Pathobiology of Avian Influenza in Wild Bird Species

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Cover: Cerebellum of tufted duck showing Purkinje cells and neurons infected with highly pathogenic avian influenza virus-H5N1 (red stained cells)
(photo: Caroline Bröjer)

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Abstract

Avian influenza viruses, especially highly pathogenic avian influenza viruses (HPAIV), affect a wide range of species, including humans and have thus become a major concern for veterinary medicine and public health. A HPAIV-H5N1 belonging to clade 2.2, originally from South East Asia, spread across Eurasia and reached Sweden in 2006. Currently the most commonly isolated HPAIV-H5N1 from wild birds belong to clade 2.3.2. There is a growing concern that the H5N1 virus has evolved in such a way that it can be maintained in the wild bird population without causing severe disease. At the same time the role of natural hosts, such as mallards (*Anas platyrhynchos*), in the epidemiology of avian influenza is an ongoing concern. In order to characterize the natural disease in free ranging birds in Sweden and to assess the pathogenicity of clade 2.3.2 viruses, histopathology, polymerase chain reaction, virus isolation and immunohistochemistry (IHC) were used to investigate lesions and viral tissue targeting of HPAIV-H5N1 in naturally infected tufted ducks (*Aythya fuligula*) and in tufted ducks experimentally infected with a clade 2.3.2 virus. Since neurotropism is a key feature of HPAIV-H5N1 infection, the encephalitis in 9 wild bird species from the Swedish outbreak was characterized in more detail. Results were compared to mallards infected with a low pathogenic avian influenza virus H1N1. The studies highlight the range and variation of the presentation of the natural disease in wild birds. Experimentally infected ducks were highly susceptible to the current HPAIV-H5N1 clade and showed similar lesions and viral antigen distribution as the naturally infected ducks. The studies suggest that there are several routes of infection and dissemination of the virus including, respiratory, hematogenous and olfactory routes. The respiratory tract is probably the main route of excretion of HPAIV-H5N1 since no viral antigen was found in the intestine. This was in contrast to the experimentally infected mallards which had primarily intestinal replication with minimal lesions. The results highlight the importance of continued investigation of the pathobiology of both low- and HPAIV infections in wild birds which is essential in the understanding of their epidemiology and, in turn, can contribute to the design and implementation of preventive and control measures to protect the health of humans and animals.

Keywords: Avian influenza, encephalitis, H1N1, H5N1, HPAI, LPAI, mallard, pathology, tufted duck, wild birds

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Dedication

To Johan, Rebecka, and Kristoffer

Discovery is seeing what everyone else has seen and thinking what no one else has thought.

Albert Szent-Gyorgi

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Bröjer, Caroline, E. O. Ågren, H. Uhlhorn, K. Bernodt, T. Mörner, D. S. Jansson, R. Mattsson, S. Zohari, P. Thorén, M. Berg, D. Gavier-Widén (2009). Pathology of natural highly pathogenic avian influenza H5N1 infection in wild tufted ducks (Aythya fuligula). *Journal of Veterinary Diagnostic Investigation*, 21, 579-587.

II Bröjer, Caroline, E. O. Ågren, H. Uhlhorn, K. Bernodt, D. S. Jansson, D. Gavier-Widén (2012). Characterization of encephalitis in wild birds naturally infected by Highly Pathogenic Avian Influenza (HPAI)-H5N1. *Avian Diseases* 56(1), 144-152.

III Bröjer, Caroline, G. van Amerongen, M. van de Bildt, P. van Run, A. D.M.E. Osterhaus, D. Gavier-Widén, T. Kuiken. Pathogenicity and tissue tropism of currently circulating HPAI-H5N1 clade 2.3.2 virus in experimentally infected tufted ducks (Aythya fuligula). (Manuscript).

IV Bröjer, Caroline[*], Josef D. Järhult[*], S. Muradrasoli, H. Söderström, B. Olsen, D. Gavier-Widén. Pathobiology and virus shedding of low-pathogenic avian influenza virus (A/H1N1) infection in mallards exposed to oseltamivir. (Submitted manuscript).

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Abbreviations

AEC	Aminoethylcarbazole
BSA	Bovine serum albumin
CT	Cycle threshold
dpi	Days post inoculation/infection
EID	Embryo infectious dose
GFAP	Glial fibrillary acidic protein
HA	Haemagglutinin
H&E	Hematoxylin and eosin stain
HIER	Heat induced epitope retrieval
HPAI	Highly pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus(es)
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
LPAI	Low pathogenic avian influenza
MDCK	Mardin-Darby canine kidney
NA	Neuraminidase
NP	Nucleoprotein
OC	Oseltamivir carboxylate
PELS	Periellipsoidal lymphoid sheaths
PLA	Proximity ligation assay
RT-PCR	Reverse transcriptase polymerase chain reaction
rRT-PCR	Real-time reverse transcriptase polymerase chain reaction
TCID	Tissue culture infectious dose

1 Introduction

1.1 Avian influenza virus

Avian influenza viruses are negative-sense single-stranded segmented RNA viruses that belong to the family *Orthomyxoviridae*. Influenza viruses are classified into the genera A, B, or C based on antigenic differences of the nucleocapsid and matrix proteins. Avian influenza viruses belong to type A and apart from domestic and wild birds, Influenza A can infect a range of species including humans, pigs, horses, mink, and marine mammals (Webster *et al.*, 1992). The influenza viruses are further classified into subtypes based on the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). Based on the antigenicity of these proteins, 16 subtypes of HA (H1-H16) and nine subtypes of NA (N1-N9) have been identified among influenza A viruses (Fouchier *et al.*, 2005). The different virus subtypes can include similar but distinct strains (or clades) based on genetic sequences and the clustering of the isolates (FAO, 2007). The strains are created by genetic mutations or via reassortment of genetic material between different viruses infecting a common host.

According to their ability to cause disease and death in chickens (*Gallus domesticus*), the influenza A viruses can further be classified into two groups: highly pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses. The viruses are classified as highly pathogenic if they produce 75% or greater mortality in intravenously inoculated chickens, have a chicken intravenous pathogenicity index of 1.2 or greater, or if they are H5 or H7 avian influenza viruses having an HA cleavage site with a polybasic amino acid sequence similar to other HPAI viruses (HPAIV) (OIE, 2009). Low pathogenic avian influenza viruses (LAPIV) are maintained in wild bird reservoirs especially aquatic birds in the orders Anseriformes and Charadriiformes and typically do not cause disease in these species (Olsen *et al.*, 2006; Webster *et al.*, 1992). In gallinaceous birds infections with LPAIV

may go unnoticed or may result in mild disease including decreased activity, decrease in egg production, and mild respiratory signs. However, more severe disease can be noticed depending on the virus strain, age of the host, and presence of concomitant disease or environmental factors (Capua, 2001). In wild birds LPAIV infections are usually also sub-clinical but there is some evidence that they can negatively affect body weight (Latorre-Margalef *et al.*, 2009) and foraging and migratory performance (van Gils *et al.*, 2007). When LPAI viruses of the strains H5 and H7 from the natural hosts come in contact with- and infect poultry, they can adapt to the new host and subsequently reassort or mutate resulting in a HPAIV. The resulting, highly pathogenic forms may affect a wide range of species (Alexander, 2000). Before 2002 outbreaks with severe systemic disease and high mortality caused by HPAI viruses were almost exclusively reported among gallinaceous birds (Stallknecht *et al.*, 2007). This scenario changed in 2002 when HPAIV-H5N1 caused clinical disease and death among captive waterfowl and free-living wild birds in Hong Kong (Ellis *et al.*, 2004). Since the 2002 event, the number of outbreaks in which wild birds were involved increased. After an outbreak among wild bird species in Qinghai Lake in China in 2005, the HPAI-H5N1 viruses began spreading among wild birds and were disseminated to various geographical locations resulting in more extensive outbreaks in the wild bird population (Chen *et al.*, 2006; Chen *et al.*, 2005).

