goitres in some human populations have been linked to goitrogens of bacterial origin that are present in drinking water (Vought *et al.*, 1974; Gaitán *et al.*, 1980; Gaitán, 1986). It is possible that some of the goitres seen in fishes have a similar aetiology. A common feature for many of the reported cases of goitres in bony and cartilaginous fishes is that they are held in captivity in circulating and filtered water systems. The filter systems rely on bacterial action to reduce the accumulation of organic materials, and it is possible that these bacteria are the source of goitrogens, which are probably metabolic by-products. Goitrogens of microbial origin may explain the thyroid lesions that have been found in salmonid species introduced into the Great Lakes of North America and in other species held in re-circulating aquarium systems (e.g. killifish, Fig. 3.22c).

Aetiology of goitres in salmon from the Great Lakes: a cautionary tale

In the 1970s and 1980s, thyroid tumours (Fig. 3.21) were reported in epizootic proportions in the salmon that had been

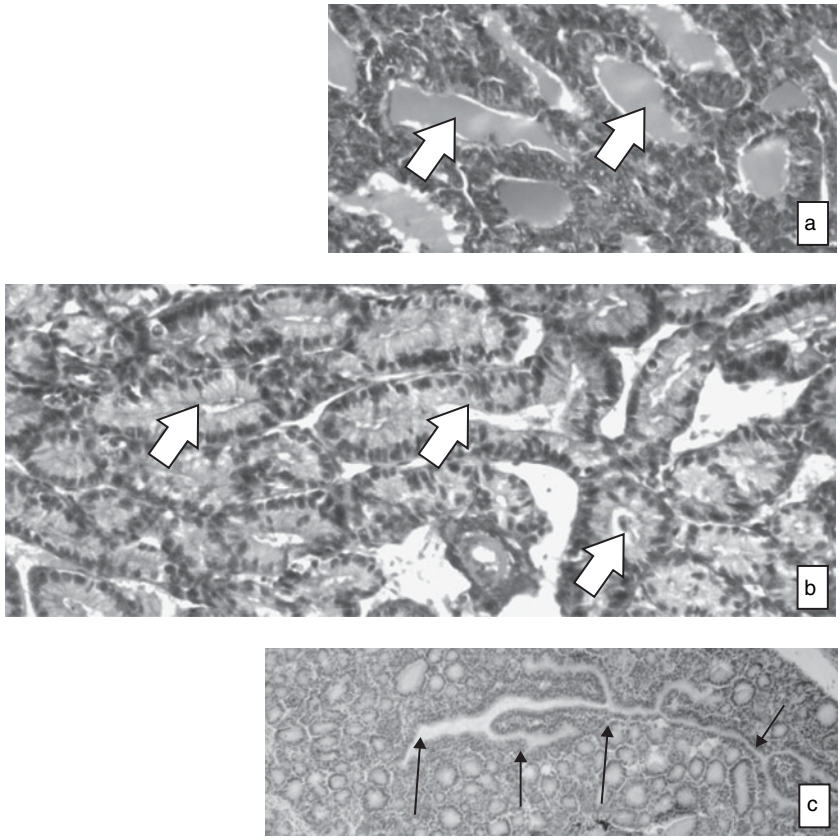


Fig. 3.22. Histological sections of thyroid tissue in several fish species. Two of the figures (a) and (b) show sections of thyroid tissue contained in the type of goitre shown in Fig. 3.21. Some of the tissue contained thyroid follicles that contained colloid (open arrows), as shown in (a), but these were restricted to small areas of the tissue. Note the large size of the thyrocytes compared with the normal thyroid tissue shown in Fig. 3.11. The vesicles in the periphery of the colloid in some follicles represent areas from which the colloid has been removed by endocytosis into the thyrocytes. The tissue shown in (b) is more common. Note the lack of colloid (examples marked by arrows) in the lumen of the follicles; also note the tubular nature of many of the 'follicles'. (c) shows part of the goitre of a killifish (*Fundulus heteroclitus*); note the tubular nature of the thyroid tissue in this preparation also (arrows).

introduced to the Great Lakes of North America as part of an effort to rehabilitate the Great Lakes and re-establish fish populations. Coho (*Oncorhynchus kisutch*), chinook (*Oncorhynchys tshawytscha*) and pink salmon (*Oncorhyncus gorbuscha*) taken from Lakes Ontario, Michigan, Erie, Huron and Superior were affected, with the prevalence of gross lesions being close to 100% in some cohorts in some study years (Leatherland, 1992). Iodide insufficiency was discounted as a causative factor, based on two observations. The total tissue iodide levels and the plasma thyroid hormone concentrations of the Great Lakes salmon were similar to those of wild salmon migrating from the Pacific Ocean into rivers in British Columbia. These two findings suggested that the Great Lakes fish were not iodide-deficient (Leatherland, 1993).

The condition was subsequently attributed to the effects of anthropogenic chemicals, such as PCBs, because these organochlorine (OC) compounds were known to induce thyroid enlargement in rats (Bastomsky *et al.*, 1976), and OC levels in the ecosystems of the Great Lakes were extremely high

(Colborn *et al.*, 1990). The very high body burdens of PCBs and other OC compounds in the salmon from some of the lakes tended to support the OC aetiology hypothesis; however, there was no correlation between the size and severity of the thyroid lesions and the OC body burden of the fish from different lakes in the Great Lakes system. For example, Lake Erie salmon had by far the lowest OC content, but the highest prevalence of large lesions. Moreover, in 'fish-to-fish' studies, in which salmon and trout were fed diets made from the 'naturally contaminated' Great Lakes salmon, and studies in which trout were fed diets containing PCBs and the pesticide Mirex (a major contaminant in Lake Ontario), thyroid lesions of the type found in the wild fish were not found. Paradoxically, 'fish-to-rodent' studies, in which 'naturally contaminated' Great Lakes salmon were fed to rats and mice, did result in the formation of goitres, and the severity of the lesions was proportional to the levels of OC contamination in the fish-based diets (Cleland *et al.*, 1988; Leatherland, 1998). Moreover, fish-eating birds in the Great Lakes region developed goitres, as did captive mink that were fed fish from the Great Lakes. Taken together, these findings suggest that the 'naturally occurring' goitres of the salmon were not caused by the accumulated OCs. However, these fish induced goitres in rodents and in fish-eating wildlife. A possible explanation for this apparent paradox is presented below.

Although the OC levels in the wild Great Lakes salmon were not correlated with the size and prevalence of the thyroid lesions, there was a strong correlation between the size of the lesions, the prevalence of gross lesions and the degree of eutrophication of the lakes. Salmon from a very eutrophic lake, such as Lake Erie, had a significantly higher prevalence and larger lesions compared with salmon from less eutrophic lakes, such as Lake Superior. This may suggest that the goitres have a microbial aetiology similar to that found in human populations, as discussed previously. In support of the hypothesis were the findings that water samples taken from Lake Erie were found to contain chemicals that inhibited iodination of thyroglobulin in an *in vitro* pig thyroid assay (E. Gaitán, J.F. Leatherland and R.A. Sonstegard, unpublished data).

The ubiquitous nature of the thyroid lesions in salmon throughout the Great Lakes system is consistent with the possibility of water-borne goitrogens being present, at different levels in all of the lakes studied. But the apparent resistance of the salmon to their OC contamination remains to be explained. Several factors may be involved: (i) differences in the metabolic rate (MR) of fish compared with birds and mammals, and the key role that thyroid hormones play in MR regulation in endothermic animals; (ii) the distribution of the contaminants in the salmon, largely in adipocytes; and (iii) the characteristics of transport of thyroid hormones in the blood of fish compared with mammals. First, thyroid hormone secretion rates in birds and mammals are considerably higher than in fish, thus any disturbance of thyroid homeostasis would be more critical to the endothermic animals. Second, because of the lipophilic nature of OCs, the vast majority of the OC body burden of the fish was in the lipid fraction, probably in adipose tissue, and not in the blood. Thus the exposure of body tissues to blood-borne OC would be low, and hence pathophysiological responses would also, theoretically, be low. In both the 'fish-to-fish' and 'fish-to-rodent' feeding trials, the post-prandial plasma OC levels would presumably be high and then fall as the lipophilic compounds were incorporated in adipocytes. The differences in responses of the recipient fish and rodents suggest that the response of the mammalian thyroid to OCs in mammals is much higher than that of the fish thyroid. A third possible explanation of the difference is the manner in which the OCs affect thyroid hormone homeostasis in fish compared with mammals. In mammals (and probably also birds), the goitrogenic action of the OCs appears to be due to competition with the thyroid hormones for binding sites on the main thyroid hormone transport protein: thyroxine-binding globulin (TBG) in most mammals and transthyretin in rodents. Under normal conditions, greater than 99.9% of the plasma total thyroid hormone in mammals is bound

to these constitutive blood proteins, but in the presence of OC, there is a reduction in the amount of hormone bound to TBG, leading to the loss of 'free' thyroid hormone via the kidney and a subsequent increase in the synthesis and secretion of TSH; the goitres seen in the rodent studies are the result of the increased TSH stimulation. The relatively low sensitivity of fish to OC exposure, compared with mammals (and birds), is possibly related to the nature of the binding proteins, or perhaps to differences in the amount of thyroid hormone that is bound to blood transport proteins. TBG is not found in the fish studied to date (mostly salmonid species); the thyroid hormones bind to albumin and pre-albumin proteins, and the per cent of free thyroid hormone is much higher than that seen in mammals (Eales and Brown, 1993). Consequently, OC-induced displacement of thyroid hormone from the transport proteins may be much less severe in fish than in mammals, and hence the absence of goitre formation in OC-exposed fish.

Disorders of the thyroid tissue associated with anthropogenic environmental chemicals

There is considerable evidence to suggest that many anthropogenic chemicals affect thyroid hormone homeostasis in vertebrates. The excellent reviews by Bruckner-Davis (1998), Rolland (2000a) and Boas *et al.* (2006) examine the range of chemicals that have been identified as having anti-thyroidal effects, as well as the endocrine responses to these chemicals in many vertebrate species. The range of chemicals that have proven effects is broad and includes, amongst others, PCBs, dioxins, dibenzofurans, flame retardants and phthalates used in the production of plastics, all of which are present at high levels in the environment. Relatively few studies have examined the effects of contaminants on thyroid function in fish. Carbamate compounds, several OCs and some heavy metals have been reported to alter plasma thyroid hormone levels (Bruckner-Davis, 1998), but in most cases the responses were small and the doses of contaminants applied were very high. Even very high levels of dietary PCBs or Mirex, both of which cause changes in plasma thyroid hormone levels and thyroid gland enlargement (goitre) in rodents, failed to cause consistent changes in plasma thyroid hormone or thyroid histology in trout or salmon (Leatherland and Sonstegard, 1979). Thus, the evidence that suggests a marked effect of anthropogenic chemicals, at levels present in impacted ecosystems, on fish thyroid function is not convincing. The relatively small reported changes could easily be argued as compensatory responses to contaminant-induced alterations in metabolism. However, other fish species may be more susceptible; for example, Adams *et al.* (2000) reported transient thyroid hormone homeostasis responses following injection of American plaice (*Hippoglossoides platessoides*) with one of two PCB congeners (77 and 126); the reported responses included changes in hepatic monodeiodination activity and plasma T_3 concentrations.

Disorders associated with the hypothalamus–pituitary gland–interrenal gland axis and immunocompetence

The response of the hypothalamus–pituitary gland–interrenal gland (HPI) axis to stressors forms the subject of Chapter 6, this volume, and will not be dealt with at length here. There is mounting evidence, however, to show that xenobiotic factors influence the function of the HPI axis and subsequently affect the immune responses of fish. For example, Norris (2000) reported an impaired stress response in brown trout (*Salmo trutta*) collected from environments containing high levels of heavy metals; Hontela *et al.* (1992) reported impaired cortisol responses in yellow perch (*Perca flavescens*) and northern pike (*Esox lucius*) collected from aquatic systems contaminated with polyaromatic hydrocarbons, PCBs and mercury; Milston *et al.* (2003) reported that a one-time (*in ovo*) exposure of chinook salmon (*O. tshawytscha*) to o,p'-DDE had long-term effects on humoral immunocompetency, and Stouthart *et al.* (1998) reported changes in whole-body ACTH, α-MSH and cortisol levels in carp embryos that had been reared from eggs treated with PCB 126 at the time of fertilization.

The immunosuppressive actions of PCBs and polyhalogenated aromatic hydrocarbons (PHAH) are reviewed by Reynaud and Deschaux (2006) and Bowden (2008) and in Chapter 9, this volume. Whilst there is no discounting the immunotoxic nature of PHAHs in fish, the effects vary greatly depending on the mode of exposure and the doses applied; in addition, the responses to the xenobiotics depended greatly on the developmental stage and age of the fish (Duffy *et al.*, 2002). Recent findings suggest that xenobiotics exert a range of actions on the immune system in fish. For example, Cuesta *et al.* (2008) studied the effects of ppDDE and lindane on the activity of head kidney leucocytes of gilthead seabream; they found that whereas there appeared to be no negative effects on cell viability, there was upregulation of eight immune-related genes (including IL-1β and TNFα). Eder *et al.* (2008) examined the effects of the insecticides chlorpyrifos and esfenvalerate on chinook salmon before and after exposure to infectious haematopoietic necrosis virus (IHNV); the pesticides did not affect mortality rates, but there were significant changes in spleen and kidney cytokine (Mx protein, IL-1β, IGF-1 and TGF-β) expression, both upregulation and downregulation depending on the cytokine; these responses were clearly indicative of altered immune response. In another study examining the effects of the organophosphopesticide diazinon and one of its metabolites on Nile tilapia, Girón-Pérez *et al.* (2008) examined proliferation and acetyl choline content of spleen cells; the lymphoproliferative response of spleen cell mitogenic activity was not affected, but spleen ACh content was suppressed, as was the ACh-driven lymphoproliferation, suggesting a role for cholinergic processes in immune responses to xenobiotics.

Reproductive and Developmental Disorders in Fish

Recognizing the problems

With the possible exception of reports of the gonadal tumours mentioned below, and intersex and sterile fish of several species collected from several locations around the globe, there are very few reports of direct gonadal dysfunction in fishes. Although this might suggest that dysfunctional conditions are very rare, it is also quite possible that the lack of published reports is due to the lack of appropriate studies. Epizootiological studies that screen species for the prevalence of specific conditions such as gonadal problems require that large numbers of fish be sampled and large numbers of gross and histological preparations be assessed. Studies of that type are rare. The broad categories of hypothalamus–pituitary gland–gonadal (HPG) axis disorders are: (i) disorders related to problems in the development of the HPG axis, commonly found in hybrids and highly inbred stocks, and often characterized by sterility due to impaired gametogenesis, sometimes together with the presence of tumours (Figs 3.23–3.25); (ii) problems linked to the actions of xenobiotic compounds, commonly exerting their effects by impairing the normal endocrine regulation of gonadal function and consequently reducing reproductive success (see below) or affecting the development of embryos and early juveniles developmental stages; and (iii) various putative reproductive disorders linked to stressors of various kinds. In addition, several anomalous conditions such as gonadal cysts (Leatherland and Ferguson, 2006) are occasionally reported, which do not readily fit into these categories.

Although certain reproductive dysfunctional states, such as sterility, gonadal tumours and ovarian cysts, have been attributed to environmental factors, either xenobiotic factors or other environmental features that negatively affect reproduction by endocrine-related pathways, the evidence tends to suggest that other factors are involved. Sterility can be the result of problems at several levels in the hypothalamus–pituitary gland–gonadal axis, and the most common form is evident in hybrid fishes or intensely inbred captive fish, and it probably has a genetic aetiology. The best studied of these is the carp × goldfish hybrid population in an area of Lake Ontario, Canada (see review by Leatherland and Down, 2001) (Fig. 3.25).

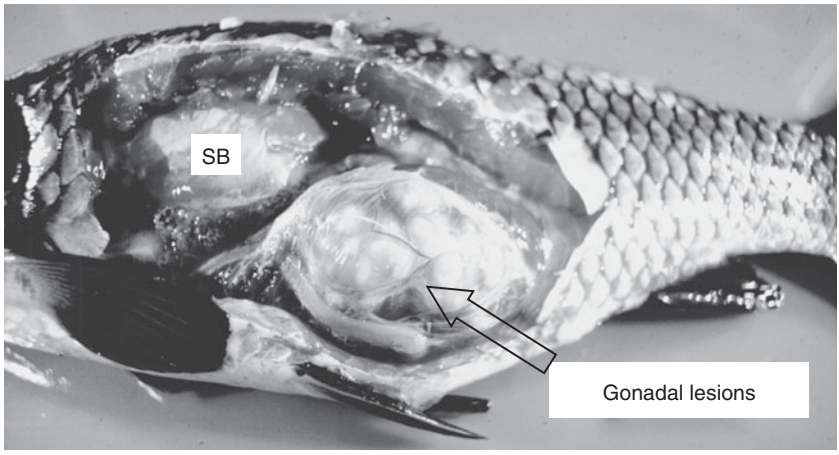


Fig. 3.23. Gross appearance of a gonadal tumour in a carp (*Cyprinus carpio*) × goldfish (*Carassius auratus*) hybrid (the abdominal wall has been removed to show the lesions). The animal is phenotypically female. Note the large mass of solid and cystic gonadal lesions; the swimbladder (SB) is labelled for reference.



Fig. 3.24. Gross appearance of a testis dissected from a phenotypic male carp (*Cyprinus carpio*) × goldfish (*Carassius auratus*) hybrid. The image shows multiple overt nodular lesions along the length of the testis.

Very commonly, these sterile conditions are associated with gonadal tumours, which have been postulated to be seminomas, dysgerminomas, teratomas and Sertoli cell tumours, and with pituitary adenomas. Ovarian cysts are rarely reported (Leatherland and Down, 2001) and where found are usually in fish that have failed to ovulate, and are therefore possibly linked to collateral endocrine dysfunction.

Stressor-related (possibly due to elevated cortisol levels) impaired reproductive function, particularly in cultured species, has been reported; the outcome is usually reported to be poor egg quality (see Reddy and Leatherland, 1998 and Chapter 6, this volume, for references). However, the studies have not always been consistent or repeatable, and some authors have been unable to demonstrate any detrimental actions of aquaculture practices or cortisol treatment on gonadal steroidogenesis or the quality of gametes (Leatherland, 1999). The literature that reports such effects shows changes in feeding activity of the stressed fish, changes in the size of oocytes (presumably linked to

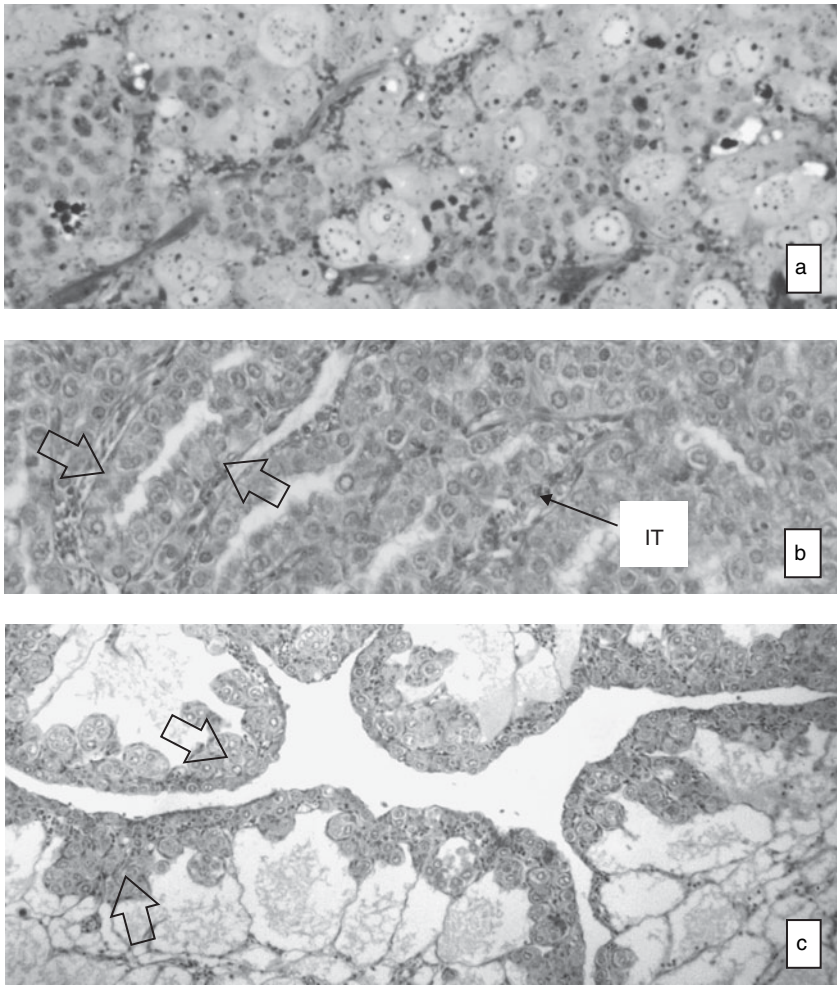


Fig. 3.25. Histological sections of the gross lesions shown in Figs 3.23 and 3.24. (a) shows the gonad of a phenotypic male – a germ cell tumour; only very early gamete cells are evident. (b) shows a Sertoli cell tumour, with enlarged Sertoli cells (open arrows) and relatively few germ cells present; IT, interstitial tissue. (c) shows part of an ovary that contains only primary oogonia (arrows).

altered feeding behaviour) and, in some cases, even outbreaks of infectious disease. The evidence for direct actions of the stress axis hormones, whether primary (i.e. elevated catecholamine levels) or secondary (i.e. elevated glucocorticoid levels), is inconsistent and not convincing (Leatherland, 1999). However, there is strong evidence to link elevated maternal cortisol levels with elevated egg cortisol levels and negative impacts of elevated egg cortisol levels on embryo development, growth and survival of salmonid fishes (Eriksen *et al.*, 2006, 2007; Mingist *et al.*, 2007), but this was not found for channel catfish (*Ictalurus punctatus*) (Small, 2004).

Most other forms of reproductive and developmental problems in fish have been attributed to the effects of environmental contaminants. Whilst this is probably true for many of the reported cases, some caution is needed in interpreting the available evidence before proposing cause–effect relationships between xenobiotic compounds

and reproductive failure. Several examples of issues related to the a priori assumption of a xenobiotic cause of reproductive failure follow.

As discussed in Chapter 1, this volume, the disappearance of fish stocks from a particular ecosystem is sometimes used (even retrospectively) as an indicator of contaminant-related reproductive dysfunction; the assumption made is that putative contaminants have had a negative impact on reproduction. However, a progressive increase in contaminant levels in aquatic ecosystems is commonly accompanied by an increased human impact on that physical characteristics of that system, such as a decrease in overall water quality, a reduced availability of forage, destruction of spawning habitats and changes in water temperature, all of which may negatively influence the choice of habitat for a particular species. The loss of a stock or a population may be indirectly related to the overall destruction of the habitat, not to chemical-induced impairment of reproductive capacity. The major loss of lake trout (*Salvelinus namaycush*) from the Great Lakes that occurred between the early 1940s and late 1950s has been linked to the increasing levels of DDT during that period, and the decline in Atlantic salmon (*Salmo salar*) stocks in the Atlantic Ocean off New Brunswick, Canada was attributed to increasing levels of nonylphenol, a known xenooestrogen in salmonid fishes (Madsen *et al.*, 1997); however, although the loss of these stocks is commonly cited as evidence of a contaminant cause–effect relationship, the evidence for a direct association is still not definitive (Rolland, 2000b).

Changes in phenotypic expression have also been postulated as an indicator of contaminant-related reproductive dysfunction. For example, epizootics of poorly expressed secondary sexual characteristics in male coho salmon in the Great Lakes were initially attributed to OC-induced impairment of gonadal steroidogenesis; however, the 'problem' was subsequently shown to be due to loss of mate competition. The gametes from all Great Lakes stocks were manually stripped from the adults, thus by-passing the normal biological mate selection for sexual characteristics. Within a few generations, the hooked jaw and coloured flanks of the adult males had been lost, and phenotypic differences in coloration between sexually mature males and females were largely absent (Leatherland, 1993).

Similarly, the death of hatchery stocks of Atlantic salmon (*S. salar*) embryos in both the North American Great Lakes and the Baltic Sea was initially thought to be caused by an unknown toxicant. The condition was separately identified in North America, where it was called Early Mortality Syndrome (EMS), and in Europe, where it was called M74, because it was first described 1974. Entire cohorts of embryos died within a very short period at the late yolk-sac absorption stage, when approximately two-thirds of the yolk has been absorbed. Subsequent studies have shown that EMS is not caused by contaminants; it appears to be a thiamine deficiency, which can be avoided by a single immersion of the embryos in a solution of thiamine (Börjeson and Norrgren, 1997). The thiamine deficiency appears to be caused by loss of preferred forage species and the salmon resorting to use alternate species that contain thiaminase, which depletes the thiamine reserves of the adult females, resulting in a reduced transfer of thiamine to the oocytes during egg formation. The result is insufficient thiamine being available for the final development of the embryos.

Impaired reproduction associated with environmental chemicals

The caveats concerning the interpretation of field studies notwithstanding, there is substantial direct and indirect evidence in support of the hypothesis that many anthropogenic chemicals present in aquatic and terrestrial ecosystems affect reproductive and developmental events in vertebrates. The reviews by Colborn *et al.* (1993) and Daston *et al.* (1997) list the range of chemicals that are suspected of impairing reproductive function in fish and other vertebrates; Short and Colborn (1999) summarize the quantity of these chemicals that are used annually in the USA. There is still controversy as to

whether these factors affect human health, but the consensus is that fish and other wildlife species are impacted (Daston *et al.*, 1997). It is beyond the scope of this chapter to review in detail all of the available literature dealing with environmental contaminant effects on reproduction and early development in fishes and the reader is directed to the excellent detailed overview of the topic by Rolland (2000b) and others cited below. Some of the best-established contaminant-associated situations and disorders are summarized in the following sections.

The contaminants most commonly cited as causative agents include the organochlorines (OCs), nonyphenols and heavy metals (Colborn *et al.*, 1993), and representatives of these chemical families are now ubiquitous in the body tissues of most animals. In addition, the inclusion of phyto-oestrogens in commercial fish diets has been found to affect gonadal function (Green and Kelly, 2008). Because of their wide distribution, cause–effect relationships between specific chemicals and specific pathophysiological responses are not always possible, particularly in field studies. Contaminated sites have varying concentrations of a range of chemicals, and each chemical may exert an effect on a particular aspect of the hypothalamus–pituitary gland–gonad axis or the transport of hormones in the blood, or affect the binding of native hormones with their receptors.

The global findings of relatively higher prevalence of impaired reproductive function in fish collected from suspected 'contaminated' compared with 'uncontaminated' sites has led to speculation about a link between impaired reproductive events and one or more of the contaminants. White croaker (*Genyonemus lineatus*) and kelp bass (*Paralabrax clathratus*) from the Pacific Ocean off the coast of California have expressed reproductive-impaired conditions that have been tentatively linked to sewage and industrial discharges (Cross and Hose, 1988; Spies and Thomas, 1997). Tentative associations between environmental OC contaminants and impaired reproductive success have been made for burbot (*Lota lota*) and cod (*Gadus morhua*) in the Baltic Sea, and English sole (*Parophrys vetulus*) in the Pacific Ocean off Washington State (reviewed by Rolland, 2000b).

Concerns over the possible impact of the release of bleached kraft mill effluent (BKME) into natural environments has led to a series of studies examining the toxicity of the complex mixture on reproductive physiology of wild and captive fishes; these studies have been underway for over 30 years. The reader is referred to the following sources for more detailed information (Servos, 1996; Braunbeck *et al.*, 1998).

BKME contains OCs such as dioxins and dibenzofurans, as well as the phytosterols that are extracted from the wood used in the pulping mills. Fish collected from BKME effluent-impacted lake systems exhibit multiple reproductive problems, including delayed gonadal maturation, reduced size of gonads, changes in steroidogenesis and impaired expression of secondary sexual characteristics; taken together these are indicative of multiple sites of action of the chemical mixtures in BKME (Rolland, 1990b; Rickwood *et al.*, 2006). The complex nature of the effluent and, in some instances, the transitory nature of the responses has made it very difficult to identify which factor (or factors) is responsible for the reproductive responses (or altered stress-responses (Hontela *et al.*, 1997)).

Intersex conditions, in which gonochoristic fish develop both male and female gametes, have been reported in several cyprinid species (Jobling *et al.*, 1998; Nolan *et al.*, 2001; van Aerle *et al.*, 2001; Faller *et al.*, 2003); the condition is most commonly associated with sewage effluent exposure, probably caused by the exposure of phenotypic male fish to oestrogen in the sewage effluent; the oestrogens may be native steroid or pharmaceutical steroid that is not removed during primary sewage treatments. Many of the reported environmentally induced intersex conditions appear in phenotypic male fish, although both sexes are sensitive to steroidal disruption, particularly at early developmental stages (Piferrer, 2001; Devlin and Nagahama, 2002). Also, some naturally occurring intersex conditions have been reported, but for the most part these also have an unrecognized xenobiotic aetiology.

The topic of intersex conditions in fish is dealt with at length in Chapter 4.

The xeno-oestrogens in sewage also induce vitellogenesis (Arukwe and Goksøyr, 2003); hepatic vitellogenesis, a key process during the growth and maturation of oocytes does not normally occur in males or immature females because the circulating levels of oestrogen are low. However, the blood VtG levels are relatively high in male (and immature female) fish exposed to sewage effluent, and this bio-indicator has been used as a biomarker of xeno-oestrogen exposure of a population. The physiological consequences of induced VtG secretion are not fully comprehended, but the energetic costs of VtG synthesis are high, and energy that is normally directed toward somatic growth may be redirected, thus affecting the growth potential of the animal. Also, the induced secretion of the phospholipoprotein at high levels will undoubtedly increase the blood viscosity and impose increased burdens on the cardiovascular system.

Contaminant-related impairment of embryo development

Fish embryos from the zygote stage to yolk-sac absorption stage, particularly the post-hatched embryos, appear to be the most vulnerable, and laboratory studies have shown that these early stages respond to toxicant levels that do not affect adult stages (Rolland, 2000b; Finn, 2007). This phenomenon is particularly problematic for fish species that produce lipid-rich yolky eggs. Lipophilic xenobiotic chemicals are transferred from the maternal blood to the lipid-rich oocytes, possibly in association with vitellogenin. During the development of the embryo, as the yolk is mobilized and metabolized, the developing embryo will potentially be exposed to the effects of the xenobiotic compound, many of which have oestrogenic or anti-androgenic properties. The xenobiotic compounds may also impair the ability of the embryo to metabolize and excrete the naturally occurring hormones that are also present in the yolk, resulting in indirect xenobiotic-related effects. When considering the effects of lipophilic contaminants on early developmental stages, there are several considerations: (i) actions that affect very early gene expression may permanently change the subsequent phenotypic outcomes, including those related to future reproductive success; (ii) the xenobiotic compounds may have a discreet period of development in which they have a detrimental effect (a phenomenon seen in responses of human embryos to Thalidomide); (iii) metabolites of the environmental xenobiotic compounds produced by the embryo may be more potent toxicants than the root chemical; (iv) the sensitivity of the embryo to contaminant insult is orders of magnitude lower than it is for the later developmental stages; and (v) our current knowledge about the processes of early development of fish embryos does not provide us with a basis for extrapolation of recorded effects to potential causes (McLachlan, 2001). The future application of molecular techniques to explore this issue may provide the framework for future interpretation, but other than mortality, contaminant–developmental impairment relationships in fish have not been demonstrated.

Several examples of xenobiotic effects that impair fish development have been reported. For example, impaired lake trout (*Salvelinus namaycush*) egg hatchability and yolk-sac embryo survival in Lake Michigan have been linked to specific PCB congeners DDT (Mac *et al.*, 1993); the field studies' findings were supported by the results of experimental laboratory studies. Similar associations between environmental OCs and embryo survival have been made for marine pleuronectid, clupeid and gadid species in Europe and North America (Rolland, 2000b).

Another type of xenobiotic effect is seen in the dioxin-induced condition called blue sac disease (BSD), a fatal condition characterized by oedema of the yolk sac and pericardium, skeletal disorders and impaired growth. Field and laboratory studies have found the condition in several species, with clear links to dioxin and bisphenol A (reviewed by Finn, 2007), and possibly also PCB (Stouthart *et al.*, 1998). Recent studies suggest that BSD is caused by an increased

permeability of the vascular endothelium, which is associated with the upregulation of CYP enzyme synthesis via the AHR/ARNT induction pathway. Immunohistochemical approaches showed the CYP enzymes to be located in the vascular endothelial cells and their presence to be associated with ischaemia, resulting in anaphylactoid complications (Finn, 2007).

Conclusions and Future Directions

The endocrine 'systems' in vertebrates are extremely complex and integrated chemical regulatory systems, and any factor that disturbs one system will inevitably influence other components of the system, possibly in a compensatory manner in which the animal can maintain homeostatic systems, but also having an indirect deleterious effect on systems other than the one that was primarily affected. Consequently, it has been difficult in many cases to determine the causes of the non-infectious disorders that have been reported in captive stocks or wild populations of fishes; most cause–effect links have been speculative and not definitive.

Undoubtedly, there are endocrine disorders that are linked to environmental contaminants, but some (e.g. M74 in Atlantic salmon and goitres in North American Great Lakes Pacific salmon species) are probably caused by ecological, rather than contaminant, factors, and others, such as the pituitary and gonadal lesions found in hybrids, are probably genetically based. When interpreting the data from field or captive situations, it is tempting to describe an 'anthropogenically' derived chemical aetiology to each dysfunctional condition, which may not necessarily be the case.

Stressor-induced or toxic chemical-induced immunosuppression in fish undoubtedly influences 'downstream' functions such as growth and reproduction, as well as making the animal more vulnerable to infectious disease. This aspect of fish dysfunction has a marked endocrine component and has significant consequences for both the aquaculture industry and fisheries management and requires further extentensive investigation.

Far more work is needed to establish the mechanism of action of those environmental chemicals that have been genuinely associated with disorders in fish. The application of genomic toolboxes as described by Bobe *et al.* (2006), Goetz and MacKenzie (2008) and several publications in special issues of *Reviews in Fisheries Science* (Sundell and Power, 2008) and the *Journal of Fish Biology* (Maclean, 2008) will enable significant advances to be made in this field, particularly in the identification of clusters of genes involved in different aspects of endocrine and reproductive function. These tools, in combination with follow-up studies of specific genes using real-time RT-PCR technology will allow us to develop a much better understanding of the 'normal' as well as of the 'disordered' situations. These findings will complement and strengthen the traditional pathological approaches that have formed the major component of studies into the nature and progression of non-infectious disorders in the past.

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4 Chemically Induced Alterations to Gonadal Differentiation in Fish

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Introduction

In several regions around the world, alterations to the sex differentiation of fish have been linked to exposure to chemical contaminants (Mills and Chichester, 2005). There are indications that complete sex reversal is occurring among some fish populations. Male-biased sex ratios were found in eelpout (*Zoarces viviparus*) collected from a marine area near the discharge of a large Swedish pulp mill (Larrson and Forlin, 2002). In the Columbia River, significant numbers of phenotypically female chinook salmon (*Oncorhynchus tshawytscha*) were observed to have the genotypic marker for the male sex (Nagler *et al*., 2001).

Gonadal intersex consisting of both oocytes and testicular tissue in the gonad of the same fish has been observed in male roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) from rivers in the UK, and this developmental alteration has been attributed to exposure to endocrine-disrupting chemicals (EDCs) in the effluents of sewage treatment plants (Jobling *et al*., 1998; Van Aerle *et al*., 2001). Gonadal intersex has also been observed in roach from rivers in Denmark (Bjerregaard *et al*., 2006). An overview of the characteristics and the population impacts of gonadal intersex in roach is included in this chapter.

Intersex gonads have been observed in several other freshwater fish species collected from locations that are impacted by industrial and domestic wastewaters, including barbel (*Barbus plebejus*) from a river in Italy (Viganò *et al*., 2001), shovelnose sturgeon (*Scaphirhynchus platyorynchus*) from the Mississippi River near Saint Louis, Missouri, USA (Harshbarger *et al*., 2000) and a catfish species (*Clarias gariepinus*) from a river in South Africa (Barnhoorn *et al*., 2004). Testicular atrophy and intersex in the gonads of male common carp (*Cyprinus carpio*) have been observed in locations impacted by urban pollution (Sole *et al*., 2003; Lavado *et al*., 2004; Snyder *et al*., 2004), as well as in other carp species (Papoulias *et al*., 2006). Intersex gonads have also been observed in marine fish species from contaminated locations, including male flounder (*Platichthys flesus*) from polluted estuaries in the UK (Lye *et al*., 1997; Allen *et al*., 1999) and male flounder (*Platichthys yokohamae)* from Tokyo Bay in Japan (Hashimoto *et al*., 2000).

Intersex gonads were observed in white perch (*Morone americana*) from urbanized and industrialized regions of the lower Great Lakes, Canada (Kavanagh *et al*., 2004). The intersex gonads observed in immature male white perch are characterized by the presence of immature (primary) oocytes distributed to varying degrees throughout the testicular

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tissue. Recently, Blazer *et al.* (2007) reported the high prevalences of intersex gonads among male smallmouth bass (*Micropterus dolomieu*) in the Potomac River and adjacent watersheds in West Virginia, areas which are impacted by intensive livestock production. Mikaelian *et al.* (2002) observed a relatively low prevalence (12%) of female whitefish (*Coregonus clupeaformis*) from the St Lawrence River, Canada with ovaries containing spermatogonia. Other effects on gonadal development, such as atresia of oocytes in female fish, have been observed at high prevalence in fish populations exposed to pulp mill effluents (Janz *et al.*, 1997).

Feminization or masculinization of fish by exposure to steroid hormones or their synthetic analogues have been used in aquaculture for many years in order to maximize the somatic growth of the cultured fish species (Johnstone *et al.*, 1978; Yamazaki, 1983; Blasquez *et al.*, 1995; Devlin and Nagahama, 2002). Intersex and other alterations to gonadal development have been observed in model fish species that have been exposed in the laboratory to EDCs. Mills and Chichester (2005) provided an excellent review of the laboratory models that have been used to study EDC-induced alterations to gonadal development. The Japanese medaka (*Oryzias latipes*) is an aquarium fish that has been used for over 50 years as a model for the chemical induction of gonadal alterations in fish (Yamamoto, 1953, 1958). The characteristics and reproductive alterations related to the induction of gonadal intersex and sex reversal in this species are reviewed in detail in this chapter. Other fish species in which complete feminization or intersex gonads have been induced by exposure to EDCs include the common carp (Gimeno *et al.*, 1997), the Japanese flounder, *Paralichthys olivaceus* (Shimasaki *et al.*, 2003), sea bass, *Dicentrarchus labrax* (Blasquez *et al.*, 1998), sheepshead minnow, *Cyprinodon variegates* (Zillioux *et al.*, 2001), the platyfish, *Xiphophorus maculates* (Kinnberg *et al.*, 2000), spottail shiners, *Notropis hudsonius* (Aravindakshan *et al.*, 2004), three-spine stickleback, *Gasterosteus aculeatus* (Bernhardt *et al.*, 2006), zebrafish, *Danio rerio* (Orn *et al.*, 2003; Fenske *et al.*, 2005; van der Ven *et al.*, 2007), the fathead minnow, *Pimepheles promelas* (Länge *et al.*, 2001) and the rare minnow, *Gobiocypris rarus* (Wei *et al.*, 2007). In a laboratory study with roach exposed to sewage effluent, disruption to the development of the gonadal duct of males was observed (Rodgers-Gray *et al.*, 2001).

By using a unique experimental approach of adding the synthetic oestrogen ethinyloestradiol for three summers to a lake in north-western Ontario, Canada, a team of researchers was able to evaluate the effects of chronic exposure to this synthetic oestrogen on vitellogenin production, gonadal development, reproductive capacity and population dynamics of several wild fish species, including fathead minnow (Kidd *et al.*, 2007), pearl dace, *Margariscus margarita* (Palace *et al.*, 2006) and lake trout, *Salvelinus namaycush* (Werner *et al.*, 2002, 2006; Pelley, 2003). The outcomes of the studies on fathead minnow and pearl dace are reviewed in a later section in this chapter.

Intersex gonads are a natural feature of gonadal differentiation in hermaphroditic fish, but intersex is not considered a normal feature of gonadal differentiation in gonochoristic fish species (Yamazaki, 1983). Figure 4.1 shows a classification of the various features of the sex phenotype in fish, which includes gonadal sex, external sex characteristics and ethological (behavioural) sex. These phenotypic features may have independent mechanisms for hormonal and environmental control of tissue differentiation and development. Among gonochoristic species, there are '"undifferentiated' species, where the gonad first develops into an ovary-like gonad and then about one-half of the fish become males and the other half become females. In 'differentiated' fish species, the gonad directly differentiates into an ovary or a testis. There is some evidence that gonadal sex is more 'labile' in undifferentiated gonochoristic species (Beamish and Barker, 2002).

In gonochorist fish species, the hypothalamus–pituitary gland–gonad (HPG) axis is probably not involved in triggering sex differentiation, but steroid hormones are key to regulating this process (Baroiller *et al.*, 1999). There is ample evidence that

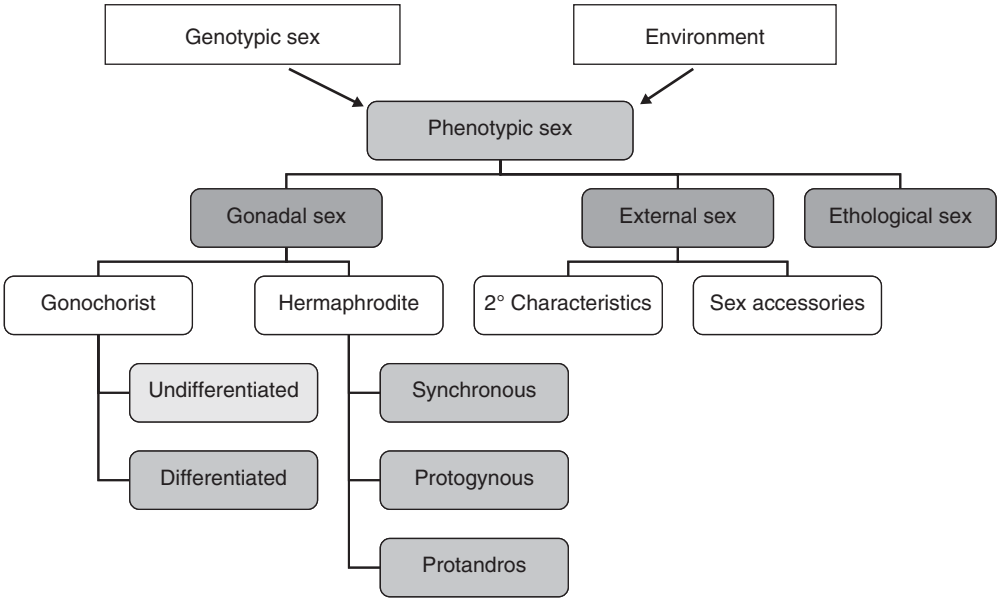


Fig. 4.1. A classification system for the different elements of phenotypic sex in fish. The development of phenotypic sex in gonochoristic species may be altered by both genotypic factors and environmental factors (e.g. temperature, disease, exposure to exogenous chemicals).

the gonadal sex phenotype can be manipulated easily in differentiated gonochoristic fish species when exposure to steroid hormones occurs around the time of sex differentiation, which, depending on the species, can occur soon after hatch or during the development of the juveniles. However, gonadal intersex has been observed in fish exposed as adults to steroid hormones, which has been interpreted as evidence of bipotential germ cells in the gonad (Shibata and Hamaguchi, 1988; Kobayashi *et al.*, 1991; Gray *et al.*, 1999a). Gametogenesis is an independent process involving maturation of the oocytes in the ovary or spermatocytes in the testis, which takes place in sexually mature fish (Grier, 1981; Iwamatsu *et al.*, 1988). Gametogenesis can take place either in a synchronous pattern (i.e. during a spawning season) or in an asynchronous pattern (i.e. continuous spawning).

Despite the previous evidence that gonochoristic fish species do not develop intersex spontaneously, there is a developing body of evidence showing that immature oocytes can be present at a relatively high prevalence in the testicular tissue of some gonochoristic fish species. However, the histological patterns and the prevalence of these gonadal alterations seem to vary among species and possibly among populations. For instance, Bernhardt *et al.* (2006) reported that 'hermaphroditic' (i.e. intersex) three-spine sticklebacks have never been observed in wild populations despite 'more than 150 years of intense scientific research in Europe, North America, and Asia'. Among European sea bass, 62% of juvenile males from aquaculture operations were observed to have 'intra-testicular oocytes', and similar examples of subtle gonadal intersex were observed in wild males from the eastern Atlantic Ocean and western Mediterranean Sea (Saillant *et al.*, 2003). Among roach sampled in rivers in the UK, gonadal intersex was observed at a prevalence of up to approximately 20% in fish collected from 'control' sites, although the condition consisted of relatively small numbers of primary oocytes distributed throughout the testis (Jobling *et al.*, 1998). An elevated prevalence of gonadal intersex was observed

in juvenile white perch from some locations in the Great Lakes (Kavanagh *et al.*, 2004), but spontaneous gonadal intersex (incorrectly described as 'hermaphroditism') has been reported sporadically for this species (Bishop, 1920; Dorfman and Heyl, 1976). A high proportion of intersex whitefish were observed in an isolated mountain lake in Switzerland (Bernet *et al.*, 2004). Among female pike (*Esox lucius*) sampled in rivers in the UK, upstream and downstream of sewage treatment works (STWs), there was a 14% prevalence of gonadal intersex, characterized by patches of male germ cells among ovarian tissue, but the prevalence of this masculinization condition was independent of whether the fish were captured above or below the STWs (Vine *et al.*, 2007).

It is not clear what causes the spontaneous development of feminized or masculinized intersex gonads in gonochoristic fish species. There is some evidence that parasitic infections that damage to the gonad can lead to the regeneration of a gonad of the opposite sex, which can then lead to sex reversal (Van Duijn, 1967). In any event, it is clear that caution must be taken when interpreting data on the prevalences of intersex gonads of wild fish or the incidence of these gonadal alterations in laboratory fish models. All numerical data should be compared with reference sites or control treatments, and information should be collected on the extent or severity of these gonadal abnormalities.

Another area of uncertainty is whether gonadal intersex or other gonadal alterations in fish can be correlated with reproductive or population-level effects. There is some evidence that fish with intersex gonads are physiologically capable of reproducing, although their reproductive capacity may be altered through other mechanisms, such as effects on spawning behavior (Balch *et al.*, 2004b). There is interest in determining whether a relatively obvious and unequivocal response such as the presence of intersex gonads in fish can be used as a biomarker for population-level effects or even extirpation of fish in areas impacted by EDCs. This chapter will review these research questions, with a focus on studies that have been conducted with a laboratory fish model, the Japanese medaka, field-based studies in the UK on roach, and a whole-lake experiment in which fish were chronically exposed to 17α-ethinyloestradiol (EE2).

Gonadal Alterations in the Japanese Medaka (*Oryzias latipes*)

The Japanese medaka is an oviparous freshwater killifish belonging to the Cyprinodont family. Although the species is indigenous to South-east Asia, several different cultured varieties of medaka have been developed. Their popularity as a model species for research is partly due to the ease with which they can be induced to breed and the short period of time between egg production and development to sexual maturity (e.g. 6 weeks). The male and female participate in a brief courtship, and 10–30 fertilized eggs are laid and entangled by chorionic fibres near the female's vent. The cluster of eggs hangs from the female for several hours and can be easily removed for subsequent studies. At a temperature of 25 °C, the time to hatch is 11–12 days, and the fry absorb their yolk sac by 18–19 days post-fertilization.

There are subtle, but clearly recognizable, differences in the external sex characteristics of male and female medaka. In mature male medaka, the rays of the dorsal and anal fins are longer and thicker than those of the females, and there is a characteristic notch at the posterior part of the distal margin of the dorsal fin. In mature females, the urogenital papilla is a prominent, paired protuberance between the anus and the oviduct opening, as compared to the less prominent, unilobed structure in males. Medaka are a differentiated gonochoristic species, and spawning is asynchronous over most of the year under conditions of temperature and light that maintain spawning. The gonad of the medaka is a single organ positioned medially beneath the swimbladder. Sexual differentiation of the gonad begins before hatch in females (Yamamoto, 1958) and after hatch (i.e. 13 days post-fertilization) in males (Yamamoto, 1953).

Yamamoto and co-workers carried out numerous studies with medaka throughout

the 1950s and 1960s to study alterations to differentiation of the gonad in response to exposure to steroid hormones (Yamamoto, 1953, 1958, 1969). According to these studies, two conditions appear to be necessary to induce complete sex reversal. First, medaka must be exposed to a heterologous hormone (i.e. androgen for genotypic females, oestrogen for genotypic males) during the critical stages of gonadal differentiation (i.e. just before hatch for females, just after hatch for males). According to these studies, exposure after the critical period for gonadal differentiation may induce temporary effects that could degenerate after exposure to the exogenous hormone ceases. Second, the dose of the heterologous hormone must be sufficient to induce complete sex reversal. Dosages below a threshold appear to induce an intersex condition. The continuum between the induction of intersex and complete gonadal sex reversal in medaka is illustrated in Fig. 4.2. The data for medaka exposed to four different concentrations of 17α-ethinyloestradiol, which was originally presented by Metcalfe *et al.* (2001), show that intersex of the gonad was induced in males exposed to lower concentrations, while complete feminization (as determined by skewed sex ratios) was induced in fish exposed to the highest concentration (Fig. 4.2a). Previously unpublished data for medaka exposed to methyltestosterone (Fig. 4.2b) show that gonadal intersex was observed in fish exposed to low concentrations of the androgen, and complete masculinization of the gonad (as determined by skewed sex ratios) was observed in fish exposed to the highest concentration. Interestingly, it was not possible to determine the sex of eight medaka exposed to the highest concentration of methyltestosterone (Fig. 4.2b), possibly because of degeneration of the gonad, which made it difficult to find this organ during histological sectioning.

Experimental alterations to gonadal differentiation

Table 4.1 lists the endogenous hormones, anti-androgens and anti-oestrogens, and synthetic endocrine disruptor compounds that have been tested to determine whether they alter differentiation or development of the gonad in the Japanese medaka. Note that sex reversals have primarily been identified through the appearance of statistically significant changes to sex ratios. The d-rR strain of medaka, originally developed by Yamamoto (1958), has a sex-linked colour marker, which has been used to evaluate changes in sex phenotype (Scholz and Gutzeit, 2000). The recent development of a new strain of medaka (*i.e.*, the FLFII strain) that has both color and pigmentation markers, as well as a definitive molecular marker for genotypic sex, has improved the capacity to quantitatively evaluate masculinization or feminization (Balch *et al.*, 2004a). Gonadal intersex, which has been variously referred to as 'testis–ova' or 'ovo-testes', has been observed frequently in these studies (Table 4.1). In medaka exposed to either androgens or oestrogens, the intersex gonad consists of oocytes varying in the stage of oogenesis, which are distributed throughout testicular tissue. Typically, the oocytes in the intersex gonad are pre-vitellogenic (Fig. 4.3), but more mature oocytes have been frequently observed. In the intersex gonad, there is often evidence of disruption to the patterns of development of the testicular tissue, ranging from extensive fibrosis within the testicular stroma to more subtle disorganization of the spermatocytic cysts (Fig. 4.3).

It must be mentioned that care must be taken in interpreting the incidence of intersex in Japanese medaka. A recent retrospective study showed that gonadal intersex was observed in medaka from control treatments in 15 of 41 studies (Grim *et al.*, 2007). While most of the 54 cases of gonadal intersex observed among the control treatments consisted of a small number of pre-vitellogenic oocytes clustered in the germinal epithelium, some more severely affected individuals had pre-vitellogenic oocytes clustered in the centre of the gonad, and, in one case, several vitellogenic oocytes were observed (Grim *et al.*, 2007). Obviously, adequate numbers of control fish should be included in experimental studies to evaluate alterations to gonadal differentiation in medaka.

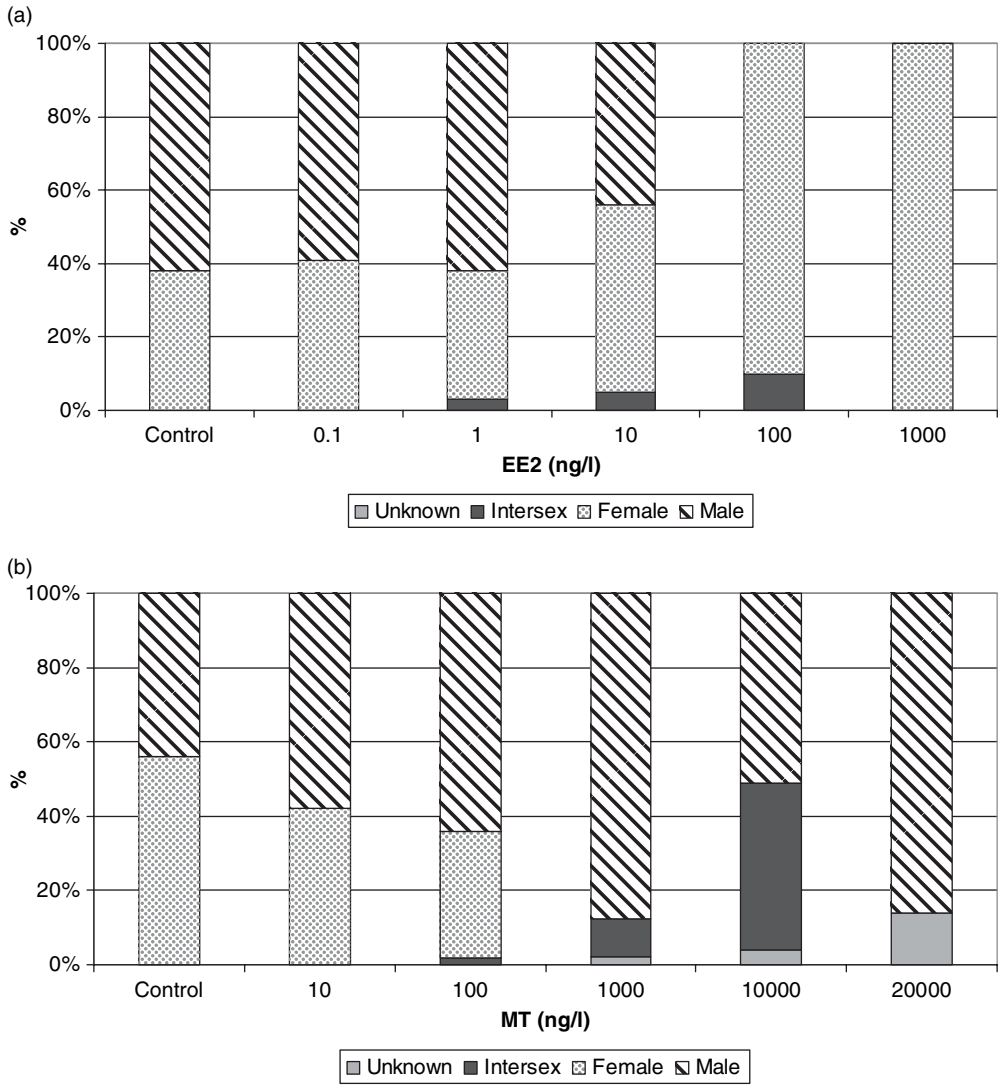


Fig. 4.2. The relative proportions of phenotypically male, female, intersex and unknown sex among Japanese medaka exposed from 1 to 100 days post-hatch to varying concentrations of: (a) 17α-ethinyloestradiol (data originally presented in Metcalfe *et al.,* 2001); (b) methyltestosterone (data previously unpublished). The sex of unknown fish could not be identified because no gonadal tissue was detected among the histological sections prepared from whole medaka.

Male medaka appear to be most sensitive to feminization of the gonads if exposure to oestrogens begins before 2 weeks post-hatch, but there is no consensus on the optimal period for induction of gonadal intersex (Yamamoto, 1953; Satoh and Egami, 1972; Gray *et al.*, 1999a; Koger *et al.*, 2000). Interestingly, intersex was not induced in male medaka by pre-hatch exposure to the oestrogenic chemical o,p'-DDT either through maternal transfer (Metcalfe *et al.*, 2000) or by *in ovo* exposure (Papoulias *et al.*, 2003). There are germ cells in the testis of juvenile and adult male medaka that retain their

Table 4.1. Results of studies conducted over the past 10 years on the effects of chemicals on the differentiation of the gonads of Japanese medaka. Information is provided on whether intersex gonads or complete masculinization (Masc) or feminization (Fem) were observed, and whether reduced reproductive capacity was noted. NE = not evaluated.

Chemical	Reference	Intersex	Masculinize or feminize	Reproduction reduced
Oestrogens				
Oestradiol	Metcalfe *et al.* (2001)	Yes	Fem	NE
	Kang *et al.* (2002)	Yes	No	Yes
	Balch *et al.* (2004b)	No	Fem	NE
	Koger *et al.* (2000)	Yes	Fem	NE
	Seki *et al.* (2006)	No	No	NE
	Tabata *et al.* (2000)	Yes	Fem	NE
Oestrone	Metcalfe *et al.* (2001)	Yes	No	NE
Ethinyloestradiol	Metcalfe *et al.* (2001)	Yes	Fem	NE
	Orn *et al.* (2003, 2006)	Yes	Fem	NE
	Seki *et al.* (2002)	Yes	No	Yes
	Balch *et al.* (2004a)	Yes	No	Yes
	Scholz and Gutzeit (2000)	No	Fem	Yes
Androgens				
Trenbolone	Orn *et al.* (2006)	No	Masc	NE
Trenbolone	Seki *et al.* (2006)	No	No	NE
Testosterone	Koger *et al.* (2000)	Yes	No	NE
Methyltestosterone	Reported here	Yes	Masc	NE
	Orn *et al.* (2003)	Yes	Masc	NE
Anti-oestrogens and Anti-androgens				
ZM 189,153	Reported here	Yes	No	NE
Cyproterone acetate	Kiparissis *et al.* (2003a)	Yes	No	NE
Industrial chemicals and pesticides				
o,p'-DDT (oestrogen)	Metcalfe *et al.* (2000)	Yes	No	NE
	Papoulias *et al.* (2003)	No	No	NE
Vinclozolin (anti-oestrogen)	Kiparissis *et al.* (2003a)	Yes	No	NE
Tributyltin	Nirmala *et al.* (1999)	No	No	Yes
	Shimasakai *et al.* (2003)	Yes	Masc	NE
Bisphenol A (oestrogen)	Metcalfe *et al.* (2001)	Yes	No	NE
	Tabata *et al.* (2000)	Yes	No	NE
Nonylphenol (oestrogen)	Gray and Metcalfe (1997)	Yes	No	NE
	Balch and Metcalfe (2006)	Yes	No	NE
	Tabata *et al.* (2000)	Yes	No	NE
Octylphenol (oestrogen)	Gray *et al.* (1999a)	Yes	No	NE
	Gray *et al.* (1999b)	Yes	No	Yes
Phytoestrogens				
Genistein	Kiparissis *et al.* (2003b)	Yes	No	NE
Equol	Kiparissis *et al.* (2003b)	Yes	No	NE

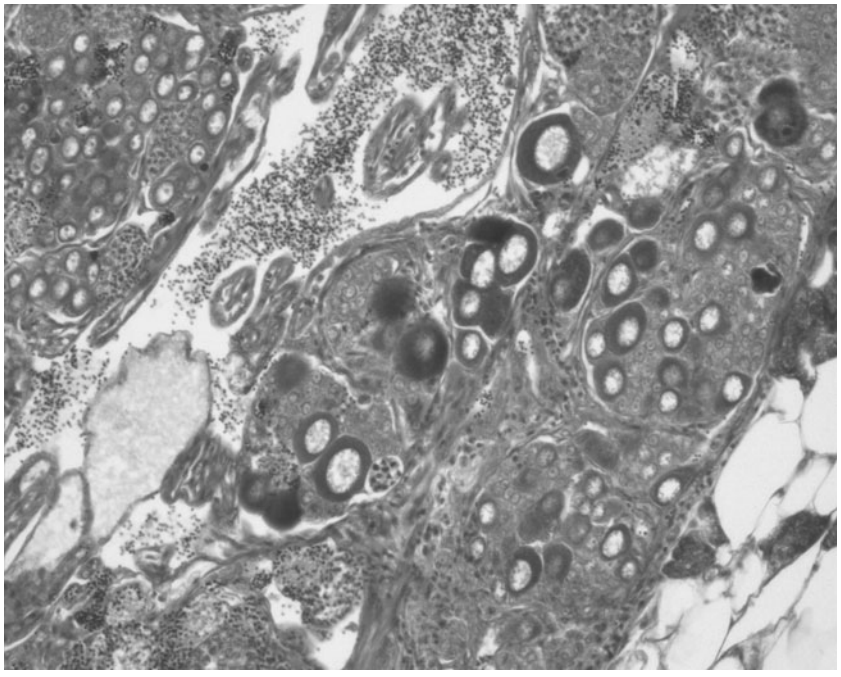


Fig. 4.3. Histological section of the gonad of a fertile phenotypically male Japanese medaka that had been exposed from 1 day post-hatch to 17α-ethinyloestradiol (10 ng/l). The section shows the intersex condition, characterized by the presence of pre-vitellogenic oocytes distributed among testicular tissue that shows mild disorganization of the spermatocytic cysts. Note the presence of spermatids in the efferent duct, but no mature spermatozoa. H&E staining, ×400. This study was originally described by Balch and Metcalfe (2006).

sexual bipotentiality long after the gonad has differentiated into a testis (Shibata and Hamaguchi, 1988). Thus, it is possible to induce intersex in mature male medaka by exposure to concentrations of oestrogens that are approximately one order of magnitude higher than the concentrations that induce a response at earlier life stages (Gray *et al.*, 1999a; Seki *et al.*, 2002). It is interesting to note that external factors, such as high temperatures, that cause testicular degeneration can promote the development of gonadal intersex in adult male medaka (Egami, 1956).

The optimal period for exposure to androgens for masculinization of female medaka has been less well studied. Yamamoto (1958) came to the conclusion that the optimal period for exposure of female medaka to androgens was just before hatch. Koger *et al.* (2000) observed gonadal intersex in female medaka when 6-day exposures began on Day 1 and Day 7 post-hatch, but intersex was not observed in treatments where exposures were initiated at pre-hatch, hatch or 21 days post-hatch. Exposure of medaka to the synthetic androgen 17β-trenbolone (50 ng/l) for 60 days, beginning at 1 day post-hatch, did not cause gonadal intersex or masculinize the fish, although this treatment did cause complete sex reversal (i.e. masculinization) in zebrafish, (Orn *et al.*, 2006). The zebrafish is an undifferentiated gonochorist fish species in which the final stage of gonadal differentiation does not occur until 20–30 days post-hatch. Previously unpublished data for medaka exposed to methyltestosterone for 100 days starting 1 day after hatch (Fig. 4.2b) shows that post-hatch exposure to this steroidal androgen

can induce gonadal intersex or complete masculinization, depending on the exposure concentration. These studies indicate that exposure to androgens immediately after hatch can induce gonadal intersex in female medaka, but the optimal period for masculinization remains to be determined. Exposure of adult medaka to trenbolone at concentrations up to 5000 ng/l induced masculinization of the secondary sex characteristics but not the gonad (Seki *et al.*, 2006).

According to Baroiller *et al.* (1999), 'nearly all attempts to masculinize or feminize fish using steroid receptor antagonists have failed'. However, in studies of medaka exposed to the clinical anti-androgen cyproterone acetate and to the anti-androgenic fungicide vinclozolin, low incidences (i.e. <10%) of intersex were observed in the gonads of exposed fish (Kiparissis *et al.*, 2003a). Previously unpublished data for medaka exposed from 1 to 100 days post-hatch to the experimental clinical anti-oestrogen ZM 189,153 (provided by AstraZeneca, Brixham, UK) indicate that gonadal intersex was induced at a low incidence in fish exposed to concentrations of 10 ng/l (Table 4.2), but the primary effects of this compound on the gonad were fibrosis of the testis and inhibition of spermatogenesis in males, as well as inhibition of oogenesis and egg atresia in females. These studies indicate that steroid receptor antagonists can masculinize or feminize the gonads of medaka, albeit at relatively low incidences.

Effects on reproduction

The Japanese medaka has been widely used as an experimental model to evaluate the impacts of oestrogens on the reproduction of fish (Gray *et al.*, 1999b; Scholz and Gutzeit, 2000; Kang *et al.*, 2002; Seki *et al.*, 2002; Oshima *et al.*, 2003; Balch *et al.*, 2004a). Exposure of male medaka to the oestrogenic chemical octylphenol from 1 day to 6 months post-hatch at nominal concentrations of 25 and 50 µg/l reduced reproductive success (i.e. production of fertilized eggs) and affected spawning behavior, but one of two male fish observed with intersex gonads was capable of fertilizing the eggs of an unexposed female (Gray *et al.*, 1999b). Seki *et al.* (2002) observed intersex gonads among adult male medaka that were exposed for 21 days to 17α-ethinyloestradiol at measured mean concentrations of 63.9, 116, 261 and 448 ng/l, but fecundity was only reduced statistically for paired medaka (i.e. female–male pairs) that were exposed to the highest of these concentrations. A similar experimental protocol with adult medaka exposed to

Table 4.2. Gonadal sex and incidence of intersex gonads and absent gonads in histological sections prepared from Japanese medaka exposed to the anti-oestrogen ZM 189,154 from 1 to 100 days post-hatch (72-h static renewal exposures).

Treatment	Conc. (ng/l)	Gonadal sex[a]				
		N	Female	Male	Testis–ova[a]	No gonad[b]
Control	–	46	23	23	0	–
	0.1	50	25	24	0	1
ZM 189,154	1	51	20	29	0	2
	10	56	32	20	2	2
	100	63	35	24	0	4

[a]Fish with gonadal intersex were not included in the table in the numbers of males or females. However, for statistical analysis of sex ratios, the intersex fish were grouped together with the females since it can be speculated that the intersex condition resulted from a disruption of ovarian differentiation by the ZM 189,154. Based on the Fisher exact test comparison of observed versus expected frequencies of females and males, there were no statistically significant deviations from the expected sex ratios in any treatments; [b]No gonadal tissue was detected among the histological sections prepared from whole medaka.

17β-oestradiol showed that intersex gonads were induced in males from all treatments (i.e. 29.3, 55.7, 116, 227, 463 ng/l), but reduced reproductive success (i.e. reduced total numbers of eggs and egg fertility) was only observed at the highest concentration (Kang *et al.*, 2002). Balch *et al.* (2004b) observed that male medaka with intersex gonads induced by exposure to EE2 at nominal concentrations of 2 and 10 ng/l were capable of fertilizing the eggs of unexposed females, although reproductive success was reduced and spawning behaviour was altered in the 10 ng/l treatment. Interestingly, reproductive success was also reduced when exposed females (10 ng/l treatment) were paired with unexposed males, despite the fact that oogenesis was normal in these exposed females. Scholz and Gutzeit (2000) observed complete gonadal feminization in medaka exposed to 100 ng/l of EE2, which, of course, prevented these fish from reproducing.

Overview

These studies with the Japanese medaka show that intersex gonads in fish may be a readily observable biomarker of reduced reproductive success. However, medaka with alterations to gonadal differentiation are still capable of reproducing. Significant reductions in the reproductive success of medaka seem to occur when fish are exposed to oestrogens at concentrations somewhat higher than those that can induce gonadal intersex. Other mechanisms may explain reduced reproductive success, including alterations to the spawning behaviour of both male and female medaka. Testicular fibrosis and effects on gametogenesis in both males and females may also explain the reduced reproductive success in exposed fish.

Field-based Studies on Roach (*Rutilis rutilis*) in the UK

The roach is usually the most common fish in lowland, relatively slow-flowing rivers in the UK. It is also found in still bodies of fresh water. Individuals can live up to 15 or more years and reach weights of 1.5 kg, though such fish are exceptional. Roach are gonochoristic cyprinid fishes. Males are usually 2 years old and weigh as little as 20g when they spawn for the first time, whereas females are usually a year older, and also larger, when they spawn for the first time. Spawning occurs in late April or May in the UK, when large aggregations of fish of both sexes congregate at traditional spawning sites. They show a 'lek-like' spawning strategy (Wedekind, 1996), with the vigorous spawning activity making it difficult to observe the reproductive behaviour of individual fish. Laboratory observations of spawning (Wedekind, 1996) have suggested that males defend territories within the spawning area, with females releasing their eggs in batches, with multi-male fertilization occurring. Such a reproductive strategy would suggest that sperm concentration would be very important for reproductive success in roach. These characteristics are important considerations when it comes to trying to determine the consequences of intersexuality on both individual roach and populations of roach, as will be discussed later.

Gonadal intersex in roach

In the early 1980s, intersex roach were first discovered in the UK. The affected fish were living in settling lagoons of sewage treatment works (STWs), where particulate matter settled out before effluent was discharged into rivers. Even at that time, the occurrence of intersexuality was considered both unusual and unexpected. At the time, its presence was, with considerable foresight, linked to the possible presence in the effluent of the pharmaceutical EE2. Unfortunately, the report containing all of this information was not made public, due to public health concerns about whether or not EE2 could be present in drinking water produced from water abstracted from the rivers receiving the oestrogenic effluent. It was not until many years later that a fisheries study reported by Jobling *et al.* (1998) was conducted, aimed

at determining the incidence and severity of intersexuality in roach.

Jobling *et al.* (1998) reported that exposure to oestrogenic effluents was linked to high prevalences of intersexuality and to increased vitellogenin concentrations in roach. Although it is not possible to be certain, because roach cannot be sexed genetically, it is likely that the intersex fish were predominantly, if not exclusively, partially feminized males. These intersex roach usually had oocytes within predominantly male gonads (testes) and/or malformed or intersex reproductive ducts. Put another way, the reproductive ducts, as well as the gonads themselves, were sometimes feminized (Nolan *et al.*, 2001). It is not possible to define a 'typical' intersex gonad. The number, distribution and developmental stage of oocytes within the testicular tissue in intersex fish varied greatly. For representative examples, see the figures in Nolan *et al.* (2001). In many intersex fish that were affected to only a small degree, a few primary oocytes, or alternatively a mixture of primary and secondary oocytes, were scattered apparently randomly throughout the testicular tissue. In other, more severely feminized individuals, large areas (sometimes even entire gonads) of ovarian tissue were present in areas separate from the testicular tissue. It is possible that some of the fish that were apparently normal-looking females with two apparently normal ovaries were actually completely feminized 'males', though this cannot be proved due to the lack of a genotypic marker for sex in this species.

The prevalence of intersexuality in roach

Perhaps even more surprising than the presence of intersexuality in roach was its prevalence; it was much more widespread than anticipated. A large national survey was conducted in 1995, involving sampling of approximately 1500 sexually mature roach (of which half were expected to be genetic males). Intersex fish were found at all sites, including 'control' sites, which were lakes and canals not receiving STW effluent. However, whereas the prevalence of gonadal intersex was relatively low at these 'control' sites (on average, 12%), it was markedly higher at all river sites, especially at sites downstream of STWs. At two downstream sites, all of the 'male' fish were intersex; in other words, no normal male fish could be found at these sites (Jobling *et al.*, 1998).

A follow-up, also extensive, survey was conducted in 2002/2003 (Jobling *et al.*, 2006). Roach were sampled from 45 sites, representing a wide range of ecosystems, from those considered relatively pristine to others that were heavily impacted by STW effluent. Nearly 600 'male' roach were assessed for intersexuality. Of these, 136 (23%) were found to be intersex to varying degrees. Intersex fish were found at most locations. As with the previous survey, both the prevalence and severity of intersexuality was greatest at the sites most heavily impacted with STW effluent. Essentially, the second survey confirmed the results of the first survey, and further suggested little if any change in the prevalence of intersexuality, which remained surprisingly high; that is, almost one in every four 'male' roach were intersex, albeit to varying degrees. In fact that figure may be higher because some of the fish designated as 'female' may in fact have been fully feminized genotypic 'males'. There is no reason to believe that the situation is any different now, a further 7 years after completion of the survey.

Why are there so many intersex roach in UK rivers?

There is good, but not incontrovertible, evidence that intersexuality in male roach is caused by their exposure to oestrogenic chemicals (Purdom *et al.*, 1994; Desbrow *et al.*, 1998; Routledge *et al.*, 1998; Jobling *et al.*, 2006). It is most likely that a mixture of oestrogenic chemicals, rather than a single compound, is responsible for the intersexuality (Sumpter *et al.*, 2006). Exposure of roach to sewage effluents induced alterations to the development of the gonadal ducts in males (Rodgers-Gray *et al.*, 2001).

These oestrogenic chemicals include natural (such as 17β-oestradiol (E2)) and synthetic steroid oestrogens (such as EE2), and a variety of xeno-oestrogens, of which the alkylphenols (such as nonylphenol and octylphenol) have received the greatest degree of attention. Although it is not possible to be sure which of these chemicals is most dominant, and hence primarily responsible for inducing intersexuality, some evidence points towards EE2 playing a significant role (Sumpter *et al.,* 2006).

Although very few large fisheries studies aimed at determining the prevalence of intersexuality in fish have been conducted in other countries, with the exception of Denmark (Bjerregaard *et al.*, 2006), it seems as though the UK has a more severe problem than most (and possibly all) other countries. A likely reason for this is that the UK is an extremely densely populated country, with large numbers of STWs discharging their effluents into relatively small rivers. Hence, the flow of many rivers can be 50% effluent under conditions of low rainfall, a figure that can rise to 90%, or even higher, in extremely dry periods. The effluent-dominated nature of many UK rivers means that the fish in these rivers are almost certainly exposed to high concentrations of oestrogens, especially natural and synthetic steroid oestrogens (derived from people rather than industry) than fish living in most other countries, where effluent is diluted appreciably once it enters rivers. The relatively high use of the contraceptive pill also plays a role in maintaining overall 'oestrogen' concentrations at a level where they can cause intersexuality.

The consequences of intersexuality

It is perhaps worthwhile to state some of the things that environmental oestrogens are *not* doing to freshwater fish populations in the UK. They are not causing crashes in the roach populations; in fact, it is generally considered that freshwater fish populations are now healthier (including larger) than they were 50 or 100 years ago. This improvement is probably a consequence of the improvement in water quality that has occurred over this period; gross pollution of the aquatic environment has been successfully controlled, although occasional serious pollution incidents still occur, leading to significant fish kills. However, this does not mean that oestrogenic chemicals are not causing adverse effects, especially at the individual fish level. Our current, but far from complete, understanding of the consequences of intersexuality is discussed below.

There are two levels at which intersexuality could have consequences: the individual fish level and the population level. Even if intersexuality is associated with significant adverse effects on individual fish, these may not have any population-level consequences, so the population could still be sustainable. Whether they do or do not would depend on the proportion of fish affected by intersex or complete feminization, and the proportion of fish of the entire population that are required to sustain the population by breeding successfully.

Currently there is limited information available on the consequences of intersexuality at the individual fish level. Intersex fish can have smaller gonads, in which spermatogenesis is delayed. Spermiation is also affected, as some severely intersex fish do not appear to produce any milt, and others have a reduced milt volume and a reduced sperm density (Jobling *et al.*, 2002a). Put another way, they produce and release less sperm and this sperm is less motile. It seems likely that the reproductive capabilities of such intersex fish are impaired, though it is very difficult to prove this beyond reasonable doubt. However, using an *in vitro* approach in which milt from intersex 'male' roach was used to fertilize eggs from normal females, it was possible to show that intersex 'males' had reduced fertility (Jobling *et al.*, 2002b). Further, fertilization success was correlated with the degree of intersexuality: the more severe the condition, the lower the reproductive success of the fish. But despite these results, which intuitively seem very plausible, it must be remembered that they were conducted using an approach in which there was no sperm competition

(i.e. sperm from two or more fish were not competing to fertilize eggs). It is possible, perhaps even likely, that intersex fish will fare less well when they compete with normal fish, which is what will presumably be occurring when roach spawn naturally in large aggregations of fish.

To provide the answers that are desperately needed, it will be necessary to conduct breeding trials in which groups of roach of both sexes are allowed to spawn naturally. Subsequently, once the eggs hatch, it will be possible using genetic tools (e.g. microsatellites) to match offspring (fry) with parents. In other words, assign parentage. A histological examination of the gonads of each adult fish, to determine whether or not a 'male' is intersex and if so to what degree, can then be employed to link intersexuality to reproductive success. These experiments are currently under way. However, even if successful, these breeding trials will only determine the reproductive fitness (i.e. ability to reproduce successfully) of each individual 'male' fish. They will not provide any information about the population-level consequences of any reduction in reproductive fitness caused by intersexuality. Population modelling studies will be required to provide predictions of the consequences of intersexuality to the long-term sustainability of roach populations. There is still a long way to go before we know if intersexuality in wild roach has adverse population-level consequences.

Whole-lake Oestrogen Addition Study

Laboratory studies with fathead minnows have linked exposure to below one part per trillion concentrations of synthetic oestrogens to reduced capacity for reproduction and feminization of male secondary sex characteristics (Kramer *et al.*, 1998; Miles-Richardson *et al.*, 1999; Parrott and Blunt, 2005) and, in one study, with intersex of the gonad (Länge *et al.*, 2001). Despite evidence from laboratory and field studies that fish are being adversely impacted by exposure to oestrogens, it remains unclear whether the presence of intersex or other gonadal abnormalities impacts the sustainability of fish populations. To address this research gap, a whole-lake experiment was conducted at the Experimental Lakes Area in north-western Ontario, Canada to assess responses of fishes at the individual- through population-levels to continuous additions of the potent oestrogen EE2. This synthetic oestrogen was added continuously over three summer seasons (2001, 2002, 2003) to a small oligotrophic lake (Lake 260) containing a typical fish population for the region: fathead minnow, pearl dace, white sucker (*Catastomus commersoni*) and lake trout. Mean summer epilimnetic concentrations of EE2 ranged from 4.8 to 6.1 ng/l over the 3 years of addition (Palace *et al.*, 2006). In these lakes, fathead minnows mature in their second year of life and spawn asynchronously several times over a 2-month period. Natural mortality is high after sexual maturity and most adults do not live past age 2, although a few 4 year olds can be found. Pearl dace also mature at age 2 but live up to 7 years, are synchronous spring spawners and will spawn for several years during their lifespan (Palace *et al.*, 2009).

Developmental effects of EE2 on fathead minnow were best examined in the spring of each year, because this was the time of year when their gonads were most developed and least subject to the effects of asynchronous spawning. Gonads from pearl dace were best examined in the autumn of each year because they spawn right at 'ice-off' in the spring. Medial sections of ovaries in females were examined for the stages of oocyte maturation and the presence of atretic follicles, lesions and gonadal intersex. Medial sections of testes in males were examined for delayed testicular maturation, inhibited spermatogenesis, asynchronous cyst maturation, seminiferous lobule deformities, replacement of generative tissue with connective tissue, and gonadal intersex.

Fathead minnow

Gonadal development was delayed in every male fathead minnow collected in the second

and third years of EE2 additions (Palace *et al.*, 2002, 2009; Kidd *et al.*, 2007). Males showed widespread fibrosis and inhibition of testicular development when compared with reference fish (Palace *et al.*, 2002, 2009). In all of these EE2-exposed males, testicular tissues were mainly spermatogonia, rather than the more mature spermatocytes that are common in pre-spawning fish, and there were few or no distinct testicular tubules or lumen. Gonadal intersex was observed in four of nine phenotypically male fish collected in the spring of 2003 (Fig. 4.4). For fathead minnows exposed in the laboratory to 4 ng/l EE2, intersex of the gonad was induced in males after 56 days (Länge *et al.*, 2001). However, in this field study, gonadal intersex was not observed in male fathead minnow until Year 3 of the study (Fig. 4.5; Kidd *et al.*, 2007; Palace *et al.*, 2009). These individuals were most likely continuously exposed to EE2, because low ng/l concentrations of the oestrogen were detected under the ice during the winter months (Palace *et al.*, 2006). Intersex was not seen in any males collected in Lake 260 at the same time of year before the EE2 additions began or from the reference lakes during the years of EE2 addition ($n > 150$) (Palace *et al.*, 2009).

These histological changes in the gonads of male fathead minnow co-occurred with several other responses at the biochemical through organismal levels of organization. EE2 exposure caused these males to produce concentrations of vitellogenin that were up to 22,000 times higher for whole-body concentrations than in reference samples (Palace *et al.*, 2009). Histological examination of the kidney of male fish showed pronounced eosinophilia. This condition of the kidney has been observed in other fish exposed to oestrogens in the laboratory, and putatively linked to the deposition of vitellogenin in the kidneys of male fish, which can result in nephrotoxicity and lethality (Zillioux *et al.*, 2001; Balch and Metcalfe, 2006).

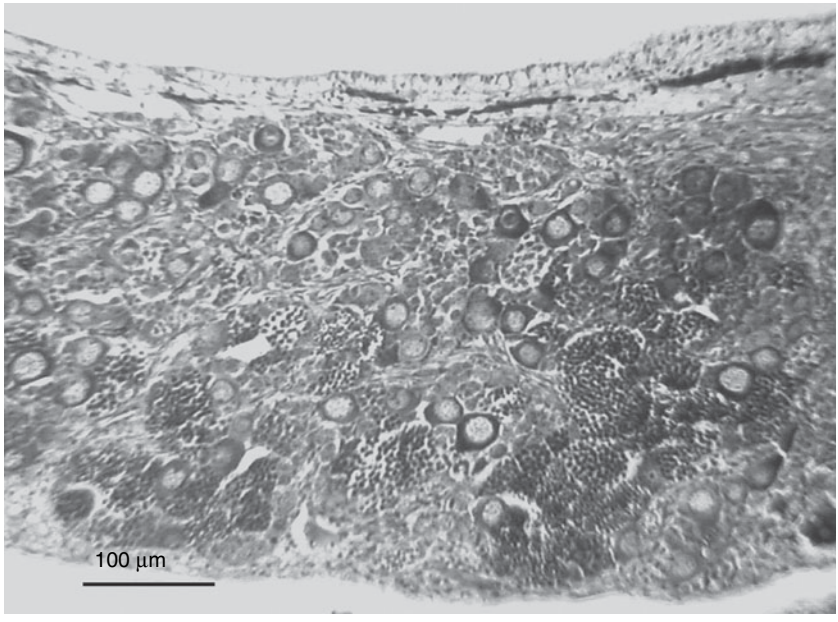


Fig. 4.4. Histological section of the gonad of a fathead minnow showing intersex (i.e. primary stage oocytes distributed throughout testicular tissue) in a phenotypically male fish collected in early May 2003 from Lake 260 after two summers of EE2 additions. H&E staining, ×100. This study was originally described by Kidd *et al.* (2007).

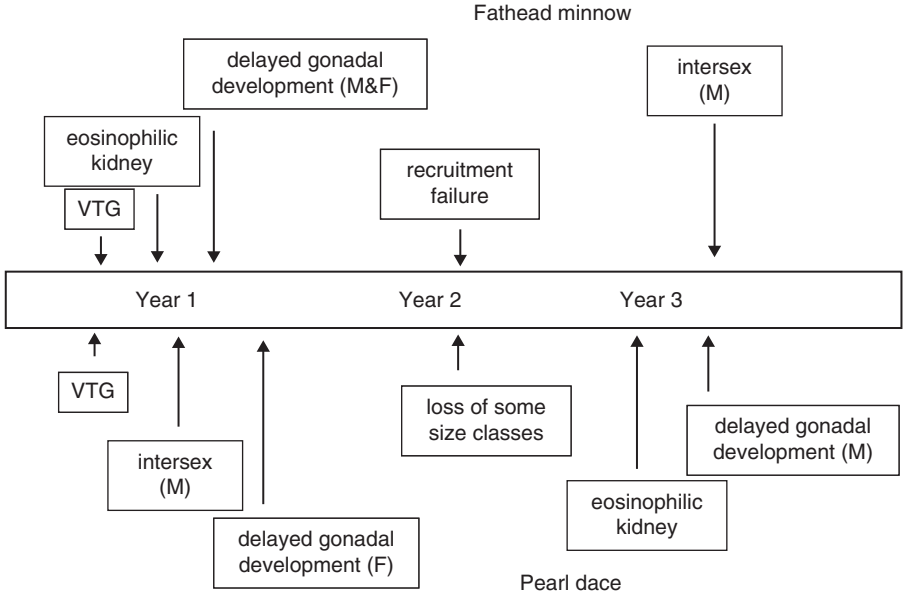


Fig. 4.5. Chronology over Years 1, 2 and 3 of alterations to the gonad and the kidney, and population-level effects in fathead minnows and pearl dace exposed to EE2 in a whole-lake addition study in Lake 260. EE2 was added to the lake in the summers of Year 1 (2001), Year 2 (2002) and Year 3 (2003). These studies were originally described for fathead minnows by Kidd *et al.* (2007) and for pearl dace by Palace *et al.* (2006).

The mean GSI (0.40 %) of male fathead minnows was significantly lower in 2002, when compared to indexes of 0.63–1.2 % from 1999 to 2001 and 2003 to 2005 (Kidd *et al.*, 2007), although the sample sizes were very limited in the latter 2 years (n = 1–3) because few fathead minnows were present in Lake 260. These fish also had no external secondary sex characteristics and prominent ovipositors. Behavioural studies and nest collections also showed that EE2 affected both the spawning behaviour of the males and the numbers of eggs and their stage of development in the nests (P. Blanchfield, DFO, unpublished data).

Gonadal development in female fathead minnow was also impacted by the EE2 additions to Lake 260. Oocytes from females exposed to one season's additions of EE2 were at a much earlier stage of development than those from reference lakes or pre-addition collections (Palace *et al.*, 2009). It is interesting to note that this delay in oocyte development was not observed in females collected the next spring.

