H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS IN WILD BIRDS: POTENTIAL FOR A NEW WILDLIFE DISEASE OR A DEAD END?

by

JUSTIN DAVID BROWN

(Under the Direction of John R. Fischer)

ABSTRACT

Mortality associated with H5N1 highly pathogenic avian influenza (HPAI) viruses has been reported in a diversity of wild avian species, but it is unknown whether these viruses can be maintained or become established in free-living avian populations. In this research, the potential for H5N1 HPAI viruses to be transmitted and maintained in these populations was determined by evaluating both host and environmental factors that could affect transmission. The two H5N1 HPAI viruses evaluated in this study generally had a shorter duration of persistence in water than the low pathogenic avian influenza viruses that naturally circulate in wild avian populations. This suggests that, at some point in the evolution of H5N1 HPAI, these viruses have lost some degree of environmental fitness.

Several species of anseriforms and charadriiforms were experimentally infected with a H5N1 HPAI virus and the data from these studies indicate that specific wild waterfowl species (primarily the swans, geese, and gulls) are highly susceptible to this virus, as evidenced by high mortality, and these species excrete high concentrations of virus for several days. Additionally, most duck species in our study were resistant to H5N1 HPAI infection and viral shedding in these species was minimal. Taken together, our data suggests that H5N1 HPAI virus epidemics in wild birds may be dependent on the presence of highly susceptible species that may represent only a small component of the total avian population. This is consistent with the field data from

the European outbreaks of 2005/2006, in which the majority of wild bird mortality occurred in a relatively few anseriform species. Additionally, our findings suggest some waterfowl species transmit virus asymptomatically for several days and, consequently, have the potential to spread H5N1 HPAI virus between limited geographic regions. Although this information provides some insight into H5N1 HPAI virus transmission within a waterfowl population, the data are not sufficient to indicate whether H5N1 HPAI viruses can or cannot be maintained wild avian populations under natural conditions, which would depend heavily on biology of the each individual wild bird species, the environmental conditions, and the species composition of the avian population

INDEX WORDS: avian influenza virus, swans, geese, ducks, gulls, H5N1, highly pathogenic avian influenza, maintenance, Order Anseriformes, Order Charadriiformes, persistence, water, waterfowl, wild bird

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DEDICATION

This work is dedicated to my wife, Holly, who always makes me smile, and to my entire family, old and new, for their endless support and love.

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CHAPTER 1

INTRODUCTION

It is widely accepted that wild birds, especially those in the Orders Anseriformes and Charadriiformes, represent the natural reservoirs for avian influenza virus (AIV) (Stallknecht and Shane, 1988; Webster et al., 1992). Historic evidence of highly pathogenic avian influenza (HPAI) virus infection, however, is rare in these populations (Suarez, 2000). Prior to 2002, wild bird mortality associated with HPAI virus infection was limited to a single outbreak that occurred in South Africa in 1961 (Becker, 1966). This outbreak involved common terns (*Sterna hirundo*) and a H5N3 HPAI virus, but there is no subsequent evidence that this virus persisted in any wildlife population. The traditional AIV paradigm is currently being challenged by recent infections with H5N1 HPAI viruses in Europe and Asia. Since 2002, mortality in a diversity of wild bird species has been associated with H5N1 HPAI viruses (USGS, 2006), and it has been suggested that wild birds are responsible for the spread and possibly the persistence of H5N1 HPAI virus in this region. Although such mortality is alarming, it is premature to accept these observations as evidence that this virus, or related HPAI viruses, can become established or can exist naturally in wild avian populations. The uniqueness of these mortality reports, however, warrants additional research. Determining the potential reservoir status of wild birds for this or any HPAI virus is extremely important, not only to understand the role of wild birds in the ecology of HPAI viruses but also to support current HPAI eradication or control efforts in Eurasia and improve future biosecurity and surveillance needs worldwide.

The goal of this research is to understand the potential for H5N1 HPAI viruses to be efficiently transmitted and maintained in wild bird populations. The studies described here were designed to evaluate this potential by evaluating three prerequisites believed to be necessary for an

AIV to be maintained in a wildlife population. These criteria are extrapolated from our current knowledge related to the epidemiology of AIV in wild avian populations and include: 1) long-term environmental persistence in water; 2) significant and persistent viral shedding by an efficient route; and 3) a population of highly susceptible birds. Justifications for these criteria are as follows:

Criteria 1: Environmental stability in water. Transmission of AIV in wild birds occurs via an indirect fecal-oral route involving fecal-contaminated water on shared aquatic habitats (Webster et al., 1978). This route of transmission is dependent on environmental persistence of AIV which is enhanced in water. Avian influenza viruses are well-adapted to water (Stallknecht et al., 1990) and all known free-living avian reservoirs utilize aquatic habitats (Krauss et al., 2004). These factors suggest that viral stability in water may represent a critical prerequisite for efficient virus transmission in these populations.

Criteria 2: Significant fecal shedding. It is well established that AIV are maintained in nature through a fecal-oral route of transmission (Hinshaw et al., 1980). For such transmission to be effective, high levels of virus must be excreted to compensate for environmental dilution associated with the aquatic habitats utilized by wildlife species. Respiratory infections and related viral shedding associated with the airways may have application to AIV in domestic poultry housed under confinement conditions. We believe, however, that such viral shedding would have little relevance to effective virus transmission in free-living avian populations under normal conditions. Respiratory infections, by contrast, may have a more significant role in the transmission of AIV under abnormal conditions that artificially increase population densities in small geographic clusters, as evidenced by H5N1 HPAI outbreaks associated with waterfowl parks (Ellis et al., 2004) or severe winter weather (Sabirovic et al., 2006).

Criteria 3: *An ample supply of highly susceptible birds.* The final prerequisite for AIV maintenance in wild avian populations is efficient transmission of virus from the environment to a susceptible host in order to complete the transmission cycle. Because dilution and time-dependent degradation will decrease viral concentrations in the environment, viral transmission itself, must be efficient. We believe such efficiency will be reflected in the ability of the AIV to infect a susceptible bird at a very low dose.

Low pathogenic avian influenza (LPAI) viruses in wild anseriforms have evolved into the perfect host-parasite relationship (Webster et. al, 1978), satisfying all three of these criteria for viral maintenance. Low pathogenic avian influenza viruses can persist for long durations in water (Stallknecht et. al, 1990); experimentally infected ducks shed high concentrations of virus for a prolonged duration via the fecal route (Webster et. al, 1978); and a high prevalence of AIV infections occur annually in the fall when susceptible juvenile ducks congregate at marshalling sites prior to fall migration (Halvorson et. al, 1985). The research described herein experimentally evaluates these maintenance criteria for H5N1 HPAI virus in wild birds to determine the efficiency of viral transmission and the likelihood of this virus persisting in free-living avian populations.

Specific objectives of this study include the following:

1. to determine the duration that H5N1 HPAI viruses can remain infective, as detected by tissue culture, in water under different experimental conditions and to compare this environmental persistence of H5N1 HPAI viruses to H5 and H7 LPAI viruses isolated from anseriforms or charadriiforms;

2. to examine species-related differences in clinical response and extent and duration of viral shedding between wild species of North American anseriforms and charadriiforms experimentally infected with H5N1 HPAI viruses; and,
3. to determine the concentration of H5N1 HPAI virus required to produce infection and death in a susceptible anseriform species.

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CHAPTER 2

LITERATURE REVIEW

AVIAN INFLUENZA: THE VIRUS

Avian influenza viruses (AIV) belong to the family *Orthomyxoviridae*, genus *Influenzavirus A*. The morphology of these viruses is pleomorphic, ranging from spherical to rod-shaped to filamentous, and is partially dependent on whether the isolate is obtained directly from the host animal or after passage in eggs or tissue culture (Lamb and Krug, 1996). Virions typically range from 80- to 120-nm in diameter, but the filamentous form can reach up to 400- to 800-nm in diameter. The genome consists of eight negative-sense, single-stranded RNA segments that code for eight structural proteins and two nonstructural proteins. The structural proteins include hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein 1 (M1), matrix protein 2 (M2), and three proteins that collectively form the polymerase complex (PB1, PB2, and PA). The two nonstructural proteins (NS1 and NS2) were previously thought to only be present in influenza-infected cells, but NS2 is now known to be a component of free-virions (Yasuda et al., 1993).

The outer surface of influenza viruses is covered by numerous viral-encoded surface glycoproteins that are embedded in the host cell-derived lipid envelope and project out from the cell surface. Influenza viruses have two distinct types of surface glycoproteins, the HA and NA. The HA is an integrated type 1 membrane glycoprotein that is a rod-shaped trimer and is required for AIV entrance into the target host cell. Viral attachment occurs at a sialic acid-containing cellular receptor, which initiates endocytosis and the entry of virus into the host cell. The low pH environment within the endosome vesicle facilitates proteolytic cleavage of HA into two smaller subunits, designated HA_1 and HA_2. In addition, the low pH of the endosome causes

an irreversible conformational change to the HA_2 subunit, which results in the exposure of a highly conserved hydrophobic region at the N-terminus, called the fusion peptide. This peptide is thought to insert into the endosome membrane, mediating a fusion between the viral and host cell membranes and, subsequently, the release of viral nucleocapsid into the cytoplasm. The NA is a tetrameric integrated type 2 membrane glycoprotein that has a mushroom shape and is arranged as clusters on the surface of the virus. The primary function of NA is to catalyze the cleavage of a terminal sialic acid residue on the host cell glycoprotein. This is thought to facilitate elution of viral progeny from the cell as well as to prevent agglutination of released viral progeny. Also embedded in the lipid envelope is an integrated type 3 membrane glycoprotein, termed M2, which functions as a proton ion channel. The ion channel activity is important for viral uncoating within the endosome.

The M1 protein, the most abundant structural protein in influenza viruses, is arranged beneath the lipid envelope. In addition to providing rigidity to the outer viral membrane, M1 is an integral protein that interacts with both the outer surface glycoproteins and the internal viral components and also participates in the budding of viral progeny from the host cell. The remaining internal components of AIV are composed of NP, polymerase complex, and the viral RNA genome, which are collectively called the ribonucleoprotein (RNP) complex. The NP is associated with the viral RNA and is necessary for cytoplasmic to nuclear transport of the viral RNP and for viral RNA synthesis. The polymerase complex is composed of the three proteins, PB1, PB2, and PA, which have different roles but function together to synthesize the viral mRNA.

The NS1 protein has multiple functions which facilitate viral replication including the inhibition of cellular mRNA processing and enhanced translation of viral mRNA. The NS2 protein functions primarily in the export of RNP out of the host-cell nucleus.

Different strains or isolates of AIV can be distinguished based on several characteristics including antigenicity and pathogenicity or through molecular or genetic testing (Swayne and Halvorson, 2003). Antigenic classification of AIV is based on serologic reactions with structural viral proteins. Influenza viruses are classified into one of three genera or types (A, B, or C) based on serologic reactions to the internal proteins NP or M1, which is typically performed with an agar gel immunodiffusion (AGID) test (Swayne et al., 1998 A). All AIV are type A influenza viruses, which can infect a wide-diversity of animals including birds, pigs, sea mammals, horses, and mink (Swayne and Halvorson, 2003; Webster et al., 1992). Type B and C influenza viruses primarily infect humans and occasionally pinnipeds and pigs. Type A influenza viruses are further classified into subtypes based on serologic reactions with the surface glycoproteins, HA and NA. The HA and NA subtypes are determined by the hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) tests, respectively (Swayne et al., 1998 A). Currently, 16 HA subtypes (H1-16) and 9 NA subtypes (N1-9) of AIV have been recognized and most combinations have been reported in wild or domestic birds (Olsen et al., 2006; Webster et al., 1992).

Pathogenic classification of AIV is based on *in vivo* testing of an isolate's virulence in experimentally infected domestic poultry (USAHA, 1994; Alexander, 1996) or *in vitro* testing that identifies, through molecular techniques, viruses that have the potential to be highly virulent in domestic poultry (Swayne et al., 1998 B). In chickens, AIV can be classified as highly pathogenic avian influenza (HPAI) or non-HPAI. Other names commonly used for non-HPAI

include low pathogenic avian influenza (LPAI) or mildly pathogenic avian influenza (MPAI) viruses. To date, only a few H5 and H7 viruses have been highly pathogenic and viruses of the remaining fourteen HA subtypes have exhibited no or mild virulence for birds (Swayne and Halvorson, 2003). Classification criteria established by the U.S. Animal Health Association define HPAI as: 1) any AIV that kills 75% or greater 4- to 6-week old chickens within 10 days after intravenous inoculation, 2) any AIV of the H5 or H7 subtype that has multiple basic amino acids at the proteolytic cleavage site of HA, or 3) any AIV that can kill one of five chickens and grow in cell culture without the addition of supplemental trypsin. Identifying AIV isolates that are HPAI or have the potential to be HPAI is important for regulatory purposes to ensure that adequate control measures are taken to eradicate these viruses from a poultry flock. Based on field data from outbreaks and experimental infection studies, recent isolates of H5N1 HPAI viruses that cause high morbidity and mortality in wild birds have been designated as "highly pathogenic" for wild birds (Hulse-Post et al., 2005). It is important to note that pathogenicity testing is specific for the host animal used in the test (Swayne and Halvorson, 2003), and some of these designations in non-gallinaceous species are not based on established criteria.

