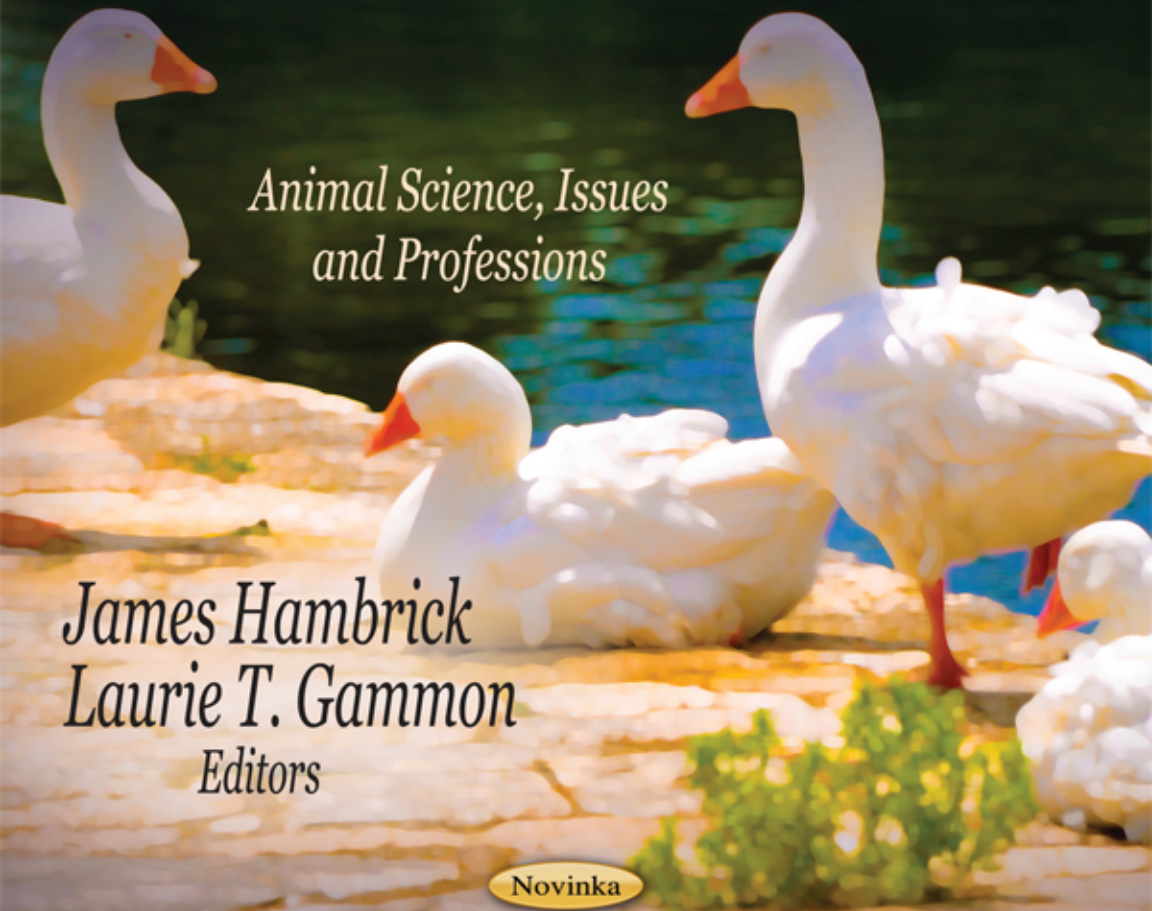
DUCKS

Habitat, Behavior and Diseases

Animal Science, Issues and Professions



James Hambrick
Laurie T. Gammon
Editors

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ANIMAL SCIENCE, ISSUES AND PROFESSIONS

DUCKS

HABITAT, BEHAVIOR AND DISEASES

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ANIMAL SCIENCE, ISSUES AND PROFESSIONS

DUCKS

HABITAT, BEHAVIOR AND DISEASES

JAMES HAMBRICK
AND
LAURIE T. GAMMON
EDITORS



New York

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CONTENTS

Preface		**vii**
Chapter 1	Bornaviruses in North American Waterfowl *Susan L. Payne, Guo Jianhua and Ian Tizard*	**1**
Chapter 2	Critical Components of Duck Research: The Origin and Genetics of Domestic Ducks *Ian Tizard and Donald J. Brightsmith*	**21**
Chapter 3	Recent Viral Diseases of Japanese Anatid with a Fatal Case of Marek's Disease in an Endangered Species, White-Fronted Goose (Anser albifrons) *Mitsuhiko Asakawa, Tetsuya Nakade, Shiro Murata, Kazuhiko Ohashi, Yuichi Osa and Hiroyuki Taniyama*	**37**
Chapter 4	Bird Schistosomes *Marta Chanová*	**49**
Chapter 5	A Brief Overview of Parasitic Nematodes Recorded from Waterfowls on Hokkaido, Japan *Tomoo Yoshino and Mitsuhiko Asakawa*	**59**
Index		**65**

PREFACE

This book studies the habitat, behavior and diseases related to ducks. Topics include the origin and genetics of domestic ducks; bornaviruses in North American waterfowl; recent viral diseases of Japanese anatid with a fatal case of Marek's disease in the endangered white-fronted goose; bird schistosomes; and parasitic nematodes in waterfowls of Hokkaido, Japan.

Chapter 1 – Bornaviruses are enveloped, non-segmented negative strand RNA viruses. They replicate in the host cell nucleus, cause persistent infections, are non-cytopathic in cell culture and are highly cell-associated. The first avian bornavirus (ABV) was identified in 2008 and is the etiologic agent of proventricular dilatation disease (PDD) in parrots. ABV replicates in many organs, especially the central nervous system. It can be intermittently detected in cloacal swabs and feather follicles. During surveys to determine the possible origins of ABV the authors examined multiple oropharyngeal/fecal swabs and brain samples from multiple species of geese, ducks and mute swans. Reverse transcription PCR was used to screen RNA samples from swab samples from 409 Canada geese (*Branta canadensis*) and 200 mute swans (*Cygnus olor*) of which 3.6% tested positive for ABV. Brain tissues were subsequently collected from 25 Canada geese and 192 mute swans and 200 ducks of multiple species; ABV was detected in 52% and 23% of Canada goose and mute swan brain samples respectively. ABV was also detected in 11% of brain samples from hunter-harvested ducks.

Of the major duck species tested, 7/84 (8.3%) of redheads (*Aythya americana*), 4/63 (6.3%) of northern pintails (*Anas acuta*) and 6/25 (24%) of gadwalls (*Anas strepera*) were ABV positive. Among other duck species, 2/9 (22%) northern shovelers (*Anas clypeata*), 1/10 (10%) mallards (*Anas platyrhynchos*), and 4/12 (33%) American widgeons (*Anas americana*) were

also positive. RT-PCR products obtained from Canada geese, mute swans and ducks were sequenced, revealing that two different genotypes of ABV were circulating in these waterfowl. ABV genotypes 4 (ABV4) was recovered from ducks in Texas while a distinct genotype (ABV-CG) was recovered from geese and swans across the United States. ABV-CG was also detected in a small number of embryonated mallard duck eggs obtained from commercial breeders. ABV-CG appears, under some circumstances, to cause acute encephalitis or proventricular dilation disease in geese and swans.

Chapter 2 – Biomedical science is increasingly focused on the fine details of DNA and molecular structure, making it vital that investigators describe accurately and precisely the origins and genetics of the animals included in their studies. The chicken and the turkey, the world's most common domesticated birds, both originated from a single species, but domestic ducks have a much more convoluted history making accurate descriptions of their origin and genetics complicated. The phylogeny of the predominant domestic species, the mallard (*Anas platyrhynchos*) is especially complex. In addition, the domestication of the mallard has, on occasion, included interbreeding with other wild duck species including the Eastern spot-billed duck (*Anas zonorhyncha)* and the American black duck (*Anas rubripes)*. An unrelated species, the Muscovy duck (*Cairina moschata*) was originally domesticated in pre-Columbian America. As a result of this complexity it is essential that any scientific papers or gene sequences pertaining to ducks, especially domestic ducks, document in considerable detail the genetic and taxonomic provenance of the birds studied. It is inappropriate to refer to these birds simply as "ducks".

Chapter 3 – In our increasingly managed environment, where the maintenance of many ecosystems, habitats and species rests largely in human hands, disease control is now an important process in the active conservation of wild waterfowl populations because individual disease outbreaks are known to have killed many tens of thousands of a wide range of wild bird species. On the other hand, most infectious agents, especially commensal viruses, are component parts of the ecosystems in which their wild avian hosts occur, they have co-evolved, and the infectious agents do not necessarily cause disease. But, some viral diseases have killed wild waterfowls including ducks, geese, swans, and captive ones, as well. Considerable environmental change has occurred in Japan over the last 150 years. With the increased availability of firearms following the Meiji Restoration (1860s), the populations of duck (*Anas* and *Aythya* spp.), geese (*Anser* and *Branta* spp.), and swans (*Cygnus* spp.) were dramatically depleted during the late 19th and early 20th centuries.

Furthermore considerable impact as a result of industrial development during the 20th century (particularly during the post 1945 rapid development phase) led to extensive environmental damage to marine, coastal, lowland, freshwater, and montane ecosystems, with extensive areas of natural habitat being entirely destroyed (coastal and lowland wetlands have been particularly hard hit). As a consequence of this, avian habitats have been greatly altered and populations mostly reduced. Although waterfowl populations steadily increased during the latter part of the 20th century as persecution has declined (Japanese Association for Wild Geese Protection 1994), continued habitat loss has led to extensive concentration of populations. Not only are waterfowl populations greatly concentrated at shrinking wetland sites, both during migratory stop-over, and during the main winter period, but they are also remaining at fewer sites for longer periods of time, thus greatly increasing the risk of, and facilitating, bird-to-bird spread of such pathogens. An understanding of the infectious and parasitic avian disease organisms that are present in Japan, their ecology, transmission, and symptoms is an essential conservation tool. The authors' aim in this chapter is, therefore, to provide an overview of viral disease organisms, their recognition, their implications for avian populations in Japan, and potential strategies for risk reduction, in the hope of helping to prevent a severe epidemic event among bird concentrations in Japan. Although viral diseases have not been recognized as major causes of illness and death in wild birds in Japan, there has been significant concern about such diseases since the 1970s in the USA because of severe outbreaks of Duck plague there, although it is not deemed indigenous there. Duck plague, or duck viral enteritis, is caused by a herpes virus (Family Herpesviridae). Although duck viral enteritis has not yet been recorded from Japan, there has been a case of another lethal herpes viral infection among wild birds in Japan, namely of Marek's disease virus (MDV) (Family Herpesviridae). In spring, prior to breeding in Russia, and in autumn, prior to wintering in Honshu, most of approximately 60,000 individual White-fronted Goose (*Anser albifrons*) spend several weeks feeding on the Ishikari River plain in west Hokkaido, near Bibai City, with the majority of them roosting each night at one particular swamp - Miyajima-numa (approximately 30 ha). From an ornithological and conservation perspective Miyajima-numa is a remarkably important site for the White-fronted Goose and for a wide range of other waterfowl species. It is also a highly significant site from an epidemiological viewpoint as the lethal case involving a tumor lesion, caused by Marek's disease, in an adult female White-fronted Goose was obtained from Miyajima-numa in November 2001. Marek's disease is caused by a highly contagious herpes virus with a

worldwide distribution, and it is normally found amongst chickens, but lesions resembling those caused by MDV also have been reported in owls, pheasants, partridge, ducks, geese, swans and a kestrel. In Japan, Marek's disease is most commonly found among domestic chickens especially among battery chickens. As MDV has been shown to be transmitted through diresct and indirect contact with contaminated aerosols, it is very risky that too highly congestion of geese in roosting sites. Hence, a preliminary epidemiological survey of 45 individuals of geese utilising Miyajima-numa was done by us in April 2002. Adding, in this chapter, the authors will show the histopathological and molecular biological (PCR) results.

Chapter 4 – Ducks are the most frequently reported waterfowl infected with bird schistosomes. Freely swimming infective larvae (cercariae) of these flukes directly penetrate the skin of birds exposed to the water environment. Penetrating cercariae transform into schistosomula, the developing stage which migrate through the host body using highly specific migratory routes. They are transported via blood circulation, except from *Trichobilharzia regenti*, which pervade through the peripheral nerves to the spinal cord and brain directly through the nervous tissue. The target locations of migrating worms differ among species and may be related to gastro-intestinal tract (visceral species) or nasal area (nasal species). At these sites parasites mature, copulate and produce eggs.

The presence of migrating stages, as well as stages of adult worms and eggs in the host body usually cause injuries in involved tissues/organs and may be of serious consequences varying from breathing or digesting disorders to the neurological symptoms and severe paralysis.

Chapter 5 – Nowadays, there have been suspicious parasitic nematode diseases on wild and/or captive waterfowls since the mid 1990s in Japan. Among them, some nematodes could infect each other among captive and wild birds, and it seems to become a proper conservation medical research and educational model as well. Hence, the present authors have continued to report parasitic nematodes of free-ranging and/or semi free-ranging captive waterfowls belonging to not only the family Anatidae but also the other families from Hokkaido, the most northern of the main islands of Japan. Among them, there appeared to be strict host/parasite relationships between the wild swans/geese and *Amidostomum anseris*/*Epomidiostomum crami*, and between wild duck species and *A. acutum*/*Ep. uncinatum*, respectively.

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Chapter 1

BORNAVIRUSES IN NORTH AMERICAN WATERFOWL

Susan L. Payne, Guo Jianhua and Ian Tizard
Department of Veterinary Pathobiology
and the Schubot Exotic Bird Health Center,
Texas A&M University College of Veterinary Medicine
and Biomedical Sciences, College Station, TX, US

ABSTRACT

Bornaviruses are enveloped, non-segmented negative strand RNA viruses. They replicate in the host cell nucleus, cause persistent infections, are non-cytopathic in cell culture and are highly cell-associated. The first avian bornavirus (ABV) was identified in 2008 and is the etiologic agent of proventricular dilatation disease (PDD) in parrots. ABV replicates in many organs, especially the central nervous system. It can be intermittently detected in cloacal swabs and feather follicles. During surveys to determine the possible origins of ABV we examined multiple oropharyngeal/fecal swabs and brain samples from multiple species of geese, ducks and mute swans. Reverse transcription PCR was used to screen RNA samples from swab samples from 409 Canada geese (*Branta canadensis*) and 200 mute swans (*Cygnus olor*) of which 3.6% tested positive for ABV. Brain tissues were subsequently collected from 25 Canada geese and 192 mute swans and 200 ducks of multiple species; ABV was detected in 52% and 23% of Canada goose

and mute swan brain samples respectively. ABV was also detected in 11% of brain samples from hunter-harvested ducks.

Of the major duck species tested, 7/84 (8.3%) of redheads (*Aythya americana*), 4/63 (6.3%) of northern pintails (*Anas acuta*) and 6/25 (24%) of gadwalls (*Anas strepera*) were ABV positive. Among other duck species, 2/9 (22%) northern shovelers (*Anas clypeata*), 1/10 (10%) mallards (*Anas platyrhynchos*), and 4/12 (33%) American widgeons (*Anas americana*) were also positive. RT-PCR products obtained from Canada geese, mute swans and ducks were sequenced, revealing that two different genotypes of ABV were circulating in these waterfowl. ABV genotypes 4 (ABV4) was recovered from ducks in Texas while a distinct genotype (ABV-CG) was recovered from geese and swans across the United States. ABV-CG was also detected in a small number of embryonated mallard duck eggs obtained from commercial breeders. ABV-CG appears, under some circumstances, to cause acute encephalitis or proventricular dilation disease in geese and swans.