1.2 Influenza virus in wild birds

Much of the available information about HPAIV-H5N1 infection in wild bird species derives from experimental studies conducted in various groups of birds including gulls (Perkins & Swayne, 2002b), gallinaceous birds (Perkins & Swayne, 2001), ducks (Keawcharoen *et al.*, 2008; Kwon *et al.*, 2010; Brown *et al.*, 2006; Isoda *et al.*, 2006; Sturm-Ramirez *et al.*, 2004), swans and geese (Neufeld *et al.*, 2009; Brown *et al.*, 2008; Kalthoff *et al.*, 2008; Pasick *et al.*, 2007) passerines (Breithaupt *et al.*, 2011; Perkins *et al.*, 2003) and pigeons (Klopfleisch *et al.*, 2006; Perkins & Swayne, 2002a). There are not as many reports on natural disease in free ranging birds that include descriptions of the pathology. These include reports in magpies (*Pica pica*) (Kwon *et al.*, 2005), sparrows (Kou *et al.*, 2005) wild waterfowl, a grey heron (*Ardea cinerea*), and black-headed gull (*Larus ridibundus*) (Ellis *et al.*, 2004), bar-headed geese (*Anser indicus*), great black-headed gull (*Larus ichthyaetus*), and brown-headed gull (*Larus brunnicephalus*) (Liu *et al.*, 2005), large billed crows (*Corvus macrorhyncos*) (Tanimura *et al.*, 2006) and swans (Pálmai *et al.*, 2007; Teifke *et al.*, 2007).

The results indicate that there is a host-species dependant range of susceptibility. While some species develop widespread severe disease, with high viral replication, others develop asymptomatic and transient infections. Despite HPAIV-H5N1 having tropism for multiple tissues, the preferential targeting of tissues appears to be related to the host-species. In many species lesions and viral antigen are mainly located in the brain and pancreas and often parts of the respiratory tract. There is usually variable involvement of other organs, especially the liver, heart, and adrenal gland. On the other hand several of the studied species only develop mild infections with mild clinical signs but still shed viruses as detected in oropharyngeal and cloacal swabs.

Low-pathogenic avian influenza viruses in wild birds are usually asymptomatic and lesions are usually confined to the intestinal tract (Slemons & Easterday, 1978; Webster *et al.*, 1978) However, as mentioned above, it is important to study these viruses since they can constitute the basis for the development of HPAI viruses or become part of human-adapted strains with pandemic potential.

1.3 Outbreak of HPAIV-H5N1 in Sweden

In February 2006, HPAIV-H5N1 outbreaks occurred in wild-living birds in many European countries simultaneously. The first confirmed case in Northern Europe occurred in a mute swan (*Cygnus olor*) on the German island of Ruegen (Weber *et al.*, 2007) in the Baltic Sea, situated about 400 km south of the Swedish coast. Three weeks later, the first case of HPAIV-H5N1 infection was detected by the National Veterinary Institute (SVA) and confirmed by the Community Reference Laboratory (CRL; VLA Weybridge) in two tufted ducks (*Aythya fuligula*) from Oxelösund on the east coast of Sweden. During the following 8 weeks the infection spread along the coast northwards up to Stockholm and southwards down to the Blekinge archipelago (between latitudes 55^{o}and 60^{o}N), involving a coastal area of approximately 900 km. During this outbreak, infection was confirmed in avian species of the taxonomical orders: Anseriformes: [tufted duck (*Aythya fuligula*), scaup (*Aythya marila*), smew (*Mergus albellus*), goosander (*Mergus merganser*), mute swan, Canada goose (*Branta canadensis*)]; Charadriiformes (herring gull (*Larus argentatus*)); Falconiformes (common buzzard (*Buteo buteo*)), Strigiformes (European eagle owl (*Bubo bubo*)) and in a wild mink (*Mustela vison*) (Zohari *et al.*, 2008). In Sweden, as in Denmark (Bragstad *et al.*, 2007), tufted ducks accounted for the largest number of identified positive cases.

At the time that the work for this thesis was begun, the available information about HPAIV-H5N1 infection was more limited than it is today.

Relevant questions for the understanding of the biological behavior of this virus and its epidemiology in the natural disease in free ranging birds in Sweden, such as distribution of virus in organs/tissues, time-development and course of the disease, pathology, clinical signs and routes of viral shedding, and the variability of these features in relation to the avian species involved remained unanswered and were the focus of this thesis. In order to further understand the pathobiology of avian influenza viruses, the results obtained from the naturally infected birds were compared with results from two experimental infections (one with HPAIV-H5N1 and one with LPAIV-H1N1) where more parameters could be controlled for.

1.4 Recent HPAIV-H5N1 strains

As mentioned above, the H5N1 virus subtypes can include various clades. Different clades seem to differ in their pathogenicity in different species (Sakoda *et al.*, 2010). Currently, clade 2.3.2 is one of the most common clades isolated from wild birds infected with influenza H5N1 suggesting a wide dispersal of this new clade (Hu *et al.*, 2011; Kajihara *et al.*, 2011; Kim *et al.*, 2011; Reid *et al.*, 2011). The degree of pathogenicity in wild birds has not been extensively studied. Post-mortem examination of whooper swans (*Cygnus cygnus*) naturally infected with HPAI-H5N1 clade 2.3.2 in Japan (Okamatsu *et al.*, 2010; Ogawa *et al.*, 2009) showed that they had severe lesions similar to those found in mute and whooper swans infected with clade 2.2 in 2006 in Germany (Teifke *et al.*, 2007) and Hungary (Pálmai *et al.*, 2007) suggesting the clade 2.3.2 viruses are highly pathogenic. However, in 2010 HPAIV-H5N1 2.3.2 was found in fecal samples from apparently healthy migratory ducks in an area of Japan where there had not been any signs of influenza before or after sampling. Subsequently the same strains of H5N1 were isolated from domestic and wild birds in different locations in Japan and these isolates were almost identical to the strains isolated from dead whooper swans in 2009 and 2010 in Mongolia (Kajihara *et al.*, 2011). Similarly clade 2.3.2 was isolated from a healthy mallard captured during routine screening in South Korea in 2010 (Kim *et al.*, 2011).

The pathogenicity of this clade has been studied in experimental infections but no pathology studies have been done. In one study a clade 2.3.2 was highly pathogenic to Muscovy ducks, Japanese quail, and mice when inoculated intranasally (Sun *et al.*, 2011). A study in which chickens, pigs, and domestic ducks were inoculated with either a 2009 clade 2.3.2 virus or a 2005/2006 clade 2.2. virus showed that both viruses were highly pathogenic for the chickens, the clade 2.3.2 virus resulted in more severe neurologic signs and

higher virus titers than the 2.2 viruses in the ducks, and the pigs showed no clinical signs with either virus but excreted virus (Sakoda *et al.*, 2010). In contrast, another study in which chickens, domestic ducks, mice, and ferrets were infected with either a 2011 clade 2.3.2 virus or a 2006 clade 2.2 virus showed that the clade 2.3.2 clade was either less pathogenic (ducks and ferrets) or had the same pathogenicity (chickens and mice) than the clade 2.2 viruses. These conflicting findings have led to concern that this clade is less pathogenic than the 2.2 clade and can therefore be maintained in the wild bird population.