The intravenous pathogenicity index (IVPI) (criteria 1, listed above) is considered the gold standard determinant of a highly pathogenic virus. However, advances in examining the genetic sequences of AIV have provided further understanding of viral characteristics, including pathogenicity, on a molecular level. Pathogenicity is a polygenic trait that requires the proper combination of all eight gene segments of the virus to manifest the full phenotype (Bosch et al., 1979; Klenk et al., 1998). The HA gene, itself, is the primary determinant of pathogenicity in chickens (Steinhauer, 1999; Swayne and Halvorson, 2003). The proteolytic cleavage of HA into HA_1 and HA_2 is required for an AIV to be infectious. The ability of different types of proteases

to recognize and cleave the HA glycoprotein is dependent on the amino acid sequence at the cleavage site. Non-HPAI viruses have a single basic amino acid (arginine) at the cleavage site, which is recognized by extracellular trypsin-like proteases found in specific anatomical sites, such as the epithelial cells lining the respiratory tract and intestines (Klenk et al., 1998; Rott et al., 1995; Swayne et al., 2003). The location of these proteases restricts HA activation, and non-HPAI infection, to the gastrointestinal and respiratory tracts. The HA cleavage site of HPAI viruses contains multiple basic amino acids (arginine and lysine) and is recognized and cleaved by the trypsin-like proteases (mentioned above) and intracellular furin proteases that are present in many cell types of most visceral organs and of the nervous system (Klenk et al., 1998; Steinhauer DA, 1999; Stieneke et al., 1992). Consequently, HPAI virus infection is widespread and viral replication occurs in many host tissues. Multiple basic amino acids at the HA cleavage site of an AIV can be used as a genetic marker to identify viruses that have the potential to become highly pathogenic based on structural properties of the HA glycoprotein. Alternatively, HA cleavability can be determined indirectly by the ability of an AIV to grow in tissue culture without the addition of supplemental trypsin. The ability of a virus to replicate and cause cytopathic effects in cell culture without supplemental trypsin is associated with HA cleavage by ubiquitous furin proteases and correlates strongly with high pathogenicity results via the IVPI test (Swayne and Halvorson, 2003; USAHA, 1994). In addition to its genetic sequence, other properties of the HA glycoprotein affect cleavability and pathogenicity potential, including the glycosylation of amino acids surrounding the cleavage site and the protein's secondary structure (Perdue et al., 1995; Perdue and Suarez, 2000; Swayne et al., 1998 B).

A standardized international system for identifying influenza virus strains was established in 1980 by the World Health Organization (WHO, 1980). Influenza virus strains are named as

follows: viral type (A, B, or C)/animal host of origin/geographic site of isolation/laboratory strain number/year of isolation, which is followed by virus subtype enclosed in parenthesis. Examples of AIV viruses include A/Ruddy Turnstone/New Jersey/828227/2001 (H5N8) and A/Mallard/Minnesota/182761/1998 (H7N3). Host of origin is excluded from human influenza viruses. The pathogenicity of a virus isolate is not included in the standardized nomenclature.

Propagation and isolation of AIV are most commonly performed in 9- to 11-day old, specific pathogen free embryonated chicken eggs (Swayne et al., 1998 A). Briefly, 0.2 ml of a suspect sample is inoculated into the allantoic sacs of three to five eggs. The inoculated eggs are incubated at 37º C for three to seven days and examined daily for embryo death (lack of movement by the embryo and a loss of vasculature in the egg) by holding a light source over the air cell of the egg. Embryo death within 24 hours of inoculation is most likely due to bacterial contamination or injury from the inoculation syringe, and these eggs can be discarded. Amnioallantoic fluid is collected from any egg exhibiting embryonic death after 24 hours and from all remaining eggs from three to seven days post-inoculation (DPI). The fluid is tested for the presence of AIV as determined by the ability of the fluid to agglutinate chicken red blood cells (HA test) (Swayne et al., 1998 A). Since other avian viruses, such as Newcastle disease virus, also have the ability to hemagglutinate, additional tests are required to differentiate AIV from these other viruses. The HI, NI, or AGID tests are commonly used to confirm the presence of AIV antigen. Avian influenza virus can also be propagated in primary tissue cultures. The most commonly used cell culture systems include chicken embryo fibroblasts, chicken embryo kidney cells, and Maden Darby canine kidney cells (Sugimura et al., 2000; Easterday et al., 1997; Swayne et al., 1998 A; Tobita et al., 1975).

AVIAN INFLUENZA VIRUS IN WILD BIRDS

AIV history in wild birds

The first reported isolation of AIV from wild birds was in 1961 from common terns (*Sterna hirundo*) in South Africa during a large mortality event attributed to a H5N3 HPAI virus (Becker, 1966). In addition to being the first isolation of AIV from wild birds, this was the only known occurrence of HPAI virus in free-living wild birds not associated with domestic poultry prior to the detection of the Asian strains of H5N1 HPAI in wild birds. However, the tern outbreak was a localized event and there is no evidence that the HPAI was maintained in or transmitted to wild or domestic birds beyond the primary site. Serologic surveys following the tern epizootic detected antibodies to AIV in several wild avian species, providing evidence that free-living birds are commonly infected with these viruses (Easterday et al. 1968, Asplin 1970, Laver and Webster 1972, Winkler et al. 1972). This was later confirmed by the isolation of AIV from shearwaters in Australia (Downie and Laver 1973) and from ducks in California (Slemons et al., 1974). The high prevalence of AIV and multiple subtypes isolated from ducks in the later study not only provided the first evidence that ducks may play an important role in the ecology of AIV, but also suggested a possible source for genetic recombination and subtype diversity in nature. Following these studies, a significant amount of research was focused on AIV in wild avian species, greatly improving our understanding of the natural history of AIV. Currently, AIV has a known global distribution; virus has been isolated from free-living birds in Australia, Africa, Asia, Europe, North America, and South America (Stallknecht and Shane, 1988; Olsen et al., 2006, Spackman et al., 2006) and serologic evidence has been documented in Antarctica (Austin and Webster, 1993; Wallensten et al., 2006).

LPAI virus in wild birds: Host range

Host range of AIV in wild birds represents the combined effects of exposure and susceptibility to the virus. Exposure to the virus is dependent on the habitat use, behavior, and population dynamics of individual species of birds. Although extensive literature exists on factors affecting exposure of wild birds, there is a limited understanding of the species-related differences in susceptibility to AIV. It is established that AIV can infect a broad diversity of taxonomic groups (Homme and Easterday, 1970; Slemons and Easterday, 1972; Alexander et al., 1978; Alexander et al., 1986; Wood et al., 1985; Kawaoka et al., 1988), but susceptibility is dependent on the individual strain of virus and on the avian species (Perkins and Swayne, 2003 A; Pantin-Jackwood and Swayne, 2007; Swayne DE, 2007). Recognizing that these viruses have the potential to infect diverse avian species is important in interpretation of surveillance data, as AIV may be isolated from species that are not normally associated with infection under natural conditions (Alexander et al., 1974; Alexander et al., 1978; Senne et al., 1983); isolation events must not be over-interpreted, unless consistently observed, as more than just spill-over events.

Naturally occurring infections of AIV have been reported from more than 100 avian species in 12 orders (Stallknecht and Shane, 1988; Hanson et al., 2002; Olsen et al., 2006; USGS, 2006). Most of these species are associated with aquatic habitats, and currently anseriforms (ducks, geese, and swans) and charadriiforms (gulls, terns, and shorebirds) are considered the most important avian groups involved in the ecology of AIV (Webster et al., 1992). Species of birds within these two orders are diverse and often occupy very different habitats, ranging from small, fresh-water marshes to pelagic, salt-water habitats (Barry et al., 2006; Heindel MT, 2006; Howell and Jaramillo, 2006). While the host range of AIV is typically discussed at the taxanomic level of 'Order', surveillance data suggest that the distribution of AIV

in wild birds is more specific and is strongly associated with particular families and, in some cases, individual species. For example, most AIV isolations from anseriforms have been reported from the subfamily Anatinae (dabbling and diving ducks), and most of these isolations have been from mallards (Hanson et al., 2002). These species congregate in large numbers on freshwater habitats, especially during the post-breeding season, facilitating AIV transmission within the population (Bellrose FC, 1976). Within the Charadriiformes, AIV have been isolated from three families, but infection is most commonly associated with species in the families Scolopacidae (shorebirds: sandpipers and turnstones) and Laridae (gulls and terns) (Hanson BA, 2002; Olsen et al., 2006). While AIV isolations from gulls and terns are relatively common and taxonomically diverse, the overwhelming majority of shorebird AIV isolations come from ruddy turnstones (*Arenaria interpres*) at Delaware (DE) Bay, USA (Stallknecht DE, 1998; Stallknecht and Brown, 2007). It is currently not known why the prevalence of AIV in ruddy turnstones is so much higher than in other shorebird species but it may be attributable to a combination of factors including behavior, species susceptibility, habitat use, or interaction with anseriform species during spring migration (Hanson, 2002).

Of the remaining avian orders from which AIV have been isolated, most are associated with aquatic habitats, including Gaviiformes (loons), Podicepediformes (grebes), Procellariiformes (shearwaters and petrels), Pelecaniformes (pelicans and cormorants), Ciconiiformes (ibis and herons), and Gruiformes (moorhen and coots) (Stallknecht and Shane, 1988; Hanson, 2002). The AIV prevalence in these orders may be due to sympatric habitats with anseriform or charadriiform hosts and enhanced viral transmission between birds in aquatic environments. In these environments shared water can serve as an efficient viral transport medium. Sporadically, AIV has been isolated from more terrestrial species in the Orders

Columbiformes, Piciformes, and Passeriformes (Boudreault et al., 1980; Stallknecht and Shane, 1988). Traditionally, surveillance efforts have included some of these species that are associated with domestic poultry environments in the sample population because of their potential contribution to AIV transmission and spread within a poultry facility. Most isolation attempts from these species, however, have proven unsuccessful (Deibel et al., 1985; Hinshaw and Webster, 1982; Nettles et al., 1985).

There are no reports of AIV isolation from some avian orders, including Trogoniformes, Apodiformes, Coraciiformes, and Spheisciformes, which may be attributable to the limited number of samples that have been tested from these birds. It is also important to recognize that surveillance for AIV within a given order or species is geographically and seasonally dependent. Successfully isolating AIV from an avian species may require that samples are collected at specific times and locations, which may differ for anseriforms and charadriiforms. With the attention and resources that have recently been devoted to AIV surveillance in wild birds, our understanding on this topic will improve and it is possible that the known host range of AIV may greatly expand.

LPAI virus in wild birds: Transmission and epidemiology

It is well-established that the transmission of AIV in wild bird populations is dependent on an indirect fecal-oral route involving fecal-contaminated water on shared aquatic habitats (Hinshaw et al., 1979; Sandu and Hinshaw, 1981; Sinnecker et al., 1983). Replication of AIV in waterfowl occurs primarily in cells lining the lower intestinal tract (Slemons and Easterday, 1977; Webster et al., 1978) and high concentrations of virus are excreted in the feces (Webster et al., 1978; Hinshaw and Webster, 1982). For example, experimentally-infected Muscovy ducks (*Cairina moschata*) shed large quantities of fecal material per hour with a high viral titer of

$1X10^{7.8}$ median embryo-infectious dose (EID_{50})/ml (Webster et al., 1978). The duration of viral shedding may also be prolonged in the waterfowl host, as evidenced by experimentally-infected Pekin ducks (*Anas platyrhynchos*) which excreted virus for over 28 days (Hinshaw et al., 1980).

The peak prevalence of AIV in North American anseriform populations occurs in late summer to early fall when high concentrations of susceptible juvenile birds congregate on waterfowl marshalling sites throughout Canada and the northern United States prior to fall migration (Hinshaw et al., 1985). During this time, AIV infection rates can exceed 30% in juvenile ducks (Hinshaw et al., 1985) but prevalence rapidly decreases as the population migrates. On wintering areas the AIV prevalence is often lower than 1-2% (Stallknecht and Shane, 1988). While much less is known about influenza in charadriiforms, preliminary data indicate that AIV infection in these birds differs spatially, temporally, and genetically from what is observed in anseriforms (Kawaoka et al., 1988; Stallknecht and Shane, 1988). The peak prevalence of AIV in North American charadriiforms occurs in the spring when shorebirds migrating along the Atlantic coast of the United States stop over at DE Bay to feed on spawning horseshoe crab eggs. Bird density at DE Bay during this time is high, which facilitates transmission of AIV within the population. This is the only time and geographic site worldwide where consistent AIV isolations from shorebirds have been reported, and prevalence rates from these species at other locations or times are generally either very low or zero (Hanson, 2002; Stallknecht and Shane, 1988; Fouchier et al., 2003). The maintenance cycle of AIV in gulls and terns is poorly understood. While there appears to be some overlap between the shorebird and gull cycles (similar AIV subtypes have been isolated from gulls and shorebirds at DE Bay), some characteristics, such as unique HA subtypes (H13 and H16), suggest that a distinct AIV cycle exists within gulls (Olsen et al., 2006).