INTRODUCTION

Bornaviruses (order *Mononegavirales*, family *Bornaviridae*) are enveloped, non-segmented negative strand RNA viruses with a genome size of approximately 8.9 kb. Borna disease virus (BDV) found in sheep and horses is the prototype member of the family. Bornaviruses encode 6 proteins, and share overall genome organization with other families in the order *Mononegavirales* including the paramyxoviruses and rhabdoviruses. However the bornaviruses are distinctive in that they replicate within the host cell nucleus, using mRNA splicing to generate a set of viral transcripts. BDV is highly cell-associated, is found in only small amounts in cell culture supernatants and spreads to cells in contact. BDV virions are enveloped particles of 80- to 100- nm, with 50- to 60- nm electron dense cores, but are difficult to visualize and no detailed structural analyses have been performed on these viruses [1]. BDV infection is non-cytopathic and persistent in cultured cells; indirect immunofluorescence assays using antibodies directed at the viral nucleoprotein (N) reveal a speckled pattern in the nucleus resulting from the close association between BDV proteins and cellular chromatin [2]. The association of viral nucleocapsids with host cell chromatin is believed to ensure that viral genomes are delivered to daughter cells during mitosis [2]. BDV causes a neurologic disease mainly affecting horses and sheep, with the largest recorded outbreaks occurring in central Europe in the 1800s [1]. Borna disease mortality can be as high as 80 to 100% in horses and 50% in sheep. The disease presents as a

progressive, nonpurulent encephalomyelitis characterized by lymphocytic infiltrates affecting the grey matter [3]. Serologic studies suggest that up to 12% of horse population in Germany may have been exposed to BDV, evidence that inapparent infections are not uncommon [3]. The disease has become increasingly rare in ungulates but cases of Borna disease continue to be sporadically reported from around the world, in a wide variety of mammals [1]. BDV has a wide host range among experimentally infected mammals [1]; a natural mammalian host, the bicolored white-toothed shrew (*Crocidura leucodon*), was identified as a reservoir of this virus in Central Europe [4]. Experimentally, the outcome of BDV infection varies with host species and age [1].

Neonatal rats become chronically infected and develop subtle behavioral abnormalities [1]. In contrast, immunocompetent adult rats develop meningoencephalitis with marked central nervous system (CNS) inflammation and loss of brain material, which is often fatal [1]. The presence of CD8+ T cells in the brain parallels the onset of neurologic dysfunction, suggesting that this CNS damage is immune mediated [1]. BDV spreads throughout the host largely through neural networks thus any route of infection that allows virus access to the nervous system will eventually lead to CNS infection and disease in susceptible adult rats [1]. For a more complete review of BDV history, molecular biology and pathogenesis, the reader is directed to recent review by Lipkin [1]. While BDV is generally considered an infection of mammals, Borna disease has been reported in birds. Malkinson and colleagues [5] identified BDV as the cause of a neurologic disease in captive ostriches (*Struthio camelus*) in Israel and more recently Berg and Johanson [6] used a PCR assay to detect the presence of BDV sequences in the droppings of mallards (*Anas platyrhyncos*) and jackdaws (*Corvus monedula*) in Sweden.

Avian bornavirus (ABV) was discovered in 2008[7, 8] during an intensive search for the etiologic agent of proventricular dilatation disease (PDD), a fatal disease of psittacine birds. Multiple genotypes of ABV have been identified [9]. In the course of the authors' studies we found that parrots can be healthy carriers and intermittent shedders of ABV [10]. This finding, in conjunction with other reports of PDD and BDV in Canada geese [11, 12] prompted us to look for ABV in populations of healthy wild waterfowl. We were able to utilize large numbers of samples collected during screening for early detection of highly pathogenic H5N1 avian influenza virus for the authors' studies. In this chapter we review recently published work and present novel findings from a screen of hunter-harvested ducks. The current phylogeny of bornaviruses is also discussed.

Proventricular Dilatation Disease

Proventricular dilatation disease, a progressive and fatal neurologic disease, has been recorded in over 50 species of captive parrots worldwide [13]. Not only is it a cause of significant losses to aviculturists but it is also a threat to highly endangered species such as the Spix's macaw, that depend upon captive breeding programs for their survival [13]. The clinical presentations of PDD are variable. In one form the disease presentation may be predominately neurological (weakness, ataxia, proprioceptive deficits, seizures, blindness) [13]. In another form of the disease, weight loss, passage of undigested food, regurgitation and delayed crop emptying result in proventricular dilatation and death by starvation [13]. Some birds present with both neurological and gastrointestinal signs [13]. Examination of tissues from PDD cases typically reveals a severe lymphoplasmacytic inflammation in central nervous tissues and within the enteric ganglia [13]. It has been assumed that damage to the enteric nervous system leads to proventricular paralysis and flaccidity [13]. While PDD was first recognized in the 1970s, its cause was unresolved until Kistler et al. [8] and Honkavuori et al. [7] used unbiased molecular methods and identified several distinct genotypes of ABV highly associated with PDD. Following initial reports, ABV sequences and/or antigen were found worldwide in the brains of birds with PDD [14-19]. Experimental challenge of parrots with PDD- brain homogenates [14] or with ABV-infected duck embryo fibroblasts [20] causes disease and PDD-like lesions.

Directed viral genome sequencing initially revealed 5 genotypes of avian bornaviruses, all distinct from the prototype member of the family, BDV [8]. These genotypes have been found worldwide, a feature that is not surprising, given the worldwide commercial trade in captive birds. To date there is no apparent correlation of ABV genotype with host species, location or disease severity. Additional genotypes of ABV have been identified from passerine birds (canary) [21] and more recently, from waterfowl [9]. In 1991Daoust and colleagues [12] reported two cases of a PDD-like disease in Canada geese (*Branta canadensis*) from Prince Edward Island, Canada. Following the identification of ABV in 2008, Delnatte et al. [22] used archival material from waterfowl recorded as suffering from ill-defined neurologic disease and detected ABV in brain samples from Canada geese and mute swans (*Cygnus olor*). ABV is now recognized as a potential avian pathogen with a widespread distribution.

ABV is most often detected in parrots and macaws with a diagnosis of PDD, however the virus is also found in apparently healthy captive parrots.

Initially we detected ABV in droppings from healthy, seropositive and seronegative, parrots housed our aviary [10]. ABV was subsequently found in droppings of cockatiels (*Nymphicus hollandicus*) purchased from a local aviary. There was no history of PDD in the aviary and the birds appeared to be in good health. However the majority of the purchased birds shed virus intermittently for at least three years, demonstrating that inapparent infection is not a rare event [10]. Subsequent investigations have shown that ABV infection is common in captive cockatiel flocks (Tizard/Payne unpublished observations). These findings, in conjunction with the earlier reports of PDD, ABV and BDV in wild waterbirds [12, 23] motivated us to screen a variety of waterfowl for the virus.

METHODOLOGY

We have tested 13 species of waterfowlfor the presence of ABV using either oropharyngeal/cloacal swabs or brain tissues for RT-PCR testing. Swab samples: We initially obtained two sample sets that were collected between 2007 and 2009 as part of an early detection program for H5N1 avian influenza virus. The first sample set consisted of combined oropharyngeal /cloacal swabs collected by the Wildlife Services Agency of USDA-APHIS from the northern United States. The samples were collected as part of an early surveillance program for highly pathogenic avian influenza viruses; after collection they were stored at -80°C in viral transport medium (brain-heart infusion broth). Three-hundred combined oropharyngeal and cloacal swabs from Canada geese were initially tested.

The majority of the samples were from Canada geese that had been designated as nuisance birds, but some geese from Oregon and Wyoming were hunter harvested [9]. Twenty-five samples were obtained from each of 12 states selected to give as broad a coverage as possible of the United States and the major migratory bird flyways. An additional 109 samples were obtained from geese captured in California, Washington and Oregon. In addition to the goose samples, 219 mute swan samples, from birds removed under wildlife damage management were also provided by USDA-APHIS. Thus we processed over 600 oropharyngeal/cloacal swabs. The swabs, in viral transport media, were shipped frozen to our laboratory and were placed at -80°C upon arrival. All samples had previously tested negative for avian influenza viruses. To prepare RNA, oropharyngeal/cloacal swabs were thawed on ice. RNA was extracted from 140 microliters of clarified transport medium using the Viral

RNA Mini kit (QIAGEN) [9]. We also screened over 500 RNA samples, from cloacal swabs, collected from hunter-harvested waterfowl in Texas during the 2007 and 2008 hunting seasons [24]. These samples also had originally been collected and processed for avian influenza virus detection and only samples negative for avian influenza virus were tested for ABV.

Brain samples: Brain tissue samples from 25 Canada geese and 192 mute swans culled from urban or suburban areas between April 2008 and August 2011, from Michigan, New Jersey, New York, and Rhode Island were tested for evidence of ABV infection [9, 25]. Brain samples were removed from previously frozen heads either by dissection or by needle aspiration. We also tested brain samples from ducks that were hunter harvested in Texas. The samples were collected between December 2011 and February 2012 and before delivery to our laboratory, handling and storage conditions varied widely.

Upon delivery to our laboratory the heads were placed in -80C storage. RNA was purified from tissue lysate using an RNeasy minicolumn (Qiagen) [9]. cDNA was generated from total purified RNA samples using the Applied Biosystems® High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), using approximately 500ng of RNA and random primers. PCR targeted the M gene (PM1F 5'-GGTAATTGTTCCTGGATGGC-3' and PM1R 5'-ACACCAATGTTCCGAAGACG-3'). PCR conditions were: 94°C, 2 min, followed by 35 cycles of 94°C, 30 sec, 55°C, 30 sec and 72°C, 30 sec and one cycle of 5 min at 72°C. PCR assays included multiple reagent controls to screen for reagent contamination [9]. PCR products of the appropriate size were confirmed by sequencing and the genotypes were determined, as we have previously described [9].

RESULTS

Canada Goose Swab Samples

Of the 409 goose swab samples tested, 12 were positive based upon the size of the PCR products by agarose gel electrophoresis. Of these 11 (2.6%) were sequenced and confirmed to be the expected products from the ABV genome (Table 1 and [9]). A somewhat expected but never the less interesting result of Canada goose swab testing revealed that infections were common within some flocks (Figure 1). For example, multiple infected birds were identified in flocks in Johnson Co., Kansas (5/17), Cheshire Co., New

Hampshire (5/25,) and Queens Co., New York (6/15). No positive samples were obtained from either Ohio (0/25) or Wisconsin (0/25).

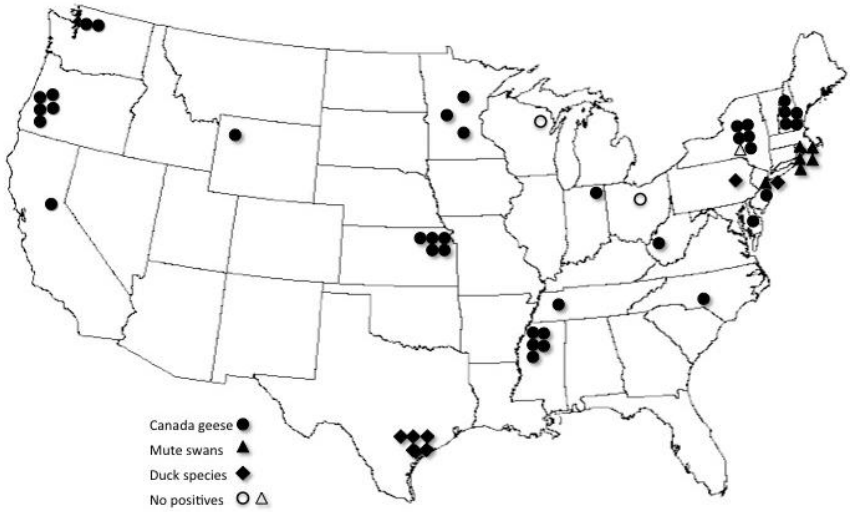


Figure 1. Sampling locations. Each filled symbol represents a single positive sample; if 5 or more samples were positive, a maximum of 5 symbols are shown. Open symbols show states from which no positive samples were obtained.

Of the 15 positive Canada geese whose gender was reported, 5/65 were males and 10/100 were females.

Mute Swan Swab Samples

Of 219mute swan oropharyngeal/cloacal swabs, 14 (6.4%) samples gave a product of appropriate size on gel electrophoresis. Four of these samples were selected for sequencing and confirmed to be ABV M-gene sequences. Evolutionary analyses of these amplified sequences indicated that the genes clustered in a single group and were closely related to the M genes of the previously described goose isolates (Figure 2).

Table 1. Detection of ABV in North American Waterfowl

SPECIES	LOCATION*	SAMPLE	TESTED	POSITIVE	GENOTYPE
Canada goose	CA, NH, NJ, NC, NY, OR, WA,WV, WY	oropharyngeal/ cloacal	409	11	ABV-CG
Mute swan	MI, NJ, RI	oropharyngeal/ cloacal	219	14	ABV-CG
Total		*oropharyngeal/ cloacal*	*628*	*25 (3.9%)*	
Mute swan	NJ, MI, RI	brain	192	45	ABV-CG
Canada goose	NJ	brain	25	11	ABV-CG
Snow Goose	KS, TX	brain	115	22	ABV-CG, ABV4
Ross's Goose	KS, TX	brain	58	6	ABV-CG, ABV4
Greater White-fronted Goose	TX	brain	10	0	
Wood Duck	TX	brain	2	0	
Mallard	TX	brain	10	1	ABV4
Gadwall	TX	brain	25	6	Not determined
Green-winged Teal	TX	brain	9	0	
American Widgeon	TX	brain	12	4	ABV4
Northern Pintail	TX	brain	61	4	Not determined
Northern Shoveler	TX	brain	9	2	ABV4
Redhead	TX	brain	84	7	ABV4
Total		*brain*	*602*	*108 (17.9%)*	

*The two letter state designations are as follows: CA (California), IN (Indiana), KS (Kansas), MI (Michigan), MS (Mississippi), NH (New Hampshire), NJ (New Jersey), NC (North Carolina), NY (New York), OH (Ohio), OR (Oregon), RI (Rhode Island), TX (Texas), TN (Tennessee), WA (Washington), WV (West Virginia), WY (Wyoming).

Texas Duck Swab Samples

We were unable to confirm any ABV positive samples from a large set of archived RNA samples prepared from ducks that were hunter-harvested from Texas wintering grounds [24]. Reasons that we were unable to detect the virus from this set of RNA samples could include: (1) The Texas wintering ground duck RNA samples were purified from cloacal swabs while the Canada goose and mute swan samples were combined oropharyngeal/cloacal swabs. It is possible that higher levels of ABV are shed via the nasopharynx rather than via the cloaca. (2) RNA purification and storage conditions were different for the two sets of samples [9].

Additional surveys of live birds should be planned with these issues in mind as, for convenience and to minimize stress, it would be preferable to carry out surveys for ABV carriage using fecal samples as the starting material. The possibility that most duck species are not ABV carriers was ruled out based on the testing results of brain samples that we describe elsewhere in this chapter.

