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Influenza A Virus in Wild Birds

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”The birds around me hopp’d and play’d,
Their thoughts I cannot measure -
But the least motion which they made
It seem’d a thrill of pleasure.”

W. Wordsworth, *Written in early spring*

To my family - Johanna, Maja,
Skrollan and the tiny one

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SAMMANFATTNING PÅ SVENSKA

Influensavirus är RNA virus och indelas i olika typer och subtyper. Influensa A virus orsakar sjukdom hos ett flertal fågel- och däggdjursarter. Vilda fåglar anses utgöra den viktigaste reservoaren för influensa A virus.

Hos människa orsakar influensa A virus årliga epidemier av luftvägssjukdom med hög sjuklighet och stora ekonomiska konsekvenser för samhället. Eftersom frekventa mutationer orsakar ändringar i virusets ytstrukturer krävs årlig vaccination med nytt anpassat vaccin för att ge skydd mot sjukdom.

Det finns många olika subtyper av influensa A virus. Dessa karaktäriseras med två av virusets ytstrukturer; hemagglutinin och neuraminidas, vilket till exempel skrivs H5N1. Virus av olika subtyper kan rekombinera och på så sätt skapa nya varianter. Om en subtyp som tidigare ej cirkulerat bland världens befolkning orsakar ett utbrott kan detta leda till en världsomfattande epidemi, en så kallad pandemi. Pandemier har drabbat mänskligheten med viss regelbundenhet genom historien och haft förödande konsekvenser. Till exempel orsakade pandemin ”Spanska sjukan” under åren 1918-1920 mer än 50 miljoner dödsfall.

Influensa A virus orsakar också förödande utbrott i fjäderfäbesättningar. Virus av vissa subtyper kan mutera till högpatogena varianter och orsaka så kallad högpatogen aviär influensa. Dessa högpatogena varianter kan även överföras till och orsaka sjukdom hos människa och andra djur vilket varit fallet under det pågående utbrott av H5N1 som startade i sydöstra Asien 2003. Alla kända subtyper av influensa A virus har isolerats i material från vilda fåglar vilka lever i vattenmiljö, framförallt från änder. Dessa arter anses därför utgöra influensavirusets reservoar i naturen. Hos änder orsakar viruset framförallt en subklinisk infektion i gastrointestinalkanalen och sprids genom faekal-oral överföring via vatten i vilket viruset kan förbli aktivt en längre tid.

Det finns fortfarande många obesvarade frågor angående influensa A virus ekologi bland vilda fåglar. I denna avhandling presenteras fem artiklar som tillför ny kunskap inom detta område. I avhandlingen styrks bevisen för att vilda änder utgör virusets reservoar i naturen dels genom en metaanalys av samtliga publicerade data rörande fynd av influensa A virus hos vilda fåglar, dels med hjälp av data från fyra års provtagning från flyttande vilda änder vid Ottenby fågelstation. Resultaten påvisar temporala skillnader i influensvirusets prevalens i den västeuroasiatiska andpopulationen jämfört med den nordamerikanska. Prevalensen i den västeuroasiatiska andpopulationen är hög under perioden augusti till december och i viss mån även under våren. Dessa fynd talar för att influensavirus kontinuerligt cirkulerar i andpopulationen.

Under studien av förekomsten av influensa A virus hos änder isolerades enbart olika lågpatogena subtyper. Subtyperna H5 och H7 var vanligt förekommande. Dessa subtyper är benägna att utvecklas till högpatogena varianter om de sprids till fjäderfäbesättningar med svåra konsekvenser som följd. Genom studier av virus släktskap visas att de virus vi isolerat från vilda änder är snarlika de som orsakat utbrott bland fjäderfä i Europa under de senaste sju åren. Detta styrker värdet av att övervaka förekomsten av influensavirus hos vilda fåglar för att på så sätt förhindra utbrott av sjukdom bland fjäderfä.

Undersökning av prover från skrattmås (*Larus ridibundus*) ledde fram till upptäckten av en helt ny subtyp av influensavirus; H16. Därmed utvidgades spektret av kända subtyper i naturen.

Influensa A virus isolerades från sillgrisslor (*Uria aalge*) i Östersjön vilket inte tidigare gjorts hos denna art i Europa. Dessa virus innehöll gener från både nordamerikanska och euroasiatiska fågelpopulationers virus. Det visar att det finns ett utbyte av virus mellan fågelpopulationerna på de skilda kontinenterna.

ABSTRACT

Influenza virus is a RNA virus that exists as different types and subtypes. Influenza A virus strains are known to cause disease in several bird and mammalian species. Wild birds are believed to constitute the natural reservoir for influenza A virus.

In humans, influenza A virus causes yearly seasonal influenza epidemics of respiratory disease resulting in high morbidity and severe economic consequences. Due to the virus' ability to change its antigenic properties by mutation, yearly vaccination is required for protection from the disease.

There are many different subtypes of influenza virus which are characterized according to two surface structures - the hemagglutinin and neuraminidase proteins - , for example; H5N1. These subtypes have the ability to recombine, and thereby creating new variant combinations. If a subtype that the living population of humans has not encountered before starts to spread among humans, it can result in a pandemic. Pandemic outbreaks have occurred at irregular intervals throughout history and have had a devastating impact on mankind. For example the Spanish influenza pandemic of 1918 is thought to have killed more than 50 million people.

Influenza A virus is also an important cause of disease in poultry where virus strains of some subtypes may change into forms that are highly pathogenic. These virus strains may transmit directly to man and multiple other species. This has been the case in the ongoing outbreak that started in South East Asia in 2003. All known subtypes of influenza A virus have been isolated from wild birds living in aquatic environments, mainly dabbling ducks. These species are considered to be the reservoir for influenza A virus. The virus causes sub clinical gastrointestinal infection in ducks. High amounts of virus are excreted in the feces and spread via the fecal-oral route through water where it can persist for a prolonged time.

There are still many unknowns about the ecology of influenza virus in the wild bird reservoir. This thesis includes five articles where data are presented that add new knowledge on this subject. We add proof that wild ducks are indeed the host for most influenza A virus subtypes by presenting data from a meta-analysis on all published screening data from wild birds and by presenting data from a four year screening of migratory ducks that were caught and sampled at Ottenby Bird Observatory. Our investigations have shown that the prevalence of influenza virus in the wild duck population of western Eurasia shows temporal differences in comparison to the results found in studies in North America. The prevalence in western Eurasian ducks is high during the period August to December and also rises in the spring. These findings are of importance for the understanding of how influenza virus is perpetuated in nature. During the course of the study only low pathogenic subtypes were isolated. Of concern is the high frequency of isolation of virus strains of the H5 and H7 subtypes that are prone to change into highly pathogenic variants in poultry. Many of the strains isolated in our study are similar to the ones that have caused influenza outbreaks in poultry in Europe during the last seven years. This indicates that wild bird surveillance for influenza A virus can be of major value as a sentinel system to prevent outbreaks in domestic poultry.

Studies on Black-headed Gulls (*Larus ridibundus*) revealed a previously unknown subtype, H16. This finding widened the spectra of known influenza A virus subtypes in nature.

Influenza A virus was also isolated in samples from Guillemots (*Uria aalge*) in the Baltic Sea. This was the first time influenza A virus was isolated from this species in Europe. The isolated virus strains contained a mix of genes, some of which must have been derived from influenza A virus strains present in the North American bird population. This finding proves that limited exchanges between the virus strains present on the American and the Eurasian continents exist, which is of concern for evaluating the risk of spread of highly pathogenic virus strains by wild birds to the Americas.

ORIGINAL PAPERS IN THIS THESIS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from Black-headed Gulls**
Ron A.M. Fouchier, Vincent J. Munster, Anders Wallensten, Theo M. Bestebroer, Sander Herfst, Derek Smith, Gus F. Rimmelzwaan, Björn Olsen and Albert D.M.E. Osterhaus
Journal of Virology 2005, 79(5): 2814-22.

II. **Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2 in Guillemot (Uria aalge)**
Anders Wallensten, Vincent J. Munster, Johan Elmberg, Albert D.M.E. Osterhaus, Ron A.M. Fouchier and Björn Olsen
Archives of Virology 2005, 150: 1685-1692

III. **Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe**
Vincent J. Munster, Anders Wallensten, Chantal Baas, Guus F. Rimmelzwaan, Martin Schutten, Björn Olsen, Albert D.M.E. Osterhaus and Ron A.M. Fouchier
Emerging infectious diseases 2005 11 (10):1545-1551

IV. **Multi-year surveillance of influenza virus type A in migratory waterfowl in northern Europe**
Anders Wallensten, Vincent J. Munster, Neus Latorre-Margalef, Mia Brytting, Johan Elmberg, Ron A.M. Fouchier, Thord Fransson, Paul D. Haemig, Malin Karlsson, Åke Lundkvist, Albert D.M.E. Osterhaus, Martin Stervander, Jonas Waldenström and Björn Olsen
Submitted Manuscript

V. **Global patterns of influenza A virus in wild birds**
Björn Olsen, Vincent J. Munster, Anders Wallensten, Jonas Waldenström, Albert D.M.E. Osterhaus and Ron A.M. Fouchier,
Science 2006, 312 (21): 384-388

ABBREVIATIONS

EID_{50}	Egg Infectious Dose 50
HA	Hemagglutinin
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A virus
LPAI	Low Pathogenic Avian Influenza
M1	Matrix protein
M2	Membrane ion channel protein
NA	Neuraminidase
NP	Nucleoprotein
NS	Non-structural protein
PA	polymerase A
PB1	polymerase B1
PB2	polymerase B2
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
vRNP	Viral ribonucleoprotein

SPECIES INDEX

English name	Scientific name	Swedish name
Arctic Tern	*Sterna paradisaea*	Silvertärna
Barnacle Goose	*Branta leucopsis*	Vitkindad gås
Black-headed Gull	*Larus ridibundus*	Skrattmås
Brent Goose	*Branta bernicla*	Prutgås
Gadwall	*Anas strepera*	Snatterand
Common Eider	*Somateria mollissima*	Ejder
Common Pochard	*Aythya farina*	Brunand
Common Shelduck	*Tadorna tadorna*	Gravand
Common Tern	*Sterna hirundo*	Fisktärna
Eurasian Teal	*Anas crecca*	Kricka
Eurasian Wigeon	*Anas penelope*	Bläsand
Goldeneye	*Bucephala clangula*	Knipa
Greylag Goose	*Anser anser*	Grågås
Guillemot	*Uria aalge*	Sillgrissla
Long-tailed Duck	*Clangula hyemalis*	Alfågel
Mallard	*Anas platyrhyncos*	Gräsand
Mute Swan	*Cygnus olor*	Knölsvan
Northern Pintail	*Anas acuta*	Stjärtand
Red-breasted Merganser	*Mergus serrator*	Småskrake
Tufted Duck	*Aythya fuligula*	Vigg

FOREWORD

This thesis summarizes four years of exciting research in the field of influenza. What seemed like an odd but fascinating multifaceted study subject on the edge of research subjects like ornithology, ecology and molecular biology soon turned into a hot topic with worldwide attention. Being a clinically working medical doctor my main interest in research on influenza is to gain knowledge that might somehow benefit the patient sitting in front of me in the examination room. Like with other preclinical research subjects it may be hard to see how the research is relevant to the practice of medicine. In this case the outcome we all want to avert is the pandemic spread of a pathogenic influenza A virus. The object of our research must ultimately be to gain knowledge that may help us avoid such an outbreak. In order for this to be possible, all stones must be turned and the whole process of the emergence of new influenza virus strains that infect humans must be elucidated.

Influenza A virus has a complex natural history and pattern of infection and we have had to limit our research to the ecology of influenza A virus in its natural reservoir, wild birds, and how the strains and subtypes vary between species, seasons and locations. In order to do this different molecular techniques have been used as tools.

I have tried my best to give a background to the other relevant aspects of influenza virus infection in the introductory overview. With the sudden focus on wild birds and influenza, up-stirred by the ongoing outbreak in South East Asia, and the transmission of these virus strains right to our shores by wild birds, the research into the field has exploded and new knowledge is being published every week. I have done my best to summarize what is known so far although I realize that old truths may be replaced with new ones as I write and that within the next few years we shall have a much better understanding of the subject. Hopefully increased knowledge about influenza A virus in the wild bird reservoir species will provide us with a tool to avert pandemics and in the end spare us the horrendous task of facing a pandemic outbreak.

1. INTRODUCTION TO THE SUBJECT

1.1 History and scientific progress

The earliest written reports of a disease that might have been caused by influenza virus were made by both Hippocrates and Livy in 412 BC. Over the centuries there have been numerous accounts of epidemics and pandemics (worldwide epidemics caused by a new influenza virus variant which did not circulate before the outbreak) that may have been caused by influenza virus. The Russian flu pandemic of 1889-1892 is the first one for which we have good enough documentation to be sure that it was caused by influenza virus (Nicholson, Webster, and Hay, 1998). An overview of recent pandemics of influenza A virus is provided in table 1.1.1.

During the years 1918-1920 one of the most devastating disease outbreaks in world history took place. It would become known as the Spanish flu pandemic and left the world in horror as it caused the death of maybe as many as 50-100 million people (Johnson and Mueller, 2002). It beats even the Great Plague in the number of people killed. It is not known where this pandemic started although British army camps in northern France during the first world war have been suggested (Oxford et al., 2005). The first documented clinical cases were found in the United States and an alternative theory states that recruits traveling to the war brought the disease over to Europe. The Spanish flu spread from continent to continent and returned in three major waves during next years with increasing virulence. This horrific event sparked research into the field that eventually led to the discovery of the viral culprit. In 1933 Smith and co-workers discovered a filterable substance that caused influenza-like respiratory disease in humans which could be transmitted between ferrets and rendered them immune to reinfection (Smith, Andrewes, and Laidlaw, 1933). Smith and Stuart-Harris were later able to fulfill Koch's postulate by isolating the influenza virus from one of the researchers throats when he developed influenza-like illness after he had accidentally been sneezed upon by one of the infected ferrets (Nicholson, Webster, and Hay, 1998). The results were published in Lancet in1936 (Smith and Stuart-Harris, 1936). By analyzing exhumed remains from of an individual buried in the arctic, where there had been permafrost since the outbreak, and by sampling tissues from victims stored in formalin, Taubenberger and Reid et al (Basler et al., 2001; Reid et al., 1999; Reid et al., 2004; Reid et al., 2002; Reid et al., 2000; Reid et al., 2003b; Reid, Taubenberger, and Fanning, 2004; Taubenberger, Reid, and Fanning, 2000;

Taubenberger et al., 2001) were able to recover enough RNA to determine the subtype of the Spanish Influenza pandemic virus as H1N1.