The AIV subtype diversity within wild birds is dependent on the avian host taxonomy and geographic location. In addition, subtype diversity within an avian population varies by year, with prevalence reportedly following a 2-year cycle (Krauss et al. 2004). Viruses of the H3, H4, and H6 hemaglutinin subtypes and the N2, N6, and N8 neuraminidase subtypes are the most common AIV isolated from North American waterfowl (Stallknecht et al., 1990 C; Stallknecht and Shane, 1988; Sharp et al., 1993, Krauss et al., 2004). Within these common waterfowl subtypes there are specific HA and NA combinations that repeatedly occur together, such as H3N2, H4N6, and H6N8. It is currently unknown why these subtypes occur more frequently than others, but it is possible that viruses with these specific combinations of HA and NA genes possess biological attributes, such as increased fitness in the environment or in the host, that allow them to out compete other subtypes. Most other HA subtypes are isolated from waterfowl. Viruses of the H5, H7, and H9 subtypes are generally uncommon in waterfowl, though higher prevalences of these subtypes occasionally have been reported at specific geographic locations or years (Hanson et al., 2003; Munster et al., 2005). Subtype diversity is not as well understood in charadriiforms. Kawaoka et al. (1988) originally reported that the H9 and H13 subtypes were most frequently isolated from charadriiforms. Subsequent surveillance efforts conducted over longer durations have shown that subtype diversity varies each year at DE Bay with little to no identifiable subtypes consistently predominating within the shorebird population (Hanson, 2002; D. Stallknecht, personal communication). Traditionally, the H5 and H7 subtypes have been uncommonly isolated from charadriiforms but, in 2006, over 75% of the AIV isolates from DE bay were of the H7 subtype (D. Stallknecht, unpublished data). The epidemiologic factors that determine subtype diversity each year at DE Bay are not known. It is

possible that the subtypes circulating each year in shorebirds at DE Bay reflect the predominant subtypes present in waterfowl and gull populations within the Atlantic flyway.

The prevalence of AIV is not high enough or consistent enough in other avian orders to accurately characterize the subtype diversity in these populations.

LPAI virus in wild birds: Reservoir hosts and the maintenance cycle

A "reservoir" includes any animal or inanimate substance upon which a pathogen depends for its survival (Martin et al., 1987). Anseriforms are a reservoir for AIV based on the global prevalence and subtype diversity, asymptomatic viral infection, prolonged and concentrated viral shedding, and large genetic diversity of AIV within their populations (Hinshaw and Webster, 1982; Stallknecht DE, 1998). As stated previously, only a subset of anseriform species probably comprise the true AIV reservoir. Mallards are a widespread host of AIV (Stallknecht and Shane, 1988; Olsen et al., 2006) and blue-winged teal (*Anas disors*), through their biology and migratory behavior, contribute to AIV transmission on wintering sites (Stallknecht et al., 1990 C; Hanson et al., 2005). While these two species may play slightly different roles in AIV epidemiology, the interactions between multi-species assemblages of anseriforms on shared habitats may represent the critical component for AIV maintenance.

Though charadriiforms are typically considered a reservoir for AIV, the nature of this reservoir probably differs from anseriforms. As stated previously, most AIV isolates are from ruddy turnstones in the spring at DE Bay; most other isolation attempts worldwide have been unsuccessful (Hanson, 2002; Fouchier et al., 2003; Stallknecht et al., 1998). Isolates from shorebirds and ducks are genetically similar (Spackman et al., 2005; Widjaja et al., 2004). Taken together, these data may suggest AIV infections at DE Bay are annual spill-over events of duck-origin AIV into a susceptible shorebird species, the ruddy turnstone.

Species in the Family Laridae may represent true reservoir hosts for some HA subtypes of AIV, including H13 and H16. Viruses of these subtypes are genetically similar to each other and distinct from other AIV, suggesting viruses of these subtypes have been isolated or segregated for sufficient duration to allow differentiation (Fouchier et al., 2005). Ducks are not easily infected with all gull-origin AIV, supporting the hypothesis that an independent AIV cycle exists in gulls and terns (Kawaoka et al., 1988; Webster et al., 1992). It is important to keep in mind, however, that only a subset of the AIV circulating in gulls and terns is of distinct Laridae-origin, and many AIV isolated from these charadriiforms are indistinct from the viruses that circulate in anseriforms (Olsen et al., 2006).

There is no evidence to suggest AIV can cause persistent infections in individual birds, but the mechanism of its maintenance in nature is not completely known. Likely, the maintenance cycle involves a combination of continuous circulation within reservoir host populations and viral persistence in the aquatic environment. Serologic and virus isolation data suggest AIV continues to circulate among ducks at low levels on the wintering grounds after fall migration (Stallknecht et al., 1990 C; Demarco et al., 2003). Early migrating species, such as blue-winged teal in North America, are thought to facilitate viral transmission on wintering sites by serving as an immunologically naïve population; blue-winged teal leave the breeding ground prior to the peak prevalence of AIV in juvenile ducks in the fall (Stallknecht et al., 1990 C). Avian influenza viruses have also been detected in ducks on the wintering ground in the early spring (Hanson et al., 2005) and on the breeding grounds in late spring (Sharp et al., 1993), providing evidence for continuous transmission of AIV in ducks. The identification of continuous AIV circulation in migratory ducks does not exclude the possibility that other mechanisms play a role in the maintenance cycle of AIV. The ability of AIV to persist in water

may contribute significantly to their perpetuation in wild birds by facilitating viral transmission between birds in aquatic habitats and, potentially, by preserving infectious AIV in frozen lakes over winter to re-infect returning waterfowl in the spring (Webster et al., 1992). As mentioned previously, multi-species interaction and co-habitation are important for transmission and may be important factors in the maintenance cycle, as well.

LPAI virus in wild birds: Morbidity, mortality, and pathology

Infection with LPAI viruses in wild birds is usually asymptomatic, and viral replication is primarily in the enteric tract (Webster et al., 1978). Clinical disease, however, has sporadically been associated with natural AIV infection in domestic and wild waterfowl (Alexander, 2000, Easterday et al., 1997; Hwang et al., 1970; Lipkind et al., 1979; Xu et al., 1999), ratites (emus, ostriches, and rheas) (Alexander, 2000; Allwright et al., 1993), and psittacines (Alexander, 2000; Mase et al., 2001). In most cases, the manifestation of clinical disease was attributable to concurrent adverse conditions, including poor husbandry, secondary infections, or high stress. Similarly, LPAI virus infection in wild birds usually results in no gross or microscopic lesions but may occasionally produce inflammation in the respiratory and enteric tracts, often associated with secondary bacterial infections (Swayne and Halvorson, 2003). A recent study found that apparent subclinical infection with LPAI viruses in Bewick's swans (*Cygnus columbianus bewickii*) resulted in reduced efficiency of pre-migratory feeding and migratory performance (van Gils et al., 2007). This would suggest that AIV infection in wild birds may impact the behavior of individual birds in certain species. Further research is warranted to better understand the effects that LPAI virus infections may have on wild birds; such effects may affect both virus transmission and wildlife health.

H5N1 HPAI virus in wild birds: History

The first reported outbreak of H5N1 HPAI virus occurred at a domestic goose farm in the Guangdong province of China in 1996 (Sims et al., 2005). Genetically similar H5N1 HPAI viruses continued to circulate in domestic geese in southern China for the following 4 years (Cauthen et al., 2000). During this time, H5N1 HPAI viruses exchanged internal genes with co-circulating AIV. This recombination resulted in a diversity of H5N1 HPAI viruses with multiple genotypes and an expanded host range that included domestic ducks (Guan et al., 2002; Chen et al., 2004). In 2002, significant mortality associated with H5N1 HPAI virus infection was reported in captive and feral aquatic birds housed within two waterfowl parks in Hong Kong (Ellis et al., 2004). Mortality was also observed in free-living grey herons (*Ardea cinerea*) and black-headed gulls (*Larus ridbundus*) spatially associated with these outbreaks. Since 2002, H5N1 HPAI viruses have continued to circulate in waterfowl, and viral infection has been associated with mortality in ducks and other wild birds (Li et al., 2004; USGS, 2006).

Domestic ducks play a key role in the epidemiology of H5N1 HPAI virus in southeast Asia. In contrast to gallinaceous poultry, H5N1 HPAI infection in ducks may be asymptomatic and viral shedding can be prolonged (Chen et al., 2004). Some mallards experimentally-infected with H5N1 HPAI virus shed virus for 11 to 17 days without morbidity or mortality (Hulse-Post et al., 2005). Li et al. (2004) reported H5N1 HPAI virus infection in approximately 25% of clinically normal domestic ducks in the live bird markets in southern China. The existing surveillance and experimental data, combined with the intermixing of waterfowl and gallinaceous poultry in live bird markets, free-grazing farming practices, and significant waterfowl trade in southeast Asia, strongly suggest domestic ducks contributed not only to the

endemnicity of H5N1 HPAI virus in poultry in this region but also to the spill-over of H5N1 HPAI virus into wild birds (Chen et al., 2004; Gilbert et al., 2006).

In June, 2005, H5N1 HPAI virus was recovered from sick and dead wild birds during a large mortality event at Qinghai Lake, China, an important breeding ground for migratory birds that winter in southeast Asia (Liu et al., 2005). Over one thousand wild birds were affected in this outbreak involving several different avian species. From late summer 2005 through early winter 2006, H5N1 HPAI viruses spread to central Asia, followed by multiple waves of outbreaks in Europe and Africa (Alexander, 2007). In total, 26 European countries were affected and mortality associated with H5N1 HPAI virus infection was reported in domestic poultry and/or wild birds (Alexander, 2007). Though not confirmed, a growing body of genetic and epidemiologic data suggests that the spread of H5N1 HPAI virus to central Asia and Europe occurred in migratory waterfowl (Sabirovic et al., 2006 A). More specifically, it appears that multiple introductions of genetically similar H5N1 HPAI viruses occurred in Europe in 2005/2006 and that the outbreaks were spatially and temporally associated with migratory waterfowl populations that were displaced due to severe winter weather. It is not currently known whether similar H5N1 HPAI epidemics will reoccur in Europe or whether these were sporadic events driven by extreme environmental conditions.

H5N1 HPAI virus in wild birds: Host range

The species affected by H5N1 HPAI virus in southeast Asia have been taxonomically diverse, including multiple terrestrial and aquatic avian species (USGS, 2006). This diversity is likely due to the combined effects of poultry production practices in southeast Asia, live bird markets, and the fact that H5N1 HPAI virus is endemic in gallinaceous poultry and domestic

ducks. A large interface exists between free-living and captive birds, providing sufficient opportunities for wild, captive, or feral avian species to be exposed to the virus.

During the European outbreaks a limited number of species in the Order Anseriformes died from H5N1 HPAI virus or were determined to be infected by virus isolation. Some affected species included mute swans (*Cygnus olor*), bar-headed geese (*Anser indicus*), whooper swans (*Cygnus cygnus*), tufted ducks (*Aythya fuligula*), and Canada geese (*Branta canadensis*) (Sabirovic et al., 2006 A; Teifke et al., 2007). The host range for H5N1 HPAI virus in Europe is likely reflective of: 1) highly susceptible waterfowl species (swans and geese) with migratory pathways that permit a direct or indirect connection (direct contact or shared habitats) with infected domestic poultry in Asia, allowing H5N1 HPAI to spill-over from domestic poultry into wild birds, and 2) climatic and environmental conditions causing abnormally high concentrations of birds in focal geographic areas facilitating the transmission of virus within the population.

Throughout the Eurasian outbreaks, wild bird species from which H5N1 HPAI viruses have been isolated generally fall into three categories, including aquatic birds, carnivorous avian species, and other species closely associated with domestic poultry environments. Although affected aquatic birds have primarily been in the Order Anseriformes, H5N1 HPAI has caused mortality in several other groups of birds such as herons, egrets, and flamingos (Ellis et al., 2004; USGS, 2006). Many of these non-anseriform species were sympatric with ducks, geese, or swans and infection in these species likely represented a dead-end spill-over event. Mortality associated with H5N1 HPAI infection also has been reported in raptors, gulls, crows, and other avian species that likely are exposed to the virus through ingestion of carcasses of domestic or wild birds that died from viral infection. Mortality in these scavenger species generally has been sporadic and involved individual birds, but focal outbreaks involving multiple birds have

occurred (Tanimura et al., 2006; Kwon et al., 2005). Occasionally, H5N1 HPAI viruses have also been isolated from non-scavenging terrestrial species commonly found around poultry rearing environments, such as Eurasian tree sparrows (*Passer montanus*) or feral pigeons (*Columbia livia*) (USGS 2006; Ellis et al., 2004).