In addition to delays in ovarian development, female fathead minnow exposed to EE2 produced higher than normal concentrations of vitellogenin (up to 80 times), relative to those measured in pre-addition samples or in fish from reference lakes. Elevated vitellogenin production was observed in individuals collected both within and outside of the spawning season in 2001 through 2003 (Palace *et al.*, 2002, 2009; Kidd *et al.*, 2007). The GSI of females was not consistently affected by the EE2 additions, although this index was lower in individuals collected in the spring of 2002 and 2004 (2.5 and 2.6%, respectively; n = 9–10), in comparison to females collected either in pre-addition years in Lake 260 or in the reference lakes during the years of the EE2 additions (4.4–8.0 %; n = 5–15; Kidd *et al.*, 2007).

Experimental additions of EE2 led to a near-extinction of the fathead minnow

population in the second season of amendments (Fig. 4.5). There was a recruitment failure that summer, with no young-of-the-year caught that autumn. In this lake, the catch per unit effort (CPUE) for this species went from a pre-addition range of 50–180, down to 0.7–2.6, in 2002 and 2003, respectively, and this population collapse persisted in the post-addition years of 2004 and 2005, with CPUE values in both years of 0.1 (Kidd *et al.*, 2007). This collapse in the fathead minnow population cannot be attributed to one particular effect of EE2 on this species and was probably due to a combination of responses at the biochemical through organismal levels. It is useful to note that gonadal intersex was observed in male fathead minnow the first spring after recruitment failure was observed, indicating that population-level effects were not linked directly or solely to the presence of this gonadal abnormality.

Pearl dace

Testicular development was also negatively affected in pearl dace exposed to EE2 (Palace *et al.*, 2006), but the timing and magnitude of alterations to the gonad were different from those observed for the male fathead minnow. For pearl dace, intersex was found in one-third of the sexually mature fish collected in the autumn of all exposure years, but never observed in any pre-addition or reference ($n > 145$) fish. Thus, intersex in this species occurred after only 20 weeks of exposure to EE2, much earlier than the intersex observed in the fathead minnow (Fig. 4.5). Susceptibility to EE2 also varied with the size of the fish; testes of smaller pearl dace were more visibly affected than the gonads of larger fish. The seminiferous tubules of the smaller fish were atrophied and lacked lumena, and they had large cysts of spermatogonia and some spermatocytes, although these latter cells were often in poor condition. The testes from larger fish were similar to reference fish, but cysts with spermatogonia were more prevalent during all years of the EE2 additions.

In addition to the effects of EE2 exposure on testicular development, male pearl dace were also affected at the biochemical, tissue and organismal levels. In each year, males exposed to EE2 produced concentrations of vitellogenin up to 15,900 times greater than were measured in reference fish, and eosinophilia was observed in the kidneys (Palace *et al.*, 2009). The GSI was lower in male fish after exposure to EE2 (0.49 and 0.50%, in autumn 2002 and 2003, respectively) when compared to fish caught in the lake before oestrogen additions began (0.78–1.32%). However, there were no changes in a secondary sex characteristic (ratio of pectoral fin to fork length) for males collected during the EE2 additions (Palace *et al.*, 2006).

Differential cell counts indicated that gonads of female pearl dace collected by mid-September typically consist of primary (66–69%) and vitellogenic (30–32%) oocytes, with a small percentage (<3%) of intermediate cortical alveolar (pre-vitellogenic) stage eggs. In the EE2-treated lake, the ovaries collected in the autumn of 2001 through 2003 had higher differential oocyte counts at the cortical alveolar stage (more than 12%) and lower counts of the vitellogenic oocytes (19–25%), which probably reduced the number of vitellogenic oocytes available during spawning the following spring. Mean vitellogenic egg size was also smaller in 2001 through 2003 (means of 436, 541 and 372 μm, respectively), when compared to dace collected in the pre-addition years of 1999 and 2000 (651 and 725 μm, respectively).

As we observed for the fathead minnow, female dace were also affected by EE2 at all levels of biological organization. The oestrogen affected steroidogenesis in the ovaries and elevated concentrations of vitellogenin (up to 115 times) in spring through autumn samples when compared to reference fish. There were also reductions in the GSI in females collected in the autumn of 2001 through 2003 (3.1, 4.4 and 2.4%, $n = 15–16$, respectively), when compared to fish collected from Lake 260 before and after EE2 was added (5.6 and 7.5%; $n = 15–16$) (Palace *et al.*, 2006). Finally, the phenotypic sex ratio for this species became heavily

skewed towards females in 2002 through 2005 (K. Mills and V. Palace, DFO, unpublished data).

At the population level, pearl dace did not respond as dramatically or as quickly as the fathead minnow. Starting in autumn of 2002, some (but not all) of the smaller size classes of young-of-the-year fish were not captured, and this led to a compression in the size range of the remaining fish (Palace *et al.*, 2006). Lower catches also occurred in the autumn of 2002 through 2004 (P. Blanchfield and K. Mills, DFO, unpublished data). We did not see a complete collapse of the pearl dace populations, as observed for the fathead minnow, although some declines in abundance of dace occurred.

Overview

Chronic inputs of EE2 to Lake 260 resulted in immediate elevation of vitellogenin concentrations and the occurrence of gonadal abnormalities in both sexes and both fish species after the first summer of additions. However, the severity and timing of the impacts on gonadal development and populations differed between these two species, which was probably due to their dissimilar life history strategies and to interspecific sensitivities to the oestrogen. For example, pearl dace developed intersex the first autumn after EE2 additions began, whereas male fathead minnow developed this abnormality after two summers of exposure to the synthetic oestrogen. The population-level effects observed in these two species were preceded by intersex only for the pearl dace and were much less severe for this species than for the fathead minnow; the latter species exhibited a near-extirpation from Lake 260. Results from this study indicate that altered gonadal differentiation (i.e. intersex, feminization) in fish is not directly related to impacts at the population level. However, gonadal intersex is one of several alterations to gonadal development, gametogenesis, steroid homeostasis and, potentially, behaviour in both sexes that are linked to population-level effects. Chronic inputs of a potent oestrogen can impact the sustainability of wild populations of fish. Shorter-lived species, such as the fathead minnow, with complex mating behaviours and asynchronous spawning, may be at greatest risk from inputs of these oestrogens to rivers and lakes as a result of discharges of domestic and municipal wastewaters.

Summary

There is convincing evidence that alterations to the differentiation of the gonad in fish (i.e. intersex, complete sex reversal) can be induced by exposure to oestrogens and androgens, and possibly by exposure to antagonists of these steroid hormones. Early life stages of fish appear to be more sensitive to these responses, but laboratory studies indicate that alterations to differentiation can be induced in adult fish exposed to high concentrations of oestrogen/androgen agonists.

However, in both laboratory model species and in wild fish, there is a background level of intersex prevalence that appears to vary with species. Therefore, care must be taken to include prevalence data from control treatments or reference populations of fish when interpreting data on the prevalence of intersex gonads. Without widely available molecular markers of the genotypic sex of fish, it is impossible to determine whether complete feminization or masculinisation of fish is taking place and whether these responses are having an impact at the population level.

It is clear that exposure to androgens and oestrogens can also affect the reproductive capability of fish species, and this could have effects at the population level. Fish species that may be at the greatest risk of population effects are those that are relatively short-lived and have reproductive strategies that involve synchronized mating behaviours between single male/female pairs of fish (e.g. fathead minnows). Effects on reproduction in fish do not appear to be directly linked to gonadal intersex, as reproductive responses have been observed independently of the development of this condition. However, the

appearance of gonadal intersex in fish is a definitive and easily recognizable histological marker of exposure of fish to androgens and/or oestrogens. Other responses, such as effects on gametogenesis in both sexes, atresia of oocytes in females or fibrosis of the testis in males, require more skilled interpretation. Therefore, an elevated prevalence of gonadal intersex in fish may be a useful 'biomarker' of exposure, even though this response cannot be directly linked to reproductive effects.

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5 Disorders of Development in Fish

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Introduction

Among physical deformities in fish, skeletal, gill and fin malformations are most common, and they can range from barely detectable to lethal. With few exceptions, the motivation among fish growers to eliminate physical malformations is strong; at the very least these deformities reduce the market value of aquaculture crops. At worst they can cause the loss of an entire cohort. The search for definitive information about the causes of deformities in fish leads us in several directions – some genetic configurations can increase the susceptibility to physical and developmental malformations, but in other cases morphologically similar deformities are clearly not heritable. Slight aberrations in the rearing environment, e.g. temperature, water flow rate or diet, can trigger high rates of deformities in a clutch of fish. Occasionally, associations are made between handling stress and an elevated incidence of deformities, suggesting that stress can disrupt a genetically predetermined plan of development. The sum of the available evidence suggests that certain fishes are more susceptible to environmentally induced aberrations of development than are others. In other words, some species appear to adapt relatively well to captive rearing and may be more suitable for culture and domestication than others. This is not surprising, considering the widely varying degrees to which other animals adjust to captivity and the relatively small fraction that have adapted well.

In the 12 years that have elapsed since the publication of an earlier edition of this volume, the basic assortment of deformities commonly ascribed to fish has not changed appreciably, and to a large extent our understanding of the causes and ontogeny of these patterns is not much more detailed than it was then. Some of the patterns of developmental deformities in fish have become clearer, and some associative trends are more apparent than they were earlier. Nevertheless, the differentiation of basic deformities in developing fish is still only superficially understood, in large measure because this remains a relatively poorly studied topic.

One minor exception to the slow progress in our understanding of the ontogeny of physical deformities in fish is that this is primarily a problem of cultured fishes; increasingly our comparative data on wild and captive fish populations leads to the conclusion that high rates of deformities are symptomatic responses to conditions that aquaculture imposes. In an undisturbed wild habitat, deformities are seldom or almost never seen. In the past this has been

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a difficult observation to reconcile biologically; it has not been possible to know whether wild populations initially produce large numbers of deformed individuals that are just not quantifiable. Gross physical deformities are frequently associated with elevated rates of mortality, which makes it impractical to compare rates of deformities in wild and cultivated populations. The imposition of mortality trends on wild populations could be used to explain why wild fish do not show appreciable rates of physical deformity; it could be argued that rates of scoliosis, for example, are genetically determined and are therefore the same in wild and cultured fish, but that differential selection pressures in these two environments mask this similarity so completely that evidence of it cannot be seen. Selection against wild fish with a spinal deformity may be so complete that large numbers of these fish could be absorbed without a trace into the food chain, although recent reports support the idea that this is seldom the case.

Wild fish probably have much lower rates of developmental deformities than the same species do when cultured. The variability of meristic parts also seems to corroborate this notion, and in wild gilthead sea bream (*Sparus auratus*), the meristic counts of vertebrae and fin rays are remarkably constant (Albuquerque, 1956; Bauchot and Pras, 1980; Bianchi, 1984; Whitehead *et al.*, 1986; Fisher *et al.*, 1987), while in captive sea bream they are much more variable (Boglione *et al.*, 2001); similar observations have also been made in red sea bream (*Pagrus major*; Matsuoka, 1987) and sea bass, (*Dicentrarchus labrax*; Marino *et al.*, 1993). Direct comparisons of rates of deformities in wild and captive populations are still impractical, but a sensible argument can be based on the observation that some deformities do not alter rates of survival in capture–recapture studies and yet they are rarely seen in wild fish. Rockfish (*Sebastes inermis*) with pelvic fin deformities performed just about as well as those with normal fins in capture and release studies, and yet this condition is associated almost exclusively with hatchery production (Murakami *et al.*, 2004). In a recently concluded study that involved frequent sampling of fertilized eggs and all ages of embryonic and larval fishes from the Gulf of Kuwait, virtually no deformed larvae appeared among the samples (C. Brown, unpublished). In addition, it has become apparent that captive fish have variable and often high rates of deformities, which in all likelihood are caused by a range of genetic, environmental and nutritional problems. References are abundant in which the morphological problems of hatchery-reared fish are recognized and attributed to problems and conditions associated with captive culture (for example, see Fraser and de Nys, 2005). It was argued years ago that a majority of cultured marine fishes in Japan suffer from some sort of developmental deformities (Fukuhara *et al.*, 1980), and although the standards of larval rearing have improved and the relative frequencies of deformities have undoubtedly been reduced, these problems still exist with cultured fishes.

Robust fingerling production remains a serious impediment to the cultivation of numerous technically difficult species of fish with otherwise good aquaculture potential (National Research Council, 1992). Captive conditions often foster irregularities early in differentiation, which are fully expressed by the time of metamorphosis in survivors (Koumoundouros *et al.*, 1997a). The production of large numbers of fry is a nearly universal goal of aquaculture, and striving to accomplish this can dramatically elevate deformity levels, both by generating fry under conditions that induce deformities and by promoting the survival of such compromised fish.

One contributor to the elevation of deformity rates in captive fish is selection under artificial conditions, which can convey some disadvantages. Cultured ornamental fishes, such as the goldfishes, exemplify this principle, in the sense that traits that would be problematic in nature are deliberately concentrated in 'true breeding' homozygous strains. Certain grossly deformed genetic strains have, in fact, become highly prized. Double or missing fins, albinism, scale, pigment and other anomalies are among the heritable traits that are mainstays of the

ornamental fish trade. Vertebral compressions, 'lion-headedness', 'veil fins' and other characters that change appearance but which can potentially interfere with mobility would be selected against heavily in wild fishes, but these features are considered to be highly desirable in some strains of ornamental fishes.

In the rather extreme ornamental fish cases mentioned above, highly visible features are incorporated by artificial selection, which would reduce the fitness of these animals if they were to escape or interbreed with wild fishes. The same principle is true to a lesser degree among some cultured edible fishes; clearly in at least some cases, the defeat of natural selection is one goal of aquaculture. The economics of farming leads fish culturists to generate progeny in relatively large numbers from a limited parental pool, thereby concentrating traits that favour growth and reproduction in a captive-rearing environment and to some extent disfavour survival and adaptability to a range of wild conditions. Captive populations of fishes are subjected not only to artificial selection but simultaneously to natural selection and genetic drift, and consequently these fishes diverge to varying degrees from the wild-type genome. Through this pattern of selection, heterozygosity may become restricted in a captive-breeding population, and for this reason, the resistance to deformities can be reduced or lost altogether by fish farmers. Consequently, some of the inherent genetic flexibility that is a benefit of heterozygosity in circumventing vertebral deformities (Shikano *et al.*, 2005) can be threatened in an aquaculture situation. Genetic and other problems accentuated by captive breeding have been perceived by some to be a significant problem in salmonid culture, in which hatchery rearing has been used for decades to supplement wild population stocks (Flagg *et al.*, 2000). The genes that either cause or impart resistance to certain heritable defects can increase in abundance as a consequence of the concentration and amplification of undesirable traits by breeding and rearing programmes (Aulstad and Kittelsen, 1971; Kincaid, 1976; Campbell, 1995; Gjerde *et al.*, 2005); some cohorts consequently can and do exhibit very high deformity rates.

In order to avoid or at least minimize inbreeding effects, genetic protocols are sometimes used in the breeding programmes of fish for restocking programmes. In these protocols (for example, see Tringali and Leber, 1999) the genetic make-up of the captive population is monitored in order to maintain the heterozygosity and other aspects of the wild genome. Under breeding and stocking programmes of this sort, deliberate efforts are made to conserve both gene frequencies and the presence of rare alleles that are found in the wild population.

Genetically derived deformities that would normally be made scarce over the course of a relatively small number of generations in wild populations can be sustained and their likelihood of expression increased as a result of human intervention and artificial selection. This is an example of directional selection – selection by culturists is generally in favour of survival, rapid growth and reproduction in captivity, which can sacrifice some of the genetic diversity that enhances adaptability, resulting in reduced disease resistance and/or resistance to developmental deformities. It has also been implied that observed or published estimates of the rates of deformities may be inaccurate because deformed fish are so much easier to catch than are intact fish (Poynton, 1987).

Causative Factors

Genetics

Careful genetic management plans are needed in conjunction with large-scale hatchery efforts involving salmonids (see Shacklee *et al.*, 1993), and the genetic constitution of other wild fish populations can also be altered in untoward ways as a result of stock enhancement efforts. Stock enhancement is a blend of aquaculture and wild stock management in which cultured fishes are released into wild populations; in the course of doing that, it is possible to alter population genetics artificially and to reduce or otherwise shift patterns of genetic diversity in mixed

cultured and wild populations. Wild genomes also intermix with genomes derived in captivity as a result of escapes or introductions of aquacultured fishes. Subsequent generations have a mixed cultured and wild genome with potentially compromised heterozygosity, which may increase the fish's susceptibility to the development of deformities.

Although some physical deformities are indisputably heritable, and inbred populations may have these problems at an unacceptably high rate, it is equally clear that many or most deformities that we see are not. In the Atlantic salmon, *Salmo salar*, the susceptibility to spinal defects can be genetically determined and at least under some conditions is considered to be heritable (McKay and Gjerde, 1986). Some spinal deformities including vertebral and opercular malformations are considered to be similarly heritable in at least one strain of gilthead sea bream (Afonso *et al.*, 2000), although a range of other studies suggest epigenetic factors may be more important (Chatain, 1987; Andrades *et al.*, 1996; Divanach *et al.*, 1996). In contrast, poorly or incompletely formed gill opercula in the tilapia, *Oreochromis niloticus*, are attributed to environmental factors and are not considered to be heritable (Tave and Handwerker, 1994). In extreme cases, genetic manipulations can grossly accelerate the rate of spinal and other deformities; triploid fishes have high rates of skeletal and gill malformations in various species (Madsen *et al.*, 2000; Sadler *et al.*, 2001). Interspecies hybrids can be very susceptible to deformities as well (Iwamatsu *et al.*, 1986).

Environmental disruptions

It was reported earlier that inappropriate conditions for the culture of larval fishes such as thermal shocks, nutritional inadequacies or other suboptimal culture conditions can cause spinal curvatures (Brown and Núñez, 1998), but the cause and effect relationships of these conditions to such deformities are not at all straightforward. Non-congenital vertebral deformities have been reported in channel catfish, *Ictalurus punctatus* (Dunham *et al.*, 1991), evidently in response to deficient environmental conditions. In recent years, reports have accumulated of more conditions that can cause developmental anomalies, such as failure to inflate the swimbladder (Chatain, 1994). Deformities are promoted by high stocking densities (Mohseni *et al.*, 2000), high current velocities (Backiel *et al.*, 1984; Divanach *et al.*, 1997), the presence of certain pathogens (Madsen and Dalsgaard, 1999; Oh *et al.*, 2002) or exposure to inappropriate dissolved oxygen concentrations in the rearing tank (Hattori *et al.*, 2004). Even small differences in salinity can alter the frequency of deformities in euryhaline sea bass (Johnson and Katavic, 1984), in a freshwater fish (*Clarius* sp., see Borode *et al.*, 2002), and in *Salmo salar*, the catadromous Atlantic salmon (Bolla and Ottesen, 1998).

The possible means by which stocking density may affect development are numerable; high stocking densities can contribute to nutritional inadequacies, water chemistry imbalances and assorted other physiological changes. In addition, high stocking densities of fishes can cause social or crowding stress, which is mediated in part through the endocrine pituitary gland–interrenal tissue axis. It has become apparent that the appearance of most deformities is of very little utility in the diagnosis and correction of a particular culture system inadequacy, since it is so often the case that a variety of different potential causative factors may result in similarly problematic developmental outcomes. Most culturists that encounter an unacceptably high frequency of one or more particular developmental anomalies cannot deduce that one specific genetic, environmental or nutritional variable is responsible, but rather they are aware that one or more elements in the culture system are probably suboptimal, and further investigation and refinement is necessary (for example, see Dores *et al.*, 2006).

Nutritional deficiencies

Dietary deficiencies in captive fishes have been associated with erratic development,

resulting in increased frequencies of abnormalities (see Mills *et al.*, 1993; Cahu *et al.*, 2003). Even some particular abnormalities that have on occasion been associated with heritability issues are known to be inducible in cases in which a nutrient, micronutrient or vitamin is available in deficient quantities. It appears that development according to the genetic programme can be comprised as a series of differentiational events that are orchestrated by way of hormonal and possibly neural signals that can fail in the absence of sufficient quantities of micronutrients, vitamins or possibly structurally important raw materials. For example, low vitamin K concentrations result in an increased frequency of bone deficiencies in common mummichug (*Fundulus heteroclitus*) (Udagawa, 2001), and vitamin C deficiency through impaired collagen formation is also implicated in the development of skeletal deformities (Santamaria *et al.*, 1994; Gapasin *et al.*, 1998; Cahu *et al.*, 2003).

Hormones

It is also known that the control mechanisms involved in the regulation of differentiation can cause disruptions. In the zebrafish (*Danio rerio*), development-promoting hormones, such as thyroid hormones, are important for normal cartilage development in the jaw (Liu and Chan, 2002), although excessive quantities of exogenous hormone can induce spinal and other developmental defects in teleost fishes (Brown and Bern, 1989). The timing of developmental signals can also be critically important; in spotted halibut (*Verasper variegates*), thyroid hormones administered at the correct time induce larval metamorphosis, but early or late endocrine signals can result in morphological or pigment (skin) anomalies (Tagawa and Aritaki, 2005).

Stress

The causes of development of common morphological disorders may be exceedingly ambiguous; in at least one case it has been proposed that handling stress is responsible for the onset of fin deformities (Martinez, 1996). Swimbladder inflation delays and consequent skeletal malformations have been attributed to high activity levels in sea breams (*S. auratus*) that have been raised in rapidly flowing water, in which continual swimming is required (Chatain, 1994). Among explanations for problems with swimbladder inflation is the possibility that gulping and swallowing of air assists or is necessary for inflation, and heavy swimming activity can interfere with the ability of larval or juvenile fishes to linger at the surface of the water sufficiently long to carry out this process (Chatain, 1994). Alternatively, inflation of the swimbladder has also been attributed to gas secretion by the *rete mirabile*, which does not require access to the surface to gulp water. Failure of swimbladder inflation is associated with pre-haemal lordosis, while a further centre of lordosis occurs in the haemal region, which has been associated in sea bass (Divanach *et al.*, 1997) and red sea bream (*P. major*) (Kihara *et al.*, 2002) with intense swimming effort in fish with an inflated swimbladder. Recent biomechanical analysis in sea bass suggests that lordotic vertebrae may be an adaptation to increased swimming activity (Kranenbarg *et al.*, 2005). However, during the development of lordosis it is uncertain whether the sequence of events was one in which excessive swimming caused a cascade of morphological problems, whether culture conditions were compromised because they were physiologically stressful, or both. Nevertheless, some authors have made a direct association of increased frequencies of developmental defects with handling stress, as in the milkfish (*Chanos chanos*) (Hilomen-Garcia, 1997) and the razorback sucker (*Xyrauchen texanus*) (Martinez, 1996). If in fact stress – as manifested in the synthesis, release and actions of interrenal glucocorticoid hormones – is an integral component of the differentiation of physical deformities in developing fishes, to the knowledge of the authors the endocrine mechanism of such an interaction has not been identified. It would seem to follow that the elimination

of deformities could hinge on not only the provision of acceptable environmental and nutritional conditions but also on the elimination or reduction of stress. For many aquatic species, eliminating culture stress is a tall order; some stresses are considered unavoidable and some species are poorly adapted to captive rearing and become stressed easily.

The bluefin tuna, *Thunnus thynnus,* presents some major challenges along these lines. It has been cultured in tanks at the New England Aquarium with assorted skeletal problems such as osteoporosis, which leads to increased susceptibility to bone fractures (Krum *et al.*, 1995). This is an unusual case, in which a skeletal deficiency has been identified as occurring well after the time of skeletal ossification, although it is not clear whether this defect can be attributed to a specific environmental flaw or to the constraints of captive rearing on this large, open-water, athletic species. Despite the problems associated with tank culture, the stress of culture in open-water cages has been described as less acute than capture stress in this species (Orban *et al.*, 2006). These and other culturists have had varying degrees of success with tunas in marine cages and pens – a culture method which evidently does not induce bone disorders. Wild tuna stocks have been plummeting because of overfishing (Castro and Huber, 2007), and because the tunas are such high-priority species for market and conservation reasons, efforts to find a practical means of cultivating them are intense and sustained. For other species to be mass-cultured, it may be productive in the long run to concentrate most culture efforts on the domestication of those species that more readily adapt to aquaculture conditions, as opposed to those that acclimate poorly or are generally stressed by captive-rearing situations.

Exposure to toxic materials

Some exposures to toxic materials can lead to physical deformations that are comparable to those seen in other non-infectious conditions. Usually reactions to toxic materials are restricted to physiological and neurological disorders rather than morphological alterations, but there are exceptions. Exposure of fathead minnows (*Pimephales promelas*) to concentrated organic chemicals induces a cluster of behavioural and metabolic dysfunctions, which are first manifested in behavioural irregularities and later in physical problems, which include scoliosis (Drummond and Russom 1990). Long-term exposure to heavy metals can also affect physiology in ways that lead to vertebral abnormalities in fourhorn sculpin (*Myoxocephalus quadricornis*) (Bengtsson and Larsson, 1986).

Commonly Seen Deformities

Skeletal disorders

Spinal deformities and other skeletal problems are a frequent occurrence among cultured fishes; either weak or deformed bones are commonly seen during captive rearing. Typically, three types of spinal curvature are detected: lordosis, kyphosis and scoliosis, which correspond respectively to ventral, dorsal and lateral curvatures. These problems can be prevalent in the rearing of relatively small larvae, and the frequency may reach especially high levels in preliminary or experimental attempts to rear marine larval fishes. Fishes that have not yet gone through skeletal ossification can be especially susceptible to disruptive influences. Factors inducing spinal curvatures can be difficult to ascribe to a particular cause, since a wide range of potential causative factors have been identified. Moreover, this problem is aggravated by the relative scarcity of studies about the development and regulation of the fish skeleton.

Spinal curvatures (scoliosis) can occur during the differentiation of the vertebral column from mesodermal tissue (See Figs 5.1 and 5.2). Mesodermal tissue differentiates into somatomeres, or concentric assemblages of mesodermal cells. The somatomeres develop into dorsally situated, segmented somites, which surround the notochord and



Fig. 5.1. Aquacultured gilthead sea bream (*Sparus auratus*) 3 months post-hatch. Vertebral column deformed and evident on external observation; cause unknown.



Fig. 5.2. Larval zebra fish (*Danio rerio*) with an acute spinal curvature. Photograph: J. Nunez.

spinal chord and are then called sclerotomes. The dorsal somite wall gives rise to segmented muscle tissue (myotomes) and the dermis, and arteries and other tissues proliferate between the segments. The condensation of sclerotomal cells on the surface of the primary notochord sheath gives rise to the future vertebrae centra and, as is characteristic of all dermal bones, calcium is deposited directly in this tissue and no cartilaginous intermediate forms. Vertebral bodies form at the juncture of adjacent sclerotomes, and myotomes form axial musculature and connective tissue to move and stabilize the column. The factors which regulate calcification of the vertebral bodies in teleosts are still poorly characterized. It seems likely that a complex interplay between endocrine factors, such as parathyroid hormone and parathyroid hormone-related proteins only recently identified in fish (Canario *et al.*, 2006), and a number of different extracellular matrix proteins, such as osteonectin, osteocalcin and members of the secretory calcium-binding phosphoprotein (SCPP) family (Kawasaki *et al.*, 2004; Estêvão *et al.*, 2005; Redruello *et al.*, 2005; Roberto *et al.*, 2006), are important in mineralization of vertebral bodies and other dermal and endochondral bone of the teleost skeleton.

The skeletal differentiation process is subject to failures at several points during the formation and ossification of the vertebrae (Fig. 5.1). Other anomalies include incomplete dorsal fusion of the vertebrae around the spinal cord (spina bifida), and segmentation errors, which can result in a series of fused vertebrae. Curvatures and compressions of the spine can also result from incorrectly formed vertebrae or vertebral musculature or from a variety of fractures. Weak and excessively porous vertebrae are prone to such fractures.

Some heritable spinal deformities have been identified. In addition, assorted environmental problems are known to induce spinal deformities, including temperature, lighting and exposure to some toxic and infectious agents. The correct differentiation of the vertebrae is also sensitive to nutritional status; controlled experiments have shown that vitamin deficiencies can induce spinal curvatures (Udagawa, 2001). Vitamins C and K, and tryptophan have each been shown to be associated with erratic skeletal development, or have been shown to be capable in some cases of preventing such deformities (Akiyama *et al.*, 1986a,b; Soliman *et al.*, 1986; Kanazawa *et al.*, 1992; Udagawa, 2001; Cahu *et al.*, 2003). The observation of skeletal deformities in goldfish fed deficient diets (Mills *et al.*, 1993) does not imply that other skeletal deficiencies seen in this or other fish species are nutritionally based, since so many deformities of this sort have environmental causes. Thermal shock and other environmental conditions not directly related to nutrient intake have been found to induce spinal curvature, and the aetiology of this problem may be associated with the sensitivity of the systems involved, e.g. muscle and bones (see Brown and Núñez, 1998; Stickland *et al.*, 1988; Koumoundouros *et al.*, 2001; Johnston and Temple, 2002; Campinho *et al.*, 2004; Sfakianakis *et al.*, 2004, 2005).

Head and jaw malformations

Problems with inadequate differentiation of the head and jaw are commonly reported, occasionally with a very high rate of incidence and with varying severity. The problems probably originate in embryos and early larvae, when the cartilage template of this region develops (Kimmel *et al.*, 1995). Moreover, such abnormalities, when they do not compromise survival, are persistent. Some of the most frequently cited problems include gross distortions such as asymmetric bites or 'crossbite' caused by a lateral shift of the inferior jaw bones (Fig. 5.3). Abnormalities of the head such as 'pugheadness', in which there is reduction of the frontal skull and upper jaw bone and reduction in the length of the upper or lower jaw (sucker mouthed) (Barahona-Fernandes, 1982). Opercular complex abnormalities can occur with a high incidence in aquacultured fishes and have also been found in wild fish in polluted waters (Sloof, 1992; Lindesjoo *et al.*, 1994). These abnormalities affect biological performance (Andrades *et al.*, 1996; Sumagaysay

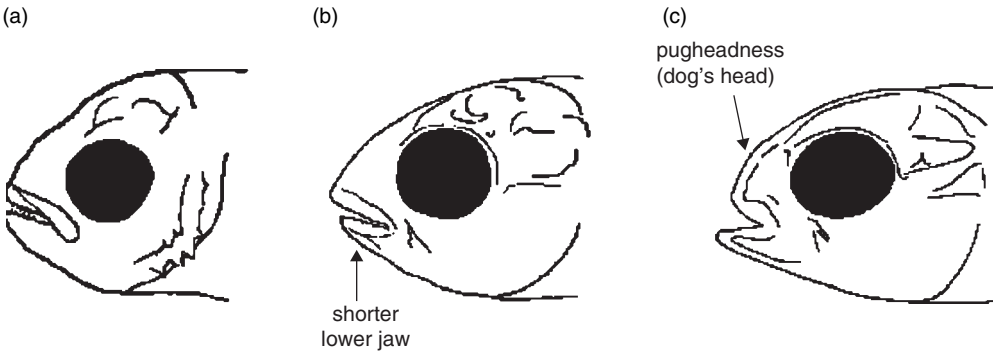


Fig. 5.3. Camera lucida drawings of head of gilthead sea bream (*Sparus auratus*) larvae. (a) normal specimen; 20.4 mm L_S; (b) deformation of lower jaw, 15.7 mm L_S; (c) deformation of frontal and upper jaw, 11.6 mm L_S.

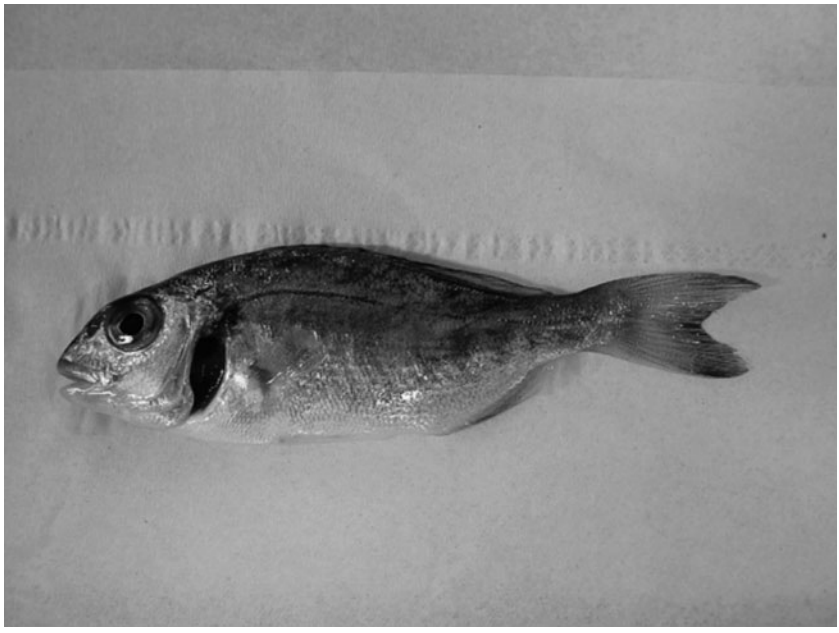


Fig. 5.4. Juvenile gilthead sea bream (*Sparus auratus*) with an incompletely formed gilloperculum; cause unknown.

et al., 1999) and are generally characterized by folding and twists of the operculum and size reduction, and are generally unilateral (Fig. 5.4; Barahona-Fernandes, 1982; Francescon *et al.*, 1988; Tave and Handwerker, 1994; Koumoundouros *et al.*, 1997a). In common with most other skeletal abnormalities, a range of different factors have been implicated in their appearance in cultured fishes, and nutritional deficiencies have been clearly linked to this problem (Gapasin and Duray, 2001; Cahu *et al.*, 2003) although unfavourable abiotic parameters and pollution also play a role.

Some head and jaw problems are environmentally induced; changes in temperature and lighting led to increased incidence of mouth deformities in Atlantic halibut,

Hippoglossus hippoglossus (Bolla and Holmefjord, 1988). The halibut is also subject to other deformities of the head and eye, which are associated with their unique pattern of larval–juvenile metamorphosis; one example is a failure of the eyes to migrate to the dorsal position (Fig. 5.5). In one cultured population of barramundi (*Lates calcifer*), the rate of improper jaw morphology was reported at 35.7%, as a consequence of shortened upper and/or lower jaws (Fraser and de Nys, 2005).

Fin disorders

Fin deformities include misshapen fins, incompletely formed fins or fins of reduced size, and these defects are often seen in conjunction with skeletal disorders. Characteristic skeletal deformities associated with fin deformities include fusion, deformation and displacement of the elements making up the fins. Most fin deformities are observed in captive-reared fishes (see Fig. 5.6); although less frequently, some incidence of this class of problems has been reported in wild-caught fishes as well (Matsuoka, 1987; Daoulas *et al.*, 1991; Marino *et al.*, 1993; Koumoundouros *et al.*, 1997b; Boglione *et al.*, 2001). Genetic factors have been associated with some fin deformities detected in medaka (*Oryzias latipes*) and tilapia (*O. niloticus*) (Ishikawa, 1990; Mair, 1992). Thermal shocks can also induce disruptions of fin development and cause skeletal defects (see Brown and Núñez, 1998). Other environmental disturbances associated with very high frequencies of fin deformations include gas hypersaturation in the rearing tank (Oritz-Delgado and Sarasquete, 2006).

Skin disorders

Flatfishes show a dorsoventrally asymmetrical pattern of pigmentation. During larval to juvenile metamorphosis, the dorsal side becomes pigmented and the ventral side loses most of its pigmentation. This has

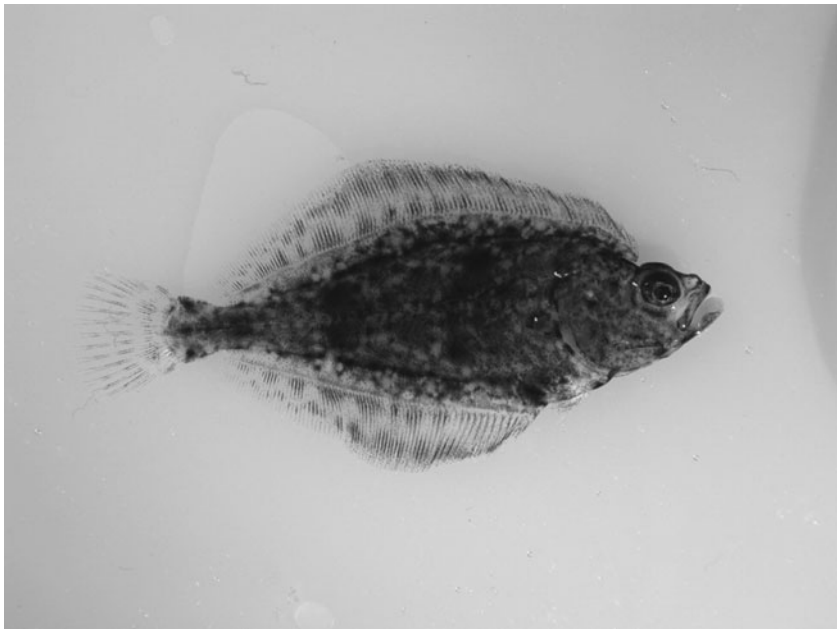


Fig. 5.5. Juvenile Atlantic halibut (*Hippoglossus hippoglossus*) with larval to juvenile body differentiation but a lack of eye migration to the right side.

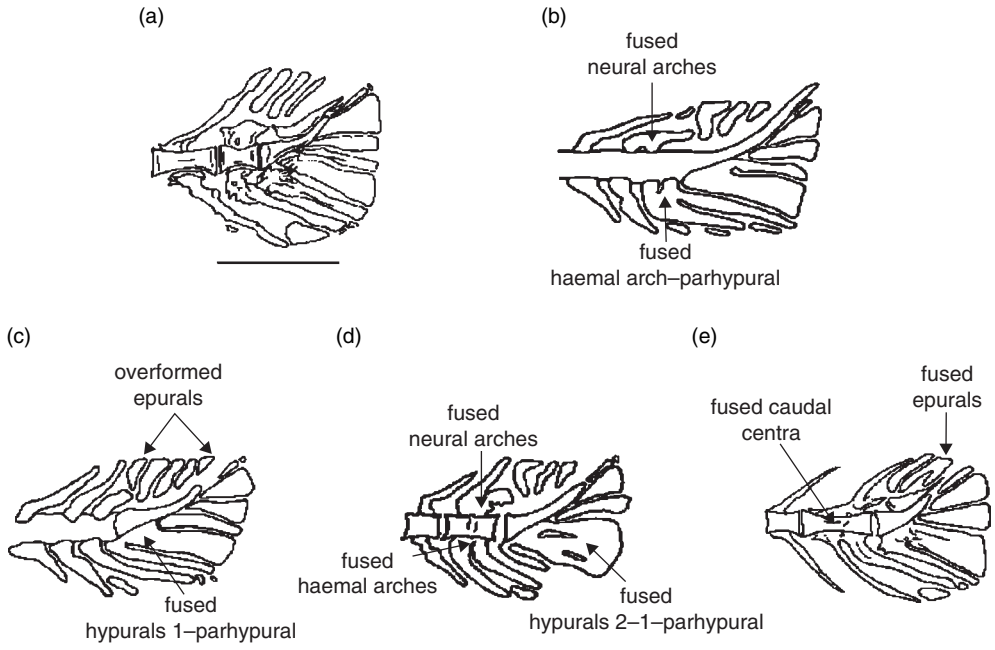


Fig. 5.6. Camera lucida drawings showing some of the more frequently seen abnormalities at the caudal region in gilthead sea bream (*Sparus auratus*) larvae. (a) normal specimen, 16.0 mm L_S; (b) 7.7 mm L_S; (c) 8.7 mm L_S; (d) 10.8 mm L_S; (e) 18.7 mm L_S.



Fig. 5.7. Juvenile Atlantic halibut (*Hippoglossus hippoglossus*) displaying irregular pigmentation.

been problematic for culturists working with flatfishes, which occasionally show erratic patterns of pigment distribution. The pattern of pigmentation in the spotted halibut appears to be determined to some extent by the secretion of thyroid hormones in a timing-dependent fashion, as related to other metamorphic events (Tagawa and Aritaki, 2005). Other captive-reared flatfishes show erratic patterns of pigmentation on occasion (Fig. 5.7), which may have a nutritional and/or neuroendocrine basis. Hypermelanosis has been observed in Japanese flounder (*Paralichthys olivacenus*) reared in captivity on diets supplemented with Vitamin D (Haga *et al.*, 2004).

In some fishes, a condition known as scale disorientation has been described, in which patches of scales are rotated into an incorrect orientation. In a wild population of pinfish (*Lagodon rhomboides*), scale disorientation amounting to up to 34% of the surface area of the skin has been documented (Corrales *et al.*, 2000). Because high frequencies of misaligned scales were found in pinfish collected from contaminated areas, those authors ascribed the skin disorder to habitat degradation (Corrales *et al.*, 2000).

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6 Stress Response and the Role of Cortisol

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Introduction

In vertebrates, generally the physiological responses to stressors serve an important survival function, and the pattern of the stress response has been highly conserved. However, repeated and chronic exposure to stressors has a detrimental effect on many aspects of the organism's physiology, including changes in nervous system function, metabolism, growth and development, reproductive function and immune system function; some of these are discussed in this chapter.

In the last couple of decades, several reviews have described the organismal and cellular stress responses in fish (Barton and Iwama, 1991; Gamperl *et al*., 1994; Wendelaar Bonga, 1997; Iwama *et al*., 1998, 2006; Barton *et al*., 2002), and although it is known that stressed fish exhibit poor growth and detrimental health effects, the mechanism(s) involved in bringing about these changes are far from clear. Indeed, a major focus of research related to aquaculture is the identification of stress markers in fish, be they molecular, biochemical or hormonal, that would accurately reflect the stress/health status of the animal. This is important as it would lead to development of husbandry practices to reduce or alleviate stress in aquaculture operations, leading to improved quality and production. New technologies, including genomics and proteomics, will undoubtedly pave the way for identifying key regulatory gene and protein networks activated in response to stressors and will generate hypotheses to test the physiological consequences associated with the activation of stress-responsive pathways. The best-studied component of the stress response is the elevation of plasma cortisol levels, and this steroid hormone is considered to be one of the best indicators of acute stress in fish. A number of reviews have been written on plasma profiles of cortisol in response to various stressors and the physiological consequences of elevated cortisol levels in fish (Barton and Iwama, 1991; Gamperl *et al*., 1994; Wendelaar Bonga, 1997; Mommsen *et al*., 1999; Barton *et al*., 2002; Iwama *et al*., 2006). This chapter will focus more on the latest developments in cortisol stress physiology and will highlight some of the areas that we believe will be useful in identifying markers that will be indicative of stress and/or health effects in fish. This work is not intended to be an exhaustive review of literature of stress and/or cortisol in fish; instead the work will focus on what we know about the mechanism of action of cortisol and its physiological implications, which may be relevant for developing markers of stress and/or health status of fish.

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The Autonomic Nervous System and the Catecholamine Response to Stressors

The autonomic nervous system (ANS), which is sometimes called the visceral nervous system, is the part of the peripheral nervous system that regulates the organ systems that are involved in maintaining homeostasis in the body of all vertebrates; these activities are generally performed without conscious

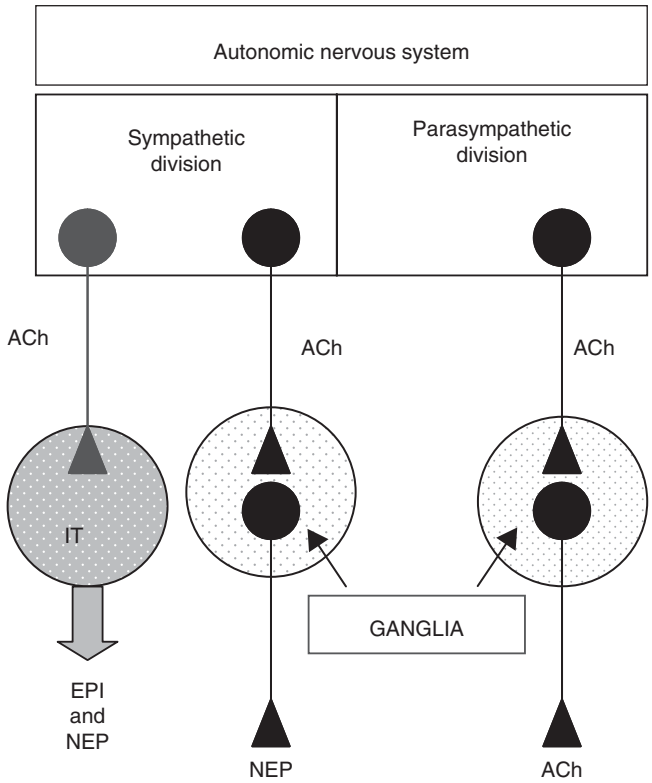


Fig. 6.1. Schematic representation of the components of the autonomic nervous system and the regulation of secretion of the catecholamines, epinephrine (EPI) and norepinephrine (NEP) by stimulation from the sympathetic division of the autonomic nervous system. The cartoons of neurons show the cell body (circle) and synapses (triangles) connected by the axon. Pre-ganglionic myelinated cholinergic neurons (using acetyl choline (ACh) as a neurotransmitter) have their cell body in the central nervous system; their axons extend from the central nervous system into the peripheral nervous system. With one exception, these axons terminate on the dendrites of neurons in a dorsal root ganglion. Action potentials arriving at the synapses cause the release of ACh, which acts on receptors in the dendrite membrane of specific dorsal root ganglia neurons (the so called post-ganglionic neurons); these are non-myelinated adrenogenic (using NEP as their neurotransmitter) neurons that innervate tissues of the cardiovascular and respiratory systems among others, regulating normal physiological function; increased activity of these neurons during a stress response increases cardiovascular and respiratory rates. The single exception is the group of pre-ganglionic neurons that do not terminate in ganglia but end in the interrenal tissue (IT) of the anterior (head) kidney, where they innervate the chromaffin cells of the interrenal tissue. The chromaffin cells are the homologue of the adrenal medulla of mammals, and the pre-ganglionic neuron innervation stimulates the cells to synthesize and release EPI and smaller amounts of NEP. The secretion of the chromaffin cells maintains normal physiological function, particularly the regulation of glucose homeostasis, but also contributes to cardiovascular and respiratory function; increased stimulation as part of the stress response brings about enhanced plasma glucose levels and increases in other forms of energy metabolites.

control, although these work together with voluntary control of some organ systems, such as ventilation of the gill surface. The ANS can be subdivided (by systems) into the parasympathetic nervous system and the sympathetic nervous system, and subdivided by functions into sensory and motor components. For further information about the function of the ANS in fish, and the neurotransmitters that play roles in the system, the reader is referred to reviews by Gibbins (1994) and Holmgren and Jensen (1994).

Acute stressors, including net-capture of fish for sampling, results in the rapid activation of the sympathetic division of the ANS, leading to increased action potential frequency in postganglionic neurons and increased release of the catecholaminergic neurotransmitter norepinephrine (NEP). These postganglionic neurons innervate muscles associated with the cardiovascular and respiratory systems and the viscera, increasing heart rate and contractility, dilating blood vessels of the respiratory system, and decreasing blood flow in the viscera. A second level of catecholamine response is the activation of the chromaffin cells of the adrenal medulla by cholinergic axons of the ANS. In fish, the chromaffin cells are distributed around the post-cardinal vein, predominantly in the anterior (head) kidney region, and together with steroidogenic cells (discussed below) form the interrenal tissue (the homologue of the adrenal gland in mammals) (Reid *et al.*, 1998). Increased cholinergic neuronal activity stimulates the release of the catecholamines epinephrine (EPI) and NEP; these hormones enter the blood and enhance the cardiovascular and respiratory effects of the postganglionic neurons; in addition, they act on the liver and other tissues to stimulate the mobilization of carbohydrate reserves, leading to an increased plasma glucose level; glucose is an important source of energy to sustain increased post-stressor activity. The increased release of catecholamine hormones from the chromaffin cells occurs within seconds of the animal's perception of a stress event and they are usually cleared very rapidly from the circulation (see Reid *et al.*, 1998 for a review on the role of catecholamines).

Hypothalamus–Pituitary Gland–Interrenal Tissue (HPI) Axis

A second layer of the response to an acute stress involves the increased secretion of glucocorticoid steroid hormones from steroidogenic cells of the interrenal tissue. The neural link between the perception of a stressor and the activation of the neurons in the paraventricular nucleus of the hypothalamus that initiate the cascade leading to the increased secretion of cortisol in fish (and other vertebrate taxa) is still poorly understood; however, *in vitro* and *in vivo* studies in mammals have shown that many different types of neurons originating from several different regions of the brain, and using different neurotransmitter substances, and multiple isoforms of neurotransmitter receptors are involved. In mammals, the factors that have been found to be involved include excitatory amino acids, such as glutamate; the catecholamines EPI and NEP; dopamine; serotonin (5-HT); gamma amino butyric acid (GABA); neuropeptides Y (NPY) and P (NPP); somatostatin; prostaglandins; nitric oxide (NO); interleukins; various growth factors; glucocorticoids; and locally synthesized proopiomelanocorticotropin (POMC); all appear to play a role in the regulation of the corticotropin-releasing hormone (CRH)-secreting hypothalamic neurons (Kiss *et al.*, 1996; Conn and Freeman, 2000; Kiss and Aguilera, 2000; Watts and Sanchez-Watts, 2002; Kasckow *et al.*, 2003; Bugajski *et al.*, 2004, 2006; Watts, 2005; Silva *et al.*, 2005; Luque *et al.*, 2006; Iranmanesh and Veldhuis, 2008). Very little is known about the regulation of the hypothalamus–pituitary gland–interrenal tissue (HPI) axis in fish.

In many fish species, elevated plasma cortisol levels are found within minutes of exposure to an acute stressor, and the hypercortisolism may be maintained for several hours. The cortisol response is also beneficial in enabling the animal to cope with the stress, in part by virtue of the gluconeogenic actions of cortisol, which allow the production of glucose from non-carbohydrate sources; however, chronic elevation of glucocorticoids has deleterious effects

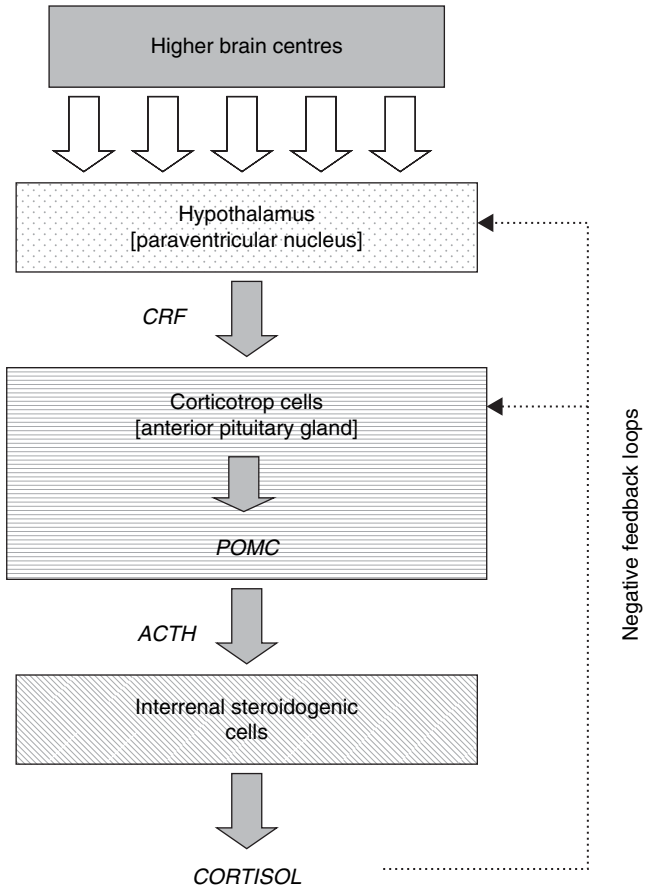


Fig. 6.2. Schematic diagram of the hypothalamus–pituitary gland–interrenal tissue (HPI) axis. Specific neurons in the paraventricular nucleus synthesize and secrete the peptide neurohormone corticotropin-releasing factor (CRF); CRF is synthesized in the cell body of the CRF-secreting neurons and transported to the anterior pituitary gland via the axons and released by exocytosis from synapses that are located close to the region of the anterior pituitary gland that contains the corticotrop cells, which secrete adrenocorticotropin (ACTH); CRF is the main stimulus for the synthesis of proopiomelanocorticotropin (POMC), the precursor for ACTH. CRF also stimulates the synthesis of convertases that catalyse the release of ACTH and β-endorphin from the larger POMC molecules. ACTH is the primary factor regulating the function of the steroidogenic cells of the interrenal tissue; ACTH activates G-protein-coupled receptors, eliciting intracellular cascades that result in translocation, via steroidogenic acute regulatory protein (StAR), of cholesterol into the mitochondria of the interrenal cells; the cholesterol is converted into pregnenolone. The translocation of cholesterol into the mitochondria is the rate-limiting step in the production of the primary end-point steroid, cortisol. Pregnenolone leaves the mitochondria and is bio-transformed by cytoplasmic enzyme systems into steroids that are precursors for cortisol manufacture; these precursors enter the mitochondria, and the final stage of cortisol formation is carried out by mitochondrial enzymes. The plasma concentration of cortisol feeds back to the CRF-secreting neurons of the hypothalamus and ACTH-secreting cells of the anterior pituitary gland and acts to reduce the secretion of CRF and ACTH to control the level of activity of the HPI axis. This is termed a negative feedback loop. As discussed briefly in the text, the overall control of CRF synthesis and secretion is far more complex than the cortisol negative feedback effect suggests. There are multiple factors involved, and very little is known about this aspect of HPI axis function in fish.

on immune system function, growth and development, and reproduction; these are discussed in this chapter.

In fish, the sensory component of the stress axis is the least studied, as most studies have focused on the hormonal response to stressor exposure. This stems from the fact that from a diagnostic standpoint it is easy to measure the release of hormones into the circulation. To this end, plasma levels of cortisol and catecholamines, more specifically EPI, are the indicators of choice to denote stressed animals. However, in fish it is very difficult to obtain resting levels of EPI because this hormone is released quickly into the circulation and is not delayed even by anaesthesia, and therefore it is not widely used as an indicator of stress. Cortisol, on the other hand, has a lag time before its release, which allows accurate measurement of resting levels and stressor-induced elevations, once the animals are anaesthetized and sampled quickly. Consequently, plasma cortisol level is the indictor of choice for detection of acute stress in fish. The cortisol response to stressors comprises the stress axis for this chapter and involves the hypothalamus, pituitary gland and the interrenal tissue. In teleost fishes, corticosteroid synthesis occurs in the steroidogenic interrenal cells, which constitute the teleost homologue of the adrenal cortex. However, these cells do not form a discrete gland and are instead located in groups, cords or strands along the walls of the posterior cardinal veins in close proximity to the catecholamine-producing chromaffin cells (Wendelaar Bonga, 1997; see also Chapter 3, this volume). The stimulation of the steroidogenic cells to secrete cortisol is under the control of the hypothalamus and the pituitary gland, which release corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH), respectively (Wendelaar Bonga, 1997). Very little is known about the sensory inputs and their activation leading to stimulation of the hypothalamus as part of the stress perception and coping mechanism; most studies have dealt with the sequence of molecular events at the hypothalamus, pituitary gland and interrenal tissue involved in the stimulation and biosynthesis of cortisol (Alsop and Vijayan, 2009b).

Recent studies on the ontogeny of the stress axis using zebrafish (*Danio rerio*) as a model suggest that the molecular components of the cortisol stress axis are developed prior to hatch, while the stressor-induced cortisol response is evident only later on in post-hatching (Alsop and Vijayan, 2008, 2009b). Alsop and Vijayan (2009b) hypothesized that this disconnect between the steroidogenic capacity of the cells and the actual perception and response to stress in zebrafish is due to the delay in the development of the neural circuitry innervating and stimulating the hypothalamus. It remains to be seen if this stressor hypo-responsive period during the critical transition phase from pre-hatched to post-hatched embryos is important for the development of the stress axis in fish. Indeed, the neural connections and stress perception is one area of research that is lacking in piscine models. The advent of genomic and proteomic technologies, along with the ease (as well as availability) of developing genetic (mutant) models in zebrafish, will pave the way for gaining further insights into the neuro-endocrine regulation of the stress axis in fish.

Cortisol biosynthesis and secretion

Adrenocorticotropic hormone (ACTH), the proopiomelanocortin (POMC)-derived peptide from the anterior pituitary gland, is the primary trophic hormone activating cortisol biosynthesis. The sequence of events involves the ACTH binding to melanocortin 2 receptor (MC2R), a G-protein-coupled receptor, and activation of adenylate cyclase and cAMP signalling cascade, leading to the transport of the steroid precursor cholesterol from the outer to the inner mitochondrial membrane. MC2R has been sequenced in fish, and increase in its mRNA levels has been observed in response to handling stress or on ACTH stimulation of interrenals *in vitro* (Aluru and Vijayan, 2008). This upregulation of MC2R mRNA levels resembles autoregulation that has been shown in mammalian models (Gantz and Fong, 2003). However, in the mammalian cell system, changes in MC2R mRNA

abundance were reported only after longer-term ACTH incubation, whereas we observed MC2R transcript upregulation with acute (2–4 h) ACTH stimulation *in vitro*, suggesting species-specific differences in the regulation of MC2R (Aluru and Vijayan, 2008). Nevertheless, activation of MC2R leads to the mobilization of cholesterol into the inner mitochondrial membrane, initiating cortisol biosynthesis. This shuttling of cholesterol is shown to be a rate-limiting step in cortisol biosynthesis and it is mediated by steroidogenic acute regulatory protein (StAR) (Stocco, 2000). In mammals, a very rapid increase in both StAR mRNA and StAR protein (reviewed by Lehoux *et al.*, 2003) occurs in response to ACTH stimulation. Moreover, plasma cortisol has been found to mirror levels of StAR mRNA (Le Roy *et al.*, 2000) and StAR protein (Liu *et al.*, 1996, Nishikawa *et al.*, 1996). StAR has been characterized in several fish species and has been shown to have similar steroidogenic function as observed in mammals. StAR transcripts have been detected in the steroidogenic tissues of rainbow trout (*Oncorhynchus mykiss*), and the levels of StAR transcripts in the interrenal cells have been shown to increase in response to severe acute stress (Kusakabe *et al.*, 2002, Geslin and Auperin, 2004) or under ACTH stimulation (Li *et al.*, 2003), suggesting that StAR is an important regulator of corticosteroidogenesis in fish. In addition to StAR, another protein, known as peripheral benzodiazepine receptor (PBR), localized in the outer mitochondrial membrane of steroidogenic cells, is also considered to play a role in cholesterol shuttling and activation of steroid biosynthesis (Papadopoulos, 1993). Compared to StAR, the precise role of PBR in fish has not been characterized. Recently, it was shown that rate-limiting steps in steroidogenesis are the targets of several contaminants, and both StAR and PBR transcript levels were downregulated by contaminants, resulting in a depressed cortisol production in response to ACTH stimulation, clearly supporting a key role for these transport proteins in corticosteroid biosynthesis (Hontela and Vijayan, 2009).

Corticosteroid synthesis from cholesterol involves a series of enzymatic steps catalysed by cytochrome P450 enzymes and hydroxysteroid dehydrogenases (HSDs) (Sewer and Waterman, 2003). Metabolism of cholesterol to pregnenolone by cytochrome P450scc (P450 side-chain cleavage) is followed by conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). This yields an active steroid, which also is a precursor for adrenal and other steroids. Progesterone is further metabolized by a combination of cytochrome P450 enzymes and steroid dehydrogenases, to give rise to cortisol. The control of cortisol secretion in teleost fishes is complex, and details of the steroidogenic pathways can be found in Kacsoh (2000). The significant reduction in cortisol release observed in hypophysectomized fish indicates that the pituitary plays the most important role in this context (Young, 1993). The pituitary produces ACTH and two other hormones which have been shown to be influential corticotropins: α-melanocyte-stimulating hormone (α-MSH) and β-endorphin. Although there is general agreement that ACTH is the main secretagogue for cortisol, recent studies in tilapia suggest that α-MSH, when potentiated by β-endorphin, may have a corticotropic activity comparable to that of ACTH (Balm *et al.*, 1993, Balm and Pottinger, 1995; Wendelaar Bonga, 1997). α-MSH has three hormonally active forms, of which the diacetylated version appears to be most important; the role of β-endorphin, which itself has no corticotropic activity, is to potentiate the activity of α-MSH (Balm *et al.*, 1993; Balm and Pottinger, 1995; Wendelaar Bonga, 1997). Many other hormones have been implicated in the regulation of cortisol secretion, most of them indirectly. These include angiotensin II, urotensins I and II, atrial natriuretic factor, growth hormone and thyroxine (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999; Hontela, 2005). Cortisol itself may exert an inhibitory effect on its secretion by modulating ACTH production via interactions with both the hypothalamus and the pituitary (Donaldson, 1981; Lederis *et al.*, 1994). Interleukin-like factors of the immune system may also have inhibitory effects, mostly via control of α-MSH release (Balm *et al.*, 1993). Finally, the close proximity of the interrenal

cells to the chromaffin cells suggests that paracrine control by catecholamines may also be involved (Reid *et al.*, 1996).

Cortisol dynamics

In mammals the majority of plasma cortisol (90–95%) is bound to a specific transporter protein, corticosteroid-binding globulin (CBG), which both controls its bioavailability (only free cortisol is biologically active) and may be involved in its delivery to target cells via interaction with CBG-binding sites (Fleshner *et al.*, 1995; Hammond, 1995). To date, CBG has not been cloned and sequenced in a piscine model, although one study did find a CBG-like protein in trout plasma (Caldwell *et al.*, 1991). Considering the importance of these proteins in the regulation of cortisol availability in mammals, further studies should be carried out to resolve this question in teleost fish. Another aspect that is not clear in piscine models is the negative feedback regulation of plasma cortisol levels. While studies have demonstrated that there may be a negative feedback that may be acting at the level of the hypothalamus and/or pituitary, as well as an ultra-short loop at the level of the interrenal tissue that regulates cortisol output, the mechanisms involved, specifically the role of corticosteroid receptors in this regulation, are unclear (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999; Hontela and Vijayan, 2009). A recent study showed that stressor-induced cortisol dynamics were altered by polychlorinated biphenyls (PCBs), and this coincided with lower brain glucocorticoid receptor (GR) protein content in Arctic charr (*Salvelinus alpinus*) (Aluru *et al.*, 2004), suggesting that corticosteroid receptor dynamics are critical for plasma cortisol regulation.

Cortisol, like other steroids, is a lipophilic molecule and is thought to cross target cell membranes by diffusion. This, however, may not be exclusive, and studies involving both mammals and fish suggest that a specific carrier may mediate transport (Porthé-Nibelle and Lahlou, 1981; Allera and Wildt, 1992; Vijayan *et al.*, 1997). Whether it interacts with receptors in target cells or not, cortisol is eventually metabolized by a number of cellular enzymes. Consistent with other lipophilic compounds, the metabolic strategy is to make the steroid molecules more hydrophilic, and this is accomplished by the actions of several cytochrome P450s, which inactivate the hormones by addition of hydroxyl groups, which facilitates steroid excretion (Pottinger *et al.*, 1992). Some steroid dehydrogenases inactivate steroids as part of an on–off switch that is important in steroid homeostasis.

Two important enzymes for this mechanism are 11β-hydroxysteroid dehydrogenase-type 2 (11 β-HSD-type 2) and 17α-HSD-type 2. 11β-HSD-type 2 catalyses the conversion of cortisol to cortisone, an inactive steroid. In addition, conjugation of compounds by glucuronidation is another pathway involved in the steroid metabolism. Uridine diphosphoglucuronosyltransferase (UDPGT) enzymes catalyse the transfer of the glucuronyl group from uridine 5′-diphosphoglucuronic acid to active endogenous and exogenous molecules having functional groups of oxygen, nitrogen and sulfur. The resulting glucuronide products are more polar, generally water soluble, less toxic and more easily excreted than the substrate molecule.

Mechanisms of action of corticosteroids

While plasma cortisol levels may be indicative of stressor intensity and duration, the target tissue response to hormone stimulation may not reflect a direct correlation with hormone concentration. A case in point is demonstrated by the strains of rainbow trout with consistently high (high responders) or low (low responders) cortisol response to stressor exposure (Pottinger and Carrick, 1999). Although the high responders showed a greater magnitude of cortisol response to stressors, the biochemical response to stressors was greater in the low responders, throwing doubt on the role of plasma cortisol levels as a direct correlate of physiological response during stress in fish (Trenzado *et al.*, 2003). This mismatch between plasma

cortisol levels and metabolic response may be related to altered receptor dynamics. While the study showed a sustained decrease in GR abundance based on binding studies in the high responders relative to the low responders (Trenzado *et al.*, 2003), a thorough investigation of spatial and temporal corticosteroid receptor (CR) abundance in response to stressors, as well as target tissue responsiveness to cortisol stimulation, is still lacking.

Most of the effects associated with cortisol in fish are thought to be mediated by genomic signalling involving cytosolic glucocorticoid receptor. The GR is a ligand-activated transcription factor, which upon activation translocates to the nucleus and interacts with specific DNA sequences, called glucocorticoid responsive elements (GREs), in the regulatory regions of target genes. The mechanism(s) involved in GR signalling is mostly based on mammalian studies as few studies have addressed this in fish. However, the rainbow trout GR, for example, has a high degree of sequence homology with the human form, except for an expanded region within the zinc finger sequence of the DNA-binding domain. This modification does not alter its transcriptional efficiency, although it appears to increase receptor expression (Ducouret *et al.*, 1995; Tujague *et al.*, 1998). However, unlike mammals, fish have multiple isoforms of GR, and they have been cloned and sequenced from a number of fish species (see reviews by Flik *et al.*, 2006; Prunet *et al.*, 2006; Bury and Sturm, 2007; Alsop and Vijayan, 2008, 2009a). Although, the two trout GR isoforms, GR1 and GR2, demonstrate different sensitivity to cortisol and RU486 binding and transactivation using *in vitro* reporter assays (Bury *et al.*, 2003; Prunet *et al.*, 2006), the functional significance of these isoforms *in vivo* is unknown. While all teleost fish examined to date have shown two isoforms of GR, zebrafish is unique in that the genome has only a single GR, similar to that in mammals (Alsop and Vijayan, 2008, 2009a; Schaaf *et al.*, 2008). Also, zebrafish is unique in showing a splice variant form of GR that is similar to the GR beta in humans (Schaaf *et al.*, 2008). Consequently, zebrafish appears to be an excellent model for studies pertaining to GR function in vertebrates, including the role of GR in human medicine (Schaaf *et al.*, 2008; Alsop and Vijayan, 2009a,b).

Similar to mammals, teleost fish also have a mineralocorticoid receptor (MR) (Sturm *et al.*, 2005; Prunet *et al.*, 2006; Bury and Sturm, 2007). However, a MR-specific ligand, including aldosterone, has not been conclusively shown in fish. It appears likely that cortisol may be the primary ligand for MR activation, while the changes in HSD2 expression suggest that a ligand in addition to cortisol may also be involved in MR signalling in fish (Alsop and Vijayan, 2008). The recent demonstration of developmental changes in GR and MR gene expression during embryogenesis led to the proposal that MR signalling by maternal cortisol may be playing a key role in the development of the cortisol stress axis post-hatch (Alsop and Vijayan, 2008). Altogether, both GR and MR signalling may be playing an important role in target tissue cortisol response in fish. However, the contribution of each receptor signalling in stress adaptation and their physiological consequences remain to be elucidated. Also, apart from the molecular structure of GR and MR, little is known about the actual role of these receptors in cortisol signalling.

Mammalian studies have clearly shown that GR is present as a heterocomplex with several other proteins and has a total mass of approximately 330 kDa, considerably more than the 85–100 kDa mass of the GR protein itself (Mommsen *et al.*, 1999). Comparatively less is known about the fish GR. Protein separation and binding experiments with tritiated cortisol in several species have isolated complexes in excess of 300 kDa, suggesting that GR is present as a heterocomplex with other proteins (Chakraborti and Weisbart, 1987; Mommsen *et al.*, 1999). Recent studies in trout clearly showed that, as in mammals, heat shock protein 90 (HSP90; a key molecular chaperone) is important for GR stability and signalling (Sathiyaa and Vijayan, 2003). Also in trout it was shown that the proteosome may be involved in GR regulation, including GR synthesis (Sathiyaa and Vijayan, 2003). This

is especially the case given the significant downregulation of GR protein in response to sustained cortisol stimulation both *in vivo* and *in vitro* using hepatocytes in primary culture (Sathiyaa and Vijayan, 2003; Vijayan *et al.*, 2003). The GR protein downregulation coincides with an elevation in GR mRNA abundance, pointing to a receptor autoregulation by cortisol. This increased GR turnover in response to stressors (elevated cortisol levels) may be a mechanism to sustain GR signalling to cope with the stressor insult.

Recently the notion that corticosteroid actions are exclusively genomic has been challenged in mammals, and evidence suggests that these hormones are capable of mediating rapid effects that depend on changes in intracellular Ca^{2+} and are insensitive to inhibitors of both transcription and translation (Wehling, 1997). In fish, similar findings have been reported for both cortisol and dexamethasone. In tilapia (*Oreochromis mossambicus*), for example, cortisol blocked both the increase in cAMP and Ca^{2+} and the stimulation of prolactin release by hyposmotic medium within minutes of administration (Borski *et al.*, 1991). More work clearly needs to be carried out in this context to elucidate the transduction mechanisms involved and determine whether GRs are distributed to cell membranes or whether the non-genomic signalling is mediated via other receptors or receptor-independent mechanisms. Clearly, non-genomic cortisol signalling is an important area of research, as from a stress standpoint rapid changes are critical for sensing and coping with stressor insults and will also lead to longer-term adaptive responses.

Target Tissue Responses to Corticosteroids

Cortisol is involved in all aspects of fish physiology, and corticosteroid receptors have been detected in all tissue types, including liver, brain, gills, gonads, intestine, muscle, red blood cells and white blood cells (Mommsen *et al.*, 1999). For this section we will focus on the role of cortisol as it pertains to growth and metabolism, reproduction and immune function.

Metabolic responses

In fish, cortisol has effects on carbohydrate, protein and lipid metabolism that are similar to those observed in mammals, albeit less pronounced and much less consistent, with species differences being considerable (Mommsen *et al.*, 1999). It is unclear how cortisol brings about the hyperglycaemia which frequently follows its administration in fish. Very conflicting evidence has been reported concerning its effects on hepatic glycogen, both increases and decreases being observed, making it impossible to draw general conclusions (Mommsen *et al.*, 1999). However, there is an agreement in studies that have reported higher activities of glycolytic enzymes after an acute stressor exposure in fish, which may be critical to cope with the increased liver energy demand, including gluconeogenesis, to re-establish homeostasis (Mommsen *et al.*, 1999; Iwama *et al.*, 2006). The rapid elevation of glycolytic genes, including pyruvate kinase and glucokinase transcripts, in response to handling stressor suggests the regulation of liver glucose uptake and oxidation in response to stressor exposure in fish (Wiseman *et al.*, 2007).

In addition, the key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK), promoting the decarboxylation of oxaloacetate to phosphoenolpyruvate, and glucose-6-phosphatase (G6Pase), hydrolysing glucose-6-phosphate into free glucose and inorganic phosphate, are also induced in response to stressors, suggesting cortisol-induced increase in gluconeogenic capacity to cope with stressors. Several studies have reported cortisol-induced increases in the activities of key gluconeogenic enzymes, including G6Pase, fructose-1,6-bisphosphatase and PEPCK (Mommsen *et al.*, 1999). The transcript levels of these genes are also shown to be elevated in the liver, in conjunction with enhanced glucose production, during recovery from

acute handling stressor exposure *in vivo* and in hepatocytes stimulated with cortisol *in vitro*. For instance, increase in liver PEPCK mRNA levels was observed both *in vivo* and *in vitro* in trout hepatocytes with cortisol stimulation (Sathiyaa and Vijayan, 2003; Vijayan *et al.*, 2003; Aluru and Vijayan, 2007). Cortisol is also thought to stimulate substrate mobilization from peripheral stores, including muscle proteolysis, thereby enhancing liver gluconeogenesis (Milligan, 1997; Mommsen *et al.*, 1999). The higher liver PEPCK in response to stressor exposure in fish (Vijayan *et al.*, 1997; Panserat *et al.*, 2001; Dziewulska-Szwajkowska *et al.*, 2003), together with our observation that cortisol upregulates PEPCK mRNA abundance in trout liver (Sathiyaa and Vijayan, 2003; Vijayan *et al.*, 2003; Aluru and Vijayan, 2007), leads us to propose that cortisol signalling plays a key role in the molecular regulation of liver metabolism, essential for fish to cope with stress. In trout, gluconeogenic substrates are predominantly amino acids (Mommsen *et al.*, 1999), and acute stress did elevate some of the genes involved in protein metabolism, further highlighting a key role for cortisol in the metabolic adjustments to stress (Mommsen *et al.*, 1999; Vijayan *et al.*, 2003; Aluru and Vijayan, 2007). Whether this transcriptional response is related to either direct cortisol signalling or indirect changes in overall metabolism remains to be elucidated.

The role of cortisol in lipid metabolism has not been clearly established in fish. Numerous studies have observed both hepatic and peripheral lipolysis in response to cortisol (Sheridan, 1988, 1994; Mommsen *et al.*, 1999). Whilst the mechanism of action has not been completely defined, cortisol probably acts to increase hormone-sensitive lipase activity, hydrolysing triacylglycerol to diacylglycerol, with the release of free fatty acids (FFAs) and glycerol. Furthermore, in hypophysectomized fish, cortisol treatment was found to restore the activity of hepatic triacylglycerol lipase, which had declined significantly following removal of the hypophysis, suggesting a lipolytic role for cortisol (Sheridan, 1994). Utilization of the increased fatty acids appears to vary considerably between species, with oxidation and re-esterification both being observed (Mommsen *et al.*, 1999).

In addition to its direct metabolic effects, cortisol is also known to modulate the activities of other metabolic hormones in both fish and mammals, although in the former case the mechanisms are poorly characterized. Most of the effects of cortisol on metabolic hormones, particularly growth hormone (GH) and insulin-like growth factor-1 (IGF-1), are permissive and subsequently modulate several physiological processes, including growth, reproduction and osmoregulation, in teleost fish. GH, in concert with IGF-1, is a major endocrine promoter of growth in salmonid fish as in other vertebrates (for review, see Reinecke *et al.*, 2005). Studies on the effect of stress on growth suggest an interaction between the HPI and GH–IGF axes (Pickering and Pottinger, 1995; Reinecke *et al.*, 2005). Cortisol administration reduces growth in rainbow trout and channel catfish (*Ictalurus punctatus*) (Davis *et al.*, 1985; Barton *et al.*, 1987), providing a direct link between cortisol and growth retardation. Similar antagonistic interactions with IGF-1 have been observed, and the genetic mechanism may indeed be important since glucocorticoid response elements (GREs) have been localized to the genes that encode for GH, IGF-1 and its receptors (Burstein and Cidlowski, 1989; Lee and Tsai, 1994; Delany and Canalis, 1995). In addition to the direct effects of cortisol on the GH–IGF axis in impacting growth, recent studies have demonstrated that chronic stressors impact growth by modulating neuropeptides involved in appetite regulation (Bernier, 2006). Recent advances suggest that the corticotropin-releasing factor (CRF) system in vertebrates plays a key role in regulating and integrating the neuroendocrine, autonomic, immune and behavioural responses to stressors (Crespi and Denver, 2004, Heinrichs, 2005; Bernier, 2006). While the results from several studies suggest that appetite-suppressing effects of physical stressors in fish may be associated with an increase in forebrain CRF gene expression, a direct link between CRF-related peptides and the regulation of

feeding following stressor exposure remains to be determined.

Effects on the hypothalamus–pituitary gland–gonad axis

Glucocorticoids markedly inhibit both growth and reproduction, the apparent logic being to delay the anabolic expenditures when these resources are being taxed by stress. Considerable evidence has been accumulated in mammals which suggests that these effects are genomic. Studies utilizing dexamethasone have demonstrated in mammals that it inhibits its hepatic transcription of the oestrogen receptor gene, destabilizes IGF-1 mRNA, and, in the uterus, inhibits oestradiol-induced IGF-1 transcription (Adamo *et al.*, 1988; Sahlin, 1995). Similar antagonistic effects of cortisol on reproduction are reported in fish (Pickering *et al.*, 1987; Teitsma *et al.*, 1998). For instance, stress-induced elevation in plasma cortisol levels are shown to cause a reduction in plasma sex steroids (testosterone and 17β-oestradiol; Pickering *et al.*, 1987; Sumpter, 1997; Contreras-Sánchez *et al.*, 1998; Schreck *et al.*, 2001) and vitellogenesis in a variety of fish species (Teitsma *et al.*, 1998). Similar effects have been demonstrated with exogenous cortisol treatment *in vitro* as well as in *in vivo* experimental studies (Carragher *et al.*, 1989; Reddy *et al.*, 1999; Pottinger *et al.*, 1991; Schreck *et al.*, 2001). The inhibitory effect of cortisol on vitellogenesis is shown to be the result of a repression of the oestradiol-induced signalling by activated glucocorticoid receptor. This is accomplished by suppressing the binding of C/EBPbeta on the oestrogen receptor promoter by protein–protein interactions and thereby preventing the oestrogen receptor (ER)-induced vitellogenesis (Lethimonier *et al.*, 2002). Recent microarray studies have further improved our understanding on the molecular basis of cortisol effects on the reproductive axis in teleost fish (Krasnov *et al.*, 2005; Aluru and Vijayan, 2007; Wiseman *et al.*, 2007). These studies provide evidence suggesting that stress-induced cortisol targets multiple sites along the hypothalamus–pituitary–gonadal (HPG) axis in fish. For instance, stress-induced cortisol levels affected transcripts of gonadotropins, sex hormone-binding globulins in the brain (Krasnov *et al.*, 2005), and oestrogen receptor and vitellin envelope protein transcripts in the liver (Aluru and Vijayan, 2007). Overall, stress-induced impairment of reproduction involves multiple targets along with the HPG axis, and available molecular evidence suggests that it involves interaction between GR and ER signalling pathways.

Also, cortisol has been shown to modulate the highly conserved cellular stress response: the heat shock proteins (HSPs) expression in fish (Iwama *et al.*, 1998, 2006). The majority of HSP studies to date, however, have focused on documenting the (HSPs) expression of HSP families and isoforms and the dynamics involved in the response to different stressors by different species, tissues and cell lines (Iwama *et al.*, 1998). These studies have established a foundation for future studies, and while there is little reason to believe that the molecular behaviour of fish HSPs and HSFs is fundamentally different from that in other vertebrates, this needs to be verified experimentally. The involvement of cortisol in fish HSP expression has been demonstrated both *in vivo* and *in vitro* using hepatocytes in primary culture (Sathiyaa *et al.*, 2001; Boone and Vijayan, 2002; Basu *et al.*, 2003; Vijayan *et al.*, 2005; Iwama *et al.*, 2006), establishing a link between the organismal stress response and the cellular stress response in fish. One hypothesis is that cortisol may increase the stress threshold of cells, as cortisol reduced the heat shock-induced HSP70 and HSP90 expression in trout (Sathiyaa *et al.*, 2001; Boone and Vijayan, 2002; Basu *et al.*, 2003; Sathiyaa and Vijayan, 2003; Iwama *et al.*, 2006).

Stress–immune interactions

Immunosuppressive functions of stress-induced cortisol levels are also well documented in teleosts (Pickering and Pottinger, 1989; Engelsma *et al.*, 2003; Metz *et al.*, 2006). Anti-inflammatory action and

immunosupression is thought to occur through their inhibition of transcription factors such as activator protein-1 and nuclear factor κB (Cato and Wade, 1996; De Bosscher *et al.*, 2000). Elevated levels of glucocorticoids thereby suppress humoral factors involved in the inflammatory response, inhibit leucocyte trafficking to inflammatory sites, and overall reduce circulating leucocytes and lymphocytes (Maule and Schreck, 1990; Ainsworth *et al.*, 1991; Engelsma *et al.*, 2003; Metz *et al.*, 2006). Chronic elevation of plasma cortisol, through hormone implantation, results in dose-dependent increases in mortality due to common fungal and bacterial diseases (Pickering and Pottinger, 1989). Furthermore, stress-induced cortisol increases susceptibility to pathogens in a variety of fish species (Maule *et al.*, 1987; Woo *et al.*, 1987; Johnson and Albright, 1992; Saeij *et al.*, 2003). However, most of these studies addressing the correlation between cortisol levels and immunosupression and increased susceptibility to diseases are based upon exogenous administration of cortisol. Recent studies have demonstrated a bi-directional communication between the neuroendocrine and immune systems, mediated by hormones and cytokines, respectively. It has been shown that the HPI axis impacts immune function primarily by modulating cytokine function (Engelsma *et al.*, 2003; Metz *et al.*, 2006). Interleukin-1β (IL-1β) is an important pro-inflammatory cytokine that mediates several immune responses (Holland *et al.*, 2002, 2003; Huising *et al.*, 2005; Metz *et al.*, 2006). In rainbow trout, acute handling stressor elevates pro-inflammatory cytokines, IL-1β and tumor necrosis factor-alpha transcripts in the liver (Wiseman *et al.*, 2007). Also, a 24-h restraint stress was shown to modulate IL-1β and its receptor expression in the head kidney and brain of common carp, leading to the hypothesis that IL-1β plays a key role in the stress-mediated peripheral immune response as well as centrally in the activation of the HPI axis (Metz *et al.*, 2006). However, cortisol suppressed LPS-induced increase in IL-1β transcript levels in the trout macrophage cell lines (MacKenzie *et al.*, 2006) and carp head kidney phagocytes and trout head kidney leucocytes (Holland *et al.*, 2003; Saeij *et al.*, 2003). These results suggest that cortisol suppresses cellular immunity by affecting inflammatory signalling pathways in a cell-specific manner. Altogether it is becoming increasingly clear that stress–immune interactions may play a major role in the susceptibility of fish to pathogens and have serious repercussions on the health and welfare of fish.

Recent Advances in Stress Physiology

In recent years, with the advent of high-throughput technologies such as microarrays there is an increased understanding of the molecular mechanisms underlying physiological function. Genomic research on salmonid fish has progressed considerably in recent years with the development of the high-density GRASP array (Rise *et al.*, 2004) and several other custom-made salmonid arrays (Bertucci *et al.*, 1999; Sneddon *et al.*, 2005; Tilton *et al.*, 2005; Wiseman *et al.*, 2007), which are sensitive, time-saving and efficient tools in determining genome-wide expression profiles and regulatory pathways. In addition, few studies have also reported on the utility of microarrays developed for humans and other species to discover new genes and pathways by heterologous hybridization (Tsoi *et al.*, 2003; Renn *et al.*, 2004). Using different array platforms, tissue-specific global transcriptional profiles have been determined in response to a variety of abiotic and biotic stressors and to understand the genetic basis of physiological traits (Table 6.1).

Until recently, most of the studies on stress physiology have focused on the plasma hormone and metabolite levels as the primary and secondary indicators of stress response, and these are necessary to cope with energy-demanding physiological adjustments to stress (Wendalaar Bonga, 1997; Mommsen *et al.*, 1999; Barton *et al.*, 2002). Increased understanding of the genetic basis of the stress response revealed that stressors impact various physiological processes, including

Table 6.1. Microarray studies in salmonids highlighting the transcriptional responses associated with aquaculture-related biotic and abiotic stressors.

Species	Area of research	References
Rainbow trout	Phosphorus deficiency of intestinal gene expression	Kirchner *et al.* (2007)
	Egg quality and developmental competence	Bonnet *et al.* (2007)
	Oocyte maturation and ovulation	Bobe *et al.* (2006)
	Social behaviour	Sneddon *et al.* (2005)
	Whirling disease infection and resistance	Baerwald *et al.* (2008)
	Toxicants exposure	Hook *et al.* (2006, 2008)
	Acute handling stress	Wiseman *et al.* (2007) Momoda *et al.* (2007) Cairns *et al.* (2008)
	Glucocorticoid receptor-mediated effects	Aluru and Vijayan (2007)
	Androgen-induced masculinization and gonadal gene expression	Baron *et al.* (2007, 2008)
	Fish meal and fish-oil-free diets on hepatic gene expression	Panserat *et al.* (2008)
Coho salmon (*Oncorhynchus kisutch*)	Transgenic GH treatment on hepatic gene expression	Rise *et al.* (2006)
Atlantic salmon (*Salmo salar*)	*Aeromonas salmonicida* infection on hepatic gene expression	Tsoi *et al.* (2003)
Baltic Salmon (*S. salar*)	M74 syndrome	Vuori *et al.* (2006)

intermediary metabolism, development, reproduction and immune response. Microarray studies not only confirmed previous observations obtained primarily using a gene-by-gene approach to decipher the function of the genes but they have also identified several new genes previously not known to be modulated by stressors. Also, these studies demonstrated that the transcriptional changes in response to acute stressor exposure were tissue- and stressor-specific (Krasnov *et al.*, 2005; Cairns *et al.*, 2008; Momoda *et al.*, 2007; Wiseman *et al.*, 2007).