The world experienced two more severe pandemics during the 20th century. Although less devastating than the Spanish flu, they still caused high morbidity and mortality with death tolls reaching 6 million worldwide (Oxford, 2000). During the years 1957-1958 there was a pandemic named the Asian flu with the H2N2 subtype, and between 1968 and 1970 the H3N2 subtype caused a pandemic known as the Hong Kong flu. After each pandemic the previously circulating strain disappeared for unknown reasons. Between 1977 and 1978 a very mild pandemic mainly affecting young people swept the world as the H1N1 subtype returned; possibly released by mistake during live vaccine trials in the Far East (Palese, 2004). This strain currently co-circulates in humans with the H3N2 subtype from the Hong Kong pandemic of 1968. An overview of the different subtypes of influenza A virus that have caused pandemics and circulated in the human during the last century is given in table 1.1.1. Based on historical patterns, pandemics can be expected to occur on average three to four times each century, but still there is no way to predict when the next pandemic will hit the world. Considering the high population density and modes of travel in today's world, a new pandemic could have devastating consequences.

Influenza A virus does not only cause disease in man but also in animals. In 1878, the disease was first identified in animals in Italy by Eduardo Perroncito. He described an initially mild disease in domestic birds that after a while turned highly pathogenic, killing virtually all the birds in the area. In 1901 two other Italian scientists, Centanni and Savonuzzi, identified "Fowl Plague", as it was then called, to be a viral disease, but it was not until 1955 that influenza virus was identified as the causative agent. Between the years 1959 and 1999, 18 outbreaks of avian influenza with high mortality (HPAI) were reported in domestic poultry around the world. These outbreaks had devastating economic consequences for the affected countries. Millions of raised birds died from the disease or were culled in order to stop the outbreaks (Capua I, 2001). In recent years the frequency of outbreaks in domestic birds has increased (Munster et al., 2005).

The first documented outbreak of HPAI in the wild bird population was in 1961, when an outbreak in Common Terns (*Sterna hirundo*) killed about 1600 birds in South Africa (Becker, 1966). This outbreak put focus on wild birds as a possible reservoir for influenza A virus. When screening wild birds in search of Newcastle disease virus (known to be spread by wild birds), during an outbreak of Newcastle disease in poultry in California in 1974, Slemons et al (Slemons et al., 1974) revealed that low pathogenic influenza A virus (LPAI) could be

isolated from wild birds. Further screening soon revealed that species living in aquatic environments such as ducks, gulls, geese and shorebirds harbored low pathogenic influenza A virus strains of many different subtypes and probably acted as a reservoir for these strains (Webster et al., 1992). It has since been shown that all known influenza strains infecting humans and other mammals originally circulated in the wild bird population (Ito and Kawaoka, 2000; Reid et al., 1999).

Research has shown that low pathogenic influenza A virus strains may, after circulation in poultry populations, sometimes mutate into highly pathogenic influenza virus strains (Alexander et al 2000). During an epizootic in Italy between 1999 and 2001, the H7N1 virus, initially of low pathogenicity, mutated within nine months to a highly pathogenic form (Zanella et al, 2001). More than 13 million birds died or were destroyed.

Influenza A virus also infects and causes epizootics in mammalian species such as horses, pigs, seals, whales, ferrets and mink (Webster et al., 1992) which will be discussed in more detail further on in the text.

It was long thought that the disease outbreaks of influenza A virus in poultry were of no concern to humans even though there had been reports of people suffering from conjunctivitis after being in contact with animals sick with influenza A virus, or when working with highly pathogenic influenza A isolates in the laboratory (Capua and Alexander, 2004). Influenza A virus was not considered to be a zoonotic disease agent of any importance until transmission from birds to humans occurred in Hong Kong in 1997. The outbreak in Hong Kong was caused by a highly pathogenic H5N1 strain that caused severe respiratory disease in 18 humans. Six of the infected people died of the disease (Chan, 2002). The cases of human infection coincided with an epizootic of HPAI in Hong Kong's poultry population, caused by the same strain of influenza A virus. Investigation of the outbreak determined that close contact with live infected poultry was the source of human infection and that the virus had been acquired directly by humans from birds. Rapid destruction of Hong Kong's entire poultry population of 1.5 million birds reduced opportunities for further direct transmission to humans.

However the world would soon again experience outbreaks of HPAI in poultry transmitting virus to and causing severe disease in humans. In 2003 an outbreak of highly pathogenic IAV of the H7N7 subtype, caused the death of one veterinarian and mild illness in 83 other humans in the Netherlands. More than 30 million birds where killed at the cost of several million euros.

The worst outbreak of HPAI in modern times is currently plaguing the world. Starting in February 2003, the outbreak has as of the 20th July 2006 led to the death or destruction of more than 100 million birds and has caused verified disease in 231 humans of which 133 have died (WHO) in ten countries. The outbreak of the same H5N1 subtype that caused disease in Hong Kong in 1997 started in South East Asia and subsequently spread to most parts of Eurasia and several countries in Africa. The virus has also transmitted to species that were previously not known to be susceptible to infection such as domestic cats, leopards and tigers (Keawcharoen et al., 2004; Kuiken et al., 2004; Thanawongnuwech et al., 2005). The H5N1 strains causing the current outbreak are thought to be related to the H5N1 strain that caused the outbreak in Hong Kong in 1997 (de Jong and Hien, 2006).

Originally, these highly pathogenic strains are believed to have been derived from low pathogenic wild bird influenza A virus strains that after infecting poultry subsequently mutated into highly pathogenic variants. These highly pathogenic strains were then transmitted back into the wild bird population on several occasions. As a result, wild birds not normally seriously affected by influenza virus infection by low pathogenic influenza A virus strains have become ill or died. There are major differences in how highly pathogenic virus strains affect different species. For example, the currently circulating highly pathogenic H5N1 virus caused high mortality in wild birds at Qinghai lake in China in an outbreak that primarily affected Bar-headed geese (*Anser indicus*) (Chen et al., 2005; Liu et al., 2005), killing several thousand of this endangered species and highlighting the risk posed to endangered species. In contrast, some duck species have been shown to survive infection by the same strain without showing clinical signs (Chen et al., 2004). Therefore ducks may have acted as long distance disease transmitting vectors (Webster et al., 2006) of this virus. After the outbreak at Qinghai Lake, the H5N1 virus was found among wild birds in many countries in Asia, Europe and Africa, indicated by increased mortality in certain bird species such as swans and raptors. In Sweden, the H5N1 virus caused deaths primarily among Tufted Ducks (*Aythia fuligula*). Thus, wild birds have been carrying the virus between different geographic areas and as a consequence there have been outbreaks in domestic poultry in many countries. However, domestic poultry trade and poultry handling in conjunction with agricultural practices have probably been responsible for local transmission and the persistence of outbreaks (Gilbert et al., 2006).

The ongoing outbreak that started in South East Asia has sparked fears of an imminent pandemic in humans. The possibility that humans, if concurrently infected with human and avian influenza strains could serve as a "mixing vessel" for the emergence of a novel subtype

that has the ability to be easily transmitted from person to person increases as more humans become infected. If this were to happen it would probably mark the start of an influenza pandemic.

Table 1.1.1 A summary of influenza A virus pandemics and the time periods that different subtypes have circulated in the human population during the last centuries. (Hilleman, 2002; Johnson and Mueller, 2002; Kilbourne, 2006; Oxford, 2000)

Pandemic name	Time period	Subtype	Estimated mortality (initial outbreak)
Russian flu	1889-1892	H2N2?	6 million
	1898-1900?	H3N2 or H3N8?	0.5 million
Spanish flu	1918-1957	H1N1	> 50 million
Asian flu	1957-1968	H2N2	4 million
Hong Kong flu	1968-	H3N2	2 million
Russian (red) flu	1977-	H1N1	?

1.2 Structure and function

Influenza virus is a negative sense single stranded RNA virus of the Orthomyxoviridae family. The genetic material is stored in eight segments of viral RNA (7 for type C) coding for ten different proteins (table 1.2.1). It exists in three types A, B and C classified by antigenic differences in viral nucleoprotein and matrix proteins. Types A and B are epidemiologically important while type C only causes a mild and rarely diagnosed disease. Type B is relatively stable, while type A is highly unstable and has the ability to mutate, recombine and exchange genetic information. Type C has been found to circulate in humans and pigs while type B has been found in humans and seals. Influenza virus Type A (influenza A virus) infects and circulates in many animal species reported elsewhere in this text and is of primary interest to research because of its pandemic potential.

The following text only refers to influenza type A virus. Influenza A virus can be further divided into different subtypes on the basis of antigenic differences of the primary antigenic structure, the HA and the secondary antigenic structure the NA. The different subtypes of these antigenic structures do not cross-react serologically and thus immunity to one subtype does not provide protection for any other subtype. The subtype is determined by which combination of the two a particular influenza A virus consists of. To date, 16 different HA subtypes and 9 different NA subtypes have been found in nature (Fouchier et al., 2005) and

they can exist in many different combinations. Influenza virus strains are described by the influenza type, the host of origin, the strain number, the year of isolation and finally the subtype (for example A/Mallard/Sweden/105/02 (H7N7)).

The enveloped influenza A virus (figure 1.2.1) contains eight gene segments, each with a ribonucleoprotein (RNP) complex made up of the polymerase A (PA), B1 (PB1) and B2 (PB2) proteins. The gene segments are encapsidated by the nucleoprotein (NP). The encapsidated particles are surrounded by a membrane derived from the plasma membrane of the infected cell. The matrix protein (M1) lines the inner surface of the membrane. Three proteins are embedded in the membrane and protrude from the surface: the hemagglutinin (HA) protrudes as a trimer of identical subunits; the neuraminidase (NA) protrudes as a tetramer of identical subunits and the matrix (M2) is made up like a tetrameric membrane channel.

All gene segments code for a single protein except the M and NS genes that code for two different proteins (M1 and M2 and NS1 and NS2 also called nuclear export protein, NEP, respectively), generated by RNA splicing. The NS1 protein is not present in the virion but only expressed in the infected cell.

Infection of a host cell is initiated when the HA attaches to sialic acids containing glycoprotein and glycolipid receptors on the host cell surface. Human influenza A virus strains preferentially bind sialic acid residues by an α2-6 linkage, while avian and equine virus strains preferentially bind to sialic acids by an α2-3 linkage (Connor et al., 1994; Rogers and Paulson, 1983). The HA is synthesized as a precursor HA_0 that needs to be cleaved by host proteases to create two subunits, HA_1 and HA_2 that are linked only by a single disulfide bond, in order to successfully infect cells (Steinhauer, 1999).

After attaching, the influenza A virus is taken up by the cell via receptor-mediated endocytosis. Catalyzed by the M2 protein channels in the viral envelope, hydrogen ions flow through these channels into the viral endosome dissociating the M1 proteins. This dissociation is necessary for subsequent migration of the viral RNP to the nucleus. Blocking the function of M2 with drugs such as amantadine and rimantadine can inhibit infection.

The lower pH of the endosome induces conformational changes in the HA that facilitates fusion of the viral and host endosome cell membranes releasing viral RNP into the cell cytoplasm. The viral RNP then enters the nucleus where replication transcription is started by the viral RNA polymerase complex.

The viral proteins are formed before new viral RNA. New nucleocapsids are formed when newly synthesized viral RNA binds to NP, possibly with the help of the M1 (Wakefield and Brownlee, 1989). M1 promotes migration out of the nucleus (Martin and Helenius, 1991) together with NEP. The NS1 protein down-regulates the host cells' antiviral interferon production by sequestering viral genomic RNA from host cell intracellular receptors (Hilleman, 2002).

At the cell membrane, the M1 covered nucleocapsids are enclosed by an envelope containing the viral surface glycoproteins (Martin and Helenius, 1991). Budding may be facilitated by interactions between the M1, M2, HA and NA (Mikulasova, Vareckova, and Fodor, 2000). The NA is involved in the release and spread of mature virions as it prevents aggregation at the surface of the infected cell by enzymatic removal of sialic acids from the virus surface (Liu et al., 1995). It may also have a role in the initial process of viral entry (Matrosovich et al., 2004b). The use of neuraminidase inhibitors such as oseltamivir and zanamivir inhibits the release of new virions, causing them to aggregate on the cell surface and thereby preventing the infection of new cells. A schematic presentation of the influenza A virus cycle of infection is found in (figure 1.2.1).

Table 1.2.1. Influenza A virus components and their main function

Genome segment	Proteins coded	Main function
1	PB2	Sub-unit of RNA-polymerase
2	PB1	Sub-unit of RNA-polymerase
3	PA	Sub-unit of RNA-polymerase
4	HA	Viral attachment and membrane fusion
5	NP	Major structural component
6	NA	Release of new virions by preventing aggregation
7	M1	Facilitating migration of viral RNP in cell
	M2	Ion channel involved in uncoating of virus
8	NS1	Post-transcription modulation, interferon antagonist
	NS2 or NEP	Mediates nuclear export of vRNAs

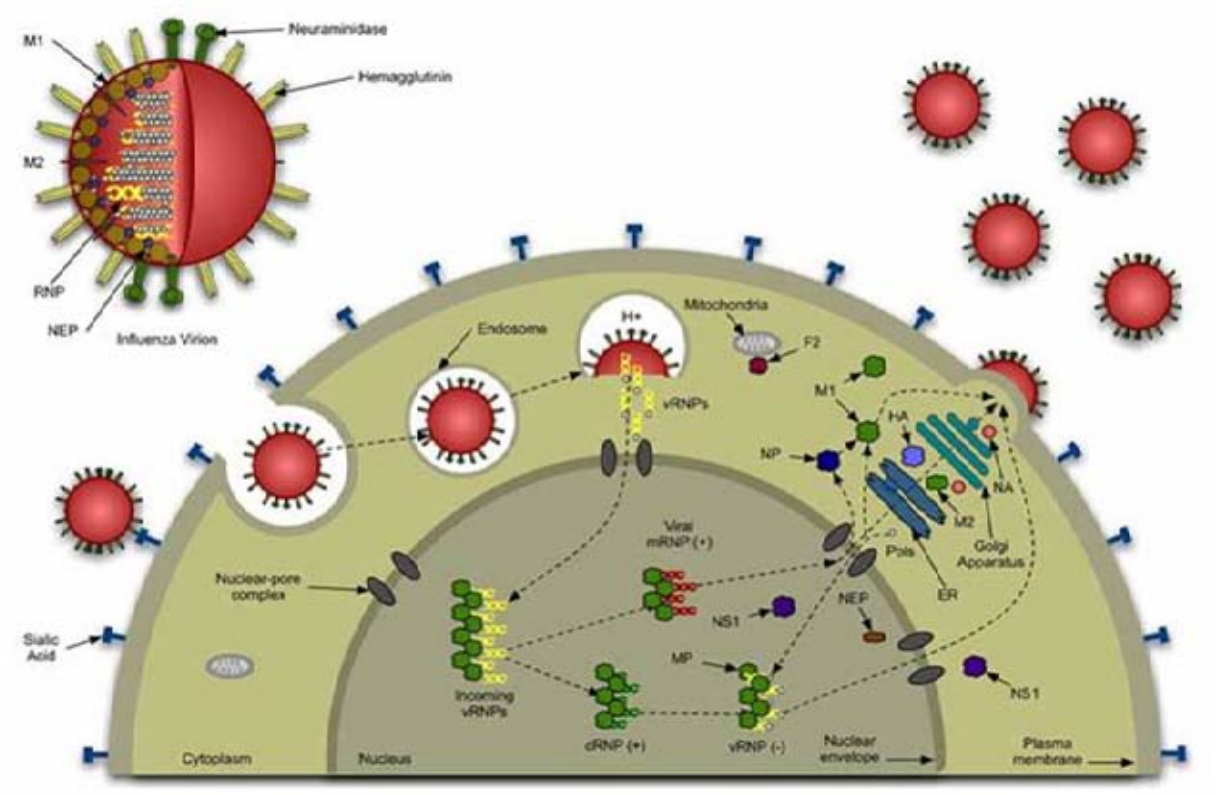

Figure 1.2.1. Schematic picture of the influenza A virus and it´s life cycle.
Source: Luo, F, Squires, B, Scheuermann, 2006. Influenza infection (Homo sapiens) in influenza life cycle. Reactome. World Wide Web URL - http://www.reactome.org

1.3 Genetic variability

Influenza A virus has been called a master of metamorphosis (De Jong et al., 2000). It uses two main ways to elude host defenses: antigenic drift and antigenic shift.