Perkins and Swayne (2003 A) evaluated the susceptibility of several avian species to H5N1 HPAI virus after experimental infection with A/chicken/Hong Kong/220/97. Based on mortality, mean death time, and widely disseminated viral antigen, gallinaceous birds and zebra finches (*Taeniopygia guttata*) were the most susceptible species, followed by domestic Embden geese (*Anser anser domesticus*), emus (*Dramaius novaehollandiae*), house finches (*Carpodacus mexicanus*), and budgerigars (*Melopsittacus undulatus*). Minimal and localized viral replication and mild or no clinical disease were exhibited by Pekin ducks (*Anas platyrhyncos*), house sparrows (*Passer domesticus*), and laughing gulls (*Larus atricilla*). Pigeons (*Columbia livia*) and European starlings (*Sturnus vulgaris*) were refractory to disease and resistant to viral replication. This study provided essential information on the range of susceptibility and pathobiology among avian species infected with H5N1 HPAI virus. However, it was conducted with an early virus isolate. No similar, large-scale studies have been conducted with viruses isolated after 2002, when H5N1 HPAI viruses that were virulent for wild birds emerged. Experimental infection studies have been conducted in ducks using recent H5N1 HPAI viruses but the studies, to date, have only focused on mallards or Pekin ducks (Hulse-Post et al., 2005; Pantin-Jackwood and Swayne, 2007). Collectively, these studies have shown that this species is susceptible to H5N1 HPAI virus, but the observed virulence is multifactorial and dependent on the interaction of several factors, including the strain of virus, age of the birds, route of exposure, concomitant infections, and environmental conditions.

H5N1 HPAI virus in wild birds: Transmission and epidemiology

Experimental infections of ducks have consistently shown that H5N1 HPAI viral shedding is predominately associated with the respiratory tract rather than the cloaca. Furthermore, viral titers appear to be positively associated with the severity of clinical disease; ducks exhibiting the most severe clinical signs shed the highest titers of virus (Sturm-Ramirez, 2004). Transmission of H5N1 HPAI virus between ducks has been demonstrated under confinement conditions and, based on the location of viral shedding, it is thought that the primary route of transmission is via the respiratory tract. Previously AIV infection in waterfowl has only been known to occur through an indirect fecal-oral route. It is unknown how this deviation from the traditional AIV paradigm will affect the transmission or maintenance efficiency of H5N1 HPAI virus in the waterfowl population.

Surveillance data indicate that H5N1 HPAI viruses are endemic in domestic poultry in southern China, including ducks and gallinaceous birds (Li et al., 2004). Within these poultry populations, H5N1 HPAI has a seasonal pattern with the majority of virus isolations occurring between October and January when temperatures drop below 20º C. The cause for this seasonal pattern is not known. The prevalence of H5N1 HPAI virus in wild birds, as detected by mortality or virus isolation, has not been consistent enough to recognize seasonal patterns of disease. During the outbreaks in 2005 and 2006, however, H5N1 HPAI virus spread to central Asia and eastern Europe from May, 2005 to January, 2006 and was reported throughout several European Union (EU) member states and sub-Saharan African nations during the first four months of 2006 (Alexander, 2007). Based on these data, the greatest risk of H5N1 HPAI virus spread in wild birds occurs during winter migration from August to November, when migrating birds move westward from Asia to Europe and Africa (Sabirovic, 2006 B). This risk is

dependent on the likelihood that the migratory wild birds move through areas that have had recent outbreaks of H5N1 HPAI and are sufficiently exposed to the virus through environmental sources or other infected birds (including domestic birds). The likelihood of these events is not known and, subsequently, the risk of viral dissemination in wild birds is uncertain.

H5N1 HPAI virus in wild birds: Reservoir hosts and maintenance cycle

Though wild bird mortality was spatially and temporally associated with the 2005/2006 spread of H5N1 HPAI virus to Europe and Africa, virus has not been detected in clinically healthy wild birds (Gauthier-Clerc et al., 2007) and there is no indication that the virus is maintained or geographically spread in these asymptomatic avian hosts. As mentioned previously, relatively few species of anseriforms are over-represented in the mortality reports, including mute swans, whooper swans, and bar-headed geese. A reliance on dead bird surveillance makes the evaluation of H5N1 HPAI virus in wild birds difficult and has left several gaps in our understanding (Nagy et al., 2006; Teifke et al., 2007). It is unknown whether H5N1 HPAI viruses originating from domestic poultry have become established in wild avian populations or whether these outbreaks will reoccur.

Mallards experimentally infected with some H5N1 HPAI viruses have had prolonged duration of asymptomatic shedding, suggesting that infected mallards could potentially contribute to the maintenance of virus in a wild avian population. In addition, transmission of H5N1 HPAI virus has been demonstrated between infected and contact mallards under penned conditions (Sturm-Ramirez, 2005); however, viral shedding in these cases, was primarily via oropharyngeal secretions. It is unlikely that similar respiratory infections would result in the same environmental contamination levels as fecal shedding and, consequently, the oropharyngeal

route may not represent an efficient means for prolonged viral transmission within a wild avian population.

Prior to the 2002 outbreaks of H5N1 HPAI virus in Hong Kong, the only wild bird mortality associated with HPAI viruses was an outbreak in South Africa in 1961 in common terns (*Sterna hirundo*), which was attributed to a H5N3 HPAI virus (Becker, 1966). There is no evidence that this or any other HPAI virus can be maintained in wild bird populations, and all information suggests that HPAI viruses evolve independent of wildlife reservoirs when introduced wild-type AIV adapt to domestic poultry populations (Suarez, 2000). Though strong field evidence supports H5N1 HPAI virus transmission over large geographic distances by migratory wild birds in 2005 and 2006, surveillance efforts in EU-member states since these outbreaks have failed to detect this virus (Sabirovic et al., 2007). The risk factors associated with H5N1 HPAI virus transmission and spread in wild birds are unclear, and there is little evidence to support or refute the possibility that these viruses are established in free-living avian populations. Until additional outbreaks occur or surveillance efforts detect H5N1 HPAI virus in clinically healthy wild birds, the reservoir status and maintenance risk of wild birds will remain undetermined.

H5N1 HPAI virus in wild birds: Morbidity, mortality, and pathology

Species-related differences in susceptibility to H5N1 HPAI viruses are apparent both in natural and experimental infections (Perkins and Swayne, 2003; Ellis et al., 2004). These species differences include prevalence of sickness and death, mean time to death, duration of clinical disease, and distribution of viral antigen and lesions. Highly susceptible species, such as gallinaceous poultry, are often found dead without exhibiting clinical signs at 1 to 2 days post-inoculation (DPI), whereas less susceptible species, such as house finches (*Carpodacus mexicanus*) and budgerigars (*Melopsittacus undulates*), do not show clinical signs until 7 DPI (Perkins and

Swayne, 2003 A). Regardless of species, clinical disease associated with H5N1 HPAI virus infection generally consists of early signs, such as weakness, inactivity, and lethargy, which progress to severe depression and neurologic dysfunction, including ataxia, paralysis, tremors, and seizures (Ellis et al., 2004; Perkins and Swayne, 2003 A).

In highly susceptible species such as gallinaceous poultry and zebra finches, viral antigen is first demonstrated in the vascular endothelium but rapidly disseminates to the parenchyma of multiple visceral organs and the brain. In particular, H5N1 HPAI virus has a predilection for neurons and glial cells of the brain, cardiac myocytes, adrenal corticotrophic and chromaffin cells, pancreatic acinar cells, and hepatocytes; virus is less commonly detected in the endothelial cells of the lungs, lymphoid tissue, and renal tubular epithelium (Teifke et al., 2007; Perkins and Swayne, 2003 B; Perkins and Swayne, 2001; Zhou et al., 2006). The distribution and severity of histologic lesions are strongly associated with the presence of viral antigen and include hemorrhage, exudation, and necrosis. Viral infection in gallinaceous poultry and zebra finches is acute and experimentally, mortality is usually greater than 75%. Infected birds often are found dead, but when clinical disease is observed, it is primarily neurologic.

In less susceptible species, morbidity and mortality are delayed and the clinical disease is prolonged (Perkins and Swayne, 2003 B; Perkins and Swayne, 2002). Clinical signs in less susceptible species range from mild inactivity and lethargy to severe neurologic dysfunction. The mortality rate is highly variable, ranging from 0 to 75%. The severity and distribution of viral antigen and histologic lesions are not as widespread as in highly susceptible species, and lesions are primarily located in the brain and, less commonly, in the heart, adrenal glands, and pancreas. Lesions in acute cases are generally necrotizing with infiltrates of lymphocytes, plasma cells, and heterophils becoming more prominent in the affected tissues as the duration of disease increases.

Some avian species are minimally susceptible or refractory to H5N1 HPAI virus infection (Perkins and Swayne, 2003; Brown et al., 2006), and in some species, the susceptibility appears to be dependent on the strain of virus. The virulence of H5N1 HPAI viruses for mallards and Pekin ducks, for example, is highly variable with mortality rates ranging from 0 to 100% (Perkins and Swayne, 2003 B; Perkins and Swayne, 2002; Sturm-Ramirez, 2005). Histologic lesions in species that are resistant to H5N1 HPAI virus infection are minimal and detectable viral antigen is scarce.

AVIAN INLUENZA VIRUS IN THE ENVIRONMENT

Research on the ecology of AIV has traditionally focused on virus-host animal interactions, including the determination of species susceptibility, identification of virus-host adaptation, and description of pathogenesis. This knowledge is important in understanding the epidemiology of AIV. An understanding of interactions between AIV and the environment, however, is needed to fully evaluate the "normal" maintenance cycle of wild-type AIV in waterfowl populations and also the "abnormal" cycles of HPAI viruses in wild avian populations. Transmission of AIV within wild bird populations occurs via an indirect fecal-oral route involving fecal-contaminated water on shared aquatic habitats (Hinshaw et al., 1979; Sandu and Hinshaw, 1981; Sinnecker et al., 1983). The efficiency of this transmission cycle is dependent on the stability of AIV in water. The initial study that examined the environmental stability of AIV was performed using A/duck/Memphis/546/74 (H3N2) and viral persistence was evaluated in both fecal material and water (Webster et al., 1978). An initial viral dose of $10^{6.8}$ EID_{50} remained infective for 32 days in fecal material (pH 7.68) at 4º C, but virus was not detected in the feces after 13 days at 22º C. Similarly, an initial viral dose of $10^{8.1}$ EID_{50} remained infective for 32 days in non-chlorinated Mississippi river water at 4º C but virus was not detected after 7 days at 22º C. This study established the ability of wild-type AIV to persist

in the aquatic environment and, based on this environmental stability, provided support for the indirect fecal-oral mechanism of transmission of AIV within a waterfowl population. Further support for this AIV paradigm was provided by field surveillance studies in which AIV were isolated from surface water at waterfowl habitats in Canada (Hinshaw et al., 1980), Minnesota (Halvorsen et al., 1985), and Alaska (Ito et al., 1995), but virus was not present in water from lakes devoid of ducks (Hinshaw et al., 1986).

With an indirect transmission strategy involving water, it is reasonable to assume that these viruses have evolved to persist in water for extended periods of time. In a laboratory evaluation of five wild-type AIV derived from wild ducks in Louisiana, the estimated persistence in water of 10^6 $TCID_{50}$ ranged from 126 to 194 days at 17° C and 30 to 102 days at 28° C (Stallknecht et al., 1990 A). In addition to documenting extended infectivity in water, this study noted significant variability in the different AIV isolates' ability to persist in water. In a subsequent evaluation of the effects of pH, temperature, and salinity on AIV persistence in water using a distilled-water model, it was determined that even subtle differences in water chemistry (differences that are present within the normal environmental habitats) can significantly influence AIV persistence (Stallknecht et al., 1990 B). In this study, the wild-type AIV isolates persisted longest in colder temperatures (4° C and 17° C), fresh water (salinity of 0 parts per thousand), and slightly basic conditions (pH = 8.2). Furthermore, the AIV persistence results obtained from the distilled water model were validated when similar laboratory results were obtained from surface water samples derived from coastal waterfowl habitats in Louisiana (Stallknecht et al., 1990 B). It is well documented that AIV transmission within wild bird populations is spatially and temporally dependent (Stallknecht and Shane, 1988). These initial water studies by Stallknecht et al. (1990 A and B) suggest that transmissibility may also be

regulated by water conditions within specific wildlife habitat types and may affect AIV prevalence in waterfowl locally and regionally.