Hepatic gene expression patterns were investigated under various stressor intensities, and one of the key findings from these studies was that several genes involved in energy metabolism were upregulated (Momoda *et al.*, 2007; Wiseman *et al.*, 2007). This is consistent with earlier findings that stress increases liver metabolic capacity, and one of the key metabolic responses to stress involves enhanced glucose production to meet the increased energy demand associated with stress adaptation (Mommsen *et al.*, 1999). In agreement, key gluconeogenic enzymes such as PEPCK and G6Pase were upregulated in response to handling stressor, underscoring the enhanced liver capacity for gluconeogenesis as an adaptive response to cope with stress (Momoda *et al.*, 2007; Wiseman *et al.*, 2007). Similarly, genes regulating proteolysis, immune function and reproduction were also shown to be stress-responsive in fish. All these studies clearly demonstrated that stressors impact various physiological pathways, and homeostatic re-adjustments involve genome-wide transcriptional changes. Stressors are known to impact phenotypic traits such as development, growth, disease susceptibility and reproductive competence. Understanding the genetic basis of stressor impacts on phenotypic traits will help us to improve the animal husbandry practices in hatchery rearing and intensive aquaculture. For this, comparative

genomics studies using species-specific microarrays should target aquaculture-related problems, including impact of temperature, photoperiod, feeding, water quality, stocking density and drugs used for disease control to obtain stressor-specific and non-specific responses across a wide range of cultured species. These studies will have direct implications to aquaculture as they will identify key gene regulatory pathways, providing a mechanistic link between phenotypic traits and husbandry practices. Furthermore, expression patterns of some of the candidate genes identified can be utilized as biological indicators of stressor exposure and/or impact, as well as markers of growth and fitness. For instance, recent studies reported the utility of microarrays to develop molecular biomarkers of effect associated with the blue sac syndrome affecting salmonid hatcheries (Vuori *et al*., 2006; Baerwald *et al*., 2008), as well as the role of various nutrients on growth in aquaculture (Kirchner *et al*., 2007; Panserat *et al*., 2008). From a mechanistic standpoint, these studies highlight the importance of a transcriptomics approach in identifying multiple signalling pathways that are modulated by stressors.

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7 Disorders of Nutrition and Metabolism

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Introduction

All aquatic animals require a continuous supply of nutrients for essential physiological functions, maintenance of health and growth. These nutrients are acquired from food and the aquatic environment, digested, absorbed and transported to specific cells within the organism and metabolized to chemical and physical forms most suitable for assimilation and biochemical synthesis by cells. Combined with the metabolism of nutrients is the degradation and excretion of endogenous and exogenous compounds. A modern definition of a nutrient by Young (2001) states that:

> A nutrient is fully characterized (physical, chemical, physiological) constituent of a diet, natural or designed, that serves as either (i) a significant energy yielding substrate, (ii) a precursor for synthesis of macromolecules and/or compounds needed for normal cell differentiation, growth, renewal, repair defence and/or maintenance, (iii) a required signalling molecule, cofactor and/or determinant of normal molecular structure /function and/or (iv) a promoter of cell and organ integrity.

The major nutrients required by all animals include protein, lipid, carbohydrate, vitamins, minerals and water. Although various aquatic and terrestrial animal species have developed many specific metabolic differences, the general qualitative patterns of required nutrients are strikingly similar throughout the animal kingdom.

The body of aquatic animals depends on a consistent supply of dietary nutrients, and it has developed regulatory biochemical mechanisms that enable it to adjust successfully to low or excessive nutrient intake; thus the metabolism of essential nutrients is under constant physiological control. The control of these processes may be within the cells or between the cells; the latter is governed by hormonal signals. When control is upset by metabolic disorders, infectious diseases, trauma and medications, or other factors, the dietary nutrient requirements are altered. Unless the dietary supply and balance of nutrients can compensate for these changes, health will deteriorate. Some nutrients cannot be synthesized adequately by fish and must therefore be obtained from the diet (e.g. essential amino acids and fatty acids and vitamin C) or the external aquatic environment (e.g. minerals). Nutritional deficiency diseases involving physiological changes can result from insufficient intakes of dietary essential nutrients. Therefore proper nutrition is one of the most important factors influencing the ability of fish to attain genetic potential for growth, reproduction and longevity. It is important to consider nutrient

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requirements and metabolism throughout their life cycle, which may vary at various stages of development.

Nutrient Deficiency Disorders

A deficiency disease develops when the concentration in the tissues of a specific nutrient normally supplied by the diet falls below a critical level. Many single-nutrient deficiencies cause clearly defined biochemical and pathological changes, which may be limited to certain tissues. Multiple-nutrient deficiencies, however, are not uncommon during starvation, infectious illness and low absorption of the nutrients due to a dietary imbalance. The length of time required for a nutrient deficiency to appear depends on the degree of deprivation and the magnitude of the tissue stores. Generally, the latter factor is more important. For example, liver and kidney stores of ascorbic acid in young fish may last only for a few weeks, whereas the normal liver contains sufficient vitamin A to supply the body's requirements for few months. The rate of utilization and excretion is also important, and if they are increased a deficiency will appear earlier.

The main causes of nutrient deficiency diseases include inadequate intake, poor digestibility and absorption (bioavailability), malabsorption from gastrointestinal interferences, increased utilization, blockage in utilization by antimetabolites in diet and excessive loss of nutrients (Fig. 7.1). There are many causes of nutritional deficiencies in an organism that are independent of inadequate food intake. Environmental stress, altered gastrointestinal activity, disease state, physiological needs, drug-induced anorexia, metabolic defects and food contaminants may all lead to malnutrition (Fig. 7.2). Often it is difficult

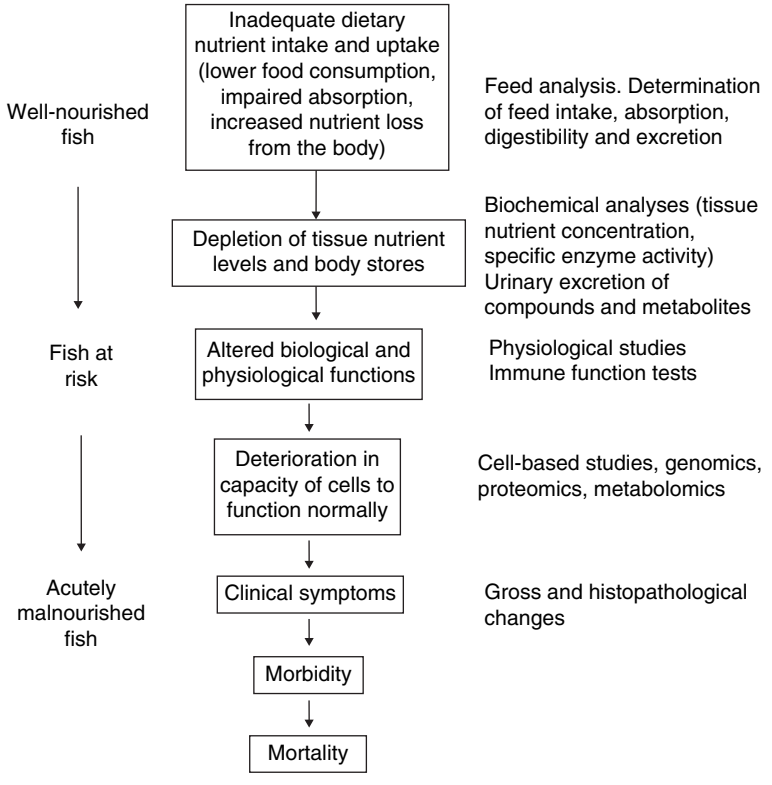


Fig. 7.1. Development of nutritional deficiency disease in fish.

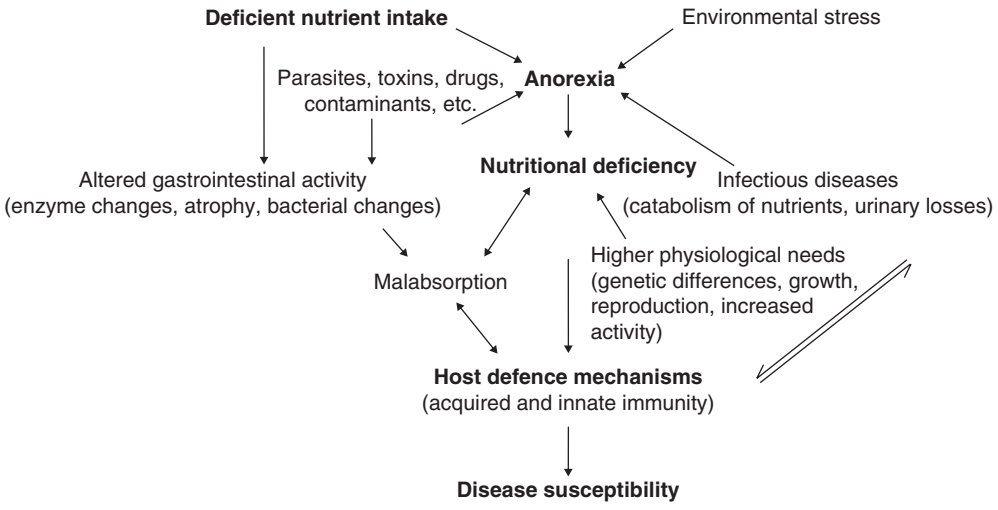


Fig. 7.2. Factors influencing nutritional status, health and immune function of fish.

to diagnose the cause of nutritional deficiencies of fish at various stages of development (larvae, juvenile, adult, broodstock) because the quantitative requirements of nutrients are mainly specified for growth. Species and genetic differences, nutrient interactions, nutrient bioavailability and ability of an organism to adapt to food deprivation may alter the magnitude of a specific nutrient deficiency. It is possible to diagnose a severe deficiency of a nutrient such as ascorbic acid, which causes scoliosis and lordosis; however, marginal deficiencies of one or more nutrients are always difficult to characterize. Generally, marginally deficient fish succumb to infection and the underlying deficiency may never be diagnosed as the cause of death. In the past three decades, the criteria of adequacy for approximately 40 specific nutrients have been made, recognizing the different requirements of different species (NRC, 1993). A minimum requirement has been established, which will prevent signs of deficiency. At higher intakes of vitamins, minerals, amino acids and essential fatty acids, increased reserve is built up in the tissues. The continued intake of certain nutrients in excess amounts causes saturation of various coenzymes. Fat-soluble vitamins and minerals are toxic when taken in excess.

Deficiencies or excesses of each of the major dietary components, including proteins, fats, total calories, vitamins and trace elements, may have profound effects on disease development and the survival of fish, largely through their effect on host defence mechanisms. Nutritional deficiencies may influence the integrity of skin and epithelial tissues and the composition of tissues and body fluids, and reduce mucus secretions, consequently predisposing the fish to infections.

Physiological response to starvation

Many fish species can withstand lengthy periods of starvation: up to 18 months in Japanese eel, *Anguilla japonica*, before death occurs. However, pathological and biochemical changes can be observed much earlier, and the order of tissue changes varies between species. Behavioural starvation is observed when wild fish, such as Atlantic salmon (*Salmo salar*), cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), walleye (*Sander vitreus*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparas* and *Pagrus* spp.) and turbot (*Psetta maxima*), are captured and maintained in captivity and

they do not recognize or refuse to accept prepared foods. Weaning of newly hatched larvae from live food organisms such as brine shrimp (*Artemia* spp.) and rotifers to dry feeds may cause some fish to starve and show large heads and slender bodies. During starvation, muscle tissue is catabolised and many gross biochemical changes are observed. The dynamics of endogenous energy use in response to starvation can be monitored by morphological indices such as the hepatic somatic index (HSI), gut somatic index and condition factor, as well as the size of perivisceral fat bodies. Gut and liver size in fish respond quickly to starvation, and they are reduced in size in fish starved for only 30 days (Love, 1980). In the gut, a progressive reduction in microvilli and the length of the intestine can be observed. Liver size, as determined by HSI, is also depleted rapidly as a result of mobilization of glycogen, lipid and protein to a minimal level. Starvation-induced changes in liver tissue can be observed histologically as reduced cell volume rather than reduced cell number. Fish generally utilize glycogen stores in the early stages of starvation but later rely on lipid as the major energy source (Plisetskaya, 1980), increasing gluconeogenesis to provide sufficient circulating sugars (Sheridan and Mommsen, 1991). Lipid is stored in perivisceral fat bodies in well-nourished fish, but it is mobilized after the liver energy reserves are depleted and readily detected microscopically. These fat bodies are capable of storing large amounts of fat (Sheridan, 1994) and their condition can also provide an indication of malnutrition resulting in obesity.

The last tissue to be catabolised extensively prior to death is the skeletal musculature. Although glycogen stores in muscle may be used early, this is not always the case. A second interspecific variation that is observed is the depletion of muscle lipid in fatty fish such as mackerel (*Scomber* spp.). In these species, the removal of muscle fat can be correlated with an increase in muscle water content. The water content of muscle is therefore diagnostic of the nutritional condition of these species. Non-fatty species such as rainbow trout (*Onchorhynchus mykiss*) (Jezierska *et al.*, 1982) and common carp (*Cyprinus carpio*) (Mazeaud *et al.*, 1977) do not show such a relationship between length of starvation and muscle water content, although such a relationship can be observed in the liver. In both liver and muscle, protein turnover is reduced in starved fish, presumably as a result of a lack of substrates, with both protein synthesis and degradation being lower in starved than in fed fish.

Nutrient Metabolism and Disorders

The understanding of nutrient metabolism is important to translate molecular events to whole-body metabolism to overall growth and reproductive performance and behaviour. In recent years new biochemical and molecular techniques have generated insights about nutrient metabolism and animal biology by identifying the molecules involved in various biological events. Approximately 24 complex nutrients are absolutely essential because they cannot be synthesized by fish in sufficient quantities from their precursors. Some compounds can be synthesized by fish, but production may not always be sufficient to meet the needs, particularly at certain times of their life cycle. The deficiency of a nutrient occurs at a metabolic level when the substrates or cofactors required for a particular biochemical reaction are not available. While we know of many of these reactions and the role of certain nutrients involved, there are probably many others, particularly those requiring micronutrients as cofactors, that are yet to be discovered. In this section the biochemical role of certain essential nutrients and their deficiency disorders are given. The deficiency and toxicity signs of various nutrients, including pathological signs associated with nutritional diseases, have been reviewed in several books and reviews (Roberts, 2002; Ferguson, 2006). The use of improved purified diets based on current nutrient requirements provides opportunities to better characterize the pathogenesis of single- or multiple-nutrient deficiency diseases; however, progress in this area has been slow in the last two decades. It was not possible to describe all the single nutrient deficiency disorders (Table 7.1), therefore this

Table 7.1. Major disorders associated with certain single- and multiple-micronutrient deficiencies, nutrient toxicities and other dietary factors in fish.

Disorders		Single- or multiple-nutrient deficiency	Nutrient toxicity or dietary factor
Eye	Cataract	Vitamin A, riboflavin, methionine, histidine (mainly in salmon smolts), tryptophan, zinc	Choline, oxidized lipid
	Exophthalmia	Vitamin A, vitamin E, pantothenic acid, folic acid, niacin	Oxidized lipid
Gills	Hyperplasia, clubbed and/or pale gills	Pantothenic acid, biotin, vitamin C, essential fatty acids	
Body surface and skin	Depigmentation	Essential fatty acids, vitamin E, riboflavin, folic acid, niacin	
	Fin and skin haemorrhage	Vitamin A, vitamin K, vitamin C, thiamin, riboflavin, pantothenic acid, niacin, biotin, vitamin K, inositol	Oxidized lipid
	Sunburn, reduced photosensitivity	Niacin	
	Oedema	Vitamin A, vitamin E	
	Fin erosion	Riboflavin, niacin, vitamin C, inositol, lysine, tryptophan, zinc	Vitamin A, lead
Blood	Anaemia	Folic acid, iron, niacin, essential fatty acids	Oxidized lipid, lead
	Prolonged blood clotting	Vitamin K	
	Erythrocyte fragility	Essential fatty acids, vitamin E	Oxidized lipid
Liver	Fatty liver	Choline, inositol, biotin, essential fatty acids, vitamin D, excessive dietary fat (mainly gadoids)	Oxidized lipid
Kidney	Nephrocalcinoisis	Magnesium	Selenium
Digestive tract	Stomach distention		Histamine, other biogenic amines, pellet stability, pellet disintegration in stomach, other physiological and dietary factors
	Intestine inflammation (mainly Atlantic salmon)		Antinutritional factors in soybean meal
	Visceral granuloma		Mycotoxins and other dietary factors
Muscle	Tetany	Vitamin D, potassium	
	Muscular dystrophy	Vitamin E, selenium	
	Exudative diathesis	Selenium	
Thyroid	Hyperplasia (goiter)	Iodine	
Skeletal deformity	Scoliosis and/or lordosis	Vitamin C, tryptophan, magnesium, phosphorus, essential fatty acids	Vitamin A, lead, cadmium, oxidized lipid

continued

Table 7.1. *continued.*

Disorders		Single- or multiple-nutrient deficiency	Nutrient toxicity or dietary factor
	Spinal deformities	Phosphorus, manganese, zinc, oxidized fish oil	Vitamin A
Neurological	Convulsions, erratic swimming, low resistance to handling	Thiamine, pyridoxine, biotin, magnesium, potassium, essential fatty acids	
	Loss of equilibrium	Thiamine	
Eating	Anorexia	Potassium, phosphorus, magnesium	Gossypol, mimosine, feed rancidity, several antinutritional factors, contaminants, certain drugs, other feed deterrents

section is mainly focused on an introduction to nutrients and their metabolism in fish.

Protein and amino acids

Proteins are needed for growth, development, reproduction and survival of fish. They are the primary constituent of structural and protective tissues (e.g. bones, ligaments, scales, and skin), soft tissues (organs, muscle) and body fluids. Inadequate amounts of protein in the diet cause a reduction or cessation of growth and ultimately withdrawal from certain less vital tissues to maintain their essential function. About 22 or more amino acids form the building blocks for all complex proteins. Therefore, a dietary requirement for protein is essentially a requirement of the amino acids contained in the protein. Amino acids incorporated in fish protein are α-amino acids, with the exception of proline, which is an α-imino acid. The terms indispensable (essential) and dispensable (non-essential) are widely used to classify the nutritional importance of amino acids in fish. The ten essential or indispensable amino acids are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and they cannot be synthesized by fish and therefore must be provided in the diet.

A few specific disorders associated with amino acid deficiencies have been reported in fish, but mainly in salmonid species. Methionine- and histidine-deficient Atlantic salmon and rainbow trout develop bilateral cataracts, which are discussed in a later section. Tryptophan deficiency results in scoliosis and lordosis in sockeye and chum salmon, apparently the result of low 5-hydroxytryptophan synthesis. Lysine deficiency may cause caudal fin erosion. The disproportionate levels of specific amino acid antagonistics, such leucine and isoleucine and others (arginine/lysine, cystine/methionine), in the diet may result in marginal or severe amino acid deficiency, particularly when fish are under certain environmental and physiological stress. Certain essential amino acids (e.g. leucine) may also be toxic when present in excess in diets (Hughes *et al.*, 1984). Toxicity signs of rainbow trout fed a diet containing 13.4% leucine included scoliosis, deformed opercula, scale loss and spongiosis of epidermal cells (Choo *et al.*, 1991). The intake of feed ingredients containing toxic amino acids, such as mimosine and L-canavanine in

plant legumes, has negative effects on growth and feed utilization.

Lipid

Dietary lipids supply essential fatty acids (EFA) and energy. Most fish cannot synthesize (*de novo*) polyunsaturated fatty acids (PUFA) and therefore they must be supplied in the diet for normal growth, reproduction and health. EFA include PUFA of the n-3 and n-6 series, e.g. α-linolenic acid, 18:3n-3, and linoleic acid, 18:2n-6. Generally, EFA requirements of freshwater fish can be met by the supply of 18:3n-3 and 18:2n-6 fatty acids in their diets, whereas the EFA requirement of marine fish can only be met by supplying the long-chain PUFAs eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) (NRC, 1993). Freshwater fish are able to elongate and desaturate 18:3n-3 to 22:6n-3, whereas marine fish, which lack or have a very low activity of Δ5-desaturase, require the long chain PUFAs, EPA and DHA (Sargeant *et al.*, 2002). The mechanisms by which fish utilize dietary lipid and EFA for metabolism, growth, development and reproduction is complex and subject to intensive ongoing investigations that involve the application of nutrigenomic and metabolomic techniques (Leaver *et al.*, 2008).

Nutritional deficiency signs experimentally produced in fish fed EFA-deficient diets include fin rot, myocarditis, reduced growth rate and feed efficiency, shock syndrome and high mortality. EFA deficiency affects the reproductive performance of male and female fish, causing poor fertilization and hatchability of eggs, embryonic deformities and a low rate of survival of offspring. Dietary lipid composition affects quality, as well as fatty acid composition, of sperm and eggs. High mortalities and several abnormalities, such as underdeveloped swimbladder and malpigmentation, have been observed in marine fish when fed live food organisms such as rotifers and brine shrimp containing low concentrations of n-3 PUFAs. High dietary concentrations of EFA may cause a deleterious effect on growth and feed efficiency in some fish. In yellowtail (*Seriola quinqueradiata*), the upper limit of n-3 highly unsaturated fatty acids (HUFA) was approximately 22% of the total dietary lipid intake (Takeuchi *et al.*, 1992). In Atlantic salmon, high intake of n-3 fatty acids caused immunosuppression and degenerative changes in the heart and skeletal muscle (Erdal *et al.*, 1991). Nutritional pathologies may also develop from the intake of toxic non-essential fatty acids, such as cyclopropenoic acids.

Twenty-carbon PUFAs derived from EFA are precursors of two groups of eicosanoids, prostaglandins and leucotrienes, which have diverse pathophysiological actions, including immune response and inflammatory processes. Eicosanoids are synthesized from di-homo γ-linolenic acid (20:3, n-6), arachidonic acid (AA; 20:4, n-6) and EPA (20:5, n-3), by the action of two oxygenase enzymes, cyclooxygenase and lipoxygenase. Prostaglandins and leucotrienes constitute a group of extracellular mediator molecules that are part of an organism's defense system. They are formed during the inflammatory process, and if the inflammation is caused by invading bacteria, the formation of prostaglandin and leucotrienes will stimulate macrophages and other leucocytes to begin the process of destroying the bacteria. Eicosanoids may be involved in the regulation of the immune system by their direct effect on cells such as macrophages and lymphocytes or their indirect effect via cytokines (Rowley *et al.*, 1995).

The nature of dietary lipids and the concentration of essential fatty acids have a direct effect on the eicosanoid metabolism and immune function. Several reports show positive effects of n-3 fatty acids on immune response of fish. Generally, diets containing high levels of n-6 PUFAs enhance the immune response due to the high levels of pro-inflammatory AA-derived eicosanoids, and diets containing high levels of n-3 PUFA may be immunosuppressive due to the high levels of EPA-derived anti-inflammatory eicosanoids. However, the impact dietary fatty acids have on the immune response is more complex and depends on several factors that influence eicosanoid production, including competition between n-3 and n-6

fatty acids during metabolism for chain elongation and saturation, the cell type involved and the source of fatty acids in the diet. Studies conducted on fish show that diets containing different levels of n-3 and n-6 fatty acids from fish and vegetable oils can modify the fatty acid composition of cell phospholipid (Bell *et al.*, 1993). Changes in the fatty acid composition of phospholipid affect the synthesis of eicosanoid precursors. When the intake of n-6 fatty acids increased, a higher level AA-derived eicosanoids was observed (Bell *et al.*, 1996). In summary, preliminary findings on the role of dietary lipid as it relates to eicosanoids metabolism and immune response of fish is an interesting area of research; however, reports on the effect of n-3 and n-6 fatty acids on immune response and eicosanoid production are not as conclusive as for other terrestrial animals.

Fish diets and tissues contain relatively higher concentrations of PUFA, which are highly vulnerable to lipid peroxidation in the absence of suitable antioxidant protection. Susceptibility and rate of oxidation depends to a large extent on the fatty acid profile of the tissue or diet: the greater the degree of unsaturation, the more easily the lipid oxidizes. Oxidation, a free-radical process, proceeds through initiation, propagation and termination steps and yields aldehydes, epoxides, ketones, diglycerides, monoglycerides and polymers. These oxidative products formed in diets or tissue can react with other nutrients (vitamins, protein and lipid), thereby causing further tissue damage or affecting nutritional value of feeds. The major pathological signs that result from feeding diets containing oxidized lipid and/or absence of vitamin E and antioxidants include the following: loss of appetite, muscular dystrophy, fatty liver, depigmentation, abdominal swelling, haemolytic anaemia, erythrocyte fragility and ceroid deposition in adipose tissue and liver. Antioxidants are commonly added to fish feeds to prevent rancidity and toxicity of oxidized lipids to fish. However, prolonged storage and storage conditions (light and increased temperature, etc.), as well as the presence of lipoxidase, haem compounds, peroxides and trace elements (iron and copper), may cause some degree of lipid peroxidation in diets.

Vitamins

Vitamins have high biological activity and are required in minute amounts for the growth and maintenance of normal cells and organ functions. They are classified into two groups: fat-soluble (A, D, E and K) and water-soluble (thamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, vitamin B_{12} and vitamin C) vitamins. Generally, fat-soluble vitamins function as an integral part of cell membranes, and some of them may have hormone-like functions. Water-soluble vitamins act as coenzymes, accelerating enzymatic reactions, and often serve as a carrier for specific chemical groupings. Diseases due to vitamin deficiencies are a gradual process. When the deficiency persists, the level in cells falls and the metabolic process involving a particular vitamin is impaired. However, the changes do not occur at a uniform rate throughout all tissues of the body, because some retain particular vitamins more strongly, whilst other tissues, by virtue of their metabolic peculiarities, are sensitive to change in vitamin availability.

Vitamin A

Generally, vitamin A activity refers to β-ionone derivatives, which have the biological activity of all-*trans*-retinol. The most significant retinoids in animal metabolism are the alcohol (all-*trans*-retinol), the aldehyde (11-cis-retinal and 11-*cis*-3-dehydroretinal) and the acid (all-*trans*-retinoic acid) forms, including retinyl esters such as retinyl palmitate and retinyl β-glucuronide. All three forms are found in two variants, with either the β-ionone nucleus or the dehydrogenated β-ionone nucleus. However, the former is both quantitatively and qualitatively more important as a source of vitamin A activity. Retinol (A_1) is found in high proportions in marine fishes, whereas vitamin 3-dehydroretinol (A_2) is the predominant

form in freshwater fish. In freshwater fish, oxidative conversion of A_1 to A_2 occurs (Goswami, 1984). Channel catfish (*Ictalurus punctatus*) convert β-carotene to vitamin A_1 and A_2 in about a 1:1 ratio (Lee, 1987). In tilapia, *Oreochromis nilotica*, liver, β-carotene and canthaxanthin are converted into A_1, whereas dihydroxycarotenoids such as astaxanthin, zeaxanthin, lutein and tunaxanthin are converted into A_2 (Katsuyama and Matsuno, 1988).

The best-understood function of vitamin A is its role in vision. Vitamin A is metabolized by fish to produce retinal, which links to a lysine residue in the protein rhodopsin, a photoreceptor in the eye. Vitamin A also has essential roles in growth, embryonic development, reproduction, normal maintenance of epithelial tissues and bone development of fish. Retinol deficiency in salmonid fishes causes poor growth, anaemia, twisted gill opercula, eye lesions, degeneration of the retina and haemorrhage in the eyes and base of fins. Signs of retinol deficiency such as anorexia, pale body colour, haemorrhagic skin and fins, exophthalmia and twisted gill opercula occur in carp. Deficiency signs in yellowtail fingerlings include arrested growth of gill opercula, dark pigmentation, anaemia, and haemorrhage in the eyes and liver, accompanied by high mortality (Hosokawa, 1989). Hypervitaminosis A in fish causes slow growth, blindness, exopthalmia, haemorrhages, anaemia, bone deformities and severe necrosis of the caudal fin. Teratogenic effects such as oedema and brain defects have been described in zebrafish embryos as a result of exposure to excess levels of vitamin A (Hermann, 1995), and enlarged liver and spleen and epithelial cell deformities have been also described.

Vitamin D

The two major natural forms of vitamin D are cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2). Although most animals, including fish, are able to synthesize cholecalciferol from 7-dehydrocholesterol in the presence of UV light, under many circumstances this occurs at too low a rate and the vitamin is thus required in the diet. Marine teleosts have large hepatic stores of vitamin D_3. Atlantic salmon, Atlantic halibut and some tissues of Atlantic cod, such as liver, kidney, gills, spleen and intestine, all produce 25-(OH)D_3, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, as well as 25,26-$(OH)_2D_3$ (Graff *et al.*, 1999). Sundell *et al.* (1993) have identified 1,25-$(OH)_2D_3$ receptors in Ca-regulatory tissues, such as the gills and intestine, in Atlantic cod and have observed increased Ca absorption after 1,25-$(OH)_2D_3$ administration *in vivo*. It appears that an interaction between vitamin D and Ca metabolism may exist, and they may be indirectly linked to phosphorus and bone metabolism.

Vitamin D, either ingested or produced in the skin, is carried through the circulatory system to the liver, where it is converted to 25-hydroxy vitamin D. This metabolite is the major circulating form and is subsequently converted at the tissue to the active form calcitriol (1,25-dihydrocholecalciferol), which binds as a typical steroid to receptors in the nucleus of enteric epithelial cells, renal cells and osteoblasts. Acting in these cells, calcitriol regulates calcium and phosphorus homeostasis by regulating gastrointestinal uptake, excretion and bone mineralization and resorption. Fish can also absorb calcium through the gill membrane; therefore gastrointestinal absorption is likely to be of limited importance to fish in water with low calcium concentrations.

The major pathological effects of vitamin D deficiency are tetany of muscle and structural changes in muscle fibres resulting from poor calcium homeostasis. Major signs of vitamin D deficiency in salmonid species and channel catfish include poor growth, elevated liver lipid, lordosis-like droopy tail and impaired calcium homeostasis manifested by tetany of white skeletal muscles. However, no hypocalcaemia or changes in bone ash have been reported in rainbow trout (Barnett *et al.*, 1982). Hypervitaminosis has been demonstrated in brook trout (*Salvelinus fontinalis*) fed a 3,750,000 IU vitamin D_3/kg diet, which caused hypercalcaemia and increased haematocrit levels but no difference in rates of growth and survival (Poston, 1969). However, diets containing

1,000,000 IU D_3/kg showed no toxic effect in channel catfish and rainbow trout (Hilton and Ferguson, 1982; Brown, 1988).

Vitamin E

Vitamin E is a generic term for eight naturally occurring derivatives of dihydrochromanol that are differentiated by the degree of methyl substitution in the ring (α, β, γ, δ) and the presence of unsaturated bonds in the phytyl side chain (tocopherol, tocotrienol). α-tocopherol has the highest biopotency among the different forms of vitamin E. Vitamin E requirement is directly related to the amount of PUFA in cell membranes. The PUFAs of biological membranes are particularly susceptible to attack by hydroxyl radicals. Once reacted with the hydroxyl radical, a PUFA itself contains a radical group, which, in the presence of oxygen, will attack other PUFAs. Thus, a single hydroxyl radical can initiate a chain reaction that will not cease until all PUFAs in the membrane have been oxidized. In biological systems, vitamin E acts as an antioxidant, inhibiting the chain reaction of free-radical propagation to protect PUFAs against peroxidation. In this role, vitamin E acts in synergy with an enzyme system that comprises superoxide dismutases and glutathione peroxidise, with selenium.

The most common vitamin E deficiency diseases are muscular dystrophy, involving atrophy and necrosis of white muscle fibres; oedema of heart, muscle and other tissues due to increased capillary permeability, allowing exudates to escape and accumulate, which are often green in colour as a result of haemoglobin breakdown; anaemia and impaired erythropoiesis; depigmentation; and ceroid pigment in the liver (reviewed by Roberts, 2002). Vitamin E is carried about the body attached to plasma lipoproteins. Since there is rapid exchange between the lipoproteins and erythrocytes, and vitamin E protects membranes, plasma vitamin E levels are inversely related to susceptibility to oxidative haemolysis and provide a good indication of vitamin E status. Erythrocyte fragility or the haemolysis test has been used to detect vitamin E deficiencies in some fish and other animals (Hung *et al.*, 1981).

Vitamin K

Vitamin K is a fat-soluble vitamin best known for its effects on blood clotting. Compounds with vitamin K activity have a common 2-methyl-1,4-naphthoquinone ring but differ in the structure of the side chain at the 3-position. This vitamin occurs in three different forms: vitamin K_1 (phylloquinones; 2-methyl-3-phytyl-1,4-naphthoquinone); vitamin K_2 (menaquinones; 2-methyl-1,4-naphthoquinones); and vitamin K_3 (menadiones). Vitamin K_1 is synthesized by plants, especially green plants. Vitamin K_2 is synthesized by bacteria and microflora in the lower intestinal track regions in terrestrial animals; however, the ability of fish to synthesize this vitamin is not known. Vitamin K_3 is a synthetic form. All three forms of vitamin K are biologically active for fish. The function of vitamin K is to serve as a cofactor for the vitamin K-dependent carboxylase that facilitates the conversion of glutamyl to γ-carboxyglutamyl residues. Its classic role involves the synthesis of several coagulation factors, including plasma procoagulants, prothrombin (factor II) and factors VII, IX and X and anticoagulants (proteins C and S). More recently, the identification of γ-carboxyglutamyl-containing proteins in bone of terrestrial animals, notably osteocalcin and matrix γ-carboxyglutamyl protein, has generated much interest in the role of vitamin K in bone metabolism and bone health of other organisms and fish. Signs of vitamin K deficiency include an increase in blood prothrombin time, anaemia and haemorrhagic areas in the gills, eyes and vascular tissues in several fish species (NRC, 1993). A vitamin K deficiency has resulted in bone abnormalities and weak bones in haddock (*Melanogrammus aeglefinus*) and mummichog (*Funduus heteroclitus)*, and has affected bone development (Udagawa, 2004; Roy and Lall, 2007).

Thiamine (vitamin B_1)

Thiamine (vitamin B_1) is phosphorylated by thiamine pyrophosphokinase in the presence of ATP to produce thiamine pyrophosphate (TPP). TPP acts as a coenzyme to pyruvate decarboxylase, the enzyme

catalysing the breakdown of pyruvate to form acetyl CoA and to release carbon dioxide. TPP is also a coenzyme in the transketolase reaction of glycolysis. The functions of thiamine are reflected in two measurable symptoms of thiamine deficiency: increased blood levels of pyruvic acid and decreased red blood cell transketolase activity. The latter was used as a tool to determine thiamine requirement of rainbow trout and turbot (Cowey *et al.*, 1975). The other physiological importance of thiamine is linked to normal function of neural tissues and myocardium and the protective effects on the gastrointestinal track. Early gross pathologies observed in relation to thiamine deficiency usually occur in the nervous system, as TPP is less stable in brain than other tissues (reviewed by Halver, 2002). They include trunk-winding, convulsions, loss of equilibrium, nervous disorders and hyperirritability. Skin-related disorders, such as pigmentation changes, congested fins and subcutaneous haemorrhage, have also been described.

Riboflavin (vitamin B_2)

Riboflavin (vitamin B_2) functions as a coenzyme in the intracellular conversion of energy from dietary fats and carbohydrates to the form readily used in muscles and tissues. The principal forms occurring in tissues and cells are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD); the latter is found in cells either H-bonded to purines, phenols or indoles or covalently bonded to essential enzymes such as succinate dehydrogenase. These flavinoid compounds act as electron acceptors in reactions catalysed by over a hundred enzymes in animal and microbial systems. The general and ubiquitous nature of these reactions means that pathologies associated with deficiency are equally general, being loss of appetite, impaired growth and reduced feed efficiency. Specific deficiency signs linked to this vitamin are cloudy lens or cataracts and loss of erythrocyte glutathione reductase activity.

Pyridoxine (vitamin B_6)

The term vitamin B_6 refers to all 3-hydroxy-2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine (3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine). The vitamin includes aldehyde (pyridoxal) and amine (pyridoxamine) forms. The metabolically active form of vitamin B_6 is pyridoxal phosphate (PLP), which functions as a coenzyme for reactions (transamination, decarboxylation, desulfhydration and oxidative deamination) involving amino acids. PLP also plays an important role in the biosynthesis of porphyrin, catabolism of glycogen, metabolism of lipid and γ-aminobutyric acid, and synthesis of the neurotransmitters 5-hydroxytryptamine and serotonin from tryptophan. The signs of pyridoxine deficiency include neurologic disorders such as erratic swimming, rapid opercular movement, hyperirritability and convulsions, which have been observed in salmonid species, channel catfish, common carp, gilthead sea bream (*Sparus auratus*), yellowtail and Japanese eel. Erythrocyte and plasma transaminase activities are also depressed in deficient animals (Jurss, 1978). The activity of certain aminotransferase enzymes that require pyridoxal phosphate as a coenzyme is also a good index of pyridoxine status in fish.

Niacin

Niacin is the generic name for nicotinic acid and nicotinamide, both of which may constitute the dietary source for this vitamin. The biologically active forms of niacin, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), function as coenzymes to many dehydrogenase enzymes found in the cytosol and mitochondria. NAD and NADP are involved in reactions of oxidative metabolism, reductive biosyntheses of fatty acids and steroids, and degradative metabolism of carbohydrates, lipids and amino acids. Thus, they are key components in several pathways of carbohydrate, lipid and amino acid metabolism. Although both act as electron acceptors, they are not interchangeable in reactions, and most enzymes have a particular specificity. While niacin present in animal tissues is readily digested and absorbed, that in many plants is complexed with peptides and

carbohydrates and is not released during digestion. Niacin may also be obtained by metabolism of tryptophan but with an apparently low relative efficiency. The most common niacin deficiency signs are associated with epithelial cell dysfunction. Other pathologies include susceptibility to sunburn, dark skin, haemorrhage and lesions on the skin; however, these deficiency symptoms may be also linked to other micronutrient deficiencies. Poor growth, ataxia, muscle spasms and high mortality are other, non-definitive deficiency signs of this vitamin.

Pantothenic acid

Pantothenic acid is a component of coenzyme A, acyl CoA synthetase and acyl carrier protein (ACP). The coenzyme form of this vitamin is responsible for acyl group transfer reactions. All known derivatives of CoA and related pantothenic derivatives are thiol esters, which participate in numerous metabolic reactions. The central role of acetyl CoA in the tricarboxylic acid cycle means that pantothenic acid is enzymatically involved in amino acid, carbohydrate and lipid metabolism. Pantothenic acid deficiency results in clubbed gill disease in a number of species; this is a readily observed as necrosis, scarring and cellular atrophy of the gill filaments (reviewed by Halver, 2002).

Biotin

Biotin is a trivial designation of the compound hexahydro-2-oxo-1H-thieno [3,4-D] imidazole-4-pentoic acid. Biotin is a coenzyme for several enzyme-catalysed carboxylation reactions that are important in carbohydrate and lipid metabolism. Some examples of these enzymes are: (i) pyruvate carboxylase, which, in conjunction with phosphoenolpyruvate carboxykinase, plays a key role in gluconeogenesis; (ii) acetyl CoA carboxylase, which catalyses the first step of fatty acid synthesis; and (iii) propionyl CoA carboxylase, which catalyses the oxidation of odd-chain fatty acids. Biotin in most feed ingredients is covalently linked to a carboxyl carrier protein through a peptide bond to the ε-amino group of lysine (as biocytin). Thus bioavailability of biotin depends upon the hydrolytic digestion of the carrier protein to release the biocytin. Biotin is also synthesized by intestinal microflora and, although labile in heat, deficiencies are rarely observed. Deficiency can be induced experimentally in fish, as in other animals, by feeding avidin, found in raw hen egg white. Avidin complexes with biotin in the gut in such a way as to prevent its absorption. Symptoms observed under these conditions reflect the generality of lipid and carbohydrate metabolism and include skin and neurological disorders and muscle atrophy. Experimentally induced deficiency signs include anorexia, lower weight gain, higher feed conversion, histopathological changes in gills, kidney and liver in salmonids, skin depigmentation in channel catfish and dark skin coloration in Japanese eels (reviewed by Halver, 2002).

Folic acid

Folate is the generic descriptor of all compounds that exhibit the biological activity of folic acid (pteroylmonoglutamic acid) and related compounds. In most animal tissues, the predominant forms are polyglutamates. These forms may contain up to eight glutamic acid residues attached to the terminal glutamate of folic acid in an amide linkage as a polyamide. Polyglutamates are reduced active forms in animal tissues. Folic acid is the completely oxidized form of the molecule and it is not found as such in nature. The molecule can be reduced to the dihydro and tetrahydro forms. Folates carry out their metabolic functions as carriers of one-carbon units in tetrahydro (FH_4) forms. The various one-carbon units carried on folate coenzymes are used to synthesize methionine and purine rings and convert deoxyuridinemonophosphate to deoxythymadinemonophosphate for DNA synthesis. Deficiencies of this vitamin occur most frequently in rapidly dividing tissue such as epithelial tissue and red blood cells. Signs of folic acid deficiency in salmonids include anorexia, slow growth, poor feed conversion, and macrocytic normochromic, megaloblastic anaemia (reviwed by Halver, 2002), characterized by pale gills, anisocytosis and

poikilocytosis. The erythrocytes are large with abnormally segmented and constricted nuclei, and a large number of megaloblastic proerythrocytes are present in the erythropoietic tissue of the anterior kidney. Poor growth, anaemia and dark skin coloration were noted in Japanese eels, yellowtail and other fish fed a deficient diet.

Vitamin B_{12}

Vitamin B_{12} refers to a group of complex compounds that contain a cobalt-centred nucleus (corrin ring) that qualitatively exhibits biological activity of cobalamin. The commercial preparation includes a cyanide group and is termed cyanocobalamin. *In vivo*, vitamin B_{12} functions as the coenzymes methylcobalamin or 5'-deoxyadenosylcobalamin. Vitamin B_{12} is required for normal maturation and development of erythrocytes, for the metabolism of fatty acids, in the methylation of homocysteine to methionine, and for the normal recycling of tetrahydrofolic acid. Vitamin B_{12} is also synthesized by gut microflora, and, as a result, deficiencies are rarely observed. Intestinal microbial synthesis of vitamin B_{12} has been demonstrated in common carp, channel catfish and Nile tilapia. Microcytic hypochromic anaemia with fragmented and immature erythrocytes is the only diagnostic pathology reported, but this has only been in salmonid fishes (reviewed by Halver, 2002). Vitamin B_{12} deficiency can be differentiated from folate deficiency as the animal responds rapidly to supplementation by the vitamin. Japanese eels require the vitamin for normal appetite and growth.

Vitamin C

Vitamin C comprises compounds exhibiting the biological activity of ascorbic acid. Most animals can synthesize ascorbic acid via the glucuronic acid pathway from glucose, with the exception of fish, a few species of mammals, birds and invertebrates. Their inability to synthesize the vitamin appears to result from the congenital absence of the last enzyme in the biosynthetic pathway: L-gulonolactone oxidase. There are a wide variety of functions described for vitamin C, most of which relate to its ability to serve as a biochemical redox system, thus allowing it to serve as an electron donor in a number of hydroxylation reactions. Ascorbic acid is a strong reducing agent and is readily oxidized to dehydroascorbic acid. Dehydroascorbic acid can be enzymatically reduced back to ascorbic acid in animal tissues with glutathione or reduced NADP. A major function of ascorbic acid is as a cofactor in the biosynthesis of collagen (the main supportive protein of bones, skin, tendon, cartilage and connective tissues). In this role, ascorbic acid serves as a cofactor to the enzymes prolyl or lysyl reductase, responsible for hydroxylation of proline or lysine residues of procollagen. Hydroxyproline and hydroxylysine bind carbohydrate groups to form cross-links in the collagen, thus providing structural integrity. Ascorbic acid is considered to be the most important antioxidant in extracellular fluids, and many cellular activities of an antioxidant nature are known for this vitamin. It has the ability to efficiently scavenge superoxide, hydrogen peroxide, hypochlorite, the hydrogen radical, peroxy radicals and singlet oxygen. Ascorbic acid protects cell membranes against peroxidation by enhancing the activity of tocopherol, the major lipid-soluble chain-breaking antioxidant. It has an important role in several hydroxylases involved in the metabolism of neurotransmitters, steroids, drugs and lipid. Ascorbic acid is a cofactor of two iron-containing hydroxylases involved in the synthesis of carnitine, which is required for fatty acid transport into the mitochondria. Ascorbic acid also facilitates the absorption of iron, thus preventing the anaemia that is often observed in ascorbic acid-deficient fish. It reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}).

Ascorbic acid deficiency signs in most of the fish studied to date show structural deformities (scoliosis, lordosis, gill and fins) (reviewed by Halver 2002; Roberts, 2002) Hyperplasia of cartilage followed by scoliosis, lordosis and deformities of the jaw and snout occur shortly after the onset of vitamin C deficiency and are readily apparent in young, rapidly growing fish. In salmonid

fishes, internal haemorrhaging preceded by non-specific signs, such as anorexia and lethargy, ascites and haemorrhagic exophthalmia, and high levels of plasma triglycerides and cholesterol have also been observed. In turbot, opacity of the cornea and kidney granulomatosis associated with hypertyrosinemia have been described as signs of ascorbic acid deficiency (Messager *et al.*, 1986). Phagocytic activity of cells of the immune system in fish produces reactive oxygen radicals that are potent microbicidal factors but also autotoxic to fish macrophages (Secombes *et al.*, 1988). Ascorbic acid appears to protect phagocytic cells and surrounding tissues from oxidative damage. An increased immune response due to high levels of ascorbic acid supplementation has been demonstrated in several fish species (reviewed by Gatlin, 2002). Dietary and environmental contaminants such as heavy metals increase the ascorbic acid requirements of fish. Reduced reproductive performance has also been reported in rainbow trout fed ascorbic acid-deficient diets (Sandnes *et al.*, 1984). Ascorbic acid reserves are rapidly depleted during embryonic and larval development of certain fish, suggesting essentiality of this vitamin during early life stages as well as a higher requirement than juveniles and adult fish. Liver and kidney ascorbic acid concentrations of less than 25 μg/g have been suggested as an indicator of ascorbic acid deficiency in salmonid fishes and channel catfish.

Choline

Choline is the trivial name for 2-hydroxy-N,N,N-trimethylethanaminium. It is widely distributed in tissues; however, in feed it is mostly in the form of phosphatidyl choline. Free choline can be oxidized by the mitochondrial enzyme choline dehdrogenase to yield betaine aldehyde, which is converted by betaine aldehyde dehydrogenase to betaine. Choline has four basic functions in animals: (i) as phosphatidylcholine it is a structural element of biological membranes; (ii) also as phosphatidylcholine, it promotes lipid transport; (iii) as acetylcholine, it is a neurotransmitter; and (iv) oxidised irreversibly to betaine, it acts as a labile methyl donor in a range of cellular reactions. Certain species of fish can meet a part of their choline needs by synthesis of choline from other methyl donor compounds. The common pathology identified with choline deficiency is fatty liver and liver vacuolization (Halver, 2002). A thinning of the intestinal wall muscle and focal degeneration of the exocrine pancreas were observed in choline-deficient sturgeon (*Acipenser transmontanus*) (Hung, 1989).

Inositol

Myo-inositol, a water-soluble, hydroxylated, cyclic 6-carbon compound (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol), is the only bioactive form of inositol. *Myo*-inositol is synthesized from glucose by most animals except fish and female gerbils, and thus in these species, inositol is a dietary requirement. *Myo*-inositol is important in lipid transport and, as phosphatidyl inositol, is an important component of biological membranes, being in high concentration in brain and kidney. As a source rich in arachidonic acid, a precursor of eicosanoids, phosphatidyl inositol may also play an important function in providing readily accessible arachidonic acid for metabolism. Inositol may be synthesized in common carp intestines (Aoe and Masuda, 1967), but not in sufficient amounts to sustain normal growth of young fish without an exogenous source of this vitamin, because younger carp require a higher level of inositol than older fish. In channel catfish, *de novo* synthesis of inositol in the liver and intestine has been demonstrated. (Burtle and Lovell, 1989). Inositol deficiency signs include poor appetite, anaemia, poor growth, fin erosion, dark skin coloration, slow gastric emptying, and decreased activities of cholinesterase and certain transaminases in rainbow trout, red sea bream (*Pagrus major*), Japanese eel and yellowtail. Rainbow trout fed an inositol-deficient diet had large accumulations of

neutral lipids in the liver and increased levels of cholesterol and triglycerides, but decreased amounts of total phospholipid, phosphotidyl choline, phosphotidyl ethanolamine and phosphotidyl inositol (Holub *et al.*, 1982).

Minerals

Aquatic animals require minerals for their normal life processes. Essential minerals are broadly classified into two groups: those required in gram amounts are called macro-minerals and those for which the requirement is much lower (mg or μg per kg) are referred to as trace elements. Macro-minerals include calcium, magnesium, phosphorus, sodium, potassium, sulfur and chlorine. Seventeen trace elements (arsenic, boron, chromium, cobalt, copper, fluorine, iodine, iron, lead, lithium, manganese, molybdenum, nickel, selenium, silicon, vanadium and zinc) are considered to be essential in animals; however, the essentiality of only a few of these elements has been demonstrated in fish. The main functions of these essential minerals include formation of skeletal structure, maintenance of colloidal systems (osmotic pressure, viscosity, diffusion) and regulation of acid–base equilibrium. Trace elements are important components of hormones, enzymes and enzyme activators. They are involved in a wide range of cellular (e.g. oxygen transport, respiration, enzyme activity) and physiological (e.g. growth, reproduction, vision, immunity) processes of fish. Unlike most terrestrial animals, aquatic organisms absorb inorganic elements from their external aquatic environment. An excessive intake of minerals through either the diet or gill uptake can cause toxicity, and therefore a fine balance between mineral deficiency and surplus is vital for aquatic organisms to maintain their homeostasis through either increased absorption or increased excretion. Major gaps exist in the knowledge of mineral requirements and their physiological functions and these have been reviewed elsewhere (reviewed by Lall, 2002, 2007; Lall and Milley, 2007).

Calcium and phosphorus

Calcium and phosphorus are the most abundant minerals in fish and their functions are closely related, particularly in the development and maintenance of the skeletal system. In addition to its structural functions in bones and scales, calcium plays an important role in the maintenance of acid–base equilibrium, muscle contraction, blood clot formation, nerve transmission, maintenance of cell integrity and activation of several important enzymes. As an important constituent of nucleic acids and phospholipid, phosphorus is directly involved in all energy-producing cellular reactions, maintaining the structural integrity of cell membranes and in various cell functions. It also plays an important role in carbohydrate, lipid and amino acid metabolism, as well as in various metabolic processes involving buffers in body fluids. The calcium requirement of fish is met in large part by absorption through gills and skin in fresh water and by drinking seawater. Although most aquatic organisms have the ability to absorb phosphorus from water, the concentration of this element is too low in both fresh water and seawater to meet the nutritional requirements.

Phosphorus deficiency signs in several fish species include poor growth, reduced feed efficiency and poor bone mineralization (reviewed by Lall, 2002). In addition, common carp fed a low phosphorus diet showed an increase in the activity of certain gluconeogenic enzymes in their liver; an increase in carcass fat, with a decrease in carcass water content; reduced blood phosphate level; and a deformed head. A low phosphorus intake by red sea bream caused curved, enlarged vertebrae; increased serum alkaline phosphatase activity; higher lipid deposition in muscle, liver and vertebrae; and a reduction in liver glycogen. A significant reduction in operculum and scale phosphorus concentration occurs in salmon and trout fed low phosphorus diets. A high level of dietary phosphorus caused a decreased in vertebra ash concentration and resulted in histological changes in the bone of the marine fish, haddock (Roy *et al.*, 2002). The amount of phosphorus in feeds must be carefully

balanced to prevent deficiency signs (e.g. skeletal abnormalities), as well as to minimize urinary and faecal excretions to reduce phosphorus discharge in natural waters.

Magnesium

Magnesium is required in skeletal tissue metabolism, osmoregulation and neuromuscular transmission. It is a prosthetic ion in enzymes, which hydrolyses and transfers phosphate groups. Hence it is essential for energy-requiring biological functions such as membrane transport, generation and transmission of nerve impulses, contraction of muscles and oxidative phosphorylation. Magnesium is also essential for the maintenance of ribosomal structure and thus protein synthesis. It plays an important role in the respiratory adaptation of freshwater fish. Magnesium deficiency signs in common carp, channel catfish, anguillid species and rainbow trout include anorexia, reduced growth, sluggishness, high mortality and reduced magnesium content. In rainbow trout, magnesium deficiency also causes calcinosis of kidney, vertebrae deformity and degeneration of muscle fibres and epithelial cells of the pyloric caecum and gill filaments. Common carp fed a low magnesium diet develop convulsions. Magnesium is the third most common element in seawater and is readily taken up by drinking seawater. Thus, Atlantic salmon, red sea bream and other marine fish reared in seawater do not show signs of magnesium deficiency.

Sodium, potassium and chlorine

Sodium, potassium and chlorine are the most abundant electrolytes in the body. Sodium and chlorine are the principal extracellular cation and anion, respectively, in the body. Sodium is important in osmoregulation, acid–base balance and the membrane potential of cells, as well as in active transport across cell membranes. Chlorine is essential in the maintenance of electrolyte balance and is also the chief anion in gastric juice. Potassium serves as the monovalent cation to balance intracellular anions and participates in neuromuscular functions. It is also an abundant mineral in muscle tissue. Sodium, potassium and chloride are abundant in the environment and in virtually all feed ingredients, thus deficiency symptoms have not been described in farmed fish. Experimentally induced potassium deficiency in chinook salmon (*Onchorhynchus tshawytscha*) caused anorexia, convulsions, tetany and death (Shearer, 1988). The Na^+, K^+-stimulated ATPase activity of gill microsomes is elevated by dietary salt supplementation of some salmonid species, thus making saltwater adaptation easier physiologically.

Iron

Iron serves several vital roles in the body related to cellular respiratory processes through its oxidation–reduction activity and electron transfer. It is found in the body mainly in a complex form bound to proteins such as haem compounds (haemoglobin and myoglobin), haem enzymes (mitochondrial and microsomal cytochromes, catalase, peroxidise, etc.), and non-haem compounds (transferrin, ferritin and iron-containing flavoproteins, e.g. ferredoxins, dehydrogenases). Food is considered to be the major source of iron for metabolic purposes; some absorption of iron takes place across gill membranes. Iron deficiencies are not generally observed under normal conditions, but can be readily induced by feeding a low iron diet. The major pathologies observed are microcytic anaemia, and low haematocrit and blood iron concentration, and the liver becomes yellowish-white. Iron deficiency reduces haematocrit, haemoglobin and plasma iron levels and transferrin saturation (Gatlin and Wilson, 1986). Dietary iron toxicity signs develop in rainbow trout fed higher than 1380 mg iron/kg (Desjardins *et al.*, 1987). The major effects of iron toxicity include reduced growth, poor feed utilization, feed refusal, increased mortality, diarrhoea and histopathological damage to liver cells.

Manganese

Manganese functions either as a cofactor activating a large number of enzymes to form metal–enzyme complexes or as an

integral part of certain metalloenzymes. Since the chemistry of the manganese ion is similar to that of the magnesium ion, many enzymes can be activated by either manganese or magnesium. Certain enzymes, e.g. glycosyl transferases, are highly specific for manganese activation. The enzymatic function of manganese in lipid and carbohydrate metabolism and brain function is widely recognized. Deficiency of manganese reduces the activities of Cu–Zn–superoxide dismutase and Mn–superoxide dismutase in cardiac muscle and liver of some fish species, as well as manganese content in bone. Manganese deficiency causes reduced growth and skeletal abnormalities in rainbow trout, carp and tilapia. Manganese deficiency also produces poor hatchability and low egg manganese levels in rainbow trout (Takeuchi *et al.*, 1981).

Zinc

The essential function of zinc in fish and other animals is based on its role as an integral constituent of a number of metalloenzymes and as a catalyst for regulating the activity of specific Zn-dependent enzymes. Zinc metalloenzymes, including carbonic anhydrase, alkaline phosphatase, carboxypeptidases A and related peptidases, alcohol dehydrogenases and cytsolic superoxide dismutase, are involved in regulation of several metabolic processes of carbohydrate, lipid and protein metabolism. The major routes of zinc absorption in fish are via the gills and intestinal track in both freshwater and seawater species (reviewed by Lall, 2002). The nutritional zinc status of fish is tightly controlled, and surplus Zn is excreted via bile, the sloughing of intestinal mucosa in faeces and through the gills (Handy, 1996). The accumulation of zinc in gills is also regulated through alteration in Zn uptake mechanisms, limiting its excessive uptake (Bury *et al.*, 2003).

Zinc deficiency causes reduced appetite and growth, high mortality, lens cataracts, erosion of fins and skin, short body dwarfism, and low bone zinc and calcium levels and serum zinc concentrations. The excess minerals (total ash) present in white fish meal may affect zinc absorption and use, resulting in lens cataract. Caudal fin zinc concentration is a good indicator of zinc status in rainbow trout (Wekell *et al.*, 1986). Diets low in zinc reduce egg production and hatchability of eggs (Takeuchi *et al.*, 1981).

Copper

Copper is a constituent of many enzymes that are involved in oxidation–reduction reactions and occurs tightly bound to proteins in the cell rather than as free ions. It is associated with cytochrome oxidase of the electron transport chain in the cell. Copper metalloenzymes are involved in protection of cells from free-radical damage (superoxide dismutase), collagen synthesis (lysyl oxidase) and melanin production (tyrosinase). Copper-bound ceruloplasmin, which occurs in the cell and plasma, is involved in iron utilization. Diet is a major source of copper for optimum growth of fish; however, gills also contribute a significant amount of copper uptake (Taylor *et al.*, 2007). An excessive amount of copper supplied in the diet does not enter the body; instead, it is retained in gut tissue by metallothionein and excreted into the faeces through sloughing off of the epithelial membrane (Clearwater *et al.*, 2000).

Signs of copper deficiency have not yet been reported for fish. A decrease in heart cytochrome *c* oxidase and liver Cu–Zn–superoxide dismutase activities have been observed in copper-deficient channel catfish (Gatlin and Wilson, 1986). Copper is widely distributed in feeds and water; therefore its deficiency would only occur in fish under extreme conditions. Copper toxicity may cause severe damage to the gills and necrotic changes in the liver and kidneys. The toxicity of this element was induced in rainbow trout fed 730 mg copper/kg of diet (Lanno *et al.*, 1985). The toxicity signs include reduced growth and feed efficiency and elevated liver copper levels.

Iodine

Iodine is required by fish for the biosynthesis of the thyroid hormones thyroxine and triiodothyronine (see Chapter 3, this volume).

Thyroid hormones regulate cellular oxidation and interact with other hormonal systems to influence growth and metabolism. Iodine is taken up in its ionic form (iodide) by fish through the gill epithelia and across the gut wall. As in other animals, goitre or hypothyroidism is the major result of iodine deficiency. However, iodine deficiencies are rare in marine or brackish-water species because seawater is relatively rich in iodide. Even in fresh water fish, iodide-deficient forms of hypothyroidism are rare because iodide in food is generally sufficient to satisfy the animal's needs. Thyroid hormone deficiency can also develop through glucosinolates when rapeseed meals are incorporated in the diet.

Selenium

Selenium is an essential nutrient required for activities of several enzymes, including various isozymes of glutathione peroxidase, thioredoxin reductase and iodothyronine 5′-deiodinase types 1, 2 and 3. It is present in most proteins in the form of selenomethionine and selenocysteine. Glutathione peroxidase can destroy hydrogen peroxide and hydroperoxides to the alcohol by reducing equivalents from glutathione, thereby protecting cells and membranes against peroxide damage. The interaction of selenium and vitamin E, polyunsaturated fatty acids and other dietary factors significantly influences the requirement for selenium. The uptake of selenium across gills is very efficient at low waterborne concentrations. Liver and kidney play an important role in the excretory process of selenium in trout; however, the major excretory routes appear to be the gills and urine (Hilton, 1989). Selenium deficiency causes growth depression in rainbow trout and channel catfish; however, selenium deficiency alone does not cause any pathological signs in these fish. Both selenium and vitamin E are required in the diet to prevent muscular dystrophy in Atlantic salmon and exudative diathesis in rainbow trout. Glutathione peroxidase activity in plasma and liver is a sensitive index of selenium status in fish and its activity decreases during selenium deficiency. Rainbow trout and catfish develop toxicity signs, including nephrocalcinosis, when fed diets containing 10 mg selenium/kg or above (Hilton and Hodson, 1983; Gatlin and Wilson, 1984). Selenium reduces the toxicity of methyl mercury; thus selenium deficiency accentuates heavy metal toxicity.

Chromium

Chromium is considered to be an essential nutrient for humans. It may have a role in activating enzymes and in maintaining the structural stability of proteins and nucleic acids, but the primary physiological role of chromium in a biologically active complex is to potentiate the action of insulin. The biological function of chromium is closely related to insulin. Chromium supplementation of common carp and Nile tilapia diets has increased glucose utilization; however, this finding has not been confirmed in other fish species. Chromium is present in food in at least two forms: as the inorganic Cr^{3+} ion and as part of a biologically active molecule. The exact structure of the biological molecule is not actively known, but it is postulated to contain nicotinic acid and some amino acids (glycine, glutamic acid, cysteine, glutathione). Pathologies in response to chromium deficiency have not been demonstrated, although toxicity of hexavalent chromium at high levels in the diet has been reported.

Other trace elements

Molybdenum, fluorine, cobalt and boron are elements known to have metabolic functions in other organisms but for which no specific deficiency symptoms have been described in fish. Molybdenum is an essential component of several enzymes, including xanthine oxidase, aldehyde oxidase and sulfite oxidase, where it occurs in the prosthetic group molybopterin. Fluorine is an essential trace element that is best known for its beneficial effects and role in protecting against dental caries. Fluorine rarely occurs in the free form in nature but combines chemically to form fluorides, which are widely distributed in nature. Fluorine is

a normal component of calcified tissues and its concentration is directly related to fluorine exposure. The only known function of cobalt relates to its role as a component of vitamin B_{12}. Approximately 4.5% of the molecular weight of vitamin B_{12} is contributed by elemental cobalt. Although vitamin B_{12} cannot be synthesized by animals, bacterial synthesis in the digestive tract provides much of the requirement for this vitamin. Addition of cobalt to diets of carp has been described as having a beneficial effect on growth and haemoglobin synthesis, presumably as a result of providing a source of the mineral for bacterial vitamin B_{12} synthesis. A role of boron in embryonic development of rainbow trout eggs has been demonstrated (Eckhert, 1998).

Nutritional Diseases

Morphological and pathological signs of nutrient deficiency and toxicity in fish have been reviewed (Roberts, 2002). In general, nutritional diseases are difficult to characterize, and disease due to a single deficiency rarely exists in fish farms. For example, nephrocalcinosis in rainbow trout is caused by magnesium deficiency, higher selenium intake, high levels of carbon dioxide in water, and other factors related to food and water chemistry. Several single-nutrient deficiency diseases in fish have been described using purified diets under experimental conditions (Tacon, 1992; NRC, 1993). Infectious diseases of unknown aetiology have been reported where certain nutrients or dietary factors may be involved. Vitamin E and selenium have been implicated in the pathogenesis of pancreas disease (McCoy *et al.*, 1994) and Hitra disease (Salte *et al.*, 1988). Vitamin E requirement is closely related to other nutrients and dietary factors, including selenium, polyunsaturated fatty acids, vitamins C and A, antioxidants, oxidative quality of dietary oil supplements and the bioavailability of vitamin E. Investigations on the cause of both diseases show that a complex nutrient interrelationship exists, and environment and husbandry-related factors may also be involved. Specific disorders in fish linked to multiple nutrients are discussed in a later section.

Fish depend more heavily on nonspecific defence mechanisms to provide protection against infection. Nutritional modulation of resistance to infectious diseases can be divided into five major groups. In the first category, one must consider a proper balance of macro- and micronutrients, including amino acids, polyunsaturated fatty acids (PUFA), vitamins and trace elements, which are essential for the development of the immune system, starting at the larval stage. Deficiencies in these nutrients may impact several development events, including the proper development of lymphoid organs. Marginal deficiencies may negatively affect the immune system at later stages of life. Severe deficiencies will increase susceptibility to disease and may result in the death of the animal. In the second category, adequate nutrition is essential for cells of the immune system to divide and synthesize effector molecules. The diet supplies the immune system with the amino acids, PUFAs, enzyme cofactors and energy necessary to support lymphocyte proliferation and the synthesis of effector (e.g. immunoglobulins, lysozyme and complement) and communication molecules (e.g. cytokines and eicosanoids). The quantitative need for nutrients to maintain a normal immune function is relatively small compared to the requirements for growth and reproduction. In the next category, it is important to consider that some nutrients provide essential substrates for the proliferation of pathogens (e.g. iron) and their presence at low concentrations in body fluids may limit the growth of pathogens within the fish. The fourth mechanism may include the indirect regulatory effects of diets on the immune system that are mediated through the endocrine system. The regulatory action of PUFAs and other nutrients (vitamins A and E) on leucocytes has been demonstrated. Eicosanoids produced from PUFAs, especially arachidonic acid, are a major component of the humoral immune system. Finally, diet composition and physical characteristics of the diet may modify the

microorganisms in the gastrointestinal tract and the integrity of intestinal epithelium. The presence of oxidized lipids, plant antinutritional factors (e.g. lectins, protease inhibitors and oligosaccharides) and fibre can affect the gut physiology, along with the make-up and size of the gut microfloral population, and thus aspects of the non-specific immune response.

An impaired nutritional status contributes to defective host resistance at all stages of development; however, larvae and juvenile fish are most susceptible to infectious diseases. Malnourished fish harbour latent infections, and certain physiological conditions (e.g. seawater transfer) and environmental stress (temperature, salinity, water quality, light and density) may predispose them to infections. Although the detrimental effects of specific dietary deficiencies upon innate and acquired immunity are well documented in experimental animals, only a few investigations have been undertaken on fish. In fish particularly, the role of vitamins A, E and C and minerals (iron and selenium) in host defence mechanisms and disease resistance is well recognized (reviewed by Gatlin, 2002) and beyond the scope of this chapter. Acute or chronic infections generally deplete the body of important nutrients, and the resultant nutritional deficits then render fish more susceptible to secondary infections. Anorexia caused by the infections or other factors will, depending on their severity, reduce the intake of dietary nutrients to varying degrees. Losses of other key intracellular elements, such as potassium, magnesium, phosphate, sulfate, zinc and body nitrogen, occur during bacterial kidney disease (*Renibacterium salmoninarum*) infection (Lall and Olivier, 1993).

Pathogenic intestinal microorganisms cause disturbances in gut motility, and destructive and inflammatory lesions within the mucosa, intestinal wall and the lymphatic system, which may interfere with absorptive functions. Intestinal parasites may also damage the intestinal mucosa and lead to a direct loss of blood and protein. Changes in the number, composition and location of intestinal microflora also interfere with digestive functions and the absorption of nutrients. Thus, impaired absorption of nutrients may be due to either direct or indirect altered gastrointestinal defects of an infectious process. A prompt attempt to correct the nutritional depletion of body stores which accompanies acute infectious diseases and short-term starvation is important for the treatment of convalescent fish. The rapid restoration of depleted nutrients will help prevent recurrent or superimposed infections, which lead to a vicious cycle common in malnourished fish.

Disorders of the Gastrointestinal Tract

The primary function of the gastrointestinal tract (GT) is to digest and assimilate nutrients ingested from food and protect the body from ingested harmful microorganisms and toxic compounds. Some distinct morphological differences exist among various fish species and digestion and absorption of nutrients. Carp and other cyprinidae have no stomach; however, most species have a stomach consisting of a descending cardiac and fundic region and an ascending pyloric region. Generally the digestive tract is longer in herbivorous than in carnivorous fish. The overall organization of the GT follows a universal pattern characterized for other vertebrates. Most fish have an acidic stomach with peptic digestion. The GT is an active metabolic organ and protects the body against harmful substances. Food selection, and to some extent sensory discrimination of feed, may prevent intake of these harmful substances before ingestion by fish. The chemical action of saliva and production of mucus, gastric acid and digestive enzymes may further alter potentially harmful substances. A complex immune system, which involves the production of luminal antibodies to neutralize many ingested parasites, bacteria and viruses, adds further protection. Other protective mechanisms include spitting out feed and vomiting, which is coordinated by chemoreceptors through the nervous system and afferent impulses of the gastrointestinal tract. The supply of essential nutrients is important to maintain mucosal blood flow,

oxidative fuel supply and the intestinal bacterial flora, which protect fish against gastrointestinal barriers' dysfunction. Maintenance of intestinal epithelial cell structure and gut-associated lymphatic tissue and certain luminal microbial populations are considered necessary to prevent translocation of toxins and harmful bacteria from the intestinal lumen to the bloodstream and other organs. Several nutrients and substrates, such as glutamine, short-chain and n-3 polyunsaturated fatty acids and nucleotides, maintain the integrity of the intestinal mucosa in mammals, but their role in fish remains to be investigated.

Deficiencies of individual nutrients on gastrointestinal pathology of fish have not been characterized. Experimentally induced essential fatty acid deficiency leads to the accumulation of lipid within the enterocytes, indicating that there is a breakdown in mechanisms for lipid absorption and transport. Pancreatic atrophy occurs in response to deficiencies in vitamin A, pantothenic acid and biotin. A white–grey intestine also occurs in response to inositol deficiency. The most common intestinal disorders are diarrhoea, fluid and electrolyte disturbances, and malabsorption. Vomiting and spitting out of food is a digestive disorder that functions to prevent the digestion of potentially harmful material and feed pellet of undesirable physical characteristics. Excessive amount of dietary lipid and infection may also induce this condition in certain fish species. Diarrhoea functions to reduce the time for which potentially toxic compounds are present in the gastrointestinal tract, but can be also triggered by other factors, such parasites and bacterial infections. Parasites damage the absorptive surface of the gastrointestinal tract, thus reducing absorption of nutrients. Stress may further intensify these conditions. Certain antinutrients, nutrient toxicity, drugs and toxic compounds can induce changes in the intestinal structure and cause dysfunction of non-immunological (salivary secretions, intraluminal gastric pH, proteolysis, intestinal bile salts, peristalsis, mucus coat, microvillus membrane and commensal bacteria) and immunological (secretory immunoglobins and lymphoid tissues) mucosal barriers in terrestrial animals and fish.

Digestive enzyme inhibitors distributed in plant products may inhibit the activity of one or more enzymes. The most important of these are protease inhibitors, which are widespread in plant seeds, particularly legumes. They form stable, inactive complexes with digestive enzymes, especially trypsin and chemotrypsin, and are referred as trypsin inhibitors. Amylase inhibitors also occur in legumes. The action of digestive enzymes on plant proteins can be also impaired by the presence of other antinutritional factors in the diet, such as non-starch polysaccharides and phenolic compounds, and by the physical barrier of indigestible plant cell walls and shellfish chitin, which impede the access of digestive enzymes to the substrates. These dietary factors reduce absorption of carbohydrate, fat, protein and micronutrients. Lipid and protein absorption is often used to probe overall adequacy of diet absorption. Impaired lipid absorption may be due to reduced fat hydrolysis, poor solubilization of the product of lipolysis, mucosal diseases and impaired transport mechanisms. Antinutritional factors in diets based on plant protein, such as soybean meal, reduce protein and lipid absorption. Pancreatic insufficiency also affects nutrient absorption, and some of the digestive enzymes are not fully functional to hydrolyse protein for absorption.

The gastrointestinal tract is colonized by a variety of microbes shortly after hatching, most of which are Gram-negative aerobic bacteria. The composition of the intestinal microflora is influenced by factors such as development stage, age, diet composition, microbial populations of water and ingestion of natural food organisms (Ringo *et al.*, 1995). Some potential contributions to nutrition from microbial digestion have been demonstrated, including the production of bacterial cellulase (Stickney and Shumway, 1974) and vitamin B_{12} (Sugita *et al.*, 1991). Also, concurrence between establishment of intestinal microflora and increased ability to digest plant material has been shown (Rimmer, 1986). In cod, hydrolysis of chitin depends on endogenous

enzymes such as chitinase and certain bacteria with chitinolytic properties. The oral administration of drugs has the potential to cause vitamin deficiencies in animals (Roe, 1985). Several drugs in experimental animals induce malabsorption of vitamins as well as affecting their synthesis by gastrointestinal microflora. Antibiotic supplements variously affect the population size and structure of enteric bacteria in a range of species (Ringo *et al.*, 1995). Chromic oxide in the diet induces changes in populations of microorganisms and reduces lipid absorption in arctic char (Ringo, 1993). The beneficial and adverse effects of microorganisms may vary among fish species cultured under wide range of environmental conditions and fed diets formulated from variety of feed ingredients and supplements.

Some other dietary factors, including nutrient interactions, food rancidity, antinutrients, drugs, food additives and toxicants, influence the gut function as well as digestion and absorption of nutrients. Severe inflammation of the stomach and intestine may develop due to feeding rancid feeds made from oxidized animal and fishery by-products (fish, slaughterhouse offal, salted fish) or feeds stored for an extended period of time. In Japan, a disease of carp, commonly known as 'sekoke', has been linked to feeding rancid foods such as spoiled silkworm pupae (Yokote, 1970). The disease is characterized by severe emaciation, skin haemorrhage, histopathological changes in the islets of Langerhans, muscle, liver and kidney, and hyperglycemia, glycosuria and chetonuria. Similar pathological conditions have also been reported in salmonid and marine fish fed a diet containing rancid feed ingredients and fish by-products. Although, vitamin E deficiency is the main cause of sekoke disease, often multiple vitamin deficiencies may be involved in pathogenesis of this disease, as well as others linked to feeding rancid food to fish.

Gastric distention

A condition of obscure aetiology in seawater-reared rainbow trout and chinook salmon has been referred to as water belly, bloat and gastric dilation air sacculitis (GDAS), as it leads to enlarged abdomens and dilated stomachs (Staurnes *et al.*, 1990; Lumsden *et al.*, 2002) and a stenosis of the pyloric sphincter (Staurnes *et al.*, 1990). Affected fish show a flaccid stomach containing large amounts of watery fluid, mixed with droplets of dietary lipid or undigested feed, and a significant increase in serum sodium osmolarity (Staurnes *et al.*, 1990). Both low water temperature and high salinity may cause osmoregulatory failure, leading to osmotic stress, which may trigger abdominal distension (Rørvik *et al.*, 2000). This condition is observed sporadically but may cause significant losses, mainly because the fish fail to survive. Under similar circumstances, regurgitation of dietary lipid has also been observed, but the relation between these two conditions, as well as the disease mechanisms, remains unclear. More recent work conducted on rainbow trout suggests that low water stability of feed pellets in the stomach causes separation and accumulation of lipid. This condition is further accentuated by osmotic stress caused by fluctuating salinity and water temperature and higher feeding rate (Baeverfjord *et al.*, 2006). Thus low water stability of the diet causes oil separation in the stomach, which may result in oil-belching in trout suffering from osmotic stress. Gastric distension has also been induced in rainbow trout fed diets containing histamine and other biogenic amines (Watanabe *et al.*, 1987; Fairgrieve *et al.*, 1994).

Antinutritional factors and enteritis

Plant proteins contain antinutritional factors, which include fibre, carbohydrates, protease inhibitors, goitrogens, antivitamins, tannins, phytic acid, saponins, lysinoalanine, oestrogens, antigenic proteins, etc. The biological effects of natural and other toxicants depend on the nature of the compound as well as the concentration or dose. The regurgitation of feeds containing a noxious substance or poor acceptability of diets may

be the initial response for some natural toxic constituents. These compounds influence the absorptive capacity of the gut by either enzyme induction or effects that may be stimulatory, inhibitory or toxic to mucosal cell growth, turnover or villus structure. The most well-characterized cause of a feed-related intestinal disorders in salmonid fishes is induced by full-fat and extracted soybean meal (Rumsey *et al.*, 1994; Beaverfjord and Krogdahl, 1996; Van den Ingh *et al.*, 1996). It causes a subacute inflammatory response in the distal intestine of Atlantic salmon and rainbow trout, and is often associated with reduced growth performance and nutrient utilization, as well as diarrhoea, in a dose-dependent manner. The inflammation is histologically detectable following short-term exposure and recedes following removal of soybean meal from the diet. Involvement of a mixed population of T lymphocytes and increased numbers of epithelial cells undergoing apoptosis, proliferation and stress responses in the affected distal intestine have also been demonstrated (Bakke-McKellep *et al.*, 2007).

Saponins possess detergent properties, and the use of feed ingredients containing high concentrations of this component may affect membrane structure and functions. Soybean lectins also induce morphological changes in the intestine and primarily bind to the small intestine in Atlantic salmon (Hendricks *et al.*, 1990), whereas soybean-meal-induced changes are primarily in the distal intestine (Van den Ingh *et al.*, 1996). The severity of the intestinal inflammation may vary among salmonid species as well as other marine fish species. The enteritis in Atlantic salmon resembles coeliac disease in humans, and it is proposed that enteritis may be an allergic reaction to soybean peptides (Beaverfjord and Krogdahl, 1996). Saponins in soybean products also interfere with micelle formation in the intestine and may affect lipid absorption. Protease inhibitors, e.g. trypsin inhibitors, affect protein digestion. The proposed mechanisms related to antinutritional factors in soybean meal in fish include: (i) decrease in nutrient absorption by reduction in their hydrolysis at the brush border of the intestine; (ii) impairment in transport capacity across intestinal mucosa; and (iii) excessive loss of pancreatic enzymes in the faeces due to reduce protein re-absorption.

Liver Disorders

The liver is a unique metabolic organ, which metabolizes and detoxifies nutrients, toxins and drugs from the blood supply. It plays a vital role in protein, carbohydrate, lipid and micronutrient metabolism and maintains nutrient blood levels at a constant level, despite variations in substrate availability. The liver synthesizes plasma proteins, non-essential amino acids and other nitrogenous compounds, glycogen and hormones, including anabolic hormones and insulin-like growth factors. The liver is also a major site for lipid metabolism, producing bile required for intestinal fat absorption. Damage to this vital organ impacts on the nutritional status, and derangements in metabolic functions develop by deficiencies and toxicities of nutrients and by malnutrition. The liver synthesizes bile acids from cholesterol, which are secreted in response to a meal. When bile acids are released in insufficient quantities, the critical micellar concentration is affected, which directly influences lipid absorption. Damage to the liver can negatively affect glycogen stores, since the liver is the major site of glucose production. Liver disorders also affect plasma amino acid concentration and poor utilization of amino acids by fish.

Metabolic liver disorders can cause discoloration of the liver and an increase or decrease in hepatosomatic index (HSI), fatty liver or other pathological signs. An essential fatty acid deficiency causes increased HSI, swollen pale liver and fatty liver in several fish species (Tacon, 1992). All salmonid and certain marine fish are susceptible to lipoid liver degeneration when fed rancid feeds containing oxidized lipid. Generally, oxidized lipid affects liver lipid metabolism and several metabolic disorders of liver, including lipoid degeneration (ceroid accumulation), depigmentaton, distension of bile duct,

and anaemic, pale and swollen liver. Liver cells are often distended by fat vacuoles. If there is heavy fat infiltration of the liver, hepatic function is impaired, and a reduction in circulating protein occurs. Cyclopropenoic acids in cottonseed products are toxic for fish and cause extensive liver damage. Feed contaminated with aflatoxins produced by the mold *Aspergillus flavus* was the major cause of liver hepatoma in rainbow trout hatcheries during 1960s (Ashley *et al*., 1965). Among different species produced by different strains of *Aspergillus*, the B_1 form was responsible for inducing neoplasmic changes in the liver with concentrations as low as 0.5 µg/kg during short duration (Ashley *et al*., 1965; Sinnhuber *et al*., 1968).

Although fatty liver infiltration of liver cells is commonly observed in farmed fish, certain wild fish, particularly gadoids, accumulate large amounts of lipid in their liver during summer months, when marine productivity of natural food organisms is plentiful. Similar fatty liver conditions with an enlarged liver and pale white or yellow colour develop in farmed gadoid fish when higher levels of lipid are incorporated in their diets. In haddock, cod and other gadoids, the primary site of lipid storage is the liver, and they retain higher than 60% lipid in the liver. The HSI in cultured gadoids often exceeds 12%, whereas in wild cod a hepatosomatic index of 2–6% is considered normal. The liver lipid in these fish consists mainly of triacylglycerols (>90%) (Lie *et al*., 1986; Nanton *et al*., 2001). Liver function of haddock is not affected by excessive amounts of lipid (>65%) present in liver or at high HSI (11–17%), but the liver is more susceptible to lipid peroxidation (Nanton *et al*., 2001). These gadoid fish, unlike salmonid fishes, have little ability to transport the large amounts of deposited lipid from the liver to the muscle for storage. Unlike wild fish, the depletion of lipid from the liver is slow when low lipid diets are fed.

Cataracts and Eye Disorders

A cataract is an opacity of the lens, causing reduction in visual function. The prevalence of cataracts has been well documented in farmed as well as wild fish (Hargis, 1991; Bjerkås *et al*., 2006). It includes opacities in the eye lens or the lens capsule that mediate an abnormal dispersion of light through the lens and cause reduced visual ability and ultimately blindness. Cataracts develop from a disruption of the normal arrangement of the lens fibres or from alterations in the conformation or water-binding capacity of the proteins of the lens (Benedek, 1997). In Atlantic salmon, cataracts are often localized in the cortex, but extensive cataracts may also affect the nucleus (Bjerkås *et al*., 1996; Wall 1998).

Cataracts in farmed fish can be caused by nutritional deficiencies (or food deprivation and rapid growth), by environmental factors such as poor water quality, toxicants, low water temperature, osmotic imbalance, parasitemias, radiation damage, physiological stress (e.g. smoltification), chemicals (medications and contaminants), stress trauma from careless handling and injuries from unsafe culture systems and by genetic factors such as hereditary predisposition and triploid constitution (reviewed by Hargis, 1991; Bjerkås *et al*., 2006). Multiple or single nutrients may be involved in the pathogenesis of cataracts. Deficiencies of eight nutrients have been linked to the pathogenesis of eye disorders: exophthalmia, clouding and severe degeneration of lens caused by vitamin A; clouding of the cornea due to thamine; degeneration of the cornea and retina by riboflavin; and lenticular opacity with no involvement of other ocular tissues by sulfur amino acids (methionine and cystine), tryptophan, histidine and zinc (Hughes, 1985; Tacon, 1992; Bjerkås *et al*., 2006). A unique pathology of the eye caused by vitamin A deficiency involves expothalmous and the retina, as well as the cornea, of rainbow trout (Poston *et al*., 1977).

The nutrient requirements of fish may vary throughout the life cycle. In Atlantic salmon, cataract develops in certain genetic strains during smoltification and the post-smoltification period (Bjerkås *et al*., 1996). Several dietary factors are implicated in the pathogenesis, including histidine deficiency (Breck *et al*., 2005) and higher growth of

smolts fed a high-energy diet containing high levels of lipid and low protein content (Waagbø *et al.*, 2003). Atlantic salmon undergoes characteristic physiological changes during smoltification before transfer to seawater. In addition to physiological and environmental stress during the smoltification period, nutritional deficiencies may further accentuate cataract problems.

Biochemical mechanisms involved in cataract formation are not well understood because multiple nutrients, and genetic and environmental factors may be involved. Excessive amounts of minerals (high ash), particularly high levels of calcium and phosphorus, reduce zinc bioavailability and cause cataract formation in salmonid fishes as well as zinc deficiency produced in other fish species. The essential function of zinc is based on its role as an integral constituent of a number of metalloenzymes and as a catalyst for regulating the activity of specific zinc-dependent enzymes, such as alkaline phosphatase and cytsolic superoxide dismutase. In Atlantic salmon smolts, dietary histidine appears to be an important factor in preventing cataracts, and the beneficial effects are related to high levels of histidine and the build up of N-acetyl histidine (NAH) in the lens, which possess buffering and antioxidant properties (Bjerkås *et al.*, 2006). In addition, NAH is possibly important in lens water homeostasis. The oxidation of lipid and protein is considered to be an important mechanism of catarogenesis in experimental animals (Varma *et al.*, 1995). Certain oxidants may elude the defensive barriers of the antioxidant system and attack components of the epithelial and lens fibre cell membranes and enzymes involved in the maintenance of electrolyte balance, eventually causing loss of the ability of these cells to maintain homeostasis. Antioxidant enzymes such as catalase and superoxide dismutase protect the lens cell membrane from oxidative stress. Oxygen activated by ultraviolet radiation and other biochemical mechanisms may oxidize lens crystallins and thereby produce protein aggregation. Vitamins (thiamine, riboflavin, vitamin A) and certain amino acids (methionine, cystine, tryptophan) require further investigation to ascertain their significance in cataract aetiology. Nutrient deficiencies remain a major factor in cataract formation; however, a multidisciplinary approach with consideration of various physiological and genetic factors may explain the series of events leading to this critical disease.

Nephrocalcinosis

Nephrocalcinosis is a kidney disorder involving granular deposition of calcium phosphate in the renal tubules and ducts. These deposits may result in reduced growth, feed conversion and kidney function. Several dietary and environmental factors such as poor water quality, particularly low oxygen and high carbon dioxide levels, magnesium deficiency (Cowey *et al.*, 1977) and toxicity of selenium (Hilton *et al.*, 1980) and arsenic (Cockell, 1991) cause nephrocalcinosis. Calcium, magnesium, bicarbonate and phosphate are not directly involved in osmoregulatory processes; however, they influence the functioning of the kidney, an important osmoregulatory organ. In various regulatory processes, respiration supplies oxygen and removes carbon dioxide, digestion maintains the level of nutrients, and osmoregulation controls the volume and composition of fluids. Higher carbon dioxide levels may interfere with normal kidney function, resulting in calcium deposits (Eddy *et al.*, 1979). In addition to calcinosis, magnesium deficiency causes other pathological signs, such as vertebrae deformity, degeneration of muscle fibres and epithelial cells of the pyloric caecum and gill filaments, convulsions and cataracts (Lall, 2002). Atlantic salmon and red sea bream do not show magnesium deficiency signs in the seawater environment because the Mg concentration is much higher than in fresh water and they obtain magnesium by drinking the seawater. However, it is not uncommon to find nephrocalcinosis in rainbow trout reared in seawater. Poor water quality (low oxygen and high carbon dioxide) during the freshwater rearing period of salmonids and other factors may induce early

signs of nephrocalcinosis, but the clinical signs develop after seawater transfer.