2 Aims of the thesis

The aim of the thesis was to obtain more information on the pathobiology of avian influenza viruses by comparing the pathology of HPAI-H5N1 virus in naturally infected wild birds in Sweden to the pathology of HPAI-H5N1 virus in experimentally infected tufted ducks and to the pathology of LPAI-H1N1 virus in experimentally infected mallards. The specific aims were:

- To describe the type and distribution of lesions in various wild bird species naturally infected with HPAI-H5N1 virus (paper **I** and **II**).
- To determine the distribution of virus in organs, tissues and cells in order to assess viral tropism and viral involvement in the development of lesions (paper **I** and **II**).
- To evaluate the neurotropic nature of HPAI-H5N1 virus by describing and comparing the meningoencephalitis seen in different wild bird species naturally infected with HPAI-H5N1 virus (paper **II**).
- To assess the pathogenicity of a currently circulating HPAI-H5N1 virus in experimentally infected tufted ducks and compare it to naturally infected tufted ducks (paper **III**).
- To describe the distribution of viral antigen and possible lesions in mallards infected with low pathogenic avian influenza and compare these to the HPAI virus infections (paper **IV**).

3 Materials and Methods

3.1 Study animals

3.1.1 Wild birds

During the 2006 outbreak of HPAI in Sweden 502 dead wild birds were sent in to the National Veterinary Institute (SVA) for post-mortem examination and PCR- screening of cloacal and /or tracheal swabs for influenza virus. Out of the 502 birds, 62 tested positive for HPAI viral RNA. The majority (37) of positive birds were tufted ducks. Therefore this species was selected for more in depth histopathological studies. For paper **I**, 20 tufted ducks were selected based on low degree of autolysis, detection of HPAI nucleic acid by reverse transcriptase polymerase chain reaction (RT-PCR) in at least one sample (swab or tissue) and availability of brain and at least one other organ for histopathological investigation. Thirteen ducks were found dead and 7 ducks were euthanized due to severe neurologic signs including head tilt, circling, loss of balance and drooping wings.

Out of the 62 positive birds 42 birds comprising 8 species were included in paper **II**. The selected birds were 19 tufted ducks (*Aythya fuligula*), four scaups (*Aythya marila*), one smew (*Mergus albellus*), five goosanders (*Mergus merganser*), six swans [five mute swans (*Cygnus olor*) and one swan whose species was not recorded but was probably also *Cygnus olor*], two Canada geese (*Branta Canadensis*), one herring gull (*Larus argentatus*), one common buzzard (*Buteo buteo*) and three European eagle owls (*Bubo bubo*). Inclusion criteria for the study were low degree of autolysis, detection of HPAI nucleic acid by RT-PCR in at least one sample (swab or tissue) and availability of brain for histopathological investigation.

3.1.2 Tufted ducks

For the study in paper **III**, eight captive-bred tufted ducks (seven males and one female), 5 months old were used. Tufted ducks were chosen because they are a migratory species that span Asia, Africa and Europe, because of their known susceptibility to HPAIV-H5N1, and because their calm nature makes them a suitable species to keep in captivity under experimental conditions.

3.1.3 Mallards

Fifty-four male, captive-bred Mallard ducks, three to six months old, were used in study **IV**. Mallards were chosen because they are considered to be one of the natural carrier species of avian influenza viruses. They are also both an abundant free-living wild migratory species as well as a commonly bred species in game farms. Thus mallards often come in contact both with other wild birds, poultry, and humans and can potentially contribute to viral transmission and dissemination.

3.2 Experimental design

3.2.1 Natural HPAIV-H5N1 infection

The studies in paper **I** and **II** were based on the investigations carried out on the dead wild birds submitted to SVA during the Swedish HPAIV-H5N1 outbreak. Concurrent with tracheal and cloacal swabbing for detection of influenza virus with PCR, routine post-mortem examinations were performed on all birds and selected tissue samples were stored fresh and/or fixed in 10% neutral buffered formalin for histopathology and immunohistochemistry. The number of tissue samples varied depending on the condition of the bird (degree of autolysis and amount of scavenging).

The study in paper **I** describes the pathology and viral tissue targeting of H5N1 and highlights the range and variation in the presentation of the natural disease in tufted ducks. The tissues available for histopathology from the selected ducks included brain (n=20), lungs (n=17), upper respiratory tract (n=17), air sacs (n=7), heart (n=14), liver (n=17), spleen (n=14), kidneys (n=15), pancreas (n=14), adrenal glands (n=10), intestines (n=14), gonads (n=10), gizzard, and proventriculus (n=9). These tissues were analyzed with routine hematoxylin and eosin (H&E) stain to detect histopathological changes and with immunohistochemistry targeting the nucleoprotein (NP) of avian influenza A to detect viral antigen. The intensity and extension of the lesions and immunostaining were assessed semi-quantitatively (negative, mild, moderate or marked).

In paper **II** encephalitis, the most common lesion in the naturally HPAIV-H5N1 infected birds, was described and compared between and within bird species. Several brain sections were available from each case. However, the sections were not all trimmed in a standardized way at the time of processing for histological evaluation, which resulted in a variation in the number of brain sections per bird. The phenotype and distribution of inflammatory cells in relation to lesions and presence of viral antigen in the brain was assessed using immunohistochemistry to visualize avian influenza A viral antigen, (anti influenza-A NP), T-cells (anti CD3), B-cells (anti CD79a), activated macrophages/microglia (Lectin RCA-1), and astrocytes (anti GFAP). A semi-quantitative scoring system (negative, mild, moderate and marked) was used to evaluate the intensity and extension of different features. The features scored were: total area of inflammation, individual inflammatory components (focal gliosis, diffuse gliosis, and perivascular cuffing), viral antigen, neuronal changes (degeneration, necrosis, and neuronophagia), and vascular changes. The relative abundance of each inflammatory cell phenotype was similarly scored. The distribution of viral antigen, gliosis, and inflammatory cells (diffuse and in perivascular cuffs) as well as the severity of the inflammatory response were mapped on schematic diagrams of coronal and sagittal brain sections.

3.2.2 Experimental HPAI-H5N1 infection

The study in paper **III** was carried out to assess the pathogenicity of a currently circulating HPAI-H5N1 virus, belonging to clade 2.3.2, in experimentally infected tufted ducks and to compare it to the tufted ducks naturally infected with a clade 2.2 virus. Four tufted ducks were inoculated with influenza virus $1X10^4$ mean tissue culture infectious dose ($TCID^{50}$) A/duck/Hong Kong/1091/2011 (clade 2.3.2), 1.5 ml intraesophageally and 1.5 ml intratracheally. Another four ducks were sham inoculated in the same way with sterile PBS and served as a control group. The birds were monitored for clinical signs and virus excretion until the day of euthanasia. The ducks were euthanized 4 days post inoculation (dpi) except one of the infected ducks that was euthanized at 3 dpi due to severe neurologic signs. After euthanasia, the ducks were necropsied and several tissue samples were taken for histology, immunohistochemistry (anti-NP of influenza A) and virology (RT-PCR and culture).

3.2.3 Experimental LPAIV-H1N1 infection

The study in paper **IV** was carried out to obtain more knowledge on the pathobiology of LPAIV in one of the viruses' natural hosts, the mallard. The

study was part of an investigation in which mallards were infected with LPAIV-H1N1 and were exposed to three low levels of the active metabolite of oseltamivir (Tamiflu®), oseltamivir carboxylate (OC), in their pool water to see if the virus would develop the viral resistance mutation H274Y during the course of the experiment.
For two of the OC concentrations (80 ng/l and 1µg/l) six ducks were inoculated intraesophageally with 1 ml of viral stock solution (10^8EID50) of influenza A/Mallard/Sweden/51833/2006 (H1N1). Four ducks were euthanized, one per day, on day 1, 2, 3, and 4 post-inoculation. To achieve transmission between ducks, two uninfected ducks were introduced to the experiment room three days after the inoculation. At 5 dpi the remaining two artificially inoculated infected ducks were euthanized. Two new ducks were introduced on day 6. New ducks were subsequently introduced every third day and kept together for two days before the preceding generation was removed and euthanized. A total of ten duck generations lasting five days each were used. For the 80µg/l experiment, two ducks were similarly inoculated intraesophageally. At 3 dpi two new ducks were introduced for contact infection. The ducks were euthanized at 7 dpi, necropsied and several tissue samples were taken for histology, immunohistochemistry (anti-NP of influenza A) and virology (RT-PCR and culture).