Very little is known about the environmental stability of HPAI viruses. An H5N2 HPAI virus isolated from the 1983/1984 outbreaks in Pennsylvania persisted in feces for longer durations at colder temperatures and when the fecal material was moist (Beard et al., 1984). The HPAI virus in this study was detected in wet feces after 35 days at 4° C, but no virus was re-isolated from wet feces stored at 22° C after two days. As with the LPAI virus studies described above, this study suggests that HPAI viruses have the ability to persist in the environment for several weeks, but viral persistence is highly dependent on ambient conditions, such as temperature and moisture. Very little is known about the environmental stability of H5N1 HPAI virus. Webster et al. (2007) reported that H5N1 HPAI viruses from 1997 were not infective in an aqueous solution of feces after 2 days at 37º C, but viruses isolated from 2003 to 2004 remained viable for 4 to 6 days under similar conditions. To date, this is the only reported persistence data that exist on H5N1 HPAI virus. While significant work has been done to improve our understanding of H5N1 HPAI viral infection in domestic poultry, much of the ecology of this virus as it relates to wild bird infection, remains unknown. The environmental stability of these viruses has not been determined and, without this information, it is unknown how efficiently these viruses will be transmitted or whether they will be maintained in free-living avian populations.

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CHAPTER 3

PERSISTENCE OF H5 AND H7 AVIAN INFLUENZA VIRUSES IN WATER[1]

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ABSTRACT

Although fecal-oral transmission of avian influenza viruses (AIV) via contaminated water represents a recognized mechanism for transmission within wild waterfowl populations, little is known about viral persistence in this medium. In order to provide initial data on persistence of H5 and H7 AIVs in water, we evaluated eight wild-type low pathogenicity avian influenza H5 and H7 viruses isolated from species representing the two major influenza reservoirs (Anseriformes and Charadriiformes). In addition, the persistence of two highly pathogenic avian influenza (HPAI) H5N1 viruses from Asia was examined to provide some insight into the potential for these viruses to be transmitted and maintained in the environments of wild bird populations. Viruses were tested at two temperatures (17 C and 28 C) and three salinity levels (0, 15, 30 parts per thousand sea salt). The wild-type H5 and H7 AIV persistence data to date indicate that: 1) H5 and H7 AIVs can persist for extended periods of time in water, with a duration of infectivity comparable to AIVs of other subtypes; 2) the persistence of H5 and H7 AIVs is inversely proportional to temperature and salinity of water; and 3) a significant interaction exists between the effects of temperature and salinity on the persistence of AIV, with the effect of salinity more prominent at lower temperatures. Results from the two HPAI H5N1 viruses from Asia indicate that these viruses did not persist as long as the wild-type AIVs.

INTRODUCTION

Aquatic birds in the Orders Anseriformes and Charadriiformes are the natural reservoir for avian influenza viruses (AIVs) (9). Infections in these avian hosts are normally asymptomatic and characterized by preferential replication in the intestinal tract with high concentrations of virus shed in the feces (16). Viral transmission in aquatic bird populations is thought to occur through an indirect fecal/oral route involving contaminated water (4, 5, 16). The maintenance of

AIV in these populations may also be dependent on or enhanced by environmental persistence. It is possible that virus shed by birds in the fall, prior to migration, could be preserved in the water over winter, and provide a source of infection to birds returning during the following spring (17). Despite the well-recognized role that contaminated water plays in the transmission cycle of AIVs in wild waterfowl populations, very little is known about the viral persistence in this medium.

Experimental data suggest that AIVs have evolved to persist for extended periods in aquatic habitats. The initial laboratory studies investigating the environmental stability of wild-type low pathogenicity avian influenza (LPAI) viruses determined that virus in fecal material remained infective in non-chlorinated water for at least 30 days at 4 C and up to 7 days at 20 C (16). A validated model system using distilled-water was later developed to evaluate the effects of different environmental parameters on the persistence of AIVs (11). Experimental studies using this system indicate the following: 1) wild-type AIVs can remain infective in water for an extended period, with an estimated persistence > 190 days for some viruses with a starting viral concentration of 10^6 mean tissue-culture infective dose ($TCID_{50}$)/ml; 2) the ability to persist in water differs between individual AIVs; and 3) viral persistence is markedly influenced by differences in temperature, salinity, and pH (based on limits encountered in natural field conditions). Wild-type viruses persist longest in cold freshwater (17 C, 0 parts per thousand (ppt)), with a slightly elevated pH (8.2) (10, 11).

To date, evaluations of environmental persistence of wild-type AIVs have not included H5 or H7 AIVs. Understanding the potential for environmental persistence of these viruses is important because H5 and H7 AIVs can become highly pathogenic in domestic poultry, causing up to 100% mortality and substantial financial losses (15), and because some AIVs of the H5 and H7 subtypes have the ability to transmit directly from the avian host to humans (3, 6, 14).

Avian influenza virus infections in wild birds are rarely associated with morbidity or mortality. In 2002, this paradigm was challenged when highly pathogenic avian influenza (HPAI) H5N1 viruses caused mortality in wild and captive birds in two waterfowl parks in Hong Kong (2, 12). Though HPAI H5N1 viruses have been isolated from wild species of waterfowl throughout Eurasia since this initial outbreak, it is unknown if the viruses can be maintained in these avian populations. Information on the environmental persistence of HPAI H5N1 viruses would greatly improve our understanding of the natural transmission cycle of the viruses and the potential risk for maintenance of these viruses in wild waterfowl populations. Currently, there is limited information on persistence of any HPAI virus in aquatic habitats, and to our knowledge, this is the first study to evaluate environmental stability of HPAI H5N1 viruses circulating in Eurasia.

The goals of this study are: 1) to experimentally determine the duration that wild-type H5 and H7 AIVs and HPAI H5N1 viruses can remain infective in water; 2) evaluate the effects of temperature and salinity on the duration of infectivity of AIVs; and 3) compare the environmental persistence of HPAI H5N1 viruses recently isolated from Asia with wild-type H5 AIVs.

MATERIALS AND METHODS

Viruses

The eight LPAI viruses used in this study were isolated from wild avian species within the Orders Anseriformes and Charadriiformes. Specific viruses included: A/Laughing Gull/DE/AI00-2455 (H7N3), A/Mallard/MN/182761/98 (H7N3), A/Blue-winged Teal/TX/578597/02 (H7N4), A/Ruddy Turnstone/DE/650635/02 (H7N3), A/Ruddy Turnstone/NJ/828227/01 (H5N8), A/Mallard/MN/182742/98 (H5N2), A/Mallard/MN/355790/00

(H5N3), and A/Ruddy Turnstone/NJ/828219/01 (H5N7). The viruses were propagated in 9- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs and infective amnio-allantoic fluid (AAF) was harvested after 96 hours post-inoculation. The AAF was stored at -70 C. All trials were performed with low passage isolates (second, third, or fourth passage). These experiments were conducted under biosafety level (BSL) 2 facilities at the College of Veterinary Medicine, The University of Georgia, Athens, GA.

The two HPAI H5N1 viruses evaluated in this study were obtained from the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, Georgia. Individual stocks of A/Whooper Swan/Mongolia/244/05 (H5N1) (Mongolia/05) and A/Duck Meat/Anyang/01 (H5N1) (Anyang/01) AIVs were prepared by second passage in embryonated chicken eggs as described above. Stock AAF of Mongolia/05 virus was diluted 1:20 in uninfected SPF AAF to approximate the Anyang/01 stock titer. All experiments with HPAI viruses were conducted under USDA-certified BSL 3-Ag facility at SEPRL (1).

Infectivity Assays

Infectivity of AIV in water samples was quantified using a microtiter endpoint titration as previously described (10). In this protocol, a 0.5-ml sample of AIV-inoculated water was diluted 1:2 by addition of 0.5 ml of 2X serum-free Eagle's minimum essential medium (MEM). Ten-fold dilutions (10^{-1} to 10^{-8}) were then made in MEM supplemented with antibiotics (100 u penicillin G with 100 µg streptomycin sulfate/ml). Each well of a 96-well microtiter plate received 100 µl of cell suspension and 50 µl of the appropriate virus dilution. Cell suspensions were prepared as primary cultures of chicken embryo fibroblasts (CEFs) from 9- to 11-day-old SPF chicken embryos. Final cell suspensions consisted of $3x10^6$ CEF/ml suspended in serum-free MEM

supplemented with antibiotics. For LPAI virus trials, each well was then supplemented with 50 µl of MEM containing 2.8 µg of highly purified trypsin (final concentration per well of 0.5 µg/ml). Preliminary studies conducted at SERPL determined that supplemental trypsin did not affect the titer of HPAI viruses using this assay (data not shown) and was therefore not required for HPAI virus trials.

For both LPAI and HPAI experiments, plates were covered and incubated at 37 C under 5% CO_2 for 96 hr. Examination for cytopathic effects was performed with light microscopy and, to confirm results, plates were stained with 1% crystal violet in 10% neutral buffered formalin (to inactivate virus) for further examination. Endpoints were recorded as 100% monolayer destruction with $TCID_{50}$ calculated as previously described (7). The minimal detectable limit of this assay is 2.2621 $TCID_{50}$/ml.

Experimental Procedures

Infective AAF was diluted 1:50 in sterile glass-filtered distilled water that was previously adjusted to the following conditions. Three salinities (0, 15, 30 ppt) were evaluated, corresponding to fresh, brackish, and salt water, respectively (11). Adjustments in salinity were made with commercially available sea salt. The pH of all three water treatments was adjusted to 7.4 with a sterile 1 *N* solution of NaOH and stabilized with 2 mM HEPES buffer. The pH of the water measured at the end of the trial did not vary more than 0.2 for all evaluated water treatments. We previously determined that the inoculation of AAF into this system at a dilution of 1:50 or greater did not affect the duration of virus infectivity (data not shown). Aliquots of inoculated water (2.0 ml) were transferred to 2.5 ml glass tubes. The samples were then evenly divided and placed in environmental chambers at 17 C and 28 C. These values represent the winter and summer temperatures, respectively, for coastal marshland in Louisiana and were

chosen to allow accurate comparison of data from this study to previous trials with non-H5 or H7 subtypes (11). Starting at post-inoculation day (PID) 0, aliquots were removed weekly, and titrations were conducted as described above, over a 60-day trial period. For three of the viruses (A/Mallard/MN/98 (H5N2); A/Mallard/MN/98 (H7N3); and A/Laughing Gull/DE/00 (H7N3)) water samples were also titrated on PID 120 and 180 to confirm long-term persistence of AIVs in this model-system and to determine whether linear regression models continue to accurately describe the data. Weekly titrations for each virus/salinity/temperature treatment group were terminated when virus was not detected in the water on two consecutive assays. To account for the minimal detectable limit of this assay, a value of 2.26 $TCID_{50}$/ml was used for the first weekly titration point that virus was not detected in each trial.

Statistical Analysis

Sequential data for each virus/salinity/temperature trial were $\log_{10}$ transformed and subjected to linear regression analysis using Microsoft Excel (Microsoft Office Excel 2003, Redmond, Washington, USA). The resulting models were used to determine the estimated persistence (days) for each virus/salinity/temperature trial with a starting viral concentration of 10^6 $TCID_{50}$/ml. In addition, the time required for each virus to reduce infectivity by 90%, as evidenced by a decrease in titer by 1 $\log_{10}$/ml, was calculated for all salinity and temperature combinations. The persistence of virus in water was evaluated using Analysis of Variance (ANOVA) with a full factorial design including the fixed effects of temperature (17, 28 C), salinity (0, 15, 30 ppt), and pathogenicity (high-pathogenicity or low-pathogenicity). The above analyses on the estimated persistence of virus were performed using the General Linear Models procedure of SAS® (SAS Inc., Cary, North Carolina, USA) (8).

RESULTS

The linear regression models, R^2, and estimated persistence for each of the virus/salinity/temperature trials are summarized in Tables 1 and 2. Persistence was highly variable between viruses within the same subtype and between individual HPAI H5N1 viruses. Although ANOVA results indicated that the persistence of virus in water was reduced by both salinity ($F_{2,48}$=9.16; p=0.0004) and temperature ($F_{1,48}$=52.37; p<0.0001), a significant interaction existed between the effects of salinity and temperature ($F_{2,48}$=4.48; p=0.0165). Figure 1 characterizes the nature of this interaction in which the effect of salinity on viral persistence is less at 28 C than at 17 C. The HPAI H5N1 viruses examined in this study did not persist as long as the wild-type AIV viruses ($F_{1,48}$=4.09; p=0.0488).

Linear regression models continued to appropriately describe the data from the three viruses that were assayed at 120 and 180 days (Table 3). The coefficients of determination for the 6-month-data were higher for all three viruses in these long-term persistence models. In addition, the slopes from the 2-month-data and the 6-month-data did not vary more than 0.01 for any of three viruses.

DISCUSSION

Wild-type AIVs in ducks are thought to have evolved over time into the perfect host-parasite relationship (16). Within this reservoir, viruses are transmitted between ducks through fecal-contaminated water. Prolonged infectivity of a virus in water would potentially enhance its transmissibility and, in such an indirect transmission cycle, it is conceivable that AIVs would adapt not only to the host but also to the aquatic environment.