Dietary selenium toxicity (13 mg/g) in rainbow trout resulted in an increased level of calcium and magnesium in kidney and elevated levels of magnesium in liver. The major renal damage was tubular (Hicks *et al.*, 1984). Chronic exposure of dietary arsenic (14 mg arsenic/g) caused nephrocalcinosis in rainbow trout (Cockell, 1991). The mechanism of selenium and arsenic toxicity as well as magnesium deficiency in the pathogenesis of nephrocalcinosis in fish is not clear.

Skeletal Disorders

Skeletal disorders in farmed fish are linked to a complex and poorly understood relationship between nutrition, environment and genetic factors. The nutrition status of several macro- and micronutrients is considered to be important for the normal development of skeletal tissues (Lall and Lewis-McCrea, 2007); however, limited information is available on the pathogenesis of bone disorders linked to specific nutrient deficiencies in fish. Morphologically, fish bones consist of the dermal head bones, internal skeleton and scales. The skeleton is a metabolically active tissue that undergoes continuous remodelling at various stages of development and growth. Bone and scales of fish consist of calcium hydroxyapatite salts embedded in a matrix of type I collagen fibres. The organic bone matrix mostly comprises collagen and hydroxyapatite, a hydroxylated polymer of calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$); however, cartilage consists of cells in an extracellular matrix, which may or may not be mineralized, depending on the cartilage type (Hall, 2005). Cartilage primarily consists of glycosaminoglycans, mainly chondriotin sulfates and proteoglycans. Bone and cartilages develop during embryonic, larval, juvenile or adult stages under normal ontogeny, as well as during pathological states, wound repair and bone regeneration. Three types of cells play a significant role in the bone remodelling process and in bone formation, resorption and mineralization: osteoblasts (bone-forming cells), osteocytes (entrapped inside the bone matrix) and osteoclasts (multinucleated bone-resorbing cells). Skeletal growth is achieved in the bone-remodelling process, during which it is repetitively reabsorbed via osteoclastic cell activity, and then reformed on a larger template by osteoclastic action. Deformities develop when bone modelling and remodelling are affected. In most skeletal metabolic diseases, bone mineralization includes re-formation of the matrix, which also involves an osteoblastic controlled function in this process. Bone resorption, formation and mineralization require several hormones, growth factors, cytokines, nutrients and other factors.

Deformities affect growth, development, survival and market value of farmed fish products. Several types of vertebral and spinal malformations, such as kyphosis (humpback, hunchback), lordosis (saddleback, swayback), scoliosis (lateral curvature with rotation of the vertebrae) and platyspondyly (short-tail, compressed vertebrae) have been reported in fish. These disorders may show fusion of vertebrae, 'neck-bend' or 'stargazer', compressed snout (pugheadness), bent jaw (crossbite), front and downwards protuberance of the jaw (harelip, reduction of lower jaw), short operculum and other defects (reduced or asymmetric fins, etc.). Often these deformities may be a combination of several deformities; however, neck, vertebral and spinal disorders are most prevalent and often linked to dietary factors.

Nutrient deficiencies or toxicities of minerals (calcium, phosphorus, zinc, selenium and manganese) and vitamins (A, D, C, E and K), as well as their interactions and lipid peroxidation, may cause pathogenesis of skeletal deformities in fish (reviewed by Lall and Lewis-McCrea, 2007). Effects of these nutrients on bone disorders have been experimentally produced, but the biochemical mechanisms involved in the pathogenesis remain poorly understood. In addition to the above-mentioned nutrients, protein, magnesium, potassium, boron, copper, silicon, vanadium, strontium and fluoride are also known to promote bone formation or

mineralization in terrestrial animals and humans but have not been studied in fish. Other B-vitamins and minerals may also be needed for metabolic processes related to bone either directly or indirectly.

Biochemical mechanisms involved in skeletal tissue metabolism of fish differ from other vertebrates. Unlike terrestrial vertebrates, bone is not the major site of calcium regulation in fish (reviewed by Lall, 2002). The regulation of calcium absorption occurs at the gill, fins and oral epithelia, and vitamin D and its metabolites have a limited role in calcium and phosphorus homeostasis (Vielma and Lall, 1998). An important vitamin D metabolite in bone metabolism of vertebrates, 1,25-$(OH)_2D_3$, had no effect on bone formation of Atlantic salmon (Graff *et al.*, 1999). Although skeletogenesis in terrestrial animals is closely linked to the dietary calcium supply and its metabolism, fish absorb Ca from water and depend on the dietary phosphorus supply for bone mineralization. Bone development and growth are highly dependent on concentration as well as the availability of dietary phosphorus. A deficiency or excessive intake of phosphorus can result in the formation of skeletal abnormalities throughout the skeleton. Common skeletal deformities induced by phosphorus deficiency include curved spines and soft bones in Atlantic salmon (Baeverfjord *et al.*, 1998), cephalic deformities in the frontal bones of common carp (Ogino and Takeda, 1976) and compressed vertebral bodies resulting in scoliosis in haddock (Roy and Lall, 2003) and halibut (Lewis-McCrea and Lall, unpublished results). Bones affected by phosphorus deficiencies are soft and brittle due to the reduced mineral content, and with muscular action the bones become twisted. Histological and histochemical examination of phosphorus-deficient haddock showed an initial increase in bone resorption, which was subsequently followed by a decrease in bone mineralization and reduced bone formation (Roy and Lall, 2003).

Skeletal disorders related to other minerals in fish have not been investigated. Magnesium influences bone mineral metabolism indirectly through its role in ATP metabolism and as a cofactor of several enzymes. Fluoride can replace the hydroxyl groups in hydroxyapatite crystal to form less-soluble fluoroapatite in bone, which influences the crystallization and bone fragility. Zinc is required for osteblastic activity, collagen synthesis and alkaline phosphate activity. Copper influences bone formation, skeletal mineralization and the integrity of connective tissues. Lysyl oxidase, a copper-containing enzyme, is essential for cross-linking of collagen fibres, thereby increasing the strength of protein forming connective tissues. Iron acts as a cofactor in enzymes involved in collagen bone matrix synthesis. Two iron-dependent enzymes, prolyl and lysyl hydroxylases, are essential in the biochemical steps before cross-linking of the matrix by lysly oxidase. Manganese is required for the biosynthesis of mucopolysaccharides in bone matrix formation and is a cofactor for several enzymes in bone tissues. Generally, zinc, manganese, copper and iron deficiencies are reflected in low vertebrae mineral (total ash) content and lower concentration of these minerals in bone (Lall, 2002). Zinc and manganese deficiencies cause short-body dwarfism and skull deformities; however, histomorphic changes in bone associated with these trace elements have not been characterized.

Among the vitamins needed for the development of the skeleton, the role of four vitamins (A, C, E and K) has been demonstrated in skeletal tissue metabolism of fish. An important function of vitamin A is the regulation of cellular differentiation and proliferation, and embryonic development and growth of aquatic organisms (Olson, 1994; Haga *et al.*, 2002). Vitamin A regulates skeletogenesis and cartilage development by controlling chondrocyte function, maturation and proliferation of cells (Koyama *et al.*, 1999). Retinoid toxicity reduces collagen synthesis and bone formation as well as increasing the number of osteoclasts, causing a net bone loss (Frankel *et al.*, 1986), and increases skeletal turnover (Hough *et al.*, 1988). Vitamin A toxicity advances chondrocyte maturation and stimulates osteoclasts, which delays the production of the bone matrix and accelerates the

development of the vertebral column through precocious mineralization, resulting in vertebral abnormalities (Iwamoto *et al.*, 1994). Precocious mineralization can cause skeletal deformities, including vertebral curvatures (Dedi *et al.*, 1995, 1997), vertebral compression (Takeuchi *et al.*, 1998), vertebral fusion (Dedi *et al.*, 1995, 1997) and jaw deformities (Haga *et al.*, 2003). This onset of skeletal abnormalities during the embryonic and first feeding stages has been extensively examined in Japanese flounder (*Paralichthys olivaceus*) (Takeuchi *et al.*, 1995). In Japanese flounder, retinoic acid stimulates abnormal pharyngeal cartilage development, since retinoic acid controls resorption and growth of cartilage through regulation of proteoglycan synthesis (Suzuki *et al.*, 1999; Haga *et al.*, 2002). In sea bass larvae, higher levels of vitamin A induced a delayed vertebral development and affected bone formation in the cephalic region (Villeneuve *et al.*, 2006). When vitamin A toxicity was induced at the later development stages in juvenile Atlantic halibut, abnormalities in the pharyngeal skeleton were observed (Lewis-McCrea and Lall, unpublished results).

Ascorbic acid (vitamin C) is essential for bone formation, collagen synthesis and connective tissue metabolism of fish (reviewed by Halver, 2002). This water-soluble vitamin is a cofactor in the hydroxylation of proline and lysine. Hydroxylation of these amino acids is necessary for the conversion of procollagen to mature collagen. Ascorbic acid-deficient fish that show skeletal malformations have underhydroxylated collagen and a reduction in the proportions of hydroxylysine and hydroxyproline (Sato *et al.*, 1982). The deficiency reduces alkaline phosphatase activity and osteoblastic activity, which results in poor bone calcification and metabolism (Johnston *et al.*, 1994). Skeletal abnormalities such as lordosis and scoliosis have been observed in several scorbutic fish species, and the vertebral column regions affected depend on the species. Lordosis is commonly present in the mid-haemal region of the vertebral column in scorbutic rainbow trout and Japanese flounder, and the caudal region in pearl cichlid (*Geophagus brasiliensis*), while scoliosis is prevalent throughout the vertebral column in Atlantic halibut (reviewed by Lall and Lewis-McCrea, 2007). Abnormalities occur more frequently in larval and juvenile fish than in older fish, as younger fish exhibit increased bone growth and turnover rates (Sato *et al.*, 1982).

Vitamin E stimulates protein synthesis, specifically the bone matrix produced by osteoblasts. In human beings, fatty acid peroxidation alters bone cell cellular membrane components, which affects the function and integrity of the cells, causing an uncoupling of bone remodelling or modelling to occur (Raisz, 1993; Xu *et al.*, 1994; Watkins *et al.*, 1997). This can result in an inhibition of osteoblasts and stimulation of osteoclasts, ultimately causing a net bone loss (Parhami *et al.*, 1997; Tintut *et al.*, 2002; Parhami, 2003). A reduction in bone formation and a stimulation of bone resorption could result in the development of skeletal abnormalities, as observed in halibut (Lewis-McCrea and Lall, 2007). In halibut, scoliosis was commonly observed in the cephalic/prehaemal and anterior haemal regions of the vertebral column (Lall and Lewis-McCrea, 2007), whereas lordosis spans the cephalic to mid-haemal regions (Lewis-McCrea and Lall, 2007). The patterns and types of abnormalities observed in halibut fed oxidized dietary lipid were similar to those of larval and juvenile fish from a commercial hatchery, possibly suggesting exposure to partially rancid feed during early development. Vitamin E supplementation at adequate levels (300 IU/kg diet) did not decrease the frequency of abnormalities observed in halibut (Lewis-McCrea and Lall, 2007), while vitamin E supplementation improved bone quality and tensile strength in adult mice that had been exposed to normal oxidative stress (Wang *et al.*, 2000). Therefore, dietary oxidative products can cause deficiencies of antioxidant nutrients, resulting in skeletal abnormalities, as previously described.

Both vitamin E and ascorbic acid are important antioxidants for optimal skeletal development. They are involved in the intracellular defence mechanism used to protect bone cells from free radicals (Xu *et al.*, 1995). Understanding the direct effect of

antioxidant deficiencies and/or the presence of oxidants in bone tissue on bone development is limited, especially in fish. In other vertebrates, α-tocopherol combats endogenous and exogenous free radicals, which can cause damage to osteoblasts and stimulate osteoclasts. Vitamin E associates with the lipid bilayer of bone cells, allowing it to be the first line of defence against free radicals (Arjmandi *et al.*, 2002).

Vitamin K deficiency affects synthesis of bone proteins in terrestrial animals. This vitamin functions as a cofactor for the vitamin K-dependent carboxylase that facilitates the conversion of glutamyl to γ-carboxyglutamyl residues. In bone, certain γ-carboxyglutamyl-containing proteins, particularly osteocalcin and matrix γ-carboxyglutamyl protein, are involved in bone metabolism (Vermeer *et al.*, 1995). A vitamin K deficiency resulted in bone abnormalities and weak bones in haddock and mummichog, and affected bone development (Udagawa, 2004; Roy and Lall, 2007).

Low intake of phospholopid and excessive amounts of PUFAs may also induce vertebral malformations in marine fish larvae (Kanazawa, 1993; Villeneuve *et al.*, 2006). Fish skeletal tissues contain a significant amount of lipid, PUFAs and micronutrients, which are particularly susceptible to lipid peroxidation. Fish bones may contain as high as 24–90% lipid (Phleger, 1991). Antioxidants (e.g. vitamin E, vitamin C, selenium and glutathione) and antioxidative enzymes (e.g. glutathione peroxidase, catalase and superoxide dismutase) scavenge free radicals and thus protect tissue against lipid peroxidation.

Other Disorders

Gill hyperplasia

Among the numerous factors which may induce gill lesions, deficiencies of pantothenic acid and other micronutrients have been identified as the cause of nutritional gill disease in rainbow trout and channel catfish. Clinically deficient fish exhibit gill hyperplasia, and clubbed gills develop due to fusion of the secondary lamellae in rainbow trout (Wood and Yasutake, 1957; Masumoto *et al.*, 1994). Nutritional gill hyperplasia is distinct from hyperplasia caused by poor culture conditions. The fusion begins at the base of the gill lamellae in pantothenic acid-deficient fish rather than at the tips of lamellae, as in gill diseases associated with poor water quality. In turbot, essential fatty acid deficiency causes gill hyperplasia and changes in gill membrane lipid composition (Bell *et al.*, 1985). The onset of anorexia precedes gill lamellar hyperplasia in rainbow trout fry fed a pantothenic acid-deficient diet (Karges and Woodward, 1984). The fusion of lamellae has functional consequences on the respiration capacity of gills.

Fin and skin lesions

Fin and skin lesions are commonly observed and are often interpreted as unspecific reactions to environmental and mechanical stress factors. A number of dietary factors, including deficiencies of lysine, tryptophan, essential fatty acids, zinc, copper, riboflavin, inositol, niacin and vitamin C; toxicities of vitamin A and lead; lipid peroxidation; and feed rancidity can cause these lesions (Tacon, 1992; Lall, 2002; Roberts, 2002). Typically, skin and fins show erosion and haemorrhages, and often multiple nutrients and environmental factors are involved. Overcrowding and overfeeding may also lead to fin and skin lesions. Often poor culture conditions and marginal micronutrient deficiencies result in an unfavourable microbiological environment, which predisposes them to secondary infections, thus leading to skin lesions. Winter ulcers characterized by round, deep skin ulcers typically located on the sides of the body develop in salmon reared in sea cages at low water temperatures. *Vibrio* spp. are often isolated from these lesions; however, limited food intake and micronutrient deficiency during long winter periods may predispose salmon to this pathological condition (Salte *et al.*, 1994).

Conclusions

The nutrition of fish is a complex subject reaching into domains of physiology, biochemistry, pathology, fish husbandry, veterinary science, genetics, environmental science and food chemistry, and often beyond these disciplines. Although the science of nutrition has developed rapidly in the past two decades, there are major gaps in the knowledge of nutrient requirements of most fish species. Nutrient requirements are better defined for terrestrial animals than fish. Nutritional disorders are often associated with multiple-nutrient deficiencies and toxicities related to certain vitamins, trace elements and natural toxins. Certain disorders, such as skeletal deformities and nephrocalcinosis in farmed fish, develop over an extended period of time, and early detection techniques are lacking. Although most micronutrient deficiencies have been reported in young fish, it is recognized that certain disorders may appear at later stages of the life cycle. Knowledge of genetic factors, stress, environmental factors, diseases and other factors that affect the susceptibility to disease, as well as nutrient requirements at various stages of development, are often necessary to resolve the problem. Certain fish model species, such as zebrafish (*Rerio danio*) and medaka (*Oryzias latipes*), can provide useful information on nutrient metabolism, particularly gene action, cell differentiation, morphogenesis, species differences in phenotypic expression of genetic abnormalities, enzyme activities associated with deposition of nutrients in tissues in response to nutrient levels and hormone actions in a relatively short timeframe. The knowledge obtained from these model animal studies, however, should be further tested to determine the effects of environmental, genetic and other factors, to confirm the mode of action of nutrients and control deficiency diseases. In characterizing specific nutritional disorders, diet composition should also be considered and given priority, since all other interactions involving genetic and environmental factors will be adversely affected by uncorrected nutrient deficiencies. Many of the nutrients and dietary factors mentioned in this chapter have been shown to produce deficiency diseases under experimental conditions, and their role must be proven by practical application of these findings in development of diets that control nutritional disorders under the diverse environmental conditions of fish farming.

Nutrition of aquatic animals must be considered as an interdisciplinary catalyst for fish physiology and biochemistry that will continue to promote the understanding of the integrative biology research directed towards disease prevention, better growth and production of high-quality fish for humans. Further investigation of the role played by nutrients and mechanisms underlying nutrient functions is likely to become clearer using advanced genomics, proteomics and metabolomics technologies in addition to traditional methodologies currently used. Recent advances in approaches used to predict the consequences of a change in nutrient intake and nutrient balance on physiological and pathological processes is a promising area, which has the potential to resolve some of the complex nutritional disorders in fish.

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8 Food Intake Regulation and Disorders

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Introduction

The past decade has seen a significant advance in our understanding of the physiological processes that control food intake. While most of the research has used rodent models and is driven by the global obesity epidemic (Morton *et al.*, 2006), increasingly fishes are also being used as models to investigate the hormonal control of food intake and the evolution of appetite-regulating systems (Lin *et al.*, 2000; De Pedro and Björnsson, 2001; Volkoff *et al.*, 2005, 2009; Song and Cone, 2007; Matsuda, 2009). In general, although some significant differences have been identified (Huising *et al.*, 2006; Matsuda *et al.*, 2009b), it appears that the same neuroendocrine signals and receptors involved in the control of food intake and metabolism in mammals are conserved in teleosts.

Given the economic importance of food intake in fish for fish in the wild and aquaculture, considerable effort has gone into identifying the factors that influence the ingestion of feed (Kestemont and Baras, 2001). While some environmental factors can stimulate food intake within certain thresholds, factors that disturb homeostasis, independent of whether they may be environmental, social or physical, are often associated with a reduction in food intake (Bernier, 2006). Similarly, anorexia is a characteristic feature of many fish diseases. Despite the recent progress in our knowledge of food intake regulation in fish, very little is known about the mechanisms that mediate the food intake disorders that are associated with stressors and infection. Therefore, as a means of providing a framework for future studies, this chapter aims to review what is currently known in fish about the regulation of food intake, the conditions that lead to anorexia, and the mechanisms that mediate food intake disorders.

Food Intake Regulation

The regulation of food intake in fish, as in other vertebrates, involves a complex neuronal circuitry that must integrate and process various types of information (Morton *et al.*, 2006; Shioda *et al.*, 2008; Volkoff *et al.*, 2009) (Fig. 8.1). In general, the current model of food intake control suggests that cognitive, visual, olfactory and gustatory cues are relayed to specific hypothalamic nuclei and integrated with both short- and long-term peripheral signals related to the energetic status of the animal. In return, the hypothalamus, together with other brain regions, regulates energy balance by governing the activity of neuronal pathways involved in food-seeking behaviour and peripheral

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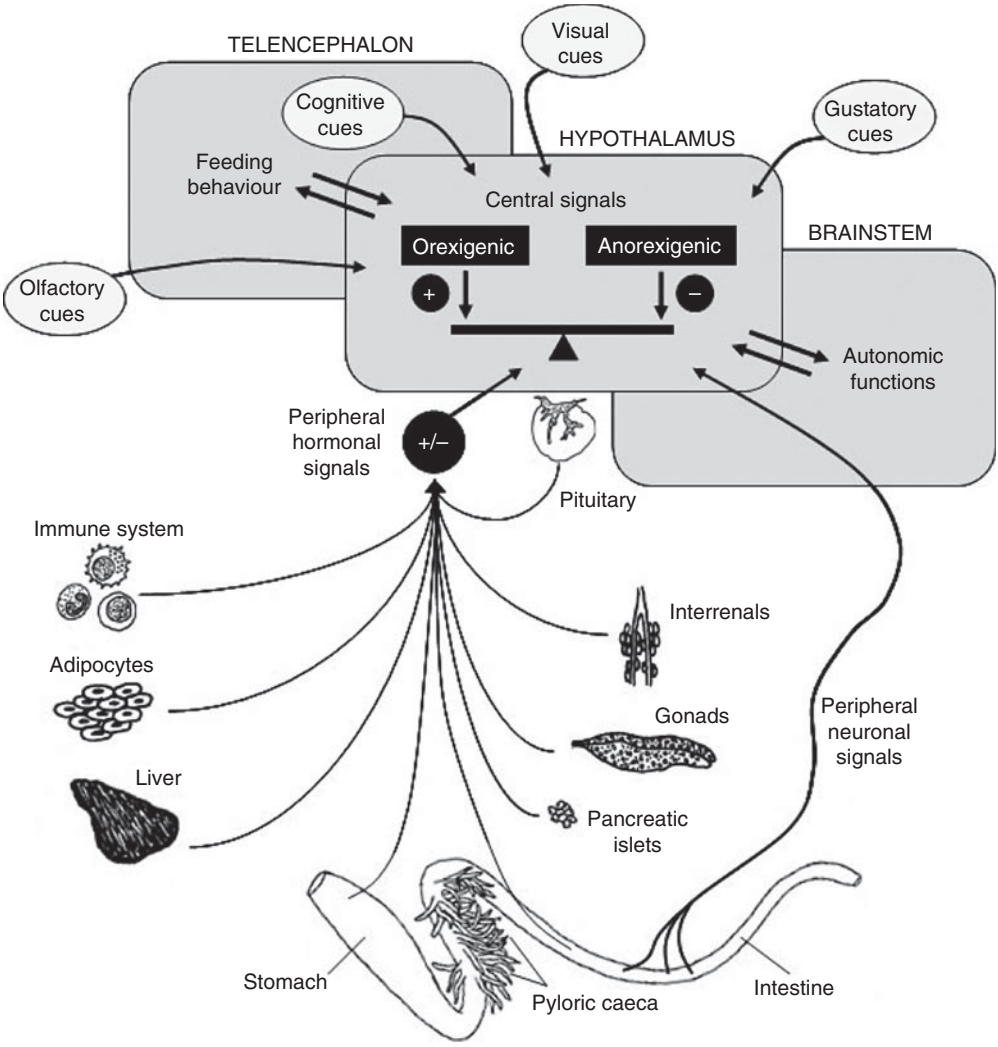


Fig. 8.1. Summary of neuronal pathways and signals that contribute to the regulation of food intake in fish. Abundant hypothalamic neurons producing appetite-stimulating (orexigenic) and appetite-inhibiting (anorexigenic) neuropeptides are considered to participate in feeding regulation. The hypothalamic circuit, with other brain regions, regulates energy balance by governing the activity of neuronal pathways involved in feeding behaviour and autonomic functions. While sensory organs relay olfactory, visual and gustatory cues, higher-order brain regions communicate cognitive cues to the appetite-regulating hypothalamic circuit. The hypothalamus also receives short-term peripheral signals of hunger and satiety, and long-term signals related to the energetic status of the fish. The peripheral signals are either hormonal or neuronal and originate from a variety of different cell types and organs.

metabolism. While the presence of food in the gastrointestinal system elicits the release of several appetite-regulating signals, endocrine signals from various other peripheral tissues also contribute to the regulation of feeding (Coll *et al.*, 2007). The peripheral signals convey information to the appetite-regulating circuits of the brain either indirectly via vagal afferents or directly across the blood–brain barrier. The appetite-regulating

pathways of the hypothalamus produce various neuropeptides with either appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) properties (Valassi *et al.*, 2008). Overall, although several hormones and neuropeptides exert similar effects on food intake in fish and mammals, clear differences are also emerging. This section will briefly review the actions of the principal central and peripheral orexigenic and anorexigenic signals in fish (Table 8.1), their interactions and proposed roles in the short-term regulation of satiation and long-term regulation of food intake.

Central orexigenic signals

Neuropeptide Y (NPY) is a potent orexigenic peptide in the brain of fish and other vertebrates. To date, intracerebroventricular (icv) injections of NPY have been shown to stimulate food intake in goldfish (*Carassius auratus*; Lopez-Patino *et al.*, 1999), channel catfish (*Ictalurus punctatus*; Silverstein and Plisetskaya, 2000) and rainbow trout (*Oncorhynchus mykiss*; Aldegunde and Mancebo, 2006), and fasting is associated with an increase in brain NPY gene expression in several fish species (Silverstein *et al.*, 1998; Narnaware and Peter, 2001; MacDonald and Volkoff, 2009). Interestingly, however, the NPY receptor subtypes mediating the orexigenic effects of NPY in fish may differ from those in mammals. Studies on the NPY receptor repertoire of fish have shown that the NPY receptor subtypes that mediate the appetite-stimulating effects of NPY in mammals, namely Y1 and Y5, have been lost from the genome of several teleosts (Salaneck *et al.*, 2008). The actions of NPY on food intake in fish may also result from complex

Table 8.1. Principal factors involved in the regulation of food intake in fish and their primary source.[a]

Orexigenic factors	Source	Anorexigenic factors	Source
NPY	Brain	CRF/UI	Brain
AgRP	Brain	Serotonin	Brain
Orexins	Brain	αMSH	Brain
Galanin	Brain	MCH	Brain
Ghrelin	Gut	CART	Brain
Growth hormone	Pituitary gland	PACAP/VIP	Brain
		Neuromedin U	Brain
		CGRP	Brain
		Intermedin	Brain
		Amylin	Brain
		PrRP	Brain
		GnRH	Brain
		CCK	Gut
		GRP/BBS	Gut
		GLP-1	Pancreas/gut
		Insulin	Pancreas
		Leptin	Liver
		Cortisol	Interrenal tissue
		T/E2	Gonads

[a]The factors involved in the regulation of food intake are generally pleiotropic and expressed in multiple locations. For example, the gut peptides are generally also expressed in the brain, and many of the brain signals are also expressed in multiple peripheral locations. Abbreviations: AgRP, agouti-related protein; BBS, bombesin; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; CRF, corticotropin-releasing factor; E2, 17β-oestradiol; GnRH, gonadotropin-releasing hormone; GLP-1, glucagon-like peptide 1; GRP, gastrin-releasing peptide; MCH, melanin-concentrating hormone; αMSH, α-melanocyte-stimulating hormone; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating polypeptide; PrRP, prolactin-releasing peptide; T, testosterone; UI, urotensin I; VIP, vasoactive intestinal polypeptide.

interactions with other appetite regulators, e.g. cocaine- and amphetamine-regulated transcript (CART) (Volkoff and Peter, 2000), leptin (Volkoff *et al.*, 2003), melanin-concentrating hormone (MCH) (Matsuda *et al.*, 2009a), ghrelin (Miura *et al.*, 2006) and others (see Volkoff *et al.*, 2009).

In mammals, the appetite-regulating NPY neurons of the arcuate nucleus co-express another orexigenic neuropeptide, agouti-related protein (AgRP) (Morton *et al.*, 2006). AgRP is an endogenous antagonist of the melanocortin receptor subtype 3 and 4 (MC3/4R), the MCRs that mediate the anorectic effect of α-melanocyte-stimulating hormone (αMSH). Indirect evidence suggests that AgRP also has an orexigenic role in fish. For example, transgenic zebrafish (*Danio rerio*) overexpressing AgRP exhibit obesity, increased growth and adipocyte hypertrophy (Song and Cone, 2007). Also, fasting upregulates hypothalamic AgRP gene expression in both goldfish and zebrafish (Cerdá-Reverter and Peter, 2003; Song and Cone, 2007).

The orexins, orexin A and B, and galanin, potent central stimulators of food intake in mammals, have also been implicated in the regulation of feeding in fish. Icv injection of orexins stimulates food intake in goldfish (Volkoff *et al.*, 1999; Nakamachi *et al.*, 2006), and fasting increases the number of hypothalamic orexin-like immunoreactive cells and the brain mRNA levels of the orexin precursor in goldfish (Nakamachi *et al.*, 2006) and zebrafish (Novak *et al.*, 2005). Similarly, central injections of galanin stimulate food intake in goldfish (De Pedro *et al.*, 1995a) and tench (*Tinca tinca*; Guijarro *et al.*, 1999), and food deprivation increases the brain mRNA levels of the galanin precursor in goldfish (Unniappan *et al.*, 2004b). As observed for NPY, the orexins and galanin appear to interact with several other orexigenic and anoreginexic signals (Volkoff and Peter, 2000, 2001b; Volkoff *et al.*, 2003; Miura *et al.*, 2007).

Peripheral orexigenic signals

Ghrelin is the only known orexigenic signal that originates from the gastrointestinal tract. In fish, as in mammals, ghrelin is primarily expressed in the stomach, with much lower mRNA levels in the brain (Unniappan and Peter, 2005; Kaiya *et al.*, 2008). While ghrelin stimulates food intake in goldfish (Unniappan *et al.*, 2004a; Matsuda *et al.*, 2006a) and tilapia (*Oreochromis mossambicus*; Riley *et al.*, 2005), equivocal results have been observed in rainbow trout (Jönsson *et al.*, 2007; Shepherd *et al.*, 2007). Fasting increases brain and gut ghrelin gene expression in some fish species (Unniappan *et al.*, 2004a; Matsuda *et al.*, 2006a; Terova *et al.*, 2008; Amole and Unniappan, 2009) but not in others (Parhar *et al.*, 2003; Jönsson *et al.*, 2007; Xu and Volkoff, 2009). Similarly, while fasting has been associated with an increase in plasma ghrelin levels in goldfish (Unniappan *et al.*, 2004a), food deprivation had an opposite effect in burbot (*Lota lota*; Nieminen *et al.*, 2003). Peripherally there is evidence that ghrelin interacts with gut satiation signals (Canosa *et al.*, 2005), and centrally the orexigenic effects of ghrelin appear to be mediated via orexin- and NPY-dependent pathways (Miura *et al.*, 2006, 2007).

In addition to its significant role in the regulation of growth and metabolism (Björnsson *et al.*, 2004; Chang and Wong, 2009), growth hormone (GH) is an orexigenic signal in fish. Implants or intraperitoneal (ip) injections of GH stimulate appetite and foraging behaviour in rainbow trout (Johnsson and Björnsson, 1994; Johansson *et al.*, 2005). Similarly, transgenic coho salmon (*Oncorhynchus kisutch*) overexpressing GH eat significantly more than their non-transgenic counterparts (Stevens and Devlin, 2005). To date, however, the mode of action by which growth hormone stimulates appetite remains largely unknown (Raven *et al.*, 2008).

Central anorexigenic signals

Acting in opposition to the orexigenic signals discussed above are a much larger number of factors which promote a decrease in food intake (Table 8.1). Among these are factors that play a key role in short-term satiation, i.e. meal termination, and factors that

are involved in long-term body-weight regulation and energy homeostasis. Corticotropin-releasing factor (CRF) and the related peptide urotensin I (UI), as part of their key role in the regulation of the hypothalamic–pituitary–interrenal (HPI) axis and the coordination of the stress response in fish (Bernier *et al.*, 2009), fall in the latter category of anorexigenic signals, which are involved in the modulation of centrally controlled metabolic functions (Kuperman and Chen, 2008). Icv injections of CRF or UI in goldfish suppress food intake in a dose-related manner, and UI is significantly more potent than CRF (De Pedro *et al.*, 1993; Bernier and Peter, 2001a). Similarly, icv treatments with CRF in tench inhibit feeding (De Pedro *et al.*, 1995b). The ability of the CRF receptor antagonist α-helical $CRF_{(9-41)}$ to reverse the reduction in food intake induced by pharmacological treatments that elevate brain CRF and UI gene expression also suggests an endogenous role for CRF-related peptides in the control of food intake (Bernier and Peter, 2001a). Moreover, in goldfish there is evidence that the anorexigenic effects of serotonin (De Pedro *et al.*, 1998b), αMSH (Matsuda *et al.*, 2008a), neuromedin U (NMU; Maruyama *et al.*, 2008) and pituitary adenylate cyclase-activating polypeptide (PACAP; Maruyama *et al.*, 2006) are at least partially mediated by CRF-related peptides.

The central serotonergic system in vertebrates modulates various behavioural responses, including food intake (Leibowitz and Alexander, 1998). In goldfish, both icv injection of serotonin (De Pedro *et al.*, 1998b) and intraperitoneal (ip) treatment with the serotonin reuptake inhibitor fluoxetine (Mennigen *et al.*, 2009) decrease food intake. Likewise, ip administration of the serotonin-releasing agent fenfluramine induces a short-term inhibition of feeding in rainbow trout (Ruibal *et al.*, 2002).

Anatomical and physiological evidence implicate αMSH in the regulation of food intake in teleosts. Expression of the prohormone for αMSH, pro-opiomelanocortin (POMC), and αMSH immunoreactivity have been localized to hypothalamic regions responsible for feeding regulation in the brain of fish (Vallarino *et al.*, 1989; Cerdá-Reverter *et al.*, 2003b; Amano *et al.*, 2005; Matsuda *et al.*, 2008a). In goldfish, while icv administration of the MC4R agonist NDP-MSH and of the non-specific agonist melanotan II (MT II) dose-dependently inhibit food intake, the specific MC4R antagonist HS024 stimulates appetite (Cerdá-Reverter *et al.*, 2003a,b). Similarly, in rainbow trout, while central administration of MTII decreases food intake, both HS024 and the MC3/4R antagonist SHU9119 have the opposite effect (Schjolden *et al.*, 2009). The αMSH signalling pathway in the hypothalamus of goldfish is also involved in mediating the anorexigenic action of melanin-concentrating hormone (MCH) (Shimakura *et al.*, 2008).

While the actions of most appetite-regulating signals appear to have been conserved between mammals and fish, recent evidence suggests that this may not be the case for MCH. In mammals, MCH is orexigenic and plays a prominent role in the regulation of feeding behaviour and energy balance (Pissios *et al.*, 2006). In contrast, icv injection of either barfin flounder (*Verasper moseri*) or human MCH exerts an anorexigenic action in goldfish (Matsuda *et al.*, 2006b), and immunoneutralization of brain MCH results in an increase in food intake (Matsuda *et al.*, 2009b). Studies into the pathways that mediate the anorexigenic action of MCH in goldfish suggest that MCH enhances the anorexigenic actions of αMSH via the MC4R signalling pathway and blocks the synthesis of NPY and ghrelin in the diencephalon (Shimakura *et al.*, 2008). In contrast, transgenic medaka (*Oryzias latipes*) that overexpress MCH have normal growth and feeding behaviour (Kinoshita *et al.*, 2001).

Originally isolated as an mRNA that is upregulated after administration of psychostimulant drugs in rodents, cocaine- and amphetamine-regulated transcript (CART) is a powerful anorexigenic signal in mammals, which acts in the hypothalamus (Valassi *et al.*, 2008). Similarly, icv administration of human CART decreases food consumption in goldfish (Volkoff and Peter, 2000, 2001a), and fasting decreases brain CART mRNA levels in goldfish (Volkoff and Peter, 2001a), Atlantic cod (*Gadus morhua*; Kehoe and Volkoff, 2007), channel catfish

(Kobayashi *et al.*, 2008) and Atlantic salmon (*Salmo salar*; Murashita *et al.*, 2009). In goldfish, the anorexigenic actions of CART may be mediated in part via inhibitory actions on the NPY and orexin pathways (Volkoff and Peter, 2000), and through an interaction with leptin (Volkoff *et al.*, 2003).

To date, although only identified in goldfish, there is evidence that several additional anorexigenic signals are involved in the central regulation of food intake in teleosts. For example, both icv and ip injections of heterologous PACAP or vasoactive intestinal peptide (VIP), two members of the secretin–glucagon superfamily of peptides, inhibit food intake in the goldfish (Matsuda *et al.*, 2005b). Moreover, excessive feeding of goldfish for 7 days increases the expression of the mRNAs for PACAP and its receptor, the PAC1 receptor (Matsuda *et al.*, 2005a). Similarly, icv injection of goldfish neuromedin U (NMU)-21 suppresses food intake in a dose-dependent manner, and fasting for 7 days induces a reduction in brain NMU-21 mRNA levels (Maruyama *et al.*, 2008). Three members of the calcitonin/calcitonin gene-related peptide (CGRP) peptide family, CGRP, intermedin and amylin, have also been implicated in the central regulation of feeding in fish. Icv injection of human CGRP, pufferfish intermedin (IMD) or rat amylin all induced a decrease in food intake in goldfish with a rank order of potency (amylin > CGRP > IMD), which is in line with the potency previously established in rodents (Thavanathan and Volkoff, 2006; Martinez-Alvarez *et al.*, 2009). In addition, the effects of amylin on food intake in goldfish are mediated in part by central cholecystokinin (CCK; Thavanathan and Volkoff, 2006). A short-term anorexigenic role for prolactin-releasing peptide (PrRP) around a scheduled meal time is suggested from the observation that icv and ip administration of goldfish PrRP elicits a dose-dependent suppression of food intake and from the increases in hypothalamic PrRP mRNA levels both post-feeding and after 7 days of food deprivation (Kelly and Peter, 2006). Finally, gonadotropin-releasing hormone (GnRH), an important neuropeptide for the regulation of pituitary gonadotropin release and sexual behaviour in vertebrates, may also serve as a link between energy homeostasis and reproduction. Icv administration of the chicken GnRH II (cGnRH II) variant at doses that stimulate spawning results in a suppression of food intake in goldfish (Hoskins *et al.*, 2008; Matsuda *et al.*, 2008b). Icv injections of cGnRH II also suppress hypothalamic orexin mRNA levels, suggesting that the anorexigenic actions of cGnRH-II in goldfish might be in part mediated by orexin (Hoskins *et al.*, 2008).

Peripheral anorexigenic signals

Anorexigenic signals involved in both the short-term and long-term regulation of food intake in fish also originate from peripheral organs such as the gastrointestinal (GI) tract, the pancreas, liver, adipose tissue, interrenals and gonads. For example, the gut–brain peptide CCK is a potent satiety signal involved in the short-term regulation of both food intake and the digestion of ingested food. Produced in response to the presence of food in the GI tract by the endocrine cells of the stomach and intestine, as well as by gut nerves and in the brain, CCK slows gastric emptying and stimulates gallbladder contraction and GI motility (Olsson *et al.*, 1999; Jönsson *et al.*, 2006; Nelson and Sheridan, 2006; Olsson and Holmgren, 2009). Injections of CCK inhibit food intake in goldfish (Himick and Peter, 1994b; Thavanathan and Volkoff, 2006) and channel catfish (Silverstein and Plisetskaya, 2000), and oral administration of CCK receptor antagonists stimulates appetite in rainbow trout (Gelineau and Boujard, 2001). Also produced by gut nerves and endocrine cells of the GI tract, and by the brain, are the structurally and functionally related peptides gastrin-releasing peptide (GRP) and bombesin (BBS). Although both GRP and BBS have been implicated in the control of digestion and gut motility in fish (Nelson and Sheridan, 2006; Olsson and Holmgren, 2009), their role in the short-term regulation of food intake remains to be established. While icv and ip injections of BBS suppress

food intake in goldfish (Himick and Peter, 1994a), in contrast to CCK, feeding does not influence plasma GRP levels in rainbow trout (Jönsson *et al.*, 2006).

The pancreatic peptides glucagon-like peptide-1 (GLP-1) and insulin may be implicated in the regulation of food intake in fish. Overall, while GLP-1 has catabolic and energy-mobilizing actions in fish, and these are generally opposed by the anabolic and energy-storing actions of insulin (Nelson and Sheridan, 2006), both peptides may have anorexigenic actions in fish, as observed in mammals (Turton *et al.*, 1996; Niswender *et al.*, 2004). Both central and peripheral injections of catfish GLP-1 suppress food intake in channel catfish (Silverstein *et al.*, 2001). Similarly, icv and ip administration of bovine insulin inhibits food intake in rainbow trout (Soengas and Aldegunde, 2004). In contrast, bovine insulin had no effect on feeding in channel catfish (Silverstein and Plisetskaya, 2000). In general, the physiological conditions under which either GLP-1 or insulin may play a role in the regulation of food intake in fish have not been established.

While leptin was discovered in 1994 and has long been recognized as a key adiposity signal that regulates food intake and energy balance in mammals (Zhang *et al.*, 1994; Morton *et al.*, 2006), the considerable sequence dissimilarity between fish and mammalian leptins delayed the characterization of fish leptins until relatively recently (Kurokawa *et al.*, 2005; Huising *et al.*, 2006; Gorissen *et al.*, 2009). While heterologous leptins have no effect on feeding in some fish species (Baker *et al.*, 2000; Silverstein and Plisetskaya, 2000), icv and ip injections of murine or human leptin inhibit feeding in goldfish (Volkoff *et al.*, 2003; De Pedro *et al.*, 2006), and treatment with homologous recombinant leptin suppresses food intake in rainbow trout (Murashita *et al.*, 2008). Whereas the anorexigenic effects of leptin in goldfish are at least partly mediated by CCK and via interactions with the NPY and orexin pathways (Volkoff *et al.*, 2003), the appetite-suppressing effects of leptin in rainbow trout are associated with changes in hypothalamic NPY and POMC gene expression (Murashita *et al.*, 2008). In mammals, leptin is produced mainly in adipose tissue and its circulating levels increase with overfeeding and decrease with starvation (Zhang *et al.*, 1994). In contrast, the major site of leptin expression in fish appears to be the liver (Kurokawa *et al.*, 2005; Huising *et al.*, 2006), and in rainbow trout plasma leptin levels increase with fasting and are not correlated with condition factor (Kling *et al.*, 2009). Similarly, neither fasting for days or weeks nor long-term feeding to satiation affects hepatic leptin gene expression in common carp (*Cyprinus carpio*; Huising *et al.*, 2006). Therefore, while the physiological role of leptin in fish may be linked to the regulation of food intake and energy balance, it does not appear to act as an adiposity signal (Huising *et al.*, 2006; Kling *et al.*, 2009; Gorissen *et al.*, 2009).

Cortisol, the principal corticosteroid secreted by the interrenal cells in teleosts (Mommsen *et al.*, 1999), is involved in the regulation of food intake in fish, but its role is equivocal (Bernier, 2006). In goldfish, while moderate chronic increases in plasma cortisol stimulate food intake, decrease forebrain CRF gene expression and increase NPY mRNA levels, larger catabolic doses of cortisol decrease CRF mRNA levels but have no effect on food intake or NPY gene expression (Bernier *et al.*, 2004). In contrast, chronic moderate and larger catabolic elevations in plasma cortisol suppress food intake in rainbow trout (Gregory and Wood, 1999). Similarly, chronic catabolic doses of cortisol decrease food intake in channel catfish (Peterson and Small, 2005). In rainbow trout, the appetite-suppressing effects of chronic hypercorticoidism are associated with increases in preoptic area CRF and NPY gene expression, decreases in hypothalamic AgRP and ghrelin mRNA levels, and a marked increase in liver leptin expression. These multiple interactions between cortisol and the central and peripheral appetite-regulating signals probably contribute to the dose-dependent and species-specific effects of cortisol on the regulation of food intake in fish.

Finally, recent evidence suggests that the sex steroid 17β-oestradiol (E2) also

impacts the regulation of food intake in fish (Leal *et al.*, 2009). In European sea bass (*Dicentrarchus labrax*), while implants containing E2 or testosterone (T) significantly inhibit self-feeding levels, implants containing 11-ketoandrostenedione (a non-aromatizable androgen) have no effect on food intake (Leal *et al.*, 2009). Therefore, while both E2 and T are anorexigenic, the inhibitory effect of T on food intake appears to be mediated by its aromatization to E2.

Short-term versus long-term regulation

The gene expression of several appetite-regulating signals appears to be entrained by mealtime in fish (Fig. 8.2). In goldfish, there is a preprandial increase and a postprandial decrease in the hypothalamic mRNA levels of the orexigenic signals NPY (Narnaware *et al.*, 2000), galanin (Unniappan *et al.*, 2004b) and ghrelin (Unniappan *et al.*, 2004a). The mealtime-associated variations in brain ghrelin are paralleled by periprandial changes in gut ghrelin gene expression and plasma ghrelin levels (Unniappan *et al.*, 2004a). In contrast to the increase in the mRNA levels of the anorexigenic signals CART (Volkoff and Peter, 2001a), PrRP (Kelly and Peter, 2006), CCK (Peyon *et al.*, 1999) and tachykinins (Peyon *et al.*, 2000). Similarly, in Atlantic cod, hypothalamic NPY, orexin and CART all display periprandial changes in gene expression that are consistent with a role for these peptidergic signals in the short-term regulation of food intake (Kehoe and Volkoff, 2007; Xu and Volkoff, 2007). The attenuation and/or absence of the above periprandial changes in fish that are unfed at the scheduled feeding time (Peyon *et al.*, 1998, 1999; Volkoff and Peter, 2001a; Unniappan *et al.*, 2004a,b; Kelly and Peter, 2006) suggest that the central neurons and peripheral cells that produce various

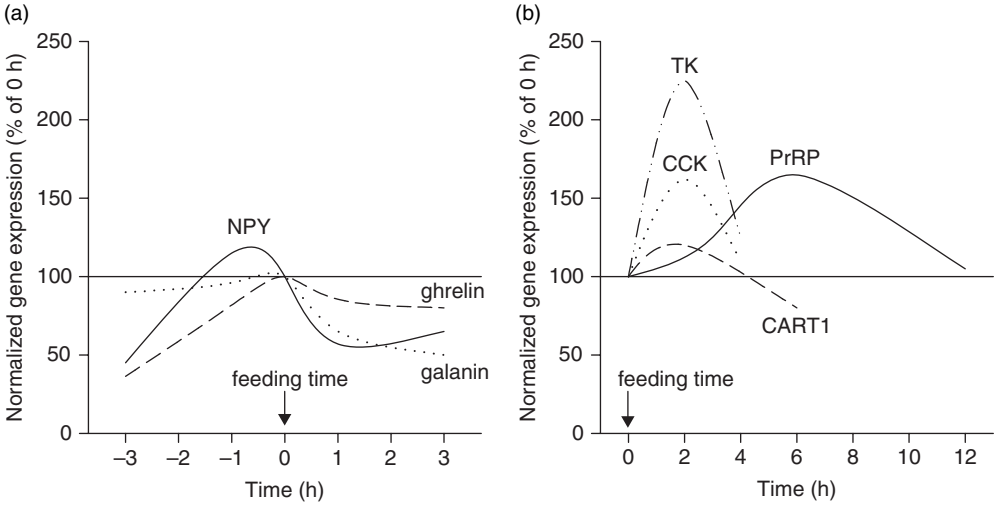


Fig. 8.2. Summary of periprandial changes in the hypothalamic mRNA levels of orexigenic (a) and anorexigenic (b) factors involved in the regulation of food intake in goldfish. The gene expression data is shown as the normalized percentage of the 0 h value (the scheduled feeding time) for each given transcript. In general, there is a preprandial increase and a postprandial decrease in the mRNA levels of the orexigenic factors neuropeptide Y (NPY; Narnaware *et al.*, 2000); ghrelin (Unniappan *et al.*, 2004a) and galanin (Unniappan *et al.*, 2004b). In contrast, there is a postprandial increase in the mRNA levels of the anorexigenic factors cocaine- and amphetamine-regulated transcript 1 (CART1; Volkoff and Peter, 2001a), cholecystokinin (CCK; Peyon *et al.*, 1999), tachykinin (Peyon *et al.*, 2000) and prolactin-releasing peptide (PrRP; Kelly and Peter, 2006).

orexigenic and anorexigenic signals are responsive to changes in nutrient levels.

In fish, as in mammals (Marty *et al.*, 2007), there is evidence implicating plasma glucose levels as a potential trigger for meal initiation and termination. In goldfish, for example, ip injections of glucose dose-dependently decrease food consumption and significantly reduce the number of cell showing orexin-like immunoreactivity in the hypothalamus (Nakamachi *et al.*, 2006). While hyperglycemic conditions inconsistently impact food intake in rainbow trout (Soengas and Aldegunde, 2004; Polakof *et al.*, 2008), both insulin-induced hypoglycemia (Polakof *et al.*, 2008) and glucodeprivation via icv administration of the non-metabolizable 2-deoxy-D-glucose (Soengas and Aldegunde, 2004) increase food intake. Several studies have also demonstrated that the hypothalamus and hindbrain in rainbow trout are glucose-sensing areas (Polakof *et al.*, 2007a,b, 2008).

The lipostatic model is the current and well-accepted paradigm for the long-term regulation of food intake and energy homeostasis in mammals. The model states that adiposity signals produced in proportion to the amount of body fat modulate food intake to maintain energy homeostasis (Henry and Clarke, 2008). Similarly, body fatness affects food intake in teleost fishes. In both salmonids (Metcalfe and Thorpe, 1992; Shearer *et al.*, 1997) and catfish (Silverstein and Plisetskaya, 2000), fat fish eat less than lean fish. In mammals, both leptin and insulin function as important signals in the feedback regulation of body fat mass (Niswender *et al.*, 2004). Whether either insulin or leptin play a similar role in fish remains to be established. To date, a direct relationship between fat stores and plasma insulin levels in fish has not been demonstrated (Silverstein and Plisetskaya, 2000; Beckman *et al.*, 2001), and leptin does not appear to act as an adiposity signal (Huising *et al.*, 2006; Kling *et al.*, 2009). Finally, various additional hormones are synthesized by adipocytes in mammals, e.g. adiponectin, resistin, visfatin (Henry and Clarke, 2008), but their physiological roles in fish have yet to be determined.

Stressors and Food Intake Disorders

An integral component of the stress response in vertebrates is a reallocation of energy away from investment activities, such as growth and reproduction, and towards activities that contribute to the restoration of homeostasis, such as oxygen delivery, hydromineral balance and locomotion. Among the non-essential physiological functions that are inhibited during the stress response are feeding and appetite (Charmandari *et al.*, 2005). Fish are no different from other vertebrates in this regard, and a characteristic feature of the response to diverse stressors in fish is a reduction in food intake (Schreck *et al.*, 1997; Wendelaar Bonga, 1997; Bernier and Peter 2001b; Bernier 2006). Beyond appetite, stressors have been shown to disrupt several aspects of the feeding behaviour of fish, including their ability to search, find and capture preys (Beitinger, 1990). This section will review how diverse stressors affect food intake in fish and the suggested mechanisms that may be involved in mediating the appetite-suppressing effects of stressors.

Environmental factors affecting food intake

Aquatic ectotherms are more prone to being exposed to temperature, hypoxia, ammonia and osmotic challenges than terrestrial animals. While each one of these disturbances is known to affect food intake, there is a unique relationship between each environmental parameter and ingestion rate. Moreover, the tolerance to variation in temperature, oxygen, ammonia and salinity varies greatly between species and also between life stages. Fishes are also routinely exposed to an increasing number of environmental contaminants, many of which have now been shown to suppress appetite.

Temperature

Temperature, by virtue of its importance in governing metabolic rate in ectotherms, is one of the most influential environmental factors affecting food intake in fishes

(Kestemont and Baras, 2001). In general, food intake increases with rising temperature, plateaus and then falls sharply near the upper lethal temperature (Brett *et al.*, 1969) (Fig. 8.3a). While fish vary considerably in their range of temperature tolerance, each species has an optimum temperature range, over which feeding increases with rising temperature (Elliott, 1981). Acute changes in temperature, however, even within the optimum temperature range, can also result in marked reductions in food intake (Elliott, 1991). While the specific endocrine mechanisms responsible for the gradual temperature-induced changes in food intake are only now beginning to be explored (e.g. Kehoe and Volkoff, 2008), sudden marked temperature changes are known to stimulate the HPI axis in fish (Strange *et al.*, 1977; Sumpter *et al.*, 1985; Van den Burg *et al.*, 2005). Therefore, given the role of CRF-related peptides and cortisol in the regulation of food intake discussed above (Bernier, 2006), it seems likely that components of the endocrine stress response contribute to the suppression of appetite observed with acute temperature changes. On the other hand, the pronounced drop in food consumption near the upper lethal temperature may be due to the limitations of the cardiovascular system in maintaining adequate tissue oxygenation and preventing hypoxaemia (Jobling, 1997; Clark *et al.*, 2008).

Hypoxia

The oxygen content of the air at 20°C is approximately 30 times higher than that of air-saturated water, and oxygen diffuses 200,000 times faster in air than it does in water (Hill *et al.*, 2008). As a result, oxygen in water can be depleted rapidly by aquatic organisms, is only slowly replenished through diffusion, and hypoxic conditions are a common feature of various aquatic habitats. Hypoxic conditions can develop seasonally in northern temperate lakes as a result of stratification and ice cover (Hasler *et al.*, 2009), and daily in tropical fresh water, tide pools and coral reefs as a result of algal respiration and isolation of waterbodies (Nilsson and Ostlund-Nilsson, 2006; Val *et al.*, 2006). Anthropomorphic activities are also a major cause of environmental hypoxia, and there are now over 400 aquatic ecosystems worldwide that have reported accounts of eutrophication-associated anoxic zones (Diaz and Rosenberg, 2008). Chronic

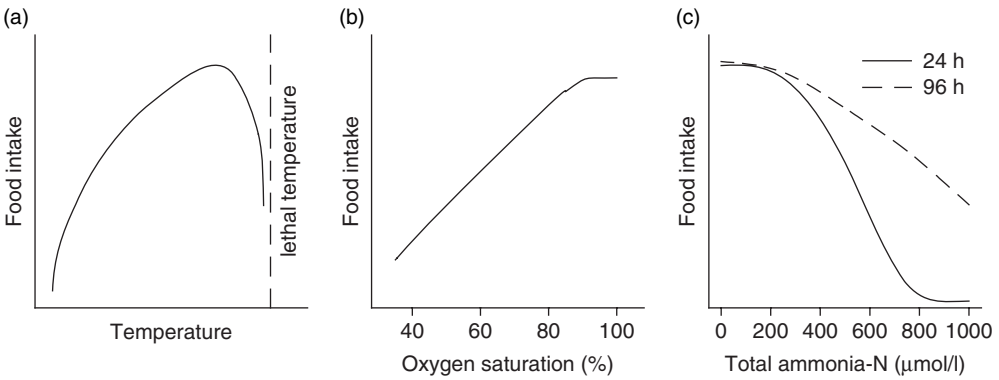


Fig. 8.3. Effects of water temperature, oxygen saturation and total ammonia on food intake in fish. (a) In general, food intake increases with rising water temperature, plateaus and then falls sharply near the upper lethal temperature (Brett *et al.*, 1969). (b) Food intake is independent of water oxygen saturation above a species-specific threshold but decreases in proportion to oxygen availability below this value (Bernier and Craig, 2005; Pedersen, 1987). (c) Food intake is independent of water ammonia levels below a species-specific threshold but decreases in proportion to the severity of the hyperammonemic conditions above this value. Chronic exposure to constant hyperammonemic conditions is associated with a partial recovery in food intake over time (Ortega *et al.*, 2005).

exposure to hypoxia has been shown to reduce food intake in several freshwater and marine hypoxia-sensitive and -tolerant fish species (Pedersen, 1987; Chabot and Dutil, 1999; Buentello *et al.*, 2000; Pichavant *et al.*, 2001; Zhou *et al.*, 2001; Bernier and Craig, 2005; Ripley and Foran, 2007). While food intake is independent of oxygen availability above a species-specific threshold, it is directly related to dissolved oxygen concentration below this value (Fig. 8.3b). In general, among the hierarchy of physiological responses associated with hypoxia in fish, a reduction in food intake is a behavioural strategy that is recruited relatively early in the overall response to decreasing oxygen levels and one that is sustained under conditions of chronic hypoxia (Boutilier *et al.*, 1988; Pichavant *et al.*, 2001; Bernier and Craig, 2005). In the short term, the appetite-suppressing effects of hypoxia are associated with a stimulation of the HPI axis in rainbow trout, and there is evidence that endogenous CRF-related peptides are involved in mediating at least a portion of the reduction in food intake (Bernier and Craig, 2005). Chronically, although the appetite-suppressing effects of hypoxia are sustained (Pichavant *et al.*, 2001; Bernier and Craig, 2005), CRF-related peptides do not appear to play a role in mediating the anorexia and the mechanisms responsible have yet to be determined.

Ammonia

Ammonia is the metabolic nitrogenous waste product excreted by most fish (Wright, 1995). Although toxic, in well-aerated flowing water ammonia is readily excreted by the gills using a combination of ionic and diffusive mechanisms (Tsui *et al.*, 2009). However, in eutrophic environments and under intensive aquaculture conditions, fish can also encounter elevated levels of ammonia (Ip *et al.*, 2001). In rainbow trout (Wood, 2004) and walleye (*Sander vitreus*; Madison *et al.*, 2009), exposure to low levels of exogenous ammonia (≤ 225 μmol/l) can stimulate growth without altering food intake. Instead, the growth-promoting effects of low ammonia concentrations have been attributed to a stimulation of protein synthesis and/or a reduction in metabolic costs (Wood, 2004; Madison *et al.*, 2009). In contrast, exposure to elevated concentrations of water ammonia suppresses growth and appetite (Beamish and Tandler, 1990; Atwood *et al.*, 2000; Wicks and Randall, 2002; Ortega *et al.*, 2005), and elicits a surge in plasma cortisol (Tomasso *et al.*, 1981; Spotte and Anderson, 1989; Person-Le-Ruyet *et al.*, 1998; Ortega *et al.*, 2005). In rainbow trout, chronic exposure to high water ammonia (≥500 μmol/l) for 96 h elicits an initial dose-dependent reduction in food intake followed by a partial recovery (Fig. 8.3c) (Ortega *et al.*, 2005). Correlated with these reductions in food intake are time-dependent and brain-region-specific changes in serotonergic and dopaminergic activities, and changes in the mRNA levels of the neuropeptides CRF and UI, which implicate these anorexigenic signals as potential mediators of the appetite-suppressing effects of ammonia (Ortega *et al.*, 2005).

Salinity

Depending on the species, life stage, season and water temperature, and both the magnitude and rate of change, alterations in salinity can have no effect, induce small changes or have a marked effect on feeding in fishes (Imsland *et al.*, 2001, 2008; Kestemont and Baras, 2001). For example, chronic exposure of stenohaline common carp to 10‰ salinity, levels close to their iso-osmotic value, reduced food intake by 70% and had adverse effects on growth and survival (De Boeck *et al.*, 2000). In contrast, in the euryhaline European sea bass, lowering the salinity over a 72 h period from 25‰ to 7‰ and 0‰ reduced food intake by 27% and 42%, respectively (Rubio *et al.*, 2005). In salmonid fishes, several studies have now shown that abrupt transfer from fresh water to seawater is associated with an osmoregulatory imbalance, an increase in plasma cortisol levels and a suppression of food intake (Usher *et al.*, 1991; Arnesen *et al.*, 1993; Craig *et al.*, 2005; Liebert and Schreck, 2006). Interestingly, while the reduction in food intake is chronic and appetite recovery can take several weeks, both plasma cortisol

levels and osmoregulatory parameters return to basal values within hours to days (Pirhonen *et al.*, 2003a; Craig *et al.*, 2005; Liebert and Schreck, 2006). Therefore, in salmonid fishes at least, there appears to be a clear separation during seawater adaptation between the osmoregulatory and feeding response.

Contaminants

Various contaminants in the aquatic environment can disrupt food intake in fish (Beitinger, 1990; Kestemont and Baras, 2001). While there is evidence that some compounds directly affect the circuitry of feeding-related peptides in the brain, others suppress feeding through actions on food palatability or digestibility, or by disrupting the ability of fish to capture prey (Samis *et al.*, 1993; Boujard and Le Gouvello, 1997; Mennigen *et al.*, 2009). Examples of environmental contaminants that can reduce feeding in fish include pesticides (Muniandy and Sheela, 1993; Samis *et al.*, 1993), herbicides (Hussein *et al.*, 1996; Nieves-Puigdoller *et al.*, 2007), metals (Lanno *et al.*, 1985; Shaw and Handy, 2006) and pharmaceuticals (Stanley *et al.*, 2007; Mennigen *et al.*, 2009). In general, while several compounds can suppress appetite, the impact of contaminants on feeding in fish will very according to dose, species, life stage, method of exposure and whether the animals are exposed to an individual compound or mixtures. Although feeding can be a sensitive behavioural indicator of low-level exposure to some agents (Beitinger, 1990), long-term exposure to environmentally realistic doses of some contaminants can have a marked impact at the cellular level without having an effect on either feeding or growth (Abalos *et al.*, 2008).

Social stressors

Social stressors, such as subordination, isolation, confinement, crowding and predator avoidance, can affect food intake in fish (Kestemont and Baras, 2001; Bernier, 2006). For example, subordination in pairs of rainbow trout (Abbott *et al.*, 1985; DiBattista *et al.*, 2006) and Arctic char (*Salvelinus alpinus*; Øverli *et al.*, 1998) results in a drastic and sustained reduction in food intake. Similarly, in larger groups of salmonid fishes, the social rank of a fish within the group's hierarchical structure correlates positively with its mean share of group meal (McCarthy *et al.*, 1992; Winberg *et al.*, 1993a). Although the dominant fish can monopolize food, the appetite inhibition in subordinates is not merely the result of interference competition, as appetite in the subordinate fish continues to be depressed for several days in the absence of the dominant fish (Øverli *et al.*, 1998; Griffiths and Armstrong, 2002; DiBattista *et al.*, 2006). Instead, the subordination-induced anorexia is associated with a chronic activation of the endocrine stress response, as well as with changes in the concentration and expression of multiple signals known to play a role in the regulation of food intake in fish (Bernier, 2006; Johnsson *et al.*, 2006; Bernier *et al.*, 2008); see earlier sections of the chapter for details). Isolation and confinement can also reduce food consumption (Øverli *et al.*, 2002) and stimulate the HPI axis (Ando *et al.*, 1999; Doyon *et al.*, 2005; Bernier *et al.*, 2008). Interestingly, however, while these milder social stressors elicit a relatively small and transient increase in plasma cortisol levels (Doyon *et al.*, 2005; Bernier *et al.*, 2008), the reduction in food intake in response to isolation and confinement can persist for several days. In rainbow trout, for example, most fish do not eat the day following transfer to isolation, and food intake only slowly and progressively recovers over 6 days or longer (Øverli *et al.*, 2002; Schjolden *et al.*, 2005). Depending on the species, crowding or high stocking density can have either detrimental or stimulatory effects on food intake. In most fish species, *e.g.* Atlantic cod (Lambert and Dutil, 2001), brook charr (*Salvelinus fontinalis*; Vijayan and Leatherland, 1988), gilthead seabream (*Sparus aurata*; Canario *et al.*, 1998), largemouth bass (*Macropterus salmoides*; Petit *et al.*, 2001) and sea bass (Sammouth *et al.*, 2009), daily food intake remains unchanged within a species-specific range of rearing

densities and decreases once an upper threshold is reached. In contrast, as a result of an inverse relationship between the incidence of agonistic interactions and rearing densities, Arctic charr reared at high densities have higher daily food intake than those reared at low densities (Jorgensen *et al.*, 1993; Jobling and Baardvik, 1994).

Potential mechanisms mediating the appetite-suppressing effects of stressors

CRF plays a central role in mediating the appetite-suppressing effects of stressors (Richard *et al.*, 2002; Bernier, 2006). Recognized as a key regulator of the HPI axis, there is also evidence that CRF in fish, as in mammals, may be involved in the regulation and coordination of the behavioural, autonomic and metabolic responses to stressors (Bernier *et al.*, 2009). Although causal relationships have seldom been established (e.g. Bernier and Craig, 2005) and the current evidence is primarily based on correlations, a variety of different types of stressors that suppress food intake in fish also elicit an activation of the HPI axis and an increase in forebrain CRF and UI gene expression (see Bernier, 2006 for review). Evidence for a role of CRF and UI in the regulation of food intake in fish also comes from the demonstration that these peptides are potent anorexigenic signals that can mediate the appetite-suppressing effects of several other regulatory hormones (discussed in an earlier section of this chapter). In rodents, all four structurally related ligands of the CRF system – CRF (Britton *et al.*, 1982), urocortin (UCN; Spina *et al.*, 1996), UCN2 (Inoue *et al.*, 2003) and UCN3 (Fekete *et al.*, 2007) – are anorexigenic, and both CRF receptor subtypes (CRF-R1 and CRF-R2; Zorrilla *et al.*, 2003) and the CRF binding protein (CRF-BP; Heinrichs *et al.*, 1996) have been implicated in the regulation of food intake, feeding behaviour and energy homeostasis. While the CRF system of all vertebrates also appears to be composed of four ligands, two receptor subtypes and a binding protein (Chang and Hsu, 2004; Lovejoy and Jahan, 2006; Alderman *et al.*, 2008), the individual contributions of the urocortin-related peptides, CRF-R1, CRF-R2 and CRF-BP to food intake regulation and disorders in fish remains to be determined.

Cortisol, the end product of HPI axis activation, also probably plays an important role in mediating and/or modulating the appetite-suppressing effects of stressors in fish (Bernier and Peter, 2001b). Although species differences exist (see the Peripheral anorexigenic signals section), cortisol has been shown to affect the gene expression of several key central and peripheral factors that regulate food intake (Bernier *et al.*, 2004; Madison *et al.*, 2009b). Moreover, both RU-486, a glucocorticoid receptor antagonist, and metyrapone, an inhibitor of cortisol synthesis, significantly affect feeding in goldfish (Bernier and Peter, 2001a). To what extent the effects of cortisol on food intake in fish are direct or indirect is not known, and future studies aimed at localizing glucocorticoid and mineralocorticoid receptors within the neuronal network of the hypothalamic feeding centre are needed.

Perception by the brain of disturbances to homeostasis, i.e. stressors, is achieved by a complex neurocircuitry that releases various stress mediators (Joels and Baram, 2009). While this stress-sensitive neurocircuitry regulates the activation of the HPA axis in mammals (Herman *et al.*, 2003), it also orchestrates complex responses at several levels of the CNS (Joels and Baram, 2009). An important group of stress mediators that are also involved in regulating feeding behaviour and energy balance are the monoamines, including noradrenaline, dopamine and serotonin (Nelson and Gehlert, 2006). While the neurocircuitry that is involved in the perception and coordination of stressors in fish largely remains to be identified (Bernier *et al.*, 2009), several appetite-suppressing stressors are known to affect the brain monoaminergic systems (Johnsson *et al.*, 2006). For example, social subordination in salmonids is associated with elevated brain noradrenergic, dopaminergic and serotonergic activity in selected brain areas (Øverli *et al.*, 1999). Handling (Winberg *et al.*, 1992), confinement (Øverli *et al.*, 2001), predator

exposure (Winberg *et al.*, 1993b) and hyperammonemia (Ortega *et al.*, 2005) also elevate brain serotonergic activity, and hypoxia depresses the activity of this monoaminergic system (Thomas *et al.*, 2007). There is also evidence that serotonin, dopamine and noradrenaline (De Pedro *et al.*, 1997, 1998a; Kaslin *et al.*, 2004; Johansson *et al.*, 2005) are involved in the regulation of food intake in fish. Thus, although much work is needed to identify their specific functions and targets, monoamines may also be important mediators of the appetite-suppressing effects of stressors in fish.

Fish Diseases and Food Intake Disorders

A clinical sign of disease in fish is a loss of appetite. Similarly, anorexia is part of the sickness syndrome in mammals, i.e. part of the endocrine, autonomic and behavioural changes that make up the normal response to infection (Dantzer *et al.*, 2008). In general, this sickness-associated change in motivational state enables ill individuals and animals to cope better with an infection (Kelley *et al.*, 2003). Indeed, several fish studies have shown that the infection-induced loss of appetite can reduce the severity of the disease and increase survival (Li and Woo, 1991; Wise and Johnson, 1998; Pirhonen *et al.*, 2003b; Damsgard *et al.*, 2004). In contrast, the sustained anorexia and associated catabolic state that characterizes chronic diseases, such as cancer, obstructive pulmonary disease or heart failure, can be life-threatening and contribute to mortality (Laviano *et al.*, 2008). This section will review the prevalence of anorexia in fish affected by viral, bacterial and parasitic infections, and the mechanisms that may be involved in mediating the appetite-suppressing effects of diseases.

Prevalence of anorexia in diseased fish

Infection of fish with several well-known viruses is accompanied by a reduction in food intake. In Atlantic salmon, while infection with infectious pancreatic necrosis virus (IPNV) can chronically inhibit both food intake and specific growth rate, changes in appetite and growth are only detected from approximately 20 days after infection, once virus titres have reached relatively high levels (Damsgard *et al.*, 1998). Moreover, while IPNV-infected freshwater fry of Atlantic salmon are characterized by greatly distended intestines filled with undigested food, infected seawater post-smolts usually fail to grow and become emaciated (Roberts and Pearson, 2005). Infections of Atlantic cod and Atlantic halibut (*Hippoglossus hippoglossus*) with nodavirus (Patel *et al.*, 2007; Mezeth *et al.*, 2009), the causative agent of viral encephalopathy and retinopathy (VER; Munday *et al.*, 2002), are associated with a loss of appetite. Similarly, Atlantic salmon infected with infectious salmon anaemia (ISA), also known as haemorrhagic kidney syndrome, are anorectic (Byrne *et al.*, 1998). While anorexia is a clinical sign of nodavirus and ISA infections, to our knowledge the specific impact of these viral diseases on individual food intake and growth in fish has not been determined.

Bacterial infections are also generally associated with a loss of appetite. For example, Atlantic salmon infected with *Vibrio salmonicida* are characterized by a transient reduction in food intake (40–50%) that peaks between 2 and 3 weeks after infection (Damsgard *et al.*, 2004). In fish infected with *Aeromonas salmonicida*, the causative agent of furunculosis, it appears that the severity of the anorexia depends on the level of infection. In rainbow trout infected with a dose of *A. salmonicida* that elicited 40% mortality, food intake was chronically depressed by about 25% for a period of 2 weeks post-infection (Neji *et al.*, 1993; Neji and de la Noue, 1998). In contrast, in chinook salmon (*Oncorhynchus tshawytscha*) infected with a dose of *A. salmonicida* that elicited only 5% mortality, food intake was unaffected (Neji and de la Noue, 1998; Pirhonen *et al.*, 2003b). Similarly, in chinook salmon, there is an inverse relationship between the proportion of fish with detectable bacterial kidney disease (BKD) p57 antigen and food intake (Pirhonen *et al.*, 2000).

Parasites can affect food intake in fish via a variety of different mechanisms. While some parasites may directly affect appetite (Woo, 2003), other parasites may reduce the stomach capacity of infected fish (Sirois and Dodson, 2000), damage the alimentary canal and the intestinal diffuse endocrine system of the intestine (Dezfuli *et al.*, 2003), affect the foraging behaviour of their host (Barber *et al.*, 2000) or affect feeding through a combination of the above. Characterized most extensively among the different parasites that are known to affect feeding in fish are the effects of the protozoan haemoflagellate *Cryptobia salmositica* on food intake in rainbow trout (Woo, 2003). Depending on water temperature, the onset of anorexia in *Cryptobia*-infected rainbow trout is ~2–5 weeks post-infection and coincides with a significant rise in parasitaemia and a decrease in haematocrit (Chin *et al.*, 2004). Maximal anorexia is reached ~1 week after the onset, is associated with a ~50–80% reduction in food intake and concurs with peak parasitaemia and minimum oxygen-carrying capacity (Chin *et al.*, 2004). The return of appetite in *Cryptobia*-infected fish is associated with the establishment of an immune response against the pathogen that significantly reduces parasitaemia and anaemia. *Cryptobia* infection also strengthens the feeding hierarchy within groups of fish, exacerbating the difference in mean share of meal between dominant and subordinant fish (Chin *et al.*, 2004). Ectoparasitic copepods such as the sea louse *Lepeophtheirus salmonis* can also cause appetite suppression in Altantic salmon (Dawson *et al.*, 1999) and exacerbate the reduction in food intake associated with seawater transfer in brown trout (*Salmo trutta*; Dawson *et al.*, 1998). Finally, infection with the microsporan parasites *Loma salmonae* in rainbow trout (Ramsay *et al.*, 2004) and *Loma branchialis* in Atlantic cod (Khan, 2005) is associated with significant (~25–45%) reduction in food intake. In rainbow trout, *Loma salmonae*-associated reductions in food intake and specific growth rate coincide with the appearance of gill lesions and xenoma onset, i.e. the presence of enlarged host cells filled with spores and developmental stages of microsporidia (Ramsay *et al.,* 2004).

Potential mechanisms mediating the appetite-suppressing effects of diseases

Despite the significant negative economic impact to the aquaculture industry of the appetite- and growth-suppressing effects of diseases, very little is known about the specific physiological mechanisms that mediate the anorexic state of diseased fish. In contrast, there is an extensive mammalian literature on the signals and pathways that mediate the transient loss of appetite associated with sickness and the anorexia that characterizes chronic illnesses (Dantzer *et al.*, 2008; Laviano *et al.*, 2008). Therefore, as a means of reference, this section will first provide a brief overview of the mechanisms involved in mediating the appetite-suppressing effects of diseases in mammals before reviewing the evidence for such mechanisms in fish.

In general, the immune system detects pathogens and signals their presence to the central nervous system (CNS). The CNS, in return, can coordinate an appropriate physiological response through neuronal and endocrine signals. In mammals, the behavioural symptoms of sickness are triggered by cytokines that are produced at the site of infection by activated accessory immune cells and detected by the brain via several parallel pathways (Dantzer *et al.*, 2008). In rodents, the main pro-inflammatory cytokines involved in sickness behaviour, including the loss of appetite, are interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Dantzer, 2001). These pro-inflammatory cytokines cause complex changes in brainstem and hypothalamic monoaminergic and peptidergic systems that regulate feeding and energy homeostasis. Specifically, the mechanism of action of cytokines involves the modulation of the serotoninergic, dopaminergic and noradrenergic systems, an inhibition of orexigenic NPY/AgRP neurons and a stimulation of the anorexigenic POMC/CART neurons (Guijarro *et al.*, 2006; Scarlett *et al.*, 2007; Laviano *et al.*, 2008;

DeBoer *et al.*, 2009). Moreover, in mammals, IL-1β and other cytokines can potently stimulate the HPI axis via multiple mechanisms, including an activation of the CRF-containing cells of the paraventricular nucleus (PVN) (Dunn, 2005). The intensity and duration of the behavioural signs of sickness are regulated by a balance between pro- and anti-inflammatory cytokines (Dantzer *et al.*, 2008), and the anorexia associated with chronic diseases results from a sustained inflammatory state and a failure of the hypothalamic pathways that control food intake and energy expenditure to respond appropriately to peripheral inputs (Laviano *et al.*, 2008).

While the overall picture is still fragmentary, cytokines also communicate pathogen recognition to the CNS and coordinate the cellular response of the immune system in fish (Verburg-van Kemenade *et al.*, 2009). Indeed, several fish studies have reported an increase in the expression of pro-inflammatory cytokines in response to viral (Tafalla *et al.*, 2005; Seppola *et al.*, 2008), bacterial (Seppola *et al.*, 2008) and parasitic (Saeij *et al.*, 2003; Gonzalez *et al.*, 2007; Wagner *et al.*, 2008) infections. The kinetics of the cytokine-mediated inflammatory reaction in fish have also been studied in response to zymosan-induced peritonitis (Chadzinska *et al.*, 2008) and lipopolysaccharide (LPS) stimulation (Engelsma *et al.*, 2002, 2003): standard models of acute inflammation. In goldfish, both icv and ip injection of LPS elicit dose-dependent reductions in food intake, and the appetite-suppressing effects of LPS given ip are associated with a decrease in telencephalon NPY expression and an increase in hypothalamic CRF, CCK and CART mRNA levels (Volkoff and Peter, 2004). Similarly, there is evidence that LPS modulates CRF content and release in the brain of tilapia (Pepels *et al.*, 2004) and that IL-1β can activate the HPI axis in rainbow trout (Holland *et al.*, 2002) and common carp (Metz *et al.*, 2006). To date, however, the direct impact of either peripheral or central administration of pro-inflammatory cytokines on food intake in fish has not been investigated. Furthermore, the phenotype of IL-1β and TNF-α targets within the brain regions that control food intake in fish have yet to be identified. So while pro-inflammatory cytokines are recruited in response to various infections and acute inflammation can induce a reduction in appetite, a direct involvement of pro-inflammatory cytokines in the regulation of food intake in fish remains to be established.

An important mechanism by which fish pathogens bring about disease is through the production of extracellular products that are highly haemolytic or that agglutinate erythrocytes (Woo and Bruno, 1999). As a result, a clinical sign of most fish diseases is anaemia (Olsen *et al.*, 1992; Mesa *et al.,* 2000; Li *et al.*, 2003; Rehulka, 2003; Woo, 2003; Rehulka and Minarik, 2007). For example, *C. salmositica* produces a metalloprotease that lyses erythrocytes (Zuo and Woo, 2000), significantly reduces the oxygen carrying capacity of the host and increases the susceptibility of the infected fish to environmental hypoxia (Woo and Wehnert, 1986). Similarly, furunculosis produces several haemolytic factors (Hiney and Olivier, 1999), and hypoxic conditions exacerbate the appetite-suppressing effects of this pathogen (Neji and de la Noue, 1998). Therefore, in addition to pro-inflammatory cytokines, mediators of the appetite-suppressing effects of hypoxic/hypoxaemic conditions in fish may play an important role in the regulation of food intake following infection with various diseases. For example, as discussed earlier, CRF-related peptides mediate at least a portion of the acute appetite-suppressing effects of hypoxia in rainbow trout (Bernier and Craig, 2005). However, although severe anaemia can be observed within days following infection with some fish diseases (e.g. Li *et al.*, 2003), it is not known whether CRF-related peptides contribute to the regulation of food intake during such acute hypoxaemic events. Another anorexigenic signal that may play an important role in the regulation of food intake in hypoxaemic fish is the class-I helical cytokine leptin. Leptin is a hypoxia-sensitive gene and its expression is stimulated by hypoxia-inducible factor 1 in response to oxygen deficiency (Grosfeld *et al.*, 2002). In rainbow trout infected with *C. salmositica*, the gradual reduction and

recovery in oxygen carrying capacity and appetite is associated with a marked increase and recovery in liver leptin gene expression. A specific involvement of leptin in mediating the appetite-suppressing effects of *Cryptobia* infection is further supported by the observation that normoxic fish pair fed to the anorexic *Cryptobia*-infected trout have liver leptin mRNA levels that do not differ from normoxic satiated controls (MacDonald *et al*., 2009). Further studies are now needed to determine the circulating levels of leptin during the course of *Cryptobia* infection and the targets of leptin within the appetite-regulating pathways of the hypothalamus, and to assess whether leptin is a common mediator of the appetite-suppressing effects of diseases in fish.

Perspectives

Significant advances have been made in the last decade in the identification of central and peripheral appetite-regulating factors in fish. In general, while significant differences have been identified, the basic properties of most of the appetite-regulating signals in fish appear to be conserved with those initially described in mammals. Among the challenges ahead is to determine the specific involvement of these various appetite-regulating factors in a model that takes into consideration the basic physiological properties of fish. While the current models of food intake regulation are based on sexually mature rodents that maintain a set body weight but also require a constant supply of energy to maintain body temperature and high metabolic rates, poikilothermic fish have much lower energy requirements, can go without food for prolonged periods of time and generally have indeterminate growth rates. Hence the physiological mechanisms and specific properties of the factors involved in signalling the status of energy reserves, appetite and satiation in fish may differ from those in mammals. Differences in the regulation of food intake between species may also be expected, given the broad diversity of diets among fish, their patterns of food availability and utilization, and the sensory modalities that they use to locate and ingest food. Most stressors, either acute or chronic, are associated with a reduction in food intake in fish. To date, although few experiments have established causal relationships, CRF-related peptides, cortisol and brain monoamines have been identified as important mediators of the appetite-suppressing effects of stressors. Finally, the mechanisms that mediate the appetite-suppressing effects of diseases in fish are poorly understood. While there is some evidence that both pro-inflammatory cytokines and leptin may play a role in regulating food intake during disease, the relative importance of these factors in mediating the anorexia associated with various viral, bacterial and parasitic infections is not known. Determining the factors involved in the pathogenesis of the appetite-suppressing effects of diseases in fish will be key to the future development of therapeutic strategies aimed at minimizing the impact of this disorder.

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9 Immunological Disorders Associated with Polychlorinated Biphenyls and Related Halogenated Aromatic Hydrocarbon Compounds*

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Introduction

The immune system protects the body from disease by detecting and neutralizing disease-causing pathogens (viruses, bacteria, fungi and parasites) and transformed (neoplastic) cells. In order for the immune system to be effective it must be capable of discriminating between what is foreign and what is not foreign, i.e. 'self'. The process of self–non-self discrimination involves intricate interactions between target cells (e.g. pathogens and tumour cells) and both cellular and humoral (soluble) elements of the immune system. Once foreign agents are detected they are subjected to a vast array of effector cells (phagocytes, granulocytes, cytotoxic cells and natural killer cells) and soluble factors (antibodies, complement) that facilitate neutralizing, killing and clearing of the inducing agent. Disruption or modulation of these interactions by drugs or chemical contaminants is the subject of immunotoxicology. Exposure to immunotoxic chemicals may result in a variety of disorders, including immunosuppression, immunopotentiation, immunodeficiency, hypersensitivity or autoimmunity (Dean and Murray, 1991). Although most of what is known about the action of immunotoxic compounds is based on the mammalian immune system, there is increasing interest in assessing effects on lower vertebrates, some of which may accumulate high concentrations of immunomodulating chemicals in the environment.

Fish immunotoxicology is an emerging field of study. Recent reviews (Weeks *et al.*, 1992; Dunier and Siwicki, 1993; Wester *et al.*, 1994; Zelikoff, 1994, Anderson and Zeeman, 1995) and symposia (Stolen and Fletcher, 1994) report on the manner in which immune functions in fish may be modulated by toxic xenobiotic compounds, especially mammalian immunotoxins, or by pollutants associated with contaminated habitats where fish health is impaired. However, compared with mammalian immunotoxicology, where efforts have been focused on relatively few, well-characterized and extensively investigated animal models, much less is known about the effects in fish. This is due, in part, to the large number of fish species studied, the lack of many fish-specific reagents (e.g. monoclonal antibodies that detect cell-surface markers on

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fish leucocytes and secretory products) and fewer researchers in the field. Nevertheless, there are published reports describing lesions in lymphoid tissues, altered immune functions or increased disease susceptibility in toxicant-exposed fish or in fish collected from contaminated areas (Tables 9.1 and 9.2). This review characterizes immunological disorders in fish associated with the widespread environmental contaminants polychlorinated biphenyls (PCBs) and related halogenated aromatic hydrocarbons (HAHs). Special attention is devoted to comparing the sensitivity of fish species, identifying sensitive immunological end points and postulating mechanisms of action.

Toxicity of Halogenated Aromatic Hydrocarbons (HAHs)

Halogenated aromatic hydrocarbons comprise a class of chemicals that induce pleiotropic effects in mammals, including immunomodulation (Vos and Luster, 1989). Polychlorinated dibenzofurans, polychlorinated dibenzo-*p*-dioxins (dioxins) and PCBs are among the most toxic HAHs (Fig. 9.1) and are also ubiquitous environmental contaminants. Because of their resistance to degradation and high lipophilicity, HAHs tend to be biomagnified in aquatic food chains. As a result, detectable concentrations of HAHs have been measured in fish throughout North America (Smith *et al.*, 1990). The most toxic member is 2,3,7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). Several fish species are very sensitive to the lethal effects of TCDD (LD_{50} 3–16 µg/kg; Kleeman *et al.*, 1988), particularly when compared with sensitive mammalian species. In fact, the early life stages of salmonid fishes are most sensitive to TCDD (LD_{50} 0.065–0.230 µg/kg; Walker and Peterson, 1991; Walker *et al.*, 1991).

The mechanism by which TCDD exerts many of its toxic and biochemical effects is believed to require binding to the cytosolic aryl hydrocarbon receptor (AhR; Poland and Knutson, 1982). Among the sublethal effects associated with AhR binding is the induction of cytochrome P450IA1, a mixed-function oxygenase responsible for HAH metabolism. PCB congeners that are structurally similar to TCDD, in that they can attain a planar configuration and are chlorinated in meta and para positions, also bind the AhR and induce P4501A1 activity. Of the 209 PCB congeners, relatively few have high affinity for the AhR (Safe, 1987). In fish only, non-*ortho*-substituted tetrachloro (3,3′,4,4′-tetrachlorobiphenyl, 3,3′,4,5′-tetrachlorobiphenyl), pentachloro (3,3′,4,4′,5-pentachlorobiphenyl) and hexachloro (3,3′,4,4′,5,5′-hexachlorobiphenyl) congeners are known to induce AhR-mediated responses (Janz and Metcalfe, 1991; Walker *et al.*, 1991; Newsted *et al.*, 1995). AhR-active PCB congeners are a small percentage of the total mass of commercial PCB formulations, such as Aroclor® 1254, which contains over 50 different congeners (Ballschmiter and Zell, 1980). Thus, compared with TCDD, greater doses of commercial PCB mixtures are required to produce similar effects (e.g. chinook salmon, *Oncorhynchus tshawystscha*, LD50: 270 mg Aroclor® 1254/kg; Arkoosh *et al.*, 1994).

Overview of the Teleost Immune System

The detection of sublethal effects of PCBs and other HAHs on the fish immune system has evolved along with the fundamental understanding of immunological processes in fish. The teleost immune system, including nonspecific and specific immunity, and humoral or antibody-producing and cell-mediated responses, is shown in Fig. 9.2. The piscine immune system as it relates to protective immunity (innate and acquired) and structure is comprehensively reviewed earlier (van Muiswinkel, 1995; Iwama and Nakanishi, 1997; Zhang *et al.*, 1999; Ewert *et al.*, 2001; Tort *et al.*, 2003; Russell and Lumsden, 2005; Boshra *et al.*, 2006; Fisher *et al.*, 2006; Magnadóttir, 2006; Noga, 2006; Reite and Evensen, 2006; Robertson, 2006; Zapata *et al.*, 2006; Hall *et al.*, 2008; Zapata and Cortés, 2008; see also Chapter 3, this volume). The intent of this chapter is to provide a framework with

Table 9.1. Laboratory studies investigating the effects of halogenated aromatic hydrocarbons (HAHs) on functional immune responses in fish.