Waterfowl Brain Samples

We processed brain samples from 25 Canada geese and 192 mute swans. Bornaviral sequences were detected in 52% and 23% of these brain samples respectively [9, 25]. Of the positive mute swan samples, 26/96 (27%) were from Michigan and 18/81 (22%) were from Rhode Island; a single sample from New Jersey was also positive. None of the 14 mute swan samples from upper New York State were positive. All of the positive Canada goose brain samples were collected in NJ [9]. Samples were obtained from 183 geese hunter-harvested geese, 115 of which were snow geese (62.8%), 58 Ross's geese (31.7%) and 10 (5.4%) greater white-fronted geese. No samples from greater white-fronted geese were positive for ABV, while 10% of Ross's geese and 19% of snow geese samples were positive. Overall 15% of brain samples from geese were positive for ABV (Table 1).

Two hundred and twelve duck brains were sampled in the study. These samples reflected the species composition in the areas hunted. Thus 39.6% were redheads (*Aythya americana*), all obtained from a hunting camp in Calhoun County TX, where this species winters in large numbers. The other samples came from ponds in the region of Ft Bend County, TX and reflect the diversity of waterfowl in that area of the state. Of the total birds collected,

28.8% were northern pintails (*Anas acuta*) and 11.7% were gadwalls (*Anas strepera*). The remaining species included northern shovelers (*Anasclypeata*), mallards (*Anas platyrhynchos*), and American widgeons (*Anas americana*).

ABV was detected in 7 of 84 redheads (8.3%), 4 of 61 northern pintails (6.5%), 6 of 25 gadwalls (24%), 1 of 10 Mallards (10%) and 4 of 12 American widgeons (33%). Virus was not detected in wood ducks (*Aix sponsa*) nor in green-winged teal (*Anas crecca*). The only common species untested was the blue-winged teal (*Anas discors*).

Thus ABV was apparently widespread in both dabbling ducks (*Anas spp*.) and diving ducks (*Aythya spp*.). Given this widespread virus distribution, with no apparent preference for specific duck species, we suggest that ABV occurs in birds living close to bodies of fresh water and is probably therefore an environmental contaminant. This suggestion is supported by the finding of ABV in the brain of another common waterside species, a yellow-crowned night heron (*Nyctanassa violacea*).

ABV Genotypes in North American Waterfowl

The first ABV characterizations were made from macaws, parrots and diverse other psittacines [8].On the basis of nucleotide and amino acid sequence identity, Kistler and colleagues [8] recognized multiple genotypes of ABV. In our studies of PDD affected psittacines we recovered ABVs that shared over 95% nucleotide sequence identity with viruses in ABV genotypes 1,2 and 4 [26]. Thus we were surprised to find that Canada geese and mute swans were infected with a unique genotype of ABV that we named ABV-CG [9]. This genotype was also identified, in geese, by Delnatte and colleagues in 2011 [22]. The Canada goose and mute swan ABV sequences that we identified are all closely related, sharing over 97% nucleotide sequence identity with each other [9, 25]. The ABV-CG cluster diverges from other bornaviruses by approximately 20- 30% [9, 25] and phylogenetic analysis (Figure 2) indicates that the ABV-CG cluster is no more related to other avian bornaviruses than to BDV. In fact a close examination of genome organization suggests that ABV-CG is more closely related to mammalian BDV than to other ABVs [9, 25]. ABV-CG was also recovered from some brain samples of Ross's geese and snow geese.

After finding ABV-CG was the predominant bornavirus among Canada geese and mute swans we were not expecting to find genotype 4 ABVs in

hunter-harvested Texas ducks, but in fact, of the duck samples genogrouped to date, we have identified only ABV4.

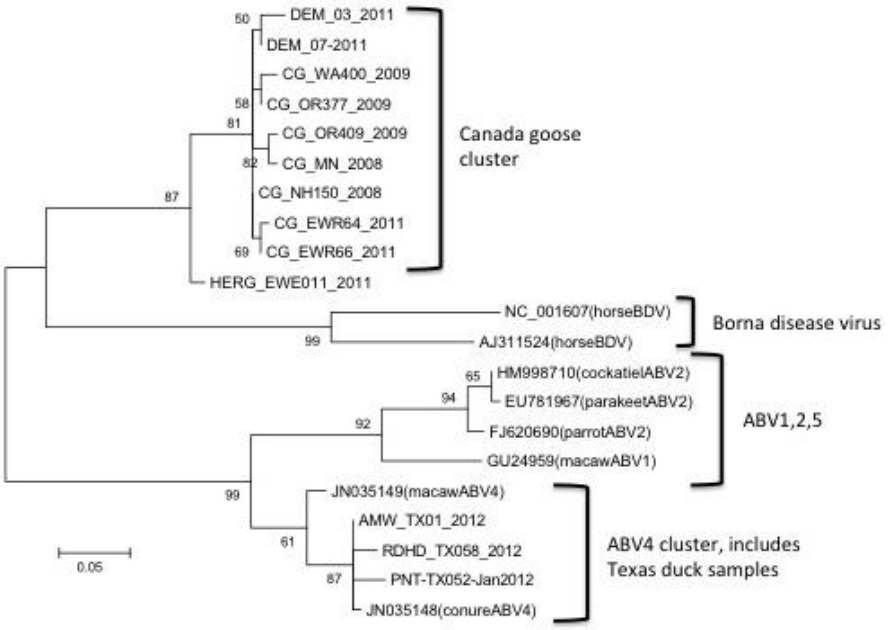


Figure 2. Phylogeny generated from a 182 nt region of the bornavirus M gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5. For ABV samples discussed in this chapter: CG, Canada goose, HERG, herring gull; PNT, northern paintail; RDHD, redhead; AMW, American Widgeon. Ganbank accession numbers are provided for other bornaviruses.

The nucleotide sequence of a short region of the duck ABV genomes placed them in a subcluster within ABV genotype 4. The duck isolate sequences are not identical to our laboratory isolates of ABV4, thus it is very unlikely that they are laboratory contaminants. We identified three distinct duck sequences that, as a group, share only 68 to 69% nucleotide sequence identity with ABV-CGs. Some ABV4 viruses were also found among Ross's geese and snow geese, thus these birds seem to play host to both ABV-CG and ABV4. We have not formally investigated the possibility of dual infections in these geese; our method of sequencing uncloned PCR products might reveal

only the predominant genotype. However, dual infection of captive parrots has been documented by others [29].

Experimental Infections

Our original breakthrough when working with ABV was our finding that it replicated in duck embryo fibroblasts (DEF). This immediately suggested that ducks (mallards) might be susceptible to this virus.Our ability to culture ABV in DEF [10, 20] and to detect it in brains from wild birds [9, 25] provided a basis for determining the pathogenicity of ABV for ducks. Specific pathogen free mallard (Khaki Campbell) ducklings were therefore challenged with a virulent strain of ABV4 grown at low passage in DEF. ABV4, strain M24, isolated from a yellow-collared macaw was grown as the challenge virus based on its ability to infect and promote disease in Patagonian conures (*Cyanoliseus patagonus*) and cockatiels (*Nymphicus hollandicus*) [20].

In the experiment, 3 ducklings, 3-5 days old were challenged intramuscularly (im), another 3 were inoculated by gavage (gi) and 3 ducklings were challenged by the intraocular (io) route. Six ducklings served as uninoculated controls. No consistent clinical signs were observed in the inoculated ducks, but fecal shedding indicated ABV infection. One duck from each infection route was necropsied at day 40 postinoculation (pi). At 20 weeks pi two additional ducks were necropsied (1 io and 1 im) and at 33-35 weeks pi the remaining inoculated ducks were tested (1 im, 2 oral). No gross lesions were detected in any of the ABV4 inoculated ducks and histopathology showed no sign of viral infection [13]. At 40 days post-inoculation the ducks were seronegative in a western blot to detect recombinant ABV N antigen but brain samples were PCR positive for ABV4. At 20 weeks pi, brain tissue was PCR positive for ABV4 sequences and the ducks were seropositive as well. At 33-35 weeks pi, the remaining three infected ducks were seropositive (PCR was not performed). Sequence analysis confirmed that the ABV recovered was the M24 inoculated strain. Thus the inoculated virus was likely able to access neural networks and reach the CNS despite different routes of inoculation.

ABV in Mallard Duck Embryos

ABV is noncytopathic, thus ABV infection of primary DEFs cannot be detected by direct visualization using light microscopy. As part of the

procedure for generating DEF for the culture of waterfowl ABV, we found that freshly prepared DEF were contaminated with a strain of ABV whose M protein gene sequence did not match any of our laboratory isolates. To follow up on this observation, eggs were purchased from four commercial duck hatcheries. Upon receipt, eggs were placed in an incubator for 8-14 days. The incubator was cleansed and disinfected between egg batches. Intact eggs were cleaned externally by swabbing with alcohol.Eggs were opened using sterile scissors and the contents emptied into a sterile Petri plate and the embryo was decapitated. If the embryo was sufficiently developed, the cranium was opened aseptically and a brain tissue sample was taken. The rest of the body was eviscerated, macerated and placed in culture medium. All samples were tested for the presence of ABV by reverse transcriptase PCR. We found evidence of ABV infection in duck eggs from 2 of 4 sources that we sampled. In both cases 2/18 eggs in each sample tested positive. Sequencing of PCR products indicated that these viruses belonged to the ABV-CG genotype. M gene sequences clustered with the goose ABV, but with minor differences in sequence from those previously cultured in the laboratory. These differences indicated that these viruses were not laboratory contaminants. Because DEFs are widely employed in avian research, as well as in some commercial vaccines, we suggest that such cells be screened for ABV prior to use, and that eggs be obtained only from hatcheries that are free of this infection.

DISCUSSION

ABV-CG and Disease

In 1991 Daoust et al. [12] reported seeing, in two emaciated Canada geese, severe proventricular dilatation and food impaction associated with a nonsuppurative encephalomyelitis and ganglioneuritis. The birds were submitted to the Diagnostic Laboratory the Atlantic Veterinary College, University of Prince Edward Island in 1988 and 1989. The authors reported that assays for lead as well as suspect alphaviruses and flaviviruseswere negative and that the gross and microscopic lesions bore striking similarity to parrots with PDD [12]. If those formalin fixed samples are still available, ABV infection might be confirmed by PCR.

At the present time we also know of results from 2 studies that support of a role for ABV in neurologic disease in Canada geese and mute swans.Smith et al. [11] conducted a retrospective study to determine whether avian

bornavirus infection might be associated with pathological changes in wild waterfowl. They identified fifteen Canada geese and three trumpeter swans (*Cygnus buccinator*) with nonsuppurative encephalitis of unknown etiology. Thebrains of 11/12 of these geese and 2/2 swans tested positive for ABV by immunohistochemistry (IHC). Six of the IHC-positive geese were also positive for the ABV N gene by RT-PCR [11]. In a subsequent communication, Delnatte et al. [22] examined 57 necropsy reports from Canada geese and trumpeter swans euthanized or found dead in southern Ontario. Of these, the brains of 20 geese and 3 swans were positive for ABV N protein by immunohistochemistry. Twelve of fourteen brains tested were also positive for ABV N gene by RT-PCR. The N gene sequences were identical and corresponded to the ABV-CG genotype. The prevalence of ABV in these dead or dying birds was considerably higher than the background level in apparently healthy birds. These results thus support the contention that under some circumstances (and perhaps in some species) ABV is a significant pathogen in waterfowl. Specific triggers for disease development are not yet known but might include environmental stress, co-infection with other pathogens, overall immune status or age.

ABV Epidemiology

The relationship between ABV in waterfowl and ABV in parrots is currently unknown. Genotype 4 is found in both and this raises the question as to whether one group of birds acquired it from the other. The molecular phylogeny data in this chapter do not clarify this issue. In North America, waterfowl and parrots do not occupy the same or adjacent territory so direct transfer is improbable. That is not the case in southern South America. In Patagonia, multiple species of waterfowl coexist with austral parakeets (*Enicognathusferrugineus)*, monk parakeets (*Myiopsittamonachus*) and Patagonian conures. It is conceivable that the jump between parrots and waterfowl occurred there. Most waterfowl are migratory, some such as Ross's geese and pintails moving between the Arctic coast in summer and the southern United States in winter. Opportunities for viral transmission abound at both ends of this migratory flyway. Canada geese and mute swans may be even more efficient viral multipliers since many populations of these birds are year-long residents of the shorelines of lakes with perennial open water. Thus many occupy power station cooling lakes where the water is slightly warmed and so provides year-round habitat and allows fecal contamination to build up.

It is also relevant to point out that a feature of many waterfowl such as Canada geese is their exceedingly short intestinal transit time. Thus grazing geese consume large quantities of grass, pass it rapidly through the gut extracting only the most readily available nutrients, and deposit the undigested material in the form of abundant, large, wet droppings. Besides being messy, these droppings may provide an effective method of viral survival.

ABV is not, of course the only virus shed in the feces of waterbirds. Avian influenza virus also cycles in the same manner, being readily shed in feces, surviving for prolonged periods in cold water, and apparently spreading readily between waterbirds during the Northern summer. However it should be noted that these viruses have very different life-styles, despite the similarity of fecal-oral transmission. Influenza virus infection is acute, with virus being shed for a relatively short period of time. As reviewed recently by Vergahen, Fouchier and Munster [30], estimates of avian influenza shedding are between 7 and 17 days. Immunity develops and shedding ceases in recovered birds.It is proposed that a yearly crop of non-immune juveniles is necessary to keep multiple strains of avian influenza viruses in circulation and it is not likely that a single infected bird could move avian influenza virus on a continental scale [30]. In contrast, studies of ABV in parrots suggest a viral life style very similar to that of BDV. ABV is neurotropic but infects other organs including the kidney, adrenals and sexual organs. Infection is persistent and in our aviary we have detected intermittent ABV shedding by healthy birds for over 3 years. Healthy shedding birds can thus transmit virus for long periods of time and individual birds could transmit ABV over long distances.

CONCLUSION

Avian bornavirus is present in the brains of a significant fraction of North American waterfowl. The high prevalence suggests that this is a persistent rather than an acute infection. Since the majority of these birds were apparently healthy at the time of sampling it is apparent that the virus is non-pathogenic much of the time. This lack of pathogenicity is consistent with the failure of the virus to cause clinical disease in experimentally infected ducklings. It is also consistent with the known lack of cytotoxicity shown by this virus. That said, it is also clear that ABV infection of waterfowl may be associated with disease, specifically encephalitis and neurologic disease. The original report of Daust [12] suggests that PDD does occur in Canada geese. Smith and her colleagues [11] have demonstrated the presence of ABV-CG in

both Canada geese and whooper swans suffering from neurologic disease. Additionally we have isolated ABV-CG from captive Canada geese during an outbreak of neurologic disease in California. However, it remains unclear under what circumstances and with what frequency ABV causes disease in waterfowl. One possible clue comes from observations on the vertical transmission of this virus and the fact that BDV-induced disease in laboratory rodents is immunologically mediated. Vertically transmitted viruses, by infecting the embryo at a very early stage of development can induce immunological tolerance. In the absence of a host response to this non-cytopathic virus, tissue lesions and hence disease may not occur. There is limited evidence from parrots that birds with the highest antibody titers are more likely to develop disease. Alternatively, ducks may have been exposed to this virus for a long time and evolved a healthy carrier state rather than disease susceptibility. This is supported by our failure to cause disease in healthy ducklings [13] although we cannot exclude the possibility that these birds, although claimed to be specific pathogen free were in fact vertically infected.