The results of the water trials with wild-type H5 and H7 AIVs were consistent with previous studies on AIVs using the same model system (10, 11). Though variability in duration

of infectivity existed between H5 and H7 viruses of the same subtype, the estimated persistence values were comparable to results for other AIVs. As in previous studies, environmental persistence was inversely proportional to both temperature and salinity. Persistence was influenced by the interaction among the two effects as well, although the mechanism of this interaction is currently unknown. However, temperature strongly impacts many physiologic processes through its effects on enzyme activation and catalytic rates (18), and these temperature-dependent changes in enzyme kinetics may account for the observed interaction in our study.

The HPAI H5N1 viruses had a shorter persistence of infectivity in the environment than the wild-type AIVs. In general, the wild-type AIVs persisted longer than Mongolia/05 at all salinity and temperature combinations. However, the earlier H5N1 isolate (Anyang/01), persisted longer than six of the eight wild-type viruses at 15 ppt, and seven of the eight at 30 ppt. It is currently unknown why this virus has a prolonged infectivity at higher salinities, but this finding emphasizes the need to evaluate environmental persistence of any AIV (LPAI and HPAI viruses) under a variety of conditions.

The HPAI H5N1 viruses circulating in Eurasia exhibit varying biologic characteristics, including antigenic patterns, pathogenicity, and variation in the extent and duration of viral shedding (13). In experimental trials it has been demonstrated that unlike wild-type AIVs, replication of the HPAI H5N1 Asian strains is primarily associated with the respiratory tract. However, fecal shedding does occur and contact transmission has been demonstrated under experimental conditions (13). The extent and duration of fecal shedding to date suggests that it is less than would be expected with ducks infected with wild-type AIVs (16). The results of this study indicate that HPAI H5N1 virus isolates also differ in their environmental persistence

profiles. Taken together, these results suggest that compared to wild-type AIVs, ducks infected with HPAI H5N1 viruses would shed less virus into the environment and that the viruses remain infective in those environments for a shorter duration of time. This may imply that the HPAI H5N1 viruses may not be as fit as the wild-type viruses to persist and transmit within wild bird populations. However the variation in salinity tolerance between the two H5N1 viruses indicates that much more information is needed before we can accurately predict the extent of environmental fitness within the diversity of aquatic habitats utilized by wild birds.

The coefficients of determination for some models in the 17 C trials (Table 1) were much lower than those obtained in previous studies using linear models to describe AIV persistence in water (10, 11). Possible factors contributing to the reduced fit of these models include a small sample size, spurious laboratory results, or changes in the slope of viral persistence during the trial. As previously discussed, minimal variation was present between the slopes of viral trials examined at 2-months and 6-months, indicating that changes in slope are an unlikely cause for the low coefficient of determination. Spurious laboratory results could produce outliers in the data that would reduce the coefficient of determination. For example, the linear model for the persistence of MN/98 (H5N2) in 0 ppt water at 17 C has a coefficient of determination of 0.28. However, if one influential data point is omitted, the coefficient of determination increases to 0.72. The effect of outliers on the coefficient of determination is stronger in experiments with a small sample size, so including additional assays in future trials should improve the ability of the linear models to explain the data. This could be accomplished by performing dual titrations at each assay (10), sampling more frequently, or extending the trial period.

The results of this study indicate the following: 1) H5 and H7 AIVs have the ability to persist in water for extended periods of time; 2) persistence is highly variable between viruses within the

same subtype and between individual HPAI H5N1 viruses; 3) an interaction exists between the effects of salinity and temperature; and 4) the two HPAI H5N1 viruses examined in this study do not persist as long as the wild-type viruses under conditions of low salinity (0 ppt). This is an important observation because freshwater habitats are of major importance to the biology of wild waterfowl, a recognized reservoir of AIVs. The ecology of AIV in the environment remains largely unknown, including the mechanism and molecular determinants of viral persistence. Though the environmental persistence of AIV has been largely overlooked up to this point, we feel it is vital to understanding the epidemiology of AIV and has important implications to human, wild animal, and domestic animal health.

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Table 3.1. Linear regression models for persistence of H5 and H7 AIVs in water at 17° C.

Viruses	Salinity 0ppt LRM*	0ppt R^2	0ppt Estimated** Persistence	Salinity 15ppt LRM	15ppt R^2	15ppt Estimated Persistence	Salinity 30ppt LRM	30ppt R^2	30ppt Estimated Persistence
LPAI Viruses									
MN/98 (H5N2)	y = 5.375 - 0.014x	0.28	429 (71)***	y = 4.695 - 0.047x	0.71	128 (21)	y = 5.057 - 0.095x	0.86	63 (11)
MN/00 (H5N3)	y = 5.318 - 0.019x	0.41	316 (53)	y = 5.549 - 0.018x	0.53	333 (56)	y = 6.000 - 0.053x	0.85	113 (19)
NJ/01 (H5N7)	y = 6.265 - 0.026x	0.57	231 (38)	y = 5.049 - 0.016x	0.61	375 (63)	y = 5.495 - 0.071x	0.84	85 (14)
NJ/01 (H5N8)	y = 4.029 - 0.021x	0.12	286 (48)	y = 3.815 - 0.048x	0.62	125 (21)	y = 4.135 - 0.103x	0.89	58 (10)
MN/98 (H7N3)	y = 6.075 - 0.028x	0.70	214 (36)	y = 6.474 - 0.045x	0.64	133 (22)	y = 6.427 - 0.065x	0.90	92 (15)
TX/02 (H7N4)	y = 6.517 - 0.034x	0.65	176 (29)	y = 5.973 - 0.045x	0.80	133 (22)	y = 6.282 - 0.056x	0.97	107 (18)
DE/00 (H7N3)	y = 5.469 - 0.009x	0.15	667 (111)	y = 5.619 - 0.042x	0.75	143 (24)	y = 5.926 - 0.057x	0.91	105 (18)
DE/02 (H7N3)	y = 5.109 - 0.031x	0.71	194 (32)	y = 4.680 - 0.043x	0.81	140 (23)	y = 5.370 - 0.035x	0.75	171 (29)
HPAI H5N1 Viruses									
Mongolia/05	y = 4.787 – 0.038x	0.77	158 (26)	y = 4.869 - 0.072x	0.88	83 (14)	y = 4.033 - 0.073x	0.77	82 (14)
Anyang/01	y = 4.950 – 0.064x	0.94	94 (16)	y = 4.445 – 0.033x	0.59	182 (30)	y = 4.724 – 0.052x	0.79	115 (19)

*linear regression model: $y = \log_{10}$ $TCID_{50}$/ml, x = persistence in days
**estimated persistence (days) for a starting viral concentration of 1 X 10^6 $TCID_{50}$/ml water
***the time (days) required to reduce the starting viral concentration by 90% (1 $\log_{10}$)

Table 3.2. Linear regression models for persistence of H5 and H7 AIVs in water at 28° C.

Viruses	Salinity 0ppt LRM*	0ppt R^2	0ppt Estimated Persistence**	Salinity 15ppt LRM	15ppt R^2	15ppt Estimated Persistence	Salinity 30ppt LRM	30ppt R^2	30ppt Estimated Persistence
LPAI Viruses									
MN/98 (H5N2)	y = 5.278 - 0.051x	0.56	118 (20)***	y = 4.691 - 0.098x	0.78	61 (10)	y = 5.310 - 0.214x	0.96	28 (5)
MN/00 (H5N3)	y = 4.767 - 0.071x	0.60	85 (14)	y = 5.650 - 0.108x	0.90	56 (9)	y = 6.184 - 0.279x	0.93	22 (4)
NJ/01 (H5N7)	y = 6.619 - 0.114x	0.90	53 (9)	y = 5.199 - 0.128x	0.99	47 (8)	y = 5.364 - 0.230x	0.84	26 (4)
NJ/01 (H5N8)	y = 4.379 - 0.167x	0.75	36 (6)	y = 4.352 - 0.143x	0.99	42 (7)	y = 4.071 - 0.143x	0.75	42 (7)
MN/98 (H7N3)	y = 5.490 - 0.086x	0.81	74 (12)	y = 6.409 - 0.205x	0.92	29 (5)	y = 6.306 - 0.188x	0.84	32 (5)
TX/02 (H7N4)	y = 6.054 - 0.100x	0.82	60 (10)	y = 6.006 - 0.320x	0.83	19 (3)	y = 5.934 - 0.209x	0.89	29 (5)
DE/00 (H7N3)	y = 5.668 - 0.090x	0.89	67 (11)	y = 5.744 - 0.233x	0.97	26 (4)	y = 6.258 - 0.269x	0.97	22 (4)
DE/02 (H7N3)	y = 5.611 - 0.252x	0.99	24 (4)	y = 5.153 - 0.242x	0.89	25 (4)	y = 5.080 - 0.231x	0.93	26 (4)
HPAI H5N1 Viruses									
Mongolia/05	y = 5.270 - 0.228x	0.95	26 (4)	y = 5.071 - 0.216x	0.93	28 (5)	y = 4.595 - 0.333x	1****	18 (3)
Anyang/01	y = 4.921 - 0.203x	0.94	30 (5)	y = 5.071 - 0.216x	0.93	28 (5)	y = 5.212 - 0.228x	0.92	17 (3)

*linear regression model: $y = \log_{10}$ $TCID_{50}$/ml, x = persistence in days
**estimated persistence (days) for a starting viral concentration of 1 X 10^6 $TCID_{50}$/ml water
***the time (days) required to reduce the starting viral concentration by 90% (1 $\log_{10}$)
****an R2 value of 1 indicate models from trials in which virus was present above the minimal detectable limit on only two sequential assays

Table 3.3. Linear regression models for persistence of three wild-type AIVs in water (0 ppt; pH 7.4; 17 C) based on data collected for 2-months and 6-months.

Viruses	2-month data			6-month data		
	LRM*	R^2	Estimated** Persistence	LRM	R^2	Estimated Persistence
MN/98 (H5N2)	y = 5.375 – 0.014x	0.28	429 (71)***	y = 5.228 - 0.010x	0.55	600 (100)
DE/00 (H7N3)	y = 5.469 – 0.009x	0.15	667 (111)	y = 5.663 - 0.017x	0.73	353 (59)
MN/98 (H7N3)	y = 6.075 – 0.028x	0.70	214 (36)	y = 5.757 - 0.017x	0.83	353 (59)

*linear regression model: $y = \log_{10}$ $TCID_{50}$/ml, x = persistence in days
**estimated persistence (days) for a starting viral concentration of 1 X 10^6 $TCID_{50}$/ml water
***the time (days) required to reduce the starting viral concentration by 90% (1 $\log_{10}$)

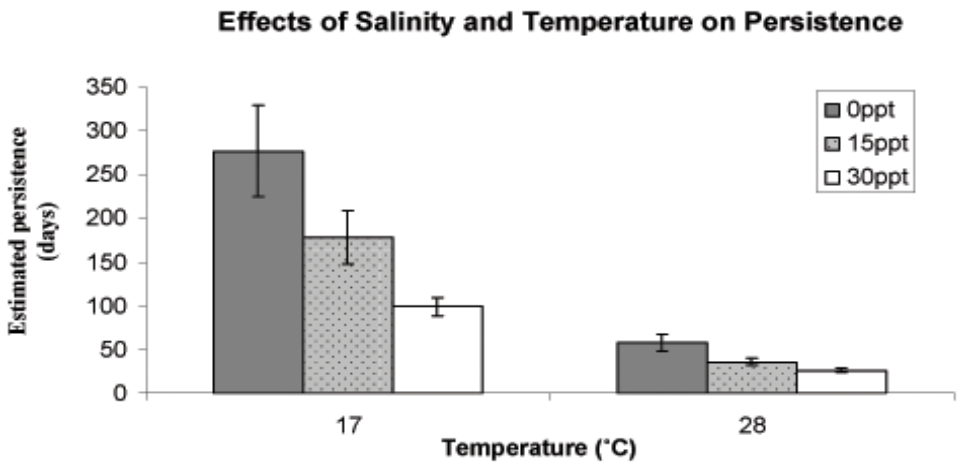

Figure 3.1. The plotted interaction between the effects of salinity and temperature on estimated persistence.