3.3 Laboratory diagnostics

3.3.1 Histopathology and immunohistochemistry

Histopathology and immunohistochemistry were key diagnostic methods in this thesis and were used in all the studies. After formalin fixation, tissue samples were processed routinely, sectioned at 4-5 µm and stained with hematoxylin and eosin (HE). Lesions were described and their degree and extension were scored semi-quantitatively (negative, mild, moderate or marked). Duplicate sections were used for immunohistochemistry (IHC) to visualize influenza-A viral antigen (papers **I-IV)** as well as T-cells, B-cells, astrocytes, and microglia/macrophages (paper **II**). The same IHC method was used for detection of influenza A in papers **I**, **II**, and **IV** but a modified method was used for paper **III**.

For papers **I**, **II**, and **IV** tissue sections were mounted on Vectabond (Vector Laboratories, Inc, Burlingame, CA) treated glass slides, deparaffinised in xylene and rehydrated. Sections were then immunostained with markers as specified in Table 1 to visualize influenza-A viral antigen, T-cells, B-cells, astrocytes, and microglia/macrophages. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min for all markers except for anti-

influenza A-NP (AI-HB65), which was blocked for 7 min. Antigen retrieval was accomplished by treating the sections with proteinase K (DakoCytomation, Glostrup, Denmark) for 6 min (AI-HB65 and GFAP) or heat induced epitope retrieval (HIER) (CD3 and CD79a). No antigen retrieval was necessary for Lectin RCA-1. Unspecific antigen staining was blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich, Sweden AB) for 20 min. Sections were then incubated at room temperature with the specific markers at dilutions as listed in Table 1 for 45 min except for AI-HB65, which was incubated for 60 min. The detection was conducted with the dextran polymer method (DAKO EnVisionTM+/HRP, DakoCytomation, Glostrup, Denmark) except for AI-HB65 which was detected with labeled streptavidin-biotin (LSAB, DAKO 0690, DakoCytomation, Glostrup, Denmark). The color was developed with aminoethylcarbazole (AEC) substrate (Vector Laboratories, Inc, Burlingame, CA). Sections were counterstained with haematoxylin.

Table 1. *Details of immunolabeling reagents used in paper II*

Primary antibody	**Producer, Product Nr.**	**Antigen or cell detected**	**Antibody dilution**	**Pretreatment**
Mouse anti-influenza A-NP, HB65	EVL, The Netherlands HB65-2007	influenza A NP[a]	1:200	Proteinase K
Polyclonal rabbit Anti-human CD3	DAKO, A00452	T-cells	1:20	HIER[b]
Monoclonal rabbit Anti-human CD79a	Thermo Scientific, RM-9118	B-cells	1:20	HIER
Polyclonal rabbit Anti-GFAP	DAKO, Z0334	Astrocytes	1:400	Proteinase K
Lectin RCA-1 Biotinylated	Vector Labs, B-1085	Microglia/ macrophages	1:750	None

[a]NP: nucleoprotein; [b]HIER: Heat induced epitope retrieval

For paper **III** slides were first incubated in 0.1% protease (P-5147, Sigma, St Louis, Missouri, USA) for 10 min at 37ºC. Endogenous peroxidase was blocked with 3% H2O2 for 10 min at room temperature. The slides were then incubated at room temperature for 60 min with the same antibody as in papers **I**, **II,** and **IV** (anti-Influenza A NP, Clone Hb65) diluted 1:400. Detection was conducted by incubating the slides at room temperature for 60 min with goat-anti-mouse IgG2a horse radish peroxidase (HRP) (Southern Biotech, Birmingham, Alabama, USA) diluted 1:400. The color was developed with AEC (Sigma Chemicals, Zwinjndrecht, The Netherlands). Sections were counterstained with hematoxylin. For all IHC methods appropriate positive and

negative control sections were included in each run. Presence of viral antigen was assessed semi-quantitatively (0: no antigen, 1: few positive cells, 2: moderate number of positive cells, and 3: many positive cells).

Immunohistochemistry is a good method for detecting the presence of antigen and to identify specific cell types. However, there are some limitations. For example, the antibody used to detect Influenza A targets the nucleoprotein, which is primarily expressed in replicating virus. Thus all antigen present may not be detected by this antibody. Furthermore, using immunohistochemical markers known to have good staining properties in some species may not work in other species. This was the case for antibodies used for phenotyping inflammatory cells in paper **II**. Several markers that are commonly used to stain tissues from humans and laboratory rodents have been used successfully in gallinaceous birds but many did not work on the wild birds in this study. For example, the Mab BLA-36 antibody that has been successfully used to stain B-cells in chicken brains (Kommers, 2002) did not stain lymphocytes in control spleens from mallards, tufted ducks and swans in the present study (data not shown). The marker that worked best was CD79a, but when tested on mallard spleens, variable staining was obtained in germinal centers and periellipsoidal lymphoid sheaths (PELS). Therefore, our technique to identify B-cells may have been sub-optimal and underestimated the numbers of B-cells present. Similarly, several cell markers for microglia and macrophages, including CD68, MAC387, and Lysozyme, were tested before finding the Lectin RCA-1 antibody that had good staining properties in the studied bird species.

3.3.2 Polymerase chain reaction (PCR)

Extraction of RNA from cloacal and /or tracheal swabs collected from dead wild birds (paper **I** and **II**) as well as from samples collected from mallards in paper **IV** was carried out by using the Virus Mini extraction kit (Qiagen, Hilden, Germany) in a Magnatrix 8000+ (NorDiag, Bergen, Norway) extraction robot. The RNA was then screened for the presence the matrix gene of avian influenza viruses by real-time reverse transcriptase PCR /rRT-PCR) (Spackman *et al.*, 2003). All positive cases were further examined with an H5 specific conventional RT-PCR (KHA, HA-gene) (Slomka *et al.*, 2007). Both PCR assays were performed according to the recommendations from the Community Reference Laboratory (CRL; VLA Weybridge). The positive H5 PCR products, covering part of the hemagglutinin gene including the H0 cleavage site, were sequenced and the cleavage site was analyzed in order to determine the pathogenicity of the viruses.

For the study in paper **III** RNA was isolated from swab and tissue suspensions using a MagnaPure LC system with the MagnaPure Total nucleic

acid isolation kit (Roche Diagnostics, Almere, The Netherlands). An ABI 7500 sequence Detection System with the TaqMan EZ RT-PCR Core Reagents kit (Applied BioSystems, Nieuwerkerk aan den Ijssel, The Netherlands) was then used to perform the real time RT-PCR analyses to detect the matrix gene of avian influenza virus.

3.3.3 Virus isolation

Since it is not possible to differentiate between presence of viable virus, defective/inactivated viruses or short segments of viruses using rRT-PCR, virus isolation was used to confirm the presence of viable virus. Virus isolation was performed on samples identified as positive for avian influenza virus by matrix rRT-PCR in studies **I** and **II** and in selected samples from study **IV**. Sample medium was inoculated into the allantoic cavity of nine to ten day-old specific-pathogen-free (SPF) embryonated hens' eggs. The allantoic fluid was harvested and centrifuged, and virus growth was confirmed by a standard hemagglutination test. The viral titer was determined by 50% Embryo Infectious Dose (EID50) (Reed & Muench, 1938). Virus isolation on samples from study **III** was carried out by inoculating tenfold serial dilutions of sample suspensions in Mardin-Darby canine kidney (MDCK) cells followed by titration as described by Rimmelzwaan (Rimmelzwaan *et al.*, 1998).