Species	Response	Chemical (dose and route)	Effect	End point – antigen	Reference
Chinook salmon	Humoral	Aroclor ® 1254	–	Primary *in vitro* AFC – TNP-KLH (T-D)	Arkoosh *et al.* (1994a)
		54 mg/kg IP	↓	Primary *in vitro* AFC – TNP-LPS (T-I)	
Chinook salmon	Humoral	Aroclor ® 1254	↓	Secondary *in vitro* AFC – TNP-KLH (T-D)	Arkoosh *et al.* (1994a)
		54 mg/kg IP	↓	Secondary *in vitro* AFC – TNP-LPS (T-I)	
Rainbow trout	Humoral	Aroclor ® 1254 3, 30, 300 mg/kg diet	–	AFC – sheep red blood cells (T-D)	Cleland *et al.* (1988a)
Rainbow trout	Humoral	Chlophen ® A50 500 mg/kg diet	↓	Antibody titre – *V. anguillarum* O antigen (T-I)	Thuvander and Carlstein (1991)
Rainbow trout	Humoral	Chlophen ® A50 40 and 80 mg/kg IP	–	Antibody titre – KLH (T-D)	Thuvander *et al.* (1993)
Rainbow trout	Humoral	Chlophen ® A50	–	Proliferation – LPS	Thuvander *et al.* (1993)
		80 mg/kg IP	↑	Proliferation – LPS (in fish previously immunized with KLH)	
Rainbow trout	Humoral	TCDD	–	Antibody tire – sheep red blood cells (T-D)	Spitsbergen *et al.* (1986)
		0.1, 1, 10µ/kg IP	–	AFC – sheep red blood cells (T-D)	
Rainbow trout	Humoral	TCDD	↓	Proliferation – pokeweed mitogen	Spitsbergen *et al.* (1986)
		10 µ/kg IP	–		
Channel catfish	Humoral	PCB 126			
		0.01 mg/kg IP	↑	AFC – *Edwardsiella ictaluri* (T-D)	Rice and Schlenk (1995)
		0.1 and 1 mg/kg IP	–		

continued

Table 9.1. *continued*.

Species	Response	Chemical (dose and route)	Effect	End point – antigen	Reference
Rainbow trout	Cellular	TCDD			Spitsbergen *et al.* (1986)
		0.1, 1, 10 µg/kg IP	–	Proliferation – concanavalin A	
Rainbow trout	Cellular	Chlophen ® A 50	–	Proliferation – PHA	Thuvander *et al.* (1993)
		80 mg/kg IP	↑	Proliferation – PHA (in fish previously immunized with KLH)	
Channel catfish	Non-specific	PCB 126			Rice and Schlenk (1995)
		0.1 and 1 mg/kg IP	↓	Oxidative burst	
Rainbow trout	Non-specific	TCDD			
		10 µg/kg IP	–	Phagocytosis	Spitsbergen *et al.* (1986)
Channel catfish	Non-specific	PCB 126			
		1 mg/kg IP	↓	NCC activity	Rice and Schlenk (1995)
Rainbow trout	Non-specific	Aroclor ® 1254 3–300 mg/kg diet	–	NCC activity	Cleland and Sonstegard (1987)

(–)No satistically significant difference between chemically treated and non-treated fish; (↓) significant decrease in response.

Table 9.2. Pathology of lymphoid tissues from fish exposed to halogenated aromatic hydrocarbons.

Species	Chemical (dose and route)	Tissue – pathology	Reference
Rainbow trout	TCDD 0.6 and 3.06 μg/kg IP	Spleen - lymphoid depletion and hyperaemia (congestion of erythrocytes)	van der Weiden *et al.* (1992)
Rainbow trout	TCDD 1μg/kg IP 10 μg/kg IP	No lesions in thymus, spleen or kidney Thymus – multiple invaginations, lymphoid-depleted cortex Spleen – lymphoid depletion Kidney – depletion of lymphomyloid elements	Spitsbergen *et al.* (1988a)
Yellow perch	TCDD 5 μg/kg IP 25 and 125 μg/kg IP	Spleen – mild to moderate lymphoid depletion Spleen – severe lymphoid depletion Thymus – thymic involution Kidney – moderate depletion of lymphoid and haematopoietic elements	Spitsbergen *et al.* (1988b)
Rainbow trout	Aroclor ® 1254 10 and 100 mg/kg diet	Spleen – reduced amount of white pulp (lymphoid elements)	Nestel and Budd (1975)
Rainbow trout	Aroclor ® 1254 100 mg/kg diet	Spleen – reduced amount of white pulp and hyperaemia	Hendricks *et al.* (1977)
Rainbow trout	Aroclor ® 1254 50 and 500 mg/kg diet	Spleen – moderate to moderately severe lymphoid depletion	Spitsbergen *et al.* (1988c)
Rainbow trout	Clophen ® A50 500 mg/kg diet	Fin erosion but no lesions in spleen, head-kidney or thymus	Thuvander and Carlstein (1991)
Rainbow trout	Clophen ® A50 40 mg/kg IP 80 mg/kg IP	No lesions in thymus or spleen Thymus – hypocellularity (depletion of lymphoid tissue) Spleen – hypocellularity	Thuvander *et al.* (1993)
Chinook salmon	Aroclor ® 1254 54 mg/kg IP	No lesions in spleen or kidney	Arkoosh *et al.* (1994a)

which to consider the implications of immunotoxic effects and not to describe in great detail all aspects of the fish immune system.

Phagocytic cells analogous to mammalian monocytes, macrophages and neutrophils (Ellis, 1977; Fänge, 1992) confer non-specific immunity by detecting, engulfing, killing and clearing pathogens. Phagocytes serve both as the first line of defence against infection and as effector cells in the humoral immune response. Natural cytotoxic cells (NCC) detect and lyse transformed target cells and protozoan parasites (Evans and Jaso-Friedmann, 1992). Like their mammalian counterpart, natural killer cells (NK), NCC induce death in target cells by necrotic and apoptotic mechanisms (Greenlee *et al.*, 1991) and are believed to play an important role in the surveillance of tumour cells. Antigen-presenting cells (APC) are phagocytic cells, typically macrophages that internalize and process antigen and present processed antigen to T cells (Vallejo *et al.*, 1992). This results in T-cell activation. The existence of T cells in fish has been based on functional criteria, including responses to mammalian T-cell mitogens (Sizemore *et al.*, 1984; Tillitt *et al.*, 1988), mixed lymphocyte reactions (Kaattari and Holland, 1990) and delayed type hypersensitivity reactions (Stevenson and Raymond, 1990); mammalian T cells are identified by the presence of specific T-cell receptors. Such receptors have yet to be

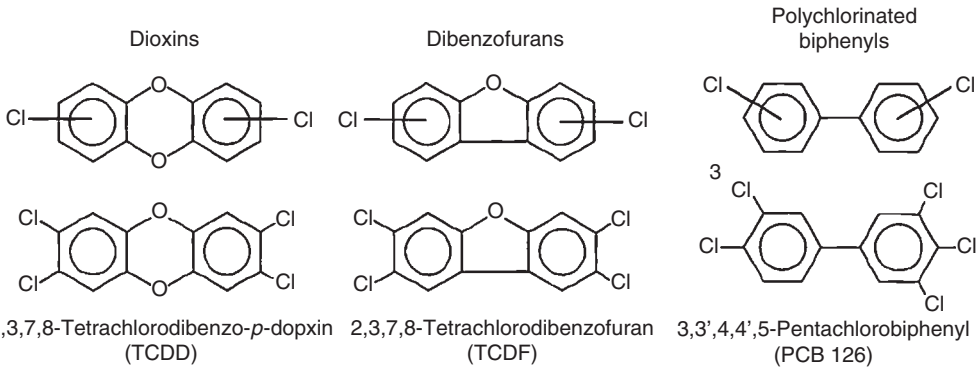


Fig. 9.1. Halogenated aromatic hydrocarbons (HAHs). General structure of dioxins, dibenzofurans and polychlorinated biphenyls, along with representative planar congeners.

characterized for fish lymphocytes (Chilmonczyk, 1992; Manning and Nakanishi, 1997). T cells, along with macrophages, function as accessory cells in the humoral immune response by secreting soluble factors, such as interleukins (ILs), that are required for B-cell activation, proliferation and differentiation (Kaattari, 1992). B lymphocytes express antigen receptors, i.e. membrane immunoglobulins (DeLuca *et al.*, 1983), and are capable of binding free (non-processed) antigen. Some polymeric antigens and mitogens can activate B cells without the participation of T cells and are referred to as thymus-independent or T-I antigens. Antigens that require T-cell involvement to activate B cells are termed thymus-dependent antigens, T-D. B cells activated by T-D antigens proliferate and differentiate into either plasma cells, which produce and secrete antibodies, or memory B cells. Antibodies circulate in the bloodstream and bind to specific antigenic features (epitopes) on pathogens that activated the B cells.

These antibody-coated (opsonized) pathogens are targeted for deletion by phagocytic cells (macrophages) or destroyed by complement-mediated cell lysis (Sakai, 1992). Memory B cells do not participate in the initial or priming exposure to antigen but respond to secondary and subsequent encounters with the specific antigen (Arkoosh and Kaattari, 1991). Secondary humoral responses to antigen occur more rapidly and with greater intensity (more antibody-producing cells and higher antibody titres) than the primary response (Arkoosh *et al.*, 1991).

The major lymphoid tissues in teleost fishes include the anterior kidney (pronephros), thymus and spleen. The anterior kidney is the principal haemopoietic tissue and also functions as a primary lymphoid tissue for B-cell maturation (Kaattari, 1992). The thymus is the primary lymphoid tissue in mammals, where T-cell differentiation and maturation occur. In fish, the thymus is believed to play a similar role, although the precise function is not as well understood (Chilmonczyk, 1992). Mature T and B lymphocytes migrate from primary lymphoid tissues into the bloodstream and concentrate in secondary lymphoid tissues (e.g. spleen). The spleen contains high numbers of lymphocytes and macrophages and it also functions as a filter to trap antigens and allow maximal contact between antigen and immunoreactive cells.

Effects of HAHs on Humoral Immunity

Humoral immune responses, particularly the antibody-forming cell response (AFC), are among the most sensitive indicators of HAH immunotoxicity in higher vertebrates (Davis and Safe, 1988; Vos and Luster, 1989; Kerkvliet and Burleson, 1994). The AFC response is a measure of the number of antibody-forming cells (plasma cells) that are produced in response to immunization

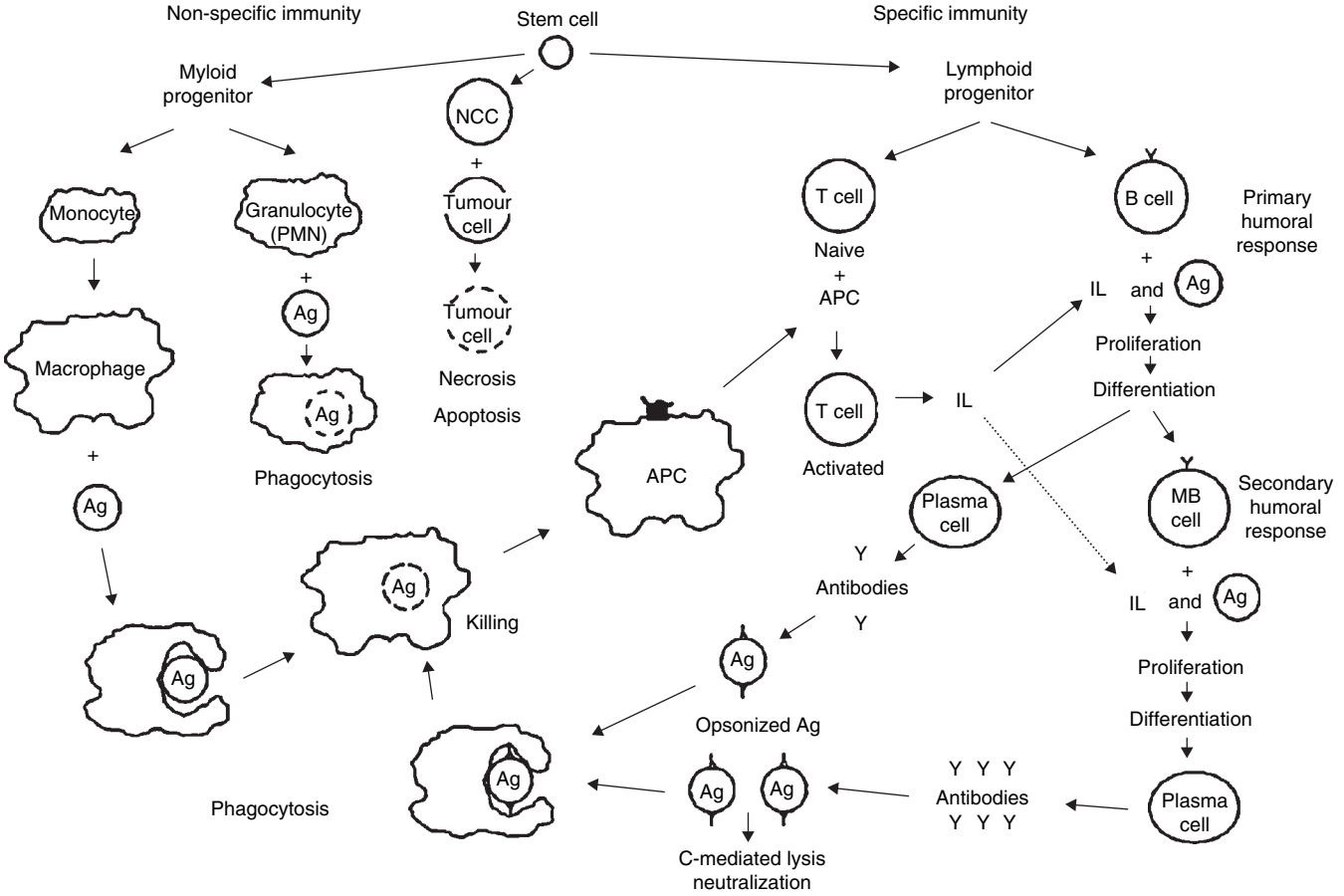


Fig. 9.2. Schematic representation of certain aspects of the teleost immune systems (sources: Ainsworth, 1992; Evans and Jaso-Friedmann, 1992; Kaattari, 1992; Sakai, 1992; Secombes, 1992; Secombes and Fletcher, 1992). Abbreviations: APC (antigen-presenting cell), Ag (antigen), C (complement), IL (interleukins), MB (memory B cell), NCC (natural cytotoxic cell), PMN (polymorphonuclear granulocytes, also referred to as neutrophils).

with antigen and therefore represents an integrated measure of B-cell and accessory cell (macrophage and T-cell) function. The degree to which HAHs affect humoral responses appears to be influenced by many factors, which include fish species, type of antigen (T-D or T-I) and mode of immunization (*in vivo* or *in vitro*).

Primary humoral responses to T-I antigens are more affected by HAHs than primary responses to T-D antigens (Table 9.1). In rainbow trout, *Oncorhynchus mykiss*, PCB treatment (500 mg Clophen® A50/kg diet) significantly reduced the humoral response (antibody titre) to *Vibrio anguillarum* O antigen, a T-I antigen (Thuvander and Carlstein, 1991); whereas, humoral responses in trout to T-D antigens were not affected by PCBs (Cleland *et al.*, 1988a; Thuvander *et al.*, 1993) or TCDD (Spitsbergen *et al.*, 1986). Similarly, the primary *in vitro* AFC response to a T-I antigen (TNP-LPS), but not a T-D antigen (TNP-KLH), was depressed in juvenile chinook salmon receiving a single dose of Aroclor® 1254 (54 mg/kg; Arkoosh *et al.*, 1994). Low doses of PCB 126 (0.01 mg/kg) actually enhanced the AFC response in channel catfish, *Ictalurus punctatus*, to a T-D antigen; yet the response was not significantly affected by higher doses (0.1 and 1 mg/kg; Rice and Schlenk, 1995). The differential effect of HAHs on humoral responses to T-D and T-I antigens in fish may reflect differences in the sensitivity of lymphocyte subpopulations. A discussion of the cellular targets of HAH-induced immunotoxicity is in a later section.

The effect of HAHs on B-cell-mediated immunity in chinook salmon indicates that secondary or amnestic responses may be more sensitive than the primary response. The primary *in vitro* AFC response of juvenile salmon to TNP-KLH (a T-D antigen) was not affected by PCB treatment (54 mg Aroclor® 1254/kg); however, the secondary response was reduced by more than 90% compared with untreated controls (Arkoosh *et al.*, 1994). Because this effect occurred at a dose that was less than half of the ED_{50} (118 mg Aroclor® 1254/kg) for HAH-sensitive mice (C57BL/6; Davis and Safe, 1989), it would appear that chinook salmon is one of the more sensitive species in terms of PCB-induced immunosuppression.

Effects of HAHs on Non-specific and Cellular Immunity

Although relatively few studies on the immunotoxicity of HAHs included non-specific and cellular immunity, there is evidence that suggests species-specific differences in the sensitivity to these compounds (Table 9.1). The phagocytic activity of peritoneal macrophages is a measure of non-specific immunity and this was not affected in rainbow trout treated with a lethal dose of TCDD (10 µg/kg; Spitsbergen *et al.*, 1986). However, the oxidative burst activity in stimulated phagocytes, another indicator of immune competence, was significantly reduced in channel catfish treated with sublethal amounts of PCB 126 (0.1–1.0 mg/kg; Rice and Schlenk, 1995). In the same study, the activity of natural cytotoxic cells (NCC) was also suppressed in PCB 126-exposed catfish (1.0 mg/kg). In contrast, NCC activity was not inhibited in rainbow trout receiving prolonged dietary exposure to Aroclor® 1254 (3–300 mg/kg; Cleland and Sonstegard, 1987). Although these studies examined the effects of different HAHs, it would appear that the non-specific and cellular immune responses in rainbow trout are more resistant to HAHs compared with channel catfish.

The proliferative response of lymphocytes to T-cell mitogens is another measure of cellular immunity. Neither TCDD (Spitsbergen *et al.*, 1986) nor Clophen® A50 (Thuvander *et al.*, 1993) significantly affect the response of rainbow trout lymphocytes to T-cell mitogens. However, in rainbow trout previously immunized with KLH, the responses to both phytohaemagglutinin (PHA; a mammalian T-cell mitogen) and lipopolysaccharide (LPS; a mammalian B-cell mitogen) were significantly enhanced following exposure to Clophen® A50 (80 mg/kg; Thuvander *et al.*, 1993). These results suggest that HAHs differentially

affect lymphocyte activity and it depends on the immune status of the fish prior to chemical exposure. Enhanced mitogen responsiveness was also observed by Faisal *et al.* (1991a) in contaminant-exposed spot, *Leiostomus xanthurus*. The authors suggested that greater LPS responsiveness of spot leucocytes may have been due to contaminant-induced inhibition of suppressor T-cell activity. PCBs have been shown to decrease T suppressor activity of murine leucocytes (Kerkvliet and Baecher-Steppan, 1988). Lymphocytes with T suppressor activity are believed to participate in the regulation of immune functions in fish (Kaattari *et al.*, 1986); however, the role of suppressor T cells in mediating HAH-induced immunomodulation has not been fully explored.

Pathology of Lymphoid Tissues

Thymic involution, or reduction in size and cellularity of the thymus, is an indication of TCDD toxicity in mammals (Vos and Luster, 1989). TCDD-induced lesions in the lymphoid tissues of fish have been detected but they usually occur at lethal or near-lethal doses (Table 9.2). Thymic lesions, characterized by multiple invaginations of the thymic epithelium extending into a lymphoid-depleted cortex, were described by Spitsbergen *et al.* (1988a) in rainbow trout receiving a lethal dose of TCDD (10 µg TCDD/kg; the 80-day LD_{50}). These fish also exhibited splenic lymphoid depletion and depletion of lymphomyloid elements in the pronephros and mid-kidney. No lesions were found in trout dosed with sublethal amounts of TCDD. Splenic lymphoid depletion was detected by van der Weiden *et al.* (1992) in rainbow trout dosed with lower levels of TCDD (0.6 and 3.06 µg TCDD/kg). These doses were near or below the lethal threshold (20% mortality at 3.06 µg TCDD/kg) and in the range where moderate hepatic EROD activity (EC_{50} 0.79 µg TCDD/kg) was induced. Differences in the sensitivity of various rainbow trout strains to TCDD have been reported for other toxicological end points (early life stage mortality; Walker and Peterson, 1991) and could have contributed to the differences in sensitivity. Percid species are also sensitive to TCDD. Mild to moderate splenic lymphoid depletion in yellow perch, *Perca flavescens*, occurred at lower doses of TCDD (5 µg/kg) than thymic involution and pronephric lymphoid depletion (>25 µg TCDD/kg; Spitsbergen *et al.*, 1988b); however, these lesions were not detected at doses below the 80-day LD_{50} (3 µg TCDD/kg).

In studies in which fish were exposed to PCBs, lesions in thymic and/or splenic tissues were not always observed. Splenic lesions were found in rainbow trout exposed to dietary levels of PCBs ranging from 10 to 500 mg Aroclor® 1254/kg (Nestel and Budd, 1975; Hendricks *et al.*, 1977; Spitsbergen *et al.*, 1988c). These levels were not reported to be lethal over the course of these studies (75 days to 12 months). Thymic and splenic hypocellularity were noted in rainbow trout injected with a sublethal dose (80 mg/kg) of Clophen® A50 (Thuvander *et al.*, 1993). However, no lesions were detected in rainbow trout fed 500 mg Clophen® A50/kg for 10 weeks, although significant effects on humoral immunity were observed (Thuvander and Carlstein, 1991). Similarly, humoral immune responses were suppressed in chinook salmon injected with 54 mg Aroclor® 1254/kg, but no lesions in lymphoid tissues were detected (Arkoosh *et al.*, 1994). Thus, lesions in lymphoid tissues are not always associated with HAH exposure or HAH-induced effects on immune functions.

Effects on Disease Resistance

The ultimate manifestation of immunotoxicity is the ability of a toxicant to increase disease susceptibility. However, relatively little is known about the effects of HAHs on disease resistance in teleost fishes, other than in rainbow trout. In this species, disease resistance has not been compromised by exposure to HAHs. Neither median time to death (MTD) nor cumulative mortality in

rainbow trout challenged with infectious haemopoietic necrosis virus (IHNV) was affected by exposure to TCDD (0.01–1 µg/kg body weight) or PCB (5–500 mg Aroclor® 1254/kg diet; Spitsbergen *et al.*, 1988c). However, lesions characteristic of IHNV-induced disease were more severe in fish treated with PCBs or TCDD, which indicates that HAHs may enhance progression of the disease without hastening mortality. Similarly, MTD in rainbow trout challenged with *Yersinia ruckeri* was not shortened following 90-day waterborne exposure to PCBs (0.23–2.9 µg Aroclor® 1254:1260/l; Mayer *et al.*, 1985). In addition, resistance of rainbow trout to *V. anguillarum* was not compromised in fish fed HAH-contaminated diets consisting of Pacific or Great Lakes coho salmon (0.02–2.3 µg PCB/g; Cleland *et al.*, 1988b). These findings are consistent with the relative ineffectiveness of HAHs at altering humoral and cellular immunity in this species. However, impaired disease resistance associated with HAH exposure has been reported for other fish species. Immunization against *Aeromonas hydrophila* was ineffective at protecting PCB-treated (70 mg Aroclor® 1232/kg) channel catfish from a challenge with the virulent bacterium (Jones *et al.*, 1979). More recently, Arkoosh *et al.* (1994) reported that juvenile chinook salmon retrieved from an urban estuary contaminated with PCBs and polycyclic aromatic hydrocarbons (PAHs) suffered higher mortality following exposure to *V. anguillarum* than salmon from a non-contaminated estuary or salmon held in a hatchery. The humoral immune response was depressed in salmon from the same contaminated estuary (Arkoosh *et al.*, 1991).

Field Observations

Establishing cause–effect relationships between a suspected chemical agent and effects observed in wild fish populations (i.e. epizootiology) can be complicated by uncontrollable factors that may potentiate, mask or independently induce the effect(s). Nevertheless, detection of strong associations between chemical contaminants and biological effects can strengthen the argument for causality when the same effects have been demonstrated in controlled laboratory studies.

Altered immune functions have been detected in feral fish from field locations known to be contaminated with HAHs and other organic and inorganic contaminants. Carlson and Bodammer (1994) found that humoral immunity was compromised in winter flounder, *Pleuronectes americanus*, inhabiting an area of Long Island Sound (Morris Cove – New Haven Harbor) that was contaminated with PCBs, PAHs and heavy metals. The authors measured the *in vitro* AFC response of splenic lymphocytes and observed that the response to both T-I (TNP-LPS) and T-D (TNP-KLH) antigens in fish from the Morris Cove site was about 50% lower than the response in fish from a less contaminated reference site. Humoral immunity was also depressed in juvenile chinook salmon that were collected from an HAH–PAH-contaminated urban estuary in Puget Sound (Arkoosh *et al.*, 1991). Although no effects were observed in the primary response, the secondary *in vitro* AFC response of anterior kidney leucocytes in salmon from the contaminated urban estuary was significantly less than the response in hatchery salmon or in salmon collected from a non-urban estuary. Several reports have also documented altered immune functions in fish from sections of the Elizabeth River (Virginia) that are heavily contaminated, primarily with PAHs but also with HAHs (Huggett *et al.*, 1992). The immunological disorders in fish from that system include diminished natural cytotoxic cell activity (Faisal *et al.*, 1991b), reduced phagocytic and chemotactic activity of kidney macrophages (Weeks *et al.*, 1990) and altered responsiveness of pronephric lymphocytes to mitogenic stimulation (Faisal *et al.*, 1991a). The abundance of macrophage aggregates in wild fish has been positively correlated with concentrations of HAHs and other contaminants in bottom sediments (Blazer *et al.*, 1994). Macrophage aggregates are accumulations of pigmented

macrophages in the spleen, kidney and liver with normal physiological and immunological functions (Wolke, 1992). Changes in abundance of macrophage aggregates may be due to contaminant-induced stress. Establishing causal relations between immunological disorders and environmental exposure to HAHs requires an understanding of the mechanisms by which HAHs modulate the immune system.

Mechanisms of Immunomodulation

Many of the pleiotropic effects attributed to HAHs are mediated by a process that requires initial binding of ligand to the AhR (Fig. 9.3). Support for the essential role of AhR-ligand binding is based primarily on two lines of evidence: (i) quantitative structure–activity relationships between AhR binding affinity and toxic potency; and (ii) the differential sensitivity of mouse strains possessing alleles encoding high- and low-affinity AhR. TCDD is the prototypical AhR agonist. The AhR binding affinity of other HAHs is greatest for planar congeners that are structurally most similar to TCDD (Poland *et al.,* 1976; Safe *et al.*, 1986). Toxic responses (weight loss, thymic atrophy and immunomodulation) and biochemical responses (enzyme induction) to HAHs are correlated with AhR binding affinity (Poland *et al.*, 1976; Safe, 1987; Davis and Safe, 1988; Kerkvliet *et al.*, 1990a). Thus, TCDD-like toxicity is observed with HAH congeners that bind with high affinity to the AhR. Similarly, mouse strains possessing the AhR allele that expresses a receptor with high TCDD binding affinity are much more sensitive to biologically active HAHs than mouse strains that express receptor with low binding affinity (Silkworth and Gaberstein 1982; Vecchi *et al.*, 1983; Tucker *et al.*, 1986; Birnbaum *et al.*, 1990; Kerkvliet *et al.*, 1990b). The TCDD binding affinity of AhR in responsive mouse strains (C57BL/6J

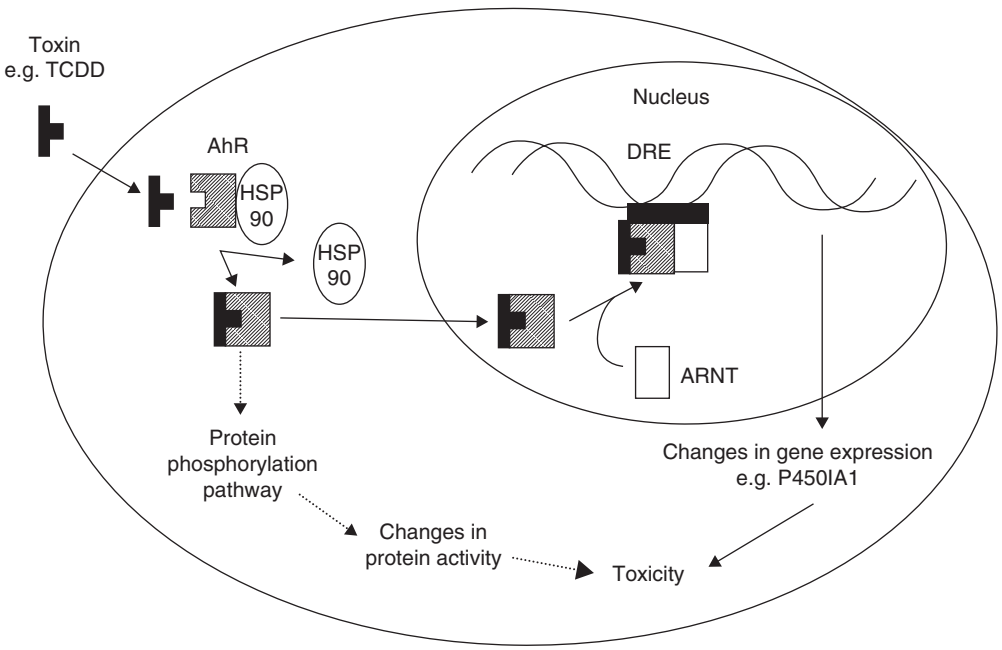


Fig. 9.3. Proposed mechanism for Ah-receptor-mediated toxins. Modified from Richter, 1995 (sources: Whitlock, 1993; Matsumura, 1994).

mice) is tenfold greater than in DBA/2J mice, a low responsive strain (Okey *et al.*, 1989). Binding of HAHs to the AhR is a prerequisite for many physiological and biochemical effects.

The most well-studied of the TCDD-related effects is the induction of cytochrome P450IA1 (a mixed-function oxygenase), which is encoded by the CYPIA1 gene (Fig. 9.3). P450IA1 induction requires initial binding of ligand (TCDD or other active HAH congeners) to the AhR, followed by a transformation of the receptor and translocation of the ligated AhR to the nucleus and binding with ARNT, the aryl hydrocarbon nuclear translocator protein (Nebert and Jones, 1989; Whitlock, 1990; Hankinson, 1995). In the nucleus, the AhR–ligand heterodimer binds to dioxin-responsive enhancer (DRE) regions in the 5′ flanking region of the CYPIA1 gene. Binding of the AhR–ligand complex to DREs enhances transcription of the downstream gene, CYPIA1. Thus, the DRE-binding form of the AhR–ligand complex functions as a transcription factor for CYPIA1, resulting in elevated CYPIA1 transcription and increased levels of the P450IA1 protein.

Induction of detoxification enzymes such as P450IA1 is an adaptive response and not necessarily a measure of toxicity. Whether TCDD acts through this same mechanism to induce toxic responses has yet to be demonstrated unequivocally. However, CYPIA1 is not the only gene that is responsive to the AhR. Sutter and Greenlee (1992) have classified a number of genes that belong to the Ah gene battery. Members of this family include growth factors (interleukin-1 and transforming growth factor-α) and intracellular proteins involved in signal transduction (phospholipase A2, protein kinase C and tyrosine kinases). It is possible that some of the TCDD-related toxic effects may involve direct interactions with DREs that regulate the transcription of growth factors or other regulators of cellular activity. There is also evidence that the AhR–ligand complex can modulate the phosphorylation of cytosolic proteins that are involved in signal transduction pathways (Puga *et al.*, 1992; Matsumura, 1994). Such alterations could affect the responsiveness of cells to extracellular stimuli. Recent studies by Masten and Shiverick (1995) suggest that the suppressive effect of TCDD on B-lymphocyte activation and antibody production may involve a direct effect of the TCDD–AhR complex on gene expression. CD19 is a membrane receptor expressed on the surface of mammalian B lymphocytes and participates in B-cell activation and differentiation (Kehrl *et al.*, 1994; Tedder *et al.*, 1994). Treatment of a human B-lymphocyte cell line (IM-9) with TCDD resulted in a 67% decrease in CD19 mRNA, indicating that TCDD may affect CD19 gene expression. The promoter region for the CD19 gene contains a binding site for BSAP, the B-cell lineage-specific activator protein (Kozmik *et al.*, 1992). BSAP regulates CD19 gene expression and is believed to play a role in early neurological development as well (Urbanek *et al.*, 1994). The DNA binding site for BSAP contains a five-base sequence identical to the DRE consensus sequence. These results suggest that binding sites for the AhR–ligand complex exist in regulatory regions for genes that modulated B-cell activation and differentiation. In the case of CD19, the AhR–ligand complex may compete with the endogenous ligand (BSAP) for binding to the BSAP binding site, resulting in reduced CD19 transcription. Fewer CD19 transcripts may result in reduced expression of CD19 on the cell surface and a diminished capacity to bind and respond to extracellular stimulation. Thus, the DNA binding activity of the TCDD–AhR complex may not only act to 'turn genes on' but may also interfere or compete with other transcription factors, thereby reducing gene expression and altering cellular functions. Despite the substantial body of evidence supporting AhR involvement in numerous HAH-induced effects, there are some notable exceptions, which indicate that HAHs may act through other mechanisms. One particular dioxin congener that lacks AhR binding affinity, 2,7-dichlorodibenzo-*p*-dioxin (Poland *et al.*, 1976), suppresses the AFC response of mouse splenocytes both *in vivo* (Holsapple *et al.*, 1986a) and *in vitro* (Holsapple *et al.*, 1986b). Unlike TCDD, the

immunosuppressive effects of 2,7-dichlorodibenzo-*p*-dioxin are not accompanied by elevated levels of hepatic P4501A1. Other dichlorinated dioxin congeners that have low AhR binding affinity, such as 2,8-dichlorodibenzo-*p*-dioxin, do not suppress the AFC response (Tucker *et al.*, 1986). Thus, 2,7-dichlorodibenzo-*p*-dioxin appears to act through a unique mechanism that does not require AhR binding in order to suppress B-cell immunity.

Results from studies with high and low AhR-responsive mouse strains also suggest that some immunosuppressive effects of HAHs may be mediated by AhR-independent mechanisms. As previously mentioned, the immunosuppressive effects of HAHs have been shown to segregate with the AhR alleles. However, Morris and coworkers (1992) have demonstrated that the exposure regime can greatly influence the responsiveness of low AhR-responsive mice. DBA/2 mice that received subchronic doses of TCDD exhibited a tenfold enhancement in humoral immune suppression compared with DBA/2 mice that received the same cumulative dose of TCDD but in an acute exposure. In addition, the severity of immunosuppression in subchronically exposed DBA/2 mice was comparable to the suppression observed in B6C3F1 (AhR responsive) mice. These findings are supported by results from *in vitro* exposures in which TCDD was equally effective at suppressing the AFC response in splenocytes from both high and low AhR-responsive mouse strains (Holsapple *et al.*, 1986b). The mechanism by which HAHs induce immunotoxic effects, independent of the AhR, is believed to involve modulation of intracellular Ca^{2+} (Holsapple *et al.*, 1991a,b). Taken together, these findings indicate that several factors can modulate the immunosuppressive activity of HAHs and that AhR involvement may be critical for many, but not all, toxic responses.

The role of the AhR in HAH-induced immunodepression in fish is not well understood. Appreciable amounts of AhR have only recently been detected in fish cells (20 fmol/mg protein; Lorenzen and Okey, 1990). However, cytochrome P450IA1 induction has been measured in fish liver, kidney and gill (Miller *et al.*, 1989; Goksoyr and Förlin, 1992). Structure–activity relationships in fish for P450IA1 induction (Janz and Metcalfe, 1991; Newsted *et al.*, 1995) and early life stage mortality (Walker and Peterson, 1991) suggest that these effects are mediated through the AhR. Although AhR agonists have been shown to affect various immune responses in fish, as discussed previously, there is insufficient information at present to determine whether these effects are dependent on AhR-mediated processes.

Several approaches have been used to identify cellular targets in HAH-induced immunotoxicity. Results from *in vitro* and *ex vivo* recombination studies with inbred mice indicate that suppression of the AFC response by TCDD is due to an alteration in the function of B cells, and not T cells or antigen-presenting cells (Dooley and Holsapple, 1988). TCDD has been shown to directly affect B-lymphocyte differentiation under *in vitro* conditions (Tucker *et al.*, 1986; Luster *et al.*, 1988). However, T cells appear to be more sensitive than B cells when the effects of dioxins on the AFC response are tested *in vivo* (Kerkvliet and Brauner, 1987). This conclusion is based on the finding that mice immunized with T-D antigens are more sensitive to the suppressive effects of dioxin (1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin; HpCDD) than mice immunized with T-I antigens. Because the AFC response to T-D antigens requires greater T-cell involvement, the logical explanation for the antigen-dependent sensitivity to HpCDD is impaired T-cell function. It is not clear why differences in dosing and immunization schemes would result in differential sensitivity of B and T cells, although Kerkvliet and Burleson (1994) suggested that dioxins might affect activated T cells *in vivo*, through indirect mechanisms. Indirect effects are known. Depletion of thymocytes associated with TCDD-induced thymic atrophy is believed to occur indirectly through cell–cell contact with TCDD-affected thymic epithelial cells (Greenlee *et al.*, 1985).

In fish, it seems B cells are a target of HAH-induced depression of the primary

AFC response, because significant effects have been demonstrated with T-I antigens. Surprisingly, responses to T-D antigens are less affected. Perhaps stimulation provided by T lymphocytes in some way protects fish B cells from the modulatory effects of HAHs. If this is so, then the activation of naive T cells would have to be less affected by HAHs. Lower T-cell sensitivity may be inferred from the study by Spitsbergen *et al.* (1986). TCDD treatment depressed the proliferative response of rainbow trout splenocytes to pokeweed mitogen, a stimulator of B and T lymphocytes, but did not significantly affect the response to Con A (a mammalian T-cell mitogen).

The heightened sensitivity of the secondary AFC response to T-D antigens observed in PCB-treated chinook salmon indicates that T-cell-mediated events may be affected in the memory response. Arkoosh *et al.* (1994) suggest that if fish have a requirement for memory T cells similar to that of mammals then PCBs may affect the transition of naive T cells to memory T cells. Such an effect would reduce the pool of memory cells available to participate in the secondary AFC response. Further progress in identifying the mechanisms of HAH immunotoxicity will undoubtedly require both *in vivo* and *in vitro* approaches, given the complexity of immune responses and the multiplicity of HAH-associated effects.

Conclusions

HAHs can disrupt normal immune functions in fish, but some species are more severely affected than others. For example, rainbow trout, one of the more thoroughly studied species, seems to be less sensitive than chinook salmon or channel catfish. Humoral immunity, particularly the secondary AFC response, is one of the more sensitive indicators of HAH immunotoxicity. Non-specific and cell-mediated responses have not been as thoroughly investigated, although some effects have been reported. Histological lesions in lymphoid tissues, similar to those described in mammals, have been observed in HAH-treated fish, but the incidence and severity of these lesions has not always coincided with impaired immune function. Immunodepression has been reported in wild fish inhabiting areas contaminated with HAHs and other organic and inorganic pollutants. However, a better understanding of the mechanisms underlying HAH-induced immunomodulation and of the sensitivity of fish species in aquatic communities will be required to assess the risk posed by environmental exposure to HAHs more accurately.

Future Considerations

One of the major limitations in identifying sensitive immunological end points of HAH immunotoxicity has been the fish-to-fish variability often encountered in measuring immune responses. In some studies the coefficient of variation (a measure of within-group variability) has far exceeded 50% (Spitsbergen *et al.*, 1986; Thuvander *et al.*, 1993). This tremendous variation increases the probability of type II error (i.e. accepting the null hypothesis when in fact there were real differences). Mammalian immunotoxicologists have the advantage of working with inbred and syngeneic strains of animals that respond more consistently. This permits greater sensitivity in detecting subtle differences. Inbred fish strains are being developed (Komen *et al.*, 1990), and this will improve the sensitivity of these studies. Alternatively, *in vitro* techniques using tissue sections (Anderson, 1992) or primary cell cultures (Noguchi *et al.*, 1994, 1996) from an individual fish will allow the effects to be measured in genetically identical cell populations. *In vitro* approaches are valuable for studying mechanisms of action and assessing the intrinsic sensitivity of individual fish, and to help identify factors that may account for variability in immune responses between fish.

HAHs and other contaminants represent only one of the many environmental factors that may affect the immune status of wild fish. Identification of HAH-specific immunological perturbations (perhaps effects on the secondary AFC response) may help to distinguish chemical-induced effects from other contributing factors, such as nutrition (Blazer, 1992), temperature (Clem *et al*., 1991) or season (Zapata *et al*., 1992). Currently, it is necessary to employ a battery of immunological and other tests (e.g. enzyme induction) to generate a profile of immunomodulation that characterizes a chemical aetiology.

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10 Disorders of the Cardiovascular and Respiratory Systems

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Introduction

Fish are in intimate contact with their environment. This intimacy is maintained in part by the respiratory and cardiovascular systems, which, although distinct from each other, work in a coordinated manner to optimize the transport of gases and ions between the aquatic environment and the tissues.

The gill secondary lamellae of most fish are the primary gas-exchange sites because of their large surface area and exceptionally high level of vascularization. The coordination of water flow and blood flow through the gill optimizes the efficiency of gas transport between blood and water. Through countercurrent flow, oxygen (O_2) is taken up from the environment across the gills and delivered to all tissues of the body, and in exchange, carbon dioxide (CO_2) and ammonia (NH_3) are transported from the tissues of the body and excreted across the gills. However, many fish species, particularly as juveniles, also conduct gas exchanges through the skin, because the skin has a high surface area relative to the gills (Rombough and Ure, 1991).

The large surface area of the gill, its delicate structure and the thin tissue barrier between the water and the fish's blood make fish particularly vulnerable to waterborne agents. Consequently, the gill epithelium is an important site of antigen entry. Under normal conditions, these antigens are neutralized or destroyed in the blood by various components of the natural and adaptive immune systems, or they are transported to various immunologically active sites such as the head kidney or spleen, where they can be processed and destroyed. While the role of the respiratory surface in antigen entry is important to recognize, the main focus of this chapter is on the respiratory function of the gill epithelium and on the ionic exchanges related to CO_2 and NH_3 excretion. The following discussion, therefore, applies to those fish in which the gill epithelium is the main site for gas and ion exchange between body fluids and the water. The discussion is divided into a description of the relatively normal states of the respiratory and cardiovascular systems, and descriptions of those systems under various stressed conditions.

Stressors from the external environment are associated more with pathological conditions of the respiratory system, whereas abnormal conditions inside the body primarily affect the cardiovascular system. Other than pathological conditions that are purely genetic in origin, all stressors ultimately originate from the external environment. For example, some causes of cardiovascular disorders are related to unbalanced diets. At the outset it is noteworthy that basic knowledge

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about many aspects of the respiratory and cardiovascular systems are still lacking, which forces us to speculate on their physiological significance. For instance, we still do not completely understand the functional significance of the secondary circulatory system in fishes. The extent of our coverage of each topic, therefore, reflects, in most part, the amount of knowledge available.

Overview of Normal Systems

Respiratory system

Fish are the most successful vertebrate group in terms of number of species. The wide variability in the respiratory systems of the more than 25,000 species of fish reflects the extensive adaptation of this group of animals to a wide range of environments. The respiratory system described here is one of a water-breathing teleost, such as a salmonid fish, which is perhaps the best-studied family of fishes with respect to respiratory and cardiovascular systems, as well as other physiological systems. The central components of the respiratory system include the water flow over the gill and the blood flow inside the gill epithelium. Water is pumped over the gills in an anterior to posterior direction, creating a flow that is countercurrent to the flow of blood through the secondary lamellae (Fig. 10.1). Countercurrent flows maintain the maximum partial pressure gradients between blood and water for the exchanged gases, as well as maximum concentration gradients for ions, throughout their transit through the gills. This maximizes the passive flux of both gases and ions between the blood and water.

Continuous and rhythmic ventilation of the gills is achieved by synchronous

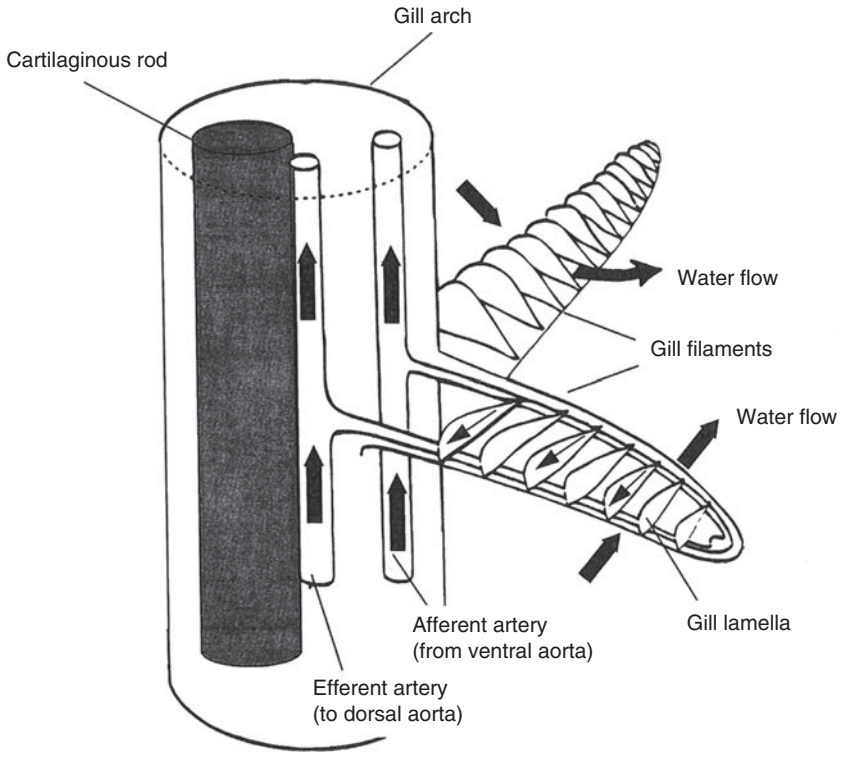


Fig. 10.1. Diagram of a fish gill arch illustrating the pattern of blood and water flows (adapted from Wedemeyer *et al.*, 1976).

activities of buccal and opercular pumps. Water flows from the mouth, over the gills and out of the operculum. The buccal and opercular pumps are driven by skeletal muscles that control the floor of the mouth and opercular covers, respectively. Lowering the buccal floor creates a negative pressure, which 'sucks' water into the mouth. At the same time the opercular cavity is expanded with the opercular covers closed to draw water from the buccal into the opercular cavity and across the gill exchange surface. Closing the mouth, while raising the buccal floor and opening the opercular covers, again drives the water across the gills under positive pressure out of the opercular opening. This cycle is repeated continuously, creating the unidirectional flow through the branchial cavity. While most fish use this rhythmic ventilation, some are ram ventilators; they ventilate the gills by keeping their mouths open and swimming forwards through the water. Salmonid fishes do this at moderate to high swimming velocities. Fish can also orientate into water currents (negative rheotaxis) and benefit from ram ventilation without locomotion. While such alternate modes of ventilation require energy to maintain the opening of the mouth, that energetic cost is probably much lower than the cost of normal rhythmic ventilation.

Although blood is the medium that the cardiovascular system transports throughout the body, it is the haemoglobin in the red blood cells that increases the capacity of the blood to carry O_2. The haematocrit (Hct) of 20–30% in fish increases the oxygen carrying capacity approximately 20-fold compared with the amount of O_2 that could be dissolved in plasma. The number of red blood cells and their haemoglobin content vary considerably among fish species and with the environment in which the fish are found. For instance, ice fish from the Antarctic are unusual in having no haemoglobin. However, they live in a cold environment (higher ambient oxygen content) and have physiological attributes such as a very large blood volume, low metabolic rate and large cardiac output, which allows them to live in that environment. Other Antarctic teleosts have a reduced Hct compared with temperate species, but can release large numbers of stored red blood cells from the spleen when either stressed or during exercise (Gallaugher and Farrell, 1998). This capability of the spleen is diminished in temperate species (Farrell and Steffensen, 2005).

The way in which O_2 binds to haemoglobin is described by an oxygen dissociation curve (Fig. 10.2). The role that the red blood cell plays in oxygen and carbon dioxide transport between tissues and the water via the blood is also shown in Fig. 10.2. As oxygenated blood arrives at tissues, its affinity for haemoglobin is reduced by the higher CO_2 tensions, which originate in the respiring tissue (Fig. 10.3). The carbonic anhydrase-catalysed hydration of CO_2 generates protons, which bind to haemoglobin, resulting in an off-loading of O_2, which then diffuses into tissues. As the deoxygenated venous blood enters the gill lamellae, it begins to bind oxygen in a saturable manner. As the partial pressure gradient drives O_2 into the red blood cell, CO_2 generated from HCO_3^- and H^+ diffuses out of the cell and into the water (Fig. 10.3).

In addition to its respiratory function, the fish gill is also an important site of ammonia excretion. Most of the ammonia that the body generates (through the deamination of amino acids) leaves the fish across the gill and as NH_3 gas (see Wright and Wood, 1985; Heisler, 1989). A carrier-mediated exchange (a NH_4^+/Na^+ exchanger) is also involved in the excretion of ammonia (Cameron and Heisler, 1983; Wright and Wood, 1985) under certain environmental conditions, such as highly alkaline fresh water (Wright and Wood, 1985; Yesaki and Iwama, 1992), where there may be a net inward gradient of NH_3. CO_2 excretion plays an important role in moderating ammonia toxicity through the acidification of the gill surface boundary layer (Randall and Wright 1989; reviewed in Wilkie 2002), as does feeding.

The gill is the primary sense organ for changes in internal and external levels of O_2 and CO_2, and fish will maintain their biological needs for O_2 through a number of cardiorespiratory reflexive behaviours (reviewed

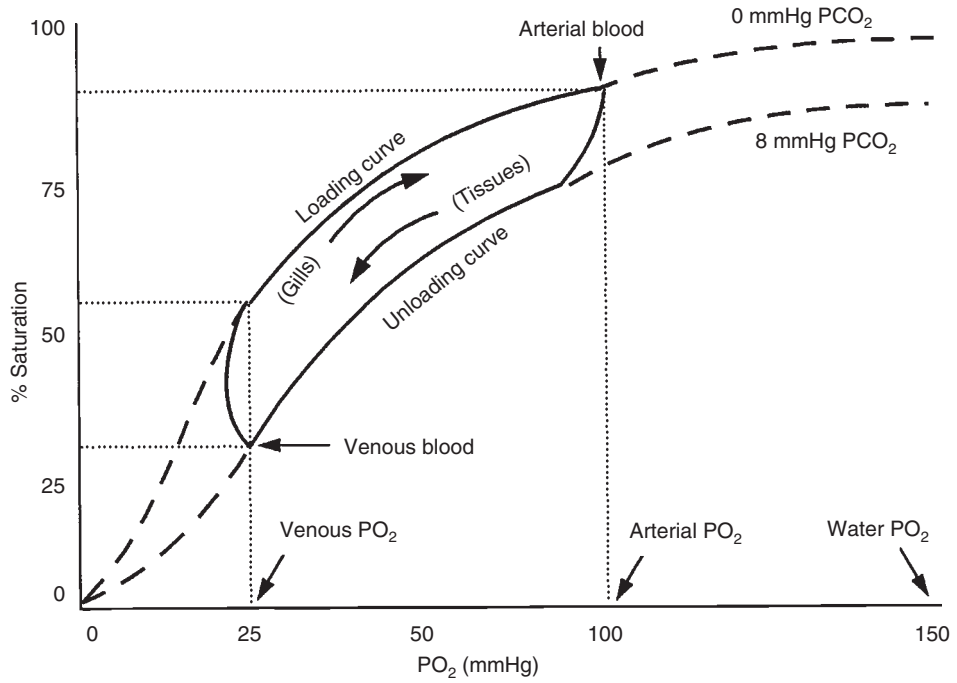


Fig. 10.2. Generalized oxygen dissociation curve for teleost blood (adapted from Eckert and Randall, 1983).

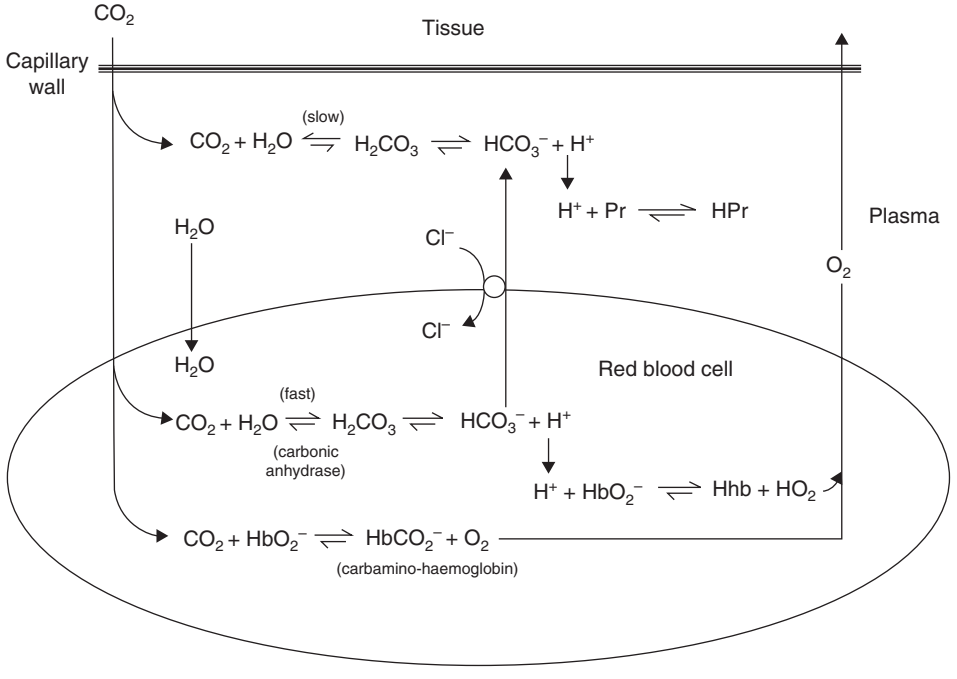


Fig. 10.3. Diagrammatic representation of the oxygen and carbon dioxide flux relationships between the red blood cell and tissue, the haemoglobin binding of oxygen, and the hydration of carbon dioxide (adapted from Eckert and Randall, 1983).

by Perry and Gilmour, 2002). In addition to being the primary organ for respiratory gas exchange, it is also vital for osmoregulatory maintenance and nitrogen excretion. Anything that alters the structure or function of the gill or its associated blood supply can have significant biological consequences in the body.

Gill structure and blood circulation

The teleost gill has four gill arches on each side of its midline and two rows of primary filaments per arch (Figs 10.1 and 10.4a). Elasmobranchs have five to seven paired gill arches. Plate-like secondary lamellae are arranged perpendicularly to the filament, somewhat like rungs of a ladder, along the upper and lower surfaces of each filament. The plate-like secondary lamellae form narrow channels, through which the water flows (Figs 10.1 and 10.5). This interlamellar space is approximately 0.02–0.05 mm wide, 0.20–1.60 mm long and 0.10–0.50 mm high. The width is particularly important, in that one half of that width is the maximum distance for gases and dissolved materials such as ions to diffuse between water and blood. The secondary lamellae consist of thin (around 10 μm) vascular sheets of lamellar capillaries, which occupy most (80%) of the lamellar surface area (Farrell *et al.*, 1980). The remainder of the lamellar surface area is taken up by contractile pillar cells, which keep the blood sheet together and adjust its thickness. A larger-diameter marginal vessel extends around the periphery of each lamella. The lamellar vascular sheet is encased by a very thin (1–10 μm) sheet of epithelial tissue, which acts as the main protective barrier between the blood and the water (see Fig. 10.11c). In addition to providing protection and support for the lamellae, which are the basic functional respiratory units, the epithelium contains a number of important cell types, such as the ionoregulatory cells, that play various roles in the maintenance of homeostasis (reviewed in Wilson and Laurent, 2002). The afferent branchial arteries distribute blood along each gill arch and feed an afferent filamental artery at the base of each gill filament (Fig. 10.5). Each afferent filamental artery, in turn, supplies blood to each of the secondary lamellae. An afferent lamellar arteriole and an efferent lamellar arteriole connect each lamella to the afferent and efferent filamental arteries, respectively. In elasmobranch fishes, a sinus-like corpus cavernosum lies between and connected to the afferent filamental artery and most of the afferent lamellar arterioles. Blood leaves the gills via efferent filamental arteries and efferent branchial arteries, and enters either the primary systemic circulation or the secondary circulation of the gills. For a more detailed review of the vascular anatomy of the fish gill, readers are referred to Olson (2002).

Cardiovascular system

There is great diversity in the organization of the cardiovascular system in fishes. For in-depth descriptions of the fish cardiovascular systems, readers are referred to publications by Olson and Farrell (2006), Olson (2002), Farrell and Jones (1992), Bushnell *et al.* (1992), Steffensen and Lomholt (1992), and Satchell (1991, 1992), as well as to Hughes (1984) and Laurent (1984) for the general anatomy and internal vascular pathways of fish gills. The following is a brief and simplified description of the cardiovascular organization in water-breathing teleost and elasmobranch fishes.

The main (branchial) heart is contained within a pericardial sac and consists of four chambers: a *sinus venosus*, an atrium, a ventricle and either a *bulbus arteriosus* in teleosts or a *conus arteriosus* in elasmobranchs (Fig. 10.4c). Venous blood returning to the heart is first collected by the *sinus venosus* and then pumped sequentially by the atrium and the ventricle into the *conus* or *bulbus* and the main artery of the primary circulation, the ventral aorta. All of the blood pumped from the ventricle (i.e. the entire cardiac output) enters the respiratory (branchial or gill) circulation via four to

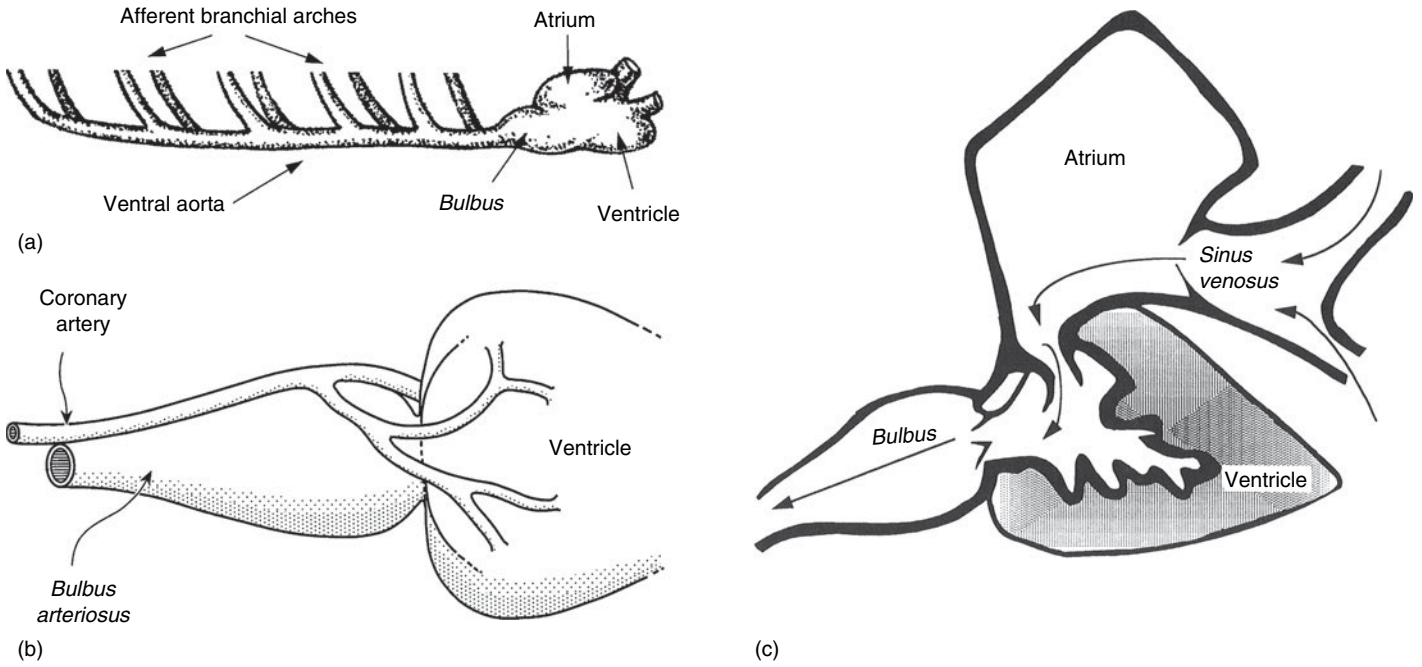


Fig. 10.4. (a) Diagram showing the branching of the four afferent branchial arteries off the ventral aorta in a teleost (adapted from Romer and Parsons, 1986). (b) Representation of the association of the coronary artery to the ventricle and the *bulbus arteriosis*. (c) Diagram of a cross-section of a trout heart.

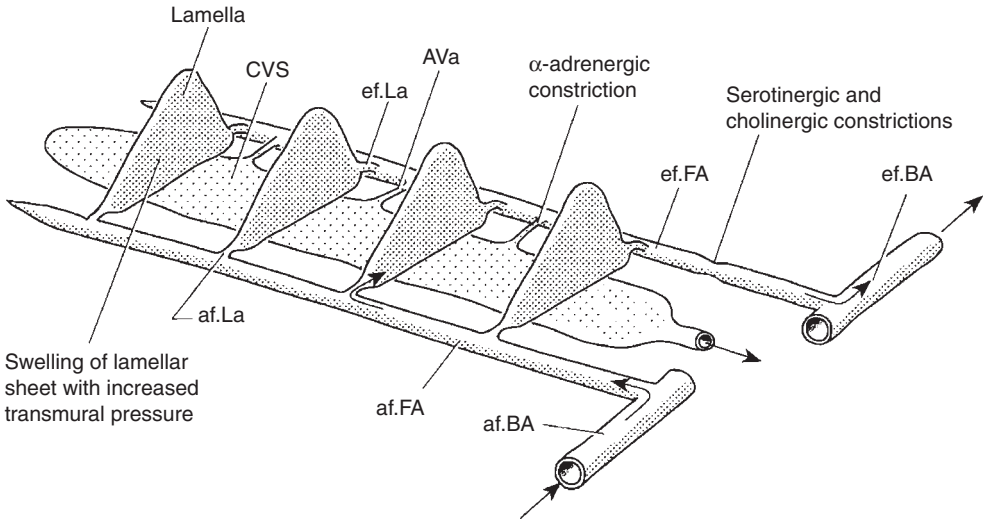


Fig. 10.5. A schematic representation of the major vascular pathways in the gill filament of a teleost fish. Some of the known sites for changes in vascular resistance or dimensions are indicated. (af, afferent; ef, efferent; BA, branchial artery; FA, filament artery; La, lamellar arteriole; AVa, arteriovenous anastomoses; CVS, central venous sinus; lamella, secondary lamella.) From Farrell (1993).

seven bilateral branches from the ventral aorta, the afferent branchial arteries. Each branchial artery serves one gill arch (Fig. 10.1). As blood passes through the respiratory-exchange area of the gills, the secondary lamellae, it loses CO_2 and becomes oxygenated. Oxygenated blood is then collected into efferent arteries for distribution to tissues through the primary and secondary circulations. Fish contrast with other vertebrates in two ways: (i) blood goes directly to the systemic circulation after passing through the respiratory circulation and does not return to the heart to be boosted around the systemic circulation; and (ii) fish are unique in possessing primary and secondary circulations while apparently lacking a lymphatic system.

The branchial heart

The four heart chambers are anatomically distinct, unlike the mammalian heart (Fig. 10.4c). The *sinus venosus* is a thin-walled venous reservoir and is also the site of the pacemaker tissue that initiates the heartbeat. The atrial wall has a mesh-like network of thin, muscular bundles (trabeculae) about 19–35 mm in diameter (Santer, 1985). Contraction of the atrium is thought to be the main means for filling the ventricle (Farrell and Jones, 1992), though this has been challenged recently by Lai *et al.* (1996) and Graham (1997).

The ventricle is the main pressure-generating chamber of the heart and hence has the greatest muscle mass in its walls of all the cardiac chambers (Fig. 10.4). Ventricular mass ranges from 0.05% to 0.4% of body mass among fishes, whereas atrial mass is generally 8–25% of ventricular mass (Farrell and Jones, 1992). Ventricular size, shape, histology and vascular supply all show considerable variability between species (Santer, 1985), reflecting, in part, substantial interspecific differences in both ejected volume (cardiac stroke volume) and pressure generation (ventral aortic pressure) and, in part, the external morphology of the fish itself. Ventral aortic pressure is lowest in elasmobranch fishes and highest in very active teleost fishes (Bushnell *et al.*, 1992).

The ventricle can have two types of muscle (myocardium): (i) *spongiosa*, a sponge-like network of muscular trabeculae, which accounts for the greater proportion of

ventricular mass in almost all fishes; and (ii) *compacta*, an outer, more compact muscle layer enclosing the inner *spongiosa* (Santer, 1985; Tota, 1989; Davie and Farrell, 1991). Most teleosts have only *spongiosa*, which contains no blood capillaries, and therefore venous blood returning from the body tissues and contained in the lumen and intertrabecular spaces of the ventricle (luminal blood) provides the only blood and oxygen supply to these types of hearts (hence the terms venous, lacunary or avascular hearts). All elasmobranch species and about one-quarter of teleost species (typically those that either tolerate environmental hypoxia or are active swimmers) have both *spongiosa* and *compacta*. In most of these teleosts a coronary circulation provides an additional oxygen supply to only the *compacta*, but all elasmobranchs and those teleost species that are very active (e.g. tuna and marlin) have coronary vessels in the *spongiosa* as well (Tota, 1989).

The *bulbus arteriosus* of teleost fishes (Fig. 10.4), an elastic chamber, expands with each heartbeat to dampen the pulsatile flow of blood ejected from the ventricle, thereby creating a more continuous flow of blood in the rest of the circulation (Bushnell *et al.*, 1992). The *conus arteriosus* of elasmobranchs performs a similar function to the *bulbus*, but it contains cardiac muscle, is contractile, and has two to six sets of valves.

Primary systemic circulation

A generalized pattern of the systemic vasculature in teleosts is presented in Fig. 10.6. Efferent branchial arteries unite to form the anterior carotid arteries (supplying the head region) and the posterior dorsal aorta (supplying the tail musculature and viscera). These arteries are the main distribution vessels for the primary systemic circulation. Blood pressure in the dorsal aorta, systemic blood pressure, is around two-thirds of that in the ventral aorta, i.e. about one-third of the blood pressure generated by ventricular contraction is lost to the resistance to blood flow encountered in the gill vessels (Bushnell *et al.*, 1992). The coeliacomesenteric artery(ies) is(are) the major distribution vessel(s) to the viscera (Farrell *et al.*, 2001). The trunk muscle is supplied by segmental lateral arteries. Paired branches from the efferent branchial arteries form the mandibular artery (supplying the pseudobranch and choroid gland) and the hypobranchial artery (supplying some of the pectoral

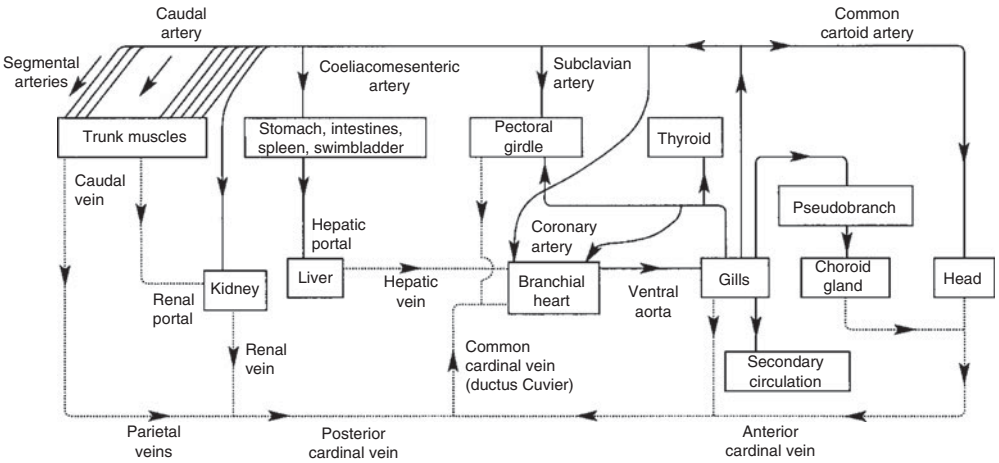


Fig. 10.6. Schematic representations of the primary arterial (solid lines) and venous (broken lines) circulations in a salmonid, as a representative of a teleost fish. Three principle veins draining the head, a singular jugular vein and the paired anterior cardinals, are shown together as the anterior cardinal. From Farrell (1993).

muscles and the cranial (cephalad) coronary circulation). The cranial coronary circulation reaches the ventricle across the surface of the *bulbus* or *conus*. An additional pectoral (caudal) coronary circulation is found in a few fish and arises from the first branch of dorsal aorta, the coracoid artery. Both anatomical origins of the coronary circulation are such that oxygenated blood is delivered to the ventricle directly from the gills and at the highest possible post-branchial blood pressure. The coronary veins drain into the atrial chamber close to the atrio-ventricular region. More thorough descriptions of the coronary circulations in fishes are presented by Tota *et al.* (1983), Tota (1989) and Davie and Farrell (1991).

The return of venous blood from the trunk muscles and gastrointestinal tract passes, respectively, through the kidney (renal portal system) and liver (hepatic portal system) (Fig. 10.6). The major central veins are the anterior jugular vein (draining the head region), the caudal vein (draining the tail) and the hepatic vein (draining the liver). The hepatic vein and anterior jugular veins empty directly into the *sinus venosus* of the branchial heart, whereas the caudal vein and jugular veins first unite to form the paired Cuverian ducts (posterior cardinal veins), which represent the main venous return route to the heart. Venous blood passing through the head kidney can pick up catecholamines released from this tissue under stressful situations. The first organ that these stimulatory hormones reach is the heart.

Blood pressures in veins of fishes are generally low and sometimes sub-ambient. Thus, accessory (caudal) hearts can be found in fish tails, and these aid in the return of venous blood to the branchial heart (see Satchell, 1991, 1992). In addition, venous blood can be aspirated (sucked) toward the branchial heart in certain fishes as a result of cardiac contraction (a *vis-a-fronte* cardiac filling mechanism).

Regulation of cardiac output in fish is achieved by changes in both heart rate and cardiac stroke volume. Both are altered through intrinsic, neural and humoral control mechanisms (Farrell, 1984; Farrell and Jones, 1992; Olson and Farrell 2006). A change in the amount of blood flow reaching a specific tissue can be a result of either a change in cardiac output or a change in blood flow distribution, or some combination. Up to a threefold increase in cardiac output is possible in some active fish. Changes in the distribution of blood flow between the various vascular circuits are brought about through changes in vascular resistance.

Secondary circulation

A unique feature of the circulatory system of fishes is the presence of a secondary circulation. The relationship between the primary and secondary circulations is illustrated in Fig. 10.7. Most investigations of the secondary circulation have focused largely on morphology and it is only recently that physiological investigations yielded some functional knowledge about this system. Distinctions between the primary and secondary circulations and the misconceptions regarding lymphatics and venolymphatics in fishes are well described by Vogel (1985), Satchell (1991), Steffensen and Lomholt (1992) and Olson (1996).

The secondary circulation arises from primary arteries at numerous gill and systemic locations as narrow, convoluted arterial vessels. These connections between the primary and secondary circulations appear to be of high resistance and 'filter out' the majority of the red blood cells. Thus, the secondary circulation is a low-pressure and low-haematocrit system and generally perfuses surface structures that exchange gases directly with the water (gills, scales and skin) and the gut. In addition, because of its large volume (it has been estimated to be between 10 and 50% of the volume of the primary circulatory system (Bushnell *et al.*, 1998; Skov and Steffenson, 2003)) and low blood pressure, the secondary circulation has a circulation time probably of the order of hours rather than minutes. Flow into the secondary circulation is controlled by the blood pressure in the primary arteries and the resistance of the connecting vessels.

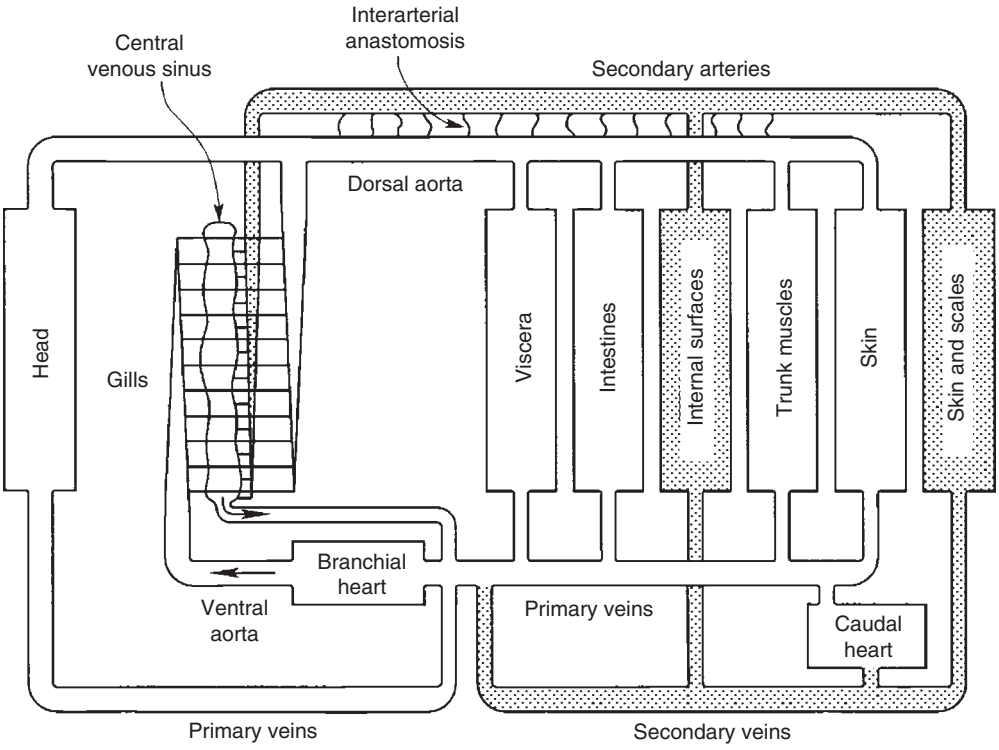


Fig. 10.7. The general distribution pattern of the secondary circulation in teleost fish and its relationship to the primary circulation. From Farrell (1993).

The secondary circulation of the gills is a highly variable and complex network of vessels (see Laurent, 1984) that previously have been incorrectly referred to as lymphatics and veno-lymphatics. A feature common in most fish gills is a central venous sinus (CVS), which lies underneath the lamellae and extends along the filament length (Fig. 10.5). The CVS has narrow arteriolar anastomoses that connect to the efferent filament artery, allowing for a significant and variable diversion of blood from the primary into the secondary circulation within the gill circulation.

Steffensen and Lomholt (1992) have described the secondary circulations to the skin, scales and intestine. Vogel (1985) considered the caudal heart to be part of the secondary circulation of fishes. This structure pumps the venous blood draining from the secondary circulation into the caudal veins of the primary circulation. Beating of the caudal heart is consistently higher (*Anguilla japonica*: 165–230 beats/min, Chan, 1971; *Anguilla* australis *schmidtii*: 90 beats/min, Davie, 1981) than the beating of the branchial heart (Hipkins, 1985). The secondary circulation of the trunk empties into the central veins of the primary circulation.

The Hct in the secondary circulation is about 3.5%, compared with the Hct in the primary circulation, being about 20–25% in rainbow trout at 15 °C (see Ishimatsu *et al.*, 1995). Steffensen and Lomholt (1992) stated that the volume of that ciculation is about 4.9% of body weight, compared with the primary circulation, representing 3.4% of body weight. This large volume must potentially have a significant diluting effect on any substance introduced into the primary circulation. Steffensen and Lomholt (1992), based on two-compartment modelling of the disappearance of labelled proteins from

the primary circulation, estimated flow rate of the entire secondary circulation as only 0.03% of cardiac output. However, 6–8% of cardiac output has been estimated to be shunted through the secondary vessels of the gill, based on studies of cardiac output partitioning in intact animals (see Ishimatsu *et al.*, 1988; Sundin and Nilsson, 1992). Thus, it is likely that there are large regional differences in flow rates within the secondary circulation perfusing different parts of the body. Estimates of pressures in the secondary circulation are generally lacking. Ishimatsu *et al.* (1992) reported values of 1.3–3.8 cm H_2O, and Farrell and Smith (1981) reported values of <10 cm H_2O. There are no reports of pressures in the systemic vessels of the secondary circulation.

There are many possible functions of the secondary circulation (see Ishimatsu *et al.*, 1995). The principal role of the primary circulation, i.e. the internal convection of oxygen, is clearly not shared by the secondary circulation. As stated above, the CVS in the gill filament is a major pool of the secondary circulation. Some possibilities for the functional significance of the CVS in the gill include: a plasma reservoir, a collecting reservoir for interstitial fluid of the gill tissues and a site of hormone degradation. It may also serve as the nutritional vasculature for the gill filamental tissue. Another possibility is that it serves an immune function. In support of this latter possibility, Ahlborn (1992) found higher lysozyme levels in fluid from the lateral cutaneous vessel of rainbow trout, compared to blood drawn from the dorsal aorta in chronically cannulated animals. Furthermore, Ototake *et al.* (1996) speculated that the secondary circulation may play a role in antigen-trapping, as endothelial cells of the secondary circulation were observed to trap experimentally introduced bovine serum albumin in rainbow trout. Due to its proximity to the location of chloride cells, the CVS may also serve in some way in the function of those cells. Several investigations have suggested that there may be an acid–base regulatory role played by the secondary circulation. Studies on intact animals with chronic catheters in the lateral cutaneous vessel (Ishimatsu *et al.*, 1992) and the branchial vein (Iwama *et al.*, 1993), which collects the fluid draining from the CVS, have shown small but detectable contributions of fluid in the secondary circulation to the accumulation of HCO_3^- in the compensation of respiratory acidoses in rainbow trout. It is unclear whether reddening of fish skin and scales is associated with a greater entry of red blood cells into the secondary circulation, giving the otherwise transparent vessel contents a red hue. Physiological investigations into the possible function of the secondary circulation are in their early days, and there are vast opportunities for research in this area.

Non-infectious Diseases

Abnormal cardiac morphology

Cardiac anomalies have been associated with a number of conditions in fish. These range from arteriosclerosis (see below) to cardiac hernia and hypoplasia (see below). In most cases, these abnormalities result in a limiting of maximum cardiac function, which may reduce tolerance to stressors.

The normal shape of the salmonid heart is roughly pyramidal, and there is a positive correlation between ventricular shape and optimum cardiac output and function (Graham and Farrell, 1992; Agnisola and Tota, 1994; Tota and Gattuso, 1996). Domesticated salmonids appear prone to the development of a more rounded ventricle with a misaligned *bulbus arteriosus* (Brocklebank and Raverty, 2002; Poppe *et al.*, 2003). Reported cardiac deformities include hypoplastic or aplastic septum transversum (Brockleback and Raverty, 2002; Poppe *et al.*, 1998), herniation (Brockleback and Raverty, 2002; Poppe *et al.*, 2002), *situs invertus* (up-side-down heart within an intact pericardial sac) (Brockleback and Raverty, 2002), and ventricular hypoplasia with ascites (Poppe and Taksdal, 2000; Brockleback and Raverty, 2002). The causes of these conditions are not known, but it has been suggested that, since no infectious

agents have been found to be associated, they are probably of either hereditary or environmental origin; elevated temperatures during incubation has been suggested as one possible factor in their development (Poppe and Taksdal, 2000). Clarieaux *et al.* (2005) investigated the relationship between abnormal cardiac anatomy and performance by examining swimming performance and cardiac pumping ability. Fish identified as 'poor swimmers' had a 26% lower maximum cardiac output and a 32% lower maximum cardiac power output than did 'good swimmers'. It was found that ventricular morphology in 'poor swimmers' was significantly more rounded than that observed in 'good swimmers'. Evidence has indicated that hatchery-raised salmonid fishes generally have a more rounded ventricle than do wild fish (Poppe *et al.*, 2003; Gamperl and Farrell, 2004). The intuitive implication of this research is that a more rounded ventricle denotes a weaker heart (species differences in ventricular shape also point to this conclusion). Such abnormalities are important because they appear to be associated with increased mortality rates in large fish during potentially stressful situations, such as grading, transportation and immersion treatments. Fish display a significant degree of cardiac plasticity (reviewed in Gamperl and Farrell, 2004) and the factors involved in abnormalities may have implications for enhancement of hatchery practices.

Pericarditis and myocarditis

The thin layer of tissue that covers the outer surfaces of the heart is known as the pericardium. It acts to anchor the heart in place, prevents excessive movement of the heart during changes in body position, lubricates the heart with pericardial fluid as it moves within the pericardium during contraction, protects the heart from infections and tumours that develop in and may spread from adjacent tissues, and may help keep the heart from enlarging. In humans, myocardial and pericardial inflammation is often a result of viral infection but may arise from other physiological conditions, such as kidney failure or as a result of some medications. Frequent cases of idiopathic myocarditis and pericarditis are reported in the mammalian literature.

It is only relatively recently that observations of myocarditis and pericarditis have been noted in farmed fish (Johansen and Poppe, 2002). Their cause, prevalence and significance are, as yet, under investigation.

Coronary arteriosclerosis

Description and prevalence

Robertson *et al.* (1961) first observed arteriosclerotic lesions in and confined to the coronary vessels of mature Pacific salmon. These lesions have since been characterized morphologically and quantitatively in a variety of salmonid species under different conditions (Van Citters and Watson, 1968; Maneche *et al.*, 1973; McKenzie *et al.*, 1978; House *et al.*, 1979; Schmidt and House, 1979; Farrell and Munt, 1983; Eaton *et al.*, 1984; Farrell *et al.*, 1986, 1990a, 1992; Kubasch and Rourke, 1990; Saunders *et al.*, 1992). Some progress has been made towards explaining the aetiology of coronary lesions in fish and, although a brief overview follows, readers are referred to Farrell (2002) for a more detailed review.

The normal histological structure of the fish coronary artery is similar to that of other vertebrate arteries: an external parenchyma surrounds a medial layer of vascular smooth muscle and an internal elastic lamina separates the media from the intima, which normally has a single layer of endothelial cells. Arteriosclerotic lesions of fish coronaries are characterized as intimal proliferations of vascular smooth muscle (VSM) with a disrupted elastic lamina (Figs 10.8a and b). The arterial changes in salmonid lesions are therefore similar to the early stages of the spontaneous arterial lesions found in chickens (Moss and Benditt, 1970; House and Benditt, 1981). Coronary lesions in salmonids consist of multifocal intimal proliferations of VSM (Maneche *et al.*, 1973; Moore *et al.*, 1976; McKenzie *et al.*, 1978; House

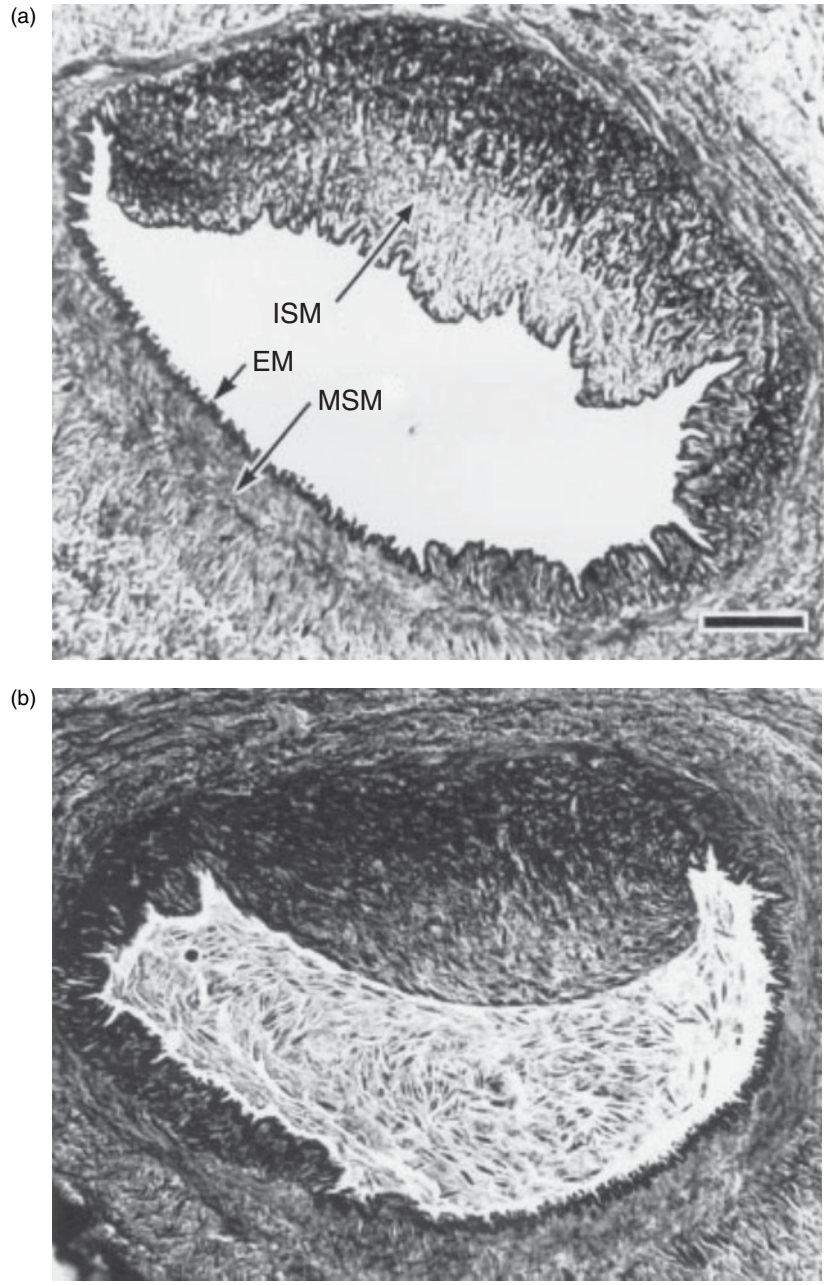


Fig. 10.8. Cross-sections of coronary arteries of mature, migratory (a) Atlantic salmon and (b) steelhead trout. In a normal artery the elastic membrane (EM) demarks the medial smooth muscle (MSM) and lumen of the artery, as shown in the lower left quadrant of panel a. In contrast, the infiltration of intimal vascular smooth muscle (ISM) beyond the elastic membrane, as well as a general disruption and fragmentation of the elastic membrane, characterize a coronary lesion, i.e. the majority of the vessel wall in these two examples. The two examples shown are representatives of lesions that would be scored as 5 in terms of severity by Farrell and co-workers. These severe forms of coronary lesions result in a significant blockage of the vessel lumen. Scale bar = 50 um. Adapted from Saunders *et al*. (1992) and Farrell and Johansen (1992).

and Benditt, 1981). Unlike the medial VSM, intimal VSM is orientated to the long axis of the artery. VSM is the main tissue component of these lesions (House and Benditt, 1981). Collagen and elastin are also present, but significant fatty deposits and calcification are absent. The internal elastic membrane is invariably split, fragmented or absent. An important distinguishing feature between coronary lesions in salmonid fishes and mammals is therefore the absence of significant fat and calcium deposits.