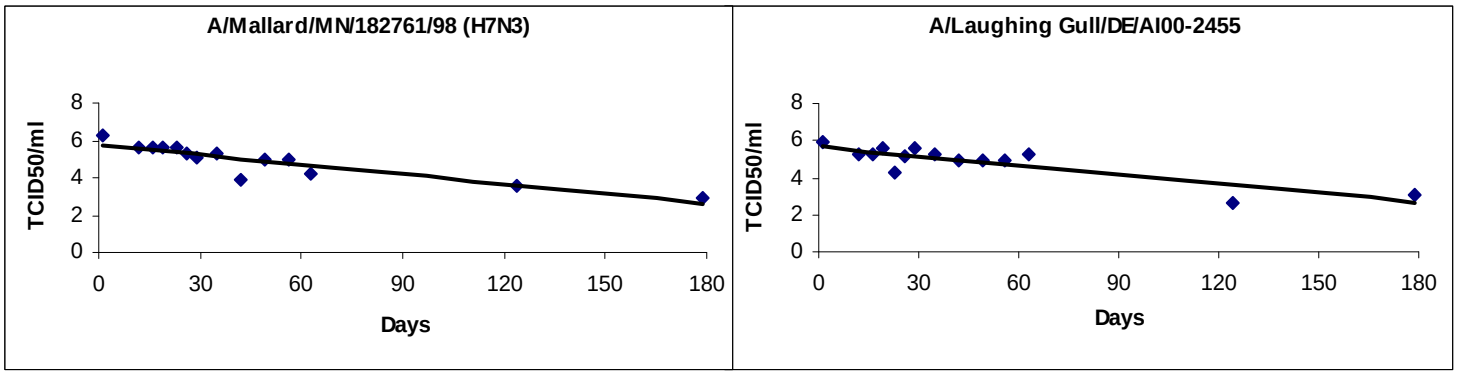

Figure 3.2. Linear regression models for the persistence of two wild-type H7N3 viruses in water (pH 7.4; salinity: 0ppt) at 17 C.

CHAPTER 4

THE SUSCEPTIBILITY OF NORTH AMERICAN DUCKS AND GULLS TO H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES [1]

[1] Brown, J.D., D.E. Stallknecht, J.R. Beck, D.L. Suarez, and D.E. Swayne. 2006. *Emerging Infectious Diseases* 12 (11): 1663-1670. Reprinted here with permission of publisher.

ABSTRACT

Since 2002, H5N1 highly pathogenic avian influenza (HPAI) viruses have been associated with mortality in numerous wild avian species throughout Eurasia. In this study, we assessed the clinical response and extent and duration of viral shedding in five species of North American ducks and laughing gulls (*Larus atricilla*) after intranasal challenge with two Asian H5N1 HPAI viruses. Birds were challenged at approximately 10 to 16 weeks of age which is consistent with temporal peaks in virus prevalence and fall migration. All species were infected, but wood ducks (*Aix sponsa*) and laughing gulls were the only species to exhibit either morbidity or mortality. Infected mallards (*Anas platyrhynchos*), northern pintails (*Anas acuta*), blue-wing teals (*Anas crecca*), and redheads (*Aythya americana*) did not exhibit clinical signs. Viral titers were higher in oropharyngeal swabs than cloacal swabs. The duration of viral shedding (1-10 days) increased with severity of clinical disease. While both the hemagglutination inhibition (HI) and agar gel precipitin (AGP) tests were able to detect post-inoculation antibodies in surviving wood ducks and laughing gulls, the HI test was more sensitive than the AGP in the remaining four species.

INTRODUCTION

Free-living birds in the Orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shorebirds) have traditionally been considered the natural reservoirs for avian influenza viruses (AIVs) (1, 2). However, prior to 2005, there was no evidence that highly pathogenic avian influenza (HPAI) viruses were maintained in wild bird populations. Rather, HPAI viruses evolved independent of wildlife reservoirs when wild-type AIVs were introduced and adapted to domestic poultry populations (3). One exception occurred in 1961 when high mortality in common terns (*Sterna hirundo*) in South Africa was attributed to a H5N3 HPAI virus without evidence of prior

infection in domestic poultry (4). However, this tern epizootic was limited and the virus did not become endemic in any wild bird population.

In 2002, significant mortality associated with H5N1 HPAI virus infection was reported in captive ducks, geese, and flamingos housed within two waterfowl parks in Hong Kong (5). Mortality was also observed in free-living grey herons (*Ardea cinerea*) and black-headed gulls (*Larus ridbundus*) associated with these outbreaks. Since 2002, sporadic mortality in wild birds associated with H5N1 HPAI has continued (6). Beginning in spring 2005, H5N1 HPAI outbreaks involving large numbers of wild birds were reported and the subsequent spread of these viruses to Europe and Africa suggests that the long-range movement of these viruses may have occurred through migratory birds. However, it is presently unknown which wild avian species are important in H5N1 HPAI movement or if establishment of these viruses in free-living avian populations will occur. The goal of this study was to determine the susceptibility of critical species of North American waterfowl to two H5N1 HPAI viruses and the potential impact of these species on the epidemiology of the viruses in North America.

MATERIALS AND METHODS

Animals

Five species of indigenous North American ducks were used in this study: mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta*), blue-winged teal (*Anas crecca*), redhead (*Aythya americana*), and wood duck (*Aix sponsa*). Species were selected to represent the diverse habitat and behavior of ducks in North America and included important AIV reservoirs (mallards), long-distant migrants (northern pintail and blue-winged teal), diving ducks (redhead), and birds that breed in both northern and southern areas of the United States (wood ducks). All ducks used in this study were captive-bred and acquired at 10 to 16 weeks of age (Howell's Exotic Waterfowl,

Muldrow, OK). This age is consistent with pre-migration staging in the late summer/early fall when AIV prevalence peaks in wild waterfowl (7). Both males and female ducks were included in each species and were approximately equal in representation.

Wild-caught gulls used in this investigation were acquired through the Southeastern Cooperative Wildlife Disease Study (SCWDS), University of Georgia (UGA), under federal permit. Nestling laughing gulls (*Larus atricilla*) were hand-caught in McIntosh County, GA by Georgia Department of Natural Resources personnel and maintained at the College of Veterinary Medicine, UGA. At 12 weeks of age the gulls were transported to biosafety level 3-agriculture (BSL 3-Ag) facilities at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA).

All birds used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (8) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at both SEPRL and UGA. All experiments were performed in the USDA-certified BSL 3-Ag facility at SEPRL (9).

Viruses

Two viruses were used in this study: A/Whooper Swan/Mongolia/244/05 (H5N1) (Mongolia/05) and A/Duck Meat/Anyang/01 (H5N1) (Anyang/01). The Mongolia/05 isolate was obtained from a dead whooper swan (*Cygnus cygnus*) and was chosen because of its known lethality in wild waterfowl. The Anyang/01 isolate was chosen based on results from previous experimental infections of Pekin white ducks (*Anas platyrhyncos*) which did not result in morbidity or mortality (10).

Individual stocks of both AIVs used in this study were produced by second passage in 9-day-old embryonated chicken eggs. Allantoic fluid from the inoculated eggs was diluted in brain-heart infusion medium (BHI) to yield a final titer of 10^6 egg infectious doses (EID_{50}) per 0.1 ml (single bird inoculum). A sham-inoculum was prepared by diluting sterile allantoic fluid 1:30 in BHI.

Experimental Design

Pre-inoculation serum was collected from each bird to confirm they were serologically naïve to influenza A viral antigens by agar gel precipitin test (AGP) and H5 influenza by specific hemagglutination inhibition (HI) testing using standard procedures (11). In addition, oropharyngeal and cloacal swabs were collected prior to inoculation to confirm an AIV-free status. The five species of ducks and laughing gulls were each separated into a control group and two virus-inoculated groups (Mongolia/05 and Anyang/01), each consisting of three birds. Ducks and gulls were inoculated intranasally (IN) with a 0.1 ml volume of the designated virus solution or sham-inoculum. All birds were monitored daily for morbidity or mortality. Due to the lack of morbidity exhibited by most ducks, experiments with these species were extended to 20 days post-inoculation (DPI) to allow adequate time for seroconversion. Cloacal and oropharyngeal swabs were collected in BHI with antibiotics (100 μg/ml gentamicin, 100 units/ml penicillin, and 5 μg/ml amphotericin B) from all birds at 1, 2, 3, 4, 5, 7, 10, and 14 DPI. Oropharyngeal and cloacal swabs were also collected on 20 DPI from the five species of ducks. At 14 DPI (gulls) or 20 DPI (ducks), serum was collected from the surviving birds for serologic testing via HI and AGP and the birds were humanely euthanized via intravenous sodium pentobarbital (100 mg/kg body weight). Serum was not collected from birds that died during the course of the study (that were not euthanized at the end of the study). Necropsies were performed on all birds and routine

tissues were collected for histopathologic and immunohistochemical evaluation. In addition, portions of heart, breast muscle, kidney, lung, brain, and oropharyngeal and cloacal swabs were collected and stored in BHI with antibiotics for virus isolation.

Histopathology and Immunohistochemistry

Tissues samples collected at necropsy were preserved in 10% neutral buffered formalin. After fixation, the tissues were routinely processed and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. Duplicate sections were immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein (NP) antigen as the primary antibody (SEPRL, Athens, GA). Procedures used to perform the immunohistochemistry (IHC) followed those previously described (12). Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen deposition in the nucleus, with or without chromagen deposition in the cytoplasm.

Virus Isolation

Oropharyngeal and cloacal swabs and tissue samples collected at necropsy were stored at -70°C until virus isolations and titrations were performed. Isolation of virus from swabs and tissues was performed using embryonated chicken eggs (11). Positive samples were titrated by determining the EID_{50}. The minimal detectable titer was $10^{0.97}$ EID_{50}/ml from swabs and $10^{1.97}$ EID_{50}/gram from tissues.

Serologic Assays

The AGP and HI tests were performed on the pre- and post-inoculation serum using standard procedures (11). The HI tests were performed using a 0.5 % suspension of chicken erythrocytes in phosphate-buffered saline.

Phylogenetic analysis

In addition to the two H5N1 viruses used in this study, A/chicken/Hong Kong/220/97 (H5N1) (Hong Kong/97) was included in the phylogenetic analysis because it is the only other H5N1 HPAI virus evaluated in multiple avian species via experimental inoculation (13). Sequence comparisons of these three viruses were conducted with the Megalign program using the Clustal V alignment algorithm (DNASTAR, Madison, Wisc.), and phylogenetic relationships were estimated by the method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc, Sunderland, Mass.) using a bootstrap resampling method with a heuristic search algorithm. Pairwise sequence comparisons were done within the Megalign program.

RESULTS

Morbidity and Mortality

Morbidity and mortality data are summarized in Table 1. Wood ducks were the only species of duck to exhibit morbidity and/or mortality after inoculation with either of the HPAI viruses. Two Mongolia/05-inoculated wood ducks developed severe clinical disease characterized by cloudy eyes, ruffled feathers, rhythmic dilation and constriction of the pupils, severe weakness, incoordination, tremors, and seizures (Figure 1). One of these ducks died at 7 DPI and the other was euthanatized at 8 DPI due to its moribund condition. Two Anyang/01-inoculated wood ducks became ill with clinical signs similar to those described for the Mongolia/05 virus group. One of these ducks died and the other slowly recovered over seven days until it was clinically normal. One wood duck in each viral group remained clinically normal for the entire trial. Clinical signs were not observed in the remaining duck species.

All three Mongolia/05-inoculated laughing gulls exhibited severe clinical signs consisting of cloudy eyes, ruffled feathers, weakness, incoordination and/or torticollis. Two of these gulls

died. The remaining gull clinically improved and stabilized over six days, but retained a head-tilt for the remainder of the trial. All Anyang/01-inoculated gulls developed severe clinical signs similar to those seen in Mongolia/05-inoculated gulls. The disease progressed to death in two of these gulls. The remaining gull exhibited clinical signs for eight days but gradually recovered until it was clinically normal.

Pathology

Viral-induced lesions were only present in the wood ducks and laughing gulls that exhibited clinical signs. Lesions were mild in birds that recovered but were severe and widespread in birds that died or were euthanatized due to severe illness. For each species, the severity and distribution of lesions were the same for both H5N1 viruses, with one exception noted below.

Gross lesions were not present in any of the recovered birds. Wood ducks that died had multiple petechial hemorrhages in the pancreas while laughing gulls had more widely distributed petechial hemorrhages in the ventriculus, apex of the heart, cerebrum, and pancreas.

Histopathologically, wood ducks that died had severe, diffuse neuronal necrosis in the cerebrum (Figure 2A) and, less commonly, the cerebellum. Other common lesions included necrotizing pancreatitis (Figure 2D) and adrenalitis (Figure 2C) and multifocal myocardial necrosis. Myocardial necrosis was only observed in wood ducks inoculated with Mongolia/05 and not with Anyang/01. Necrotizing pancreatitis and cerebral neuronal necrosis were the most common lesions in gulls that died during the study. Necrotizing adrenalitis was also observed in gulls that died but was less common and milder than the changes in the pancreas and cerebrum. Microscopic lesions in wood ducks and laughing gulls that recovered were less severe than in

those that died. In both species, the most common lesions in recovered birds were lymphoplasmacytic perivascular encephalitis and heterophilic pancreatitis.