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Chapter 2

CRITICAL COMPONENTS OF DUCK RESEARCH: THE ORIGIN AND GENETICS OF DOMESTIC DUCKS

Ian Tizard and Donald J. Brightsmith
The Schubot Exotic Bird Health Center, College of Veterinary Medicine,
Texas A&M University, College Station, TX, US

ABSTRACT

Biomedical science is increasingly focused on the fine details of DNA and molecular structure, making it vital that investigators describe accurately and precisely the origins and genetics of the animals included in their studies. The chicken and the turkey, the world's most common domesticated birds, both originated from a single species, but domestic ducks have a much more convoluted history making accurate descriptions of their origin and genetics complicated. The phylogeny of the predominant domestic species, the mallard (*Anas platyrhynchos*) is especially complex. In addition, the domestication of the mallard has, on occasion, included interbreeding with other wild duck species including the Eastern spot-billed duck (*Anas zonorhyncha)* and the American black duck (*Anas rubripes)*. An unrelated species, the Muscovy duck (*Cairina moschata*) was originally domesticated in pre-Columbian America. As a result of this complexity it is essential that any scientific papers or gene sequences pertaining to ducks, especially domestic ducks, document in considerable detail the genetic and taxonomic provenance of the birds studied. It is inappropriate to refer to these birds simply as "ducks".

INTRODUCTION

Modern biomedical sciences, especially molecular genetics and related subjects such as immunology, are increasingly focused on the fine details of DNA and protein sequence and structure. As a result, the data obtained tend to be specific to individual animal species and strains within that species. For example, it is clear that the genetic diversity of the laboratory mouse has a critical influence on its resistance to infectious agents (Marquis and Gros, 2008). As a result, scientific reports on the mouse are required to describe the genetic background of the animals employed. Such detailed genetic information is not available for other species although this is changing as more genomic data accumulate. One essential requirement however is the need to work within a single, defined, taxon so that future researchers can replicate the work and understand its biological context and relevance.

The world's two major domesticated birds each originated from a single wild bird species: the chicken from the red jungle fowl (*Gallus gallus*) and the turkey from the wild turkey (*Meleagris gallopavo*). Genetic and molecular studies on these birds can therefore be related directly to a single biological species. However, the third most important group of domestic birds, domestic ducks, are derived from at least four wild duck species; the mallard (*Anas platyrhynchos*), the eastern spot-billed duck (*Anas zonorhyncha*), the American black duck (*Anas rubripes*) and the Muscovy duck (*Cairina moschata*). Two of these species, the mallard and the spot-billed duck are genetically complex and their taxonomy and subspeciation remains in dispute (Livezey 1991, Donne-Gousse et al, 2002.). In addition, ducks have been domesticated independently on at least three occasions, on three continents, and at different times. Thus mallards were domesticated in China about 1000 BC, and in Europe around 1200 AD, spot-billed ducks may have been domesticated in Southeast Asia around the same time, while Muscovy ducks were domesticated in Central and South America prior to 1492 (Silverman and Isbell, 2008).

As a result of this diversity it is not possible to generalize regarding all domestic duck breeds and species. Results obtained in one breed or species of domestic duck may be significantly different from results obtained in another species. To cite one specific example, Cagle and her colleagues (2011) demonstrated that Muscovy ducks produced lower viral antibody titers than Peking ducks when vaccinated with a killed influenza vaccine. When challenged with highly pathogenic H5N1 influenza vaccine, Muscovy ducks developed disease earlier and had more severe neurologic signs than Peking

ducks. They also showed significant differences in innate immune responses between the two duck species, the Peking ducks had higher body temperature responses and blood nitric oxide. Peking ducks also developed a greater increase in RIG-1 expression while Muscovys showed a greater increase in IL-6 expression.

In a related study, Phuong and colleagues (2011) compared the susceptibility of domestic Muscovy ducks with an undefined line of domestic mallards to infection with avian influenza H5N1. They found that the morality in Muscovys was at least 80% regardless of the strain of virus used. In contrast, mallard mortality ranged from 0 to 100% suggesting that, in general, Muscovy ducks were more susceptible to this virus. They also found higher virus titers in the organs, especially the brain, of Muscovy ducks as compared to mallards. As a result, investigators reporting on molecular or genetic data derived from domestic ducks must take great care to ensure that the originating species or breed is accurately defined.

The Phylogeny of Ducks

The Anseriforms are a biologically diverse order of birds that contains about 150 recognized species (Carboneras, 1992). They include two suborders, the Anhimae (the screamers), found in the Neotropics, and the Anseres (the ducks, swans and geese) that are distributed worldwide. The fossil record indicates that the first Anseriformes (*Presbyornis*) were present in the Upper Eocene (40-50 mya) in North America. However their major radiation occurred during the Miocene, (5-23 mya) and some even as recently as the mid Pleistocene (Olson, 1985). Because some radiation events are so recent, interspecies genetic variation is minimal in some groups. As a result, the phylogeny of some members of the order is disputed and has been subjected to multiple, frequent revisions (Livezey 1991, Donne-Gousse et al. 2002). Like all phylogenies, it was originally based on anatomic and morphological analysis of museum specimens (Delacour and Mayr, 1945). More recently, waterfowl have been subjected to molecular phylogenic analysis (Johnson and Sorenson 1999, Donne-Gousse et al. 2002). The predominant methodology employed has focused on a comparative sequence analysis of the D loop and associated promoters (DLP) within the mitochondrial control region genes. Mitochondrial DNA is maternally inherited, it doesn't recombine and in effect it is haploid. The control region is the least conserved portion of the

mitochondrial genome and as a result, this procedure appears to provide a relatively consistent and robust phylogeny.

The suborder Anatidae has traditionally been considered to consist of three families, the Anseranatidae (the magpie goose), the Anserinae (the geese and swans) and the Anatinae, the ducks (Livezey, 1991, Carboneras et al. 1992). However mitochondrial DNA-based phylogenies provide a somewhat different picture. For example, Donne-Gousse et al. (2002) analyzed the complete mitochondrial control region of 45 waterfowl from 24 different genera. Two of these genera *Chauna* (the screamers) and *Dendrocygna* (the whistling ducks) formed distinctly separate deep branches. The remainder of the anseriformes formed two clades corresponding to the Anserinae (geese and swans) and the Anatinae (ducks) (Donne-Gousse et al. 2002).

Within the Anatinae, there are five clearly recognizable Tribes, The Anatini containing 41 species of dabbling ducks such as the pintails, wigeons, teal and the mallards; the Aythadi containing 15 species of diving ducks such as the pochards; the Cairinini containing 13 species of perching duck including the Muscovy duck; the Mergini (Mergansers) and the Tadornini (Shelducks). The Shelducks diverged early from the Anatini, followed by the Mergansers, followed by a group that includes the other 3 clades (Donne-Gousse et al, 2002).

A phylogeny has been constructed of the Anatini using both the cytochrome-b and the NADH dehydrogenase subunit 2 (ND2) mitochondrial gene sequences. These molecular data strongly support its monophyly (Johnson and Sorenson, 1999). All domestic duck breeds except the Muscovy ducks belong to the Anatini.

The most diverse genus within the Anatini is *Anas*, consisting of the dabbling ducks. Of the 41 species within the Anatini, 38 belong to this genus. Although monophyletic, this is a complex genus. Hybridization of species within the genus is frequent and the hybrids are often fully fertile. As a result, speciation is obscure and mtDNA sequence data may be of limited value in resolving their relationships. Thus, mitochondrial gene sequences must be treated conservatively and require large sample sizes if they are to be interpreted with confidence. Conversely, nuclear DNA sequences have evolved too slowly to resolve their phylogeny. The members of the genus are found on all continents except Antarctica. This global distribution reflects their high dispersal ability and it is probable that much of their speciation has been driven by this dispersal and subsequent genetic isolation (Johnson et al, 2000).

The Mallard

The mallard is found throughout the northern hemisphere and is the most widespread and abundant duck worldwide - it is the prototypical surface-feeding "dabbling" duck (Kraus et al. 2011). In general, mallards breed in northern temperate regions and where necessary, migrate southward in winter driven by the freezing of fresh water lakes, ponds and rivers. The presence of warm, unfrozen lakes such as those associated with power station cooling enables some northern mallard populations to be non-migratory. Likewise birds living in more temperate regions do not migrate. In North America these birds tend to follow clear migratory flyways but this pattern is not so obvious in Europe (Kraus et al. 2011).

While phenotypically uniform, mallards are in fact genetically diverse, as would be predicted for such a widespread species (Avise et al. 1990, Johnson and Sorenson, 1999). In addition, mallards are closely related to a variety of other, very similar duck species. While male mallards are brightly colored, their females and both sexes of many allied species share a dull mottled brown plumage. Based on both structure and hybridization, nominate *A platyrhynchos* is considered to form a superspecies with the American black duck, (*A. rubripes),* Meller's duck, *(A. melleri)* from Madagascar, and the African yellow-billed duck (*A. undulate)* (Carboneras et al, 1992).

The picture is further complicated by the presence of a multitude of morphologically distinct taxa that freely interbreed with the Holarctic archetypical mallard including the mottled duck (*A. fulginosa*), the Mexican duck *(A.diazi),* the Hawaiian duck *(A.wyvilliana)* and the Laysan duck (*A. laysanensis)*. In the ongoing disputes about mallard classification, these have been considered full species or subspecies of the mallard depending upon the taxonomists' preference (Carboneras et al, 1992. McCracken et al. 2001). In summary, the wild members of the mallard clade can be classified into the sexually dimorphic Holarctic mallard (*A platyrhynchos),* the monomorphic North American birds (*A. fulginosa*, *maculosa*, *fulvigula*, *rubripes* and *diazi*), and the isolated island mallard populations (*A. wyvilliana* and *laysanensis)*. The sexual dimorphism of the mallard itself may be a relatively recent event perhaps occurring first in eastern Asia (Johnson et al. 2000). What is abundantly clear is that the members of the mallard superspecies evolved relatively recently into multiple biogeographical lineages leaving many taxa with relatively minor phenotypic and genotypic differences, high levels of genetic compatibility, and great potential for interbreeding.

There is also evidence that sorting of populations is occurring between the Eurasian and North American mallards. Mitochondrial control region sequencing demonstrates that mallards belong to one of two major mitochondrial haplogroups; group A found across northern Eurasia and the western Pacific; and group B in North America (Avise et al, 1990).. The sequence divergence between these phylotypes is very low (0.58%) and separation appears to be recent (Johnson and Sorenson, 1999).

Kulikova and her colleagues (2005) studied wild mallard populations from western Russia to mainland Alaska. They found that group A haplogroups (80.3%) are common and widespread in mallards from Western Russia to Alaska. Group B haplogroups (19.7%) in contrast, occurred in highest frequency in Alaska and infrequently in Northeast Asia. Group B birds were found in mainland Alaska at high frequency (77.4%). This declined somewhat in the Aleutian Islands and in the Russian Far East and group B birds were not found elsewhere in Asia. In general Kulikova found little additional genetic structure within Asian mallards. Aleutian birds were somewhat different from those in either Alaska or Asia and appear to be forming a new isolated population. (Kulikova et al. 2005) All Chinese domestic and wild mallards belonged to haplogroup A (He et al. 2008)

Avise and colleagues (1990) had originally examined the relationship between the mallard and the American black duck by mtDNA control region sequencing of multiple birds. They found that their nucleotide identity was 99.2%. They also found that their genotypes clustered into two distinct haplogroups they termed A and B. The B haplogroup was present in both species whereas the A haplogroup was found only in mallards. The authors interpreted this to suggest that some haplogroups pre-dated species separation and that the mallard-black duck divergence was a very recent event. They did not believe it to be due to recent hybridization for several reasons. For example, isolated breeding populations of black ducks that are not believed to have yet encountered mallards show the same haplotype diversity. Likewise, given that mtDNA is of maternal origin, if hybridization had occurred it would imply that female mallards have been interbreeding with male black ducks. This is not what usually happens in nature where male mallards breed with female black ducks. Haplogroup B is also present in Californian mallards where black ducks do not occur naturally. Thus, they conclude that black ducks likely developed from type B mallard/black ancestors. Group B also clusters with the other North American Anas species. There are no base substitutions between most of these taxa and the mottled duck, *A. fulvigula* differs from the others in only two positions. Eastern-spot billed ducks may

also belong to either haplogroups A or B and it is possible that they are most closely related to the mottled duck (Avise et al. 1990).

Mallards hybridize extensively with other members of their genus. In Australia and NZ they hybridize with the Pacific black duck (*A. superciliosa*) (Carboneras et al. 1992). In North America they hybridize with the American black duck (*A. fulginosa*). The Philippine duck (*A. luzonensis*) hybridizes so freely with mallards that the decline of the former has been blamed on this hybridization Mallards also commonly hybridize with spot-billed ducks (*A. poecilorhyncha*)(Melville 1999, Brazil 1991).

It is thought that speciation within the mallard group may have emerged in one of two ways. One suggestion is that an ancestral dimorphic mallard gave rise to both the Asian spot-bills and to the monomorphic mallard relatives found in North America. Complete lineage sorting may not have had time to occur so paraphyly is limited. An alternative suggestion is that a dimorphic mallard ancestor may have originated elsewhere, invaded North America and hybridized with the closely related monomorphic species already there. This may have resulted in partial introgression of haplogroups from the monomorphic species into the mallard (Avise et al. 1990).

The Spot-billed Ducks

The spot-billed duck (*Anas poecilorhyncha*) is distributed widely across southeast and east Asia. It is of similar size and structure to the mallard. Spot-bills form a superspecies with the Pacific black duck (*Anas superciliosa*) and as with other members of the genus, the spot-bills hybridize regularly with other *Anas* species such as the Pacific black duck, Philippine duck (*A. luzonica*) and mallard and these hybrids are fertile. Some domestic ducks may look very similar to wild spot-bills.

As with the mallard, its classification is disputed. It is currently divided into three subspecies; the Indian spot-billed duck (*A. p. poecilorhyncha*), the Burmese spot-billed duck (*A.p. haringtoni*) and the Eastern spot-billed duck (*A.p. zonorhyncha*). The three subspecies differ in their overall color (Indian ducks are grayer while Eastern ducks are browner). The Indian subspecies has a red spot on the bill that the Eastern lacks. The Indian duck has a green speculum while the Eastern duck has a blue one. The body plumage of the Eastern spot-billed duck is very similar to *A. superciliosa* (Madge et al, 1988). The Indian and Eastern taxa are also monomorphic and closely resemble immature and female mallards. As their names imply, the Indian subspecies is

found from Pakistan and India into southern Japan. *A. p. haringtoni* is restricted to Myanmar. The Eastern subspecies summers in northeast China but migrates to Southeast Asia in the winter. This subspecies has expanded its range northward significantly in the past century, perhaps in response to climate change. Many ornithologists consider the Eastern spot-billed duck a separate species.

Eastern spot-billed ducks belong to both mallard haplogroups but their B haplogroups clearly form a subclade (Li et al. 2010). mtDNA control region and ornithine decarboxylase intron 6 sequence data suggest that *A. p. zonorhyncha* is more closely related to the north American monomorphic mallard group that includes the mottled and American black ducks than to the dimorphic mallard. It has been argued that the phenotypic similarities between the spot-billed ducks and American monomorphic mallards may be due to convergence and that the spot-bills have evolved from the point where the North American and Asia/Pacific mallard clades diverged.