It is now clear that coronary lesions are most prevalent and most severe in mature migratory salmonid species belonging to the genera *Oncorhynchus* and *Salmo* (e.g. Pacific salmonid fishes, Atlantic salmon and steelhead trout). Coronary lesions are typically found in more than 95%, and often 100%, of a sample population of migratory salmonid fish (Robertson *et al.*, 1961; Maneche *et al.*, 1973; Farrell *et al.*, 1986, 1990a; Saunders *et al.*, 1992). For a given individual, lesions are typically found to occupy 66–80% of the length of the main coronary artery. Furthermore, the lesions are severe enough to occlude the vessel lumen by, on average, 10–30%, but occlusions of 50% of the artery have been observed (Maneche *et al.*, 1973; Moore *et al.*, 1976; Farrell *et al.*, 1986, 1990a) (Fig. 10.8b). This severe level of coronary arteriosclerosis normally takes 2–5 years to develop in wild fish, but as little as 29 months in faster-growing cultured fish. It is entirely possible that more severe states of coronary arteriosclerosis develop but have not been observed because they go undetected due to mortality not directly ascribed to the lesions (see below). The progression of lesions described above was recently confirmed for Atlantic salmon, *Salmo salar* (Seierstad *et al.*, 2008).

Coronary lesions are less severe in nonmigratory salmonid fishes and absent or less severe in other fish species (Vastesaeger *et al.*, 1965; Santer, 1985). In elasmobranch fishes, for example, lesions are not found in the main coronary arteries lying on the *conus* (Farrell *et al.*, 1992), but lesions are found in smaller intraventricular arteries (Garcio-Garrido *et al.*, personal communication). The reason why arteriosclerotic lesions are restricted to coronary vessels, and more particularly to migratory fish, is not entirely clear, but it may be related to the mechanism(s) underlying the lesion formation (see below).

Aetiology

A complete picture of the aetiology of coronary lesions in salmonids is still emerging. Robertson *et al.* (1961) first suspected that sexual maturation was the primary factor, based on the observations that lesions are absent in juveniles and appeared in mature fish. Then House *et al.* (1979) found that lesions increased in juvenile trout following injections of the sex hormones human chorionic gonadotrophin, testosterone and oestradiol. Lesions were also found in sexually precocious steelhead trout (Schmidt and House, 1979). However, because well-developed lesions were found well in advance of maturation in Atlantic salmon (Farrell *et al.*, 1986; Saunders *et al.*, 1992) it has been concluded that sexual maturation is probably only a secondary factor in lesion aetiology (Farrell *et al.*, 1986).

Moore *et al.* (1976) first proposed that diet could influence lesion development. A dietary cholesterol supplement produced a greater incidence of lesions in mature Atlantic salmon held in fresh water, as well as increasing total plasma cholesterol and low-density lipoproteins (LDL) levels in the plasma (Farrell *et al.*, 1986). Eaton *et al.* (1984) have also reported a positive correlation between plasma LDL levels and coronary lesions in mature Great Lakes salmon. Whether these observations reflect arteriosclerotic mechanisms similar to those found in mammals is unknown. A potential role for dietary polyunsaturated fats in lesion aetiology is described in more detail below.

Factors related to growth have also been implicated in coronary lesion development. The prevalence and severity of coronary lesions accelerates in parallel with rapid bodily growth in the ocean (Kubasch and Rourke, 1990; Saunders *et al.*, 1992). Furthermore, when Atlantic salmon are grown faster under culture conditions, they attained a similar level of lesion prevalence

as wild salmon but in a shorter time period (Fig. 10.9). In addition, slower-growing varieties of cultured salmon accumulated lesions at a lower rate (Saunders *et al.*, 1992). The mechanism underlying this correlation between lesion development and fish growth is unexplained at this time. However, the correlation between growth rate and lesion formation may account for the observation that lesions are fewer and less severe in the slower-growing, landlocked species of salmonids compared with the migratory varieties.

Finally, it is possible that coronary lesions are initiated primarily as a result of mechanical injury (Saunders *et al.*, 1992). Vascular injury is one of the principal mechanisms for initiating coronary disease in mammals (Ross and Glomset, 1973; Ross, 1984). In fact, direct mechanical abrasion of the coronary artery in rainbow trout results in a substantial increase in vascular smooth muscle mitotic activity, as indicated by increased incorporation of ^{3}H-thymidine *in vitro* (Gong and Farrell, 1995). Saunders *et al.* (1992) envision a direct link between stress and coronary injury, which is based on the salmonid coronary artery lying on a highly compliant outflow tract from the heart (i.e. the *bulbus arteriosus* and ventral aorta), which overexpands during stress. During the hypertension associated with stressful activities (systolic blood pressures in ventral aorta may exceed 100 mmHg), the outflow tract is overdistended and the coronary artery on its surface is excessively disturbed through stretching, distortion and alteration to its blood flow pattern. Consistent with this hypothesized mechanism for initiated vascular damage in salmonid fishes is the

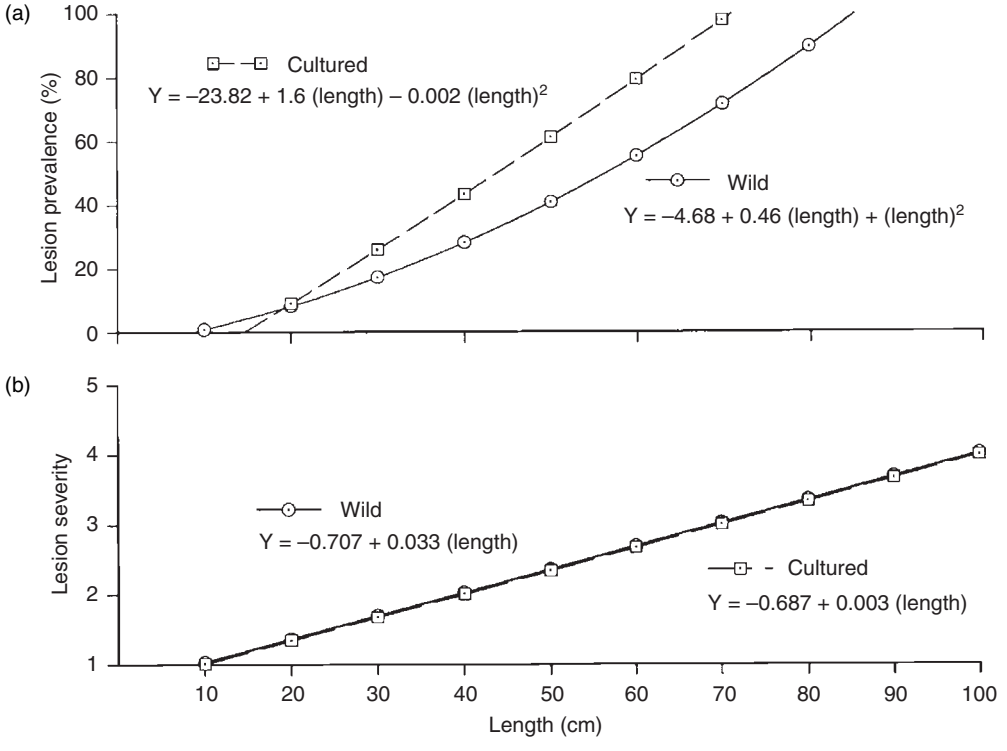


Fig. 10.9. Prevalence (a) and severity (b) of arteriosclerotic lesions in coronary arteries of wild (n = 517) and cultured (n = 908) Atlantic salmon at various life stages, based on a grouping of fish into 5-cm length classes with varying numbers (4–97 cultured, 3–190 wild) in each group. Adapted from Saunders *et al.* (1992).

observation of a different pattern of lesion accumulation in sharks. Lesions are absent in straight segments of the main coronary artery (Farrell *et al.*, 1992), which is consistent with the *conus* of sharks being less elastic than that of the *bulbus* of salmonid fishes, with the result that the main coronary artery of sharks is not distorted as much during hypertension. Instead, coronary lesions in sharks are restricted to intraventricular arteries and branch points in the main coronary artery, and these are sites of considerable wall stress, which could lead to vessel injury (Garcio-Garrido *et al.*, personal communication).

Thus, it is likely that the progressive accumulation of coronary lesions in migratory salmonid fishes reflects the sum total of: (i) the various natural stresses, such as feeding and avoiding predation, which would lead to hypertension and coronary vascular injury and thereby initiate focal lesions; (ii) the various vascular repair mechanisms (which are not understood for fishes); and (iii) the various factors, such as sexual maturation, diet and growth rate, that apparently affect the rate of progression of lesion development. If rapid growth of salmonid fish in nature is a result of a more dominant, more aggressive and stressful lifestyle, then a consequence of faster growth may well be a faster accumulation of coronary lesions. This effect may contribute to the observed higher incidence of coronary lesions in cultured fish compared with wild fish (Fig. 10.9). Given such a scenario, it is expected that stressful activities such as enforced swimming would lead to more coronary lesions. Although this idea has not been tested directly, enforced swimming has been demonstrated to act as a mitogenic stimulus for coronary VSM explants from rainbow trout (Gong *et al.*, 1996). Slower, more continuous swimming regimes did not stimulate coronary VSM mitosis under culture conditions.

Consequences of coronary arteriosclerosis

The impact(s) of coronary arteriosclerosis on salmonid fishes is largely a matter for speculation. Since the lesions are in a main arterial conduit, the potential exists for the lesion to restrict coronary blood flow by increasing the resistance to flow. Whether or not cardiac ischaemia (insufficient coronary blood flow) actually occurs is unclear (Farrell *et al.*, 1990a). However, we do know that cardiac ischaemia in salmonid fish is unlikely to be immediately life-threatening, in that the coronary circulation only supplies oxygen to the *compacta*, about half of the ventricular mass. This suggestion is supported by an experimental finding that when the coronary artery is surgically tied off, rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) do not die immediately (Farrell and Steffensen, 1987; Farrell *et al.*, 1990b). Instead, maximum swimming performance of these fishes is reduced to 70–80% of the normal capacity. Thus, myocardial ischaemia (if it does develop as a result of coronary arteriosclerosis in fish) is more likely to affect the long-term survival of salmon in the context of life-sustaining activities related to swimming performance, e.g. migrating, feeding and avoiding predators. This contrasts with the situation in mammals.

Lesion accumulation is most severe just prior to the death, at spawning, of Pacific salmon. Therefore, it could be argued that coronary arteriosclerosis has little selective value. Farrell *et al.* (1990a) did note, however, that lesion accumulation was generally higher in coho, sockeye and chum salmon than in steelhead trout. Steelhead trout, like Atlantic salmon, have the potential to survive their maiden spawning and become repeat spawners. Therefore, a critical question is: Do steelhead trout and Atlantic salmon carry with them the severe level of coronary lesions accumulated during the maiden spawning run? Van Citters and Watson (1968), for steelhead trout, and Maneche *et al.* (1973), for Atlantic salmon, presented data to support the idea that coronary lesions were lost (regressed) when individuals returned to the sea for repeated spawning. In other words, coronary lesions did not represent an accrued disadvantage for repeat-spawning species. Moreover, the observations raised the possibility that

salmon might be a natural model for coronary lesion regression. Unfortunately, this idea of lesion regression in repeat-spawning species has been refuted by more comprehensive studies with Atlantic salmon (Saunders and Farrell, 1988) and steelhead trout (Farrell and Johansen, 1992). The latter studies provided convincing evidence for a progression rather than a regression of coronary lesions in repeat-spawning species. Whether or not lesion accumulation is a factor limiting the number of repeat spawns, i.e. a low level of coronary lesions is advantageous to repeat-spawning species, has not been studied.

Effects of dietary fatty acids

Human diets rich in fish and fish oils are associated with reduced risk of cardiovascular disease and atherosclerosis (Bang and Dyerberg, 1972, *et seq.*; Kromhout *et al.*, 1985; Phillipson *et al.*, 1985). The benefits are apparently related to a lower intake of saturated fatty acids and a higher intake of long-chain polyunsaturated fatty acids (PUFA), especially eicosapentaenoic (EPA; 20:5 ω-3) and docosahexaenoic (DHA; 22:6 ω-3) acids. Linoleic acid (18:2 ω-6) is the predominant PUFA in the North American diet. Diets rich in ω-3 PUFA have at least three potential benefits in humans. First, plasma triglyceride levels are reduced (Harris *et al.*, 1983). Second, clotting time and platelet aggregation time are increased, probably as a result of ω-3 PUFAs in fish oils displacing arachidonic acid (AA) from tissue phospholipids and causing a shift in the metabolic end products of prostaglandin (PG), leukotriene (LT) and thromboxane (TX) synthesis (Needleman *et al.*, 1979; Lands, 1986). A third potential benefit relates to the inhibition of growth factor(s) that affect VSM (Fox and DiCorleto, 1988).

Gong *et al.* (1997) assessed the mitogenic activity of PUFAs on coronary VSM explants from rainbow trout. EPA, AA and eicosatrienoic acid (ETA) at concentrations greater than 80 μM were all mitogenic. However, the effects of lower concentrations were more complex. At around 20 μM, AA was an extremely potent VSM mitogen, in fact considerably more so than at higher concentrations. In contrast, 20 μM EPA had no effect and 20 μM ETA inhibited mitotic activity. The interactive effects of the PUFAs may be noteworthy. An equimolar concentration of EPA could completely inhibit the potent mitogenic effect of 20 μM AA, whereas ETA only partially suppressed AA-stimulated VSM mitosis. The authors suggested that these PUFA-mediated effects and interactions on coronary VSM mitosis probably involved PG, LT and TX synthesis. The general significance of these findings to coronary arteriosclerosis in fish is still undetermined.

As a result of the benefits of dietary intake of ω-3 PUFA to human health, there has been increased interest in manipulating the fatty acid content of cultured fish with specialized diets. Saturated fatty acids (SFA) and highly unsaturated fatty acids (HUFA) have been shown to impact metabolic rate and hypoxia tolerance in fish (reviewed by McKenzie, 2001), although the mechanisms of action are unknown. It has been suggested that low dietary ω-3 HUFA/SFA and ω-3 HUFA/AA ratios may negatively affect swimming performance (Wagner *et al.*, 2004), but that this may be offset by linoleic acid. It is now clear that experimental diets enriched with ω-3 PUFA can alter the muscle lipid composition of cultured salmonid fishes (Bell *et al.*, 1991a; Higgs *et al.*, 1995). It is equally clear that these ω-3 PUFA-enriched diets also have important physiological effects that are beneficial to the fish. In particular, there can be considerable shifts in the membrane phospholipid fatty acid compositions and the eicosanoids produced from them (Bell *et al.*, 1991a,b, 1992).

One of the remarkable effects of an insufficient dietary intake of ω-3 PUFA (i.e. a low ω-3/ω-6 ratio diet produced by using a sunflower oil rather than a fish oil dietary supplement) was that heart size was significantly reduced in cultured post-smolt Atlantic salmon (Bell *et al.*, 1991a). In severe cases a marked depletion in the amount of *compacta* and *spongiosa* of the ventricle made the ventricular wall exceedingly thin. Moreover, these fish became

more susceptible to transportation-induced shock syndrome (a 30% mortality was observed). A similar shock syndrome is also described for essential fatty acid-deficient rainbow trout (Castell *et al.*, 1972). Clearly, then, ω-3 PUFA appears to be an important dietary component for heart development and survival in post-smolt Atlantic salmon. However, it is unclear what the underlying mechanisms are and why severe levels of coronary lesions accrue in wild and cultured salmon, even though they receive a high dietary level of ω-3 PUFA. While dietary fats may affect cardiac development, feeding Atlantic salmon either 100% fish oil or 100% vegetable oil had no effect on the progression of coronary lesions, independent of whether the salmon were reared in fresh water or seawater (Seierstad *et al.*, 2008).

Cardiomyopathy syndrome

Very little is known about the causes of cardiomyopathy syndrome (CMS), and the condition is frequently referred to as 'acute heart failure' (Ferguson *et al.*, 1990). CMS has been described in marine-farmed Atlantic salmon stocks in Norway and the Faeroe Islands (Kent and Poppe, 1998; Poppe and Taksdal, 2000) that were otherwise in good condition, and similar cases have been observed in farmed Atlantic salmon in British Columbia (Brocklebank and Raverty, 2002). Common clinical signs of the condition are a haemopericardium due to atrial wall rupture, lesions described as largely restricted to the spongy portion of ventricle and atrium and comprising myocardial degeneration and necrosis, variable degrees of endocardial-associated hypercellularity and leucocyte infiltration (Ferguson *et al.*, 1990). Brun *et al.* (2003) examined the occurrence and risk factors associated with CMS and found that approximately 11.5% of all groups of salmon in the study displayed the condition. These authors regard CMS as a chronic disease, although sudden death is often characteristic. While infectious agents, such as infectious pancreatic necrosis virus (Ferguson *et al.*, 1986), nodavirus (Totland and Kryvi, 1997) and *Diphylbothrium dendriticum*, have been associated with CMS in farmed fish, these are commonly regarded as exceptions, and it is suspected that the condition is a metabolic or production aetiology rather than an infectious disease (Poppe and Taksdal, 2000). The CMS literature was recently reviewed (Kongtorp *et al.*, 2005).

Pompe-like disease

In humans, Pompe disease is an autosomal recessive genetic disorder resulting in deficiency of acid alpha-glucosidase, a lysosomal enzyme involved in cellular glycogen degradation. The resulting metabolic effects lead to an accumulation of glycogen within the lysosome, leading to its classification as a lysosomal storage disorder (Hers, 1963). The progressive accumulation of glycogen leads to disruption of cellular architecture and function, resulting in progressive organ enlargement and dysfunction (e.g. cardiomyopathy). The disease may occur at any time during life and the symptoms are progressive. The early-onset infantile disease is associated with hypotonia, generalized muscle weakness and a hypertrophic cardiomyopathy, generally culminating in cardio-respiratory failure or respiratory infection (Chen and Amalfitano, 2000; Hirschhorn and Reuser, 2001). Symptoms of juvenile onset include progressive weakness of respiratory muscles and an intolerance to exercise, while adult onset involves generalized muscle weakness and wasting of respiratory muscles. Individual prognosis varies according to onset and severity of symptoms, but the disease is particularly lethal in infants and young children. While Pompe disease is classified as a lysosomal storage disorder, it is also categorized as a neuromuscular disease, a metabolic myopathy and a glycogen storage disease. Because of the important cardiac involvement, the infantile form of Pompe is also considered a cardiac disorder. Readers are referred to Kishnani and Howell (2004) for a more thorough review of the disease.

In a recent Norwegian study a cardiomyopathy that strongly resembles Pompe disease (glycogenosis type II) in humans was observed in farmed rainbow trout (Fig. 10.10). Fish had been per-orally treated with testosterone to produce all-female progeny, but similar symptoms were subsequently observed in untreated fish from the same population, and it was hypothesized that testosterone treatment had aggravated an existing condition. Affected fish displayed abnormal behaviour, severe circulatory disturbances with exophthalmia, ascites and ventral petecchiation. Necropsy revealed alterations in cardiac shape, with distended atria and rounded ventricles. Histological examination revealed an abnormal cardiac tissue arrangement, where the inner *spongiosa* was visible between patches of outer *compacta* myocardium. Severe vacuolation of cardiac myocytes, partial absence of outer compact myocardium, extensive vascularization of the epicardium and myocardial necrosis along the outer margin of the *spongiosa* were also common. Strongly PAS-positive material was demonstrated in the walls of the vacuoles and saliva-diastase pre-treatment of serial sections abolished PAS staining, indicating the presence of glycogen (T. Poppe, MS in preparation). The cause of this condition in rainbow trout is unknown, but the lesions in cardiac myocytes bear a striking resemblance to glycogenosis type II (Pompe disease) and further investigations will probably lead to further insights on the nature of the condition.

Effects of red tide planktons

Red tides occur globally as a result of rapid growth (a bloom) of various planktonic organisms. Economic losses in aquaculture due to blooms of these organisms are in the order of millions of dollars each year. While there are many species of red tide organisms, the patho-physiological effects of two genera of such organisms are discussed here. To some extent, some of the pathological effects that they cause might be generalized to other planktonic organisms. These examples were selected on the grounds that the physiological investigations have led to



Fig. 10.10. Gross morphological characteristics of a rainbow trout heart displaying Pompe-like (glycogenosis type II) disease. Image courtesy of T.T. Poppe (2006).

some speculation about the mechanisms by which fish kills occur.

Red tide blooms of the *Chattonella* species (*C. marina* and *C. antigua*) continue to kill millions of yellowtail (*Seriola quinqueradiata*) in Japan, especially in the Seto Inland Sea. While it effectively kills fish, published evidence regarding the mechanism by which death occurs is still much debated, and a number of hypotheses have been put forward to explain their toxicity. Some studies have examined production of reactive oxygen species (ROS) and their role in damage to the gills (Oda *et al.*, 1992a); some have focused on the role that free fatty acids (FFA) play in toxicity (Okaichi *et al.*, 1989), while others have focused on anoxia, mucus production, and respiratory and cardiovascular physiology (Ishimatsu *et al.*, 1990). While it has been shown that these particular plankton species produce brevetoxin, a powerful ichthyotoxin (Ahmed *et al.*, 1995), it is still debated what role the brevetoxin, ROS or FFA, or some combination of these, plays in toxicity (Marshall *et al.*, 2003).

Fish exposed to great densities of this organism (*ca.* several thousand per ml) showed neither clogged nor visibly impaled gills that would lead to bleeding and mechanical damage (Ishimatsu *et al.*, 1990). Most investigations record a decrease in blood oxygen tensions as a result of exposing fish to heavy concentrations of *C. marina*, although this did not happen until the latter stages of exposure, just before death (Ishimatsu *et al.*, 1990). In contrast, the phytoplankton *Chaetoceros concavicornis* has long, barbed spines, which do result in the clogging and physical impaling of the respiratory epithelium of exposed fish. This can, and has, resulted in massive fish kills on salmon farms in British Columbia, Canada (Bell, 1961; Albright *et al.*, 1992). While exposure to the two plankton species might be expected to elicit different responses, due to the differences in physical characteristics, the histopathological responses have been shown to be similar in many regards.

The effect of red tide plankton exposure on mucus production at the gill can be varied. Shimada *et al.* (1983) described both a decrease in the number of goblet cells in the gill epithelium and a degeneration of the mucus cell membrane on the afferent ridge of the primary filament of yellowtail exposed to *C. antigua* within 1 h of exposure. In contrast, Endo *et al.* (1985) reported that the number of mucus cells on the primary filament of yellowtail decreased in proportion to the length of exposure to *C. marina*. Yang and Albright (1992) also observed an increase in both goblet cell number and mucus quantity in the gills of rainbow trout exposed to *C. concavicornis*. There is some contradictory evidence regarding the stimulation of mucus secretion by the gill epithelium of yellowtail as a result of *C. marina* exposure. While Shimada *et al.* (1983) reported that *C. marina* caused a stimulation of mucus secretion, and an associated disappearance of the stable mucus coat by the gill epithelium of yellowtail, Ishimatsu *et al.* (1990) did not observe such excessive mucus secretion in the yellowtail. The disappearance of the mucous covering of the gill epithelium has also been reported in yellowtail exposed to two other red tide organisms, *C. marina* (Endo *et al.*, 1985) and *Gymnodinium* (Shimada *et al.*, 1982). This disappearance of the mucous layer may be due to a stimulation of the goblet cells to produce mucus and an eventual degeneration of those cells (Shimada *et al.*, 1983). In yellowtail exposed to *C. marina*, Endo *et al.* (1985) found a significant reduction in the carbonic anhydrase (CA) activity of the cells at the tips of the secondary lamellae, which were swollen and had the least amount of mucous covering. They speculated that the reduction in CA activity was primarily due to the exposure of that part of the lamella to a greater amount of water flow. However, they also speculated that Br^- and I^-, which are abundant in many species of plankton, may have inhibited CA activity directly, as these anions have been shown to have such an effect (Pocker and Stone, 1967).

Extensive oedema of the epithelium has been reported in yellowtail exposed to red tide organisms (Shimada *et al.*, 1983; Endo *et al.*, 1985; Toyoshima *et al.*, 1985), as well as in the secondary lamellae of

rainbow trout exposed to *C. concavicornis* (Yang and Albright, 1992). The oedema in yellowtail was characterized by shrinkage of the undifferentiated cells underlying the surface of the primary filament and an expansion of intercellular spaces (Shimada *et al.*, 1983; Toyoshima *et al.*, 1985), as well as swelling of the pavement cells at the surface (Endo *et al.*, 1985). Yang and Albright (1992) observed severe hyperplasia and hypertrophy of the cells of the secondary lamellae, as well as a collapsed pillar cell system and detachment of the cells of the respiratory epithelia on the secondary lamellae from the blood capillaries, in rainbow trout. They also noted some haemorrhaging in the secondary lamellae. Most of these investigators speculate that the oedema resulted from an osmoregulatory disturbance caused by the elimination of the mucous coat from the epithelial surface. This implies that the mucous coat imparts a barrier for seawater entering the intercellular spaces, such that its disappearance would cause an influx of hypertonic seawater into the intercellular spaces. This would result in the osmotic shrinking of the cells within the epithelium (Shimada *et al.*, 1983). In contrast, the swelling of the pavement cells exposed to the water must be due to a breakdown of the ionic, and associated osmotic, regulatory mechanisms. While it is possible that the mucous coat provides osmotic protection to these cells, it is also possible that it protects the epithelial surface cells from the toxic substances of certain red tide species, which could directly inhibit active ion exchange processes that maintain the ionic and osmotic integrity of those cells. This latter possibility is supported by observations that the response of the gill tissue to toxic metals in the water is very similar to the responses described here for red tide plankton exposure (see discussion below and Fig. 10.11).

Toyoshima *et al.* (1985) described profound changes in the ultrastructure of chloride cells in the yellowtail exposed to *C. antigua*. They observed that the intralamellar gill epithelium changes from having numerous, characteristically long, cellular extensions at the apical surface to having a surface with fewer and shorter extensions. Since these cells play a central role in the transfer of Cl^-, as well as other ions, between blood and water (Foskett and Scheffey, 1982), a likely consequence of damage to the chloride cells is impaired ionic and osmotic regulation to the cells themselves, as well as to the whole animal.

The structural changes described above involve physiological consequences to both the cardiovascular and respiratory systems. Work by Ishimatsu and co-workers (Ishimatsu *et al.*, 1990, 1991) with yellowtail corroborates previous data showing a reduction in arterial pH and oxygen tension (Po_2) with exposure to high densities of red tide plankton. The fall in blood Po_2, an increase in ventilatory pulse pressure (Ishimatsu *et al.*, 1990) and a moderate increase in plasma catecholamine concentrations (Tsuchiyama *et al.*, 1992) were the initial changes as a result of *C. marina* exposure, followed by a relatively stable physiological profile until the final stages of life. After about 3 h of exposure, most of the measured variables changed drastically just before death. That stage was characterized by hypoxaemia, hypercapnia, plasma and erythrocytic acidoses, increases in plasma concentrations of Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, bradycardia and large increases in noradrenaline and adrenaline (see Ishimatsu *et al.*, 1990, 1991; Tsuchiyama *et al.*, 1992). Tsuchiyama *et al.* (1992) attributed the increased catecholamine concentration to the severe hypoxaemia. Rainbow trout exposed to *C. concavicornis* also exhibited stress, through elevated cortisol, glucose and lactate concentrations, as well as hypoxaemic blood (Yang and Albright, 1992). Yang and Albright (1992) also observed a progressive acidosis, increased ventilation frequency and lower oxygen consumption with continued exposure to that red tide organism. Endo *et al.* (1988) also reported bradycardia as a response of the sea bream, *Pagrus major*, exposed to *C. marina*; this was probably due to the hypoxaemic state of the blood. They observed that the bradycardia was due primarily to an extension in the interval between T and P waves of the electrocardiograms, and that

it was associated with a period of struggling and low Po_2.

The hypoxaemic state induced by exposure to red tide plankton is probably due to the oedematous state of the epithelial tissues. Reduced oxygen transfer (Pärt *et al.*, 1982) and reduced swimming performance (Nikl and Farrell, 1993) have been associated with increases in diffusion distances between blood and water, as would be the case in the oedematous epithelium. The bradycardia would be expected to contribute to the lower blood Po_2, through decreased uptake rates of oxygen from the water. Furthermore, it might be expected that consumption rates, especially by muscle tissues, would increase with a rise in metabolism with stress or struggling, both of which have been reported to accompany exposure to red tide organisms.

The mechanisms by which various algal blooms kill fish probably vary among the major algal groups. Clearly, the morphological and physiological changes described above (gill oedema, haemorrhage, hypoxaemia, alterations to chloride cell morphology and ionic disturbances) would be stressful enough to kill fish. Also, it is generally accepted in most cases that they are the consequences of a more primary action of the plankton, i.e. release of toxic compounds, physical damage (clogging or impalement), on the fish. However, further work is needed on the exact aetiology of these effects. For example, brevetoxin is a polyether toxin that interferes with site 5 of the voltage-gated Na^+ channel and therefore can have devastating consequences if it reaches the central nervous system. This does not mean that acute sensitivity of central neural tissue to brevetoxin is the root cause of the observed morphological and physiological changes. Peripheral effects are plausible. In fact, it is possible that the introduction of the toxin into gill tissue causes a general inflammatory response. In this regard, the work of Oda and colleagues (Oda *et al.*, 1992a,b) clearly shows that superoxide radicals and hydroxyl radicals are generated from *C. marina*. The cytolytic action of free radicals (see Dean, 1987) and the possible consequence of breakdown in ionic and osmotic homeostasis in the cells that make up the surface of the gill epithelium has been suggested as a primary process by which this red tide organism begins the degradative processes that can lead to the death of the fish (Oda *et al.*, 1992b). It has also been suggested that when *C. marina* cells come into contact with the gill surface, the plankton's glycocalyx may be discharged and that a continuous accumulation of the discharged glycocalyx may be responsible for ROS-mediated gill tissue damage, ultimately leading to the fish's death (Kim *et al.*, 2001). While Yang and Albright (1992) suggested that death from exposure to *C. concavicornis* is due to suffocation as a result of impaired O_2 uptake, Yang (1993) has also shown that such exposure makes the fish susceptible to secondary infections, probably due to enhanced pathogen entry through the sites of puncture by the spines of the plankton, and a generally immunosuppressed state caused by stress, as evidenced by elevated cortisol concentrations in the blood.

Parasites

Protozoan and metazoan infections are covered in detail in Volume 1 of *Fish Diseases and Disorders*, and while Volume 2 is intended to focus on non-infectious agents, we felt it worthwhile highlighting that many organisms normally associated with fish may have direct or indirect effects on host physiology; not all of these are obvious, nor are their impacts. Many parasites can colonize the gills (e.g. *Loma*, *Ichthyobodo*, *Trichodina*, *Henneguya*), and their attachment and grazing can cause direct mechanical damage to the gill epithelia, compromising the respiratory organ, but not all are associated with disease.

The normally free-living *Paramoeba pemaquidensis* is an opportunistic organism and the environmental conditions that lead to its proliferation on fish gills are not known (Kent and Poppe, 1998). The transfer of salmon into the marine environment is associated with structural gill changes and hyperplastic lesions (Nowak and Munday,

1994; Nowak and Lucas, 1997) and this probably predisposes fish to colonization by organisms such as *Paramoeba*. Gill damage unrelated to pathogens often provides a point of entry/attachment for parasites, and their activities may cause gill hypertrophy and/or hyperplasia, altering the available gas-exchange surface area. Similarly, mechanical irritation caused by parasites often leads to excess mucus production (Lin *et al.*, 1994), resulting in decreased gas transfer and associated respiratory stress. For example, the gill louse, *Salmincola*, attaches directly to the gills and causes extensive damage to gill filaments through both its attachment and grazing. Heavy infection levels are common in cage culture, creating serious problems where fish are kept at high densities (Sutherland and Wittrock, 1985), particularly in summer, when temperatures can rise and oxygen levels can drop. In wild populations the prevalence and intensity of infection are usually low and therefore generally have a low impact on fish (Bowen and Stedman, 1990). The gills represent a particularly vulnerable tissue and attachment of any parasite will result in alterations to both respiratory and osmoregulatory ability (Finstad *et al.*, 2000). Interestingly, many parasites elicit no inflammatory response from the host when intensity of the infection is low.

Parvicapsula minibicornis is a myxosporean parasite that sporulates in the kidney but the trophozoites are found in the glomeruli capillaries (Kent *et al.*, 1997; Raverty *et al.*, 2000). While mortality due to this parasite is generally attributed to loss of kidney function and resultant osmotic imbalance, it has been suggested that this imbalance would be exacerbated in migrating adult salmon due to increased water uptake across the gills during swimming (Gallaugher *et al.*, 2001), thereby placing an additional load on kidney function (Wagner *et al.*, 2005) and presumably the cardiovascular system as well.

Relatively few external parasites affect the heart, but one group of notable exception is the pennellid copepods (e.g. *Haemobaphes* and *Lernaeocera*), which attach to the gill arch. From this attachment point, the cephalothorax of the parasite traverses the gills and invades the circulatory system, growing through the ventral aorta, often reaching as far as the *bulbus arteriosus* or ventricle of the heart (Matthews, 1998; Begg and Bruno, 1999). The invasive feeding may cause significant damage, anaemia or occlusions of the aorta or blood vessels. While parasites have been recognized as damaging to their hosts, there has been relatively little research to investigate the interactions between fish and their parasites, and much more research is needed.

Toxicants

Various pollutants and heavy metals have been shown to cause changes in the morphology of the gill in fishes. It is neither within the scope of this chapter, nor is it an objective here, to describe in detail the responses of the respiratory and cardiovascular systems to all studied toxicants. However, we describe here those responses that are common to a range of different toxicants. Qualitative descriptions of changes in the fish gill in response to toxicant exposure are numerous. In general, the tissue reaction to being exposed to many environmental toxicants resembles an inflammatory response (Fig. 10.11).

Fish exposed to heavy metals, detergents and nitrophenols show a separation between the epithelial cells and the underlying pillar cell system, which can lead to a collapse of the structural integrity of the secondary lamellae (Skidmore and Tovell, 1972; Fig. 10.11). In response to zinc exposure, for example, Skidmore and Tovell (1972), in rainbow trout, and Mathiessen and Brafield (1973), in stickleback (*Gasterosteus aculeatus*), showed a swelling of the secondary lamellae and a detachment of the lamellar epithelium from the pillar cell system, and a sloughing of the epithelial cells, respectively. In severe case, the interlamellar spaces, through which water is normally channelled, can be completely clogged due to hyperplasia of the epithelial cells located on the primary filament and mucus

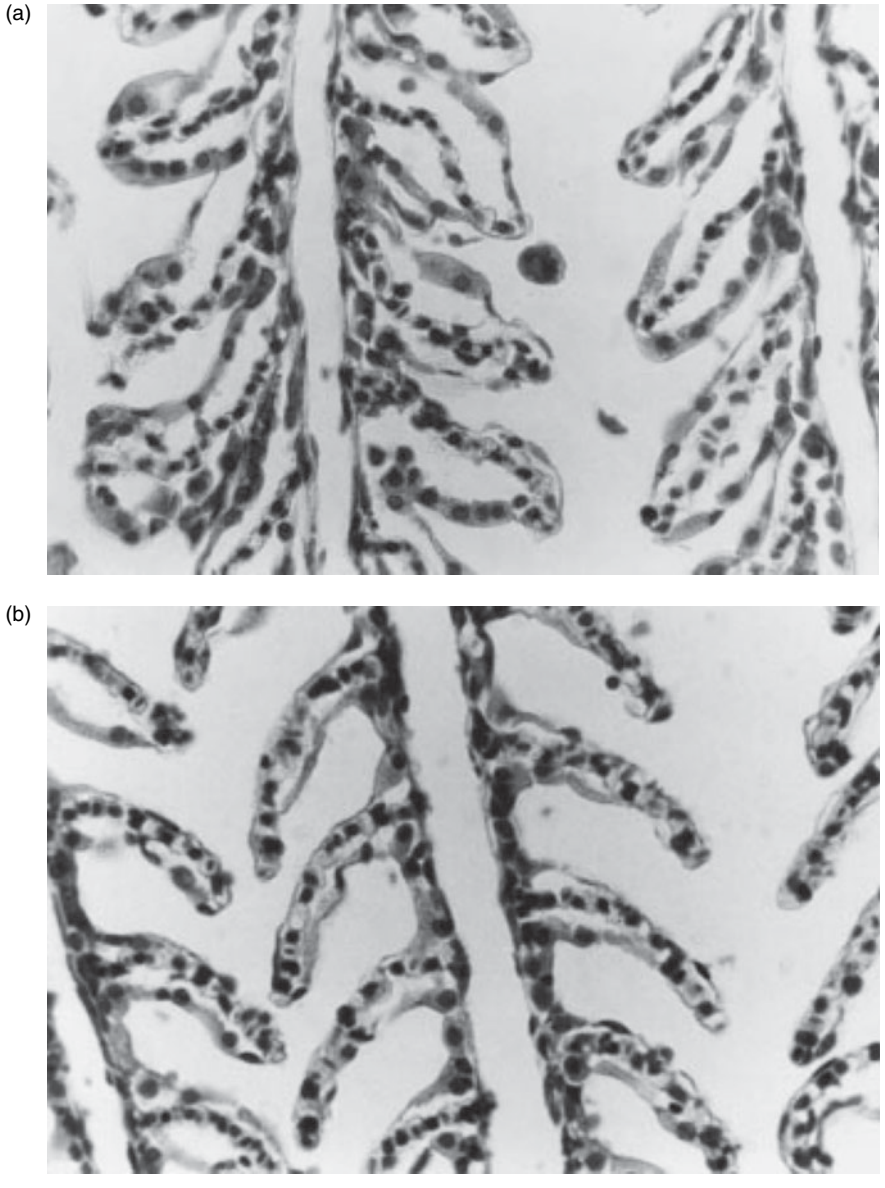


Fig. 10.11. Transverse sections though a gill filament from chinook salmon exposed to (a) control conditions and (b) 20 μg/l 2-(thiocyanomethylthio) benzothiazole at a stage where fish could not maintain equilibrium. Wax embedded 7-μm sections stained with haematoxylin and eosin (×400). Diagrams of transverse sections of the secondary lamellae of rainbow trout (c) before exposure to zinc; (d) after 60% of the estimated survival time upon exposure to zinc; (e) at a stage where equilibrium had been lost with zinc exposure; and (f) at a stage where there was no mobility in the operculum. BM, basement membrane; C, chloride cell; CBS, central blood space; E, epithelial cell; F, pillar cell flange; G, granulocyte; M, mucous cell; MC, marginal channel; ME, marginal endothelial cell; P, pillar cell; PC, proximal channel; R, red blood cell; S, subepithelial space; SE, stretched epithelial cell. Chinook salmon from Nikl and Farrell (1993). Rainbow trout from Skidmore and Tovell (1972).

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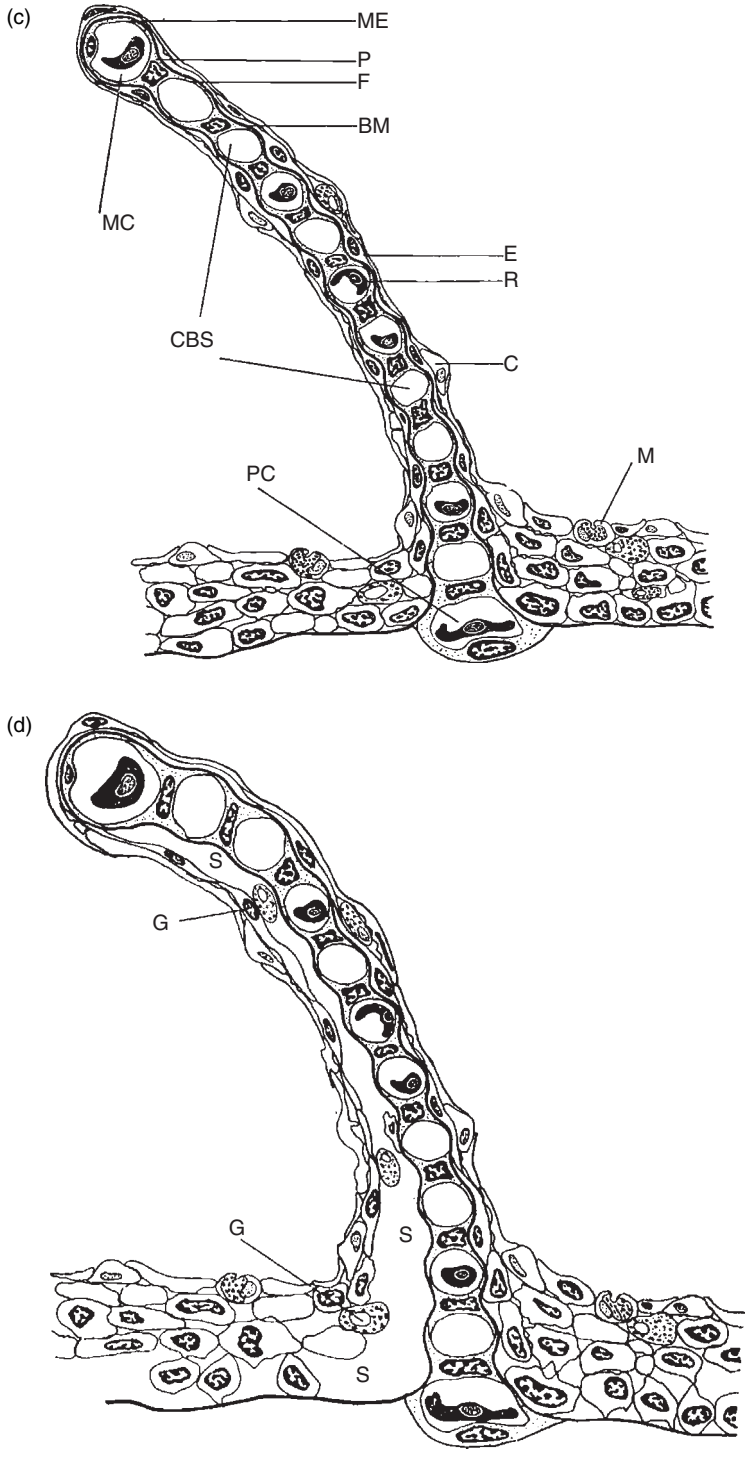


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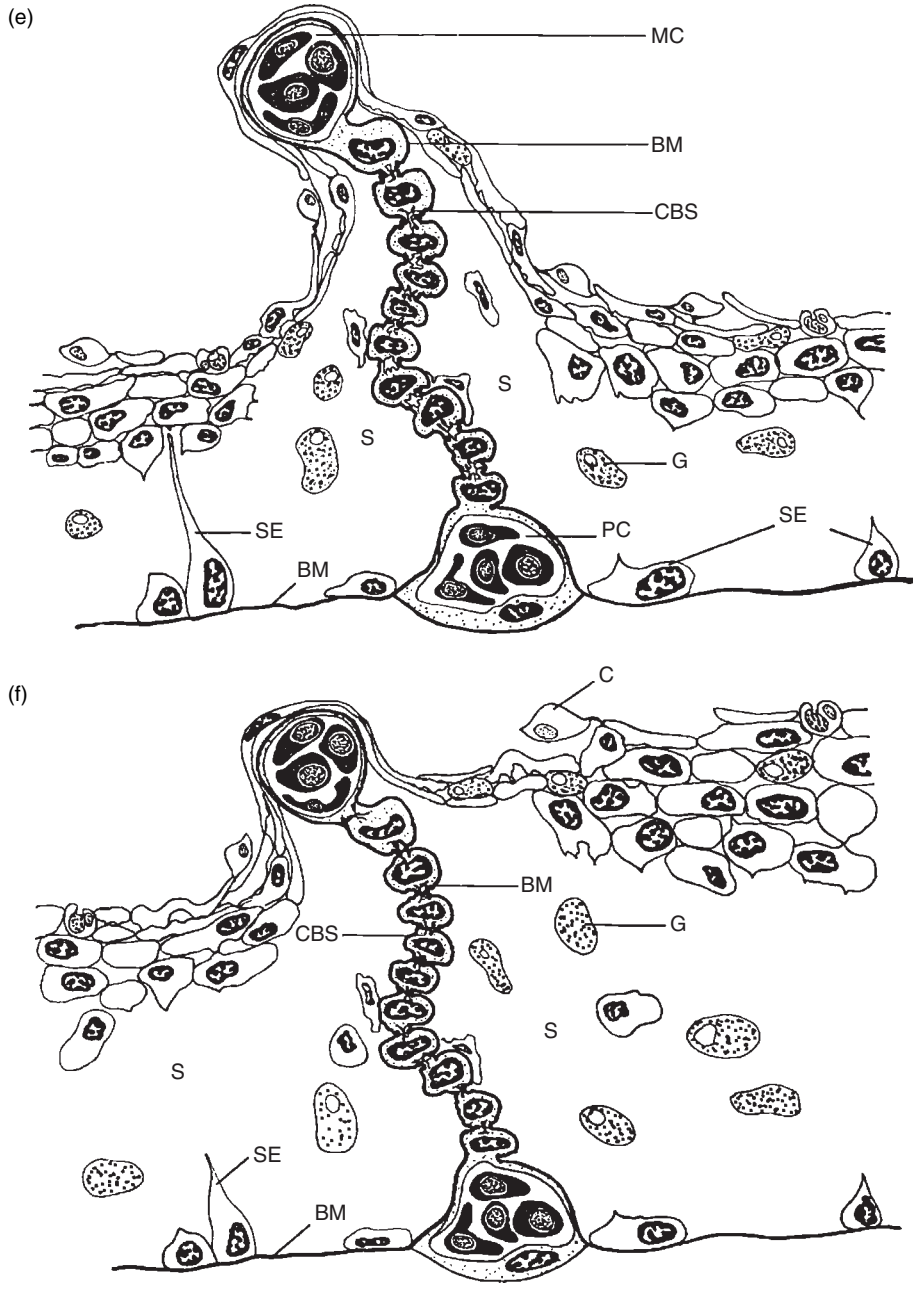


Fig. 10.11. *Continued.*

production. This general reaction of the tissue seems a direct consequence of the exposure, as opposed to a result of a systemic reaction. In the experiments of Skidmore and Tovell (1972), the internal organs of the fish exposed to zinc seem normal in appearance and equivalent amounts of zinc injected into the animals neither damaged the respiratory tissue of the gill nor debilitated the fish. Brown *et al.* (1968) also described the tissue response of rainbow trout exposed to zinc and a synthetic detergent (soft alkyl benzene sulphonate) as a typical inflammatory response to local injury. They observed the clinical signs noted above and described a loss of fluid from the blood, as well as a loss of leucocytes through the vascular walls. Furthermore, there was a fusing of the tips of the secondary lamellae, which resembled the response of rainbow trout exposed to diatomaceous earth (Hebert and Merkens, 1961), as well as the fusion of secondary lamellae of rainbow trout exposed to nickel (Hughes and Perry, 1976).

Hughes and Perry (1976) described a method by which morphological changes could be described in a quantitative manner. They applied the method to demonstrate that rainbow trout exposed to various nickel concentrations in the water showed a greater distance between blood and water and a fusion of the secondary lamellae that resulted in a 1.78- and 4.78-fold reduction in surface area of the secondary lamellae in fish exposed to 2.0 and 3.2 mg/l nickel, respectively, compared with control fish. Also, the thickness of the vascular portion within the swollen secondary lamellae of fish exposed to those nickel concentrations was reduced to 91% and 69%, respectively. Using the methods described by Hughes and Perry (1976), Tuurala and Soivio (1982) described similar changes in the morphology of rainbow trout exposed to zinc and dehydroabietic acid (DHAA), a toxic component of bleached kraft pulp mill effluent. Exposure to both substances induced an increase in Hct (zinc 30%; DHAA 40%) and a vasoconstriction in the secondary lamellae that was caused mostly by a reduction in plasma volume. The data suggested a shift in fluid from the blood vessels to the extracellular space between the blood vessels and the cells of the surface epithelium, which resulted in the volume of that space (which included the space as well as the pillar cell system) increasing by 147% in response to zinc exposure. Zinc exposure also caused both a detachment of the surface epithelium from the pillar cells and a fusing of the secondary lamellae; the latter effect caused an overall reduction in the free gas-exchange surface area by 60%, compared with that in control fish. In addition to the water shift to the extracellular space in the secondary lamellae, Tuurala and Soivio (1982) described an extreme swelling of the epithelial cells, which increased the blood to water distance by 13%, in DHAA-exposed fish. The total effect of the increase in the extracellular space and the increase in epithelial cell size was a 31% increase in the total tissue volume. They calculated that this reduced the ratio of the outer epithelial surface area to tissue volume by 67%. Such hypertrophy is similar to the oedema described above in the gills of fish exposed to red tide plankton. Such studies have provided more detail as to the possible mechanisms in the reduction of oxygen transfer, and consequent reduction in blood Po_2, as a result of the exposure of fish to noxious substances in the water. The vasoconstriction and increased diffusion distance between blood and water contribute to these effects. It seems likely, furthermore, that the loss of osmoregulatory ability by the epithelial cells is another common effect of exposing the gill to agents that injure that tissue. For example, a near-lethal exposure to copper at pH 5.0 caused gill damage, ionic imbalances and respiratory impairment (Wilson and Taylor, 1993).

Other than the lethal effects of toxicants on fish, there are many sublethal effects that have been documented in the literature. While the response of branchial tissue to any biological or abiotic irritant will probably have properties that are unique to that agent, the common response to many

irritants seems to be characterized by a basic inflammatory response. The evidence points to one of the major causes of trauma as being the loss of ionic and osmotic regulation by the epithelial cells of the gill. The resulting changes to cell shapes, epithelial thickness and fluid shifts between blood, water and cellular compartments depend on the magnitude and duration of the insult. The impairment of normal gas and ion transfer processes as a result of those physical alterations induce stress as well as osmotic and ionic regulatory failure. Reduced swimming performance, for example, in chinook salmon exposed to the wood preservative 2-[thiocyanomethylthio]benzothiaxole (TCMTB) has been demonstrated by Nikl and Farrell (1993). It is noteworthy that a 60% reduction in interlamellar distance resulted in the reduction of swimming performance of only 20% (Fig. 10.12). Therefore, there can be substantial increases in

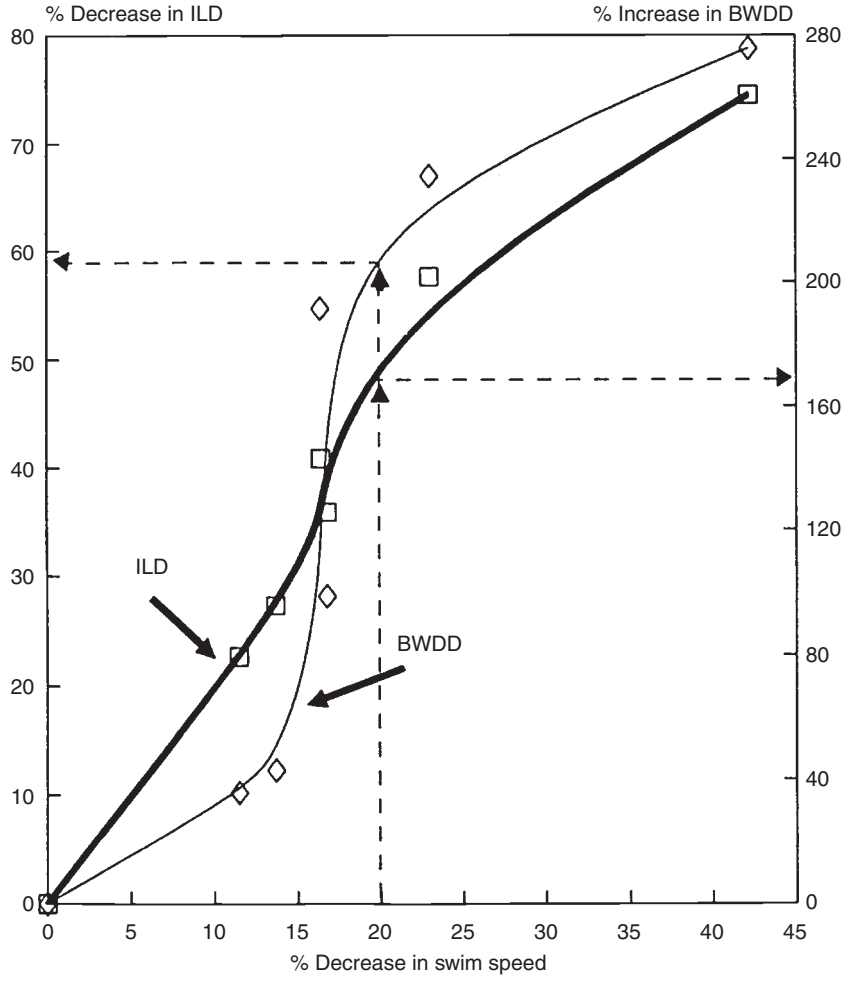


Fig. 10.12. Relationships among the changes in interlamellar distance (ILD), changes in blood–water diffusion distance (BWDD) and the reduction in swimming speed in chinook salmon exposed to a toxicant (2-(thiocyanomethylthio) benzothiazole) known to damage the gill epithelium. Dashed lines indicate measured histological changes related to a 20% reduction in critical swimming speed. From Nikl and Farrell (1993).

the diffusion distance between water and blood before O_2 transport is reduced enough to compromise swimming performance. Conversely, sublethal exposure to copper at low pH impairs ionic regulation, but the reduced arterial oxygen content and damage to gill structure evident with higher concentrations are not present (Beaumont *et al.*, 1995). Nevertheless, swimming performance remained impaired.

In many cases, such tissue damage from metal exposure is reversible. For example, the gill damage in rainbow trout exposed to 3.2 mg Ni/l was completely recovered after 19 days in clean water (Hughes *et al.*, 1979). Likewise, rainbow trout exposed to copper recovered their swimming performance after a 30-day exposure (Waiwood and Beamish, 1978). Juvenile brook trout exposed to sublethal aluminium levels at low pH show some recovery over a period of several weeks (McDonald *et al.*, 1991). However, Audet and Wood (1988) found that adult rainbow trout did not acclimate to low pH. As is the case with many toxicants, water quality affects the toxic actions. Increasing water calcium concentration, for example, ameliorates the acute toxic action of zinc and low pH to the gill tissue and whole fish (Mathiessen and Brafield, 1973; Graham and Wood, 1981). Waiwood and Beamish (1978) also showed that the influence of a copper concentration on swimming performance decreased inversely with water hardness.

Concluding Remarks

This review of the cardiovascular and respiratory systems in fish has focused on the pathological conditions that can result from non-infectious sources. We have concentrated on describing abnormal conditions that can occur in the anatomical structures due to a number of causes, but have not addressed the pathology of the regulatory centres of these systems. There is a great lack of knowledge about many aspects of both the physiology and the regulation of the cardiovascular and respiratory systems of fish. Until the resting states of the physiological systems are well described, the pathological descriptions can have no sound reference.

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11 Hydromineral Balance, its Regulation and Imbalances

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Introduction

Osmoregulation includes the processes by the fish to maintain a relatively constant (homeostatic) interior osmotic environment for the organs, while ion balance includes the regulation of interior Na^+, Cl^-, Ca^{2+} and the acid/base balance portions of homeostasis. Hydromineral balance encompasses the whole of osmoregulation and ion balance. Hypo-osmoregulation includes the osmoregulatory processes wherein the blood of the fish is hypotonic to the environment (e.g. in seawater (SW)); hyper-osmoregulation is the reverse, where the blood is more concentrated compared with the environment, as in fresh water (FW). Hydromineral balance consumes about 5–10% of the total metabolic output of the animal (Boeuf and Payan, 2001), a small but essential expenditure of metabolic energy, mostly based on direct carbohydrate sources (Tseng and Hwang, 2008) but supported by general caloric intake. Diseases that injure barrier functions of epithelia such as the gills, skin and intestinal endothelium can quickly result in osmoregulatory difficulties that, if uncorrected, will almost certainly be lethal. Most teleost fishes that die of disease-based causes suffer a combination of osmoregulatory and respiratory failure, and for this reason it is important to understand the mechanisms that govern hydromineral balance in fish and the factors (external stresses and internal regulatory factors) that impinge on osmoregulatory mechanisms. Fish hydromineral balance and its regulation have been extensively reviewed from several perspectives: gill functions (Evans *et al.*, 2005; Evans, 2008), mitochondrion-rich cells (Hwang and Lee, 2007; Evans, 2008) hormonal regulation (Manzon, 2002; McCormick, 2001; Sakamoto and McCormick, 2006), rapid regulation (Kültz, 2001; Marshall, 2007), transport mechanisms (Marshall, 2002; Evans, 2008) and larval osmoregulation (Varsamos *et al.*, 2005; Finn, 2007). This chapter provides an overview relying on recent reviews and featuring topical research since 2001.

Ion and Water Balance in Marine Teleost Fishes

Drinking and the role of the intestine

Seawater has an osmolality of 1250 mOsm/kg, while the typical osmolality of marine teleost blood and interstitial fluid is approximately 300 mOsm/kg. Because the body wall has a finite osmotic permeability, there is osmotic water loss, which must be

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compensated for to maintain osmotic balance (Marshall and Grosell, 2006). Gill apical membranes have low osmotic permeability, compared with the basolateral surface of the epithelial cells (Hill *et al.*, 2004). Marine teleost fishes drink seawater and absorb the fluid across the oesophagus and intestine, initially by passive permeability in the anterior sections, notably the oesophagus (Hirano and Mayer-Gostan, 1976; Takei and Yuge, 2007). In more distal portions of the intestine, fluid is absorbed by active ion (NaCl) reabsorption, which draws water into the blood using the local osmotic gradient favouring uptake.

Control of drinking is reflexive from the central nervous system, responding to the chlorinity of the environmental fluid and even to small increases in the osmolality of the blood. Drinking itself is intermittent intake of small volumes of seawater at a fairly constant hourly rate, rather than less frequent episodes of large volumes (Takei, 2000). Drinking can be initiated by intravenous infusion of a salt load via the dorsal aorta. Drinking satiety is apparently controlled by the osmolality of the blood and stimulated by the renin–angiotensin system, such that restoration of the normal lower osmolality can reduce drinking rate (Takei, 2000). Animals that take on an exceptional salt load, such as by swallowing considerable seawater along with food, will thus decrease or cease drinking for an interval of a few minutes to hours.

The role of the oesophagus in the Japanese eel (*Anguilla japonica*) is well studied (Hirano and Mayer-Gostan, 1976). The oesophagus of seawater eels has a high osmotic permeability and ionic conductance, such that initially salt (and water) are both absorbed passively (Hirano and Mayer-Gostan, 1976). The water channels, aquaporins, are responsible for intestinal osmotic permeability and fluid absorption (Cutler *et al.*, 2007). The aquaglyceroporin AQP3, an aquaporin that is also permeable to glycerol and urea (Ishibashi *et al.*, 1997), is expressed at high levels in eel oesophagus but not in posterior intestine (Lignot *et al.*, 2002; Cutler *et al.*, 2007), suggesting that AQP3 may be the operational water channel of the oesophagus. In cases where ion permeability is low, there can also be an osmotic water flux *into* the oesophageal lumen with little ion reabsorption, thus making this segment effectively a 'diluting' segment (Marshall and Grosell, 2006).

In the balance of the intestine, the overall absorbate is approximately isotonic (Fig. 11.1). More posterior in the intestine, there is a shift to active electroneutral NaCl uptake (Frizzell *et al.*, 1979) and higher osmotic permeability, which brings more fluid across the epithelium by isosmotic absorption (Hirano and Mayer-Gostan, 1976). In this type of absorption (Diamond and Bossert, 1967), salt taken up across the apical membrane is pumped by laterally located Na^+,K^+-ATPase into the lateral intercellular spaces, creating a local zone of high osmolality. The presence of the water channel AQP1 at higher levels in the posterior intestine of SW-adapted sea bass, compared with FW-adapted animals (Giffard-Mena *et al.*, 2007), and the lack of AQP3 imply that AQP1 imparts the osmotic permeability to the posterior intestine. Local osmotic permeability in these areas allows water to respond to the local osmotic gradient for water to enter the lateral intercellular space, thus forcing fluid out the open basal end of the lateral intercellular spaces and eventually into the blood. Meanwhile, the low osmotic permeability and low ionic conductance of the tight junctions that join the epithelial cells together means that backflow of ions and water is minimized. This is the 'standing gradient hypothesis', which explains many of the observed transepithelial fluid transport phenomena of the gallbladder, kidney, intestine and other structures (Diamond and Bossert, 1967). The system has been supported by epithelial studies for 40 years; notably, it requires considerable recirculation of osmolytes, particularly of the major ions, as demonstrated by recent modelling approaches (Larsen *et al.*, 2002).

The posterior intestine in many marine teleosts is also the location of bicarbonate and carbonate secretion into the lumen (Wilson and Grosell, 2003; Wilson *et al.*, 2009). The bicarbonate combines with calcium and particularly Mg^{2+} to levels so great

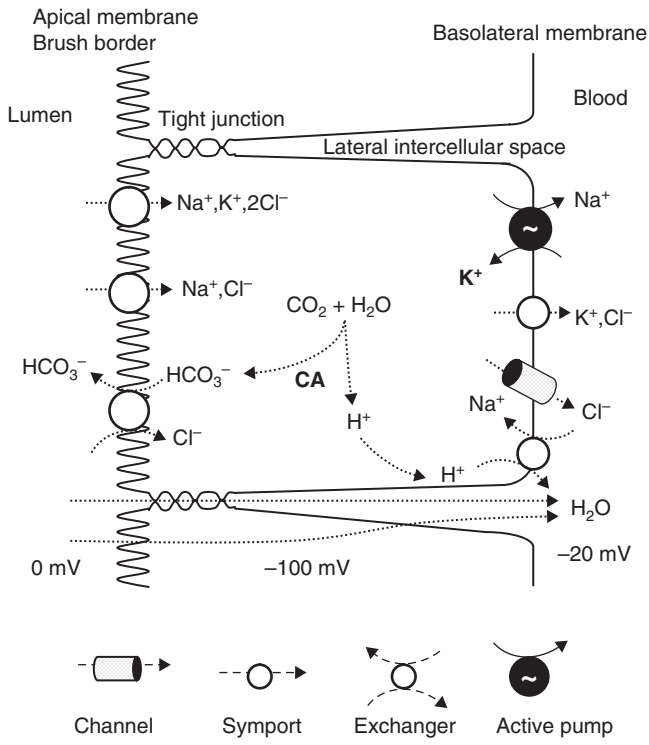


Fig. 11.1. Intestinal salt and water uptake in marine teleost fishes that drink seawater. Initially the lumenal fluid is diluted, then active ion uptake, driven indirectly by the transmembrane Na^+ gradient maintained by Na^+,K^+-ATPase, drives isotonic fluid absorption of NaCl and water. NaCl uptake may be via NCC- or NKCC2-type symports, while Cl^- uptake may be linked to bicarbonate secretion, which in turn arises from action of carbonic anhydrase (CA) on carbon dioxide and water. The absorbate is isotonic with water entering the lateral intercellular spaces via transcellular and/or paracellular pathways, using aquaporin water channels to maximize osmotic permeability of the system, thus also to maximize water uptake. The legend for channels, symports exchangers and active pumps is the same for all figures.

that a precipitate of divalent salts forms in the posterior intestinal lumen. Marine fish thus significantly contribute to global ocean carbon cycles (Wilson *et al.*, 2009). The bicarbonate secretion eliminates equivalents of base from the blood; the precipitation of magnesium carbonate fixes the magnesium in a form that cannot be reabsorbed; and the precipitation effectively removes osmotic activity from the lumen so that more NaCl and water can be reabsorbed to conserve body water. Whole-body acid/base balance thus is affected and encourages secretion of acid equivalents at the gill to balance the base secretion by the intestine. Also in the posterior intestine, fluid and mineral *secretion* can be induced (Marshall *et al.*, 2002a), and the parahormone guanylin is thought to evoke this response physiologically (Marshall *et al.*, 2002a; Takei and Yuge, 2007). Although apparently anomalous for normal hydromineral balance, this response could be analogous to temporary secretory diarrhoea and functional in purging the intestinal contents in case of infection. In sum, the normal reabsorption of NaCl and water by the oesophagus and intestine recovers the water needed for osmoregulation but loads the body with NaCl. It is therefore crucially important for the NaCl load to be excreted elsewhere, specifically at the gill epithelium.

Salt excretion and role of the gills

The gill epithelium is an extremely large surface area that is highly perfused by blood, making it the organ where the animal is most intimately exposed to the environment (Marshall, 2002; Evans *et al.*, 2005; Marshall and Grosell, 2006). Even though the gills have a low osmotic permeability, the large area that must be available for gas exchange provides a significant avenue for osmotic water gain, accounting for the majority of the total osmotic water flow in the animal (Evans *et al.*, 2005).

The gill epithelium essentially is constructed of relatively non-differentiated, flattened epithelial cells that are tightly joined to each other by low-permeability tight junctions, 'pavement cells' (Evans *et al.*, 2005). These tight junctions restrict ion and water permeability and thus slow the diffusive gain of salt and osmotic loss of fluid in seawater (Marshall and Grosell, 2006). Tight junctional proteins, claudins from Tncldn genes, are present in fish kidney, gills, skin and intestine. Renal and intestinal tissues express all four Tncldn3 genes, while the gills and skin specifically express Tncldn3a and Tncldn3c (Bagherie-Lachidan *et al.*, 2008). Interspersed between pavement cells in the interlamellar region of the gill filaments are numerous mitochondrion-rich (MR) cells, also known as ionocytes and chloride-secreting cells (Marshall, 2002; Marshall and Singer, 2002; Evans *et al.*, 2005; Marshall and Grosell, 2006). These MR cells also appear in the skin and opercular epithelium of euryhaline species, such as gobies (e.g. *Gillichthys mirabilis*), killifish (e.g. *Fundulus heteroclitus*), blennies (e.g. *Blennius pholis*) and mudskippers (e.g. *Periopthalmodon schlosseri*), where these accessory osmoregulatory structures aid in hydromineral balance. The seawater MR cells (Fig. 11.2) have a vastly elaborated basolateral membrane surface area in the form of a micro-tubular system of invaginated basolateral membrane (Marshall and Grosell, 2006). In this membrane is the sodium pump (Na^+,K^+-ATPase), which indirectly provides the driving force for salt secretion by developing and maintaining a high transmembrane Na^+ gradient, favouring Na^+ entry into the cell across the basolateral membrane (Mancera and McCormick, 2000; Marshall, 2002; Evans *et al.*, 2005). Secondarily, and with the aid of basolateral K^+ channels, there is a negative inside electrical potential, predicted to be –60 to –80 mV but as yet not directly measured. Also present in the basolateral membrane is the Na^+,K^+,2Cl^-cotransporter (NKCC1), a symporter that translocates Cl^- into the cell using the driving force of the Na^+ gradient (Marshall, 2002). Thus Cl^- accumulates above its electrochemical equilibrium point in the cell. Salt secretion is effected by a second step at the apical membrane, where anion selective channels, cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall *et al.*, 1995; Singer *et al.*, 1998), are present in a patchy distribution across a cup-shaped apical membrane surface, known as the apical crypt, which is much smaller in area than the basolateral membrane (Marshall and Singer, 2002; Marshall *et al.*, 2002b; Evans *et al.*, 2005). Chloride ions exit through these channels following the favourable electrical gradient and 'uphill' into the higher concentration of the environmental seawater. In this way Cl^u exits the animal. To maintain electroneutrality, Na^+ follows but by a different route: that of the paracellular pathway.

The paracellular pathway in the MR cell complex exists as a localized zone of cation-selective leaky junctions that are between MR cells and their immediate neighbours, accessory (or adjacent) cells (Sardet *et al.*, 1979). The accessory cells are less mitochondrion rich and may represent immature MR cells in this dynamic multicellular salt gland (Evans *et al.*, 2005). Na^+ in the lateral intercellular space is driven out of the animal by a favourable electrical potential of approximately +40 mV, sufficient to effect the secretion of Na^+ down its electrical gradient into seawater (Guggino, 1980). Measured trans-body electrical potentials in seawater (Marshall and Grosell, 2006) are usually positive with respect to the environment, but are lower than +40 mV because of the shunting effect of the large surface area of the gill.

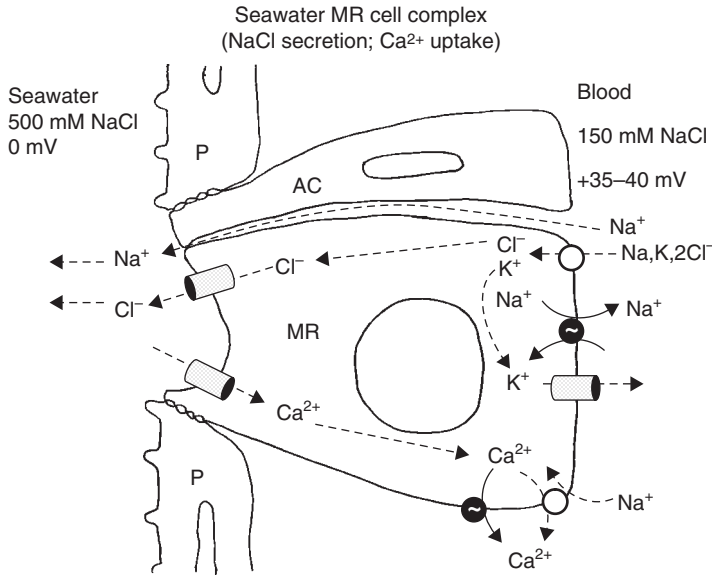


Fig. 11.2. Seawater mitochondrion-rich (MR) cell of a strong hypo-osmoregulator (e.g tilapia (*Oreochromis mossambicus*), killifish (*Fundulus heteroclitus*), sea bass (*Dicentrarchus labrax*), salmon (*Salmo salar*), flounder (*Platichtys flesus*), eel (*Anguilla anguilla*)). The basolateral Na^+,K^+,$2Cl^-$ symport (NKCC1) translocates a neutral complex of the four ions, driven by the transmembrane Na^+ concentration gradient. The Na^+ gradient is in turn established by the basolateral Na^+,K^+-ATPase. Because the K^+ taken up by this process recycles across the basolateral membrane through K^+ channels and Na^+ is pumped out across the basolateral membrane, the net effect of NKCC1 operation is to increase intracellular Cl^- levels. Cl^- then diffuses to the apical membrane and down its electrochemical gradient through CFTR anion channels and into seawater at the apical crypt of the cell. The sodium ion follows a paracellular pathway between MR cells and adjacent cells (AC) through cation-selective leaky intercellular junctions down the electrochemical gradient of approximately +40 mV. The salt secretion is highly regulated by phosphorylation/activation of NKCC and CFTR and by the ability of these cells to shut down secretion by retraction of the cell and paving over by pavement cells (P), an effect that minimizes ion loss in dilute environments. Also shown here is a parallel pathway for Ca^{2+} uptake (also shown in the freshwater MR cell models).

Regulation of salt transport in seawater

Rapid regulation of NaCl secretion includes many potential hormones and neurotransmitters (Marshall, 2007). In addition, MR cells are known to be osmosensitive, inhibiting NaCl secretion if the cells are osmotically swollen (Marshall *et al.*, 2000, 2005b) and stimulating NaCl secretion if the cells are shrunken osmotically (Zadunaisky *et al.*, 1995). These volume changes require a high osmotic permeability to the cell membrane and apparently are aided by basolateral expression of aquaglyceroporin AQP3 (Cutler and Cramb, 2002; Lignot *et al.*, 2002; Watanabe *et al.*, 2005; Cutler *et al.*, 2007). Consistent with this lack of AQP3 expression, the apical membrane of the gill epithelium has lower osmotic permeability than the basolateral membrane (Hill *et al.*, 2004). Agents, hormones and neurotransmitters that augment cAMP all increase the rate of NaCl secretion, including urotensin I, β-adrenergic agonists, arginine vasotocin (AVT), glucagon and vasoactive intestinal polypeptide (VIP). Downregulation of salt secretion is mediated physiologically by α-adrenergic agonists including adrenalin, as well as by hormones and neurotransmitters thus far only potentially physiological, including acetylcholine, urotensin II, nitric oxide (NO), prostaglandin E2 and endothelin

(McCormick *et al.*, 2000; Evans *et al.*, 2005; Marshall, 2007). Importantly also, hypotonic shock rapidly and directly inhibits salt secretion, a response that is protective of body ions for animals that move into dilute environments and experience dilution of the blood as a result (Marshall *et al.*, 2005b).

These rapid responses overlie the more fundamental adaptive hormonal responses that change the total cellular composition of the gill epithelium. Seawater acclimation involves a multihormonal response, with primary elevations in plasma cortisol along with growth hormone and mediators of growth, such as insulin-like growth factor (IGF1) (McCormick, 2001). For instance, in an *in vitro* system of sea bream (*Sparus sarba*) gill, growth hormone and IGF1 caused an increase in expression of Na^+,K^+-ATPase subunits α and β and augmented enzyme activity (Deane and Woo, 2005). In seawater adaptation, cortisol is both a stress-responsive hormone and a seawater-adaptive hormone. Cortisol apparently acts in seawater acclimation through glucocorticoid-like receptors sensitive to RU486, as opposed to mineralocorticoid receptors sensitive to spironolactone (Marshall *et al.*, 2005a; Shaw *et al.*, 2007). Glucocorticoid receptors are upregulated by adaptation of tilapia (*Oreochromis mossambicus*) to seawater (Dean *et al.*, 2003). Elevations of cortisol by artificial administration can evoke increases in MR cells even in freshwater animals, but, in concert with growth hormone and seawater exposure, evoke upregulation of necessary seawater-adaptive transport proteins, such as Na^+,K^+-ATPase, CFTR and NKCC1 (McCormick *et al.*, 2000; McCormick, 2001).

Role of the kidney in seawater adaptation

Marine teleost fishes produce small volumes of isotonic urine in renal systems that often lack glomeruli in the kidney. The renal system is secretory in the proximal regions of the tubule and tends to be reabsorptive in the distal regions. In particular, the proximal tubule actively secretes Mg^{2+} (Beyenbach, 2000) and SO_4^{2-} (Pelis and Renfro, 2004) (Fig. 11.3). The urinary system also appears to aid in secretion of organic acids. Urine production volume is minimized to conserve body water, and the kidney tubules in seawater have higher expression of AQP1 than in freshwater kidney (Giffard-Mena *et al.*, 2007), consistent with isosmotic volume reabsorption. Meanwhile, renal AQP3 expression seems insensitive to salinity change (Deane and Woo, 2006). The urinary bladder (for the species that have this structure) is an operational extension of the kidney and may be useful in recovering NaCl and water before urine in released.

Ion and Water Balance in Freshwater Teleost Fishes

Water balance and the role of the kidney and urinary bladder

Freshwater teleost fish gain water osmotically across the large surface area of the gill and through fluid absorbed from food across the intestine. The osmotic gain of water, mostly from branchial osmotic flow, must be compensated by excretion of fluid elsewhere. The kidney of freshwater fish has glomeruli and a high glomerular filtration rate, with the glomerular filtrate being isotonic with the plasma (Marshall and Grosell, 2006). Freshwater kidney tubules have lower osmotic permeability than their seawater counterparts, resulting in only small amounts of water reabsorbed and a urine flow rate that is effectively governed by glomerular filtration rate. The water channel AQP1 is expressed at a lower level in freshwater than in seawater kidney and AQP3 is absent (Giffard-Mena *et al.*, 2007), consistent with the lower osmotic permeability. NaCl reabsorption via NKCC2α and NCC symports (Cutler and Cramb, 2008) results in the production of large volumes of dilute urine, typically with approximately 10–20 mM NaCl (Fig. 11.4). Most freshwater fish have a urinary bladder, which operates as an accessory site for the further recovery of NaCl without further reabsorption of water (Marshall and Bryson, 1991; Marshall

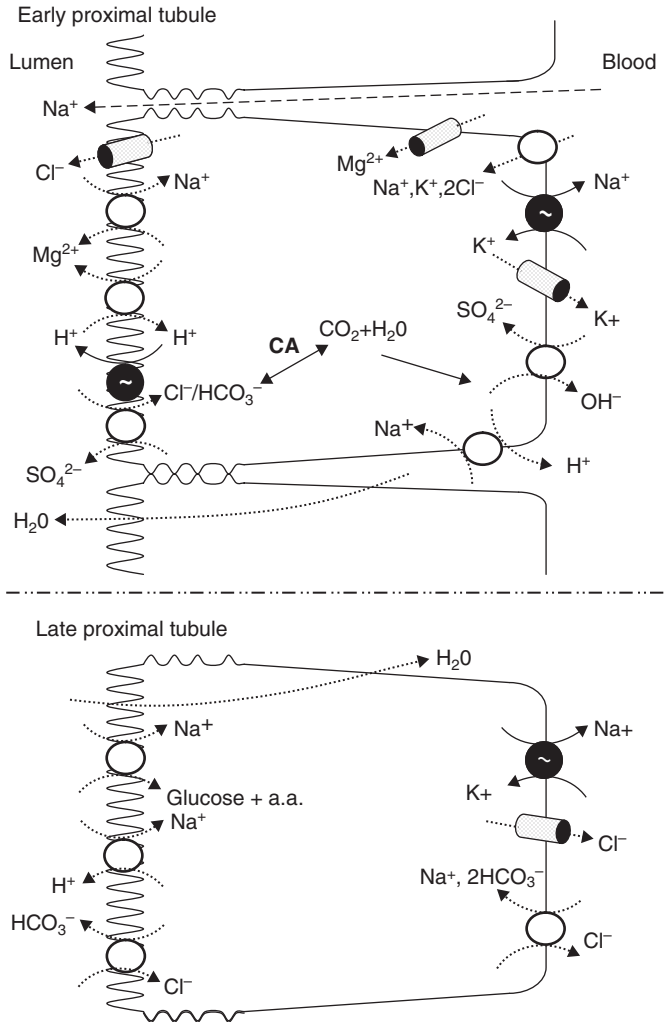


Fig. 11.3. Renal salt and water transport by the proximal tubule of marine fish is secretory. In the early proximal tubule (upper panel), secretion of Mg^{2+} and sulfate is linked to fluid secretion in this segment. In the late proximal tubule (lower panel) there is Na^+ uptake linked to glucose and amino acid uptake and fluid reabsorption and Cl^- uptake linked to bicarbonate secretion, with carbonic anhydrase (CA) as the source of bicarbonate. The basal Cl^- channels are as yet unidentified. The extra NaCl load absorbed here is presumably excreted by the gill.

and Grosell, 2006). The electroneutral NaCl uptake in the urinary bladder, a model for the operation of the distal nephron, apparently involves Na^+–H^+ exchange in parallel with a neutral NaCl (Marshall, 1986; Marshall and Bryson. 1991) mediated in eels by NKCC2β (Cutler and Cramb, 2008). The osmotic permeability of the urinary bladder is extremely low, such that the calculated concentration of the absorbed fluid in brook trout is strongly hypertonic, 1.56 M NaCl (Marshall, 1987). After this final salt uptake in the urinary bladder, the resulting release of urine often has very low NaCl levels, 2–5 mM, so that the net effect for the animal is that excess fluid absorbed

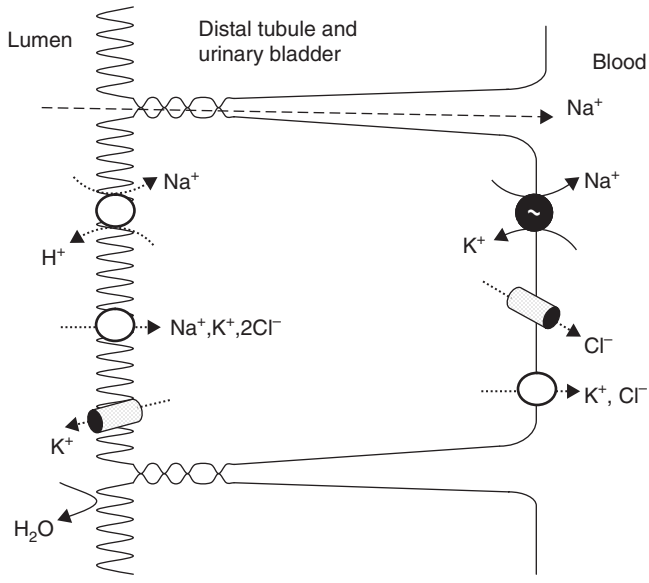


Fig. 11.4. Electroneutral Na^+ and Cl^- uptake in the distal tubule and urinary bladder involves Na^+,K^+,$2Cl^-$ co-transport (NKCC2), in part, and possibly NaCl neutral transport (NCC). Part of the Na^+ uptake is amiloride sensitive, suggesting involvement with Na^+–H^+ exchange. Ion uptake continues with a minimum of accompanying water, as aquaporins are absent. This process can draw down salt content in the final urine to less than 1.0 mM NaCl. The effective absorbate concentration is much higher than that of the blood (up to 1.5 M NaCl).

osmotically at the gill is effectively excreted by the kidney with a minimum of salt loss from the body.

Salt uptake and the role of the gills

Unlike the single accepted mechanism for NaCl secretion in marine teleosts, to absorb NaCl to freshwater fish, several different types of NaCl uptake appear to have evolved. Some of these mechanisms are sufficient to support active ion uptake from extremely dilute and ion-poor fresh water, while other mechanisms are only operational for NaCl uptake in low-level brackish water and hard fresh water. In other extreme cases, such as alkaline (Wilkie and Wood, 1996) and acidic (Gonzalez *et al.*, 2002) fresh water, more unique specializations may be revealed.

The accepted mechanism for NaCl uptake by salmonid, cyprinid and anguillid fishes uses a combination of the vesicle-type proton ATPase (V-type H^+-ATPase) and Na^+ channels in one cell type and Cl^-–HCO_3^- exchange and base secretion in another cell type to absorb NaCl (Fig. 11.5). V-type H^+-ATPase is present in the apical membrane of an acid-secreting subpopulation of gill epithelial MR cells (Goss *et al.*, 2001; Galvez *et al.*, 2002). These cells are distinguished as being peanut lectin-negative (PNA–) cells, which can be separated from peanut lectin-positive (PNA+) MR cells by their binding to peanut lectin and magnetochromatographic separation (Galvez *et al.*, 2002)). H^+-ATPase pumps acid equivalents across the apical membrane and out of the animal while generating a large, theoretically up to 100 mV, transmembrane electrical gradient. A parallel sodium channel in the same membrane then allows Na^+ in the boundary layer of mucus overlying the epithelium to be reabsorbed passively. A second pump step at the basolateral membrane recovers the Na^+ into the blood via Na^+,K^+-ATPase (Galvez *et al.*,

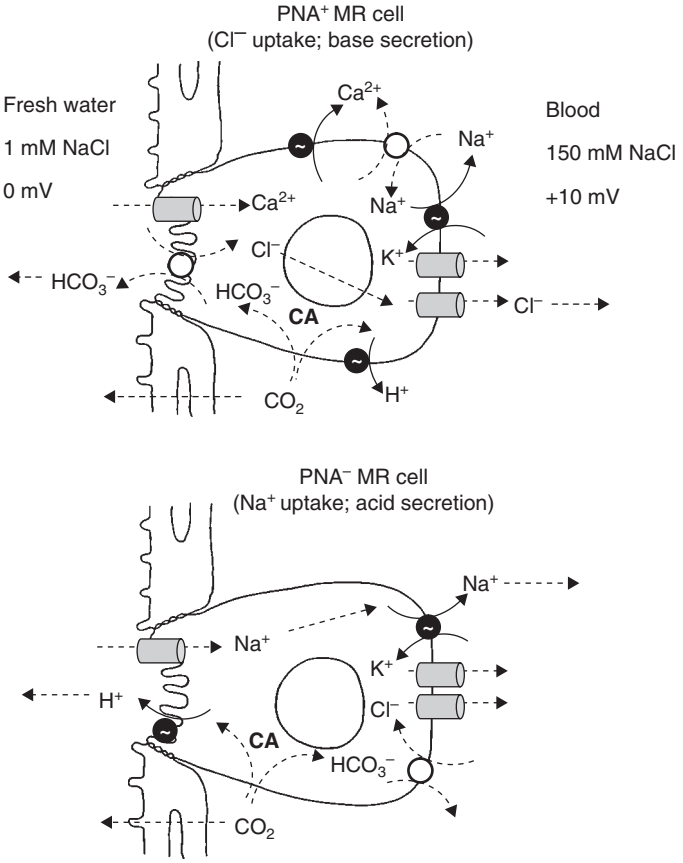


Fig. 11.5. Freshwater mitochondrion-rich (MR) cells of a typical strong hyper-osmoregulator, such as salmonid fish, goldfish (*Carassius auratus*), tilapia (*Oreochromis mosambicus*) and zebrafish (*Danio rerio*). There is a high-affinity salt-uptake mechanism in the gills, divided between two types of specialized ion-uptake cells: one that binds peanut lectin (PNA–) and links acid secretion with sodium uptake (upper panel), and the other (PNA$^-$) cell type, which links base secretion with chloride uptake (lower panel). The two operate at approximately the same rates to maintain acid/base balance, but can be experimentally manipulated to operate unequally by acid or base loading of the animal. The high affinity of the Na^+ uptake system allows these animals to adapt permanently to fresh water with environmental Na^+ concentrations less than 1 mM and with low water hardness. The PNA+ cells are also thought to be involved in Ca^{2+} uptake. CA, carbonic anhydrase.