Antigenic drift is characterized by slight changes in the antigenic structures of the virus that renders neutralizing antibodies, produced by the host immune system during the encounter of previous strains by infection or vaccination, obsolete. These antigenic changes take place frequently as influenza A virus lacks mechanisms for the "proof reading" and repair of errors that occur during replication (Webster, Shortridge, and Kawaoka, 1997). These errors result in variant strains that may become dominant and replace older strains, if they are better at evading the host immune response. Dysfunctional strains will die out but many variants will not, and several subpopulations may co-exist in an infected individual (De Jong et al., 2000; Katz and Webster, 1988). Antigenic drift has been shown to take place in humans and other mammals at rapid rates and is the reason why influenza vaccination with updated strain content must be given yearly in order to provide protection from disease. Phylogenetic analyses have revealed that antigenic drift of influenza A virus does not take place to the same extent in wild birds. The strains that exist in wild birds appear to be in a relative evolutionary stasis, as shown by the fact that strains collected from wild birds more than 80 years apart were almost identical (Reid et al., 2003a; Webster et al., 1992). This might imply that an optimal host-parasite relation has been reached and that any change in the genetic make up will result in less functional strains that will eventually become extinct.

The segmented genome of the influenza A virus allows for another potent way of evading host defenses. If a single cell is infected by two IAV strains of different subtypes, genetic reassortment can take place. The reassortment can result in the creation of a novel combination of HA and NA and thus a subtype different from both the parental virus strains. This is known as antigenic shift. If a HA or NA subtype that has previously not been circulating in a population is created and for which there is no herd immunity, a pandemic spread might be the result. Antigenic shift that gives rise to a new subtype infecting humans has long been thought to require the involvement of pigs since pigs are susceptible to infection with both avian and mammalian virus strains. They could thus serve as a "mixing vessel" for the reassortment of genetic material from human and avian virus strains resulting in the emergence of a novel subtype that has retained its ability to transmit easily between humans but includes a new antigenic subtype of the HA or NA structures. More recent

research has shown that pigs may not be the only possible mixing vessel since recent events have shown that for at least some influenza A virus subtypes circulating in bird populations, humans themselves could serve as the "mixing vessel" (De Jong et al., 2000).

1.4 Host specificity

There are many factors determining whether a species can act as a host for an infection. The most obvious being sufficient contact between the host and the pathogen for infection to occur. This makes some species more likely to become infected than others. It is illustrated by the fact that since transmission is thought to mainly occur via water, aquatic bird species (species that live in an aquatic environment) are found to be infected by low pathogenic influenza A virus strains more often than non-aquatic species (Olsen et al., 2006).

Successful attachment to a host cell is the next required step for infection to occur. As mentioned earlier, avian influenza A virus strains preferentially use α2,3 linked sialic acids as cellular receptor while human influenza A virus strains preferentially bind to α2,6 linked sialic acids. Before reaching and binding to the epithelial cells, however, there are other host barriers such as mucus and alveolar macrophages to pass (Kuiken et al., 2006). For example in the secretions protecting the eyes and respiratory tract, different mucins containing sialic acids are present that specifically bind and clear virus before they reach the epithelial cells. These mucins express different sialic acid linkages in different species and also in different organ systems of the same species. Humans are better at clearing avian influenza A virus from the respiratory tract than from the eye since the mucins of the respiratory tracts are rich in α2,3 linked sialic acids while the secretions of the eye are rich in α2,6 sialic acid linkages. The situation is reversed in chimpanzees since their respiratory tract secretions are rich in α2,6 linked sialic acids that make them partly resistant to infection by human influenza A virus (Olofsson et al., 2005). On the cellular receptor binding level there are major differences in sialic acid linkage content between species, as well as differences within organ systems, and even between cells of the same organ systems. Taken together these differences may determine where, if at all, infection of the host may occur. Ducks that express α2,3 linked sialic acids in the intestinal system are primarily affected by infection of the cells lining the intestinal tract (Ito et al., 1998). Within the human body α2,3 linked sialic acids have been found to be predominant in the eye. In the respiratory system, α2,6 sialic acid linkages are predominant in the upper part whilst α2,3 linkages are present in the lower part, where

Influenza A virus has been shown to bind preferentially to pneumocytes type II (Shinya et al., 2005; van Riel et al., 2006). Although not predominant, α2,3 receptors are also found on ciliated cells of the upper respiratory tract (Matrosovich et al., 2004a). Human influenza virus targets non-ciliated cells that express α2,6 linked sialic acids (Matrosovich et al., 2004a). This might explain why there is only limited transmission of avian influenza virus to humans, why conjunctivitis has been a common symptom (Fouchier et al., 2004) and why the respiratory infections in humans are rare but severe when they occur (Beigel et al., 2005).

It was previously thought that pigs (known to express both α2,3 and α2,6 linked sialic acids in their respiratory epithelium) were unique in their potential to act as a mixing vessel host species, where pandemic virus strains could arise by the recombination of avian and human influenza virus strains infecting the same cell (Ito et al., 1998; Scholtissek et al., 1985). However, the finding that both humans (Matrosovich et al., 2004a) and chickens (Kim, Ryu, and Seo, 2005) harbor the different receptor types in different cells indicates that theoretically this could happen in other animal hosts as well. Further research has shown that although avian influenza A virus strains preferentially bind α2,3 linked sialic acids, a further refinement of specificity exists that differs between avian species. The refinement is based on recognition of differences in the inner part of the oligosaccharide receptor (Gambaryan et al., 2005).

Successful attachment to a cell does not necessarily imply that infection can occur since the virus must also be able to enter the cell and cause it to replicate genetic material. In this process the internal genes of the virus are the determinants. It has been shown that there are host specific lineages of all the different internal genes indicating species adaptation and optimization of each gene (Baigent and McCauley, 2003). Some of these differences have been analyzed in detail and found to be important. For example the PB2 gene of the virus polymerase complex plays a major role. Research has shown that in avian influenza A virus the amino acid residue 627 of the PB2 protein differs from mammalian virus strains in that avian virus strains have glutamic acid at this site whereas mammalian strains have a lysine and that this is of major importance for host range restriction (Subbarao, London, and Murphy, 1993). This difference has been associated with optimal replication at different temperatures. Human influenza virus strains replicate in an environment of about 33 ºC in the trachea, while avian strains are adapted to replication in the intestinal tracts of birds at a temperature close to 41 ºC (Massin, van der Werf, and Naffakh, 2001). It has also been shown experimentally that a change from glutamic acid to lysine at this site results in increased virulence for mice (Hatta et al., 2001) and this change has been seen in H5N1 virus strains

and H7N7 virus strains that have caused severe disease in humans (Fouchier et al., 2004; Hatta et al., 2001).

Once successful replication has taken place, the newly constructed virus must be released from the surface of the infected cell to invade new cells and like the hemagglutinin, the neuraminidase of avian virus strains preferentially operates by cleaving the sialic acids that are α2,3 linked, while human neuraminidases prefer α2,6 linked sialic acids (Baigent and McCauley, 2003).

Even if replication and release of new virions has been successful, there are still factors that determine whether or not the infection will remain localized in the organ of entry and if it will prevail. First of all, the immunity of the host must be dealt with. In order to hinder the host's innate immunity to produce interferons that put infected cells into an antiviral state, the influenza A virus' NS1 polypeptide sequester double stranded RNA away from certain protein kinases and suppress host cell posttranscriptional processing of mRNA so that the infected cell remains undetected (Baigent and McCauley, 2003; Geiss et al., 2002; Hilleman, 2002; Kuiken et al., 2006). Research has shown that there are host specific differences in the NS1 gene of different strains. For example; the human NS1 gene is not optimal when introduced into mice strains (Palese, 2004).

The factors determining the ability of influenza A virus to produce systemic rather than localized infection in mammals are not fully understood (Kuiken et al., 2006). In poultry, however, the ability of the virus to invade other organs depends on whether or not the HA of the virus can be cleaved by ubiquitous extra cellular proteases or only by specific proteases that are restricted to the respiratory and gastrointestinal tract. This ability is determined by the amino acid sequence at the cleavage site. Poultry strains can thus be characterized as highly pathogenic or low pathogenic based on the presence of multiple basic amino acids at the cleavage site. These highly pathogenic influenza A virus strains are thought to arise from low pathogenic virus strains. How this occurs is not well understood (Banks et al., 2001; Wood et al., 1993).

1.5 Persistence and modes of transmission

The Influenza A virus, as other viruses, cannot replicate outside a host cell. In order to infect new individuals it needs to persist for some time in the environment. It seems that influenza A virus is well adapted to persist in water. Under experimental conditions, avian influenza A

virus strains stored in distilled water at 28 °C could remain infective for 100 days, at 17 °C for 200 days and possibly for as long as 1000 days at 4 °C (Stallknecht et al., 1990b). However, under natural conditions, persistence of active virus is limited by the effects of pH, salinity, UV-radiation and presence of biologically active material such as degrading enzymes, bacteria and other microorganisms.

Human influenza A virus strains are stable at a pH from neutral to 8.5. Infectivity decreases rapidly below pH 6.0. Avian influenza A virus strains exhibit more stability than human influenza A virus strains and can persist and remain active at pH 4.0 whereas human isolates do not persist pH below 5.0 (Webster et al., 1978). Infectivity is inversely related to salt content of water for avian influenza A virus (Stallknecht et al., 1990a).

In open air, human strains of influenza A virus can spread via aerosol. The persistence and infectivity of these strains in open air is promoted by low humidity. Aerosols containing influenza A virus may remain infective for up to 24 hours or more at low humidity but only for an hour at high humidity (Hemmes, Winkler, and Kool; Schaffer, Soergel, and Straube, 1976). Other limiting factors in open air are UV-radiation and wind. Some strains of influenza A virus can also be spread via fomites on hard surfaces such as stainless steel, where it can survive for up to two days (Bean et al., 1982).

Thus, many factors determine the suitability of different environments for persistence, infectivity and transmission of influenza A virus.

1.6 Influenza A virus in man

In interpandemic years the strains of influenza A virus that circulate in humans mainly cause respiratory disease and preferably infect the epithelium lining the airways. They spread by respiratory secretions. Currently, the H3N2 and H1N1 subtypes circulate in the human population. Influenza epidemics occur mainly in the winter season from October to April in the northern hemisphere and from May to September in the southern hemisphere. In tropical regions, influenza may occur throughout the year. Influenza A virus strains are isolated somewhere in the world every month and the infection is sustained and perpetuated in the human population (Cox and Subbarao, 2000). The seasonal fluctuations (outside of the tropics) are probably a result of factors promoting virus survival and spread, like the fact that people spend more time indoors and that the humidity is low (Nicholson K.G., 1998).

Influenza A virus infection is most often a self-limiting disease with abrupt onset of high fever, malaise, cough and head and muscle ache. It causes symptoms for up to 2 weeks and requires on average 3-4 days of bed rest. The disease can be severe and sometimes lethal in young children, the elderly, those who are under immune suppression and people with underlying disease such as cardiac disease or asthma (Mandell et al., 2005).

Considering the high attack rate of influenza A virus, it may have a huge impact on national economies worldwide, depending on the severity of the epidemic. In temperate climates, between 2-15 percent of the populations becomes infected. Even during years with mild influenza epidemics a large number of people die. In Sweden, it is estimated that the number of people that die as a consequence of infection is between 1000 and 4500 depending on the strain. Many more are sick, and the costs for health care and sick leave are also high (Läkemedelsverket, 2002).

When a subtype of influenza A virus, which the human population has not experienced before and thus has no immunity against starts to spread, the spread may be rapid and cause concurrent outbreaks around the globe resulting in a pandemic. Pandemics in humans caused by influenza A virus occur at irregular intervals, as have been described above. The severity of the pandemic varies with strain and pandemic strains may behave differently to the seasonal epidemic strains. For example, the strain that caused the Spanish flu was responsible for high death rates in healthy young individuals and spread during the summer months.

Treatment of influenza is mainly symptomatic although the development of neuraminidase inhibitors has created a way to shorten and perhaps limit disease if given early in the infection. Neuraminidase inhibitors are without the side-effects and problems with resistant strains that hampered the earlier antivirals Amantadine and Rimantadine (Jefferson et al., 2006).

Vaccination against influenza has been used for many years. In Sweden, elderly and groups at risk are encouraged to be vaccinated. In some countries vaccination of school children that are the principal spreaders of infection have been tested. The main problem with vaccination is that the fast antigenic drift of influenza A virus renders the antibodies produced in response to earlier vaccinations obsolete. Therefore, in order to provide protection from disease, the vaccine has to be altered every year to adjust to the changes in the antigenic sites. Since the development and production of a vaccine takes months, qualified guesswork is used to decide which strains to include in order to match the strains of the coming season.