The American Black Duck

The American black duck (*Anas rubripes)* was historically the predominant breeding mallard-type duck found in Eastern North America. Prior to the 20th Century, nominate *A. platyrhynchos* was primarily a western species but expanded eastward within the last 100 years (Avise et al. 1990). This expansion may be due to an increase in available habitat in the form of large numbers of man-made ponds. At the same time, there has been a progressive decline in American black duck numbers. There are many possible reasons for this. They include habitat loss, competitive exclusion, overhunting or simply genetic swamping as mallards breed with black ducks (Conroy et al. 2002). The species hybridize relatively frequently and the hybrids are fertile. Indeed, the Cayuga duck breed was derived by such interbreeding (See below). This interbreeding and the haplogroup data described above raise questions as to whether the black duck is a valid species.

MALLARD DOMESTICATION

Mallards have been domesticated on at least two separate occasions in two widely separate locations, China and Europe. In both locations, different wild duck populations or species provided the founder material. This raises the question, is *Anas platyhynchos* subspecies *domestica* a single entity or a genetically diverse hybrid population derived from multiple *Anas* species?

Domestication in China

It is believed that the first step in domestication was the capture of wild mallards that were caged and bred. With appropriate handling, the offspring of these birds would have become tame and relatively easy to handle and manipulate. Subsequently, these domesticated mallards would have been subjected to selection with respect to behavior, as well as the production of meat and eggs. These original domesticated ducks were, in some cases hybridized with other wild species to generate breeds with desirable properties (Tanabe 1992).

Ducks were domesticated in China as early as the West Zhou Dynasty around 1066 BC. (Li and Li, 1994). This most likely occurred in the river valleys of the Yangtze and Xi (Pearl). Large scale duck farms had been developed by the time of the Eastern Zhou Dynasty (514-495 BC) (Qu et al, 2009). Force-feeding of ducks is featured in "Qi Min Yao Shu" a book written during the Northern Wei Dynasty (386-524 AD) (He et al. 2008, Qu et al. 2009).

China has a rich and diverse group of at least 27 different domestic duck breeds. Most of these breeds were developed from wild mallards in the classical duck-rearing areas of China: along the lower reaches of the Yangtze, Huai and Xi rivers and along the coast (Li et al. 2010, Zhang et al. 2007). Some of these breeds were derived, in part, by hybridization between mallards and eastern spot-billed ducks. The relative contribution of these two species varies greatly among breeds and within different regions in China. For example, the domestic duck breeds originating from the Yangtze and Huai river basins appear to be solely mallard in origin. Zhang et al. (2007) sequenced the mitochondrial DNA D-loop control region of 106 ducks of nine breeds found in the Yangtze-Huai region. 31 haplotypes were identified in these birds. The haplotype diversity (Hd) and average nucleotide diversity (pi) were 0.798% and 0.28% respectively. Only 1.9% genetic variation occurred between breeds. (Less than that found in wild mallards.) Indeed the genetic variance between these breeds was insignificant. Their phylogeny demonstrated the presence of only mallard haplogroup A (Avise et al. 1990). There was no evidence for the presence of *A. zonorhyccha* group B haplogroup in these birds (Zhang et al. 2007). He and his colleagues also sequenced the mtDNA control region from 19 Chinese indigenous breeds and 70 wild mallard samples. They also sequenced 52 samples for cytochrome b. They found that all the domestic ducks and wild mallard haplogroups tested

were indistinguishable and argued that the domestic and wild samples could be grouped into a single haplogroup. (He et al. 2008)

In contrast, Chen et al. (1999) established a molecular phylogeny using the 667bp mtDNA CR and examined samples from 26 indigenous breeds, 25 wild mallards and 9 spot-billed ducks. Comparison of mtDNA polymorphisms using restriction fragment length polymorphism showed that both mallards and eastern spot-billed ducks contributed to the domestic duck gene pool in Fujian Province in Southeast China. Li et al. (2010) also examined the mtDNA CR of multiple Chinese duck breeds as well as wild mallards and eastern spot-billed ducks. They found a Hd of 0.645 and a pi of 0.115%, a very low figure. They were also able to demonstrate a contribution of eastern spot-billed ducks to some indigenous duck breeds, especially breeds from Fujian province. Fujian is in southern China, well away from the major duck-producing regions in the northern river valleys. It is also located south of the major mallard wintering range - where the predominant wild duck species is the eastern spot-bill. As expected, most ducks tested belonged to mallard haplogroup A.

Tanabe (1992) reported that a major domestic Japanese breed, the Kairyo Osaka breed originated from a hybrid between "Japanese native ducks and Peking". Both mallards and spot-billed ducks are common in Japan in winter.

Peking Ducks

The most important domestic duck breed worldwide is the Peking duck. This is a large, upright-standing white duck used for both meat and eggs. The breed originated as its name suggests in Northern China. It is the predominant domestic duck in the United States and about 95% of duck meat consumed in the United States is from this breed. It appears to have originated, like so many Chinese breeds, in the canals in the Nanjing-Beijing area. Peking ducks were first imported into the United States in 1873 to Long Island, NY where they form the basis of a major industry. It is unclear whether Peking ducks originated from natural wild white mutants or were developed by selective breeding of domesticated ducks during the Ming (1368-1644) or Qing dynasties (1644-1911) and bred into Peking ducks for the imperial court (Qu et al, 2009). Genotyping of Peking ducks, other domestic breeds and some wild mallards using 15 microsatellites showed that they are significantly different from other Chinese domestic breeds found in the same region (Qu et al. 2009). As expected, Peking ducks belong to mallard haplogroup A. On this basis it has been concluded that Peking ducks have been genetically separated from

other Chinese duck breeds for a long time although they originated from wild mallards (Qu et al, 2009).

Domestication in Southeast Asia

Large numbers of domestic ducks are kept in Southeast Asia, a region where mallards are uncommon winter visitors. Some domestic duck breeds such as the Indian Runner Duck appear to have originated in Indonesia and specifically on Bali. These are regions where mallards do not occur naturally but where spot-bills are abundant (Wei et al. 2009).Therefore, the local source of wild ducks would have been spot-billed ducks (Wei et al. 2009). Certainly, many domestic ducks in Laos, Cambodia and Vietnam phenotypically resemble Eastern spot-bills (R.J. Tizard, pers. com.). Tanabe demonstrated by electrophoretic techniques that multiple Indonesian domestic duck breeds were distinctly different from Chinese or European mallards and Peking ducks suggesting that they probably had a very different origin (Tanabe 1992). Although it is possible that all Southeast Asian and Indonesian domesticated ducks were introduced from China, it seems much more likely that they would have originated from the locally abundant spot-billed ducks. While genetic tests of this hypothesis are needed, Southeast Asia may have been an additional center of duck domestication.

Domestication in Europe

Domestication of the mallard occurred much later in Europe than in China and did not necessarily occur at the same time as the domestication of the goose (Harper, 1972). Nevertheless, wild ducks may have been captured and kept for food at the time of the Romans. The first records of keeping captive ducks occur in 37 BC. True domestication probably occurred much later. Records of livestock used to pay rents in French manors first begin to mention ducks around 800 AD. Nevertheless, ducks are rarely mentioned in medieval farming documents until the 12th century. This may simply be because wild ducks were abundant and there was little incentive to keep them in captivity. Decoy ducks were however widely employed. It is of interest to note that the first illustrations of captive ducks do not appear in Europe until the mid 17th century.

European domestic ducks appear to be derived exclusively from the mallard as determined by mitochondrial sequencing. D-loop sequences show minimal genetic differentiation between European domestic ducks and Eurasian wild mallards. There are no genetic breed or population clusters in Eurasian domestic ducks or mallards (Hou et al. 2011). Kraus and his colleagues (2011) also examined the origins of Eurasian ducks and found that about 20,000 SNPs are shared between wild and domestic birds out of a total of 101,000 SNPs also implying that relatively little genetic divergence has occurred.

The Muscovy Duck

The second major group of domesticated ducks is composed of descendants of the Muscovy duck (*Cairina moschata*) a forest duck whose natural range extends from Uruguay to the United States border in south Texas. The term Muscovy means from Moscow while the genus name *Cairina* means from Cairo, both reflecting confusion about the bird's origin when it first arrived in Europe.

Domestication of the Muscovy Duck

The Muscovy duck was domesticated by Native Americans before the arrival of Columbus. Sites in Panama and western Ecuador include Muscovy remains that date prior to 1000 AD but since it is not possible to distinguish wild from domesticated Muscovy duck bones found in archeological sites, the precise timing of this domestication is unclear. The duck may have been domesticated at multiple sites including the southern Caribbean, Paraguay or the Chaco region (Silveman and Isbell, 2008). Given that Muscovys are omnivores, some have speculated that they may have been used to control insect pests in agricultural communities. In the 1490's Columbus reported seeing natives keeping large ducks when he arrived in the West Indies. Amazonian villagers were reported to be keeping wild-caught Muscovy ducks in the 1500's by the Spanish conquistadors. It is unclear that these were truly domesticated since many Native American tribes keep wild birds as pets (Erikson 2000). In the 16th century, Spaniards and Portuguese took some of these ducks to Europe. They were popular table birds both in England and America until largely replaced by the Peking duck (Qu et al, 2009). As a result

of this domestication there are clear phenotypic differences between wild and domesticated Muscovys including plumage color and facial caruncles. Muscovy ducks are not suspected to have contributed to any other breeds of domestic ducks as crossing Muscovy ducks with mallards results in a large infertile hybrid called the mulard duck often used for meat and paté production.

Modern Duck Breeds

As with other domestic species, multiple breeds are recognized. They differ in posture, plumage and conformation. Unlike other species however, these breeds have not only been derived by phenotypic selection but also by hybridization with wild duck species. For example, in the United States, the Cayuga duck breed appears to have originated as a result of deliberate hybridization between domestic mallards and American black ducks (*Anas rubripes*). Tradition suggests that this breed originated completely from two wild male black ducks that were captured and bred with a domestic flock, presumably mallards. Likewise, the "Australian spotted" duck (a breed that originated in the United States) was apparently hybridized with northern pintails (*Anas acuta*) at one stage of their development. It has already been pointed out that the eastern spot-billed duck contributed to some Chinese duck breeds. As Muscovys do not prduce fertile hybrids when crossed with other duck species, there is only one Muscovy breed despite the great differences in plumage between wild and domestic Muscovys.

CONCLUSION

It is abundantly clear from the above discussion that the origin of domestic ducks is very complex and includes founders from at least five different species. In addition, the phylogeny of both wild and domestic ducks of the genus *Anas* is very complex. The history of the domesticated Muscovy duck, in contrast, is relatively simple. However, while it is clear that the mallard was the predominant contributor to most domestic *Anas*-based breeds, the Eastern spot-billed duck, the American black duck and the northern pintail have also contributed genes. Much of this contribution, especially if it was derived from male ducks would not be apparent in mitochondrial genomes. From a scientific viewpoint it is clear that investigators working on ducks, especially at the

genome/proteomics level must, of necessity define their animals very carefully. This has not always been the case and it has been common for investigators to describe their study birds simply as ducks. Thus, many gene sequences placed in the genomic and sequence databases are of very limited usefulness. The time has come for journal editors to require a careful definition of the birds employed in much the same way that mouse researchers are required to define the strains of mice they use.

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Chapter 3

RECENT VIRAL DISEASES OF JAPANESE ANATID WITH A FATAL CASE OF MAREK'S DISEASE IN AN ENDANGERED SPECIES, WHITE-FRONTED GOOSE (*ANSER ALBIFRONS*)

Mitsuhiko Asakawa,[1] Tetsuya Nakade,[1]
Shiro Murata,[2] Kazuhiko Ohashi,[2]
Yuichi Osa[3] and Hiroyuki Taniyama[1]

[1]School of Veterinary Medicine, Rakuno Gakuen University,
Ebetsu, Hokkaido, Japan
[2]Department of Disease Control, Graduate School of Veterinary Medicine,
Hokkaido University, Sapporo, Hokkaido, Japan
[3]Institute of Environmental Sciences, Hokkaido Research Organization,
Sapporo, Hokkaido, Japan

ABSTRACT

In our increasingly managed environment, where the maintenance of many ecosystems, habitats and species rests largely in human hands, disease control is now an important process in the active conservation of wild waterfowl populations because individual disease outbreaks are known to have killed many tens of thousands of a wide range of wild bird

species. On the other hand, most infectious agents, especially commensal viruses, are component parts of the ecosystems in which their wild avian hosts occur, they have co-evolved, and the infectious agents do not necessarily cause disease. But, some viral diseases have killed wild waterfowls including ducks, geese, swans, and captive ones, as well. Considerable environmental change has occurred in Japan over the last 150 years. With the increased availability of firearms following the Meiji Restoration (1860s), the populations of duck (*Anas* and *Aythya* spp.), geese (*Anser* and *Branta* spp.), and swans (*Cygnus* spp.) were dramatically depleted during the late 19th and early 20th centuries. Furthermore considerable impact as a result of industrial development during the 20th century (particularly during the post 1945 rapid development phase) led to extensive environmental damage to marine, coastal, lowland, freshwater, and montane ecosystems, with extensive areas of natural habitat being entirely destroyed (coastal and lowland wetlands have been particularly hard hit). As a consequence of this, avian habitats have been greatly altered and populations mostly reduced. Although waterfowl populations steadily increased during the latter part of the 20th century as persecution has declined (Japanese Association for Wild Geese Protection 1994), continued habitat loss has led to extensive concentration of populations. Not only are waterfowl populations greatly concentrated at shrinking wetland sites, both during migratory stop-over, and during the main winter period, but they are also remaining at fewer sites for longer periods of time, thus greatly increasing the risk of, and facilitating, bird-to-bird spread of such pathogens. An understanding of the infectious and parasitic avian disease organisms that are present in Japan, their ecology, transmission, and symptoms is an essential conservation tool. Our aim in this chapter is, therefore, to provide an overview of viral disease organisms, their recognition, their implications for avian populations in Japan, and potential strategies for risk reduction, in the hope of helping to prevent a severe epidemic event among bird concentrations in Japan. Although viral diseases have not been recognized as major causes of illness and death in wild birds in Japan, there has been significant concern about such diseases since the 1970s in the USA because of severe outbreaks of Duck plague there, although it is not deemed indigenous there. Duck plague, or duck viral enteritis, is caused by a herpes virus (Family Herpesviridae). Although duck viral enteritis has not yet been recorded from Japan, there has been a case of another lethal herpes viral infection among wild birds in Japan, namely of Marek's disease virus (MDV) (Family Herpesviridae). In spring, prior to breeding in Russia, and in autumn, prior to wintering in Honshu, most of approximately 60,000 individual White-fronted Goose (*Anser albifrons*) spend several weeks feeding on the Ishikari River plain in west Hokkaido, near Bibai City, with the majority of them roosting each night at one particular swamp - Miyajima-numa (approximately 30 ha). From

an ornithological and conservation perspective Miyajima-numa is a remarkably important site for the White-fronted Goose and for a wide range of other waterfowl species. It is also a highly significant site from an epidemiological viewpoint as the lethal case involving a tumor lesion, caused by Marek's disease, in an adult female White-fronted Goose was obtained from Miyajima-numa in November 2001. Marek's disease is caused by a highly contagious herpes virus with a worldwide distribution, and it is normally found amongst chickens, but lesions resembling those caused by MDV also have been reported in owls, pheasants, partridge, ducks, geese, swans and a kestrel. In Japan, Marek's disease is most commonly found among domestic chickens especially among battery chickens. As MDV has been shown to be transmitted through diresct and indirect contact with contaminated aerosols, it is very risky that too highly congestion of geese in roosting sites. Hence, a preliminary epidemiological survey of 45 individuals of geese utilising Miyajima-numa was done by us in April 2002. Adding, in this chapter, we will show the histopathological and molecular biological (PCR) results.