2002; Evans *et al.*, 2005; Marshall and Grosell, 2006). This dual-pump system can move Na^+ up very large apparent transepithelial gradients and effectively allows these animals to live in ion-poor fresh water. This system is depicted in Fig. 11.3. Thus, in one cell type both acid secretion and Na^+ uptake occur.

To balance the uptake of Na^+ and secretion of acid by the PNA– cells (above), a parallel set of base-secreting PNA+ cells accounts for the secretion of base and the uptake of Cl^- (Fig. 11.5). The uptake of Cl^- occurs in exchange with HCO_3^- via the well-known anion-exchange process that is sensitive to disulfonic stilbenes (DIDS). The Cl^- accumulates intracellularly and thence passes into the blood across the basolateral membrane via a system of anion channels. This is believed to occur through the PNA+

cells of the gill epithelium (Galvez *et al.*, 2002). Thus far the identity of these basolateral anion channels is unknown but could be either CFTR or members of the CLC anion channel family.

The high level of expression of AQP3 in freshwater gill epithelia (Cutler and Cramb 2002; Deane and Woo, 2006; Cutler *et al.*, 2007; Giffard-Mena *et al.*, 2007), which is present especially in the basal area of MR cells (Lignot *et al.*, 2002), suggests that there is high water permeability in MR cells, but as there appears to be no expression in the apical membrane, the overall osmotic permeability of the epithelium can still be held to low levels. Because teleost fish have some urea metabolism and as AQP3 is an aquaglyceroporin permeable to urea (Ishibashi *et al.*, 1997), the expression of AQP3 may aid in urea excretion by the gill (McDonald and Wood, 1998).

Dietary salt and the role of the intestine

The role of diet in freshwater osmoregulation is especially important and has been recently reviewed (Ferreira and Baldisserotto, 2007). Freshwater teleost fishes do not drink (Marshall and Grosell, 2006), thus minimizing gastrointestinal water uptake, but the food has significant water content. The posterior intestine of FW-adapted sea bass expresses less AQP1 than does the SW counterpart, and the anterior gut has no AQP1 expression (Giffard-Mena *et al.*, 2007), indicating low water permeability of the gut. In addition, AQP3 expression in FW intestine is not in the enterocytes, but rather in other cell types (Lignot *et al.*, 2002). As a result, intestinal osmotic permeability is low and water reabsorption from food by the freshwater intestine is limited. Thus, the principal osmoregulatory role of the intestine in freshwater fish is to absorb salt.

Dietary salt is generally beneficial to freshwater fish osmoregulation (Ferreira and Baldisserotto, 2007). The uptake of Na^+, K^+ and Cl^- by the stomach and intestine results in 80–90% reabsorption of K^+ and Cl^- but only negligible net absorption of Na^+ (Bucking and Wood, 2006). Sodium chloride uptake may be aided by basolateral expression of the anion channel CFTR (Marshall *et al.*, 2002a). Uptake is presumably via passive processes at the apical membrane driven indirectly by the Na^+ and K^+ gradients established by Na^+,K^+-ATPase at the basolateral membrane of the enterocytes and by processes not materially different from NaCl uptake in marine fish (see above). There is efficient Ca^{2+} intestinal absorption; hence dietary calcium can reduce the need for calcium uptake by the gills (Ferreira and Baldisserotto, 2007). Dietary NaCl can evoke seawater-type changes to the gill, indicating that the animal can respond exclusively to internal salt balance changes (Perry *et al.*, 2006) .

Classical experiments on salinity acclimation have been performed on animals denied food. Recently the role of diet in osmoregulation has attracted more attention, especially with reference to caloric intake and dietary salt intake. There are studies now emerging of manipulation of salt acclimation by alterations in dietary salt. Dietary salt is thought to be protective during stresses of low pH, when passive NaCl loss is increased and Na branchial uptake reduced (D'Cruz and Wood, 1998; Morgan *et al.*, 2000). Dietary salt can reduce uptake and toxicity of heavy metals such as copper (Kamunde *et al.*, 2005). It is generally appreciated that augmentation of diet is beneficial to animals subjected to stresses of salinity change or low pH. The dietary supplementation presumably fulfils the energy requirements of cell growth and replacement in transporting epithelia (Morgan *et al.*, 2000). Dietary supplements also aid the smolting process (see below) particularly, as this process is a more general morphogenesis involving many tissues. Euryhaline teleost fish are often unable to adapt to ion-poor environments unless they receive dietary salt supplements.

Hormones of freshwater osmoregulation

The major hormone associated with the low permeability of the gill and skin, as well as with enhanced NaCl uptake, is prolactin, which has more than 300 functions ascribed

to it (McCormick, 2001; Manzon, 2002). On hormone binding, prolactin receptors dimerize, and signal transduction occurs via the JAK/STAT signalling pathway. The main action of prolactin in fish is freshwater osmoregulation, although it has also been implicated in reproduction, behaviour, growth and immunoregulation (Power, 2005). Transfer of euryhaline pufferfish (*Takifugu rubripes*) to dilute media upregulates prolactin gene expression, while downregulating GH mRNA (Lee *et al.*, 2006). In sea bass (*Dicentrarchus labrax*), ovine prolactin reduces gill Na^+,K^+-ATPase, while cortisol increases gill Na^+,K^+-ATPase, consistent with the freshwater function of prolactin (Mancera *et al.*, 2002). Exposure of flounder to hyposmotic conditions causes dilution of the plasma, which apparently evokes increased expression of prolactin receptors and AVT receptors (An *et al.*, 2008). Cortisol, under some conditions, may promote proliferation of freshwater-type MR cells and ion uptake, and interacts with prolactin during acclimation to fresh water (McCormick, 2001). A recent review (Manzon, 2002) summarizes the functions and introduces modern data using measurement and effects of homologous prolactin. AVT is known to enhance NaCl secretion and renal water conservation in teleost fish (Balment *et al.*, 2006).

Ion and Water Balance in Euryhaline Teleost Fishes

Euryhaline teleost fishes can quickly adapt to large changes in salinity. Only a small number of species have this physiological ability but among them are commercially important anadromous salmonid, clupeid and anguillid fishes. These animals change salinity just a few times in their life cycle, generally to move upstream into fresh water to spawn and migrate downstream into seawater as juveniles or smolts. This evolutionary strategy appears to take advantage of the lower number of predators of larvae in these habitats. Estuarine resident euryhaline fish, such as gobies, killifish, flounders (e.g. *Platichthys flesus*), sculpins (e.g. *Leptcottus armatus*), mudskippers (e.g. *P. schlosseri*) and stickleback (e.g. *Gasterosteus aculeatus*) instead have the ability to change salinity on a daily basis, often driven by voluntary movements to feed (Marshall, 2003) as well as seasonally to spawn. Often in a taxonomic group, such as the genus *Fundulus*, there are various species with different ranges of salinity tolerance, ranging from freshwater stenohaline through to weakly and strongly euryhaline (Griffith, 1974). In exceptional cases, species, e.g. killifish, *F. heteroclitus*, within these groups have evolved the ability to deal with strongly hypersaline conditions (Griffith, 1974).

The cellular mechanisms and organ function for euryhaline teleost fishes to adapt to seawater and hypersaline conditions are largely the same as for stenohaline marine teleosts, except that strongly euryhaline species can overexpress MR cells and survive hypersaline conditions (Evans *et al.*, 2005). However, the strategies to adapt to low salinities are substantially different from that for stenohaline freshwater animals. One major difference is in the placement of the H^+-ATPase enzyme in the basolateral membrane; as is true of killifish (Katoh *et al.*, 2003) and euryhaline elasmobranch fishes (Fig. 11.6). Also, this manifests as a reduced ability to adapt to soft, ion-poor fresh water, because the ion uptake pathways have low affinity (Patrick *et al.*, 1997; Burgess *et al.*, 1998). Another difference is the well-developed osmotic responses in euryhaline fish, such that the MR cells respond to changes in plasma osmolality (Marshall, 2003; Marshall *et al.*, 2005b, 2008b; Fiol and Kültz, 2007). Euryhaline teleosts serve as ideal models for regulation of hydromineral balance, as the act of salinity transfer evokes the requisite hormonal, osmotic and transporter changes and attendant changes in gene expression (Burnett *et al.*, 2007). Changes in blood osmolality evoke upregulation of transporter (CFTR, Na^+, K^+-ATPase and NKCC) expression as well as important regulators, such as glucocorticoid-inducible kinase (SGK) (Shaw *et al.*, 2007), and transcription factors osmotic stress transcription factor 1 (OSTF1) and transcription factor II (TFIIB) (Fiol and

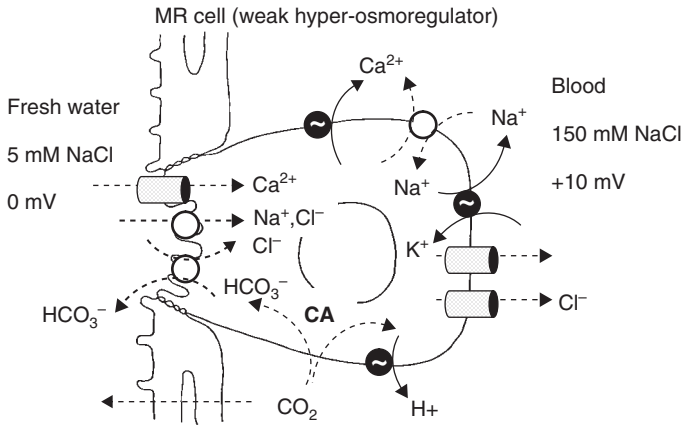


Fig. 11.6. Freshwater mitochondrion-rich cells of a typical weak hyper-osmoregulator, such as euryhaline teleost fish (killifish (*Fundulus heteroclitus*), sea bass (*Dicentrarchus labrax*), flounder (*Platichthys flesus*), gobies (*Gillichthys mirabilis*), pufferfish (*Tetraodon nigroviridis*) and sculpin (*Leptocottus armatus*)), have a variety of ion-uptake mechanisms. Here the hypothetical uptake by the gills of euryhaline estuarine animals faced with ion regulation in dilute environments is depicted. The apical membrane NaCl co-transport (possibly by NKCC2 or NCC) operation is linked with active transport at the basolateral membrane (Na^+,K^+-ATPAse). In some euryhaline teleost species, H^+-ATPase exists in the basolateral membrane, which presumably creates a large transmembrane potential, which can drive Cl^- uptake from the cells into the blood with the aid of a basolateral anion channel (CFTR or CLC type, as yet unidentified), even if the intracellular Cl^- is at low levels. The ion-uptake mechanisms are low affinity overall and require environmental NaCl above 5 mM. In addition, these animals may require high levels of water hardness and dietary salt input to cope in these dilute environments. The MR cells are also involved in Ca^{2+} uptake. CA, carbonic anhydrase.

Kültz, 2005, 2007). In killifish, exposure to hypotonic conditions reduces blood osmolality, which results in shutdown of salt secretion by MR cells but also their retraction below the surface of pavement cells so that the passive ion permeability as well as salt secretion pathways are eliminated during the temporary excursions into fresh water (Daborn *et al.*, 2001). Having a commercial species that is euryhaline is sometimes an advantage, as salinity change can be used to help condition the animals for market (such as transfer of trout to seawater to improve appearance, taste and texture) or to treat animals against possible parasites or pathogens (Marshall *et al.*, 2008a).

Osmoregulation in Hatched Embryos

Surface area issues

Prior to hatching, the vitelline membrane and chorion protect the developing embryo from environmental variations in salt and osmotic pressure (Finn, 2007). After hatching, the embryos must be prepared to osmoregulate immediately. The very large surface area to volume ratio of the embryos serves in favour of the animal in terms of gas exchange, but quite the reverse for ionic and osmotic homeostasis. Post-hatch, the yolk-sac membrane was thought to be a passive barrier to ion exchange between the embryo and the environment, but the membrane is actively involved very early in ion transport and control of osmotic permeability. The embryo must maintain as low osmotic permeability and ionic conductance as possible. The skin epithelium covering the embryo and yolk sac accordingly is made up of pavement cells with well-developed tight junctions. The pavement cells are not involved to any large extent in ion transport but would be suitable for gas exchange, given their flattened shape. Ion-transporting mitochondrion-rich cells first occupy the skin and yolk sac (Kaneko *et al.*, 2002;

Varsamos *et al.*, 2002), and as the gill develops the progressively more MR cells appear in the gill (Pisam *et al.*, 2000). The osmoregulatory challenge of embryos is an important early stress for the embryo.

Role of the chorion

The chorionic membrane serves as a surrogate gill osmoregulatory structure in yolk-sac embryos, where the gills are unformed and the kidney is a pronephros (Lin and Hwang, 2004; Varsamos *et al.*, 2005). In marine animals the chorionic membrane is populated by MR cells and is fully operational in salt secretion. The MR cells are effectively indistinguishable from those that later appear in the gill epithelium. In some unique experiments, the yolk sac was separated from the embryo, the so-called 'yolk ball' preparation, and continued to secrete salt, similar to the condition *in situ* (Shiraishi *et al.*, 2001). These yolk balls can respond to salinity changes and to hormonal stimuli (Hiroi *et al.*, 2005). The voltages measured across the yolk-sac epithelium in the absence of the gill surface area, which is a pathway for diffusive ion fluxes, are thought to approximate the 'real' transepithelial voltage across the intercellular tight junctions of the paracellular shunt pathway that is the Na^+ exit pathway. Because these yolk-sac transepithelial voltages in seawater, which are not partially shunted by the gill, are +40 mV or more (Guggino, 1980), there clearly exists plenty of electrical driving force to propel Na^+ exit from a plasma Na^+ activity of 160 mM through the localized leaky junctions of the paracellular pathway and into full-strength seawater at 1200 mM.

The osmotic (water) permeability of the yolk sac is very low, thus protecting the embryo from osmotic water gains and losses (Hagedorn *et al.*, 1997). The transition from yolk-sac embryo to juvenile is critical to survival, as the intestine, feeding, renal, circulatory and gill functions all come into increased functionality in short order (Varsamos *et al.*, 2005). Shortly after hatching, when the gastrointestinal tract becomes operational, the intestinal enterocytes are already expressing essential transport enzymes such as Na^+, K^+-ATPase (Giffard-Mena *et al.*, 2006). Failure especially to feed at this stage results in death, probably through metabolic and osmoregulatory failure.

Recently, zebrafish embryos have proven to be powerful sources of identification of freshwater ion transporters because of the genetic manipulations possible. The NaCl uptake transporter is now identified as SLC12A10.2, expressed in a special cell type (NCC) separate from the H^+-ATPase-rich (HR) ionocytes (Wang *et al.*, 2009), demonstrating that strong hyperosmoregulators can function with NaCl uptake instead of the Na^+ channel model (Fig. 11.5). H^+-ATPase in freshwater ionocytes (Figs 11.5 and 11.6) can be upregulated by exposure of zebrafish embryos to pH 4 water, specifically in the HR cells (Horng *et al.*, 2009). Now the identity of the anion channel responsible for Cl^u uptake across the basolateral membrane of freshwater ionocytes (Figs 11.5 and 11.6) has been identified as the SLC26 anion channel, again using the zebrafish embryo system (Bayaa *et al.*, 2009).

Smolting in Salmonid Fishes

The importance of salmonid aquaculture, and especially the introduction of cage- and land-based culture of naturally anadromous Atlantic (*Salmo salar*) and Pacific (e.g. chinook, *Oncorynchus tshawytscha*) salmon, including rainbow (steelhead) trout (*Oncorynchus mykiss*), puts emphasis on the parr–smolt transformation, thus the area has been well reviewed (McCormick, 2001; Björnsson and Bradley, 2007). The parr–smolt transformation (also called smolting or even 'smoltification') occurs in young river-inhabiting salmonid parr prior to downstream migration in the spring from freshwater rivers through estuaries and into seawater as smolts (Björnsson and Bradley, 2007). Whereas some salmonid species undergo smolting at a large body size, the rapid development of seawater osmoregulatory ability in early juveniles of some salmon species (pink (*Oncorynchus gorbuscha*), chum (*Oncorynchus keta*) and

sockeye salmon (*Oncorynchus nerka*)) demonstrates that the protracted smolting process that chinook, coho (*Oncorynchus kisutch*), Atlantic (*S. salar*) and masu salmon (*Oncorynchus masou*) undergo is not the only successful developmental pattern. Also, landlocked salmon (Nilsen *et al.*, 2007) do not develop seawater tolerance at all, as they fail to augment sufficiently the transporters and enzymes needed for salt secretion. Aquaculturists controlling parr movement must give the right cues to initiate the smolting process and introduce the animals to seawater at the correct time. Advancing photoperiod (Björnsson 1997; Boeuf and Le Bail, 1999; Handeland and Stefansson, 2001) and increasing temperature (McCormick *et al.*, 2000; Bottengard and Jorgensen, 2008) are the major natural cues for the process. Smolting involves diverse changes for the animal to adapt to the marine habitat, including silvering of the skin, preadaptation of the gills for salt secretion, renal changes, gastrointestinal alterations and even changes in eye pigment. Smolting is a metamorphic change controlled by multiple hormones (McCormick, 2001), primarily thyroid hormone, growth hormone (GH) and insulin-like growth factor (IGFI). Pivotal to the process is the early spring preadaptive development of the capacity in the gill epithelium to secrete salt, through the development of seawater-type MR cells (Nilsen *et al.*, 2007). This preadaptation has been frequently monitored by measuring gill Na^+, K^+-ATPase, as *de novo* expression of this transport enzyme is essential to salt secretion (Borgatti *et al.*, 1992; D'Cotta *et al.*, 2000). Arctic charr (*Salvelinus alpinus*) respond to increased temperature not by smolting but by somatic growth (Bottengard and Jorgensen, 2008), pointing to advancing photoperiod as an important cue for the process. In smolts, expression of the protein and its appearance in the basolateral membrane of MR cells follows the upregulation of the corresponding gene mRNA after a long delay, about 11 days (D'Cotta *et al.*, 2000). Concomitant rises in NKCC and CFTR follow that of Na^+, K^+-ATPase (Nilsen *et al.*, 2007). Without this preadaptation stage, animals transferred prematurely to seawater suffer loss of water and lethal rises in plasma NaCl. Post-smolts introduced to estuaries move rapidly to sea on ebb tides and maintain groupings (Lacroix *et al.*, 2004, 2005).

Hormones of the parr–smolt transformation

The suite of hormones involved in the smolting process speaks of its complexity. Thyroid hormone activity increases progressively during smolting, and failure of the thyroid to activate can cause failure of the process and death as parr. As the photoperiod increases in spring (Boeuf and Le Bail, 1999), in nature the animal moves downstream, but regardless of whether the animal is captive or free, cortisol, GH (Pelis and McCormick, 2001) and IGF-1 become elevated and initiate the changes necessary in the gill epithelium (development of, but not yet emergence of, seawater-type MR cells), changes in the skin (particularly thickening and deposition of guanidine to produce silvering of the skin) and rapid somatic growth (Björnsson *et al.*, 2002). GH appears to act locally at the target tissue level to stimulate IGF-1 autocrine/paracrine action, and on the liver to increase plasma IGF-1 levels (Björnsson *et al.*, 2002). By the end of May the pre-smolts are ready, indeed preadapted, to enter the estuary and to operate in seawater as hypo-osmoregulators. At this point the thyroid activity plateaus and cortisol subsides, while GH surges, feeding behaviour becomes more aggressive and the animals grow quickly (Björnsson, 1997).

Failures of smolting

Premature introduction of parr or early pre-smolts to full-strength seawater is generally lethal, associated with the inability of these animals to secrete NaCl, and they die of osmoregulatory failure. However, if pre-smolts are held in fresh water into the summer, the preadaptation steps taken during the smolting process become at least partially reversed, and if the animals are exposed at this stage to seawater they

similarly cannot osmoregulate properly and die of osmoregulatory failure. Hence there is a window, developmentally and in time, that ensures success in smolt transfers to seawater. Premature release of smolts results in depressed appetite, which further compromises survival (Toften *et al.*, 2003). Exposure to acid stress also is highly detrimental to later survival in seawater (Staurnes *et al.*, 1996). In nature, the animals may undergo test exposures to high salinity in the estuary, but all indications are that the post-smolts move through even large estuaries in 12 h to a few days from first downstream migration (Lacroix *et al.*, 2004, 2005).

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12 Disorders Associated with Exposure to Excess Dissolved Gases

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Introduction and Historical Perspectives

Gas bubble disease (GBD) is a syndrome comprising a range of clinical signs and lesions arising as a sequel to the presence of excess dissolved gases in water. This disorder affects both aquatic vertebrate and invertebrate species (Goldberg, 1978; Elston, 1983) exposed to uncompensated, hyperbaric total dissolved gas pressure (TGP) (Bouck, 1980). In a previous review of GBD, Weitkamp and Katz (1980) summarized much of the earlier literature on GBD and GBD research. Much of this research dealt with GBD in fish that were downstream from hydroelectrical projects.

Since the 1970s the focus of GBD investigations has shifted. The potential for GBD to serve as an *in vivo* model for hyperbaric human medical problems has promoted an interest in understanding the physiological mechanisms of gas bubble formation and the subsequent pathophysiology. Second, the phenomenal growth in aquaculture has encouraged research into the practical management (and implications) of GBD within commercial enterprises. Although GBD persists as a problem in aquaculture, surprisingly little research on GBD has taken place within the last 15 years. An emerging area of interest is the role that high levels of dissolved oxygen may play in producing a GBD variant.

Environmental Situations in which Fish are Exposed to Elevated Total Dissolved Gas Pressure (TGP)

The three most abundant atmospheric gases and their respective partial pressures are nitrogen (78%), oxygen (21%), and argon (1%). Their solubility in water is determined by inherent factors, such as their mass and partial pressure, and environmental factors. For example, gas solubility relates inversely to water temperature and directly to hydrostatic pressure. Early studies suggested that nitrogen alone was the causative agent of GBD. The work of Rucker and Kangas (1974), Meekin and Turner (1974) and Dawley and Ebel (1975) provided a basis for Weitkamp and Katz (1980) to conclude that TGP was a better index than nitrogen partial pressure for determining the potential for GBD.

There are a variety of mechanisms through which water can develop TGP sufficient to cause disease in fish. Weitkamp and Katz (1980) referred to reports of elevated rates of photosynthetic activity leading to GBD. This is presumably generally due to elevated oxygen contributions to TGP. A specific demonstration of this has been reported by Doulos and Kindschi (1990). Air injection or entrainment of air into water is a frequently cited mechanism leading to GBD (Colt, 1986). Air injection/entrainment can

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occur accidentally through pipe valves, pipe fittings and incompletely submerged intakes leading to aquaculture facilities (Harvey and Smith, 1961). Additionally, air injection technology is becoming increasingly used in aquaculture, particularly with the current trends to increase stocking densities, loading rates and use of recirculated or serial-reuse waters. Multi-gas transfer models indicate that aerating systems with high transfer efficiency for oxygen also have high transfer efficiency for nitrogen and argon (Colt and Westers, 1982). Effectively, efficient aerating devices can cause elevated TGP. Paradoxically, oxygen-injection systems are becoming widely used as a means of decreasing nitrogen and TGP to below 100% while increasing oxygen levels (Marking, 1987). As a further concern, Edsall and Smith (1991) demonstrated that an oxygen-injection system could cause a GBD variant in rainbow trout directly through supersaturation of water with oxygen alone; this mechanism is partially reviewed in a very interesting paper by Salas-Leiton *et al.* (2008), in which they examine the physiological response of juvenile Senegal sole (*Solea senegalensis*) to hyperoxic conditions through a proteomic study in an effort to detect biomarkers specific to hyperoxia. Given the differences between GBD arising from inert gas as compared with physiologically available gas (such as oxygen), the GBD variant arising from excess oxygen will not be further reviewed here.

Weitkamp and Katz (1980) discussed air entrainment problems created by hydroelectric projects. Spillways mix water and air and carry the air into the depths of the plunge basin. The increased hydrostatic pressure in the plunge basin increases the gas solubility. As this water (frequently large volumes) flows away from the plunge basin into areas of less hydrostatic pressure, supersaturation develops (Harvey and Cooper, 1962; Westgard, 1964).

Bodies of water receiving intermittent thermal effluent from industry frequently become supersaturated. This reflects the inverse relationship of solubility with water temperature. Similarly, temperature manipulations in aquaculture settings create the same scenario. For example, cold saturated water brought into a facility and warmed in a closed delivery system will become supersaturated. Delivery of this water to fish-rearing vessels before gas equilibration leads to GBD.

An interesting case report by Hauck (1986) details an episode of GBD in pink salmon (*Oncorhynchus gorbuscha*) fry caused by rapid decompression from altitude changes during air transport. Helicopter transport of fry is a common means of stocking remote sites with juvenile hatchery-reared salmon. In addition to the typical signs of GBD, Hauck (1986) also described swimbladder hyperinflation, leading occasionally to rupture. Swimbladder deflation and negative buoyancy occurred during descent and recompression.

Spring and well water (groundwater) offer many advantages to fish culturists compared with surface water from streams, rivers and lakes. However, groundwater is frequently saturated with nitrogen (Weitkamp and Katz, 1980), which begins to come out of solution as the water naturally depressurizes when pumped to the surface. If this water is delivered to a fish farm through closed pipes, release of this excess nitrogen is not possible until the water reaches the fish-rearing vessel. Equilibration here leads to GBD in the fish. Seasonal fluctuations in TGP within groundwater are common and can occur after periods when aquifers have been replenished. Water flowing downwards into an aquifer can carry, or aspirate, air with it (Weitkamp and Katz, 1980). Accordingly, supersaturation problems stemming from the use of groundwater are often intermittent.

Clinical Manifestations of Gas Bubble Disease (GBD) in Fish

Population level

In an aquaculture setting, GBD generally presents as a population problem, with markedly variable expression within tanks and between tanks. Bouck (1980) demonstrated the differences between median and average survival time, suggesting a highly skewed population response, which would

become particularly pronounced with low levels of supersaturation. Different ages and sizes of fish react differently to elevated TGP (Weitkamp and Katz, 1980). Differences between species have also been noted, due to either anatomical differences, such as ability to regulate swimbladder volume (Chamberlain *et al.*, 1980), or apparent ability to detect and avoid supersaturated regions (McCutcheon, 1966; Gray and Haynes, 1977; Stevens *et al.*, 1980). In a tank of fish of uniform age and size, some may exhibit gross manifestations of the disease, others may appear unaffected. Bouck (1980) concluded that extreme levels of supersaturation, or very long exposure periods, would be needed to kill all members in a population.

Morbidity and mortality rates associated with GBD are largely dependent on the degree of supersaturation, the duration of exposure and husbandry methods used during recovery. Unchecked high levels of TGP can virtually depopulate a fish farm. Several such cases involving rainbow trout, brown trout and lake trout were referred to by Machado *et al.* (1987). Generally a diagnosis of the problem is made and corrective measures are put in place to reduce TGP. Exposed populations may continue to exhibit mortalities directly attributed to GBD or may succumb to secondary infections caused by stress or anatomical damage to the body surface; this mechanism was felt to be responsible for an outbreak of systemic streptococcosis on a South African trout farm (Huchzermeyer, 2003). Batzios *et al.* (1998) followed the effects of GBD within a trout farm and documents economically significant changes to growth rate and weight–length ratios stemming from a conversion from allometric growth to isometric growth. Accordingly, the prognosis varies with each case.

The relationship of levels of TGP with GBD varies with different fish species and ages. These were reviewed in Weitkamp and Katz (1980). Complete elimination of supersaturated conditions downstream from hydroelectric projects and from incoming groundwater to fish facilities is difficult. This has prompted interest in studying fish tolerance to minimally elevated TGP. Chronic exposure (weeks to months) to TGP at 102% of saturation or above is considered sufficient to cause GBD in at least some fish in a population. Higher levels are associated with a more rapid rise in morbidity rates. Discrepancies in the literature for tolerance levels reflect the influence of environmental factors during the bioassays (Bouck *et al.*, 1980). One consideration is depth of the holding tank during the exposure and the ability of fish to move to different depths in an attempt to avoid regions of supersaturation (Bouck, 1980; Knittel *et al.*, 1980; Stevens *et al.*, 1980; Lund and Heggberget, 1985). There are conflicting reports over the comparative tolerance of different life stages of fish. However, it is generally accepted that eggs are quite tolerant to elevated TGP (Weitkamp and Katz, 1980).

Individual level: fry

Sac fry with GBD are often forced to the water surface because of the imparted buoyancy. Gas bubbles can form between the yolk and the perivitelline membrane, as well as in the abdominal cavity, fins and cranium (Henly, 1952; Jones and Lewis, 1976; Stroud *et al.*, 1975; Cornacchia and Colt, 1984). Depending on the location of the bubble, affected fry could be head-up, tail-up or belly-up at the water surface. Henly (1952) describes the presence of gas bubbles in the lumen of the gut of herring larvae.

Individual level: juveniles and adults

It is common for fish dying of acute GBD to die without showing visible lesions (Machado *et al.*, 1987). Clinical behavioural signs in acutely affected animals include a sharp reduction in feeding, lethargy, loss of equilibrium and buoyancy, aimless swimming, sideswimming, whirling with interspersed periods of inactivity, and spasmodic convulsions (Lund and Heggberget, 1985; Machado *et al.*, 1987). Detecting gas bubbles in tissue of these fish can require subgross dissection and examination.

Uni- or bilateral exophthalmus is a classic subacute and chronic clinical sign of GBD in juvenile and adult fish with GBD (Fig. 12.1). However, in some experimental

and natural cases of GBD, exophthalmia was not evident (Edsall and Smith, 1991) or was not described as a significant feature (Pauley and Nakatani, 1967). When exophthalmus exists, it is frequently accompanied by bubbles in all chambers of the eye and in the sclera. Bilateral lesions result in blindness, which can lead to starvation. An interesting feature frequently noted in chinook salmon with GBD is the presence of gas bubbles within the blood vessels and soft tissues of the oral cavity – usually the roof of the mouth (Fig. 12.2); this causes fish to cough and ventilate heavily.

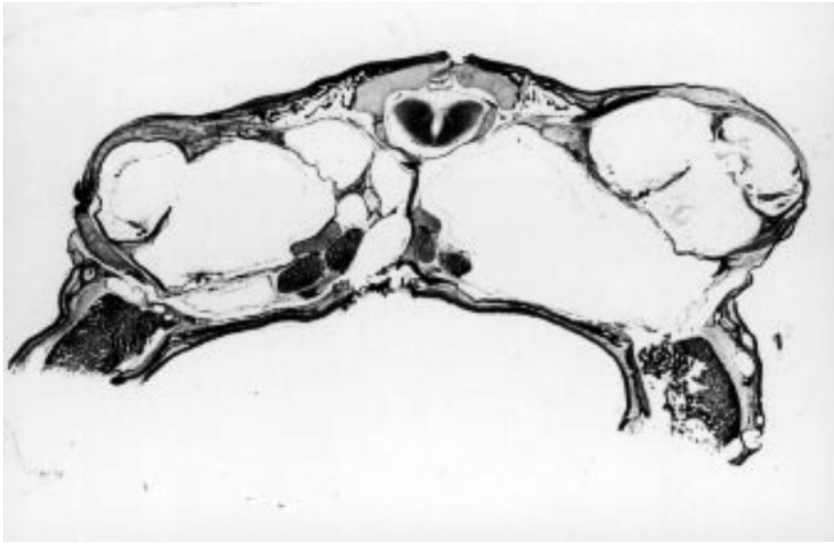


Fig. 12.1. Transverse section through the cranium of an arctic charr fingerling with subacute GBD. Bilateral severe exophthalmia and compression of the globe are due to large retrobulbar gas bubbles.



Fig. 12.2. Gross appearance of the numerous gas bubbles developing in the mouth of a chinook salmon fingerling with GBD.

Physiological events leading to the formation of gas bubbles

As pointed out by D'Aoust and Smith (1974), a critical feature that distinguishes GBD from decompression disease (DCS) of divers is that the supersaturation gradients are in reverse during the period in which clinical signs develop. In GBD, supersaturated water gradually supersaturates the fish. In DCS, tissues which have taken on excess gas during a dive, release the gas when they are 'decompressed'. This difference is a critical feature for evaluating GBD as model for the study of DCS pathology and disease management in man. It may be less critical for studying some of the basic phenomena of bubble initiation and growth, and secondary effects common to both DCS and GBD.

The physical aspects of intravascular gas bubble formation and growth have been reviewed by Strauss (1979). Initial formation of intravascular gas bubbles requires either a stable nucleus, such as a pre-existing small stable pocket of gas, or a zone of decreased surface tension. The latter would include an aqueous–lipid interface. Bubble growth requires that the total gas pressure inside the bubble exceeds the combination of forces restricting its growth. The latter include surface tension of the bubble itself, ambient atmospheric pressure and pressure exerted by surrounding host tissues. Once a bubble forms and grows, the gas within it develops an equilibrium with gas in the surrounding medium or tissue (Strauss, 1979). Thus bubble growth or shrinkage is affected by perfusion and clearance rates of gases from different tissues.

Strauss (1979) characterized body tissues as being either 'fast' or 'slow' in their rate of uptake (and subsequent loss) of gas from circulation. Tissues that are well perfused, such as the brain, are described as fast. Conversely, poorly perfused tissues, such as fat, are described as slow. Based on these differences it is predicted and noted that during acute decompression, gas bubbles initially develop in 'fast' tissues. Of interest, although bubbles develop more slowly in 'slow' tissues, they are more persistent once they develop. This is because of reduced rates of clearance (reflecting reduced blood flow) in addition to local and systemic redistribution of gases from fast to slow tissues.

Extending from the previously listed mechanisms for bubble formation, growth and persistence, the contrasting nature of the distribution of gas bubbles in fish with (typical) GBD with that in mammals with DCS is predictable. Most cases of DCS involve a single event (or several repeated but temporally distant events) of acute exposure to markedly elevated levels of inert gas supersaturation in blood and tissue. Accordingly, bubbles develop in the vasculature and preferentially in well-perfused tissues. Following decompression, excess gases are eliminated. Clinical signs relate either to the acute pathophysiological events associated with vascular damage or to primary tissue destruction (for example in the CNS) from space-occupying lesions (SOLs). Most cases of GBD in fish result from chronic (or intermittent/repeated) exposure to minimal elevations of TGP. Work by Machado *et al.* (1987) showed that at saturation levels typically associated with GBD in aquaculture situations, mortalities did not begin until several days after exposure began. Bubbles will initially develop in vasculature and in well-perfused tissues (Fairbanks *et al.*, 1969; Machado *et al.*, 1987) as they do in DCS. Additionally, there is a greater chance in GBD, as compared with DCS, of slow tissues becoming the site for bubble development and persistence, and particularly for bubble growth stemming from redistribution of excess gas from fast to slow tissues. An example of this is the sequential ocular pathology during experimental chronic low-level GBD in salmonid fishes (see later section in this chapter). In the eye, SOLs arise in highly perfused vascular tissues in the acute stages of GBD, but subacutely and chronically are replaced by SOLs in poorly perfused connective tissues (Speare, 1990).

Pathophysiological effects and sequelae related to gas bubbles in selected organs

Vasculature

Intravascular gas emboli develop during natural and experimental GBD in fish (Renfro,

1963; Smith, 1988; Edsall and Smith, 1991) (Fig. 12.3). Vascular occlusion of large branchial vessels by gas bubbles has been cited as the cause of death during GBD (Smith, 1988; Edsall and Smith, 1991). In DCS, vessel occlusion has also been cited, but generally only involving vessels of small diameter, such as in the skin and joints. In DCS, vascular pathology and evoked clotting and inflammatory cascades, rather than vessel occlusion, has been advanced as a major pathophysiological event (Levin *et al.*, 1981; Tanoue *et al.*, 1987; Francis *et al.*, 1989). Continued study of both diseases will probably suggest more similarities than differences.

Thrombogenesis and endothelial damage

Cellular thrombi are now known to accompany intravascular gas bubbles in DCS (Philp, 1974; Levin *et al.*, 1981; Tanoue *et al.*, 1987) and GBD (D'Aoust and Smith, 1974; Smith, 1988; Speare, 1990, 1991) (Fig. 12.4). Whereas clinical disease in DCS is mechanistically related to the formation and effects of both gaseous and cellular thrombi (Levin *et al.*, 1981; Tanoue *et al.*, 1987; Francis *et al.*, 1989), the implications of cellular thrombi forming during GBD remain hypothetical (Smith, 1988; Speare 1990, 1991).

During DCS, cellular thrombi are believed to be triggered by endothelial damage (Warren *et al.*, 1973). Additionally, activation of clotting mechanisms, leading to various degrees of disseminated intravascular coagulation (DIC), has been described during DCS (Levin *et al.*, 1981; Tanoue *et al.*, 1987). Casillas *et al.* (1975) have shown a similar activation of clotting cascades in fish with GBD. The link between intravascular gas bubbles, development of cellular thrombi and DIC may represent several factors acting alone or in concert in both diseases. Direct effects of gas bubbles on endothelium and platelets, as well as indirect effects of factors released from damaged cells, have been advanced for DCS as a trigger mechanism for DIC (Warren *et al.*, 1973; Levin *et al.*, 1981; Tanoue *et al.*, 1987). For example, there is ample experimental evidence to show that intravascular gas bubbles arising during DCS can directly damage endothelium (Warren *et al.*, 1973; Mason and Balis, 1980). Endothelial damage also



Fig. 12.3. Intravascular dermal gas bubbles typical of GBD. SEM, ×200.

occurs directly adjacent to intravascular gas bubbles during experimental GBD of fish (Speare, 1991) (Figs 12.5–12.7). Damage to endothelium, particularly widespread damage, is a classically recognized trigger for DIC, either indirectly through exposure of subendothelial collagen or directly by activation of the clotting cascade.

Local indirect effects of gas bubbles may also contribute to endothelial damage. For example, endothelial damage during DCS has been linked to the action of leucocytes

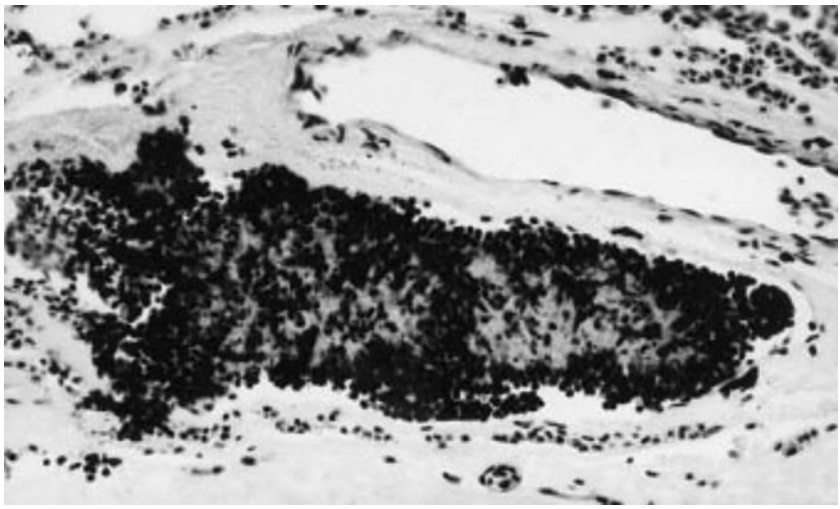


Fig. 12.4. Thrombus within the retinal vein of a fingerling chinook salmon with GBD. Haematoxylin and eosin stain, ×196.

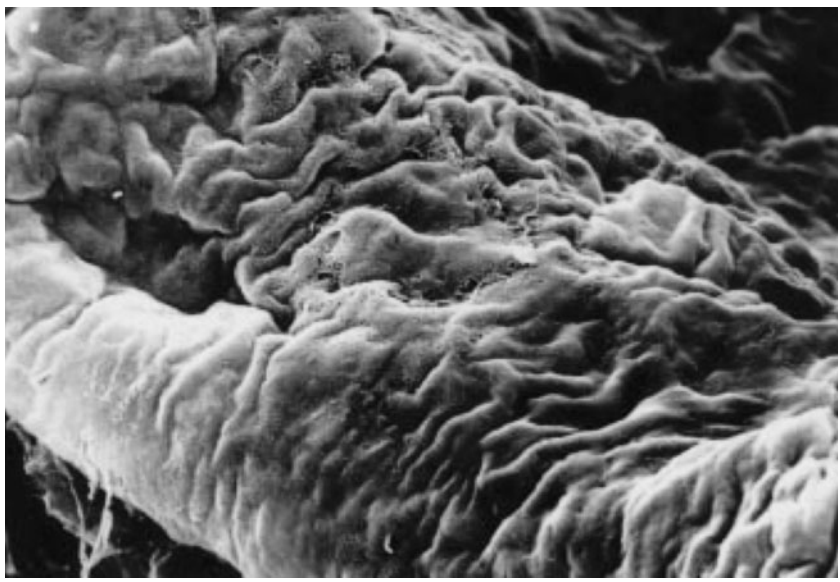


Fig. 12.5. Endothelium of a dermal blood vessel distended by a gas bubble. Surface pitting is pronounced on some degenerate endothelial cells and intercellular junctions are attenuated. SEM, ×2000.

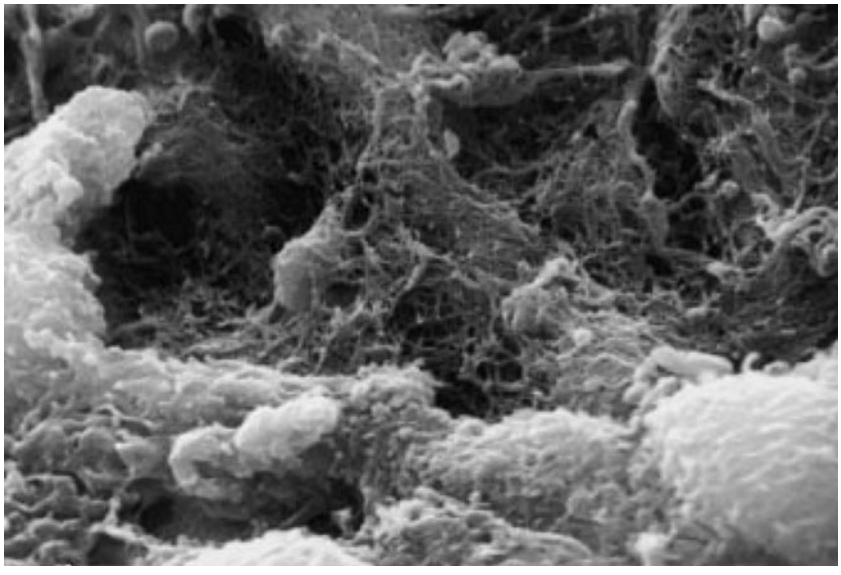


Fig. 12.6. Exposure of subendothelial connective tissue in a dermal blood vessel distended by a gas bubble. Remaining endothelial cells are severely swollen and vesiculated. SEM, ×4100.

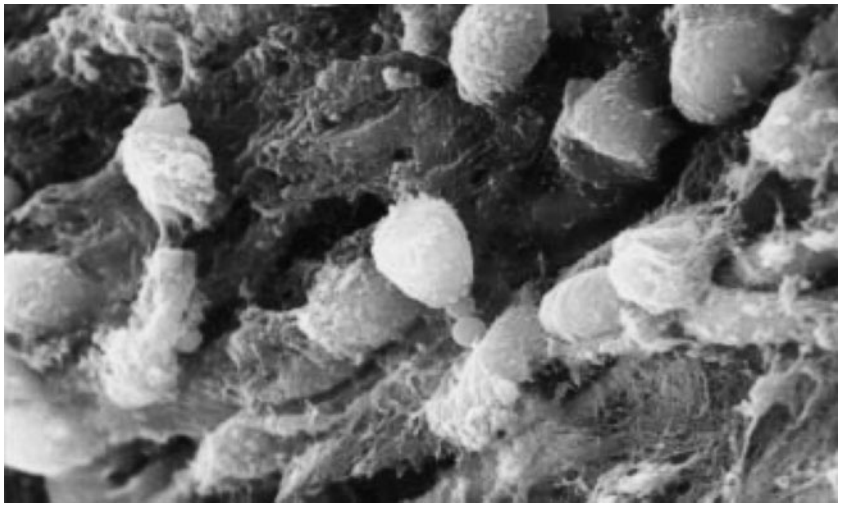


Fig. 12.7. Exposed subendothelial connective tissue sparsely covered by small round cells and strands of fibrin-like material. SEM, ×12,300.

and platelets that become adherent to the endothelium (Flick *et al.*, 1981; Levin *et al.*, 1981). Endothelial damage is also related to leucocyte emigration through vessel walls proximate to arrested bubbles (Stewart *et al.*, 1974). Catron *et al.* (1984) have advanced microvascular permeability resulting from leucocytic damage as the cause for pulmonary oedema during DCS. A substantial perivascular leucocytic response with oedema has also been shown proximate to gas emboli in GBD in fish (Speare, 1991),

which suggests a similar pathogenesis as in DCS.

An alternative mechanism for endothelial damage is that large gas bubbles (when comprising inert gases) may completely inhibit blood flow, leading to anoxic injury to the subjacent endothelium. This suggestion is supported by the apparent sequential cellular pathology of endothelial cells undergoing degeneration and death proximate to intravascular gas bubbles during GBD. This included marked exocytotic vesiculation and pitting of the apical membrane with cell swelling (Speare, 1991) (Figs 12.5 and 12.6), which is typical, although not pathognomic, for endothelial anoxia (Mason and Balis, 1980).

Further study would be useful to elucidate the mechanisms involved in vascular injury during GBD in fish, both as an advancement of GBD as an *in vivo* model for DCS and to determine points of therapeutic intervention for outbreaks of GBD.

Eyes

In determining the cause and effects of ocular lesions attributed to GBD, it is useful to categorize lesions into: (i) those directly attributable to supersaturation and the resulting SOLs, i.e. primary lesions; and (ii) those lesions that represent secondary host responses.

Grossly apparent ocular lesions include exophthalmia (uni- or bilateral), corneal and lenticular degeneration, haemorrhage and enucleation. Histological descriptions further this by demonstrating keratitis and uveitis (Hoffert *et al.*, 1971; Speare, 1990), retinal separation and degeneration (Smith, 1988; Speare, 1990), SOLs within the choroid of the posterior uvea (Hoffert *et al.*, 1971; Machado *et al.*, 1987; Smith, 1988; Speare, 1990), and optic neuritis (Speare, 1990).

The temporal progression of these lesions helps to illustrate the relative roles of supersaturation and secondary host responses. The following is based on occurrences of GBD in farm-reared salmonids (rainbow trout, brook trout, arctic charr and chinook salmon) as well as an artificially recreated and maintained GBD (115–124% TGP) episode affecting rainbow trout and chinook salmon (Speare, 1990).

ACUTE CHANGES (1–4 DAYS). Acute lesions included mild exophthalmia accompanied by a minor expansion of the equatorial anatomical axis arising from the compressive effect on the globe of SOL in the choroid gland of the posterior uvea. This is similar to the acute lesions described by Machado *et al.* (1987). SOLs displaced the retina and choroid anteriorly into the vitreous cavity. True retinal separation, defined as displacement of the retina from the retinal pigment epithelium, was not noted. However, SOLs that developed subjacent to the basement membrane of the retinal pigment epithelium (RPE) led to separation and anterior displacement of the RPE (and attached retina) from the remainder of the posterior uvea.

SUBACUTE CHANGES (5–10 DAYS). The equatorial anatomic axis of the eye became moderately expanded and accompanied dramatic exophthalmia. Large retrobulbar gas bubbles developed and replaced bubbles in the posterior uvea (Fig. 12.1). The orbit became detectably compressed along its anterior–posterior diameter (Fig. 12.1). Anterior displacement of the iris against the corneal endothelium occurred, and this was accompanied by focal or extensive anterior synechia anchored by proliferated fibrocytes (Fig. 12.8). Lenticular cataracts developed during this phase, characterized by hydropic degeneration of lens epithelium and separation of subjacent lens fibres. Suppurative panuveitis was also a feature of this subacute phase. The cornea became moderately spongiotic, mildly eroded and infiltrated with a small number of neutrophils. Periocular dermis, in contrast, was more richly invaded by neutrophils, particularly around dermal SOLs.

CHRONIC CHANGES (2–6 WEEKS). The degree of exophthalmia continued to worsen with chronicity. This led to a range of lesions not previously noted in subacute phases, such as marked attenuation of the optic nerve,

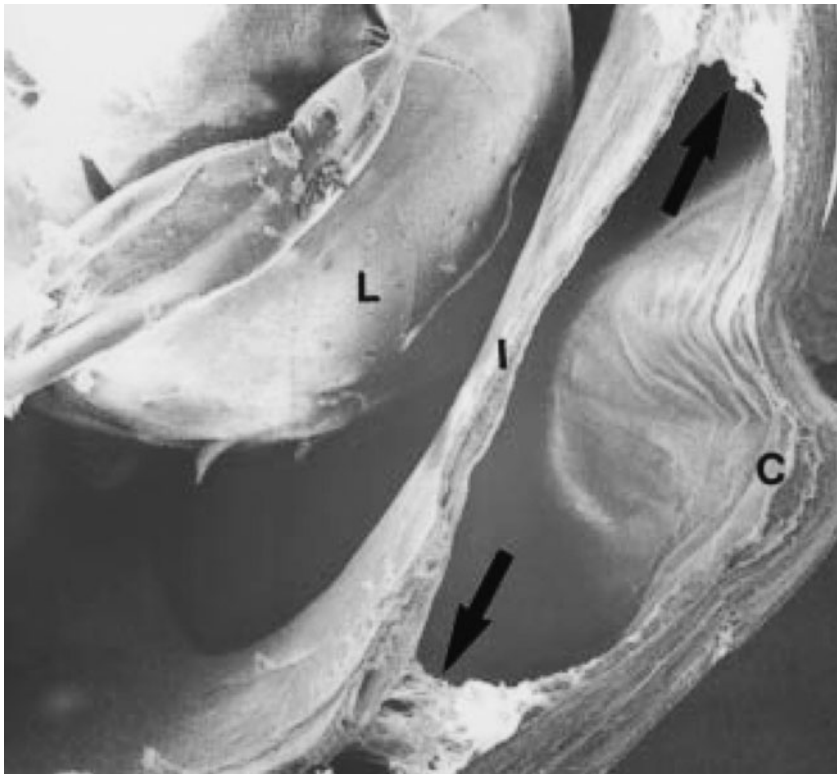


Fig. 12.8. Cryofractured section of an eye from a rainbow trout with GBD in which anterior synechiae (arrows) have developed between the anterior surface of the iris and the inner surface of the cornea. SEM, ×34. L, lens; I, iris; C, cornea.

retinal artery and retinal vein. In some cases, the globe was markedly deformed due to dramatic posterior coning of the retina, subjacent choroid and sclera. Optic neuritis was noted. Retinal changes also developed and included thinning of the nerve fibre layer and a reduction of the cell numbers in the ganglion cell layer (Fig. 12.9), as had also been described by Smith (1988).

Corneal lesions advanced to a suppurative stromal keratitis with neovascularization, scattered pigmentation and, in some cases, corneal ulceration. More advanced lenticular lesions also developed and included necrosis of lens epithelium with subjacent cataractous fragmentation of lens fibres into Morgagnian globules (Fig. 12.10). Lysis of the lens with rupture of the lens capsule and release of lens contents into the ocular cavities was a sporadically noted feature and was always accompanied by a suppurative endophthalmitis. Fish with phthisis bulbi (Fig. 12.11) and either uni- or bilateral enucleation were encountered. Ulcerative keratitis with perforation and eversion of globe contents, accompanied by suppurative and fibrosing panophthalmitis and formation of staphylomae, was typical for phthitic globes.

PERSISTENT EYE LESIONS NOTED DURING RECOVERY FROM GBD. Some fish managed to survive despite either phthisis bulbi or ocular enucleation. These changes resolved via extensive fibrotic replacement of the globe and finally re-epithelization of the defect. Other less dramatic sequelae during recovery from GBD included persistent anterior synechia (iris to corneal endothelium), persistent corneal

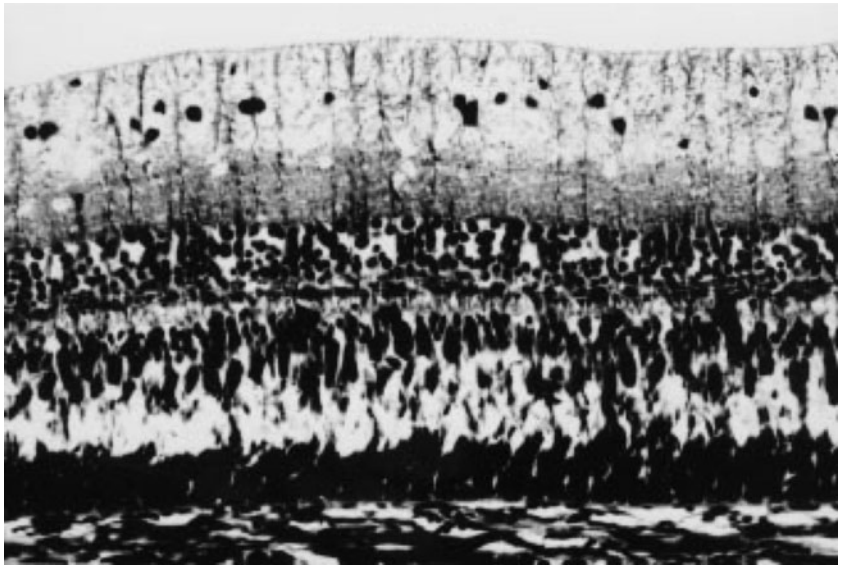


Fig. 12.9. Attenuated nerve fibre layer and reduced cellularity within the ganglion cell layer of the retina, associated with exophthalmia in a fingerling rainbow trout. Haematoxylin and eosin stain, ×175.

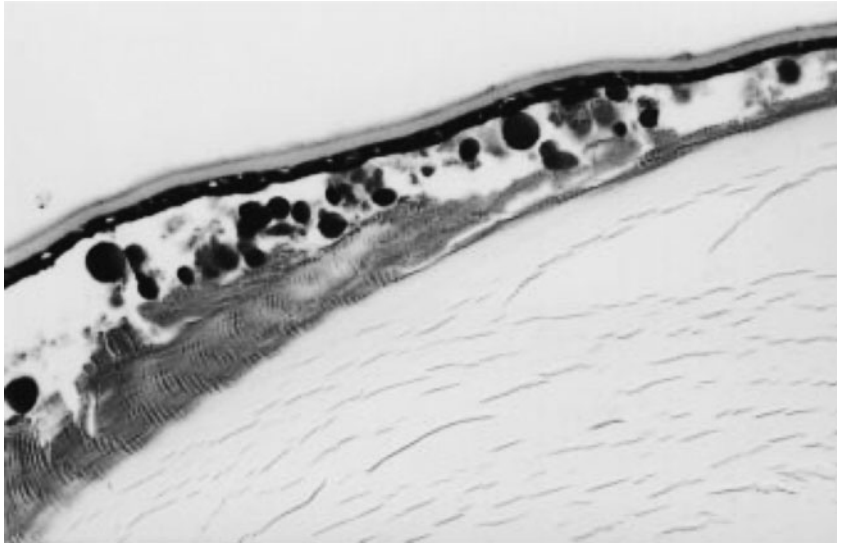


Fig. 12.10. Fragmentation of peripheral lens fibres into Morgagnian globules. Haematoxylin and eosin stain, ×280.

cataracts, and suppurative panophthalmitis with hyphema. Separation of the RPE from the choroid was noted to be persistent in some fish during recovery, particularly where SOLs had become filled with haemorrhage.

Persistent retro-orbital changes were common during recovery. These included fibrosis, suppurative perineuritis and thrombosis of the retinal artery and vein, accompanied by perivasculitis.

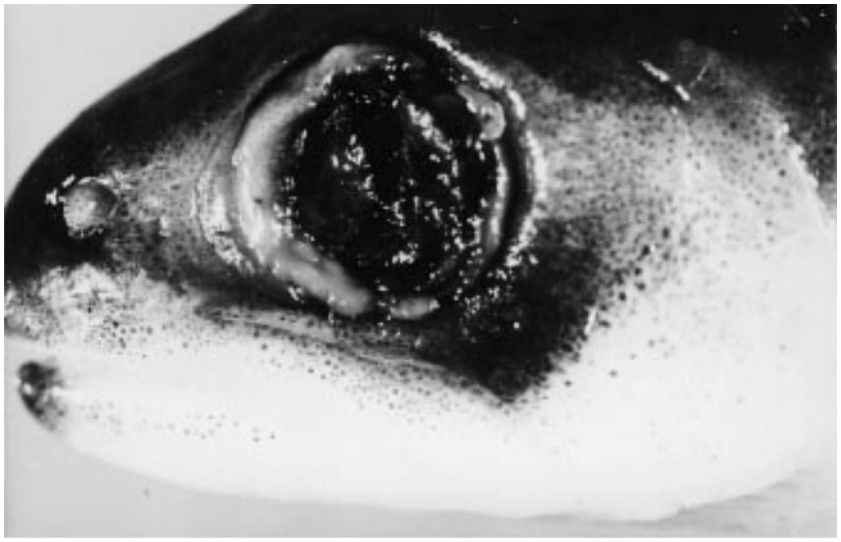


Fig. 12.11. Phthisis bulbi in a chinook salmon chronically affected with GBD.

Much of the sequential ocular pathology noted during and following GBD episodes reflects stereotypical patterns of ocular pathophysiology (Speare, 1990) common to many serious ocular diseases. As such, detection of the tissue responses alone during diagnostic investigations is suggestive rather than pathognomic for GBD. Detection of intraocular gas bubbles, in the absence of evidence of ocular infections with gas-producing bacteria, is pathognomic but is not invariably present.

Gills

SOLs frequently develop within the afferent and efferent vasculature, as well as the central venous sinusoid of the gill, during GBD (Rucker, 1953; Machado *et al.*, 1987; Smith, 1988; Edsall and Smith, 1991). These facilitate diagnosis because they are readily detected. Other gill lesions from GBD are less well defined. Pauley and Nakatani (1967) described apparent oedematous separation of the epithelial layers of the gill lamellae during GBD. This compares well with DCS, in which pulmonary oedema develops through increased vascular permeability. Whether or not the same mechanism applies to GBD is unknown.

Outbreaks of infectious gill disease are not uncommon during recovery from GBD (L. Hammell, Department of Health Management, Atlantic Veterinary College, personal communication). Whether this reflects primary or secondary damage to gill epithelium (currently not described) or reduced resistance to disease in general, through physiological stress, is unknown.

Skin

The presence of SOLs within the skin or dermis is common in fish with GBD. Dermal SOLS are present intravascularly and extravascularly (Fig. 12.3) and, in both locations, elicit a neutrophilic inflammatory response (Speare, 1991). Scanning electron microscopy of the epithelium overlying dermal SOLs detected epithelial erosions several cell layers thick (Speare, 1991) (Fig. 12.12). Epithelial erosion may provide a mechanistic link to explain the reported relationship of episodes of GBD and subsequent outbreaks of infectious skin diseases (Rucker, 1953; Stroud *et al.*, 1975; Stroud and Nebeker, 1976), particularly when opportunistic agents are involved. GBD was determined to be a risk factor for *Tetrahymena* sp. infections of guppies, *Poecilia reticulata*, at a

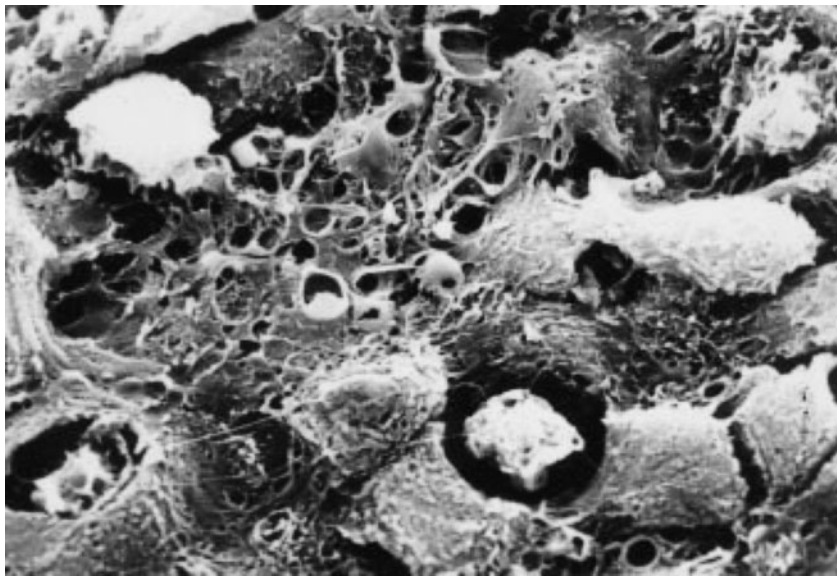


Fig. 12.12. Severely eroded skin overlying a gas bubble. SEM, ×2000.

commercial ornamental fish farm (Pimenta Leibowitz *et al*., 2005).

Summary and Perspectives

Diseases associated with supersaturation in fish have established themselves as common and persistently recurring problems associated with water management and delivery strategies. This applies to feral fish stock and fish held in captivity for aquaculture or research. Because of the pathophysiological sequelae related to GBD, this disease can have major economic consequences to fish producers and can be a major source of artifact in fish physiology and production research. Consequently, management of saturation levels to avoid GBD is strongly recommended in any situation where supersaturation (even if intermittent) is predicted. Data-logging and acquisition systems are available to enable documentation of saturation levels throughout the day and over weeks and months. Workers experienced with these systems stress the intermittent (daily and seasonal) nature of supersaturation problems. Retrofitting of most existing facilities to incorporate degassing systems is possible. Design of new facilities should incorporate degassing and gas monitoring as integral parts of the system (Bouck *et al*., 1980).

Similarities between GBD and DCS should continue to be examined. As an *in vivo* model of DCS, GBD has the potential to be a useful model to study the effects of intravascular SOLs on endothelium and also the physiological cascades initiated by SOL–endothelial interaction.

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13 Welfare and Farmed Fish

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Introduction

Until relatively recently there was little concern over the welfare of farmed fish stocks, but with the rapid expansion of this food sector and more public concern over the welfare of farmed animals, farmed-fish welfare has become an important consideration. There is now a much greater awareness of the requirements for fish welfare by the aquaculture industry, government, pressure groups, researchers and the public.

Much of the interest in fish welfare was driven in the UK by a report by the Farm Animal Welfare Council (FAWC) in 1996, and a review of fish welfare was carried out by the United States Department of Agriculture in 2003 (USDA, 2003). The FAWC report was critical of many aspects of the farming of fin fish, particularly regarding stocking densities, environmental conditions and killing practices. This report was very influential in driving change within the aquaculture industry and also focusing attention on the paucity of research into fish welfare. The report also helped to raise awareness with government bodies and retailers, which has led ultimately to the establishment of various retailer and industry codes of practice, such as the Code of Good Practice for Scottish Finfish Aquaculture and the inclusion of fin fish in recent UK welfare legislation – The Animal Welfare Act (2006) (Department for Environment Food and Rural Affairs, London)–which requires that keepers of animals ensure that their welfare needs are met.

Fish welfare has also been the subject of much recent scientific research; for example, the work of Sneddon *et al.* (2003) investigating pain responses in fish has very convincingly demonstrated that fish have the capacity not only to feel pain but to show conscious awareness of that pain, i.e. that the pain response in fish is not just an automatic reflex action. The need to concern ourselves with the welfare of the fish under our care has therefore been stimulated by increased public awareness, scientific research, and ethical concerns of various bodies, and also with the understanding that, as with other food animals, paying attention to the welfare of farmed fish results in improved health and productivity, lower levels of damage and disease, and fewer mortalities, which ultimately increases profitability.

The welfare of farmed fish has recently been reviewed by Branson (2008), and the reader is referred to this volume for an overview of the current state of knowledge. Fish welfare science is still in its infancy, and establishing parameters for monitoring the welfare of farmed fish can be very difficult; however, it is now generally accepted that

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fish should be viewed as being no different from other vertebrate animals when it comes to looking after their welfare and, although farmed fish present unique challenges, there are basic principals of animal welfare that can equally be applied to fish as to other animals.

An important framework for animal welfare was initiated by the Bramble (1965) report (Report of the Technical Committee to Ensure the Welfare of Animals Kept Under Livestock Husbandry Systems), when the principle of the 'five freedoms' of animal welfare was established, which was further developed by the newly established Farm Animal Welfare Council (FAWC) around 1979. Basically this principle asserts that the welfare of an animal can be addressed by applying five freedoms:

- Freedom from hunger, thirst and malnutrition
- Freedom from fear and distress
- Freedom from discomfort
- Freedom from pain, injury and disease
- Freedom to express normal behaviour.

These principles have recently been translated into 'five needs', namely the need to provide a suitable environment, the need to supply a suitable diet, etc. The 'five freedoms' can be applied to the welfare of farmed fish and are helpful in judging the welfare status of the fish under our care. There may be some crossover between some of the 'freedoms', e.g. conditions causing fear and distress may also cause discomfort or pain. There can also be some conflicts between the 'freedoms', e.g. allowing fish to exhibit normal behaviour may expose them to conditions in which they are more susceptible to pain and injury. These principles are therefore used as a general guide to allow a holistic approach to animal welfare. Many of the conditions and practices of aquaculture can have an impact on the welfare of the fish, ranging from direct damage due to poor handling to stress from the presence of predators, and this chapter sets out the welfare challenges of aquaculture in terms of how they have an impact on the 'five freedoms'.

Freedom from Hunger, Thirst and Malnutrition

Hunger

Farmed fish are reliant on their stock keepers to supply them with an adequate diet in terms of quantity and quality; the opportunity for feeding on natural foodstuffs in the aquatic environment is, in most circumstances, very small.

Fish may be deprived of appropriate or adequate feed through:

1. Underfeeding due to underestimation of biomass or feeding rate.
2. Inappropriate feeding practices limiting access of some fish to feed.
3. Competitive behaviour due to the presence of dominant fish, resulting from inadequate grading and excessive size variation.
4. Inappropriate physical character of the diet, such as pellets too large or too fast-sinking.
5. Insufficient knowledge of the requirements of novel aquaculture species.

Feed deprivation will lead to poor growth performance, lowered condition factor, increased susceptibility to disease and damage, and potentially increased aggressive behaviour; all are indicators of poor welfare.

Fish may be deliberately deprived of food for some management procedures, such as prior to transport or grading; prior to carrying out a treatment feed may be withdrawn for up to 48 h to reduce oxygen consumption and minimize faecal contamination of the water. This may well have welfare benefits in reducing stress to the fish but does highlight possible conflicts between the five freedoms. An occasion when feed is always withdrawn from farmed fish is immediately prior to harvesting, when the fish receive no feed for a period, which could be many days depending on harvesting practices. The reason feed is withheld is mainly to ensure that the intestine is empty of any food or faecal material and so avoid contamination during the gutting process and also possibly to give a firmer texture to the flesh (Einen and Thomassen, 1998). This practice is in direct conflict with the five

freedoms and, although it may be justified on the grounds of food safety, it does not give any obvious welfare benefit. To minimize the impact on fish welfare, feed must be withdrawn for a maximum period only to allow the gut to become empty; in salmon this period is no longer than 3 days (Robb, 2008).

Thirst

It may be thought that fish cannot suffer thirst, but there are situations where fish can become obviously dehydrated and this state can be equated with thirst. Dehydration may arise with fish in seawater when there is a disturbance of normal osmoregulatory ability. Normally, fish will balance osmotic water loss by drinking an equivalent amount of water, but Atlantic salmon (*Salmo salar*) that have not undergone complete smoltification before being transferred to seawater appear to be unable to control their water balance in this way and consequently become dehydrated; this is visible as a 'crinkling' down the body of the fish (author, personal observation). These fish will either die post-transfer or become 'failing smolts' with very poor growth and survival. To avoid this situation it is imperative that only fish that have completed smoltification be transferred to seawater and that appropriate monitoring be carried out prior to transfer. Sick or damaged fish, or those with skin deficits such as bacterial ulceration, may also suffer osmoregulatory disturbance, leading to dehydration in seawater (Stoskopf, 1993).

Malnutrition

Farmed fish require the provision of an adequate supply of the appropriate nutrients: namely, a correctly formulated diet to ensure that they do not suffer from deficiencies or an imbalance of essential nutrients. This topic is dealt with at greater length in Chapter 7, this volume. For salmonid species and the more established aquaculture species, there is a great deal of knowledge of the nutritional requirements of the animal, but with some of the species that are relatively new to aquaculture, nutritional information may be lacking, leading to potential malnutrition.

Even with apparently correctly balanced diets, deficiencies of certain nutrients can arise – risk factors appear to be rapid growth rates and increase in water temperature increasing the demand for some nutrients, which then become insufficient to meet the biological demand of the animal, and consequently deficiencies and related pathologies arise. An example of this is a jaw deformity in Atlantic salmon known as 'screamer disease', where the lower jaw is fixed in a gaping position. The cause has been identified as limited availability of phosphorus and vitamin C due to rapid growth rate and high water temperature (Roberts *et al.*, 2001). Rapid growth rates and high temperature have also been identified as major contributory factors in some cases of spinal deformity and cataracts (Fig. 13.1). It has been suggested that rapid growth rates override the availability of limited nutrients, leading to restricted skeletal development, which cannot 'keep up' with muscle growth, and spinal deformities, such as shortened tails ('stumpies') and humpbacks, occur as a consequence.

Other restrictions may be placed on the level of certain nutrients in the diet, leading to malnutrition; for example, there may be a requirement by environmental agencies to limit the level of dietary phosphorus in order to minimize the amount of phosphorus that is being discharged into the environment through waste feed and faeces and thus reduce potential eutrophication of a body of water (Stead and Laird, 2000). However, this level of dietary phosphorus may be insufficient to support skeletal development and deformities may occur (Baeverfjord *et al.*, 2009).

There is an increasing demand to replace the fish meal and fish oil in fish feed with more sustainable raw materials, such as soya protein or rape seed oil. It must be borne in mind, however, that some of these replacement ingredients may be unsuitable for some fish species, particularly carnivorous fish. Intestinal pathologies have been identified relating to the use of some alternative raw ingredients in salmon diets (Fig. 13.2), with

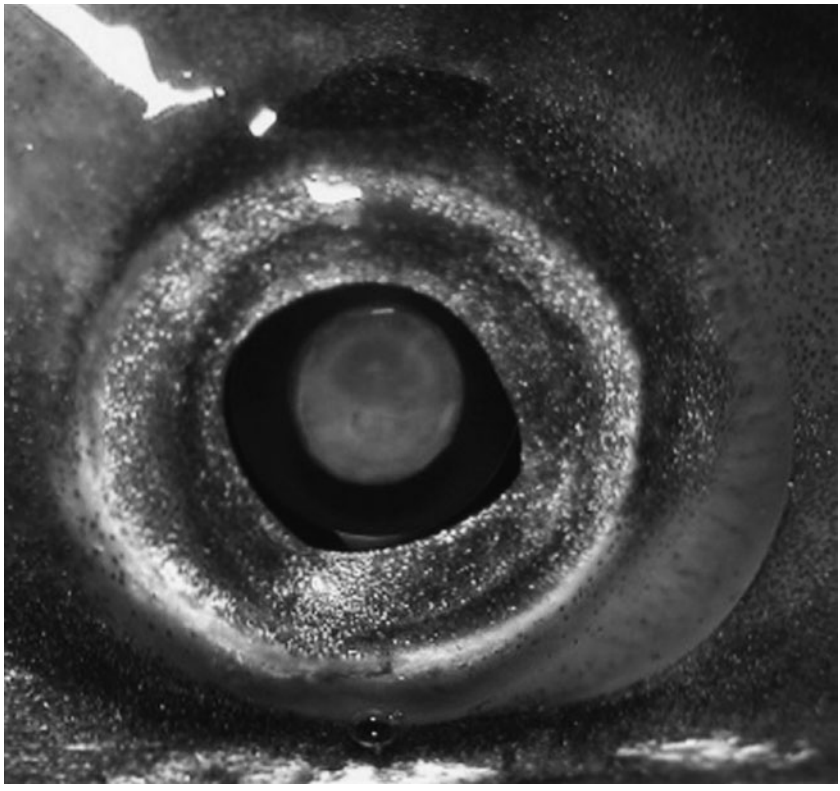


Fig. 13.1. Cataract in Atlantic salmon (photo credit: Tony Wall).

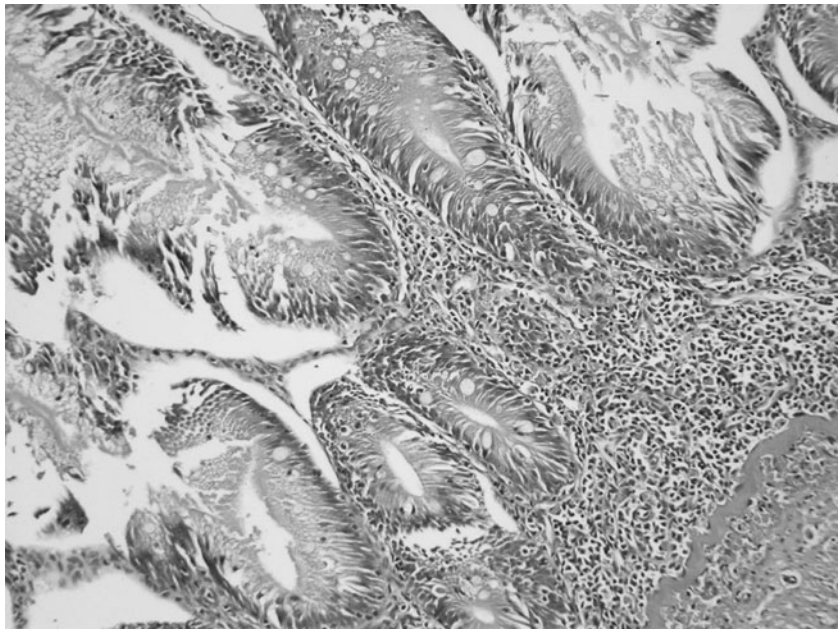


Fig. 13.2. Inflammatory cell infiltration into gut submucosa related to inappropriate diet composition.

the potential to cause poor growth and survival. Great care must therefore be exercised when formulating these diets to ensure that there are no potential health and welfare issues relating to the use of alternative raw ingredients.

Freedom from Fear and Distress

In 2008, Ashley and Sneddon stated that:

> We must take an ethical approach to the welfare of fish and, since there is significant evidence to suggest that their well being is adversely affected by potentially painful and fearful situations, it is our moral responsibility to reduce any possible suffering and discomfort.

There is a wealth of evidence to indicate that fish show fear and distress, and the normal response to this is to escape from the fearful situation (the usual response of fish to stress is 'flight' rather than 'fight'), but in aquaculture situations fish have very little escape opportunity, usually limited to swimming to the other side of the enclosure or as deep into the enclosure as possible; they are therefore usually forced to endure the fearful situation and suffer the welfare implications. We therefore have a responsibility to minimize fearful and distressing situations as much as possible. It is inevitable that many normal aquaculture operations may induce a fearful response; even human activity around a fish enclosure can evoke an escape response (although it can be argued that there should be a lot of human activity around the fish to allow the fish to become habituated, and this may be better for their welfare than more remote management systems, where there is only occasional contact between fish and human beings).

There are many ways in which fear and distress can be caused.

1. The presence of predators: just the predator in the vicinity of a stock of fish can evoke fear, stress and escape responses.
2. The presence of irritant or toxic algae or jellyfish: in addition to being directly damaging the very presence of the organisms can be stressful.
3. Sudden changes in lighting, such as in hatcheries with photoperiod control, are stressful and can cause a panic response and consequent damage; any changes in lighting must be carried out gradually, and transferring fish from areas of low-light to bright-light conditions should be avoided.
4. Sunlight and moonlight: fish tend to avoid bright sunlight and crowding fish to the surface on sunny days can be stressful and evoke an escape response. Even bright moonlight is thought to increase activity and stress.
5. All handling procedures, such as crowding prior to grading or harvesting, if not carried out with due regard to the welfare of the fish can cause an acute stress and escape response (Fig. 13.3).
6. Harvest and killing procedures, including pumping, removal from water and the killing method itself, are potentially very stressful to the fish.

The escape behaviour evoked by the fear and stress can be directly damaging to the fish as they try to get out of their enclosure, resulting in traumatic injuries to the body, fins, snout and eyes; this in turn can lead to secondary effects of osmoregulatory upset and secondary infection (Fig. 13.4), thus compromising another two of the five freedoms.

Humane killing of fish

All harvest and killing activities must be carried out as humanely and with as little suffering as possible. The killing method must render the fish immediately insensible until death, with no prior excitement; in several jurisdictions there are regulatory controls on this aspect of the aquaculture industry. The welfare of animals at slaughter in the European Union is protected by Directive 93/119 1993, which states that 'all animals bred for the production of meat … must be spared any avoidable excitement, pain or suffering during slaughter or killing and related operations inside or outside the slaughterhouse.' This Directive has been implemented in England by the Welfare of Animals (Slaughter



Fig. 13.3. Example of an escape response in Atlantic cod.



Fig. 13.4. Tail, fin and snout damage.

and Killing) Regulations 1995 (WASK) with subsequent amendments.

The humane killing of fish is an area beset with difficulties. With several species it has proved difficult to develop an efficient, effective way of rendering the fish immediately insensible. With individual larger fish it is not so difficult to percussively stun either manually or, more commonly nowadays, using automated equipment. This method

basically delivers a sharp blow to the head of sufficient force to cause shearing forces in the brain, which brings about unconsciousness; death can then take place either as a direct result of the brain damage or from subsequent bleed-out by cutting the gill arteries. If the stun is carried out accurately and efficiently then the fish will become immediately unconscious, although an inaccurate or ineffective blow may cause acute damage and pain (Wall, 2001; Robb, 2008).

Some species, such as Atlantic halibut (*Hippoglossus hippoglossus*), do not lend themselves easily to percussive stunning – the head shape means that 'standard' percussive stunners and effective manual percussive stunning using a 'priest' can be difficult due to the very small 'target area' where the blow has to be struck to achieve unconsciousness and also the prominence of the eyes in this area, where severe eye damage can be caused by an inaccurate blow (Wall, 2001; Humane Slaughter Association, 2008). Some fish such as Pangasius catfish (*Pangasius hypophthalamus*) have very thick skulls, making effective stunning difficult to achieve humanely (Figs 13.5a and b).

It is with the killing of large numbers of relatively small fish, such as portion-size rainbow trout (*Oncorhynchus mykiss*) or common carp (*Cyprinus carpio*), that more problems can be encountered where it is not possible to kill fish individually but where a method for bulk killing has been difficult to find. Early methods of bulk killing included suffocation in air, exposing the fish to water saturated with carbon dioxide or placing them in an ice/water 'slurry'. All three methods are acutely aversive to the fish, causing stress and escape response, and none achieves immediate insensibility. Some of these methods are still used in some areas; for example, Mediterranean (European) sea bream (*Sparus aurata*) and gilthead sea bass (*Dicentrarchus labrax*) are frequently killed by placing in ice slurry to achieve a so-called chill-kill; the sudden drop in temperature is meant to cause rapid loss of consciousness, but this is not the case and the fish remain conscious for several minutes (Smart, 2001; P. Varvarigos, personal communication).

There have been several attempts at developing an effective method of electrical bulk stunning/killing of fish. Some of these are now successfully employed and, provided they are set up correctly, deliver an electrical stun rendering the fish immediately insensible (in this situation it is a stun that kills the fish). There are some problems with electric killing methods. They are more difficult to use for marine fish due to the high conductivity of the water (meaning that the charge preferentially passes through the water rather than the fish), requiring high levels of charge. If the charge is too great then there can be serious problems with broken backs and acute haemorrhages (Wall, 2001).

It is very important that consideration must be given to developing an acceptable method of humane killing for any species being developed for aquaculture. In the past we have seen new species being introduced to farming, such as Atlantic halibut, without there being any initial idea of how these fish were going to be humanely killed.

Culling and emergency killing

There are occasions when it is necessary to cull fish from a population; there may be individual sick, damaged or deformed fish or 'rejects' from a grade, etc. Exactly the same welfare conditions should be given to these fish as to fish that are harvested for consumption; they should be killed humanely by a method that renders them immediately insensible with no prior excitement. This applies equally to hatched embryos ('yolk-sac fry') as to later production stages. Acceptable methods of culling would include an anaesthetic overdose, if the fish are not to be consumed, or a manual percussive stun/kill.

There are also occasions when it may be necessary to carry out emergency killing of whole populations, such as when it is necessary for disease control. Again the killing must be carried out humanely, and this can be difficult when presented with a large number of fish that need to be killed and removed rapidly. Electrocution or anaesthetic overdoses are probably the most acceptable methods for emergency killing.

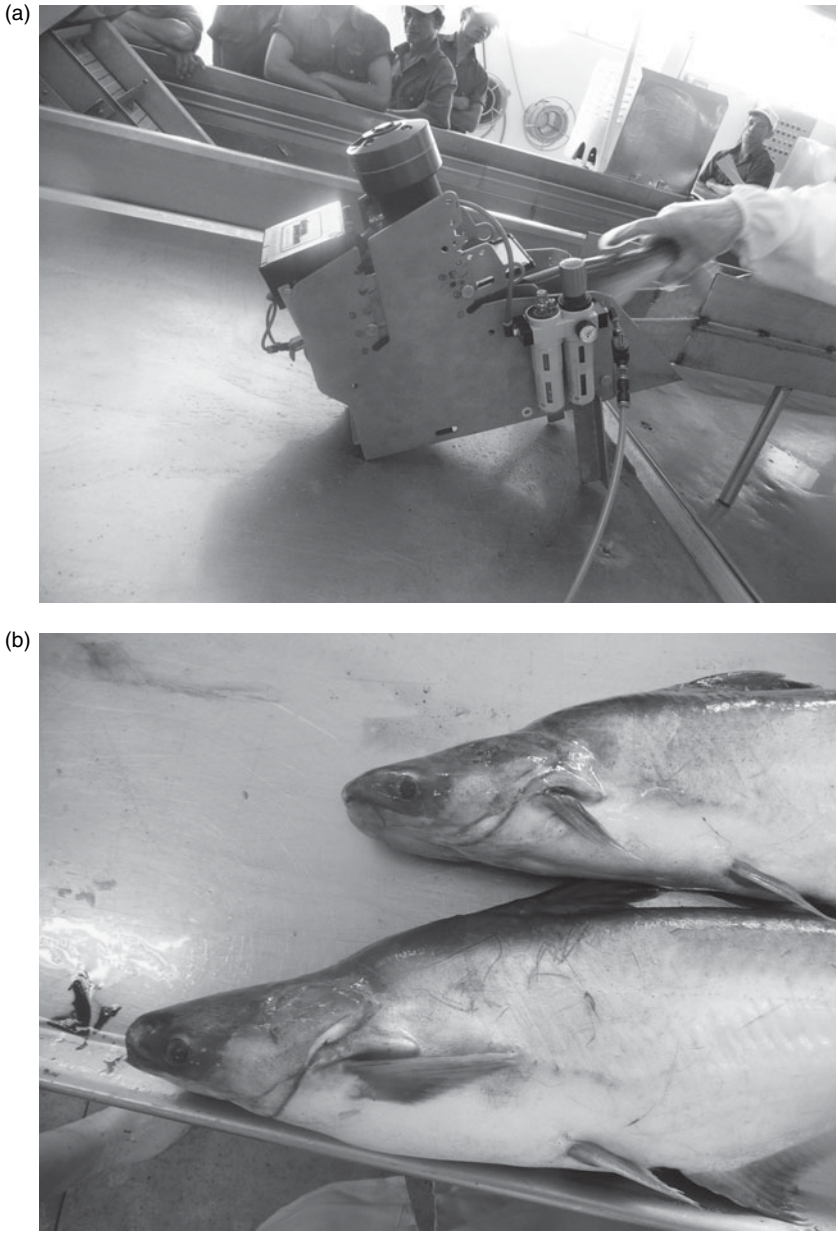


Fig. 13.5. (a) Percussive stunner for Pangasius catfish and (b) stunned catfish.