3.3.4 ELISA

In the experimental infections the tufted ducks (paper **III**) and mallards (paper **IV**) were checked for the presence of antibodies against avian influenza viruses before inclusion in the respective studies. Serum samples were tested for antibodies targeting avian influenza nucleoprotein (NP) using commercial influenza A virus antibody ELISA kits (European Veterinary Laboratory, Woerden, the Netherlands and Pourquier, France).

4 Results and discussion

The outbreak of HPAI-H5N1 that killed many wild birds in Qinghai Lake in China in 2005 (Chen *et al.*, 2005) prompted an increase in research in the field of avian influenza and has resulted in numerous publications concerning the pathogenicity of the virus and the role of wild birds in the maintenance and spread of the virus. The results of this thesis make a further contribution to the understanding of the pathobiology of HPAI by describing the tropism of the virus and its associated lesions in naturally and experimentally infected wild bird species. The role of wild bird species in the spread of avian influenza viruses is further elucidated by comparing the pathogenicity of the HPAI viruses to that of a LPAIV in one of its natural hosts, the mallard.

4.1 Pathogenicity of HPAIV-H5N1 in wild bird species

4.1.1 Tufted ducks

The tufted ducks naturally infected with HPAIV-H5N1 (clade 2.2) had a range of mild to severe, acute to sub-acute inflammatory lesions with varying amounts of viral antigen that affected between one and six organs per bird, which confirmed the within-species variation of tissue tropism in HPAI-H5N1 infections (paper **II**). The main histological lesions associated with presence of avian influenza antigen were found in the brain, pancreas, and upper respiratory tract. This was in agreement with natural HPAIV-H5N1 infection in other wild bird species (Pálmai *et al.*, 2007; Teifke *et al.*, 2007; Tanimura *et al.*, 2006; Kwon *et al.*, 2005; Liu *et al.*, 2005; Ellis *et al.*, 2004) and with experimental infection of wild birds, including tufted ducks (Keawcharoen *et al.*, 2008). Other tissues in which influenza antigen was variably found included liver, lung, adrenal gland, kidney, and peripheral nerve ganglia.

Results from the experimental infection of tufted ducks with clade 2.3.2 HPAI-H5N1 (paper **III**) revealed that this clade was also highly pathogenic for

tufted ducks and had both similar antigen distribution and caused similar lesions as those found in tufted ducks naturally infected (paper **I**) and experimentally infected (Keawcharoen *et al.*, 2008) with a clade 2.2 virus. As for the natural infection, viral antigen was found primarily in the brain, followed by the respiratory tract and more seldom in other tissues. The variable localization of viral antigen in organs such as liver, kidneys, and adrenal glands in both the natural and experimental infections suggests that the virus has pantropic potential, but other factors seem to affect the distribution of virus and lesions in different organs. In the naturally infected birds there were many unknown variables that could affect the distribution of virus. These include age of the bird, route and dose of viral exposure, duration of infection as well as previous exposure to other influenza viruses which could modulate the effect of the HPAI-infection (Costa *et al.*, 2011; Kalthoff *et al.*, 2008). Interestingly, in the experimental setting the tufted ducks were of the same age, received the same viral dose via the same routes, were euthanized the same day, and did not have antibodies against influenza virus; nonetheless there was a variation in distribution with one duck having more widespread dissemination of the virus than the others. This type of individual variation is commonly reported for other species both naturally and experimentally infected with HAPIV-H5N1 (Brown *et al.*, 2008; Pantin-Jackwood & Swayne, 2007; Teifke *et al.*, 2007).

The respiratory tract probably plays an important role in the pathogenesis of the HPAI-H5N1 infection in tufted ducks, especially early in the course of infection. Virus was isolated from pharyngeal swabs of all of the experimentally infected ducks from 1dpi to 4 dpi (day of euthanasia) and most of the naturally infected birds were identified as infected based on presence of viral RNA in tracheal swabs suggesting viral excretion from the respiratory tract. Despite the presence of congestion and inflammatory changes in the lungs of many of the naturally infected tufted ducks, mild antigen staining was only found in the lungs of three out of 18 ducks and viral antigen was not found in the trachea or air sacs. Similarly in the experimentally infected ducks virus was not consistently isolated and/or identified with IHC in tissues from the respiratory tract. It is possible that many infected epithelial cells, especially from the air sacs are sloughed and therefore not seen on IHC but picked up with the pharyngeal swabs. Furthermore, the samples collected with pharyngeal swabs contain mucous secretions with trapped particles which have been transported to the pharynx by ciliated cells in the nasal cavity, trachea, primary bronchi and roots of the secondary bronchi (Fedde, 1998) so one could expect a higher concentration of virus in the swabs than in the individual tissue samples. Interestingly, presence of viral antigen with or without accompanying inflammation in nasal epithelium was a common finding in both

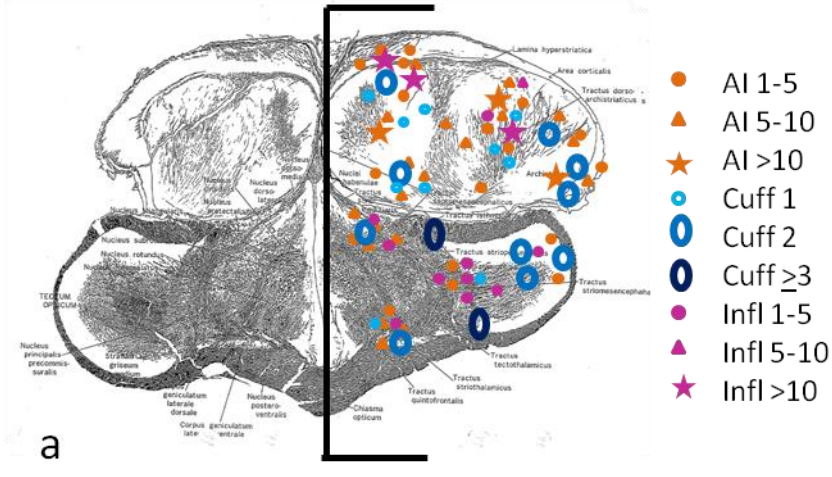
experimentally and naturally infected tufted ducks. It is thus possible that a significant amount of the virus detected in the tracheal swabs originated from the nasal cavity. The low number of antigen-positive lungs is in agreement with descriptions in naturally infected mute and whooper swans in which only vascular endothelium in the lung of 2 out of 18 swans contained viral antigen (Teifke *et al.*, 2007) as well as in magpies where only pulmonary endothelial cells were positive (Kwon *et al.*, 2005). Similarly, experimentally infected mallards, teals, and wigeons did not have any antigen in the respiratory tract whereas tufted ducks, pochards, and gadwalls had viral antigen in lungs and air sacs but not in trachea or extrapulmonary bronchus despite frequent isolation of HPAIV-H5N1 from all of these respiratory tissues (Keawcharoen *et al.*, 2008). The discrepancies between isolated virus from tissues and pharyngeal swabs (in experimentally infected ducks) compared to virus detected in tissues by IHC may also be due to differences in the susceptibility of individual ducks.

4.1.2 Encephalitis and neurotropism in wild birds

Although full necropsies were carried out for most wild birds submitted during the HPAI-H5N1 outbreak and all available collected organs from them were screened histologically, the brain was chosen for a more in depth study since meningoencephalitis was a key feature found in these birds. The lesions in the brain were characterized by non-suppurative encephalitis with prominent mononuclear perivascular cuffing, multifocal to diffuse areas of gliosis, and inflammatory cell infiltrates dominated by lymphocytes interspersed with macrophages primarily in the grey matter. Neuronal degeneration, neuronophagia, and in some cases, non-suppurative meningitis were also observed. The foci of microgliosis contained a mixed population of T-cells (CD3 positive) and activated microglia/macrophages (lectin RCA-1 positive). The perivascular cuffs were predominantly composed of T-cells but the thickest cuffs and cuffs close to areas of marked inflammation contained more macrophages. Few B-cells (CD79a positive) cells were detected in perivascular cuffs and some cells in the cuffs remained unstained. It is possible that there were more B-cells present since the CD79a proved not to be optimal in staining these cells in the wild birds investigated. The unstained cells could be other cells, such as natural killer (NK) cells or could be B-cells, T-cells or macrophages that were not detected by the antibodies used.