Experimentally, avian influenza virus strains from wild birds do not replicate well in humans (Beare and Webster, 1991) and human strains do not replicate well in waterfowl

(Hinshaw et al., 1983). Until the outbreak in Hong Kong in 1997, the occurrence of transmission of avian strains was believed to be a rare event only causing conjunctivitis in the few affected cases (Katz, 2003). However, a serological survey in rural China suggests that infection with avian subtypes has not been uncommon in people who have had close contact with domestic ducks and poultry (Shortridge, 1992). In recent years, outbreaks of highly pathogenic strains that have evolved in poultry have occurred rather frequently. The symptoms of disease and the disease pattern have been variable depending of strain. In some cases, the disease has only caused conjunctivitis and mild influenza like illness, with no evidence of human to human spread, such as in the Canadian H7N3 poultry outbreak of 2003 where two people were affected (Tweed et al., 2004). In the Dutch outbreak of 2003, conjunctivitis and influenza-like illness were also the most common symptoms, but there was also one case of fatal pneumonia. The Dutch outbreak affected at least 84 people although serological evidence suggests that as many as 1000 people were infected (Enserink, 2004). During the outbreak there was also evidence of human to human transmission in some cases (Fouchier et al., 2004; Koopmans et al., 2004). The H5N1 outbreaks in Hong Kong in 1997 and the ongoing H5N1 outbreak in Eurasia and Africa have so far caused disease in very few confirmed cases in comparison to the number of persons that have been exposed to sick birds. However, an epidemiological investigation suggests that there may be more undiagnosed cases (Thorson et al., 2006). The disease in the confirmed cases has been severe, often fatal. The most common symptoms have been high fever and lower respiratory tract symptoms of pneumonia progressing to acute respiratory distress. The disease has in some cases presented with atypical symptoms such as diarrhea, vomiting, bleeding from the nose and gums and encephalopathic signs. Leucopenia has been a common laboratory finding. Transmission between humans has probably occurred during this outbreak, but has not been common (Beigel et al., 2005; de Jong and Hien, 2006; Tran et al., 2004; Ungchusak et al., 2005).

So far, the development of a highly pathogenic strain in domestic birds has been a prerequisite for human infection, but there is increasing evidence that direct infection may occur (Shinya et al., 2005). Low pathogenic H9N2 virus has been isolated in two children (Lin et al., 2000) with mild influenza symptoms. H9N2 virus strains are considered to be even more likely than H5N1 to become the cause of a pandemic since the strains that circulate in domestic chicken and ducks worldwide (Choi et al., 2004) have already acquired receptor specificity to prefer α2,6 linked sialic acids which are found on human cells (Li et al., 2003).

1.7 Influenza A virus in other mammals

Influenza A virus infects and on some occasions creates stable lineages in several mammalian species. This has been shown both experimentally (Hinshaw et al., 1981) and in nature as described for the species below. Highly pathogenic virus strains, such as the currently circulating H5N1 virus that originates from South East Asia, have been found to infect many species that had previously not been considered vulnerable. Thus, the range of species-infectivity is heavily dependent on strain type.

1.7.1 In pigs

Influenza A virus is a common cause of respiratory disease in pigs. Pigs harbor pig-adapted strains such as the classic swine-like influenza virus H1N1, but are also susceptible to human and avian influenza virus strains (Webster et al., 1992). This is partly due to the presence of both α2,3 and α2,6 linked sialic acids in the respiratory epithelium of pigs as discussed previously (Ito et al., 1998). Avian virus strains have been found in pigs on a couple of occasions. For example, a H4N6 strain caused respiratory disease in pigs in Canada (Karasin et al., 2000). In further Canadian porcine surveys avian H1N1 and H3N3 were also isolated (Karasin et al., 2004). Avian H1N1 has been isolated in China (Guan et al., 1996) where it caused a severe outbreak in pigs 1979-1980 (Schultz et al., 1991) and has remained in the pig population since that time. Experimentally, even those avian strains that did not at first replicate in pigs could be made to replicate after reassortment with swine-like virus strains in pigs that were co-infected (Kida et al., 1994). Several studies have reported human H3N2 strains in pigs after the antigenic shift in the human population in 1968 (Ito and Kawaoka, 2000).

Studies have also shown that both avian-like and swine-like H1N1 strains circulate at the same time in pigs (Scholtissek et al., 1983) as well as human-like H3N2 and avian-like H9N2 (Peiris et al., 2001). The different strains allow for reassortment. Such reassortment does take place in pigs where it has been shown to create variants such as H1N7 and H1N2 (Brown et al., 1994; Brown et al., 1998). Reassortants between human-like H3N2 and avian-like HIN1 also occurs (Castrucci et al., 1993).

Humans may be affected by strains transmitted by pigs. Direct transmission of swine-like H1N1 to humans occurs and has been fatal (Rota et al., 1989). Reassortants between swine-like and human-like influenza A virus strains may also cause disease in humans. In 1992 such

a reassortant was isolated from sick children in the Netherlands (Claas et al., 1994). Fortunately, this virus was unable to spread between humans. These examples show that pigs are indeed a mixing vessel for human and pig virus strains as suggested (Scholtissek et al., 1985) albeit possibly not the only one.

1.7.2 In horses

Influenza A virus strains in horses are thought to be of avian origin. Different subtypes have been found to infect horses and antigenic drift creates distinct lineages within the subtypes. At least two subtypes have created stable lineages; H7N7, H3N8. (Berg et al., 1990a; Guo et al., 1995; Oxburgh and Klingeborn, 1999; Ozaki et al., 2001). Some strains have been suggested to be recent introductions from wild birds (Guo et al., 1992).

1.7.3 In canines

Historically, canine species have not been found to carry influenza virus. However, in 2004 an outbreak in racing greyhounds was found to be caused by the H3N8 influenza subtype and is thought to be an equine influenza A virus variant that had adapted to spread in canines (Yoon et al., 2005). During the current outbreak in South East Asia a recent investigation has isolated H5N1 influenza virus from dogs and has also found that antibodies to H5N1 are common in Thai dogs suggesting that they have previously been infected (Butler, 2006).

1.7.4 In felines

Feline species were not considered particularly susceptible to influenza virus prior to the current outbreak of avian influenza H5N1 that started in South East Asia in 2003. However, after captive tigers and leopards (Amonsin et al., 2006; Keawcharoen et al., 2004) became ill and died after having been fed infected chicken carcasses several investigations were performed. It was shown that there was not only direct transmission from the contaminated food but also probable transmission between tigers (Thanawongnuwech et al., 2005). Experimental infection of domestic cats has shown that cats infected with the H5N1 highly pathogenic strain develop lethal systemic infection and excrete virus in both the respiratory and digestive tract secretions. The cats in the experiment could also infect each other (Kuiken et al., 2004; Rimmelzwaan et al., 2006). In Europe, cats have also been found to be infected by the H5N1 virus in areas where there have been outbreaks in wild birds (Anonymous, 2006).

1.7.5 In mink and ferrets

Mink and ferrets have been found to be susceptible to influenza A virus in experiments (Okazaki, Yanagawa, and Kida, 1983). Mink have also been found to be naturally infected by avian Influenza A virus of the subtype H10N4 during an outbreak in farmed mink in Blekinge, Sweden (Klingeborn et al., 1985). Further investigation revealed that the virus strain causing the outbreak in the Swedish mink was most likely of wild bird origin. Although the virus was very similar to avian virus strains, it was adapted to spread in-between mink (Berg et al., 1990b; Englund, 1997; Englund, 2000; Englund and Hard af Segerstad, 1998; Klingeborn et al., 1985; Reinhardt and Scholtissek, 1988).

1.7.6 In seals and whales

In the winter of 1979-80 seals off the coast of eastern USA died of hemorrhagic pneumonia. The cause of disease was found to be influenza A virus of the subtype H7N7. The virus contained avian-like genes, but biologically behaved as a mammalian strain (Geraci et al., 1982; Webster et al., 1981b). During the autopsies and handling of experimentally infected seals, people handling the animals developed conjunctivitis. Influenza virus could be isolated from eye swabs of the affected peoples' eyes (Webster et al., 1981a). In a subsequent outbreak among seals during the season 1982-83, another even more avian-like virus was recovered from seals suffering from pneumonia. This virus belonged to the H4N5 subtype (Hinshaw et al., 1984). Further surveys of seals in the area have also found H3N3 virus strains to be present in seals (Callan et al., 1995).

Whales have been found to be infected with strains closely related to the H13 strains of gulls (Hinshaw et al., 1986; Mandler et al., 1990).

Investigations of the receptor specificity of the sialic acids in whale and seal lungs showed the presence of α2,3 linked sialic acids (Ito et al., 1999) and only a weak association with α2,6 linked sialic acids (Matrosovich et al., 2000). This might explain why these seals and whales are susceptible to infection by avian virus strains. However, seals have also been shown to be infected by influenza B virus of human origin (Osterhaus et al., 2000) and have been found to have antibodies against human influenza A virus of the H3 subtype (Ohishi et al., 2004), proving that they are susceptible to influenza A virus strains of mammalian origin.

1.8 Influenza A virus in domestic birds

Influenza A virus causes a wide spectrum of symptoms in birds, from mild illness to a highly contagious and fatal disease resulting in severe epidemics. The latter is known as highly pathogenic avian influenza (HPAI). This form is characterized by severe illness, rapid death and a mortality in the affected populations that approaches 100 percent within 72 hours. Many different species of reared birds including chickens, turkeys, quail and ostriches are susceptible to epidemics of rapidly fatal influenza (Perez et al., 2003).

The main difference between infection with highly pathogenic virus strains and low pathogenic virus strains is systemic contra localized infection (Suarez and Schultz-Cherry, 2000). The hemagglutinin of strains causing HPAI can be cleaved by ubiquitous proteases and is thus not restricted to cells in the respiratory tract. All outbreaks of the highly pathogenic form have been caused by the subtypes H5 or H7. Influenza A virus of these subtypes may enter into domestic bird populations as low pathogenic strains that only cause mild disease. From these low pathogenic strains highly pathogenic strains then arise by mutation. This is probably due to the extremely high propagation rates in dense flocks. Birds that have already been infected and survived infection with the low pathogenic influenza A virus strain have protection against infection with the highly pathogenic variant (van der Goot et al., 2003). Several mutations may add to the pathogenicity of strains causing HPAI but the accumulation of basic amino acids at the cleavage site is diagnostic for disease outbreaks.

Direct or indirect contact of domestic flocks with wild migratory waterfowl has been implicated as a cause of epizootics (Webster et al., 1992). Spread between farms during an outbreak is most likely caused by the movement of people and the transport of goods (Gilbert et al., 2006). Outbreaks of HPAI are often difficult to control since the virus can persist and remain active for some time in the environment and because it is highly transmissible. In areas with dense poultry populations and or limited resources for surveillance and control, outbreaks have lasted for years. This has been the case in Mexico (1992-95), Italy (1999-2000) and with the ongoing outbreaks in South East Asia and Africa.

1.9 Influenza A virus in wild birds

1.9.1 The wild bird reservoir

To date the search for a reservoir of influenza A virus in nature has pointed towards wild birds as being pivotal in the transmission and persistence of the virus. It is thought that all influenza virus strains infecting mammalian species originate from wild birds (Webster et al., 1992). The evidence for the existence of a wild bird reservoir is strong. Influenza A virus has been isolated from birds on all continents except Antarctica from where there is only serological evidence (Austin and Webster, 1993; Morgan and Westbury, 1981; Wallensten et al., 2006). However, most studies have taken place in developed countries and the situation in Africa, South America and parts of Asia is much less explored. All 16 HA and 9 NA subtypes in the majority of possible combinations have been isolated from avian species (Alexander, 2000). Most influenza virus strains found in wild birds have been defined as low pathogenic. Although low pathogenic influenza A virus strains have been isolated from more than 105 species from 26 different families of birds (Olsen et al., 2006; Stallknecht and Shane, 1988) almost all isolates come from the families *Anseriformes* and to a lesser extent *Charadriformes* and *Larida*e. These families include birds such as ducks, geese, swans, waders and gulls, which although they are different species evolutionary speaking, share the trait of being adapted to life in an aquatic environment. Isolations of low pathogenic virus strains from pure land-dwelling birds are on the contrary rare.

Indirect evidence that wild birds constitute a reservoir for influenza A virus comes from studies on viral evolution. These studies have shown that influenza A virus strains in wild ducks show only limited evolution over time. It has therefore been suggested that influenza A virus exists in an evolutionary stasis in the reservoir species (Bean et al., 1992; Webster et al., 1992). In support of this suggestion, analysis of strains recovered from wild ducks that had been preserved in museums since the early 20th century showed almost no antigenic drift when compared to modern avian strains (Reid et al., 2003a). This situation is very different from the situation when influenza A virus is introduced into mammals. Avian strains that are introduced into a new host species are evolving at high rates (Zhou et al., 1999). The low rate of viral evolution in ducks suggests that adaptation to the host has reached an optimum. New variants have no survival advantage and are thus not successfully sustained. It has also been

found that co-infections of different influenza virus strains are detected less frequently in ducks than in other species, suggesting that host adapted strains prevent co-infection by other strains (Sharp et al., 1997). Considering that the *Anseriformes* species have existed for millions of years; influenza virus has had ample time for this adaptation to take place (Shortridge, 1992).

There are many reasons why species connected to aquatic environments are good reservoir species for the virus. These are elaborated on in more detail further on in the text, but the implication for the wild bird reservoir is mentioned here.

The populations of *Anatidae, Charadriformes* and *Larida*e species in the world are large, the Mallard (*Anas platyrhynchos*) population by itself is estimated to 27 million birds (Kaleta, Hergarten, and Yilmaz, 2005). Most populations present in different areas are also connected as these birds travel over large distances and congregate in large numbers at specific important staging sites during migrations (Arzel, Elmberg, and Guillemain, 2006). Wild duck populations could therefore be hypothesized to support the perpetuation of even short-lived infections such as influenza, as there are enough susceptibles at any given time. The minimum population to support the perpetuation of the measles virus has been estimated to be 500 000 humans (Nathanson, 2005), and although the attack rate of influenza A virus in ducks might be lower, the population size of ducks is probably still large enough. Also, certain aspects of duck demography and ecology might make the minimum population needed even lower. First of all, the populations of ducks have high turn over rates. In Mallards about one third of the population is replaced each year, implying that this proportion is immunologically naïve (Bentz, 1985). Further, as ducks infected with low pathogenic influenza A virus strains do not appear to be severely affected by the disease, the infection does not seem to limit an infected bird's interaction with other birds or the environment. Nor does infection with low pathogenic strains seem to limit the capability for long distance flights that could transport the virus long distances to new susceptible flocks. Since virus is shed by infected birds in high quantities into an environment where it can survive for an extended time, the feeding and social behavior of the reservoir species makes it likely that susceptible individuals are exposed to the pathogen.

Wild birds living in aquatic environments are thus a reservoir for influenza A virus in nature. Questions that remain to be answered are to what extent different species of birds are involved and indeed if wild birds are the only reservoir in nature?