1. Background-Fluctuation of Anatid Population Size from Historical Viewpoint

So many winter birds inckuding most of endemic Far Eastern anatid species fly to Japan to spend winter time. Hence, in increasingly managed environment, where the maintenance of many ecosystems, habitats and species rests largely in human hands, disease control is now an important process in the active conservation of such waterfowl populations by government and/or non-governnmental organizums, because individual disease outbreaks are known to have killed many tens of thousands of a wide range of wild bird species including endemic ones restricted to East Asia (Brazil 1991; Ornithological Society of Japan 2000).

Surely, most infectious agents, especially commensal viruses, are component parts of the ecosystems in which their wild avian hosts occur, they have co-evolved, and the infectious agents do not necessarily cause disease. But, considerable environmental change is faster than such evolutionary process. Especially, in Japan, the change has occurred since only ca. 150 years ago. With the increased availability of firearms following the Meiji Restoration (1860s), the populations of anatid (Anatidae: Aves), for example, duck (*Anas* and *Aythya* spp.), geese (*Anser* and *Branta* spp.), and swans

(*Cygnus* spp.), were dramatically depleted during the late 19th and early 20th centuries. Furthermore considerable impact as a result of industrial development during the 20th century, particularly during the post 1945 rapid development phase, led to extensive environmental damage to marine, coastal, lowland, freshwater, and montane ecosystems, with extensive areas of natural habitat for anatid species being entirely destroyed. (Brazil 1991).

2. AIM OF THIS CHAPTER

However, the waterfowl populations steadily increased during the latter part of the 20th century as persecution has declined (Japanese Association for Wild Geese Protection 1994), and, on the other side, continued habitat loss has led to extensive concentration of populations. Not only are the waterfowl populations greatly concentrated at shrinking wetland sites, both during migratory stop-over, and during the main winter period, but they are also remaining at fewer sites for longer periods of time, thus greatly increasing the risk of, and facilitating, bird-to-bird spread of such pathogens. Since an understanding of the infectious avian disease organisms that are present in Japanese drastic environtal change is an essential model of epidemiology or ecology of anatid diseases, we pulished summary on the field including both anatid and non-anatid species (Asakawa et al. 2002). And now, our aim in this chapter is to provide a overview of viral disease organisms from anatid in the last ten years, and to give a prominent case report of Marek's disease in an endangered anatid species. Finaly, potential strategies for epidemic risk reduction of the family Anatidae will be presented from a viewpoint of conservation medicine.

3. BRIEF OVERVIEW OF ANATID VIRAL DISEASES RECORDED IN THE LAST TEN YEARS

Among RNA viral diseases, wild birds including especially migrating anatid have been the focus of concern as a source for H5N1 highly pathogenic avian influenza (HPAI) viruses infections in poultry and humans in Japan since 2004 when HPAI outbreaks occurred in Japan (Okamatsu et al. 2010; Sakoda et al. 2012). And, in April and May 2008, whooper swans (*Cygnus cygnus*) were found dead caused by HPAI infection in Hokkaido (Usui et al.

2009). Acctually, the fatal cases were restricted, but reduced health levels may contribute negatively to incidental mortality (by facilitating easier predation), prevent effective migration, or even reduce the capacity to compete and breed. Newcastle disease virus (NDV) is severely infectious agents among wild birds as well (Ritchie and Carter 1995; Friend and Frason 1999), and NDV is capable of infecting a wide range of avian species. In addition to domestic poultry, amongst which it is particularly virulent, more than 230 species of birds from more than half of the avian orders have been found to be susceptible to natural or experimental infections with NDV. Epornitic outbreaks of NDV appear to occur every 10 to 12 years. In a well-documented outbreak of NDV in the Great Lakes region of Canada and the United States during 1990 and 1992, total mortality attributed to the disease exceeded 20,000 wild birds including Double-crested Cormorants (*Phalacrocorax auritus*) (Friend and Frason 1999). Because NDV has been reported from pintail (*Anas acuta*) resently in Japan (Jahangir et al. 2009), it is needed to continue to perform monitoring survey on this viral disease in nature. In the mosquito-borne flaviviruses, West Nile viruse has been regarded as one of the most emerging zoonotic agents, and antibodies to the viruses were positive in mainly wild anatid (*Anas* spp.) in both Hokkaido of Japan and Far Eastern of Russia (Saito et al. 2009; Murata, R. et al. 2011). However, tests used with mosquitoes and birds for the viruses themselves and/or WNV RNA provided no evidence until now (Shirafuji et al. 2011; Hoshino et al. 2012). Among DNA viral diseases in the captive anatid, there has been a single recent case of goose parvoviral disease in Muscovy Duck (*Cairina moschata*) (Takehara 2000). Hence, DNA viral diseases have not been recognized as major causes of illness and death of wild birds in Japan, however, there has been significant concern about such diseases in the USA since the 1970s, because of severe outbreaks of Duck plague there (Friend and Franson 1999), although it is not deemed indigenous there. Duck plague, or duck viral enteritis, is caused by a herpes virus (Family Herpesviridae). It has been described as occurring among captive and free-ranging populations of various Anseriformes including dabbling ducks (*Anas, Aix, Cairina*), diving ducks (*Aythya, Bucephala*), sea ducks (*Somateria, Mergus*), geese (*Anser, Branta*), and swans (*Cygnus*) (Ritchie and Carter 1995). It is considered most likely that the duck plague virus was introduced to North America and Asia from Europe among imports of infected waterfowl (Ritchie and Carter 1995). So far, there have been no records of this disease in Japan, however, its occurrence both in the USA and China (Nuttall 1997) indicates that Japanese anatid populations may also be at risk, thus monitoring for its presence should be instigated at wetland sites.

4. CASE REPORT- LYMPHOMA CAUSED WITH MAREK'S DISEASE VIRUS IN A WHITE-FRONTED GOOSE

Above mentioned, fortunately, duck viral enteritis has not yet been occurred, but there has been a case of another lethal herpes viral infection in Japan. White-fronted geese (*Anser albifrons*) migrate to the main Japanese Honshu island, which is one of wintering areas for the eastern Asian population, from the breeding areas of northern Russia to breed. Each year about 60,000 White-fronted geese rest for several weeks at Miyajima Swamp (about 30 ha area; Figure 1) in Bibai City, located in the central part of Hokkaido, before flying on to Honshu (Japanese Association for Wild Geese Protection 1994). Epidemiological surveys of infectious diseases of white-fronted geese resting at Miyajima-numa Swamp should provide important information for the conservation of this avian species in Japan.

A lethal tumor caused by Marek's disease was found in a goose captured at Miyajima-numa Swamp. Because of its epidemiological significance, this case was briefly and quickly reported in a newsletter (Zoo and Wildlife Medical Group of Rakuno Gakuen Univ. et al. 2001) and home page (http://jjzwm.com/blog/marek/) of the Japanese Society of Zoo and Wildlife Medicine. In the present report, more details of histopathological and etiological findings of the case are described.

A female adult white-fronted goose (WAMC As No. 2631) uncapable to move was found at Miyajima-numa Swamp on October 26, 2001 (Figure 2), and presented to Rakuno Gakuen University Veterinary Teaching Hospital in Ebetsu, Hokkaido. Radiographic examination revealed luxation of the right shoulder joint, and external fixation was performed. However, the goose lost its appetite and died three days later. The carcass was kept in a refrigerator at 4 °C, and autopsy was performed on the day of death.

Body length and wing span of the goose were 730 mm and 1440 mm, respectively. Many greyish white nodules of various sizes were found on the surface of the intestinal tracts and peritoneum (Figure 3-1). The liver, kidney and ovary were markedly enlarged and white in color (Figure 3-2). These visceral organs were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (HE). Histopathological exami nation revealed diffuse accumulaion of neoplastic lymphocytes with indistinct cell borders in the peripheral nerves of intestines (Figure 3-3), liver (Figure 3- 4) and kidney (Figure 3-5), which was identical

to the characteristic microscopic lesions associated with Marek's disease infection. Although several echinostomid trematodes were obtained from the small intestine, the intestinal content was normal. PCR analysis using a specific primer pair revealed the presence of a Marek's disease virus serotype 1 (MDV1)-specific gene, *meq* (Murata, S. et al. 2007, 2011), in DNA samples prepared from the liver and tail skin indicating that the goose was indeed infected with oncogenic MDV1.



Figure 1. A herd of white-fronted geese at Miyajima Swamp in Bibai City, Hokkaido, in May, 2003.



Figure 2. A female adult white-fronted goose uncapable to move, found at Miyajima-numa Swamp on October 26, 2001.

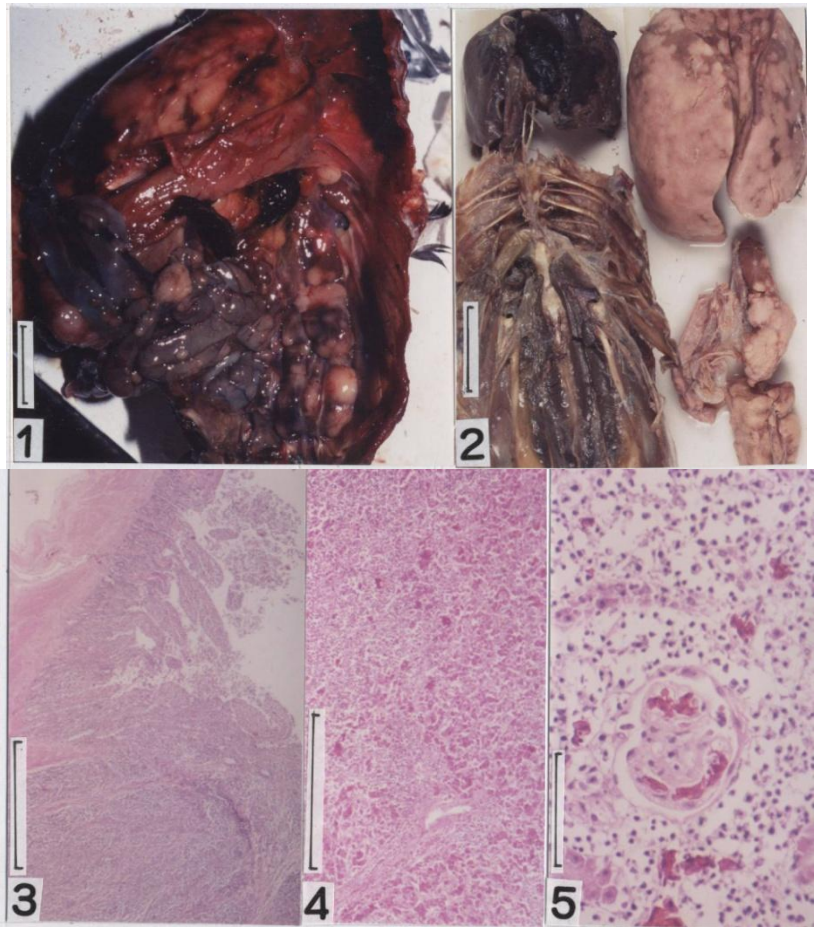


Figure 3. Gross and histopathological lesion of lymphoma caused with Marek's disease virus in a white-fronted goose.

-1: Many greyish white nodules found on the surfaces of the intestinal tract and peritoneum. Bar=30 mm.
- 2 : Markedly enlarged and white colored liver and kidney of the present case (right), comparison with liver
and kidney of another normal goose (left). Bar=30 mm.
- 3: Accumulaion of neoplastic lymphocytes in the peripheral nerves of the small intestine. HE stain. Bar=500
μm.
- 4: Neplastic cells having indistinct cell borders in the liver. HE stain. Bar=300μm.
- 5: Neplastic cells having indistinct cell borders in the kidney. HE. Bar=50 μm.

Marek's disease is caused by a highly contagious avian herpesvirus and is distributed throughout the world, including Japan. The disease is common in domestic chickens, but the similar lesions have been reported also in wild or captive avian species including owls, pheasants, partridges, ducks, geese, swans and kestrels (Friend and Franson 1999; Ritchie and Carter 1995). However, these cases were diagnosed only histopathologically, not by a molecular biological method such as PCR. Hence, the present bird is the first case of etiologically confirmed Marek's disease in wild avian species. Furthemore, the present case is valuable for considering the prevalence of Marek's disease in free-ranging wild birds (Friend and Franson 1999).

CONCLUSION

Successful disease outbreak prevention will need to be based not only on an understanding of anatid ecology but also of virus ecology (Asakawa et al. 2002). And, in order for medical researchers to be able to prepare for future epidemic outbreaks amongst birds, they must perform epidemiological surveys of the probable agents mentioned above in wild bird populations both in Japan and its surrounding areas; international and inter-agency cooperation is, therefore, essential in disease prevention. Very careful attention should be paid to the possibility of infection by zoonotic agents during monitoring surveys.

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Chapter 4

BIRD SCHISTOSOMES

Marta Chanová*
Institute of Immunology and Microbiology, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

ABSTRACT

Ducks are the most frequently reported waterfowl infected with bird schistosomes. Freely swimming infective larvae (cercariae) of these flukes directly penetrate the skin of birds exposed to the water environment. Penetrating cercariae transform into schistosomula, the developing stage which migrate through the host body using highly specific migratory routes. They are transported via blood circulation, except from *Trichobilharzia regenti*, which pervade through the peripheral nerves to the spinal cord and brain directly through the nervous tissue. The target locations of migrating worms differ among species and may be related to gastro-intestinal tract (visceral species) or nasal area (nasal species). At these sites parasites mature, copulate and produce eggs.

The presence of migrating stages, as well as stages of adult worms and eggs in the host body usually cause injuries in involved tissues/organs and may be of serious consequences varying from

* Corresponding author: Marta Chanová. Institute of Immunology and Microbiology, 1st Faculty of Medicine, Charles University in Prague, Studničkova 7, 128 00 Prague 2, Czech Republic; E-mail: martikam@centrum.sk.

breathing or digesting disorders to the neurological symptoms and severe paralysis.

REVIEW

Bird schistosomes (Digenea; Schistosomatidae) are dixenic parasitic helminths using water snails and birds as intermediate and definitive hosts, respectively. They occur worldwide and include 9 genera - *Allobilharzia*, *Austrobilharzia*, *Bilharziella*, *Dendritobilharzia*, *Gigantobilharzia*, *Jilinobilharzia*, *Macrobilharzia*, *Ornithobilharzia* and *Trichobilharzia* of approximately 58 species totally; last genus including 40 species is most abundant among schistosomes.

Usually, fresh water pulmonate snails serve as intermediate hosts; some species use marine or freshwater opisthobranchs and prosobranchs.

As definitive hosts, bird schistosomes infect birds of several orders, including e.g. Ciconiiformes, Charadriiformes, Gruiformes, Passeriformes, Pelecaniformes, Podicipediformes, and, most frequently, Anseriformes. Specificity for definitive host differs among parasite genera from those found in birds of only few families (*Dendritobilharzia* sp.; Vande Vusse, 1980) to those developing in birds of several unrelated orders (*Trichobilharzia*, *Gigantobilharzia* and *Bilharziella*; e.g. Horák et al. 2002; Jouet et al. 2009, Kolářová et al. 1997).