The severity of the inflammatory response (perivascular cuffs, diffuse and focal infiltration of lymphocytes, macrophages/activated microglia, as well as gliosis) and the viral distribution showed both intra- and interspecies variation among the naturally infected wild birds. Swans and Canada geese had the most severe inflammatory response followed by moderate inflammation in tufted

ducks, scaups, and eagle owls. The goosanders, the smew, and the buzzard generally had mild inflammation and the herring gull had no signs of inflammation. The largest amount of viral antigen was detected in swans, Canada geese, and eagle owls. In most of the birds microscopic lesions and viral antigen had a widespread distribution even though not all parts of the brain were affected in each individual. The pattern of inflammation and viral antigen distribution in the goosanders, smew, and herring gull differed somewhat compared to the other investigated birds. In the goosanders and the smew viral antigen was primarily found in the choroid plexus and in ependymal cells lining the ventricles and the inflammatory response was generally mild. Antigen was also found in the choroid plexus of a few other birds of other species but then it was accompanied by widespread inflammation in other parts of the brain (Figure 1). The herring gull had no detectable inflammatory response nor could any viral antigen be detected with IHC despite that virus was cultured from the brain.



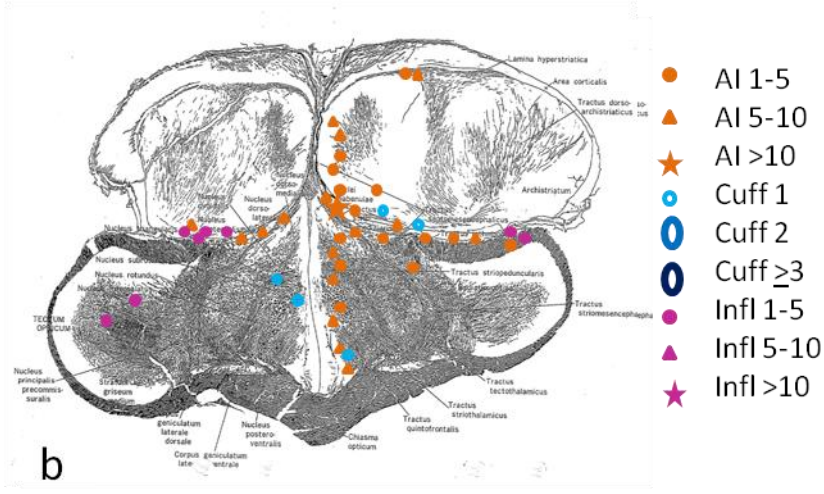


Figure 1. Schematic comparison of the distribution and quantity of viral antigen (orange), perivascular cuffs (blue), and inflammatory infiltrate (pink) in a swan (a) and a goosander (b) infected with HPAIV-H5N1. The antigen and inflammation primarily follows the ventricles and choroid plexus in the goosander whereas the distribution is more generalized in the swan. AI (n): approximate number of cells with viral antigen; Cuff 1-3: thin, medium, and thick cuff respectively; infl (n): approximate number of inflammatory cells. Diagram of brain: (Yoshikawa, 1967).

As noted above, meningoencephalitis was also the most prominent lesion in the tufted ducks experimentally infected with the 2.3.2 clade. As in the naturally infected tufted ducks these also had a widespread distribution of lesions that varied in severity and amounts of antigen. However, the experimentally infected ducks generally had even more antigen in the brain, especially in ependymal cells lining the ventricles (figure 2) and central canal of the spinal cord as well as in the epithelial cells of the choroid plexus. In one of the ducks the inflammatory response was also more diffuse and severe than in the naturally infected ducks.

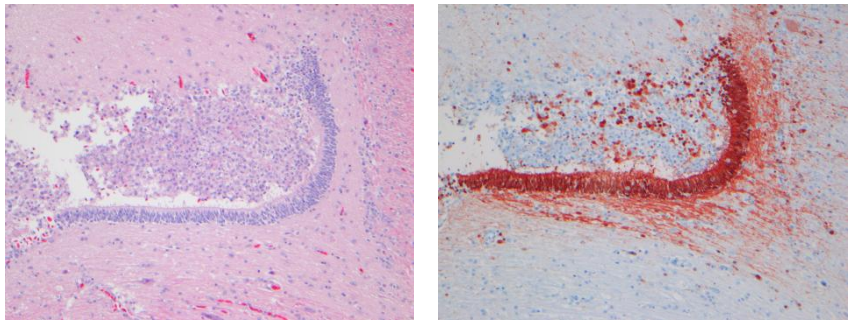


Figure 2. Aqueductus cerebri of a tufted duck experimentally infected with HPAIV-H5N1 influenza A/duck/Hong Kong/1091/2011 (clade 2.3.2). Large numbers of macrophages and epithelial cells are present in the ventricular space (a); stain: H&E. A large amount of viral antigen is present in ependymal cells and sloughed epithelial cells (b); stain: IHC-anti-influenza A-NP. Original magnification: 10X.

In addition to the brain, viral antigen was also found in other neural tissues in both the naturally and experimentally infected tufted ducks reflecting the neurotropic nature of the virus. Neural tissues in which viral antigen was detected included myenteric and submucosal (Meissner) plexa of the intestine, ganglion cells peripheral to the adrenal gland, and olfactory neurons. The affinity of the virus to neural tissue and the species variation in degree of encephalitis and general susceptibility to the virus has also been documented in other natural outbreaks among wild birds (Szeredi *et al.*, 2010; Teifke *et al.*, 2007; Tanimura *et al.*, 2006) as well as in experimental infections (Keawcharoen *et al.*, 2008; Breithaupt *et al.*, 2011; Kwon *et al.*, 2010; Neufeld *et al.*, 2009; Brown *et al.*, 2008; Pasick *et al.*, 2007; Brown *et al.*, 2006). Since many of the studied species were represented by only a few individuals, it is possible that some of the differences observed could be related to duration of the infection before death, individual variation in susceptibility or other factors such as age of the bird, route of exposure, viral dose, and environmental factors (Pantin-Jackwood & Swayne, 2007).

4.2 Routes of infection, dissemination, and shedding of HPAIV-H5N1

The natural route of infection in wild birds probably varies with species, environmental conditions, density of birds, and virus strain. Based on studies **II** and **III** as well as experimental studies on several duck species (Keawcharoen *et al.*, 2008) the oral route does not seem to be an important route of infection

of HPAI viruses in Anseriformes since viral antigen was not detected in intestinal epithelium despite deposition of virus in the esophagus in the experimental infections. This is in contrast to what is observed in Anseriformes infected with LPAI virus, in which most replication takes place in the intestinal epithelium (Webster *et al.*, 1978) suggesting that the oral-fecal route of infection plays the most important role. On the other hand, it cannot be excluded that HPAI-H5N1 virus can enter through the intestine to the myenteric and submucosal plexa. Furthermore, small amounts of infected food or water could enter the respiratory tract at the time of swallowing thus enabling infection. Raptors and scavenging birds, such as herring gulls, probably can get infected via the oral route by eating infected dead prey (Brown *et al.*, 2008) as well as through the nares when handling their prey.