Freedom from Discomfort

Freedom from discomfort is usually interpreted as providing an appropriate environment for the animal; for fish this is principally the provision of optimum water-quality conditions for the species concerned. Providing appropriate water quality is critical for the well-being of the fish, and these conditions must be stable, i.e. there must be minimal changes or fluctuations in the quality of the water; the more rapid any changes

the more likely are they to cause discomfort and stress. Poor water quality and environmental conditions can result in poor growth, direct pathologies, such as environmental gill disease, and an increased susceptibility to disease. Each species of fish has an optimum range for any water-quality parameter, outside of which the fish suffers discomfort and stress. The degree of discomfort suffered by the fish is often difficult to judge, but by direct observation of their behaviour we know that they will attempt to escape from areas of poor water quality or sudden temperature change. Placing fish into water saturated with carbon dioxide or into ice slurry for harvesting purposes provokes a very strong aversive reaction, which indicates a very high level of discomfort caused by the acidity of the water in the case of the carbon dioxide and the acute temperature change with the ice slurry (Robb, 2001, 2008).

Some of the environmental conditions that cause discomfort if they are outside the acceptable limits for the species are likely to be: low dissolved oxygen, inappropriate or change in pH, inappropriate or change in temperature, high carbon dioxide levels, high levels of irritant suspended solids, and high levels of nitrogenous waste products, particularly ammonia. Fouling on nets, dirty tanks and accumulated wastes can all contribute to poor environmental conditions, discomfort and poor welfare, as can irritant algae or jellyfish or parasitic activity.

Discomfort can also result from other mechanical environmental factors, such as exposure to vibrations and physical shocks. Fish are very sensitive to vibration and mechanical activity, and performance suffers if they are reared in the vicinity of machinery exposing them to noise and vibration. Shocks from mechanical activity, fireworks and explosions are known to cause suffering and even acute mortalities. Similarly, aquaculture practices of handling, netting and transportation of fish stocks, if not directly damaging, may be uncomfortable to the fish; it is said that holding a fish in the hand is uncomfortable not only through being out of water and from the physical contact but also from the heat of the hand. During transportation fish may suffer discomfort from the movement of the transport vehicle and possibly poorer water quality and higher stocking densities, which cause more physical contact. Motion or altitude sickness may also be possible in animals with such sophisticated balance and buoyancy mechanisms. Finally, adverse weather systems causing turbulence, which disrupts swimming behaviour and stirs up material from the substrate, also impose stress on captive fish.

Freedom from Pain, Injury and Disease

There is a degree of crossover between the five freedoms and many of the conditions listed above; causing fear and distress and discomfort may also lead to pain, injury and disease. To ensure appropriate fish welfare, the animals must be protected as far as possible from pain, injury and disease. Many aspects of fish farming have the potential to cause pain and injury, including the application of damaging or poorly maintained equipment or the inappropriate use of equipment in aquaculture practice (Fig. 13.6). Overenthusiastic crowding techniques, inaccurate stunning, activity of predators and many other situations that can injure fish should be avoided, and it is the responsibility of the stock person to ensure that all activities on the farm are designed to minimize the risk of pain and injury, that appropriate facilities and equipment are in place and that they are maintained correctly, and that there are adequate numbers of personnel trained and capable of carrying out tasks to the highest welfare standards.

For ethical reasons, captive fish must be protected from pain and injury at all times and this can be quite difficult, particularly in sea sites, where the enclosures are subject to all weather conditions, algal blooms, predator attack, etc. The fish are not capable of escaping from these conditions and we have a duty of care, through suitable site selection, provision of protective equipment, etc., to prevent, as far as possible, the exposure of the fish to these adverse conditions. This is one of the biggest welfare challenges facing

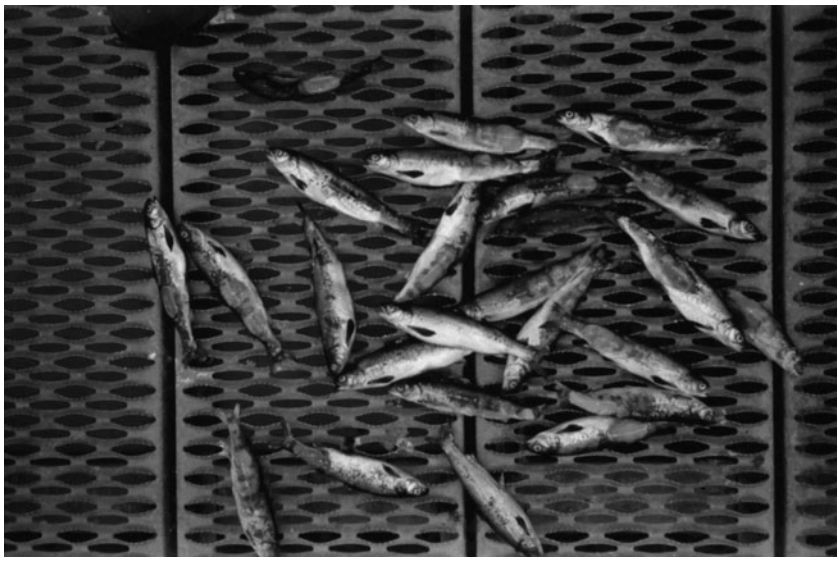


Fig. 13.6. Traumatic damage in newly transferred smolts.

the fish farmer; exposure to, for example, a significant bloom of irritant algae such as *Chaetocerus* sp. can cause extreme discomfort, pain, injury and pathology of the gills and skin. The fish display acute irritation and stress responses, and, despite early-warning systems, the deployment of barriers and air diffusers, once an algal bloom of such severity hits a farm, there is often little the farmer can do to protect his stocks.

In addition to the acute injuries that fish can suffer due to mishandling, damaging equipment, etc., they may suffer more chronic or subtle injuries due to persistent adverse conditions. Fin damage or erosion is a very common finding in farmed fish and has indeed been identified as a useful welfare indicator that can be monitored on a farm. The causes of fin erosion are complex and multifactoral and include overstocking, poor water conditions, infection and aggression from other fish (Latremouille, 2003). In order to reduce the chance of fish suffering pain, injury and disease, all aquaculture enterprises should have adequate protective measures in place, including means of disease prevention, diagnosis and treatment.

Disease prevention

A major part of disease prevention is biosecurity, ensuring that the risk of the introduction of pathogens into fish stocks is minimized by the appropriate use of disease-free stocks, biosecurity barriers and appropriate hygiene and disinfection of equipment and personnel. Nevertheless, biosecurity on fish farms can be a challenge when the source of incoming water cannot be adequately treated to rid it of potential pathogens (e.g. river-supplied tank farms and all marine enclosures); thus, despite biosecurity measures being in place, fish may be exposed to a range of potential pathogens and parasites, many of which are ubiquitous in the aquatic environment. On a positive note, there are now many effective vaccines available for many common fish pathogens and an appropriate vaccination programme is essential to disease prevention.

Diagnosis

It is highly recommended that farms have a system of rapid disease diagnosis and

stockmen must have appropriate training in the early recognition of signs of disease so that timely intervention can take place. Appropriate veterinary health planning must be in place to identify disease risks, along with appropriate monitoring and diagnostic techniques, laboratory and veterinary support, and chains of command and actions, including appropriate medicines and treatment regimes.

Medicines

There is a very limited range of effective treatments available for fish diseases and there are a number of common infectious diseases for which there is either no or very limited therapy. This can lead to major welfare issues if the disease is causing suffering. There are also a number of restrictions on the use of medicines that are available; often there is a limit on the quantity of the medicine that can be 'discharged' into the environment, and this may limit the ability to treat a population effectively. There is a also a withholding time for any medicine that has been administered, meaning that the fish cannot be harvested or consumed until the drug has cleared from the tissues down to an acceptable level for human consumption (the maximum residue level or MRL). With some medicines (e.g. oxytetracycline) this can be a prolonged time, especially at low water temperatures, and the consequence of this may be that the fish have to remain in the water for an extended time, possibly with implications for creating unacceptably high stocking densities and the possible welfare consequences of this, or the farmer may be reluctant to treat because of harvest commitments. There also may be further restrictions placed on the use of medicines, such as by some organic production systems, where a farmer may decide not to treat a condition in case (s)he loses organic status, thereby imperilling the welfare of the fish. Some organic schemes also increase the withholding time following treatment, which again may have implications for fish welfare (Farm Animal Welfare Council, 2008).

With the very restricted availability and use of medicines in fish, the control of disease falls back on the requirement for good disease prevention, biosecurity, hygiene, vaccination, good management and good welfare. Poor welfare in itself will make the fish more susceptible to disease. Damaged and injured fish are more prone to secondary infections, and chronic stress from any cause will have an immunosuppressive effect and make the fish more vulnerable to disease. This aspect of fish welfare is dealt with at greater length in Chapter 6, this volume.

Production diseases

There are several conditions that appear to be caused by the management and husbandry of the fish themselves, often as a result of the 'intensification' of the aquaculture industry. These are often grouped together under the term 'production diseases' because they are related to production techniques. Rapid growth rates, causing a limitation on available nutrients and consequent skeletal abnormalities and cataracts, have been described under malnutrition above. Skeletal and soft tissue abnormalities, such as inverted hearts, missing transverse septum and liver abnormalities, have also been attributed to high egg incubation temperatures (Branson and Turnbull, 2008). In addition, haemorrhagic smolt syndrome (HSS) (Fig. 13.7) has also been attributed to rapid growth rate and hatchery conditions; the aetiology of HSS remains unclear, but it may involve a virus infection (A. Wall, personal communication). Whatever the true cause, production diseases have the potential to have an adverse impact on fish welfare. Fish with heart abnormalities have a higher susceptibility to stressful management procedures (Branson and Turnbull, 2008); fish with deformed jaws and operculi are more susceptible to poorer water quality and low oxygen and less able to feed efficiently (Branson and Turnbull, 2008); likewise, fish with a spinal deformity are at a disadvantage when competing for food and space (Branson and Turnbull, 2008).



Fig. 13.7. Example of haemorrhagic smolt syndrome.

Production disease is not uncommon in other forms of agriculture. For example, leg weakness in broiler chickens is a result of industry practices. It is important that lessons should be learned from other animal production systems, that factors leading to production disease are identified and these factors eliminated as far as possible. For example, having identified high egg incubation temperature as a major factor in the prevalence of deformities, subsequent reduction in these temperatures has resulted in a concomitant reduction in deformities, albeit at the cost of longer incubation periods (A. Wall, personal communication).

Freedom to Express Normal Behaviour

It is not always easy to understand normal behaviour in fish or to try to provide adequately for that behaviour in the aquaculture environment. It is difficult to empathize with a fish and understand the animal's needs, and it is assumed that as long as it is swimming and feeding 'normally' then its behavioural requirements are being catered for. Sufficient space is needed within any fish enclosure to allow for adequate swimming behaviour and also to give some escape room in the face of a stressor. To a certain extent this is addressed by setting maximum stocking densities, but the relationship between stocking density and fish welfare is very complex and depends on many factors, including the behaviour of the fish and the environmental conditions (Adams *et al*., 2007).

Some farmed fish, such as Atlantic salmon, undergo migrations of thousands of miles. The shoaling and swimming that occurs in sea enclosures probably replicates these distances, but the animals are nevertheless being restricted from their normal swimming behaviour. With salmon there is no evidence to suggest that this is detrimental to their welfare until they mature, and their instinct would then be to migrate to fresh water. This is addressed by harvesting prior to maturity or taking maturing broodstock back to fresh water.

In some circumstances apparently giving the fish the ability to express their normal behaviour may even conflict with good welfare. In one study in which rainbow trout were held at low stocking densities, which would be assumed to improve their welfare, hierarchical behaviour and stress in the

subordinate population actually increased (North *et al*., 2006). This, of course, still does not replicate the 'natural' situation and was not necessarily 'normal behaviour', but it does point up the very complex nature of normal behaviour in an aquaculture environment. Fish behavioural science is still in its infancy, and there is a paucity of research in the subject; until more work is done, it is impossible to judge and cater for more than the very basic behavioural needs of fish.

Environmental stimulation

There is an increasing interest in environmental stimulation (also called environmental enrichment) in animal production systems; for example, the 1985 amendments to the United States Animal Welfare Act included provisions for the psychological well-being of non-human animals, resulting in the establishment of environmental-enrichment programmes for all animal species (Kulpa-Eddy *et al*., 2005). The concept is to add something to the animals' environment to stimulate its interest and to give the animal a more complex environment with which to interact. In other animal production systems this has been achieved by providing an 'enriched' environment, such as rooting material for pigs and straw bales for hens, rather than a bare environment of plain stalls or empty cages. It has been found that these systems produce a more 'contented' animal, with less stress, fewer losses, better growth and lower disease incidence. Very little work has been done in this area with fish, but there is no reason why the concept of a more enriched environment rather than bare tanks or cages shouldn't also improve the well-being of the fish. The provision of ropes hanging into the cages on a cod farm did seem to provide them with something interesting to 'play' with and chew, although no real assessment of the effect on their welfare was made – at least it stopped them chewing the nets (personal observation)!

Conclusion

From the foregoing it is obvious that there are many ways in which the management and husbandry of our farmed fish can have a significant impact on their welfare. There remain many welfare challenges in aquaculture, particularly in relation to environmental insults, humane killing and understanding the needs of novel aquaculture species. With increasing knowledge and improvements in technology, it should be possible to cater more effectively for the welfare needs of the fish under our care.

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Glossary

Accessory cells: Present in the gills and skin of seawater fish: mitochondrion-rich cells pair with smaller cells with fewer mitochondria, which connect with the former by cation-permeable intercellular junctions.

ACE: Angiotensin-converting enzyme: the enzyme is found in vascular tissue and converts the decapeptide angiotensin I into the octapeptide angiotensin II, a potent vasoconstrictor. ACE inhibitors, such as Viagra, prevent the production of angiotensin II, thus allowing vasodilation.

Acidemia: An unusually low blood pH.

ACTH: Adrenocorticotropic hormone (synonym: adrenocorticotropin): the hormone released from anterior pituitary gland corticotropic cells; ACTH binds to receptors (melanocortin 2 receptor) on the steroidogenic cells of the interrenal gland and is the major tropic hormone regulating cortisol biosynthesis.

Acute-phase response: A series of physiological responses elicited by the body to tissue injury or infection. This is thought to be part of the innate immune response. The hallmark of acute-phase response is the secretion, or lack thereof, of a suite of proteins, predominantly from the liver, termed the acute-phase proteins. These proteins play a protective role by defending against trauma, tissue damage and pathogen-related injury.

Adaptive stress: Physiological response to changes in environmental parameters, enabling relative stability of the internal environment (blood); associated with the concept of homeostasis.

Additive effect: The combined effects of two or more toxicants.

Adenomas: A benign neoplasm (tumour) in which the cells form a glandular structure or arise from a glandular epithelium.

Adipocytes: Cells that are specialized for the storage of triglycerides.

Adrenocorticotropic hormone: See **ACTH**.

Aetiology: The cause of a disease or disorder.

Agouti-related protein: See **AgRP**.

AgRP: Agouti-related protein or agouti-related peptide is a neuropeptide produced in the brain; the peptide acts via specific isoforms of the melanocortin receptor (MCR) to increase appetite and decrease metabolism and energy expenditure, causing obesity in some vertebrates.

AhR: See **Aryl hydrocarbon receptor**.

Alkylphenols: Partial degradation products of the alkylphenol ethoxylate class of surfactants that have oestrogenic activity; includes nonylphenol and octylphenol.

Amylin: Also called islet amyloid polypeptide (IAPP); it is a peptide hormone secreted by the same pancreatic cells that secrete insulin.

Anadromous: Literally upstream, refers to upstream migration of fishes and a general term for fishes that migrate into bodies of fresh water to spawn.

Anaemia: A common disorder of blood produced by several underlying causes. It is characterized in several ways, including on the basis of morphology of red blood cells (RBCs), underlying aetiological mechanisms and discernible clinical spectra. The most common procedure to detect mild or severe anaemia is to measure the total number of circulating RBCs or the haemoglobin content of the erythrocytes. Typical causes can include chronic or acute bleeding (internal or external), excessive erythrocyte destruction, insufficient erythrocyte or haemoglobin synthesis, poisoning or nutritional deficiencies/toxicities.

Anaplastic: Loss of cellular differentiation.

Androgen: An agonist for the androgen receptor in vertebrates. Note that natural androgens in fish can be testosterone, methyl testosterone, hydroxy-testosterone or 11-ketotestosterone.

Angiotensin: See **JG apparatus**.

Angiotensin-converting enzyme: See **ACE**.

Anorexigenic: Appetite suppression.

Anoxia: Lack of sufficient oxygen (see **Hypoxia**).

Antagonistic action of hormones: Hormones that work together in the regulation of a common physiological process but have opposite actions.

Anthropogenic: Produced or caused by human activity.

Antibody: A type of protein produced by B-lymphocyte cells that have been stimulated by an antigen; antibodies are capable of combining with antigens that induced their formation.

Antibody-forming cells (AFC): B lymphocytes that have been stimulated to produce antibodies.

Anticarcinogen: A substance that counteracts the tumourigenic actions of carcinogens.

Antigen: A molecule that can induce an immune response and/or react with an antibody.

Antinutritional factors (ANFs): Substances often found in food and feed components that can have negative effects on the intake, digestion and physiological utilization of nutrients and can also be toxic. Many common plant feedstuffs contain ANFs, such as alkaloids, haemaglutinins (lectins), phenolics, phytates, phyto-oestrogens, saponins, tannins and protease inhibitors. These ANFs can severely restrict the use of plant feedstuffs in animal and fish feeds.

Apoptosis: Sometimes called programmed cell death; the process of cell death occurring as a result of intracellular events in the normal life history of a cell.

Aquaglyceroporin: Intramembrane protein with hydrophilic pore and the capability to allow movement of water and some uncharged solutes (especially urea and glycerin) down their electrochemical gradient.

Aquaporin: Intramembrane protein with hydrophilic pore and the capability to allow movement of water down its osmotic gradient.

Arginine vasotocin: See **AVT**.

Arteriosclerosis: A focal growth of tissue (a lesion) on the inside of a blood vessel, typically an infiltration of vascular smooth muscle. Deposition of fat in such lesions requires the use of the term atherosclerosis.

Aryl hydrocarbon receptor (AhR): A receptor within cells that binds compounds that possess certain structural features shared by aromatic hydrocarbons (e.g. dioxins, polychlorinated biphenyls and polycystic aromatic hydrocarbons). The AhR–ligand

complex can induce the expression of specific genes and can also modulate cellular activity through interactions with other proteins in the cell.

Astrocytes: Synonym: gangionic gliocytes; support cells in the central nervous system, maintaining the position of the neurons.

Asynchronous spawning: A reproductive strategy among fish and other vertebrates in which males and females continuously produce gametes and do not mate at a specific synchronized time.

Atresia: Degeneration of developing ova in ovarian tissue.

Atrial natriuretic factor: A polypeptide hormone secreted by heart muscle cells. It is involved in the regulation of salt and water balance.

Autocrine: Hormone or growth factor secreted by cells that exert their actions on the cells that secrete the hormone; these autocrine factors exert local control over cell and tissue function.

AVT: Arginine vasotocin: the major posterior pituitary gland (synonym *pars nervosa*) hormone, which is synthesized in neurons in the hypothalamus and released from synaptic junctions in the *pars nervosa*.

Basophilia: In haematology, describes an increased number of basophils in the blood or tissues. In histology, describes cells that are darkly stained by basic histological dyes such as haematoxylin.

Biliary: Associated with the bile duct or gall bladder.

Bioaccumulation: The uptake of a chemical into an organism through one or more environmental pathways (via intestinal tract or gills).

Bioassay: Measurement of the effect of a treatment using a biological response as the indicator; an example is the use of vitellogenin production by male fish as a measure of environmental xeno-oestrogen levels.

Bioavailability: The portion of a toxicant that is available for interactions with organisms.

Bioindicator: Any tool – biological, physiological or genetic – that is used to detect a biological response.

Biomagnification: An increase in the concentration of chemicals in organisms as the chemicals pass up through a food chain.

Biomarker: See **Bioindicator**.

Biotransformation: A change in the structure of a chemical that occurs through enzyme-mediated metabolic pathways in organisms.

Bipotential germ cells: Undifferentiated germ cells within gonadal tissues that have the capacity to develop into either oogonia or spermatogonia.

Blood–brain barrier (BBB): The separation of circulating blood and cerebrospinal fluid (CSF) in the central nervous system (CNS). Endothelial cells restrict the diffusion of microscopic objects, such as bacterial and large or hydrophilic molecules, into the CSF, while allowing the diffusion of small hydrophobic molecules (oxygen, some hormones, carbon dioxide). Cells of the barrier actively transport metabolic products such as glucose across the barrier using specific protein transporters.

Bombesin: A 14-amino acid neuropeptide found in the central and peripheral nervous system; it stimulates gastric release from intestinal mucosal cells and, together with CCK, activates receptors in regions of the brain that inhibit feeding behaviour.

Branchial: Associated with the gill in aquatic organisms.

Branchial heart: The cardiac muscle generates blood pressure, which is used to drive blood flow through the gill circulation and then through the systemic circulation.

Branchoses: Degenerative conditions of the gill.

Brockman bodies: Endocrine pancreas found in some species of fish as a grossly glandular structure comprising cells that produce glucagon-like peptide, insulin and somatostatin. In most fish species the endocrine pancreatic cells are scattered among the exocrine pancreas.

Calcitonin: A peptide synthesized in the ultimobranchial gland, pituitary gland and brain of teleostean fish. The peptide plays an essential role in calcium regulation in mammals, but does not appear to play a similar role in fishes. Calcitonin may function as a neuropeptide in the regulation of feeding.

Calcitonin gene-related peptide (CGRP): Genes encoding for the peptide are expressed in several regions of the brain, pituitary gland and many peripheral tissues in fish. The widespread distribution of CGRP production suggests that the peptide is involved in the regulation of many diverse physiological functions in fish.

Carbonic anhydrase: An enzyme that rapidly facilitates the reversible reaction of carbon dioxide with water.

Carcinogen: An agent that causes neoplasia.

Carcinoma: A malignant neoplasm arising from epithelial cells.

Cardiac output: The volume of blood pumped out by the heart per unit time per unit body mass of the fish.

Cardiomyopathy: A general term for any pathology that affects cardiac muscle.

Cardionatrin: Also called natriuretin, formerly atrial natriuretic peptide (NAP); polypeptide hormone produced in heart involved in aspects of water regulation.

CART: Cocaine and amphetamine-regulated transcript (CART) peptides are neurotransmitters that are associated with the inhibition of feeding behaviour and body-weight regulation in vertebrates. CART peptides and their mRNA transcript are found in many brain regions and in peripheral tissues that are involved in feeding, and many animal studies implicate CART as an inhibitor of feeding.

Catadromous: Literally downstream, referring to downstream migration of anadromous fishes.

Cataract: A common degenerative condition that is characterized by a clouding in the eye lens that may either partially or completely impair the passage of light and result in reduced visual ability and ultimately blindness. Cataracts develop from a disruption of the normal arrangement of the lens fibres or from alterations in the conformation or water-binding capacity of the proteins of the lens. In Atlantic salmon, cataracts are often localized in the cortex, but extensive cataracts may also affect the nucleus.

Catecholamine: Neurohormones and/or neurotransmitter substances that are derivatives of tyrosine, including epinephrine (synonym: adrenalin), norepinephrine (synonym: noradrenalin) and dopamine.

Cathepsin D: Cathepsins are ubiquitous lysosomal proteases, most of which contain an active-site cysteine residue. The main physiological role for cathepsins is lysosomal proteolysis. There are several members of this family, which are distinguished by their structure and the proteins they cleave. These proteins are activated at low pH in the lysosomes. Cathepsin D is one of the ubiquitously distributed proteases, and in addition to the general proteolytic action in lysosomes, it is also thought to be involved in cell proliferation and activation of different prohormones.

Caudal neurosecretory system (CNS): Also called the urophysis. A collection of neurons located in the caudal region of the central nervous system of fish. Urotensins (UI and UII) and peptides related to CRH are synthesized in the neuron cell bodies and transported to synaptic axonal endings. It was originally believed that these peptides were found only in fish, but they are widely distributed among vertebrate taxa and play important roles in the regulation of cardiac function, ventilatory function and some aspects of motor function in all vertebrate taxa studied; some roles in reproduction have been proposed for the peptides in fish.

CCK: Cholecystokinin is a peptide hormone of the gastrointestinal system involved in the digestion of lipid and protein; the hormone is secreted by specialized cells of the mucosal epithelium and stimulates the release of digestive enzymes. It also acts on the central nervous system to decrease feeding.

Ceroid deposition: The accumulation of a naturally occurring golden, waxy polymer of oxidized lipid pigment in various tissues (heart, liver, gastrointestinal tract and brain), which is thought to be caused by severe vitamin E deficiency. It is often referred to as 'brown bowel syndrome' when afflicting intestinal tissues.

CFTR: Cystic fibrosis transmembrane conductance regulator is an anion channel of low single-channel conductance (7 pS) and activation through phosphorylation of the regulatory domain.

CGRP: See **Calcitonin gene-related peptide**.

Channel: An intramembrane protein with hydrophylic core that allows permeation of certain-sized charged solutes by a gating mechanism.

Chloride cell (also called chloride secreting cell): A general term referring to mitochondrion-rich cells in fish gills (or in the opercular epithelium of some species); the cells play roles in ion transport.

Cholangiocarcinoma: A malignant tumour of the biliary epithelium.

Cholangioma: A benign tumour of the biliary epithelium.

Cholecystokinin: See **CCK**.

Chromaffin cell: The homologue of an adrenal medulla cell of the mammalian adrenal gland. These cells are components of the interrenal gland found in the anterior region of the kidney (the so-called 'head kidney'). The chromaffin cells get their name from their staining properties; they secrete the hormone epinephrine together with some norepinephrine; they are innervated by cholinergic neurons of the sympathetic division of the autonomic component of the central nervous system.

Chromatophoroma: A benign tumour of pigment cells (e.g. melanophores, erythrophores, xanthophores).

Claudin: Structural proteins that contribute to tight intercellular junctions between epithelial cells.

Cocaine and amphetamine-regulated transcripts: See **CART**.

Cocarcinogen: A substance that acts concurrently with a carcinogen to increase the number of neoplasms produced.

Complement: A group of serum proteins, activated during the process of inflammation, that facilitate opsonization, cellular activation and cell lysis.

Condition factor: The condition factor or coefficient of condition is a relative measure of the robustness of the animal. It is usually represented by the letter K when the fish is measured and weighed in the metric system. The formula most often used is: $K = [10{,}000 * W]/L^3$, where W = the weight of the fish in g and L = the total length of the fish in mm.

Congeners: Use to describe members of a family of compounds; commonly used to describe the various forms of the polychlorinated biphenyl (PCB) family.

Coronary circulation: A circulation of blood that is dedicated specifically to the myocardium.

Corpuscles of Stannius: See **CS**.

Corticotropic cells: ACTH-secreting cells of the *rostral pars distalis* of the anterior pituitary cells. The corticotropic cells produce the peptide glycosylated polypeptide proopiomelanocorticotropin (POMC); enzymes produced by the cells specifically cleave the POMC peptide to release ACTH and β-endorphin.

Corticotropin-releasing hormone: See **CRH**.

Cotransporter: Intramembrane protein that binds two or more charged or uncharged solutes and translocates them down their combined electrochemical potential and across the membrane.

CRF: See **CRH**.

CRH: corticotropin-releasing factor (also abbreviated as CRF). A 41-amino acid peptide synthesized in the cell body of specific hypothalamic neurons, transported through the hypothalamus via CRH cell axons and released at synapses associated with the corticotropic cells in the *rostral pars distalis* of the anterior pituitary gland, stimulating

them to synthesize and secrete ACTH. The peptide has also been related to the regulation of feeding behaviour

CS: Corpuscles of Stannius groups of encapsulated cells called Stanneocytes forming nodules (corpuscles) in the renal parenchyma along the edges of the midsection of the kidney in fish; the corpuscles of Stannius are responsible for the synthesis and secretion of the glycoprotein hormone stanniocalcin, which regulates calcium and phosphate homeostasis in fish through its actions on the gills and kidneys.

CYP: An abbreviation for cytochrome P450; a very large and diverse superfamily of haemoproteins that catalyse a large number of chemical reactions, including many of the biotransformations of cholesterol and steroid hormones that occur in the steroidogenic cells of the interrenal tissue, testis and ovary.

Cystadenoma: An adenoma with cystic structures.

Cystic fibrosis transmembrane conductance regulator: See **CFTR**.

Cytolytic: The process of rupturing a cell.

Depigmentation: Also called hypopigmentation: a disorder of the skin, mucous membranes, hair or retina. The pigment melanin, which is produced from tyrosine by specialized cells known as melanocytes, is negatively affected or destroyed. The condition may develop due to deficiency of certain micronutrients, hyperthyroidism, adrenocortical insufficiency, alopecia, anaemia, certain infectious diseases or excessive sun exposure.

Dexamethasone: A synthetic analogue of glucocorticoid hormone that binds with high affinity to the glucocorticoid receptor.

Diffusion distance: The total distance a molecule or gas moves down its electrochemical or partial pressure gradient.

Diluting segment: Portion of a renal tubule or gastrointestinal tract that results in the dilution of the contents either by addition of fluid or by removal (uptake) of salt.

Dioxins: A group of chlorinated aromatic hydrocarbon chemicals that are formed during incomplete combustion and as by-products during the production of some industrial and agricultural chemicals; some dioxin congeners, such as 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) are extremely toxic.

Dysgerminoma: A malignant neoplasm of the germinal tissue of the ovary.

Early mortality syndrome: Large-scale mortalities of Atlantic salmon and Pacific salmon stocks in the Great Lakes of North America; the mortalities occurred in late embryonic stages, just prior to completion of yolk absorption. Also called M74 syndrome.

Electrochemical gradient: The algebraic sum of the electrical and concentration differences measured across a membrane, which govern the driving force for transmembrane solute movement.

Embryo: For teleost fish the term refers to life history stages from the zygote until the point at which the yolk is absorbed.

Endochondral: Calcified bone that is formed by the ossification of cartilage

Endocrine-disrupting chemicals: Chemicals present in the environment that interfere with normal hormonal action in organisms.

Endocrine system: The series of systems that comprise secretory cells, sometimes gathered together in glandular tissue (e.g. thyroid gland) and sometimes scattered throughout other tissues (e.g. gastrointestinal endocrine tissues). The endocrine systems synthesize and secrete chemicals called hormones, which are released into the blood and act on 'target' tissues that are distant from the source of the hormone. The hormones may be amino acid derivatives (e.g. thyroid hormones), peptides of various sizes (e.g. ACTH and TRH), proteins (e.g. GH and PRL), glycoproteins (e.g. TSH and GtH) or derivatives of fatty acids (e.g. prostaglandins).

Endorphins: A family of opiod polypeptides produced by the brain and pituitary gland; members of the family are natural relievers of pain. One of the common endorphins is the beta-endorphin and it is a cleavage product of pro-opiomelanocortin (POMC) produced in the pituitary.

Endothelins: Vasoconstricting peptides (21-amino acids) produced primarily in the endothelium of blood vessels; they play a key role in vascular homeostasis; in mammals, endothelins are implicated in vascular diseases of several organ systems, including the heart, general circulation and brain.

Enteritis: An inflammation of the lining of the small intestine. If both small and large intestine are affected, it is termed 'enterocolitis'. A subacute inflammatory response (enteritis) in the distal intestine of Atlantic salmon and rainbow trout fed soybean meal is also associated with reduced growth performance and nutrient utilization, and diarrhoea in a dose-dependent manner.

Enterocyte: Columnar absorptive and secretory cell type of the intestinal mucosal epithelium.

Eosinophilia: Increased staining of cells by the acidic stain eosin using standard histopathology staining procedures for fixed tissues, which indicates alterations to cellular organelles that may indicate a pathological response.

Ependymoblastoma: A malignant tumour composed of poorly differentiated ependymal cells of the brain and spinal cord.

Epigenetic: Developing in gradual stages of differentiation; changes that influence phenotype without altering the genotype.

Epinephrine: The major catecholamine hormone produced by the chromaffin cells of the interrenal gland. The hormone epinephrine (formerly called adrenalin) is involved in the regulation of glucose homeostasis; increased release of epinephrine as part of the stress response is a factor promoting the increase in blood glucose levels; it may also contribute to the increased activity of the cardiac and ventilatory systems.

Epithelium: The general tissue type that covers the outside of a fish.

Epizootic: A disorder or disease affecting a population of non-human animals (as compared with epidemic, which refers specifically to a disease affecting human populations).

Erythrocyte fragility: Refers to the susceptibility of erythrocytes (also known as corpuscles or red blood cells) to cell rupture when subjected to hypotonic solutions. Several tests have been developed to test the fragility of erythrocytes for the diagnosis of anaemia and other metabolic disorders involving oxygen transport.

Euryhaline: See **Stenohaline**.

Exchanger: Intramembrane protein that binds one solute on one side of the membrane and another solute on the other side of the membrane and effects a translocation of the two molecules simultaneously, releasing the solutes on the opposite sides, generally without metabolic intervention.

Exophthalmia: A condition involving the abnormal protrusion or bulging of the eyeball outside of the eye socket. Most commonly it is caused by enlargement of the choroid gland or degeneration of the extra-ocular musculature. Exophthalmia has been linked to dietary niacin deficiency and to overproduction of thyroid-stimulating hormone (TSH), or increased sensitivity of tissues to TSH.

Exophthalmus: See **Exophthalmia**.

Exotic species: A non-native or introduced species.

Extracellular space: A fluid-filled (lymph) region between cells, which swells during oedema.

Fibroma: A benign neoplasm composed primarily of fibrous connective tissue.

Fibrosarcoma: A malignant neoplasm of fibroblasts.

Fin erosion: A general term used to describe necrotic loss of fin tissues resulting in fins that appear to have gaps or holes, or look shredded. The fins typically appear white at the

edges and reddish internally, due to associated inflammation. This erosion can occur as a result of physical damage from abrasive tank walls or encounters with other fish, nutritional deficiencies or pathogenic bacterial and/or fungal infections. Fin erosion left untreated results in poor growth, widespread disease outbreaks and fish death.

Follicle-stimulating hormone: See **FSH**.

Follicostatins: See **Inhibins**.

FSH: Follicle-stimulating hormone and its related gonadotropin, luteinizing hormone (LH), are glycoprotein hormones synthesized by and secreted from gonadotropic cells of the *proximal pars distalis* of the anterior pituitary gland; the hormones were originally named after their demonstrated function in the mammalian ovary. The fish homologue GtH-1 (now also called FSH in fish) acts together with GtH-2 (now also called LH in fish) to regulate gonadal (testicular and ovarian) steroidogenesis and gonadal maturation.

Furosemide: A pharmcological blocker of NKCC cotransport function, generically a 'loop diuretic' for its action on the loop of Henle of mammals.

Galanin: A 29–30 amino acid neuropeptide present in some vertebrates, involved in a range of physiological processes, including the regulation of food intake, metabolism and reproduction; neurotransmitter and hormone release; and intestinal contraction and secretion. Galanin, acting through its receptor, is predominantly an inhibitory, hyperpolarizing neuropeptide that inhibits neurotransmitter release; it is often co-localized with other neurotranmitters, such as acetylcholine, serotonin, norepinephrine and other neuromodulators such as neuropeptide Y and substance P.

Gametogenesis: The process of differentiation and maturation of male or female gametes. See **Spermatogenesis** and **Oogenesis**.

Gastric distention: A condition of obscure aetiology in seawater-reared rainbow trout and chinook salmon and has been refereed to as water belly, bloat and gastric dilation air sacculitis (GDAS), as it leads to enlarged abdomens and dilated stomachs and a stenosis of the pyloric sphincter.

Gastrin-releasing peptide: See **GRP**.

Genomic receptor: See **Nuclear receptor**.

Genotoxic: Causing DNA damage.

GH: Growth hormone, the hormone synthesized by and secreted from the somatotropic cells of the *proximal pars distalis* of the anterior pituitary gland. GH acts on hepatocytes to stimulate the synthesis of IGF-1, which acts together with GH to stimulate somatic growth via incorporation of amino acids. GH has also been implicated in aspects of osmotic and ionic regulation, and immune system function. GH and IGF-1 are also synthesized in peripheral tissues (i.e. non-pituitary gland); this occurs in very early embryos. The locally produced hormone may play autocrine or paracrine roles, which may be particularly significant during early ontogeny.

Ghrelin: A hormone produced mainly by specialized cells lining the stomach and possibly also pancreatic cells; it may play a role in appetite control. Ghrelin is also produced in the hypothalamus and may be involved in the regulation of GH synthesis from the anterior pituitary gland.

Gill (branchial) circulation: A collective term for the blood vessels comprising the respiratory system (gills) in fishes.

Gill hyperplasia: A condition in which the secondary gill lamellae swell and thicken, restricting the water flow over the gill filaments. It can result in respiratory problems and stress and create conditions for opportunistic bacteria and parasites to proliferate. Elevated levels are a common precursor to bacterial gill disease.

GLP: Glucagon-like peptide secreted by α-cells of pancreatic tissue; the physiological roles of the peptide in fish are currently not well established, although it may be a factor involved in the regulation of feeding behaviour.

Glucagon-like peptide: See **GLP**.

Glucocorticoid: A general term for the group of adrenal (interrenal) steroids that have actions on carbohydrate metabolism. The primary glucocorticoid released from the interrenal steroidogenic tissue in most fish species is cortisol, with smaller amounts of cortisone and 11-deoxycorticosterone being released.

Glucocorticoid receptor (GR): A ligand-activated transcription factor belonging to the steroid hormone family of receptors, to which glucocorticoids bind with high affinity and either activate or repress target genes. GR is expressed in almost all the tissues and regulates a wide variety of physiological processes, including development, reproduction, growth, metabolism and immune function.

Glucocorticoid response element (GRE): A short sequence of DNA within the promoter of a gene to which glucocorticoid receptor complex binds and regulates transcription.

Gluconeogenesis: A metabolic process by which glucose is generated from non-carbohydrate carbon substrates such as certain amino acids, glycerol and lactate. Most gluconeogenesis occurs in the liver during periods of fasting or starvation, and glucocorticoids, such as cortisol, play an essential role in regulating these metabolic processes.

GnRH: Gonadotropin-releasing hormone, a neuropeptide that is synthesized in the cell body of specific neurons in the hypothalamus; one of the hypophyseotropic neuropeptides that regulate the synthesis and release of hormones from the anterior pituitary gland. In fish, GnRH has been found to influence the secretion of gonadotropins and GH, and may play a role in the regulation of food intake.

Goblet cell: An epithelial cell specialized for the production of mucus.

Goitre: Enlargement of thyroid tissue by an increase in the number and size of the thyrocytes. Goitres may be associated with hypothyroidism in fish. In mammals, goitres may also be associated with hyperthyroidism, most commonly caused by antibodies autoproduced against endogenous TSH activating the TSH receptors on the thyrocytes; to date, this has not been demonstrated in fish.

Gonadosomatic index (GSI): The ratio of gonad weight to body weight expressed as a percentage.

Gonadotropic hormone: See **FSH**.

Gonadotropin-releasing hormone: See **GnRH**.

Gonochoristic: From the noun gonochorism, which describes sexually reproducing species in which there are at least two distinct sexes, which are usually genetically determined and do not usually change throughout the animal's lifetime (i.e. the term does not apply to species that show sex-reversal reproductive strategies).

Granulation tissue: New tissue formed during wound repair; primarily composed of capillaries and fibroblasts.

Granuloma: A chronic inflammatory lesion typically consisting primarily of modified macrophages (epithelioid cells).

Granulomatous inflammation: An inflammatory response in which macrophages predominate.

Granulosal cell: One of the two major types of steroidogenic cells (together with thecal cells) of ovarian tissue. In fish the thecal and granulosal cells form a dual layer of cells that overlies the *zona pellucida*. The function of both granulosal and thecal cells is regulated by FSH and LH, and the major products are oestrogens, androgens and progestogens, depending on the stage of ovarian maturation.

Gross lesions: Tumours, deformities or other tissue or organ damage that can be discerned by the naked eye.

Growth hormone: See **GH**.

GRP: Gastrin-releasing peptide is a 27-amino acid peptide that has been implicated in regulating a number of physiological processes in vertebrates, such as various functions of

the gastrointestinal and central nervous systems, including release of gastrointestinal hormones, smooth muscle cell contraction and epithelial cell proliferation.

GtH: See **FSH**.

Guanylin: A biologically active peptide that stimulates salt and water flows across intestinal epithelium by acting on receptors on the apical (lumenal) side of the epithelium.

Gynogenic: A reproductive strategy by which a sperm is needed to activate the oocyte, but penetration of the sperm does not occur.

Haemangioendothelioma: A benign neoplasm primarily composed of endothelial cells and prominent blood vessels.

Haemangioma: A benign neoplasm formed from blood vessels.

Haemangiopericytoma: A benign neoplasm composed of pericytes forming whorls around newly formed blood vessels, which may be inconspicuous.

Haematocrit: The percentage of packed red blood cell volume in a blood sample.

Haemopericardium: The unusual presence of red blood cells in the normally clear pericardial fluid surrounding the heart.

Head kidney: A general term to describe the anterior section of the kidney of fish, which contains largely haematopoietic (red blood cell-producing) tissue. The interrenal tissue, comprising steroidogenic and chromaffin cells, is also contained within the head kidney.

Heat-shock factor (HSF): Transcription factors that regulate the expression of genes that encode for heat-shock proteins.

Heat-shock protein: See **HSP**.

Hepatic: Pertaining to the liver.

Hermaphrodite: Organisms that produce functional male and female gametes; most hermaphroditic fish species do not self-fertilize. The term is sometimes erroneously used to describe abnormal conditions where small numbers of oocytes may be present in the testis or where foci of testicular tissue are present in the ovary. The conditions may be toxicant-induced or may have a genetic cause, such as in some hybrids. Unless there is evidence that the two types of gametes are functional, such conditions are usually termed 'intersex'.

Heterozygous: An individual possessing different alleles at a particular chromosomal locus.

Homozygous: An individual possessing two copies of the same allele at a chromosomal locus

Hormone: See **Endocrine system**.

HRE: Hormone response element; the sequence of nucleotide bases in the promoter region of specific genes to which steroid hormone, thyroid hormone and retinoid receptor proteins attach and act as transcription factors that regulate the expression of those genes. Most steroid hormone receptors have to be activated by binding with their ligand before they can bind to the HRE. For thyroid (TR) and retinoid (RXR) receptors the non-activated receptor heterodimer (TR-TXR) can bind and acts to suppress gene expression; activation of the TR with its ligand results in activation of most of the genes that contain the thyroid response element (TRE) in its promoter region; however, some TRE responses are associated with gene suppression activity.

HSP: Heat-shock proteins; a highly conserved family of chaperone proteins, with a wide range of molecular mass, which are constitutively present in cells and critical for protein homeostasis. Some members of this family are induced specifically in response to stressors that impact the protein machinery and protect the cells against damage and re-establish protein homeostasis. Hence these proteins are commonly used as markers of cellular stress response.

Hydromineral balance: The combination of osmotic and salt movements that govern the homeostatic balance of the content of the blood and interstitial fluid around cells of the body.

Hypercapnia: An unusually high carbon dioxide concentration in the blood.
Hypercortisolism: Excessive levels of cortisol in the blood.
Hypermelanosis: Diffuse hyperpigmentation.
Hyperplasia (hypercellularity): An increase in organ size or tissue mass as a result of an increase in the number of constituent cells that are not neoplastic; hypoplasia refers to an underdevelopment of a tissue.
Hypersaline: An environment with salinity levels higher than that of seawater (32‰ or g/l).
Hypertrophy: An increase in organ size or tissue mass as a result of an increase in the size of constituent cells.
Hypervitaminosis: A condition caused by excessive ingestion of one or more vitamins. The condition is more common with the fat-soluble vitamins (namely A, D, E and K) than with the water-soluble vitamins, as it is more difficult for the body to excrete high levels of fat-soluble vitamins and so they are retained in tissues longer. Some examples of symptoms are growth depression, renal tubular mineralization, skin erosion, lameness, cataracts and skeletal deformities.
Hypocalcin: See **Stanniocalcin**.
Hypophyseotropic hormone: Neuropeptides synthesized in specialized neurons in the hypothalamus and transported via their axons to the anterior pituitary cells; the neuropeptides are released from synaptic terminals in the anterior pituitary gland and act to regulate ACTH, GtH, TSH, PRL, GH, MSH and MCH synthesis and secretion.
Hypothalamus: The brain region lying below the thalamus and above the pituitary gland; it regulates anterior pituitary gland function by the secretion of hypophyseotropic neurohormones, contains neurons that synthesize AVT and contributes to many autonomic nervous system functions.
Hypoxaemia: An unusually low oxygen concentration in the blood.
Hypoxia: Deficiency in the amount of oxygen reaching body tissues.

IGF: Insulin-like growth factor. There are two isoforms, IGF-1 and IGF-2. In post-embryonic fish IGF-1 is synthesized by hepatocytes under the regulation of GH. Both isoforms are also produced locally in many tissues, where they play, as yet undefined, autocrine or paracrine roles; IGF-2 appears to play important roles in early embryo development. IGF also works in concert with GH to effect cell growth.
Immunosuppression: A factor or a condition that reduces the functioning of the immune system. Stress is thought to depress immune function, which in turn can lead to disease pathogenesis. The term immunosuppression is used even though immune function may not be totally repressed.
Inhibin: Inhibin and follistatin (also called activin) are closely related proteins and members of the transforming growth factor-β (TFG-β) family. They play a number of autocrine roles in cellular biology of many tissues; in addition they play an endocrine role enhancing (follistatin) and decreasing (inhibin) the synthesis and secretion of FSH from the pituitary gland.
Insulin-like growth factor: See **IGF**.
Interleukins: A group of cytokines secreted by immune cells in response to a stressor or insult.
Intermediary metabolism: Biochemical reactions involved in storage as well as generation of metabolic energy for use in cellular processes.
Interrenal tissue (gland): Located in the head kidney of teleost fishes, the tissue comprises chromaffin cells, which secrete catecholamines (largely epinephrine), and steroidogenic cells, which secrete glucocorticoids (largely cortisol).
Intersex: The presence of male gametes in an ovary or female gametes in a testis, which is normally thought to be caused by exposure to extrinsic agents (e.g. toxicant, or parasite). This condition has also been referred to as 'ovo-testes' or 'testis–ova', respectively.

Interstitial cell: See **Leydig cell**.

Ionoregulatory cells: In the gill, specialized epithelial cells express specific protein channels and transporters that control the movements of ions between the fish and its aquatic environment, e.g. chloride and mitochondria-rich cells. These cells differ in their roles in freshwater and seawater fishes since the ionic and osmotic challenges are very different

Ischaemia: Loss of blood flow to a region of tissue or an organ system.

Iteroparous: An iteroparous organism is one that reproduces more than once in its lifetime, either within a season or in several years.

JGA: Juxtaglomerular apparatus, which is located in the kidney and is a component of the renin–angiotensin system (RA). The JG apparatus, comprising cells of the afferent arteriole, is in contact with sensory cells, the densa macula cells in the wall of the nephron. Reductions in blood pressure cause the cells in the afferent arteriole to release the enzyme renin, which acts on a blood protein angiotensinogen to produce the decapeptide angiotensin I; ACE in smooth muscles of the vascular system converts angiotensin I into the octapeptide angiotensin II, which causes vascular constriction (see **ACE**).

Juxtaglomerular apparatus: See **JGA**.

Kallekrein–kinin system: A poorly delineated system of blood proteins that plays a role in blood pressure regulation (vasodilation), inflammation and coagulation control; examples include bradykinin and kallidin.

Kyphosis: An abnormal spinal curvature causing the upper back to protrude.

Larva: The developmental stage of some species of fishes, beginning at the time of yolk absorption of the embryo and extending until the completion of differentiation; usually associated with a significant change in body form; sometimes incorrectly used to describe post-hatched embryos.

Lateral intercellular space: The space between the sides of epithelial cells, which forms a closed-end, water-filled space where electrochemical and osmotic gradients can accumulate, causing local salt and water flows.

Leiomyosarcoma: A malignant neoplasm originating from smooth muscle cells.

Leptin: A protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism. In fish a major source of leptin is the liver and the hormone has an inhibitory effect on feeding.

Leukaemia: A malignant neoplasm characterized by increased numbers of leucocytes in the blood and haematopoietic tissue.

Leydig cell: Steroidogenic cells of the testis (synonym: interstitial cells). The cells are located in the interstitium in-between the seminiferous tubules or lobules. In most teleostean fishes the cells synthesize and release androgens, including testosterone and 11-ketotestosterone.

LH: See **FSH**.

Ligand: The molecule that binds to a receptor or a transport protein; natural ligands include hormones and cell growth factors.

Lipid peroxidation: The degradation of unsaturated fatty acids (particularly the polyunsaturated fatty acids that contain multiple double bonds) with the formation of multiple oxidized products. It is a chain reaction process whereby free radicals abstract electrons from the unsaturated fatty acids to form a free radical (initiation), which, in turn, reacts with oxygen to form a peroxide and a new free radical (propagation). This chain reaction continues to play out until the generated free radicals begin to react with themselves to yield inactive products (termination). It results in cell membrane and tissue damage.

Lipoma: A benign neoplasm composed of adipocytes.

Liver disorders: Metabolic liver disorders can cause discoloration of the liver and an increase or decrease in hepatosomatic index (HSI), fatty liver or other pathological signs. An essential fatty acid deficiency causes increased HSI, swollen pale liver and fatty liver syndrome in several fish species. The liver is the main energy storage organ in cod and haddock, and their HSI is directly related to dietary lipid consumption. The total liver lipid in these species may range from 40 to 60% without any pathological changes.

Lordosis: A skeletal disorder characterized by an abnormal forward curvature of the spine in the lumbar region of the vertebrae, which results in a concave appearance when viewed from the side. This is a common sign of micronutrient deficiencies such as vitamin C, vitamin D, tryptophan and copper deficiencies.

Lower lethal limit: See **Tolerance range**.

Luteinizing hormone: See **FSH**.

Lymphatics: A system of vessels that drains the fluid (plasma minus protein, plus white blood cells) that results from filtration of the blood at the capillaries.

Lymphocytopenia: The reduction in the absolute number of circulating lymphocytes in the blood.

Lymphokines: A group of cytokines secreted by T lymphocytes, which act as chemical signals and induce growth and differentiation of white blood cells including other lymphocytes (also known as interleukins).

Lymphoma: Malignant neoplasm of lymphoid tissue (synonymous with lymphosarcoma).

Lymphosarcoma: A malignant neoplasm of lymphoid tissue.

M74: See **Early mortality syndrome**; M74 described the mortalities of Baltic Sea Atlantic stocks that were first recognized in 1974.

Malpigmentation: Also referred to as 'hypomelanosis' and is characterized by a lack of pigment that provides normal colour to skin, hair and eyes. In human beings, the condition often leads to eventual skin cancer. In some marine fish, it is thought to arise due to an imbalance of essential fatty acids (arachidonic acid, eicosapentaenoic acid) during the pigmentation window period of larval development.

MCH: Melanin-concentrating hormone (also called melanocyte-concentrating hormone); the cyclic heptadecapeptide is secreted by cells of the *pars intermedia* and stimulates melanin granule aggregation within melanophores, causing paling of the skin of many fishes.

Medulloepithelioma: A neoplasm composed of the cells that normally line the ventricles of the brain.

Melanocortin receptor (MCR): Also sometimes abbreviated as MR. This refers to a family of G-protein-coupled receptors. There are five members, MC1R to MC5R, in this family with varying specificities for melanocortins. MC2R, also known as ACTH receptor, binds ACTH and stimulates the synthesis of cortisol in the interrenal tissue. MCRs also play important roles in immune system function and the regulation of feeding behaviour.

Melanoma: A malignant neoplasm arising from melanocytes.

Melanophore-concentrating hormone: See **MCH**.

Melanophore-stimulating hormone: See **MSH**.

Melatonin: A tryptophan derivative synthesized by the pineal gland. Many tissues have melatonin receptors and respond to daily cycles of melatonin secretion; melatonin secretion is high during the dark phase of the 24-h daily cycle and is suppressed during the light phase; the plasma melatonin rhythms provide tissues with information about the length of the dark period (daily cycles) and changes in the length of the dark phase (seasonal cycles).

Membrane receptor: Most hormone receptors (and many growth factor receptors) are found in the plasma membrane of target cells. The hormone binds to a specific receptor protein; the binding site is on the exterior surface of the plasma membrane. The binding

of the hormone (the ligand) to its receptor causes a configurational change in the receptor, which initiates an intracellular cascade leading to changes in cellular metabolism or gene expression.

Meristic: An aspect of ichthyology that counts body features that occur in series and can be counted (e.g. myomeres, vertebrae, fin rays) in fish. Meristic traits are often described in a shorthand notation, called a meristic formula.

Meristic characters or parts: The serially repeated countable structures occurring in series.

Mesoderm: The middle embryonic germ cell layer, situated between the ectoderm and the endoderm. It gives rise to the skeleton–muscular system, connective tissue, the blood and internal organs.

Metaplasia: An adaptive response in which one mature cell type is replaced with another.

Metastasis: The dissemination of disease, including neoplasia, from one part of an organism to a distant site within the same or different organ.

Microarrays: A high-throughput technology used to determine simultaneously the expression of thousands of genes. A microarray works by exploiting the ability of a given mRNA molecule to bind specifically to, or hybridize to, the cDNA template from which it originated. By using an array containing many cDNA samples printed on a glass slide, the expression levels of thousands of genes within a cell or tissue sample can be determined by fluorescently labelling the RNA samples and hybridizing it to the array slides. The expression level can be quantified and is proportional to the mRNA abundance.

Mitochondrion-rich cells: Epithelial cells of the skin and gill that are specialized for salt and water transport and which have specialized microstructure and numerous mitochondria.

Mitogen: An agent or chemical that stimulates cell division (mitosis).

Mitosis: Cell replication by division.

Mixed-function oxidases (MFOs): A family of metabolic enzymes associated with P450 cytochromes, such as cytochrome P450 monoxygenases, that catalyse the oxidative biotransformation of various substrates, including chemical contaminants.

Monodeiodinase: Enzymes present in several tissues that are able to remove iodide from the thyroid hormones; there are three isoforms (D1, D2, D3), which act on different iodide units on these molecules either to activate hormone activity by converting T_4 to T_3 or to deactivate thyroid hormones by producing an inactive form of T_3 (reverse T_3) or the further deiodination of T_3 into thyronine ($T_3 \rightarrow T_2$ (diiodothyronine) $\rightarrow T_1$ (monoiodothyronine) $\rightarrow T_0$ (thyronine)).

MR: Mineralocorticoid receptor. MR, like GR, is a ligand-activated transcription factor belonging to the steroid hormone family of receptors. It is a nuclear receptor with high affinity for corticosteroid and aldosterone. However, in MR-expressing cells cortisol is inactivated by the enzyme 11-beta-hydroxysteroid dehydrogenase 2 (11b HSD2), thereby allowing aldosterone to activate the receptor. In fish aldosterone is missing, and a specific ligand for MR activation *in vivo*, other than cortisol, is currently unknown.

MSH: Melanophore-stimulating hormone, also called melanocyte-stimulating hormone or melanocorticotropin. The hormone is a peptide formed as part of the POMC peptide by MSH-secreting cells of the *pars intermedia* of the pituitary gland. MSH causes darkening of the skin in some fishes by causing dispersal of melanin granules in the melanophores of the skin. The presence of the hormone in species that do not show a skin response to MSH administration suggests that the hormone is involved in processes other than skin pigment regulation. Because MSH and ACTH both bind to receptors of the corticotrophin receptor family, MSH can affect cortisol secretion by the interrenal tissue; also, ACTH can affect skin pigmentation in species that do have endocrine control of pigmentation.

Mutagenicity: The property of being able to produce DNA damage.

Myocarditis: Inflammation of the muscular walls of the heart (myocardium). Generally caused by abnormal lipid metabolism or viral or bacterial infections; it typically causes heart failure and/or sudden death.

Myocardium: Cardiac muscle cells in general or cardiac muscle tissue. The term cardiomyocyte usually refers to a single cardiac muscle cell.

Myotome: A part of an embryonic somite in a vertebrate embryo that differentiates into skeletal muscle.

Na^+,K^+-ATPase: Intramembrane protein that actively transports three Na^+ out of the cell and two K^+ into the cell with each conversion of ATP to ADP + P_i; the sodium pump.

Necrosis: The process of cell death by degenerative events involving the loss of cellular integrity.

Neoplasia: A disease in which cells have escaped from normal growth regulation because of genetic alteration.

Neoplasm: An abnormal mass consisting of genetically altered cells that are typically not completely differentiated and to some extent are structurally and functionally independent of normal growth regulation.

Nephroblastoma: Neoplasm composed of embryonal kidney elements including poorly differentiated tubules and glomeruli.

Nephrocalcinosis: A condition characterized by the precipitation of calcium phosphate in the tubules and ducts of the kidney. These deposits may result in reduced growth and feed conversion efficiency and impaired renal function.

Neurilemmoma: See **Peripheral nerve sheath tumour**.

Neuroblastoma: A malignant neoplasm composed primarily of cells resembling embryonic cells that develop into neurons.

Neurofibroma: See **Peripheral nerve sheath tumour**.

Neuropeptide Y: A 36-amino-acid neurotransmitter peptide found in the brain and autonomic nervous system. NPY has been associated with a number of physiological processes in the brain, including the regulation of energy balance by increasing food intake, decreasing physical activity and increasing the proportion of energy stored as fat. NPY involvement in the hypothalamus regulation of pituitary hormone secretion has also been found.

Neurotransmitter substance: Molecules released by the synaptic regions of neurons that bind to receptors on the subsynaptic membrane and activate or suppress action potential generation in the subsynaptic membrane. Examples include excitatory amino acids (such as glutamate), acetyl choline (ACh), gamma amino butyric acid (GABA), serotonin (5-HT) and norepinephrine.

NIS symporter: Sodium iodide symporter (the 'N' refers to the symbol for sodium, Na^+). The symporter is found in the basilateral membrane of thyroid follicle cells (thyrocytes) and allows iodide, needed for the formation of the thyroid hormones, to move across the thyrocyte membrane; the influx of Na^+ across the membrane provides the energy for iodide uptake against its concentration gradient.

Nitric oxide (NO): A gaseous membrane-permeable neurotransmitter with biological activity.

NKCC: An intramembranous cotransport protein that translocates a neutral complex of one Na^+, one K^+ and two Cl^- ions simultaneously down their summed chemical gradients.

Norepinephrine: Formerly noradrenaline; see **Catecholamine**.

NPY: See **Neuropeptide Y**.

Nuclear receptor: Also called genomic receptor. These receptors exert their action by attaching to specific regions of the promoter regions of genes – see **HRE**. Steroid hormone receptors are generally present in the target cell cytoplasm in association with chaperone proteins. The steroid ligands enter the target cell cytoplasm and attach to their receptor, which causes the separation of the receptor from the chaperone protein. The activated

receptors form a homodimer, which migrates into the nucleus, attaches to a HRE that is specific for that receptor and activates the expression of specific genes. For thyroid hormone (T_3), the receptor complex is a heterodimer of the thyroid hormone receptor (TR) associated with a rentinol receptor (RXR), and the heterodimer is attached to the thyroid response element (TRE) in the nucleus without the TR being activated by T_3; without activation of the receptor heterodimer, the transcription factor has a gene silencing action. With the activation of the TR by T_3, the transcription factor plays a role in regulating the expression of the genes that contain a TRE.

Oedema: An unusual build-up of lymphatic fluid in the interstitial spaces of tissues, such as in the pericardial sac, intrapleural spaces, peritoneal cavity or joint capsules. It can have numerous causes, but some common causes are increased fluid pressures caused by venous or lymphatic obstructions, resulting in heart or renal failures.

Oestrogen: An agonist for the oestrogen receptor in vertebrates. Note that natural oestrogen in most teleostean fishes is 17β-oestradiol.

Ontogeny: The course of embryonic development of an individual organism.

Oocyte: A cell in the ovary from which an ovum develops by meiosis; a female gametocyte. The oocyte goes through a process of maturation and enlargement, which is recognized by classification as a primary oocyte, secondary oocyte, etc.

Oogenesis: The process of differentiation and maturation of female gametes in the ovary.

Oogonia: A precursor to the oocyte; derived from a primordial germ cell in the ovary.

Opsonization: The process in which antibodies or complement bind to antigens and promote phagocytosis.

Orexigenic: Appetite stimulating.

Orexins: Also called hypocretins; these are the common names of a pair of excitatory neuropeptide hormones (orexin-A and orexin-B) that stimulate food intake and are found in all vertebrates

Osmoregulation: The processes that control the osmotic activity of the blood and interstitial fluid surrounding cells.

Osteocalcin: A protein secreted by osteoblasts, which plays a role in bone mineralization.

Osteonectin: A glycoprotein that binds calcium, thereby contributing to bone ossification.

Oviparous: Fish that release eggs with no embryonic development within the mother. Almost all non-oviparous fish are ovoviparous, where the embryos hatch from eggs inside the mother's coelomic cavity. In the case of the sea horse the eggs are inserted into the brood pouch of the male by the female; the eggs are fertilized inside the brood pouch and the embryros are incubated within the male's brood pouch until hatch.

Ovo-testis: See **Intersex**.

Ovoviviparous: See **Oviparous**.

Oxidative stress: Free radicals (molecules with unpaired electrons), including superoxide radicals (an oxygen molecule with an extra unpaired electron), can be generated in mitochondria by the leakage of electrons from the electron transport system. These reactive oxygen species can cause damage to the cell and exert an oxidative stress on the cell and on the organism. Antioxidants such as glutathione and ascorbic acid are examples of free radical scavengers.

Oxygen carrying capacity of blood: The amount of oxygen contained in a given amount of blood, which is largely determined by the concentration of haemoglobin in blood, which in turn is related to haematocrit.

P450: See **CYP**.

PACAP: Pituitary adenylate cyclase-activating polypeptide is so named because its ligand-activated receptor increases cAMP levels in target cells. PACAP is a hypophyseotropic hormone and functions as a neurotransmitter and neuromodulator.

Papilloma: A benign epithelial neoplasm that has a vascular connective tissue stroma and forms finger-like projections from the epithelial surface.

Paracrine: Hormones or growth factors secreted by cells into the interstitial fluid that exert their actions on cells that are adjacent to those that secrete the hormone; these paracrine (as well as autocrine) factors exert local control over cell and tissue function.

Parr: Juvenile freshwater stage in the life cycle of salmon and some trout species, which undergo transformation to the smolt stage.

***Pars distalis*:** Part of the anterior pituitary gland (along with the *pars intermedia*) in teleost fishes. The *pars distalis* comprises the *rostral pars distalis* and *proximal pars distalis*; each region contains different cell types. The *rostral* region comprises PRL and ACTH cells, together with TSH cells in some species; the *proximal* region comprises GtH and GH cells, together with TSH cells in some species.

***Pars intermedia*:** Part of the anterior pituitary gland (along with the *pars distalis*) in teleost fishes. The *pars intermedia* comprises cells that secrete MSH and MCH in many species.

***Pars nervosa*:** Also called the neurohypophysis or posterior pituitary gland. The *pars nervosa* comprises the ending of axons that originate in neurons in the hypothalamus and end on the vascular complex that characterizes this gland. The primary hormone, AVT, and smaller amounts of other octapeptides are released into the intercellular fluid surrounding the blood capillaries.

Parthenogenesis: An asexual form of reproduction found in females, where growth and development of embryos occurs without fertilization by a male; the offspring produced by parthenogenesis are always female in species that use XY sex determination.

Pavement cells: Flattened polyhedral cells of the skin and gill epithelium that are joined to each other by tight junctions forming a barrier between the blood and the environment.

PBR: Peripheral-type benzodiazepine receptor is a membrane-associated protein found in many tissues. In steroidogenic cells PBR is involved, together with StAR protein, in the transfer of cholesterol from the cell cytoplasm into the mitochondria. As the cholesterol enters the inner mitochondrial compartment, it is converted to pregnenolone by the mitochondrial enzyme, cytochrome P450 side-chain cleavage (P450scc).

PCB: See **Polychlorinated biphenyls**.

Pericarditis: Inflammation of the pericardial space surrounding the heart.

Pericytoma: A benign tumour composed of pericytes; differs from haemangiopericytoma by the lack of new blood vessel formation.

Peripheral nerve sheath tumour: A neoplasm arising from the cells that form the covering of peripheral nerves. Neurilemmoma, neurofibroma and schwannoma are types of peripheral nerve sheath tumours but are considered synonymous by some fish pathologists.

Peripheral-type benzodiazepine receptor: See **PBR**.

Permissive action of hormones: A hormone (H1) that has a permissive action on another hormone (H2) allows the full expression of the effect of H2. The interaction may take the form of H1 being needed for the synthesis of H2 or its receptor, or H1 may stimulate the synthesis of factors that are required for the process regulated by H2.

Phthitic: A shrinkage and wastage of an organ.

Phyto-oestrogen: A diverse group of naturally occurring non-steroidal plant compounds that, because of their structural similarity with 17β-oestradiol, have the ability to interact with oestrogen receptors (ERs) and cause oestrogenic or anti-oestrogenic effects. Phyto-oestrogens mainly belong to a large group of substituted phenolic compounds such as flavenoids.

Pituitary adenylate cyclase-activating polypeptide: See **PACAP**.

Plasma membrane: The semi-permeable lipid and protein mosaic membrane that defines the cell margin and separates interstitial fluid from the cell cytoplasm (cytosol).

Pleomorphic: Variable in size and shape.

Polychlorinated biphenyls (PCBs): A group of industrial chemicals that possess great thermal and chemical stability. Commercial PCB mixtures contain different combinations of the 209 possible PCB congeners.

Pre-vitellogenic oocytes: Oocytes in which vitellogenin egg protein has not been deposited: see **VtG**.

Primordium: An organ or tissue in its earliest recognizable stage of development.

PRL: Prolactin; a protein hormone secreted by the somatotropic cells (prolactin-secreting cells) of the *rostral pars distalis* of the anterior pituitary gland. The hormone is named after its lactogenic role in mammals, but in fish the hormone plays an important role in osmoregulation in freshwater teleostean species and has some metabolic roles.

Procarcinogen: A carcinogen that is inactive until it has been metabolized.

Prolactin: See **PRL**.

Prolactin-releasing peptide: See **PrRP**.

Promoter: An agent that, when administered after a carcinogen, increases the number of neoplasms produced but does not cause neoplasms when administered alone.

Promoter region of a gene: A region of the gene that is involved in and necessary for initiation of transcription and to which gene regulatory proteins (transcription factors) may bind.

Prostaglandins: Modified fatty acids produced by many cells, which function as chemical messengers.

Proximal pars distalis*:** See ***Pars distalis.

Proximate carcinogen: A carcinogen that results from the metabolism of a procarcinogen.

PrRP: Prolactin-releasing peptide; A hypophyseotropic neuropeptide involved in the regulation of the secretion of prolactin, SL and possibly also GH and other hormones in the anterior pituitary gland.

Pugheadness: Anomalous head anatomy with variable distortions, which can include disproportional jaw and cranial anatomy, resulting in a relatively smaller upper jaw as compared with the lower jaw.

RA: See **JGA**.

Rathke's pouch: During embryo development, the anterior pituitary gland forms as an up-pushing of the roof of the mouth, which then separates as a hollow ball of cells, called Rathke's pouch, which migrates dorsally to meet the down-pushing of the floor of the hypothalamus. Rathke's pouch is the primordium of the anterior pituitary gland (*pars distalis* and *pars intermedia*); the down-pushing of the hypothalamus is the primordium of the posterior pituitary gland (*pars nervosa*).

Receptor protein: Proteins that have binding sites for specific ligands; attachment of the ligand with its protein will activate the protein and initiate a range of intracellular events, which may include ion transport, phosphorylation of enzyme systems and activation of transcription factors that alter gene expression. The ligands include hormones, growth factors and neurotransmitter substances.

Red tides (blooms): An unusual rapid growth of certain planktonic organisms that are known to be toxic to fishes and other animals. More common during spells of hot, wind-free weather, which create aquatic surface warming.

Renal: Pertaining to the kidney.

Renin: See **JGA**.

Resistance range: See **Tolerance range**.

Rhabdomyosarcoma: A malignant neoplasm of striated muscle.

Rheotaxis: Behavioural orientation to water flow.

Rostral pars distalis*:** See ***Pars distalis.

RU486: Mifepristone, a drug that blocks steroid receptors with some selectivity for glucocorticoid receptors.

RXR: See **Nuclear receptor**.

Sarcoma: A malignant neoplasm originating from mesenchymal tissue.

Schwannoma: See **Peripheral nerve sheath tumour**.

Sclerotome: A component of the mesodermal somite that will develop into the cartilage of the vertebrae.

Scoliosis: A skeletal disorder characterized by an abnormal lateral curvature of the spine, which results in a concave appearance when viewed from the front. It is a common symptom of impaired muscle growth and/or muscle imbalance, often caused by certain metabolic diseases, chronic improper posture, genetic factors and nutritional deficiencies or toxicities.

Secondary circulatory system: In addition to the primary circulatory system as found in other vertebrates, fishes have a secondary circulatory system, which is characterized by a low content of red blood cells, as well as a low flow rate and blood pressure. Skin and scales are well invested with this circulation, but this is not obvious because of the near absence of red blood cells. Red blood cells can enter the secondary circulation under stressful conditions, contributing to the red coloration of the skin.

Secondary lamella (plural lamellae): Leaflet-like protrusions on the gills of fishes that are highly vascularized and create a large exchange surface area for the diffusional exchange of gases, ions and water, as well as a surface for antigen attack. Changes or damage to these structures will impede normal physiological exchanges.

Sekoke disease: A condition characterized by reduced appetite, poor growth rate, muscle flesh necrosis and lesions in the kidney and pancreatic tissues of fish fed diets containing oxidized oils. Feeding diets supplemented with additional biological antioxidants (such as vitamin E) helps mitigate the problem.

Semelparous: Describes an organism that reproduces just once during its lifetime, after which it dies; examples include most Pacific salmon species.

Seminoma: A malignant neoplasm of the germinal tissue of the testis.

Sertoli cell: Cells that make up the seminiferous epithelium, and thereby the seminiferous tubules or lobules. The cells form part of the blood–testis barrier, which provides protection of the haploid gametes from attack by the parental immune system. The seminiferous lumen contains fluid that is rich in androgen-binding protein, which maintains a high concentration of androgens, particularly 11-ketotestosterone, within the tubules; this is necessary for spermatogenesis and spermiogenesis. In some species, the Sertoli cells synthesize P450 aromatase, which converts androgens (particularly testosterone and androstenedione) into oestrogens, which may also be needed for normal sperm maturation.

SGK: Serum and glucocorticoid-indicible kinase, a ser/thr kinase responsive to cortisol.

SL: Abbreviation for somatolactin, a hormone of the same family as GH and PRL. The hormone is secreted by selective cells in the *pars intermedia* and appears to play a role in calcium homeostasis of some fish species.

Smolt: Developmental stage of young salmonid fishes when the animals move downstream and become adapted to living in seawater (see also **Parr**).

Sodium ion iodide symporter: See **NIS symporter**.

Somatolactin: See **SL**.

Somatomeres: Mesodermal material that gives rise to somites.

Somatostatin: See **SRIF**.

Somite: A segment of mesoderm tissue in vertebrate embryos that develops into muscles, vertebrae and the dermis.

Spermatocyte: A cell in the testis from which spermatozoa develop by meiosis; a male gametocyte. The spermatocyte goes through a process of maturation, followed by development into spermatids, and finally spermatozoa.

Spermatogenesis: The process of differentiation and maturation of male gametes in the testis.

Spermatogonia: A precursor to the spermatocyte, which is derived from a primordial germ cell in the testis.

Spermiation: The release of mature spermatozoa from the testis, typically in association with seminal fluid.

Splice variant: Messenger RNA sequences that result from cutting and resealing of an RNA transcript by precise breakage of phosphodiester bonds at the 5′ and 3′ splice sites (exon–intron junction).

Squamous cell carcinoma: A malignant neoplasm arising from flattened (squamous) epithelial cells.

SRIF: Somatotropin release inhibiting factor, also called somatostatin. The peptide somatostatin-14 is one of the neurohormones produced by specialized cells in the hypothalamus; it acts on the somatotropic (GH) cells of the *proximal pars distalis* to inhibit GH secretion. Another form of SRIF, SRIF-28, is produced by the endocrine pancreas and other tissues and plays roles related to cellular metabolism, secretion of the *pars nervosa*, paracrine roles in the regulation of endocrine pancreatic function and possibly also in the inhibition of GH secretion.

Stable isotope: Most elements have different isotopes based on their atomic weight. Some isoforms are unstable and emit energy of different forms; these are radioactive isotopes. Other isoforms are stable, and the ratios of different forms of stable isotopes in body tissues can be used as indicators of food sources of a population and provide other forms of information about the growth of organisms, including fish.

Standing gradient hypothesis: A thermodynamic transport model that predicts solute and water flows across epithelial membranes based on microscopically local osmotic and ionic gradients in the lateral intercellular spaces.

Stanniocalcin (STC): A polypeptide hormone produced in bony fish by the corpuscles of Stannius, which are located in the kidney parenchyma; the hormone is involved in calcium and phosphate regulation, acting locally in the kidney and gut to modulate calcium and phosphate excretion; it is a major antihypercalcaemic hormone in fish. Because corpuscles of Stannius are not found in mammals, the discovery of a mammalian homologue, STC1, was surprising and intriguing; STC1 displays a relatively high amino acid sequence identity (~50%) with fish and is expressed in many tissues, including kidney.

Stanniocytes: Cells of the corpuscles of Stannius that secrete the hormone stanniocalcin.

Stanza: A term applied to describe the different growth rates seen during different stages of ontogeny of fishes.

StAR: Steroidogenic acute regulatory protein.

Stellate cell: Non-granulated (non-hormone secreting) cells in the *pars distalis*, which appear to act as support cells for hormone-secreting cells.

Stenohaline: The term describes fish that physiologically cannot adapt readily to major changes in environmental salinity. The term describes both species that are adapted to fresh water and species that are adapted to seawater. Fish that inhabit coastal estuaries and species that can adapt to a wide range of salinities are referred to as euryhaline.

Steroidogenic acute regulatory protein: See **StAR**.

Stress: A physiological response to adverse conditions.

Stressor: An environmental stimulus or stimuli that could bring about a change or disturbance in the homeostasis of an animal.

Stress response: The molecular, biochemical and physiological adjustments in response to a stressor that allow the animal to re-establish homeostasis.

Swimming performance: A general term for the maximum prolonged aerobic swimming speed of a fish.

Symport(er): See **Cotransporter**.

Synergistic action of hormones: The working together of two or more hormones that brings about a response that is greater than the sum of the effect of the group of hormones: in effect, a biomagnification of the response.

Systemic circulation: In mammals, a collective term for the portion of the cardiovascular system that carries oxygenated blood away from the heart to the body and returns deoxygenated blood back to the heart. The term is contrasted with pulmonary circulation, which carries deoxygenated blood away from the heart and returns oxygenated blood back to the heart. In fish the term describes the single blood circulatory system, which carries deoxygenated blood away from the heart toward the gills, where it becomes oxygenated; the oxygenated blood then passes to body tissues and deoxygenated blood is returned to the heart.

T_3: Triiodothyronine is the major biologically active thyroid hormone, acting on nuclear receptors to regulate the expression of specific genes. Some T_3 is released from the thyroid gland, but most of the T_3 in the circulation is produced by peripheral tissues such as liver and kidney by the monodeiodination of T_4. Some organ systems, such as the brain, produce sufficient T_3 to meet their own local needs; others, such as the liver and kidney, produce T_3 that is released via thyroid hormone transport proteins back into the blood to act on other target cells.

T_4: Thyroxine or tetraiodothyronine: the major thyroid hormone product; it acts as a prohormone for the production of T_3 (see **T_3**). In mammals, recent findings suggest the presence of a T_4-specific receptor that is involved in the formation of blood vessels (angiogenesis); it is currently not know whether this is also true for fish.

Target cells: A commonly used term to describe the cells that respond to a particular hormone; 'target cells' contain the particular receptor to respond to a specific hormone.

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin): The most toxic dioxin congener and prototypical AhR ligand.

Teratoid neoplasm: A neoplasm derived from more than one embryonal layer and consisting of a variety of tissue types.

Teratomas: A kind of encapsulated neoplasm (tumour) (usually benign) containing tissue or organ components resembling normal derivatives of ectoderm, mesoderm and endoderm.

Testicular atrophy: A reduction in the size of the testis, typically as a result of the loss of germinal tissue.

Testicular fibrosis: Replacement of germinal tissue in the testis by fibrotic (connective) tissue.

Testis–ova: See **Intersex**.

Tetany: An abnormal condition characterized by sharp flexion of the wrist and ankle joints (carpopedal spasm), muscle twitchings, cramps, numbness of the extremities and convulsions, sometimes with attacks of stridor. It is due to abnormal calcium metabolism and occurs in parathyroid hypofunction, magnesium and vitamin D deficiencies, alkalosis and the result of the ingestion of alkaline salts.

Tetraiodothyronine: See **T_4**.

Thecal cell: One of the two types of steroidogenic cells of the ovarian follicle; during gonadal maturation the thecal cells are stimulated by gonadotropins to synthesize androgens; the androgens enter the second type of steroidogenic cell, the granulosal cells, where they are converted into oestrogens by P450 aromatase.

Thymoma: A neoplasm arising from the thymic epithelial cells.

Thyrocyte: The scientific name for the cells that comprise the follicle epithelium of the thyroid gland. The cells carry out the uptake of iodide, synthesis of thyroglobulin (Tg) and secretion of Tg into the lumen of the follicle, where the oxidative iodination of Tg occurs. The thyrocytes also take up droplets of Tg from the lumen and fuse the droplets with primary lysosomes to digest the Tg and release the iodinated tyrosine and thyronine compounds, which include the thyroid hormones T_4 and T_3.

Thyroglobulin: Thyroglobulin (Tg) is a large protein molecule that is synthesized by thyrocytes under the stimulus of TSH. The protein is transferred by exocytosis from the thyrocyte cytoplasm to the lumen of the thyroid follicle (or tubule). Oxidative iodination of the tyrosine units of Tg occurs on the luminal surface of the apical thyrocyte membrane, forming mono- (MIT) and diiodotyrosine (DIT) elements within the Tg molecule; the thyroid-specific enzyme, thyroid peroxidase (TPO), catalyses the reaction. A second oxidative reaction, also involving TPO, causes condensation of the MIT and DIT units to form triiodothyronine (MIT + DIT) or tetraiodothyronine (DIT + DIT); these are the future thyroid hormones, T_3 and T_4, respectively, but they are still components of the Tg molecule. The release of the hormones occurs in the cytoplasm of the thyrocytes – endocytosis of Tg (a mixture of iodinated and non-iodinated) occurs, followed by proteolysis of the Tg to release T_4 and T_3, together with any MIT and DIT that has not been condensed.

Thyroid-stimulating hormone: See **TSH**.

Thyronine compounds: Compounds that are derivatives of the amino acid thyronine. In thyroid physiology the term is used to describe the iodinate thyronine compounds thyroxine (T_4) and T_3.

Thyroxine: See **T_4**.

Tolerance range: A term related to the concept of homeostasis. The range or the limits of environmental challenge within which the animal is able to regulate and maintain its normal physiological equilibrium; at the upper and lower limits of the tolerance range the animal will resist further changes (termed the 'resistance ranges'), but there will be some destabilization of the characteristics of the 'inner environment' – extracellular fluid. The extreme limits of the upper and lower resistance ranges are the upper and lower lethal limits, respectively, at which point the animal will die.

Toxin: A poison produced by another organism.

Transcripts: Commonly used term for copies of RNA

Transcription: Making an RNA copy from a sequence of DNA (a gene). Transcription is the first step in gene expression. Both RNA and DNA use complementary language, and the information is simply transcribed, or copied, from one molecule to the other. The DNA sequence is enzymatically copied by RNA polymerase to produce a complementary nucleotide RNA strand, called messenger RNA (mRNA), because it carries a genetic message from the DNA to the protein-synthesizing machinery of the cell. This process occurs in the nucleus.

Transcription factor: Proteins, sometimes hormone receptors, that bind to response elements in the promoter region of genes, which either enhance or impair the expression of specific genes.

Transcriptomics: Study of large-scale or global gene expression patterns.

Translation: The process of converting messenger RNA (mRNA) into protein. Translation occurs in the cytoplasm, where the ribosomes are located. Ribosomes are made up of a small and a large subunit, which surrounds the mRNA. In translation, messenger RNA is decoded to produce a specific polypeptide according to the rules specified by the genetic code.

Transport proteins: (i) Intramembrane proteins involved in the transmembrane movement of charged (ions) or uncharged solutes (e.g. urea, water) by passive or active processes; (ii) the term is also used to describe proteins that are involved in the transport of hor-

mones and other factors in the blood. They serve several purposes, including maintaining a reservoir of available hormone (only unbound hormone can react with its receptor on a target cell), and they protect small molecules, such as steroid and thyroid hormones, from being lost by filtration via the kidney glomerulae, and thereby excreted.

Triiodothyronine: See **T_3**.

Triploidy: An individual possessing three sets of chromosomes in their somatic cell nuclei rather than two (diploid).

TR: Thyroid hormone receptor; it generally refers to the nuclear receptor protein that binds preferentially to T_3.

TRE: Thyroid hormone response element; the sequence of nucleotide base units in the promoter region of specific genes to which the TR-RXR heterodimer attaches and acts as a transcription factor (see **Nuclear receptor**).

TSH: Thyroid-stimulating hormone, a glycoprotein hormone synthesized by and released from the thyrotropic cells of the *pars distalis* of the pituitary gland. TSH stimulates the thyrocytes to: (i) synthesize NIS transporters for the uptake of iodide; (ii) synthesize thyroglobulin (Tg); (iii) carry out the exocytosis of Tg to the thyroid follicle/tubular lumen; (iv) synthesize thyroid peroxidase (TPO), which is needed for the iodination of Tg and the condensation of iodinated tyrosine compound to form iodinated thyronine compounds; (v) stimulate the endocytosis of Tg droplets from the lumen; (vi) synthesize primary lysosomes, which are needed for the proteolysis of the Tg to release the thyroid hormones; and (vii) synthesize transmembrane transport proteins, which allow the movement of the thyroid hormones out of the thyrocyte into the interstitial fluid.

Tumour: A neoplasm. Also used historically by some authors to include non-neoplastic tissue masses.

Tyrosine compounds: Compounds that are derivatives of the amino acid tyrosine. In thyroid physiology the term is used to describe the iodinate tyrosines, monoiodinated (MIT) and diiodinated tyrosine (DIT), which are oxidatively condensed by the activity of thyroid peroxidase to form the iodinated thyronine compounds T_4 and T_3.

UB: Ultimobranchial gland; a gland derived from the fifth branchial pouch in embryos; in adults it lies in the traverse septum that separates the heart from the abdominal cavity. The gland is made up of small follicles of cells that secrete the hormone calcitonin, which may be involved in some aspects of calcium ion homeostasis.

Ultimobranchial gland: See **UB**.

Upper lethal limit: See **Tolerance range**.

Urogenital papillae: A protuberance around the urogenital opening in fish and other lower vertebrates; usually more pronounced in females.

Urotensin: Vasoactive cyclic neuropeptides (I and II) secreted by the urophysis (caudal neurosecretory system) of teleost fish, which have structural similarity to SRIF. The vasoconstriction potency of urotensin II is an order of magnitude greater than that of the endothelins. Urotensin I may also play a role in the regulation of food intake and act together with a related peptide, CRH, in the hypothalamic regulation of pituitary adrenocorticotrop function.

Uveitis: Inflammation of the vascular networks of the eye.

Vitellogenesis: Synthesis of vitellogenin by hepatocytes in response to oestradiol stimulation.

Vitellogenic oocytes: Oocytes after vitellogenin egg protein has been deposited. See **VtG**.

Vitellogenin: See **VtG**.

Viviparity: Mode of reproduction found in some taxonomic groups of fishes, in which fertilization and full embryonic development occur within the maternal reproductive tract, leading to the birth of free-living progeny.

VtG: Vitellogenin, a phospholipoprotein synthesized by hepatocytes under the influence of oestrogenic compounds. VtG is incorporated into the developing oocytes during the 'vitellogenic' phase of maturation, and together with lipids is the major source of nutrients for the developing embryo. Significant amounts of VtG are normally present in the plasma of female fish only during the vitellogenic phase of gonadal (oocyte) maturation, when oestrogen secretion is at a high level. Normally, trace amounts of VtG are found in the plasma of male fish and sexually immature female fish; however, higher VtG levels in males and immature females are found in fish exposed to environmental oestrogens (xeno-oestrogens), and this has been used as a biological indicator of the presence of environmental oestrogenic contaminants.

V-type H^+-ATPase: Vacuolar-type proton adenosinetriphosphatase (ATPase), an intramembrane enzyme that translocates acid equivalents from one side of a membrane to the other, linked to catalysis of ATP to ADP, named for the cellular location of discovery in plant vacuolar membranes.

Yolk-sac membrane: The body of protein and lipoprotein food (yolk) for embryos encapsulated within an epithelial membrane, which operates as a gill-like structure before the gills have developed.

Xenobiotic: A chemical present in an organism that is not normally produced or expected to be present; the term may also be used to describe chemicals that are present at much higher levels than expected. In fish, the term is usually used to describe compounds taken up from the environment, such as oestrogenic compounds, or other pollutants, such as dioxins and PCBs.

Xeno-oestrogen: A naturally occurring xenobiotic compound or a xenobiotic contaminant that has oestrogenic properties, i.e. it is able to bind to the oestrogen receptor and have an agonistic action. The term has sometimes been used to describe xenobiotic compounds that have either an agonistic or an antagonistic effect on the oestrogen receptor.

***Zona pellucida*:** Also called the *zona radiata* in fish. In each developing ovarian follicle, the *zona pellucida* is overlaid by a layer of steroidogenic cells, an outer layer comprising thecal cells and an inner layer comprising granulosal cells.

Zona radiata*:** See ***Zona pellucida.

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