1.9.2 Geographic constraints

Even though influenza A virus in the wild bird reservoir has been said to be in “evolutionary stasis”, there have been slight changes over time. Different genetic lineages of influenza A virus have evolved in bird populations with that are separated by oceans. There are only a limited number of wild bird species that migrate across the Atlantic or the Pacific Ocean, resulting in limited interaction between the populations of Eurasia and the Americas. Avian influenza A virus strains of the Americas can thus be separated from those in the rest of the world (Donis et al., 1989; Schafer et al., 1993; von Hoyningen-Huene and Scholtissek, 1983). There are also interregional differences in the prevalence of subtypes in different duck populations that use different migratory routes within continents (Hinshaw et al., 1985), again probably as a result of limited interaction with the other populations. Lately, however, limited transmission of genes between the North American and Eurasian populations have been reported (Liu et al., 2004; Makarova et al., 1999; Schafer et al., 1993; Wallensten et al., 2005) indicating that the interaction that does take place is sufficient for exchange to occur. Knowledge on the interaction and spread of pathogens between different fly-ways (figure 1.9.2.1) and continents may be crucial in estimating the spread of highly pathogenic variants between different areas of the world. Therefore, increased knowledge on this topic is urgently needed.

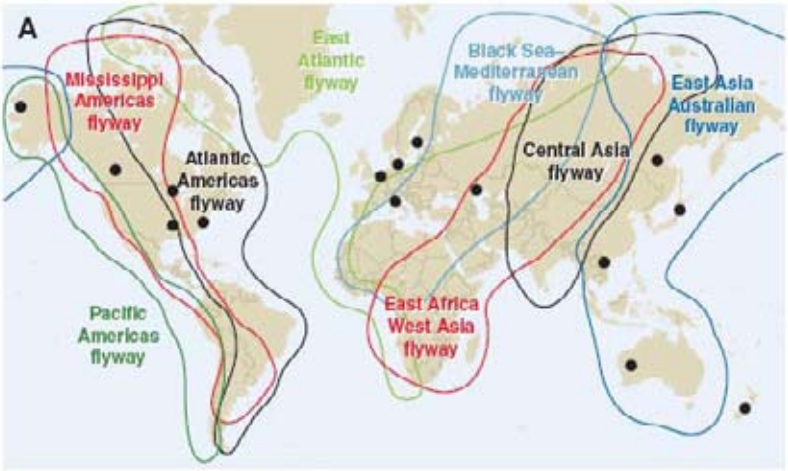

Figure 1.9.2.1. Map showing the general migratory fly-ways of wild birds (adapted from an original produced by Wetlands international and reproduced from the article Global patterns of influenza A virus in wild birds (Olsen et al., 2006). Black dots mark locations were screening of wild birds for influenza A virus has taken place.

1.9.3 Species preference

The species preference of influenza A virus is likely to be determined by the mode of transmission, i.e. by the fecal-oral route via contaminated water. Species that feed in shallow calm waters, where influenza A virus is found in the highest concentrations, run the highest risk of becoming infected. In line with this argument species like ducks, geese, waders and gulls have the highest prevalence of influenza A virus (Olsen et al., 2006). Scavenger species such as raptors that may feed on diseased birds are also likely to become infected, but not to take part in efficient transmission as they do not dwell in water.

Although almost all possible subtype combinations have been found in the wild bird reservoir, some subtypes of HA have only been isolated from certain species (Fouchier et al., 2005; Sharp et al., 1993). In addition, isolates from shorebirds and gulls do not always replicate in ducks. This indicates that there are species preferential lineages within the wild bird reservoir (Kawaoka et al., 1988), although this species specificity may not be relevant for all genes of the influenza A virus (Widjaja et al., 2004).

1.9.4 Modes of transmission

Wild ducks readily get infected with avian strains of influenza A virus through the intake of contaminated food and water. The virus still remains active after passage through the low pH of the duck gizzard and produces infection in the cells lining the intestinal tract (Shortridge, 1992). The infection may also affect cells in the lungs of ducks. During the period of infection, large amounts of virus of up to 10^8 EID^{50} is shed in the duck feces for about seven days (Webster et al., 1978) and sometimes for as long as 21 days (Kida, Yanagawa, and Matsuoka, 1980). Progeny virus is to a lesser extent also shed from the trachea. The fact that infected birds shed high amounts of influenza A virus via feces implies that birds living in aquatic environments will contaminate the water where they live. Influenza A virus of different subtypes has been isolated in concentrations of up to $10^{2,8}$ EID^{50} /ml of water from unconcentrated water in lakes where wild ducks congregate (Hinshaw, Webster, and Turner, 1979; Ito et al., 1995). Since the virus remains viable for some time in water, it permits transmission to other birds in the area that ingest contaminated water. Influenza A virus strains was even recovered from lake water for about a month after the birds in the lake had migrated south for the winter, indicating that lakes may be a source of infection for other birds for a long time. These data indicate that fecal-oral transmission via water is the most likely and efficient mode of transmissions for low pathogenic strains in wild birds.

Highly pathogenic strains may transmit in other ways. Unpublished data from recent research presented by dr. Fouchier at the FAO/OIE conference on Avian influenza and wild birds held in Rome 30-31 May 2006, show that the highly pathogenic H5N1 strain causing the ongoing outbreak that started in South East Asia is primarily excreted from the respiratory tract of infected birds.

1.9.5 Clinical picture and immunity

All birds are thought to be susceptible to infection with influenza A virus, although some species are more resistant to infection than others. Depending on the subtype, strain, host species and individual, the disease caused by infection with influenza virus may range from non-pathogenic to lethal (Laudert, Sivanandan, and Halvorson, 1993). Even within the duck family there are many different species of wild and domestic ducks and they may also show different responses to infection with influenza A virus (Suarez and Schultz-Cherry, 2000).

Infections by low pathogenic strains in ducks have traditionally been considered un-harmful, as there are no evident clinical signs of disease. This notion may not hold as more investigations into the effects of infection by these strains are studied. Clinical symptoms may be hard to detect. It is difficult to evaluate if the birds are completely free of symptoms or actually sick in a subtle way. The latter suggestion is supported by experiments in infected ducks that showed histopathological signs of mild pneumonia even though no clinical signs of disease were evident (Cooley et al., 1989).

Highly pathogenic strains behave differently and strains that are highly pathogenic for chicken may cause milder disease and different signs of disease in other species (Perkins and Swayne, 2003). They may even show no signs of disease in some more resistant species of ducks (Alexander et al., 1978; Cooley et al., 1989; Kishida et al., 2005) and gulls (Perkins and Swayne, 2002). Due to the lack of evident effects on the birds' health status, ducks and gulls may act as carriers of some highly pathogenic strains. If these strains persist in the wild bird reservoir, the eradication of such strains from domestic bird populations will be difficult due to the risk of re-introduction from wild birds.

Experimental infections of ducks have revealed that ducks only produce a short-lived low level humoral immune response and a weak cell mediated immune response as seen by suppressed T-cell function and enhanced macrophage phagocytic activity (Laudert, Sivanandan, and Halvorson, 1993). Studies have also shown that ducks may be reinfected with the same strain after only two months (Kida, Yanagawa, and Matsuoka, 1980) indicating that the protection of acquired immunity is poor. However, in large-scale studies, juvenile

ducks are found to be infected with influenza A virus more frequently than adult birds (Webster et al., 1992), indicative of some sort of acquired immunity or improved immune response. Ducks are readily co-infected by different strains and subtypes paving the way for reassortment between different influenza A virus strains and creation of new variants (Sharp et al., 1997).

Other bird species such as chicken, pheasant, turkey and quail do show a humoral response with Ig M and Ig Y production (Suarez and Schultz-Cherry, 2000). In poultry, previous infection with a low pathogenic strain provides protection from disease caused if the low pathogenic strain subsequently evolves into a highly pathogenic strain (van der Goot et al., 2003).

1.9.6 Enzootic cycle

A clear picture of the enzootic cycle of influenza A virus in wild birds does not exist. More research is needed to elucidate the different stages involved in the interaction between the virus, the host and the environment. It seems likely, however, that nestlings and juvenile birds are exposed to and probably contract several infections with different influenza A virus strains present in the surrounding environment early in their life. During subsequent migration the birds may encounter even more strains as they mingle with other flocks of birds and are exposed to virus contaminated environments.

As prevalence of influenza A virus has been shown to be higher in juvenile than in adult birds; the input of juvenile and thus immunologically naïve birds are most likely of key importance for upholding the number of susceptible birds in the population. As mentioned earlier, ducks and the other reservoir species have a high input of juveniles each year and few birds get old ensuring a high number of susceptible individuals.

Another key issue of the enzootic cycle is the persistence of the virus in different environments as the infection primarily passes on from one bird to another through fecal contamination of the environment and the subsequent ingestion of infective material. The interaction between different host species and the environment must therefore be of crucial importance in the enzootic cycle in addition to the interaction between different bird species and different flocks of the same species. These determinants are likely to be important for the distribution of virus strains around the globe and deserve more attention.

One of the enigmas of influenza A virus ecology is how so many subtypes can circulate in the wild bird populations and persist from year to year, when some of these subtypes are isolated rarely and since the prevalence in the studied bird populations differ greatly between

studied species, place and time of year. Studies in North America have shown that the prevalence in some species of ducks is highest in the fall when up to 30 percent of the ducks may be infected (Webster et al., 1992). These numbers dwindle to levels close to or below one percent during winter (Stallknecht et al., 1991) (although, more recent studies suggest that this may not be true for all areas of North America (Hanson et al., 2005)) and remain low during spring and summer. In other North American studies a reversed prevalence picture has been found in shorebirds that have high prevalence during the spring and low prevalence during the fall (Kawaoka et al., 1988; Krauss et al., 2004). While some subtypes are frequently isolated, others have been isolated only rarely in specific places or in specific species, such as the H13 and H16 subtypes that have almost exclusively been isolated from gulls. A lot of these variations and peculiarities are likely to be explained in the future when more data on the prevalence of different subtypes in different species and at different locations is available, but to date, the explanations remain elusive. Some suggestions have, however, been put forward as to how different influenza A virus subtypes and strains are perpetuated.

The first one suggests that influenza A virus is continuously circulating within the reservoir species. This requires that a sufficiently large number of individuals in the wild bird population are infected at any given time to pass on the specific strain or subtype to new individuals. Differences in prevalence of different subtypes may be explained by the immune status of different populations of birds. When herd immunity in a population of birds falls the birds will once again be susceptible to infection and the prevalence of the particular subtype causing infection will rise again (Webster et al., 1992).

A second suggestion claims that influenza A virus may survive and remain active for extended periods in the environment. The virus may for example persist frozen in lakes on the birds' breeding grounds and be passed on to susceptible birds when they return from wintering in other areas. In support of this, influenza A virus has been isolated from lake water several months after the birds in the lake had migrated south for the winter (Ito et al., 1995). However, virus has not been isolated from lakes in the spring before the migratory birds return.

A third suggestion has been that different species that have high prevalence of influenza A virus during different seasons of the year and share the same habitat may interact and pass on the infection to each other (Kawaoka et al., 1988). It has been proposed that ducks and waders interact in such a way since it has been found in North American studies that the prevalence of influenza A virus infection in shorebirds is high during the spring and low in the fall, while in ducks it is high during the fall and low in the spring.

More research is needed to determine which of these theories, or if a combination of them, holds true.

1.9.7 Spread by wild birds

Most low pathogenic influenza A virus strains do not seem to hinder birds from migrating. Thus, these virus strains may be carried over large distances by the birds either through non-stop long distance flights or in a relay pattern where one bird carries the virus a short distance and another carries it further. Until the present outbreak of H5N1 that started in Asia it was not believed that wild birds could be infected with highly pathogenic virus strains and still perform long distance migrations. As some species of ducks have been shown to be resistant to these strains this belief has had to be reviewed. The sudden outbreaks in wild birds in China and the subsequent appearance of H5N1 in wild birds in Russia, the Middle East, Europe and Africa have shown that the transport of highly pathogenic strains by wild birds is a reality.

Primary introduction of influenza A virus into poultry and domestic animal holdings are likely due to fecal contamination by wild birds either directly by contamination of the holdings or indirectly through contaminated water supplies or feed. Holdings where wild birds and domestic birds share the same habitat due to agricultural practices are at the highest risk for outbreaks (Gilbert et al., 2006) suggesting that wild bird transmission is the most common route. Transmission to new holdings in an area where an outbreak has occurred may well be the result of spread by the movement of contaminated goods, animals and people.

Further research needs to clarify to what extent wild birds are responsible for the transmission of influenza A virus strains around the globe and into domestic bird populations in relation to the smuggling and transport of infected animals and goods. By studying these underlying mechanisms accurate bio-security measures can be taken to hinder such transmission.

2.0 AIMS OF THE STUDY

i) To increase our knowledge on the ecology of influenza A virus in wild birds with special emphasis on

- which wild bird species carry influenza A virus?
- which are the different subtypes and strains of influenza A virus in these species?
- which is the seasonal variation of influenza A virus prevalence?
- are there geographical differences in prevalence?
- how is influenza A virus perpetuated in wild birds?

ii) To gain a better understanding of the connection between influenza A virus in wild birds and outbreaks in other species such as poultry and if surveillance of influenza virus in wild birds can be of predictive value and aid in hindering such outbreaks.

iii) To integrate ecological, ornithological and molecular virological methods and knowledge in order to get a more complete understanding of influenza A virus in the wild bird reservoir.

Figure 3.1.1. Ottenby Bird Observatory and the lighthouse on the southern tip of the island Öland, Sweden. (photo: Ingvar Eliasson)

3.0 POPULATIONS, SAMPLING SITES AND SAMPLING TECHNIQUES

3.1 Populations

Data for the studies in the thesis was gathered from fecal samples taken from wild birds. The main sample site was Ottenby Bird Observatory on the island Öland in southern Sweden (paper I, III and IV). Additional samples were gathered at the island Bonden outside Umeå in northern Sweden (paper II) and from sites such as Lekkerkerk and Krimpen an der Lek in the Netherlands (paper I and III). The locations of the sampling sites are shown in figure 3.1.2. Ottenby Bird Observatory (figure 3.1.1.) is situated on the southern tip of the narrow island Öland where birds congregate during migration. In the fall, it is their last chance to rest before a long crossing over open sea and during the spring a good place to land after having crossed the sea. Banding (ringing) of birds at Ottenby Bird Observatory has been ongoing since 1946 and banding of ducks caught in a trap was previously carried out between 1962 and 1982 (von Schultz, 1986).

The data gathered by the banding activities and the subsequent recoveries of banded birds provide us with good knowledge on the migratory patterns for different bird species. The Mallard samples gathered for paper III and IV can therefore be said to have been taken from birds that belong to populations that breed in northern Fennoscandia, Finland and Russia and winter in western Europe (Denmark, northern Germany, The Netherlands, England and northern France). Recoveries of Mallards banded at Ottenby Bird Observatory are shown in figure 3.1.2. The Black-headed Gull population studied in paper I breed mainly in the Baltic region and winter in north-western Europe. Finally, the Guillemots sampled in paper II breed on the island Bonden and mainly winter in the southern Baltic Sea although some individuals migrate as far as the southern part of the North Sea.