The mechanisms of neuroinvasion and dissemination of the virus in the brain in wild birds have not been clearly elucidated but it is probable that the route of infection also affects the route of dissemination. Experimental studies on mice, ferrets, and chickens have suggested that influenza viruses can enter the central nervous system hematogenously (Chaves *et al.*, 2011; Swayne, 2007), via peripheral nerves (Shinya *et al.*, 2011; Jang *et al.*, 2009; Matsuda *et al.*, 2004; Tanaka *et al.*, 2003; Park *et al.*, 2002) and via the olfactory route (Schrauwen *et al.*, 2012; Bodewes *et al.*, 2011; Park *et al.*, 2002). The variation in available brain sections per bird in study **II** made it difficult to compare exact location and extent of lesions between the cases but trends in distribution patterns were observed. Study **II** and **III** suggest several possible routes of viral spread to and within the brain.

First, multifocal distribution of the virus throughout many parts of the brain in most species, suggests that the virus spreads hematogenously. In general, hematogenous invasion of the brain can occur either via infection of endothelial cells or by crossing the blood- brain barrier. Endotheliotropism is often reported in gallinaceous species (Swayne, 2007). Although endotheliotropism has not been reported as a feature in most Anseriformes (Kuiken *et al.*, 2010) and raptors (Hall *et al.*, 2009), it has been reported from experimental infections of some waterfowl such as black swans (Brown *et al.*, 2008), mute swans (Kwon *et al.*, 2010; Kalthoff *et al.*, 2008), and wood ducks (Brown *et al.*, 2006) as well as in laughing gulls (Brown *et al.*, 2006). Viral antigen was seen in endothelial cells of the lung, spleen, bone marrow, and Peyer's patches but not in the brain of naturally infected mute and whooper swans (Teifke *et al.*, 2007). Endotheliotropism was not observed in the brain in any of the birds in studies **I** and **II**, including the mute swans, nor in the experimentally infected tufted ducks suggesting that the virus was not disseminated into the brain via endothelial cells. Thus the virus could instead

have infected the brain by disrupting the blood-brain barrier or entering the brain via fenestrated endothelia in the choroid plexus or circumventricular organs (Chaves *et al.*, 2011; Duvernoy & Risold, 2007). Antigen distribution observed in the choroid plexus and in ependymal cells along the ventricles of several of the naturally infected species as well as in the experimentally infected tufted ducks supports the latter route of entry and suggests that the virus perhaps also can spread via the cerebrospinal fluid.

Secondly, presence of virus in peripheral autonomic ganglia was found in studies **I** and **III** and has been described in naturally infected wild birds (Szeredi *et al.*, 2010). This supports the possibility of a neural pathway as described in mouse models (Shinya *et al.*, 2011; Jang *et al.*, 2009; Matsuda *et al.*, 2004; Tanaka *et al.*, 2003; Park *et al.*, 2002) but the routes of viral trafficking are still unclear. Although parts of the peripheral nervous system were included in the studies of this thesis, it was not extensively sampled and should be looked at in future studies. Lastly, viral antigen was occasionally present in the olfactory bulb in tufted ducks in both study **II** and study **III** as well as in olfactory mucosa including olfactory neurons in study **I** and **III** suggesting that the virus can enter the brain and perhaps spread through the olfactory route.

Virus shedding via the oropharynx is consistently reported from birds infected with HPAIV-H5N1. However, cloacal shedding seems to be variable both in naturally and experimentally infected birds. Cloacal shedding may be affected by the amount of viral particles trapped in mucous secretions in the respiratory tract, moved to the pharynx by ciliated epithelium, swallowed and then shed with feces (Fedde, 1998). The degree of infection of organs such as liver and pancreas may also affect cloacal shedding since these organs are probable sources of virus in the feces (Keawcharoen *et al.*, 2008).

A major concern in several reports on mortality events involving wild birds infected with HPAIV-H5N1 is whether they can act as reservoirs for the virus and contribute to the spreading of the virus not only to other wild birds but also to poultry and secondarily to people. The high pathogenicity and acute nature of the HPAI-H5N1 viruses observed in the naturally and experimentally infected tufted ducks studied in the scope of this thesis suggests that they probably cannot serve as long distance vectors of these viruses and can serve as an indicator species. However, the seemingly varied routes of infection and shedding suggests that when they become infected they can help perpetuate the disease in areas with high densities of birds where they are likely to have direct contact with each other and thus can spread the virus. It is more difficult to predict the role of the other wild birds studied in this thesis, since only few individuals from each species were studied, all organs were not analyzed in

detail, and the duration of infection before death was not known so the degree of pathogenicity could not be determined.

4.3 Pathogenicity of LPAIV-H1N1 in a natural host

One purpose of the LPAIV study in mallards (study **IV**) was to determine whether influenza A/H1N1 virus in mallards develops drug resistance when exposed to different concentrations (80ng/l, 1µg/l and 80µg/l) of the active substance of oseltamivir, oseltamivir carboxylate (OC). The results of that part of the study are published elsewhere (Jarhult *et al.*, 2011). Briefly, the study showed that at the 80ng/l concentration no drug resistance was detected, at the 1µg/l concentration two out of 127 samples (eight and 23 days after the start of the experiment) contained a mixture of wild type genotype and strains carrying the resistance mutation H274Y in the NA gene. At the 80µg/l concentration, only the H274Y genotype was detected from three days post-inoculation of the first ducks and during the rest of the experiment. A second purpose, described in study **IV**, was to use histopathology and immunohistochemistry (IHC) in combination with PCR to evaluate the localization and dynamics of the viral replication and to determine whether it causes any lesions.

Influenza infection, replication and transmission were successful in the mallard model as shown by q-PCR from fecal and intestinal samples and positive IHC. The LPAI virus infection was localized to the intestinal tract and cloacal bursa except in one mallard whose infection was located solely in the lung. Viral antigen was mostly found in epithelial cells located near the tip of the intestinal villi and in scattered mononuclear cells in the lamina propria but not in epithelial cells of the intestinal crypts. Viral antigen was occasionally found in the surface epithelium of Meckel´s diverticulum. More proximal segments of the intestine were IHC positive in early samples suggesting a "proximal to distal" progression of the LPAI virus infection. This was further supported by the q-PCR analysis from intestinal contents where a majority of the viral load was found in proximal parts of the intestine at 1 and 2 dpi. There was no histologic evidence of damage to intestinal epithelial cells. However, in the jejunum of one case (2 dpi) and the ileum of another case (2 dpi) there were mononuclear cells in different stages of degeneration as well as mild infiltration of heterophils in the lamina propria. Immunohistochemistry of the same area showed many cells with viral antigen in the nucleus as well as diffuse positively staining granular material corresponding to the degenerating cells. Most ducks also had at least one segment of intestine with mild infiltration of heterophils and ten of them had moderate to marked infiltration of heterophils amongst epithelial cells and in the lamina propria of at least one

intestinal segment. However, the heterophil infiltration was not always associated with presence of viral antigen. Therefore, a clear association between intensity of heterophil infiltration and presence of viral antigen could not be established.

These results are in accordance with previous studies in which the intestine and cloacal bursa have been shown to be the main sites of replication for LPAI viruses in dabbling ducks (Daoust *et al.*, 2011; Kida *et al.*, 1980; Slemons & Easterday, 1978; Webster *et al.*, 1978). The primarily intestinal infection is in contrast to what is observed in HPAIV-H5N1 infections (as described above) where the intestinal epithelium does not seem to be involved in the replication cycle of the virus in naturally or experimentally infected birds despite intraesophageal deposition of virus in the experimentally infected birds. In previous studies on mallards and Pekin ducks experimentally infected with LPAIV (Ito *et al.*, 2000; Kida *et al.*, 1980; Slemons & Easterday, 1978) as well as on naturally infected mallards (Daoust *et al.*, 2011) no sign of inflammation or cell injury was detected despite the presence of viral antigen. In contrast, it is interesting to note that in this study (**IV**) there was co-localization of degenerating cells and viral antigen in some birds and that there seemed to be more heterophils in the intestines of birds with more viral antigen as detected with IHC. However, since one of the control birds also had heterophil infiltration, it would be necessary to conduct additional studies with more animals per group in order to verify the significance of these findings.