Snails become infected by direct penetration of miracidium, a swimming ciliated larva hatched from schistosome egg in water environment. Through the penetration, miracidium releases surface ciliated plates and transforms into a mother sporocyst, the stage providing asexual multiplication. Mother sporocyst migrates from penetration site into deeper layers of head-foot region and its numerous germinal cells develop each into a daughter sporocyst. These, containing germinal cells as well, emerge from mother sporocysts, migrate to hepathopancreas and give rise to next developmental stage – cercariae. Matured cercariae escape from mother sporocysts, migrate through the snail tissues and leave the snail by penetration of its body-wall. First cercariae are emitted by snail approx. 4-6 weeks post penetration of miracidium, depending on particular parasite species and environmental conditions, mainly the temperature (low temperature extends the period of cercariae development). Production of cercariae continues more or less intensively through the rest of the snail life with no significant impact on

length of its survival. Thus, single miracidium may become a source of thousands of cercariae.

Released cercariae actively search for potential definitive host. They posses light-sensitive eye spots, numerous sensitive organs for host molecule detection and bifurcated tail for swimming. They exhibit several modes of behavior, including active and passive phases according to detected stimuli (passing shadows, warmth, chemical signals; for details on cercariae host-finding strategies see Haas, 1994). As no energy-intake takes place at this developmental stage, the activity and survival in water are limited and ability to successfully infect definitive host decrease rapidly after several hours.

After finding a host, cercaria attaches to the skin, creeps to a suitable penetration site and empties penetration glands. Employing spiny oral sucker and penetration movements together with hydrolytic action of released glands products, cercaria penetrates into stratum corneum (Haas and Van de Roemer, 1998). Complete penetration occurs within several minutes; cercaria detach the tail, shed the surface antigenic layer (glycocalyx) and transforms into schistosomulum, the migrating larva pervading into the host tissues.

Newly transformed schistosomula are localized in the skin where they pass toward deeper layers of dermis. Subsequent migration differs among species.

Schistosomula of most species use circulatory system, i.e. enter blood capillaries in the dermis and migrate with blood stream through blood vessels towards right heart and lungs. Then, they continue in using blood vessels for migration towards species-specific target location.

Schistosomula of one particular species, *Trichobilharzia regenti*, represent an exception among schistosomes due to the neurotropism. In the skin, they enter peripheral nerves instead of blood vessels, and continue in extravascular migration through the nervous tissue. They migrate through peripheral nerves, enter spinal cord and continue to the brain, where finally enter meningeal blood vessels, and reach the target location using blood stream (Hrádková and Horák 2002; Chanová and Horák 2007).

Target location is a place inhabited by adult worms and is closely related to the outer environment through gastro-intestinal tract (visceral species; most of bird schistosomes) or nasal cavity (eight nasal species). Adult worms of visceral species are localized either intravascularly (e.g. *Allobilharzia visceralis*, Kolářová *et al*, 2006; *Bilharziella polonica*, Baysade-Dufour *et al.*, 2006); *Dendritobilharzia purverulenta*, Vande Vusse, 1979; *Trichobilharzia. parocellata*, Islam and Copeman, 1986; *T. brantae*, Farr and Blankemeyer, 1956;) or extravascularly (e.g. *T. szidati*, Neuhaus, 1952, *T. ocellata*, Bourns *et*

al., 1973). Intravascularly localized worms (except for arterial specialists of *Dendritobilharzia* spp.) inhabit venous system (Vande Vusse, 1979). Adults of nasal schistosomes were found in blood vessels (Fain, 1955, 1956a, 1956b; Blair and Islam, 1983; Islam, 1986), except for *T. regenti*, localized mostly extravascularly in soft nasal tissues (Chanová and Horák, 2007).

At target location, mating and oviposition take place. One egg is developing in female uterus at time; the egg production is continuous and covers a period of several days to weeks. Eggs are laid immature, containig several germinal cells. These cells form a miracidium which becomes fully matured, ciliated and motile after several days. In certain cases, eggs may become ectopically disseminated with blood stream. Typically, however, developing eggs of visceral species pass into an intestinal lumen through the intestinal wall and are drained with feces. Once eggs come into contact with water, miracidia hatch and are ready to localize and infect intermediate host. In the case of nasal species, miracidia hatch directly in nasal cavity and are rinsed with water in a manners common for waterfowl.

Prevalence of bird schistosomiasis in waterfowl reported from field studies reaches high numbers, e.g. more than 35% in Lower Michigan (Guth et al., 1979), 6-22% in central Europe (Rudolfová et al., 2007), 18% in Iran (Athari et al., 2006), and exceed 50% in certain areas, e.g. in Iceland (Kolářová, 2007; Skirnísson and Kolářová, 2008). Throughout the results of prevalence studies, ducks of family Anatidae are most frequently infected birds.

Bird schistosomes as a cause of mortality or sickness (manifested mostly with emaciation and weakness) of waterfowl are reported sporadically worldwide (Farr and Blankemeyer, 1956; Levine et al. 1956; Pence and Rhodes, 1982; Wilson et al. 1982; Wojcinski et al. 1987; van Bolhuis et al. 2004, Acagami *et al.*, 2010).

Host-parasite interactions were shown to vary with host age and with host species. Young birds express more severe clinical symptoms and higher infection prevalence than adults; e.g. Rau *et al.*, 1975; Guth *et al.*, 1979; van Bolhuis, 2004. Newly introduced or migratory bird species may suffer with severe symptoms when infected with parasites that they do not normally encounter, contrary to asymptomatic endemic birds (Wojcinski *et al.*, 1987). However, differently permissive hosts are distinguished also among endemic waterfowl coexisting at biotope (van Bolhuis, 2004; Jouet *et al.*, 2010). In species-specific "normal" hosts, adult worms are found mostly at usual oviposition sites with viable embryonated eggs in large numbers and milder associated pathologies. In contrast, non-typical distribution of worms and/or

few eggs in various organs/tissues accompanied by severe injuries are known in "abnormal" hosts (Vande Vusse, 1979).

Pathologies are related either to adult worms or to deposited eggs in naturally infected birds. Extensive injuries due to adult worms presence are less common, and were reported e.g. for *A. visceralis* (Kolářová *et al.*, 2006), *T. brantae* (Wojcinski *et al.*, 1987) and *T. filiformis* (van Bolhuis, 2004). Vascular inflammation characterized as endophlebitis or periphlebitis with mononuclear cell infiltrations develops in vascular lumen. Hyperplasia of vascular intima, obliteration of vascular lumen, perivascular hemorrhages and trombosis in veins also occure. Adults of *T. physellae*, localized ectopically in hepatic portal triads caused fibrosis and periphlebitis (Pence and Rhodes, 1982). As egg-induced pathology, mainly formation of granulomas with predominance of macrophages, eosinophils, heterophils, and giant cells is reported. Granulomas are formed around eggs, typically in mesenterial veins or in mucosa and/or submucosa of particular intestine part according to species-specific oviposition site. Further, villous atrophy, fibrosis, edema and congestion due to distention of capillaries occur. More severe reactions appear around eggs incidentally entrapped in surrounding tissues on their way to intestinal lumen than around easily passing ones; even more in case of ectopical egg locations, e.g. *D. purverulenta* in brain (Levine *et al.*, 1956) or *T. filiformis* in lungs (van Bolhuis, 2004). Thus, species laying eggs in the submucosa tight to intestinal lumen represent lower risk of egg-induced tissue injuries because of minimal possibility of both, egg entrapment and ectopic dissemination with blood stream (Bourns *et al.*, 1973).

Contrary to adults and eggs, developing schistosomula are overlooked in natural infections and no schistosomula-related lesions are reported. This is probably because of their small size and presence in tissues/organs other than those usually searched for schistosomes. However, experimental infections showed that also schistosomula migrating through host body cause serious pathologic changes.

Host-parasite interactions of few *Trichobilharzia* species were studied in detail under experimental conditions, using black ducks *A. rubripes*, white Pekin ducks *A. platyrhynchos* f. domestica and Muscovy ducks *Cairina moschata*. Recently, neurotropic *T. regenti* in duck presents the most intensively studied model of bird schistosomiasis. Extensive experimental studies provide an insight into parasite behavior during pre-patent phase of infection and allow completing the knowledge on bird schistosome interactions with definitive hosts.

Unique life-strategies and exceptional pathogenic impact on host were revealed for *T. regenti.* Neurotropism and ingestion of host nervous tissue particles by migrating worms are obligatory, causing degenerative changes in the nervous tissues and, as a consequence, leg paralysis and balance disorders (Horák et al. 1999). In common, the spinal cord is more injured than the brain, and tissue damage is stronger at sites with numerous parasites. Pathology is mainly a result of host immune cell reactions against parasite presence in central nervous tissue; eosinophilic meningitis and inflammatory reaction with infiltrates of eosinophils and heterophils in white and gray matter (around parasites, perivascularly and also around the central canal of spinal cord) occurs. Occasionally also dystrophic and/or necrotic neurons in the spinal cord are found (Kolářová et al. 2001).

Significant pathogenic impact of schistosomula of visceral species was confirmed, too. Infection with *T. ocellata* leads to respiratory distress, compulsive swallowing and listlessness in early prepatetnt phase (Rau *et al.*, 1975); similar symptoms were observed due to *T. szidati* (unpublished). Histopathologic observation revealed inflammatory reaction with infiltrates of lymphocytes, heterophils, eosinophils and macrophages around pulmonary blood vessels after parasite establishment. Later, with further obligatory migration (schistosomula leave blood vessels, enter gas-exchange tissue of lungs, pass the wall of secondary bronchi and spread through free air-space into parabronchi; Bourns *et al.*, 1973; Chanová *et al.*, 2007), infiltrates of above mentioned composition forms also in the gas-exchange tissues, and an extensive inflammation of secondary bronchi and parabronchi with oedema and haemorrhages in their lumen develops. After specific migration, schistosomula re-enter blood vessels and leave the lungs, however pneumonia, alveolar hyperplasia and haemorrhages in peripheral parabronchi are present for several days (Chanová *et al.*, 2007).

Besides birds, bird schistosomes are able to infect also mammals, including human. Primary infection under experimental conditions leads to partial migration of schistosomula with shortened life-span and failure of life-cycle. Repeated exposition to cercariae initiates a protective immune response which destroys transforming schistosomula in the skin. Both, primary and repeated expositions to cercariae are of serious pathogenic impact on accidentally infected mammals. Special complication represents human skin immune response (manifested as cercarial dermatitis or "swimmers itch"), a re-emerging disease with local outbreaks of worldwide occurrence (for review see Horák *et al.*, 2002). Being a serious health, and also, if occurs in tourist

areas, an economical problem, human cercarial dermatitis attracted to bird schistosomes also the attention of many non-veterinarian authors.

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Chapter 5

A BRIEF OVERVIEW OF PARASITIC NEMATODES RECORDED FROM WATERFOWLS ON HOKKAIDO, JAPAN

Tomoo Yoshino[1,2*] and Mitsuhiko Asakawa[1#]
[1]School of Veterinary Medicine, Rakuno GakuenUniversity,
Ebetsu, Hokkaido, Japan
[2]Kyushu Regional Environment Office,
Ministry of the Environment,
Kumamoto, Kumamoto, Japan

ABSTRACT

Nowadays, there have been suspicious parasitic nematode diseases on wild and/or captive waterfowls since the mid 1990s in Japan. Among them, some nematodes could infect each other among captive and wild birds, and it seems to become a proper conservation medical research and educational model as well. Hence, the present authors have continued to report parasitic nematodes of free-ranging and/or semi free-ranging captive waterfowls belonging to not only the family Anatidae but also the other families from Hokkaido, the most northern of the main islands of

* TomooYoshino: School of Veterinary Medicine, Rakuno GakuenUniversity, Ebetsu, Hokkaido 069-8501, Japan. Kyushu Regional Environment Office, Ministry of the Environment, Kumamoto, Kumamoto 862-0913, Japan.

Corresponding author: Mitsuhiko Asakawa. School of Veterinary Medicine, Rakuno GakuenUniversity, Ebetsu, Hokkaido 069-8501, Japan. E-mail: askam@rakuno.ac.jp.

Japan. Among them, there appeared to be strict host/parasite relationships between the wild swans/geese and *Amidostomum anseris*/ *Epomidiostomum crami*, and between wild duck species and *A. acutum*/*Ep. uncinatum*, respectively.

INTRODUCTION

The most northern island of the Japanese main lands, Hokkaido, is situated on the East Asian Flyway and an important transit area for migratory waterfowls including the order Anseriformes during stopovers on migration and during the main winter period (Miyabayashi, 1994). With the increased availability of firearms following the Meiji Restoration (1860s), the populations of a wide range of species ducks (*Anas* and *Aythya* spp.), geese (*Anser* and *Branta* spp.), and swans (*Cygnus* spp.) were dramatically depleted during the late 19th and early 20th centuries. Furthermore, considerable impact as a result of industrial development during the 20th century led to extensive environmental damage to marine, coastal, lowland, freshwater, and mountain areas, with extensive areas of the natural habitat being entirely destroyed in the island. As a consequence of this, avian habitats have been greatly altered and populations mostly reduced. Although waterfowl populations of Hokkaido steadily increased during the latter part of the 20th century as persecution declined continued habitat loss has led to the considerable concentration of certain populations.

Both during stopovers on migration and during the main winter period, but they are also remaining at fewer sites for longer periods of time, thus greatly increasing the risk of bird-to-bird spread of parasitic nematodes. In this chapter, parasitic nematodes have been recorded from not only anatids but also non-anatids from Hokkaido, Japan, in order to obtain a baseline of preventing an outbreak among waterfowl caused by fatal nematodiasis.

PARASITIC NEMATODES RECORDED FROM WATERFOWLS FROM OUTSIDE OF HOKKAIDO

Uchida et al. (1991) listed ca. 170 trematode, 110 cestode, 70 nematode, and 20 acanthocephalan species, respectively, from ca. 100 species of Japanese birds. However, especially, from 1960 to 1990, no new nematodes have been added to the list. During the 1990s, several fatal cases were

reported, e.g., Little Grebe *Tachybaptus ruficolllis*, which died from a severe nematode infection with *Eustrongylides tubifex* (Murata et al., 1997). *Thelazia (Thelaziella) aquillina* was obtained from a captive Oriental White Stork *Ciconia boyciana*, endangered species restricted to East Asia, in Hyogo Prefecture (Asakawa et al., 2000). The genus *Thelazia* is well-known as a pathogen causing conjunctivitis. After 2000, some nematode genera including *Amidostomum* and *Epomidiostomum* regarded as pathogenic agent for waterfowls were recorded in some area of Honshu (Nakamura et al., 2003; Sato et al., 2005).