The birds studied in this thesis not only show different migratory behavior, but also belong to different feeding guilds. Mallards, being a dabbling duck, feed mainly on water plants, insects and mollusks by sieving water in shallow waters (Arzel, Elmberg, and Guillemain, 2006). Black-headed Gulls are scavengers feeding on all sorts of food from mollusks to fish. Guillemots feed solely on fish.

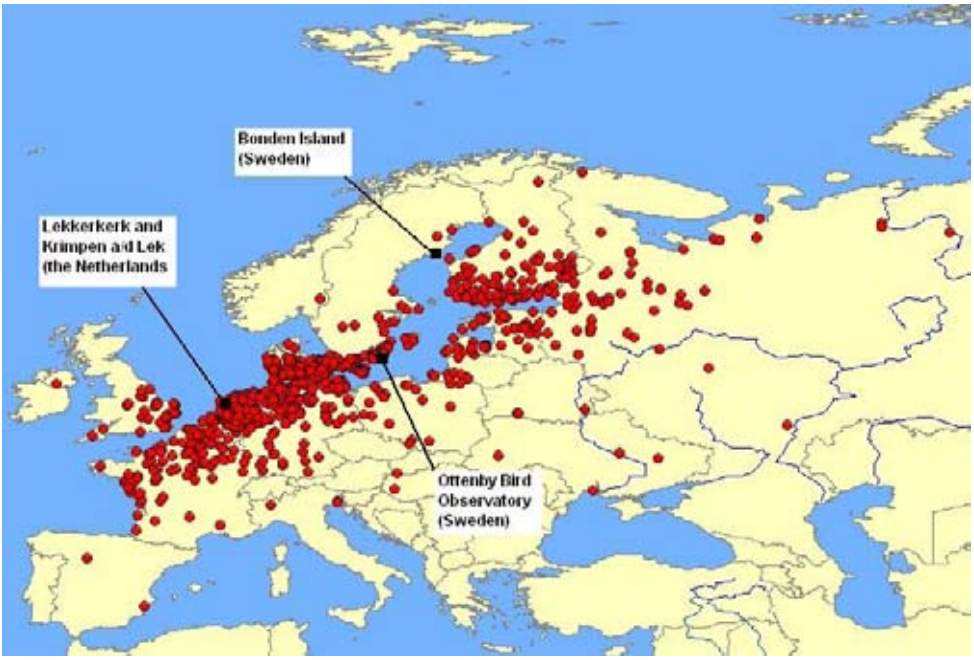

Figure 3.1.2 Map showing the sampling sites used in the studies and places from which recoveries of Mallards banded at Ottenby Bird Observatory have been reported (Marked by dots**).**

3.2 Trapping methods

The trapping of ducks at Ottenby Bird Observatory was resumed as part of our project starting in the fall of 2002. The trap used is a large funnel placed partly on land and partly in water (figure 3.2.1). The ducks were being attracted to the trap by live ducks kept captive in a separate compartment as well as by decoy ducks outside the trap, and the feeding with wheat. Ducks that swam into the trap were then sampled. Gulls were caught in walk-in traps used for capturing wader birds (figure 3.2.2). Guillemots were caught using hand nets.

Figure 3.2.1. The funnel trap used to catch waterfowl. (photo: Ottenby Bird Observatory)

Figure 3.2.2. The cages used to catch shorebirds and gulls. (photo: Ottenby Bird Observatory)

3.3 Measurements and sampling

All birds were identified regarding species, sex and when possible age. They were banded and biometrical data such as wing and beak length, weight and body fat measurements were collected. Fecal samples were collected from all birds since fecal samples have been considered the sample of choice when sampling avian species (Webster et al., 1978). At Ottenby Bird Observatory the collection of fecal samples was mainly done by putting the birds into boxes where they left droppings. In between each bird, the bottom layer was

exchanged in order to avoid cross-contamination. This method, being the least stressful for the birds was preferred to cloacal sampling with cotton swabs, which was done when this sampling method was not applicable. All fecal samples were put into vials containing a balanced salt solution and antibiotics. At Ottenby Bird Observatory samples were immediately frozen in a -70 °C freezer. Samples collected during field trips (paper II) were immediately stored on dry ice until they could be put in a freezer. The fast freezing and rapid analysis of samples appear to be critical for the conservation of RNA in the samples (Stone et al., 2004) and great effort was made to keep an intact cold chain.

When serological samples were collected, blood was drawn either from a neck vein or a wing vein.

Figure 3.3.1. Black-headed Gull (top) (photo: Ingvar Eliasson) and Guillemot (bottom) (photo: Owe Fredriksson)

4.0 METHODOLOGICAL CONSIDERATIONS

4.1 Screening for influenza A virus: RNA-isolation and virus detection

The screening of patient samples for the presence of influenza A virus using reverse transcription-polymerase chain reaction (RT-PCR) has been evaluated in many studies and been found to have a high specificity and sensitivity (Hindiyeh et al., 2005; Smith et al., 2003; Stone et al., 2004). Some studies have also evaluated methods more adapted to suit the detection of avian influenza virus strains (Cattoli et al., 2004; Fouchier et al., 2000; Lee and Suarez, 2004; Spackman et al., 2003a; Spackman et al., 2003b).

The fecal samples collected in studies I-IV were screened using RT-PCR. Different RT-PCR-methods were used at the different laboratories that cooperated in paper IV. In brief, RNA was isolated from the samples using commercially available RNA-isolation kits according to the manufacturers' instructions and from 2003 and onwards by the use of automated procedures. A reverse transcriptase (RT)-step creating a cDNA amplicon was carried out. Influenza A virus RNA was detected using primers directed at conserved regions of the M-gene of the influenza virus and amplified using PCR. From 2003 onwards all samples were screened with real time PCR technology allowing for quantification analyses and minimizing the risks of cross-contamination of samples caused by post amplification sample handling (Spackman et al., 2003a). At the Erasmus Medical Center in Rotterdam, the Netherlands, a real time PCR with Taqman™ probes was used, developed by Fouchier et al (Fouchier et al., 2000) (papers I, II, III and IV) while at the Swedish Institute for Infectious Disease Control (SMI) and at Kalmar University a real time PCR-method, developed and described by Karlsson and co-workers (Karlsson et al., submitted), using SYBR® Green was used. The Taq-man method uses a probe that is designed to bind in between the nucleotide sequence determined by two primers. When the polymerase replication takes place, the probe is cleaved and fluorescent light emits. SYBR-green is a dye that only binds to double stranded DNA. When the PCR product determined by the primers is amplified and hybridized the dye binds and emits light. Both these methods can be used to measure the amount of the desired PCR products as the amount of light emitted is proportional to the PCR product. The amount of nucleotide template in the original sample can also be determined, since the more template

molecules present at the beginning of the reaction, the fewer cycles it takes to reach the point at which the fluorescent signal is first recorded. The specificity of the reactions was also controlled by the evaluation of melting curves and when using the SYBR-green technology, samples were analyzed in duplicates or triplicates. The different methods were evaluated in in-house tests and the two different SYBR-green methods were shown to have similar sensitivity. Using the Taq-man approach with the combination of specific primers and a specific probe should theoretically be more specific than using SYBR-green technology. It is, however, more expensive than the SYBR-green method, since more primers and probes are needed. The SYBR-green method might have an advantage when it comes to detecting different variants of influenza A virus as it may allow for the amplification of strains with more variations in the nucleotide sequence. It might, however, be less specific due to the fact that unspecific binding may occur to non-specific reaction products including primer-dimers.

4.2 Isolation of influenza A virus

Virus isolation was performed on all samples that were positive by RT-PCR. Egg culturing was used since it works better than culturing on existing cell lines (Nicholson, Webster, and Hay, 1998) and since it renders infectious progeny virus. In these studies, 200µl of the original samples was inoculated into the allantoic cavities of 11 day old embryonated hens' eggs. The allantoic fluid was harvested after 2 days and influenza A virus was detected by using hemagglutination assays with turkey erythrocytes. In 1951, Hirst discovered that red blood cells in suspension fail to sediment and instead agglutinate forming a lattice. Since then the hemagglutination phenomenon has been used for the detection and characterization of influenza virus (Nicholson, Webster, and Hay, 1998). If no Influenza A virus was detected, the allantoic fluid was passaged once again in embryonated hens' eggs for one more isolation attempt.

Only about 50 percent of the PCR- positive samples could be isolated, indicating that there are limitations of this method. These limitations could be due to different reasons. The virus propagation step might require a certain amount of virus in the original sample, or the virus might not grow in the egg, or perhaps the RNA- fragments amplified were not parts of functional virus. Another limitation when using egg culturing is that it may not always propagate all strains present in a sample. Studies have shown that more than one influenza virus strain might be present in fecal samples (Hinshaw et al., 1980). Multiple strains can not

be told apart in the initial PCR step and when grown on eggs one strain might be better suited for replication and dominate the culture, in which case the other strain may not be detected.

4.3 Characterization of influenza A virus

4.3.1 Hemagglutinin and neuraminidase inhibition tests

The virus isolates obtained by egg culture were characterized to HA-subtype with a hemagglutination inhibition assay using turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all known HA subtypes (paper I, III and IV) and by RT-PCR and sequencing (paper II).

Virus isolates were characterized to NA-subtype by using RT-PCR and sequencing (paper II, III and IV), and in paper I also by the use of a neuraminidase inhibition assay with subtype-specific hyperimmune rabbit antisera raised against all known NA subtypes.

4.3.2 Serology

In paper I and III, sera from our samples were set to react in hemagglutination tests with cultured influenza A virus strains.

4.3.3 Immunization

DNA immunization of rabbits was carried out by repeated injections of HA gene segments cloned in expression plasmids (paper I).

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4.3.4 Double immunodiffusion assays

Antisera and antigens were loaded in wells stamped in a gel approximately one cm apart. The gels were then put in a humidified room for 48 hours at room temperature. After a washing step, they were covered with filter paper and dried overnight. The dried gels were then stained with Coomassie brilliant blue and analyzed.

4.3.5 Nucleotide sequencing and phylogenetic trees

RT-PCR was performed using primers either for conserved non-coding regions of separate genes such NA (paper I, III and IV) and HA (paper I and III), or for all genes (paper II). PCR products were purified from agarose gels and sequenced using a sequencing robot. The specific programs and statistical algorithms used are described in the individual papers.

Phylogenetic trees were constructed in papers I, II and III to show the relationship between different subtypes and different strains of Influenza A virus.

5.0 RESULTS AND GENERAL DISCUSSION

5.1 Influenza A virus in different species of wild birds (papers I, II, III, IV, V)

Since the first findings of low pathogenic influenza A virus among wild birds in 1974 (Slemons et al., 1974), investigations have pointed to wild birds as being a reservoir for many different influenza A virus strains. Early sampling showed that the largest amounts of influenza A virus isolations could be made from samples taken from birds living in aquatic environments such as ducks, geese, waders and gulls (Webster et al., 1992). We performed a meta-analysis on data from all published surveys (known to us) of isolations of influenza A virus from wild bird species. Extracts are presented in tables 5.1.1. and 5.1.2. From this analysis we conclude that isolations are by far most frequent from dabbling ducks (9.5 %) and to a lesser extent from other species living in aquatic environments (>1.7 %). Isolations from non-aquatic species, represented here by the passerine species, exist (0.7 %), but are not common. It should be noted, however, that most existing data have been gathered from aquatic species and from species that are easy to catch.

Much of today's knowledge of the ecology of influenza A virus in wild birds is derived from large studies on the prevalence in wild birds carried out in North America (Krauss et al., 2004). In order to assess the prevalence and distribution of influenza A virus in wild birds in Sweden, and thus a western Palaearctic bird population, we performed a large study at Ottenby Bird Observatory. There we caught and sampled Mallards, and to a lesser extent other dabbling ducks such as Eurasian Teals (*Anas crecca*), Northern Pintails (*Anas acuta*) and Shelducks (*Tadorna tadorna*). When analyzing the material from four years of sampling (described in paper IV), we found that the dabbling duck population had a similarly high prevalence of influenza A virus infection as had previously been found in other large surveys. Out of all Mallard samples collected at Ottenby Bird Observatory 14.5 percent were positive for influenza A virus as compared to 22.2 percent in wild ducks in the North American study (Krauss et al., 2004) and 8.7 percent in a German study (Suss et al., 1994).

Our data thus strongly support the idea that dabbling ducks are the main reservoir for influenza A virus in nature. However, there may be reservoir species that are not dabbling ducks and dabbling ducks may not be a reservoir for all subtypes. Therefore, we sampled other species that we thought were potential carriers.

In paper I we describe the findings of influenza A virus isolated from samples collected from ten juvenile Black-headed Gulls caught in August 1999. Six samples were positive for influenza A virus by PCR. Out of these, five influenza A virus strains could be isolated by egg-culture. Only one of these strains, an H13 strain, could be characterized using a panel of antibodies directed towards subtypes known at that time. After confirmatory tests, and by using additional techniques, the un-characterizable virus strains were designated as belonging to a new subtype, H16. This new subtype was most closely related to the H13 subtype, previously reported to exist almost exclusively in gulls (Kawaoka et al., 1988). Whether or not the H16 subtype is yet another gull specific influenza virus subtype remains to be seen.

In paper II we describe the results of our investigations in Guillemots. The Guillemot is a species belonging to the *Alcidae* or Auk family of birds. Previous studies have demonstrated antibodies in auks and a single isolation of a human-like H2N3 influenza virus has been published from an investigation in eastern Russia (Sazonov et al., 1977). In our study, we screened the Guillemot population at Bonden Island in the northern Bothnian Gulf for the presence of influenza A virus by collecting fecal samples. Samples from 3 out of 26 captured birds tested positive for influenza A virus. One influenza A virus strain could be cultured and isolated from these three samples, and another one could be partly sequenced. Further analysis revealed that these two virus isolates were of the H6N2 subtype.

Since the Russian influenza A virus strain isolated in a Guillemot was similar to concurrently circulating human strains, our findings represent the first isolation of a non-human type influenza A virus from an auk. It also represents the first recorded isolation from Guillemots and the *Alcidae* family of birds in Europe.

If auks represent a reservoir for influenza A virus, or if they are simply accidental hosts remains to be elucidated.

In conclusion, the results from our screening show that influenza virus and sometimes new subtypes of influenza virus can be found in multiple species of birds connected to aquatic environments. The studies strengthen the notion that dabbling ducks are the primary reservoir of influenza A virus in nature and that gulls may harbor separate subtypes of the virus. Some of the other species investigated and found to be positive for influenza A virus might be accidental hosts and not natural hosts that are part of the virus' enzootic cycle.

Table 5.1.1. A summary of the prevalence of influenza A virus in different families or subgroups of birds according to all published studies, where a total of at least 500 tested birds was a criterion for inclusion.