One duck had bronchointerstitial pneumonia with focally extensive infiltration of heterophils. Viral antigen was found in close association to the inflammation in the respiratory epithelium of secondary bronchi, the epithelium covering the parabronchial septa, and in epithelial cells of air capillaries. The pneumonia was similar to that found by Cooley (Cooley *et al.*, 1989) in mallards inoculated intratracheally with five different strains of LPAIV. However, contrary to the present study, using IHC Cooley found scattered viral antigen only in epithelial cells lining airways and no staining within lung parenchyma. Although no adverse reaction was noted in the duck at the time of inoculation, it is possible that it regurgitated some of the inoculum which then flowed into the respiratory tract or that the inoculum was accidentally deposited in the trachea. This theory is supported by the fact that pneumonia in conjunction with presence of viral antigen in the lung has, to our knowledge, not previously been reported in mallards with intraesophageal/oral inoculation. Even if the inoculum reached the respiratory tract by mistake, it is interesting to note the marked inflammatory response accompanied by the large number of IHC positive cells after only 1 dpi. In contrast, Slemons and Easterday (Slemons & Easterday, 1978) were able to isolate virus from lung

tissue 1 and 2 dpi but did not find viral antigen in the lungs using fluorescence in aerosol infected Pekin ducks. Similarly, Daoust et al. (Daoust *et al.*, 2011) found positive lung tissue using q-PCR but could not detect positive cells with IHC in naturally infected mallards. In our study the q-PCR analysis on fecal material from this mallard was negative but interestingly the sample from the ventriculus was positive (CT-value 26) indicating that at least some virus reached the gastro-intestinal tract either at the time of inoculation or by swallowing respiratory exudates. All other portions of intestinal content were PCR negative consistent with the negative IHC results. No tracheal or oropharyngeal swab was taken so excretion from the respiratory tract could not be evaluated.

Viral shedding as detected with qPCR of daily fecal samples varied between birds and over time. Most ducks began shedding virus at 1 dpi and continued shedding some virus until the day of euthanasia with a peak at 2 dpi. The shedding patterns were similar when comparing infection with strains carrying the H274Y resistance mutation to wild type strains and when comparing mallards exposed to 80 ng/l to 80 µg/l of OC. The cloacal shedding of virus is in contrast to what is seen with HPAIV infections where cloacal shedding is variable. Apart from the difference in replication sights, a possible explanation for the variable cloacal excretion in the HPAIV infected birds could be the virus dose; the tufted ducks were inoculated with 10^4 $TCID_{50}$ whereas the mallards received 10^8 TCD_{50}. Unfortunately oropharyngeal swabs were not taken from the experimentally infected mallards so that shedding pattern cannot be compared to the HPAIV-infected birds.

Although many wild birds probably are not good long distance vectors of HPAI viruses because the infection results in acute mortality, mallards may play an important role in spreading the virus. Except for one dead mallard in Germany and one dead mallard in Sweden (which was not necropsied), there are, to the best of our knowledge, no reports of dead mallards during the HPAIV-H5N1 outbreak in Europe in 2006. Furthermore, HPAI-H5N1 has been isolated from fecal samples from apparently healthy mallards. Additionally, experimental work on different duck species suggest that mallards can shed HPAI-H5N1 virus without showing clinical signs (Keawcharoen 2008). This information and the fact that mallards are efficient spreaders of LPAI viruses, emphasize the need for constant surveillance of the wild bird population.

4.4 Diagnostic challenges and limitations of the studies

During the course of the studies included in this thesis several diagnostic challenges and queries were encountered. One of the first things noted when

analyzing samples from the birds collected during the Swedish HPAIV-H5N1 outbreak was that birds that histologically seemed to be infected, not always had positive PCR-results on swabs collected from them. Out of 45 HPAI-PCR-positive birds that were also analyzed histologically (not all were analyzed since some were in bad condition), 10 were PCR-negative in tracheal swabs. However, they had histopathologic changes consistent with HPAI-infection including encephalitis and in some cases pancreatitis. They were re-tested by PCR on brain and lung tissue, and 5 of them were positive. Thus PCR failed to detect at least 11% of positive cases. When tracheal swabs were substituted by oropharyngeal swabs, there was better agreement between PCR-negative swabs and IHC-negative tissues.

Another diagnostic challenge arose when trying to adapt IHC methods to species in which species-specific antigens were not available. This was encountered when trying to stain different inflammatory components in the brain of various wild bird species. Many antibodies had to be tried before adequate and specific staining was obtained for B-cells, macrophages/activated microglia, and GFAP. A specific antibody that would stain macrophages of the investigated birds was not found. Therefore, the lectin RCA-1, which is an unspecific marker of macrophages and activated microglia, was used. Other things that had to be taken into account were that CD3 antibodies used to identify T-cells, also stained Purkinje cells and RCA-1 also stained endothelial cells, which had to be differentiated based on localization and morphology.

Since some of the tissues from the wild birds found dead not always were fresh, we were concerned that the "normal" IHC method might not be sensitive enough to detect all of the antigen present in the tissue. We therefore tried a complementary method to detect viral antigen, namely a proximity ligation assay (PLA). This method has been used to identify avian influenza viruses in biological specimens (Schlingemann *et al.*, 2010) and has been adapted for histological material. The PLA method worked well on the tissue samples we tested but we did not find any differences in sensitivity compared to the IHC.

Limitations of the studies included in the thesis mainly concern the number of animals included in the different studies. For study **II** there were few individuals representing each studied bird species, for study **III** only four tufted ducks were infected, and for study **IV** few animals were euthanized on 1-4 dpi. The number of animals is a frequent limitation of detailed pathology studies. Another limitation of study **IV** was that the experiment was originally designed to investigate whether the influenza virus could mutate under the pressure of OC in a live mallard model. When we decided that it was also interesting and important to look at the pathology, it was not possible to change the number of animals to be included in the experiment but we felt that

important information could still be gained from the pathology studies. Lastly, in the studies with naturally infected wild birds (**II** and **III**) sampling was not always standardized due to several different pathologists performing the post-mortem examinations as well as time constraints during the outbreak.

5 Concluding remarks and future perspectives

Despite numerous investigations concerning avian influenza viruses including their phylogeny, distribution, pathogenicity, host range, etc, many questions still remain unanswered. The results of this thesis contribute to the knowledge base by providing information about distribution of the virus in organs, types of lesions, course of the disease, routes of viral shedding, clinical signs and the variability of these features in relation to the avian species involved. Based on information from the studies in this thesis, future studies could include more detailed IHC studies, for example using CD4 and CD8 antibodies to differentiate between different types of T cells, using a better B-cell marker, and performing double labeling studies to identify virally infected cells. To better assess the route of entry and dissemination of the virus, experimental infections where birds are inoculated via different routes and euthanized earlier in the course of infection are necessary. Furthermore, the peripheral nervous system should be studied more extensively in order to try to determine the routes of dissemination of the virus. In order to continue deciphering the pathobiology of avian influenza viruses, continuous research in this field is necessary. It is of great importance to continue to monitor avian influenza among wild birds and to continue performing post-mortem examinations on animals and humans that die of natural HPAIV-H5N1 infections and to compare the findings with those acquired from experimental infections. As a whole, good knowledge of the pathobiology of avian influenza infection in wild birds is essential for the understanding of its epidemiology, and contributes to the design and implementation of preventive and control measures to protect the health of humans and animals.

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