RECENT NEMATODE RECORDS FROM HOKKAIDO

After then, we have reported parasitic nematodes of 29 species of wild waterfowls belonging to the families Anatidae, Ardeidae, Rallidae, Scolopacidae, Phalaropodidae, Laridae and Stercorariidae from Hokkaido, Japan (Inc. 64.3%), and a total of 27 nematodes (*Eucoleus contortus, Capillaria anatis*, Capillariidae gen. sp., *Baruscapillaria mergi*, *Amidostomum anseris*, *A. acutum*, *A. fulicae*, *Epomidiostomum crami*, *Ep. uncinatum*, *Cyathostoma lari*, *C. microspiculum*, *Contracaecum rudolphii*, *C. microcephalum*, *Porrocaecum reticulatum*, *P. semiteres*, *Inglisonema* sp., *Madelinema* sp., *Tetrameres fissispina*, *T. scolopacis*, *Desportesius equispiculatus*, *Skrjabinoclava horrida*, *Paracuaria adunca*, *Cosmocephalus obvelatus*, *Stegophrus stercorarii*, *Echinuria uncinata*, *Streptocara crassicauda*, *Sarconema eurycerca*) was recorded (Nakamura and Asakawa, 2001; Yoshino et al., 2009a, 2009b). Among them, most of the nematode species were the first local records in even Japan.

The spirurid genera also include gastrointestinal parasites such as *Tetrameres*, *Paracuaria*, *Echinuria*, *Cosmocephalus*, *Skrjabinoclava*, *Streptocara* and *Stegophorus*, which can cause ulceration, hemorrhaging, and proliferative inflammatory nodular lesions on the mucosal surface of the gastrointestinal tract. Furthermore, some of the trichostrongylids can cause serious intestinal disorders in both domestic and wild waterfowls (Asakawa et al., 2002). Recently, *A. acutum* was found from captive *Aigamo* duck as well (Yoshino et al., 2011). And there discovered that there appeared to be strict host-parasite relationships between the wild swans/geese and *A. anseris*/*Ep. crami*, and between wild duck species and *A. acutum*/*Ep. uncinatum*. Hence, spatial epidemiological analysis of the parasitic nematodes helminths from the anatids was conducted by a K-function method, using the spatial analyzing

module, "S+SpatialStats" (Yoshino et al., 2008). The spatial epidemiological analysis of infectious agents has been performed in human medicine, but the analysis was the first in wildlife medicine in Japan. Hence, a study on the parasitic nematode epidemiology of the waterfowls in Hokkaido has just begun.

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INDEX

#

20th century, ix, 38, 40, 60

A

access, 3, 12
acid, 10
acute infection, 15
adaptations, 56
adults, 52, 53
aerosols, x, 39
Alaska, 26
amino acid, 10
ancestors, 26
antibody, 16, 17, 22
antigen, 4, 12
Argentina, 17
Asia, 22, 25, 26, 27, 28, 31, 36, 39, 41, 61
aspiration, 6
assessment, 47
asymptomatic, 52
ataxia, 4
atrophy, 53
autopsy, 42
avian, vii, viii, 1, 3, 4, 5, 10, 13, 15, 17, 18, 19, 23, 34, 36, 38, 39, 40, 42, 45, 47, 55, 56, 60, 62
avian bornavirus (ABV), vii, 1
avian influenza, 3, 5, 15, 23, 34, 36, 40, 47

B

bacteria, 46
base, 24, 26
Beijing, 30
biogeography, 35
birds, viii, ix, x, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 23, 25, 26, 29, 32, 34, 36, 38, 39, 40, 45, 46, 47, 49, 50, 52, 53, 54, 56, 57, 59, 60, 62, 63
blindness, 4
blood, x, 23, 49, 51, 52, 53, 54, 55, 56
blood circulation, x, 49
blood stream, 51, 52, 53
blood vessels, 51, 52, 54, 55, 56
bones, 32
brain, vii, x, 1, 3, 4, 5, 6, 8, 9, 10, 12, 13, 17, 23, 49, 51, 53, 54
Brazil, 27, 34, 39, 40, 46, 62
breathing, x, 50
breeding, ix, 4, 26, 28, 30, 35, 38, 42

C

Cairo, 32
Cambodia, 31
canals, 30
Caribbean, 32
CD8+, 3
cDNA, 6

cell culture, vii, 1, 2
census, 36
Central Europe, 3
central nervous system (CNS), vii, 1, 3, 12, 56, 57
cercaria, 51
chemical, 51
chicken, viii, 21, 22
China, 22, 28, 29, 30, 31, 35, 36, 41
circulation, x, 15, 49
classification, 25, 27, 35
climate change, 28
clinical presentation, 4
clinical symptoms, 52
cloaca, 9
cloacal swabs, vii, 1, 5, 7, 9
clusters, 26, 32
College Station, 1, 21
color, 27, 33, 42
commercial, viii, 2, 4, 13, 34
communication, 14
communities, 32
compatibility, 25
complexity, viii, 21
conjunctivitis, 61
conservation, viii, x, 35, 37, 39, 40, 42, 46, 59, 62
contaminant, 10
contamination, 6, 14
convergence, 28
cooling, 14, 25
cranium, 13
culture, vii, 1, 2, 12, 13
culture medium, 13
cycles, 6, 15
cytochrome, 24, 29
cytotoxicity, 15
Czech Republic, 49, 57

D

dermatitis, 54, 55, 56, 57
dermis, 51
detection, 3, 5, 6, 17, 51
dilation, viii, 2
dimorphism, 25
diseases, vii, ix, x, 38, 40, 41, 42, 46, 59, 62
distress, 54
distribution, x, 4, 10, 17, 18, 24, 39, 52
divergence, 26, 32
diversity, 9, 22, 26, 29, 35, 36
DNA, viii, 19, 21, 22, 23, 24, 29, 34, 41, 43
domestication, viii, 21, 29, 31, 32, 34, 36
Duck plague, ix, 38, 41

E

East Asia, 39, 60, 61
ecology, ix, 38, 40, 45
Ecuador, 32
edema, 53
egg, 13, 50, 52, 53, 57
electron, 2
electrophoresis, 6, 7
encephalitis, viii, 2, 14, 15, 17, 18, 58
encephalomyelitis, 3, 13
endangered, vii, 4, 40, 61
endangered species, 4, 61
endocrinology, 36
energy, 51
England, 32
enteritis, ix, 38, 41, 42
entrapment, 53
environment, viii, x, 37, 39, 49, 50, 51
environmental change, viii, 38, 39
environmental conditions, 50
environmental stress, 14
eosinophils, 53, 54
epidemic, ix, 38, 40, 45
epidemiology, 40, 62
etiology, 14
Eurasia, 26
Europe, 2, 18, 22, 25, 28, 31, 32, 41, 52
evidence, 3, 6, 13, 16, 26, 29, 35, 41
evolution, 35, 36
exclusion, 28
experimental condition, 53, 54
external fixation, 42

F

families, x, 2, 24, 50, 59, 61, 63
farms, 29
feather follicles, vii, 1
feces, 15, 52
fibroblasts, 4, 12
fibrosis, 53
firearms, viii, 38, 39, 60
follicles, vii, 1
food, 4, 13, 31
formation, 53
freezing, 25
freshwater, ix, 38, 40, 50, 60
fungi, 46

G

gastrointestinal tract, 61
gel, 6, 7
gene pool, 30
genes, 7, 23, 33
genetic background, 22
genetic diversity, 22, 35
genetic information, 22
genetics, vii, viii, 19, 21, 22, 35, 47
genome, 2, 4, 6, 10, 18, 24, 34, 46
genotype, viii, 2, 4, 10, 11, 13, 14, 18
genre, 56
genus, 24, 27, 32, 33, 34, 50, 55, 56, 58, 61
Germany, 3
goose, vii, 1, 5, 6, 7, 8, 9, 10, 11, 13, 24, 31, 41, 42, 43, 44, 47, 56
granulomas, 53
grass, 15
gray matter, 54
grazing, 15

H

habitat, vii, ix, 14, 28, 37, 38, 39, 40, 60
haploid, 23
haplotypes, 29
health, 5, 41, 54
hemisphere, 25
herpes, ix, 38, 41, 42
herpes virus, ix, 38, 41
history, viii, 3, 5, 11, 21, 33, 35, 36
Hong Kong, 36
horses, 2
host, vii, x, 1, 2, 3, 4, 11, 16, 49, 50, 51, 52, 53, 54, 56, 57, 60, 61
host cell nucleus, vii, 1, 2
human, viii, 37, 39, 54, 62
human skin, 54
Hunter, 58
hunting, 6, 9
hybrid, 28, 30, 33
hybridization, 19, 25, 26, 27, 29, 33
hyperplasia, 54
hypertrophy, 55

I

Iceland, 52, 57
identification, 4, 35
identity, 10, 11, 26
immune response, 23, 54
immunofluorescence, 2
immunohistochemistry, 14
imports, 41
in situ hybridization, 19
incubator, 13
India, 28
individuals, x, 39
Indonesia, 31
induction, 17
industry, 30
infection, ix, 2, 3, 5, 6, 12, 13, 14, 15, 16, 18, 23, 38, 40, 42, 43, 45, 52, 53, 54, 57, 61
infectious agents, viii, 22, 38, 39, 41, 62
inflammation, 3, 4, 53, 54
influenza, 3, 5, 6, 15, 22, 23, 34, 36, 40, 46, 47
influenza a, 46
influenza vaccine, 22
influenza virus, 3, 5, 6, 15, 36, 47
ingestion, 54

injuries, x, 49, 53
inoculation, 12
intestinal tract, x, 42, 44, 49, 51
intestine, 43, 44, 53
intima, 53
intraocular, 12
intron, 28
Iran, 52, 55
Islam, 51, 55, 56
islands, x, 26, 59
isolation, 17, 24, 46
Israel, 3

J

Japan, vii, viii, x, 28, 30, 34, 37, 38, 39, 40, 42, 45, 46, 47, 55, 59, 60, 61, 62, 63

K

kidney, 15, 42, 44

L

lakes, 14, 25
Laos, 31
larva, 50, 51
larvae, x, 49
lead, 3, 13
lesions, x, 4, 12, 13, 16, 39, 43, 45, 53, 61
life cycle, 55
light, 12, 51
liver, 42, 44
livestock, 31
lumen, 52, 53, 54
lymphocytes, 42, 44, 54
lymphoma, 44

M

macrophages, 53, 54
Malaysia, 36
mallard, viii, 2, 12, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36
mammals, 3, 54
management, 5
matter, 3, 54
meat, 29, 30, 33
medical, x, 45, 59
medicine, 40, 62
meningitis, 54
methodology, 23
mice, 34, 35, 56
microsatellites, 30
microscopy, 12
migrating worms, x, 49, 54
migration, 41, 51, 54, 55, 60
minicolumn, 6
Ministry of Education, 45
Miocene, 23
miracidium, 50, 52
mitochondrial DNA, 19, 24, 29, 34
mitosis, 2
molecular biology, 3
molecular structure, viii, 21
morphological variations, 55
morphology, 35, 55, 56, 57
mortality, 2, 23, 41, 52
Moscow, 32
mosquitoes, 41, 47
mRNA, 2
mtDNA, 24, 26, 28, 29, 30, 35
mucosa, 53
mute swan brain samples, vii, 2
Myanmar, 28

N

NADH, 24
nasopharynx, 9
Native Americans, 32
nematode, x, 59, 60, 61, 62
nervous system, vii, 1, 3, 4, 18, 56
neural networks, 3, 12
neurons, 54
Nile, 41, 46, 47
nitric oxide, 23

nodules, 42, 44
non-segmented negative strand RNA viruse, vii, 1, 2
North America, v, vii, 8, 10, 14, 15, 23, 25, 26, 27, 28, 35, 41
Northeast Asia, 26
nucleoprotein, 2
nucleotide sequence, 10, 11
nucleus, vii, 1, 2
nutrients, 15

O

oedema, 54
organs, vii, x, 1, 15, 23, 42, 49, 51, 53
ornithine, 28

P

Pacific, 26, 27, 28
Pakistan, 28
Panama, 32
Paraguay, 32
paralysis, x, 4, 50, 54
parasites, x, 49, 50, 52, 53, 54, 60, 61, 62
parasitic diseases, 46, 62
pathogenesis, 3, 17
pathogens, ix, 14, 38, 40
pathology, 53
PCR, vii, viii, x, 1, 2, 3, 5, 6, 11, 12, 13, 14, 39, 43, 45
peritoneum, 42, 44
pests, 32
physiological, 56
pneumonia, 54
Poland, 57
polymorphism, 30
ponds, 9, 25, 28
population, 3, 26, 28, 32, 35, 42
poultry, 40, 47
predation, 41
prevention, 45
protein sequence, 22
proteins, 2
proteomics, 34
prototype, 2, 4
proventricular dilatation disease (PDD), vii, 1, 3
purification, 9

Q

query, 17

R

radiation, 23
reactions, 53, 54
recognition, ix, 38
relatives, 27
relevance, 22
researchers, 22, 34, 45
resistance, 22, 35
response, 16, 28, 54
restriction fragment length polymorphis, 30
reverse transcriptase, 13
rhabdoviruses, 2
risk, ix, 38, 40, 41, 47, 53, 60
risk assessment, 47
river basins, 29
RNA, vii, 1, 2, 5, 6, 9, 19, 40
rodents, 16
routes, x, 12, 49
Russia, ix, 26, 38, 41, 42, 46

S

schistosomiasis, 52, 53, 55, 56, 58
sequencing, 4, 6, 7, 11, 26, 32
sexual dimorphism, 25
sheep, 2
shorelines, 14
shrinking wetland sites, ix, 38, 40
signals, 51, 56
signs, 4, 12, 22
skin, x, 43, 49, 51, 54, 56
small intestine, 43, 44
SNP, 35

Solomon I, 35
South America, 14, 22, 36
Southeast Asia, 22, 28, 31
specialists, 52
speciation, 24, 27
species, vii, viii, x, 1, 2, 3, 4, 5, 9, 10, 14, 18, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 33, 37, 39, 40, 41, 42, 45, 49, 50, 51, 52, 53, 54, 60, 61, 62
spinal cord, x, 49, 51, 54
starvation, 4
state, 8, 9, 16
states, 5, 7
sterile, 13
storage, 6, 9
stress, 9, 14
structure, viii, 21, 22, 25, 26, 27, 35
submucosa, 53
substitutions, 11, 19, 26
surveillance, 5, 46, 47
survival, 4, 15, 51
susceptibility, 16, 23
Sweden, 3
symptoms, ix, x, 38, 50, 52, 54

T

T cell, 3
target, x, 49, 51, 52
taxa, 25, 26, 27
taxonomy, 22
techniques, 31
temperature, 23, 50
territory, 14
testing, 5, 6, 9
tissue, x, 6, 12, 13, 16, 18, 49, 51, 53, 54
trade, 4
transcription, vii, 1
transcripts, 2
transmission, ix, 14, 15, 16, 17, 38, 56
transport, 5
triggers, 14
tropism, 18
tumor, ix, 39, 42

U

UK, 46
United States (USA), viii, 2, 5, 14, 16, 18, 30, 32, 33, 38, 41, 47
Uruguay, 32
USDA, 5
USGS, 46
uterus, 52

V

vaccine, 22, 34
variations, 55
vertebrates, 57
vertical transmission, 16
vessels, 51, 52, 54, 55, 56
Vietnam, 31
viral diseases, vii, viii, 38, 40
viral infection, ix, 12, 38, 42
virus infection, 15
viruses, vii, viii, 1, 2, 5, 10, 11, 13, 15, 16, 36, 38, 39, 40, 46, 47
visualization, 12

W

Washington, 5, 8, 34, 46
water, x, 10, 14, 15, 25, 47, 49, 50, 51, 52, 56
weakness, 4, 52
weight loss, 4
West Indies, 32
western blot, 12, 17
wetlands, ix, 38
wildlife, 5, 62
Wisconsin, 7
wood, 10
worms, x, 49, 51, 52, 53, 54