Family or subgroup	n. sampled	n. positive	Prevalence %
Dabbling ducks	28,995	2755	9.5
Diving ducks	1,011	16	1.6
Geese	4,806	47	1.0
Swans	5,009	94	1.9
Gulls	14,505	199	1.4
Terns	2,521	24	0.9
Waders	2,637	21	0.8
Rails	1,962	27	1.4
Petrels	1,416	4	0.3
Cormorants	4,500	18	0.4
Passeriformes	3,678	26	0.7

Table 5.1.2. Numbers sampled and prevalence in dabbling duck species caught at Ottenby Bird Observatory between the years 2002-2005

English name	Scientific name	n. sampled	n. positive	Prevalence %
Mallard	Anas platyrhynchos	4106	575	14.0
Eurasian Teal	Anas crecca	62	8	12.9
Northern Pintail	Anas acuta	30	3	10.0
Common Shelduck	Tadorna tadorna	511	14	2.7

5.2 Subtype distribution in wild birds (papers I, II, III, IV, V)

In paper IV we describe the distribution of subtypes found during two years of surveillance of ducks caught during migration at Ottenby Bird Observatory. We isolated low pathogenic influenza A virus strains from 134 culturable fecal samples. All of these were from Mallards except five (three virus strains of the subtype H3N8 and one of the subtype H3N3 were isolated in samples from Eurasian Teals, and one virus strain of the subtype H6N2 was isolated in a sample from a Northern Pintail). Influenza A virus of all 9 NA subtypes and 11 out of 16 recognized HA subtypes were found among the isolated strains. The NAs and HAs were found in 40 different combinations (table 5.2.1). In specific the HA-subtypes H4, H6, and H7 were isolated most frequently, followed by H1-H3, H5 and H10, H11 while the H8 and H12 subtypes were isolated only rarely. The subtypes H9 and H13-16 were not isolated. Of the NA subtypes found, N2, N6 and N7 were most common, while N4 and N5 were uncommon. The most prevalent subtype combinations were, H4N6, H7N7, and H6N2.

The great diversity of virus subtypes and subtype combinations detected in our study was striking, but not very different from results obtained in long term studies performed in North America and Germany (Krauss et al., 2004; Suss et al., 1994). Differences in the prevalence of subtypes and seasonality of influenza A virus prevalence were found as discussed below. A summary of this comparison is found in table 5.2.2. It should be kept in mind that the differences found may be an artifact due to the limited scale of these studies and the limited number of sampling sites used in the studies. The differences may disappear as more studies are performed. Recent studies carried out in other parts of North America than the long term study by Krauss and co-workers found results that are more in line with our data (Hanson, 2003; Hanson et al., 2003; Hanson et al., 2005).

When compared with the large study performed in Germany between the years 1977-1989, the general frequencies of isolation of different HA and NA subtypes in our study were similar, but some subtypes like H1-H3 were isolated less frequently while H6 was isolated more often. Of the subtypes known to cause HPAI outbreaks in poultry, the H7 subtype was similarly common in both studies, but the relative frequency of H5 isolations was higher in our study. When comparing the NA isolations we found that N1 and N3 were less prevalent and N2 and N7 more prevalent. We found that the subtype combinations that were the most prevalent in the German study were also the most prevalent in our study indicating that the prevalence of different subtype combinations remains similar over time.

In comparison with the North American study performed in Canada the frequency of isolations of different HAs and NAs was similar, but H5 and H7 virus strains were isolated more often in our study than in the Canadian study. The most common subtype combinations were also similar in the two studies with the exception of H7N7 that seems to be rare in North America. It is intriguing that some subtype combinations not only seem to be prevalent throughout the years, but also in different geographic areas of the world. This is true despite the fact that geographic obstacles have resulted in separate influenza A virus lineages in North America and Eurasia due to the limited contact between the bird populations of the two continents.

The prevalence of different subtypes found in our study varied between the years and within the different seasons. If we combine this information with information on duck migration as shown in figures 5.2.1 a and b, we can conclude that different subtypes appear to arrive with different populations migrating from different breeding areas. Mallards show a diverse migration pattern complicated by the fact that male ducks are less likely to return to the same breeding grounds during subsequent years since they follow the females and may switch mate

between years. However, we used the banding data to draw maps of recoveries of female Mallards banded at different times of the year. These maps revealed that Mallards banded in southern Sweden during the fall, belonged to a different migratory population than Mallards banded in the same place during summer. Females present during October – December were usually recovered the following breeding season (May – August) east of the Baltic Sea, in Finland, Russia and the Baltic States (5.2.1.A). In contrast, females banded during summer were usually found in the same area of Sweden or in Denmark during the following breeding season (Figure 5.2.1.B). Both groups of females wintered mainly in the coastal areas of western Europe, from southern Sweden to France and Great Britain, with the mean recovery position of females banded during late fall more to the southwest than those banded in summer. Subtypes appearing later in the fall thus arrive with ducks breeding further east. More data is needed to verify this information but it could give us new insight into the perpetuation of different subtype patterns in different geographic regions.

The subtypes H13-16 appear to be rare and perhaps not associated with disease in ducks. H13 is most often isolated from gulls and this may also be the case for H16 according to our data (paper I). More investigations of the prevalence of different influenza A virus subtypes of gulls are needed to verify this hypothesis. Likewise, the finding of H6N2 in Guillemots is so far only an indication of prevalence of subtypes in that species (paper II).

Paper IV provides information about the prevalence of the H5 and H7 subtypes in wild birds in Europe. Out of 172 virus isolates collected in Sweden and the Netherlands in the period October 1999 to January 2002, 33 samples contained HA genes of subtypes H5 and H7. Thus 19 percent of all isolated strains were from subtypes that are known to be prone to change into HPAI when introduced into poultry. The knowledge of the prevalence of these subtypes may be of great importance for the evaluation of the risk of introduction of these strains into domestic birds.

During the course of our studies, we did not find any highly pathogenic strains in the samples from wild birds. This thesis only contains samples collected until December 2005 and only subtyping data from samples collected until July 2004. Therefore, we could not expect to find the highly pathogenic H5N1 influenza A virus strain that has spread from Asia and subsequently caused outbreaks in wild birds in Europe during most of the time of our study. We did not even isolate any low pathogenic H5N1 strains indicating that the H5N1 subtype has not been a common subtype in the studied duck population during the years of the study. It also highlights that highly pathogenic virus strains have been uncommon in the wild bird reservoir.

Table 5.2.1. Hemagglutinin (HA) and Neuraminidase (NA) subtypes and subtype combinations found in Mallards sampled at Ottenby Bird Observatory 2002-2004

	NA									Total
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
HA										
1	5	2				1				8
2	2		7		1					10
3		1				1		5		7
4		3				19				22
5		5	3			1			3	12
6	2	12			1	1		3		19
7							16		1	17
8				4						4
9										0
10		2		1	1	2	2	2	3	13
11	1	1	2		1	1		1	7	14
12					2				1	3
13										0
14										0
15										0
16										0
Total	10	26	12	5	6	26	18	11	15	129

Table 5.2.2. Comparison of findings between longitudinal studies of ducks in North America (Krauss et al., 2004), Germany (Suss et al., 1994) and Sweden (present study).

Study Region	**Sweden**	**Germany**	**North America**
Prevalence during fall (%)	15,0	8,7	22.2
Prevalence during spring (%)	4,0	No data	0,03
Most prevalent HA-Subtypes	H4, H6, H7	H4, H2, H1, H6, H7	H6, H3, H4
HA-Subtypes not found	H9, H13-16	H5, H12-16	H13-16
Most Prevalent NA-subtypes	N2, N6, N7	N1, N3, N6	N8, N2, N6
NA-subtypes found	N1-9	N1-9	N1-9
Most prevalent subtype combinations	H4N6, H7N7, H6N2	H2N3, H4N6, H1N1, H6N2, H7N7	H3N8, H6N2, H4N6

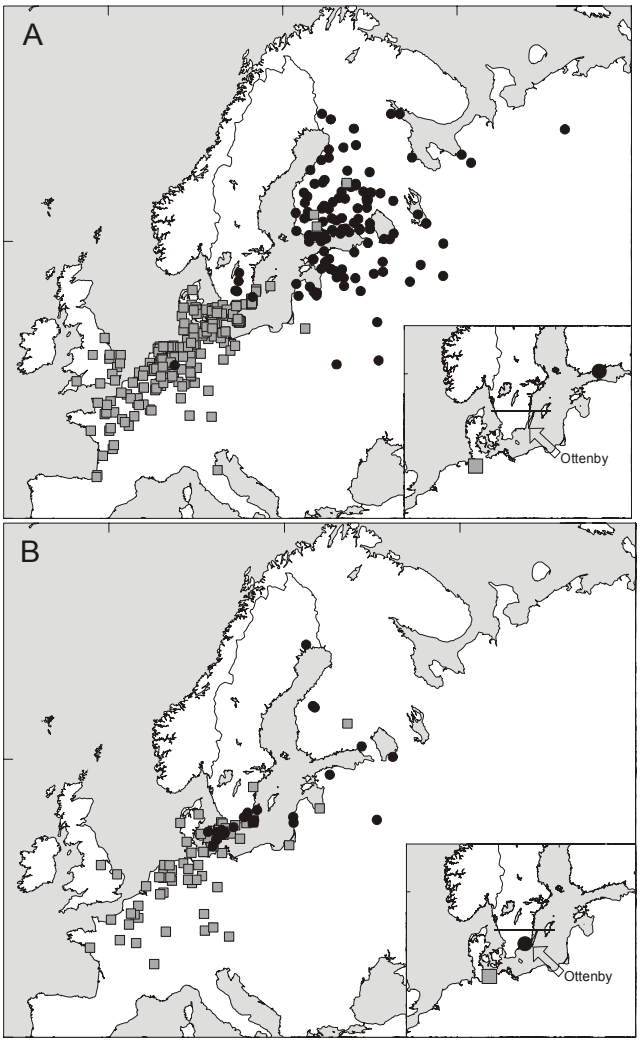

Figure 5.2.1. Female Mallards banded in Sweden south of 57º30'N (indicated by solid line in the inserted figures) during the period Oct-Dec (A) and May-Sept (B), and recovered during winter (Nov-Feb, n=255 and n=98) and summer (May-Aug, n=135 and n=53). Black dots represent summer recoveries while grey squares represent winter recoveries. Mean positions and the location of Ottenby Bird Observatory are shown in the inserted figures.

5.3 Seasonality and perpetuation in the wild bird reservoir (papers IV, V)

Increased knowledge of the seasonality and perpetuation of influenza A virus can only be achieved through large-scale systematic sampling of relevant bird populations over an extended period of time. To date, only two previous studies can be regarded to have approached this question in such a manner (Krauss et al., 2004; Suss et al., 1994); One of these studies was carried out in North America and the other one in Germany. The results of these studies have shown that the prevalence of influenza A virus in wild ducks is less than one percent during most parts of the year, but rises to high prevalence numbers of around 30 percent during the months August and September. In our studies (paper IV) we show that the prevalence of influenza A virus in Mallards migrating through Sweden is similar to those mentioned in these two studies, but with notable differences as to seasonality and subtype distribution.

We sampled birds during all times of the year except during winter, when ice and low bird numbers hindered sampling. We found a rise in prevalence of influenza A virus during both spring and fall. During the spring migration the maximum monthly prevalence was 9.5 percent. During the fall a prolonged rise in prevalence of influenza A virus was seen in our study (when compared to the North American study), which started in July and lasted to December with an average prevalence of 15 percent (figure 5.3.1)

These findings have consequences for the understanding of the perpetuation of influenza virus in nature. The low prevalence numbers during most parts of the year in the large North American studies have raised questions as to how influenza A virus and the great variation of subtypes can be perpetuated from year to year. Alternative theories have been put forward:

Since influenza A virus has been shown to persist for long times in cold water (Stallknecht et al., 1990b) one suggestion has been that the virus can persist frozen in lakes at the duck breeding grounds and then re-infect ducks as they return to these lakes in the spring (Ito et al., 1995).

Another explanation suggests that waders are a bridge species. In the Delaware Bay area, waders such as ruddy turnstones have been found to have relatively high prevalence of influenza A virus in the spring. Therefore, the suggestion has been put forward that there could be overlapping enzootic cycles between waders and ducks. Ducks having their peak influenza season in the fall could infect the waders during the fall and waders that have high prevalence of the influenza A virus in the spring could re-infect the ducks returning to the

breeding grounds in the spring. To date, the isolation attempts of influenza A virus from waders in Europe have not provided support for this theory (Fouchier et al., 2003).

Our data from the Ottenby study, covering information on the prevalence in Mallards during spring and fall migration, in conjunction with findings of a prevalence of 8 percent on the breeding grounds in Siberia (Okazaki et al., 2000) and of around 4 percent on wintering grounds in Italy (De Marco et al., 2003) raise the possibility that the ducks themselves have a high enough prevalence all year round to be the sole perpetuators of the virus. The differences, if real, between the situation in Eurasia and in North America may be a result of geographical differences between the study regions. In North America, the studies have been carried out in in-land lakes in Canada with high concentrations of birds. The duck populations breeding and migrating in western Eurasia (the population sampled in our studies) do not congregate to the same extent on lakes as migration takes place mainly along the coast. The migratory season is also more prolonged due to milder weather in coastal climates. Virus transmission can therefore be assumed to be more efficient among the ducks in the Canadian lakes resulting in a sharp rise and decline of influenza A virus prevalence. During the latter part of the year the prevalence of influenza A virus would, as a consequence, be low as only a small proportion of Canadian ducks remain susceptible to the particular strain of the influenza A virus. Studies carried out in other parts of North America than the long term study by Krauss and co-workers suggest that there are major differences between regions in North America (Hanson et al., 2005).

Our studies have thus shed light on the seasonality of the influenza A virus in ducks migrating through Sweden. The results support the theory of a continuous circulation of influenza virus throughout the year in the reservoir species. Therefore these results may help in explaining the perpetuation of influenza A virus in wild birds.

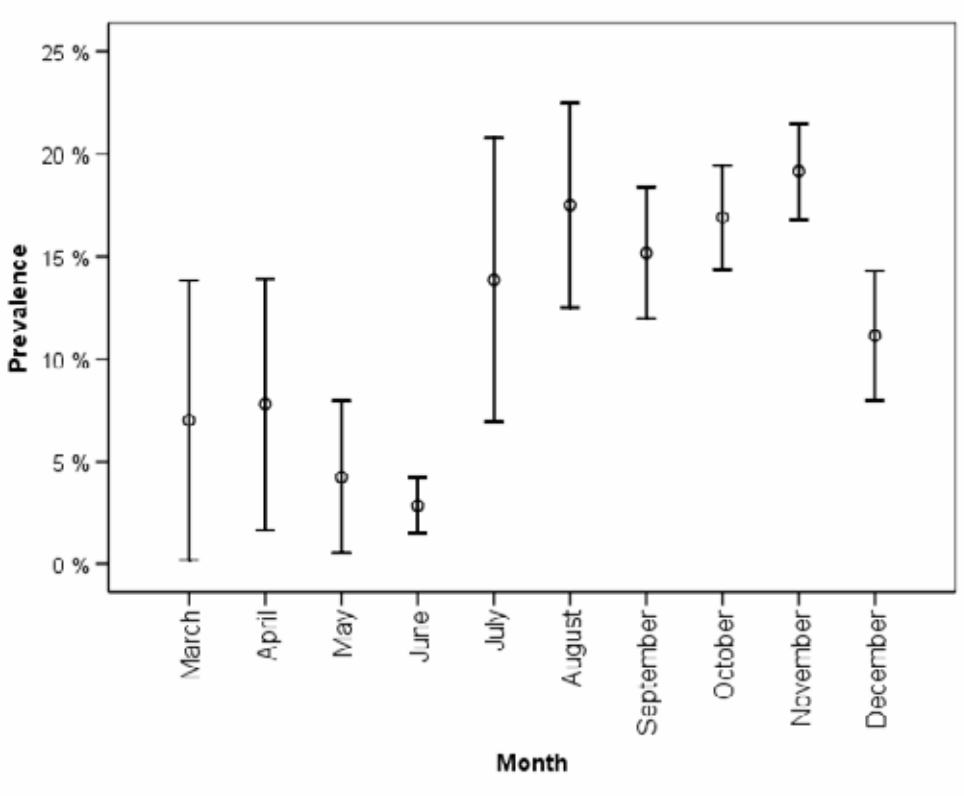

Figure 5.3.1. Influenza A virus prevalence in Mallards (n = 3,534), 2002–2005, with means and error bars indicating standard deviations (SD). Data is not shown for months when only incomplete trapping took place, yielding ≤5 samples

5.4 Geographic differences between wild bird populations (papers II, IV, and V)

Large surveys investigating the prevalence of influenza A virus in wild birds have only been carried out in a few areas of the world. Knowledge of the situation in Africa and South America is practically non-existent. Existing data suggest that there are important differences between different regions of the world as to the prevalence of influenza A virus. As discussed above, the prevalence of the influenza A virus during different parts of the year was found to differ between different regions of the world. Some bird species are only found in certain parts of the world and as a consequence influenza A virus has been isolated from different species in different regions. The differences found between different geographic regions are

likely to occur as a result of the limited interaction between birds on different continents or different regions. As mentioned before, different genetic lineages of avian influenza virus strains have developed in North America and Eurasia. In addition there are differences in subtype prevalence both between fly-ways of different continents, and also between different fly-ways within continents (Hinshaw et al., 1985). This reflects the importance of birds as reservoirs and the fact that geographic barriers limit the interaction between different bird populations. The most apparent division between regions is the separation of the Americas from Eurasia by the Atlantic and Pacific Oceans. There are only a few species that migrate between these continents and this mainly occurs in the Bering Strait area. However, we show that there must be occasional direct or indirect contact between the different bird populations on the Atlantic side as well. The Guillemots that were sampled at Bonden Island, as described above, were infected with a strain of influenza A virus that contained genes from both the North American and the Eurasian lineages (paper II). Phylogenetic analysis with the basic local alignment search tool (BLAST) available from Genbank revealed that five gene segments; HA, PB2, PB1, MA and NS of A/Guillemot/Sweden/3/00 strains belonged to the American lineage while three gene segments, the NA, PA and NP showed the highest percentage nucleotide and amino acid identity with strains of Eurasian origin (table 5.4.1). This was the first time such a chimeric virus was described in western Eurasia. How this genetic mix could occur is somewhat of a mystery as banding studies have shown that Guillemots that breed in the northern part of the Baltic Sea rarely migrate outside the Baltic. This suggests that a different species that has had contact with birds on the other side of the Atlantic must have been involved in the transfer of the genes. One possible candidate for such a transfer could be the Arctic Tern. It migrates from breeding grounds on northern latitudes to wintering grounds in the Antarctic region. In Antarctica terns from both sides of the Atlantic Ocean may meet and interact.

In paper IV we discuss our findings indicating that different populations of ducks that breed in different areas and later migrate across Sweden carry different subtypes of influenza A virus. The results are based on analysis of migration patterns of ducks according to banding and recovery data and the subtyping data gathered from samples collected from ducks at Ottenby Bird Observatory between the years 2002 and 2004, as discussed above. The fact that different populations carry different subtypes of influenza A virus could reflect that certain subtypes of the virus are constantly prevalent in certain geographic areas and infect the birds that breed in the area. It seems more likely, however, that flocks breeding in the same area will carry different subtypes of influenza A virus in different years as a result of their

immunological status. Longer time series of subtyping data from flocks breeding in different areas are needed to settle this issue.

In conclusion, our findings verify that there are separate lineages of influenza A virus in the New and Old World. However, we also show that there are occasions when influenza A virus of these different lineages recombine and as a result create mixed virus. We have also described that flocks breeding in different areas carry different subtypes of influenza A virus during their fall migration. If this is a reflection of the fact that they breed in a certain area or because of the immunological status of different flocks remain to be investigated through long term surveillance studies.

Table 5.4.1. Table showing to which lineage, the American or the Eurasian influenza A virus lineage, the different genes of A/Guillemot/Sweden/3/00 belong based on their nucleotide and amino acid identity with strains of the different lineages.

Gene segment	Lineage	Nucleotide identity %	Amino acid identity %
PB2	American	97	98
PB1	American	96	98
PA	Eurasian	98	99
HA	American	97	98
NP	Eurasian	97	99
NA	Eurasian	97	98
MA	American	98	99
NS	American	98	96

5.5 Relation between wild birds and outbreaks in poultry (papers III, IV and V)

Outbreaks in poultry of highly pathogenic influenza is thought to happen when influenza virus of the H5 or H7 subtypes gains entry into populations of domestic poultry from their reservoir among wild birds (Webster et al., 1992). This has been shown to happen on a couple of occasions but evidence is still scarce.

In study IV we describe how different subtypes are carried with ducks migrating from different breeding areas during the fall season. In the fall of 2002, there was an increase in the numbers of ducks infected with H7N7 virus strains and only a few months later an H7N7 epizootic broke out in poultry in the Netherlands. An outbreak that also affected humans as described in the introduction. The timing of the finding of the increase of H7N7 in ducks

during southern migration and the subsequent outbreak in the wintering areas of these ducks suggests that there might be a causal relation.

In paper III we have investigated the relationship of influenza A virus strains causing outbreaks in poultry with 33 low pathogenic isolates of the H5 and H7 subtypes cultured from fecal samples of Mallards caught at Ottenby Bird Observatory and collection sites in the Netherlands. These samples were characterized by sequencing and phylogenetic trees were generated on the basis of their amino acid sequences and all available sequences of H5 and H7 from public databases (figures 5.5.1 and 5.5.2). These trees showed the typical separation into the Eurasian and American lineages. The isolates from our collection clustered in the Eurasian section. Looking at the trees in more detail, we found that strains from this collection clustered close to strains causing recent HPAI outbreaks in poultry in Europe. DNA maximum likelihood trees were constructed using prototypic strains of the HPAI outbreaks in Europe during the last decade (H5N2 Italy 1997, H7N1 Italy and H7N7 the Netherlands 2003). In our limited sample collection we found isolates with close homology (96-98 % nucleotide and 98-99 % amino acid) to these outbreak strains. This genetic information was then confirmed by serological studies with post infection ferret antisera. Hemagglutinin inhibition assays were performed and the Mallard H5 and H7 samples were well conserved and did not differ significantly (up to 4-fold) from the prototypic strains causing the HPAI outbreaks.

Our studies thus suggest that surveillance of wild birds may be important as a way of assessing the relative prevalence of the different subtypes of influenza A virus in order to perform risk assessments and risk management. Another important conclusion is that wild bird surveillance may be a tool for obtaining strains of the influenza A virus that can be used for vaccine development as well as diagnostic tests and reagents as they are indeed similar to outbreak strains.

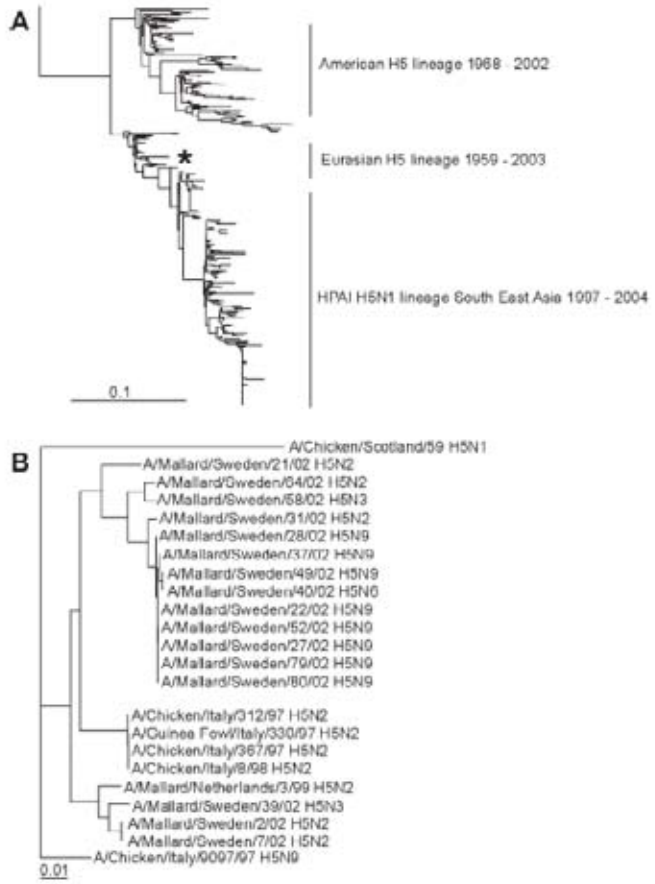

Figure 5.5.1. Phylogenetic trees of H5 sequences.
A) Phylogenetic tree based on the amino acid sequence distance matrix for the HA1 open reading frames of all H5 sequences available from public databases. The scale bar represents 10 % of amino acid changes between close relatives. * Represents the locations of the H5 influenza A virus strains isolated from Mallards.
B) DNA maximum likelihood tree for the European highly pathogenic avian influenza virus H5 strains and the low pathogenic avian influenza H5 influenza A virus strains from migrating Mallards by using A/Chicken/Scotland/59 as outcrop. The scale bar represents 1 % nucleotide changes between close relatives.

Figure 5.5.2. Phylogenetic trees of H7 sequences.
A) Phylogenetic tree based on the amino acid sequence distance matrix for the HA1 open reading frames of all H7 sequences available from public databases. The scale bar represents 10 % of amino acid changes between close relatives. * Represents the locations of the H7 influenza A virus strains isolated from Mallards.
B) DNA maximum likelihood tree for the European highly pathogenic avian influenza virus H7 strains and the low pathogenic avian influenza H7 influenza A virus strains from migrating Mallards by using A/FPV/Dutch/27 as outcrop. The scale bar represents 10 % nucleotide changes between close relatives.

6.0 CONCLUDING REMARKS

This thesis summarizes the knowledge gained from our studies of influenza A virus in wild birds. The information in the included articles has broadened the knowledge of influenza A virus subtypes and strains present in the wild bird population of Sweden and thus of the north-western Palaearctic. The studies have led to

- the discovery of a new hemagglutinin subtype H16 isolated from Black headed Gulls.
- the isolation of influenza A virus from Guillemots, a species not previously known to carry the virus in our region.
- a better understanding of the prevalence of influenza A virus during different seasons that in part contrasts to findings in North American studies.
- the discovery that the subtypes of influenza A virus known to cause outbreaks of HPAI are common in ducks migrating across Sweden and that the circulating strains are similar to those that indeed have caused outbreaks in European poultry holdings during the last decade.
- the isolation in wild birds of subtypes H1-H3 that are known to have caused pandemics in humans.
- the first western Eurasian discovery of a chimeric virus made up of genes from both the Eurasian and North American lineages of influenza A virus strains and thus showing that there is genetic exchange between North America and Eurasia.
- the isolation of strains that may be of use for development of vaccines and diagnostic tests.

Taken together we now have a better understanding on the circulation of influenza virus in wild birds. An understanding that may be valuable not only for the common goal of increasing the knowledge of pathogens in the world but also of importance for the risk assessments, risk management and vaccine development needed to limit spread of influenza virus strains to humans and domestic species thereby averting another influenza pandemic.

EPILOGUE

A personal reflection on the consequences of influenza A virus for man and animals

As we cannot rid the world of influenza virus due to its diverse reservoir in wild birds, the threat of a new pandemic will loom above us well into the future and outbreaks in poultry will continue. Although influenza virus strains are present in the wild bird populations and may transmit and cause outbreaks in other animals the current situation of world-wide spread of a highly pathogenic virus is a man-made problem. The large populations of poultry needed to feed the human population have created an environment where different viruses have an unprecedented opportunity to evolve. In order to halt this development, vast changes in the husbandry practices as well as the slaughtering and distribution processes are needed to minimize the contact between wild and domestic birds as well as the contact between sick birds and humans (Gilbert et al., 2006; Webster, 2004). These changes will have severe cultural and economic implications for many countries and will not take place unless richer countries assist the poorer ones. An alternative solution to protect us from new pandemics would be the development of a vaccine that can provide protection for any type of influenza virus. This has so far been proven to be difficult to develop. But even if such a vaccine could be manufactured, the sense of security would be false as it would still not protect us from other diseases such as SARS that may emerge due to the present day interaction between wild animals and domestic animal production and distribution.

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The work was made possible by a number of institutions and people. I would especially like to thank the people at Kalmar County Council, *Bertil Isaksson*, *Bengt Öberg, Helena Bergström,* and *Lars Brudin* to name a few, for granting me research time and for continued encouragement and support of research projects in Kalmar. I am also grateful that the people working at Smedby Health Center and my clinical supervisor *Anders Erikson* have been generous and supportive in spite of the fact that my scientific studies have not been of much help in managing the daily routines at the Health Center. Hopefully, the fact that Smedby Health Center and Kalmar County Council now figure in articles in well renowned research papers may be compensation in part.

I have been registered as a PhD-student at Linköping University. I would specifically like to thank *Lennart Svensson*, one of my two co-supervisors as well as *Eva Eigel* and *Carina Ewerlöf,* for arranging the compulsory parts of the education and answering my questions. I am thankful for the flexibility shown by Linköping University in allowing the dissertation to take place in Kalmar.

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I am grateful for the practical assistance I have received from the staff at the Kalmar County Hospital Medical library and the IT-help desk.

When I started this project, it was just Björn and me, a lot of exciting ideas, but no lab. Nowadays, things are quite different. A functioning laboratory has been set up at Kalmar University, great people have been employed to work there and Björn Olsen's research group has expanded. *Jonas Waldenström, Patrik Ellström, Neus Latorre-Margalef, Diana Axelsson-Olsson, Petra Griekspoor, Jonas Bonnedahl* among others - It has been very nice to work with you all!

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