Wood ducks that died during the study had viral antigen in numerous organs, including the brain (Figure 2B), adrenal glands, testicles, kidney, liver, small intestines, heart, skeletal muscle, pancreas, and air sacs. Viral antigen was most frequently present in cardiac myocytes, parasympathetic ganglia in the submucosal and muscular plexus of the small intestines, and numerous cell-types in the brain, including glial cells, ependymal cells, endothelial cells, neurons, and gitter cells. Viral antigen was also detected in the pancreatic acinar cells and cortical and medullary cells of the adrenal gland, though less often than the aforementioned sites. Minimal amounts of viral antigen were detected in the kidney and testis in one and two wood ducks that died, respectively. The one wood duck that recovered had a scant amount of viral antigen in the cerebellar neurons. Laughing gulls that died during the study had viral antigen most frequently detected in the neurons, endothelial cells, glial cells, and ependymal cells in the brain, pancreatic acinar cells, and cortical and medullary cells of the adrenal glands. Laughing gulls that died also had minimal amounts of viral antigen present in other organs including the heart, lungs, air sac, thymus, kidney, small intestines, and eye. Laughing gulls that recovered contained small amounts of viral antigen in the pancreatic acinar cells and cerebral and cerebellar neurons.

Virus Isolation and Serology

The virus isolation results are summarized in Table 1 and 2. Viral titers were higher in oropharyngeal swabs than cloacal swabs in all species and with both H5N1 viruses. Viral titers on cloacal swabs were low except from birds that died of AIV infection. Oropharyngeal swabs from all species collected at 1 and 2 DPI were positive on virus isolation. Wood ducks and

laughing gulls had higher viral titers on oropharyngeal and cloacal swabs and shed virus longer than any of the other species. Virus was isolated from numerous organs in the wood ducks and laughing gulls that died.

Serologic testing results are summarized in Table 3. Both the AGP and HI tests detected post-inoculation antibodies in all surviving wood ducks and laughing gulls. However, the effectiveness of these tests in the remaining duck species was variable and dependent on host species and inoculated virus. The HI test detected post-inoculation antibodies in multiple avian species that had little to no serologic response as determined by the AGP test (Anyang/01-inoculated mallards, redheads, and northern pintails and Mongolia/05-inoculated mallards). Both serologic tests were least effective in northern pintails and mallards.

Molecular Biology

In comparing the three viruses (Hong Kong/97, Anyang/01, and Mongolia/05) genetically, the hemagglutinin genes are all clearly in the Goose/Guandong/96 lineage. At the amino acid level they vary by 3.5 - 4.8%. They all have the HA cleavage compatible with a HPAI phenotype. The cleavage site is the same for Hong Kong/97 and Anyang/01, but the Mongolia/05 virus has two amino acid changes at the cleavage site. Phylogenetically, the Hong Kong/97 and the Anyang/01 are in or close to clade 3 and the Mongolia/05 strain is in clade 2 (14). The Mongolia/05 strain appears to be a representative isolate from the wild bird viruses that have been reported in Asia, Europe, and Africa.

Comparison of the other seven gene segments demonstrates evidence of reassortment. The viruses from the 1997 outbreak in Hong Kong have a unique subtype 1 neuraminidase gene as compared to any of the other H5N1 viruses. The Anyang/01 and Mongolia/05 N1 genes are from the same lineage, and both have an identical 20 amino acid stalk deletion. For the other 6

internal genes, the Anyang/01 and Mongolia/05 viruses in general were more closely related to each other than the Hong Kong/97 virus. Except for the H5 gene, the Hong Kong/97 and viruses isolated in Hong Kong in the same year appear to be a unique constellation of genes that has not been seen again. Although the Anyang/01 and Mongolia/05 viruses were more closely related, the internal genes are most likely the result of reassortment with other influenza viruses and not the result of progressive sequence in a single lineage of viral genes.

DISCUSSION

Data from this study indicate that wood ducks and laughing gulls are highly susceptible to infection with H5N1 HPAI viruses as evidenced by widespread microscopic lesions, prolonged and highly concentrated viral shedding, and seroconversion. In addition, these species are likely to exhibit clinical disease and/or death associated with H5N1 virus infection. In a previous study, 2- to 3-week-old laughing gulls inoculated with A/chicken/Hong Kong/220/97 (H5N1) and A/tern/South Africa/61 (H5N3) did not exhibit morbidity or mortality (15). Viral replication in these birds was minimal and restricted to the respiratory tract. Since 2002, some emergent H5N1 viruses have exhibited unique characteristics including lethality for waterfowl (16). Consistent with previous studies on ducks (17), the more recent isolates of H5N1 viruses used in our study caused high morbidity and mortality in gulls while the earlier H5N1 isolate, mentioned above, did not. To our knowledge, this is the first experimental inoculation of wood ducks with any HPAI viruses. Our results are consistent with field data that also indicate wood ducks are highly susceptible to H5N1 HPAI viruses. In an investigation of H5N1 virus outbreaks in two waterfowl parks in Hong Kong, 18 of the 26 wood ducks present on the lakes died (5). Of these wood ducks that died, 16 were positive for H5N1 virus via culture.

Traditionally, ducks asymptomatically shed high concentrations of wild-type AIVs in their feces (18). In this paradigm, ducks can transmit AIV over great distances as they migrate and these viruses can remain infectious for prolonged periods of time in water (18, 19). This fecal-oral mechanism is efficient at maintaining these viruses within duck populations and also transmitting AIVs from wild ducks to domestic poultry. Predominant oropharyngeal shedding has been consistently demonstrated with these H5N1 HPAI viruses (20), as it was in our study, and it is uncertain what impact this shedding pattern may have on the extent of environmental contamination, the persistence in aquatic habitats, and the transmission between birds (both wild and domestic).

An efficient surveillance system is central to any preparedness program aimed to detect H5N1 in North America. Our data indicate that wood ducks and laughing gulls would be sensitive indicators of the presence of H5N1 circulating in wild birds. Wild avian species have previously been included in monitoring programs for other infectious diseases such as the use of crow mortality for detection of West Nile virus (21). It is likely that other wild avian species in North America would also serve as sensitive indicators, but it is impossible to predict which species without experimental inoculations and/or consistent morbidity and mortality data from outbreaks.

In relation to wood ducks and laughing gulls, the remaining four duck species were much less susceptible to H5N1 HPAI virus infection and were refractory to disease. While it is possible that these species could contribute to viral transmission in wild avian populations, their role in the spread or maintenance of H5N1 HPAI virus is probably minimal. However, it is important to emphasize that our experimental results are based on small sample sizes (n=3) that are inadequate to fully evaluate potential individual bird variation in response to H5N1 challenge.

Morbidity, mortality, and viral shedding were significantly less in our study than what has previously been reported for experimental inoculation of ducks with H5N1 HPAI virus. Possible explanations for the reduced pathogenicity include the age of birds used in the study and the variability between different H5N1 HPAI viruses. An age-dependent reduction in lethality was present between 2- and 4-week-old ducks IN inoculated with H5N1 HPAI virus (22). Similarly, previous experimental infections of mallards between the ages of 2 and 6 weeks with H5N1 resulted in higher mortality than we observed with 10- to 16-week-old ducks (23, 17, 20). Experimental infections of very young ducks may overestimate the susceptibility of a species and the results may be incongruent with morbidity and mortality field data. The reduced pathogenicity and infectivity could also be characteristic for the specific H5N1 viruses used in this study.

One wood duck and one laughing gull reacted positively for pre-inoculation antibodies to AIV via the HI test. However, both of these birds were positive at the lowest detectable limit of this test and it is possible that these results are false positives due to non-specific hemagglutination. The wood duck did not become sick after inoculation with the Mongolia/05 isolate. The laughing gull did become ill after inoculation with the Anyang/01 virus, but completely recovered. If these serologic results are true positives, it is possible that the low antibody titers provided some immunologic resistance for these birds.

Serologic techniques commonly used in domestic poultry have limitations in ducks. The results of this study suggest there is variation between wild duck species in the ability of the AGP and HI tests to detect antibodies to Type A influenza virus and H5 AIVs, respectively. While the HI test was more sensitive than the AGP in detecting antibodies in our study, both serologic tests failed to detect antibodies to AIV in some post-inoculation serum samples from

experimentally infected ducks. Furthermore, usage of duck red blood cells (RBC) in place of chicken RBC for the HI test also failed to detect antibodies in some of the duck samples (Justin Brown, unpublished data). Surveillance systems relying on these serologic techniques to detect H5N1 HPAI virus in ducks may significantly underestimate the prevalence of virus. In addition, it should be noted that false positives are possible if HI testing is used in H5N1 surveillance as positive results for H5 AIVs indicate previous infection with H5N1 HPAI virus or any other H5 wild-type AIV. Further information is necessary to evaluate the efficacy of these serologic assays in other wild avian species to allow correct interpretation of serologic field data.

The genetic sequence information indicated that all three evaluated H5N1 HPAI viruses were genetically distinct from each other, although the Anyang/01 and Mongolia/05 viruses were overall more closely related to each other than to Hong Kong/97. The only gene segment that all three viruses shared as part of a single viral lineage was the hemagglutinin gene. All three viruses had cleavage sites compatible with HPAI viruses, and experimental inoculation showed them to be extremely virulent in chickens (24, 10, David Swayne, unpublished data). Because of the large sequence differences, it is not possible to identify which genetic changes account for the virulence or host specificity differences that were observed. It has been shown through reverse genetics that single amino acid differences can greatly affect virulence, such as the change of glutamine to lysine at position 627 in the PB2 gene. This single difference can greatly increase the virulence of Hong Kong/97 viruses in mice (25).

The results of this study indicate that there is significant species-related variation in susceptibility, clinical disease, and antibody response to H5N1 virus infection in wild birds. It is impossible to predict this susceptibility beyond the species examined in this study. Wood ducks and laughing gulls were highly susceptible to H5N1 HPAI viruses with high morbidity and

mortality. If H5N1 were to be introduced into North America, these species may serve as effective indicator species in a surveillance program.

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Table 4.1. Morbidity, mortality, and virus isolation data from five species of ducks and laughing gulls intranasally inoculated with two different H5N1 HPAI viruses.

Host[*]	Morbidity (sick/total)	Mortality (dead/total)	Virus Isolation (oral swab)			Virus Isolation (cloacal swab)		
			Prevalence (positives/total)	Duration (days)	AMT[¶] (log_{10} EID_{50}/ml)	Prevalence (positives/total)	Duration (days)	AMT (log_{10} EID_{50}/ml)
Mongolia/05								
BWT	0/3	0/3	3/3	2	3.8	1/3	1	1.0
RD	0/3	0/3	3/3	1-4	2.8	2/3	1	1.2
WD	2/3 (5)[†]	2/3 (7,8)[§]	3/3	4-6	4.6	2/3	2,3	3.8
MD	0/3	0/3	3/3 (1)	1-3	3.1	1/3 (1)	1	1.0
NP	0/3	0/3	3/3	1-2	1.5	1/3	1	1.0
LG	3/3 (2-5)	2/3 (7,8)	3/3	7-8	4.2	3/3	4-7	2.6
Anyang/01								
BWT	0/3	0/3	2/3	1,2	2.0	0/3	-	-
RD	0/3	0/3	2/3	4	4.0	0/3	-	-
WD	2/3 (6)	1/3 (8)	3/3	7	5.0	2/3	4,5	2.8
MD	0/3	0/3	3/3	1-2	2.1	1/3	1	1.0
NP	0/3	0/3	2/3	1,4	1.1	0/3	-	-
LG	3/3 (3-5)	2/3 (6-10)	3/3	6-10	5.0	3/3	3-6	2.0

[φ]IN sham-inoculated control birds for each avian species lacked clinical, serologic, virologic, and pathologic evidence of AIV infection.

[*]BWT=blue-winged teal, RD=redhead, WD=wood duck, MD=mallard, NP=northern pintail, LG=laughing gull, Mongolia/05=A/Whooper Swan/Mongolia/244/05, Anyang/01=A/Duck Meat/Anyang/01

[¶]Average maximum titer is the average peak titer for birds that shed virus (log_{10} EID_{50}/ml)

[†]The first day post-inoculation that clinical disease was apparent.

[§]Day of death

Table 4.2. Mean viral titer for tissues from wood ducks and laughing gulls[φ] that died after inoculation with two different HPAI H5N1 viruses.

Host-virus[*]	Brain	Heart	Lung	Skeletal Muscle	Kidney
WD-Anyang	3.7[¶]	3.5	5.1	ND[†]	2.9
WD-Mongolia	6.6	2.7	7.1	2.5	6.7
LG-Anyang	4.8	4.7	5.2	2.5	4.2
LG-Mongolia	6.3	2.5	3.3	4.2	2.5

[φ]No virus was isolated from the internal organs of the other four avian species inoculated with H5N1 viruses and all of the sham-inoculated control birds

[*]WD=wood duck, LG=laughing gull, Anyang=A/Duck Meat/Anyang/01, Mongolia=A/Whooper Swan/Mongolia/244/05

[¶] $\log_{10}$ mean embryo infectious dose per gram ($\log_{10}EID_{50}$/gram)

[†]No virus was detected

Table 4.3. Serology data from five duck species and laughing gulls inoculated with two different H5N1 HPAI viruses.

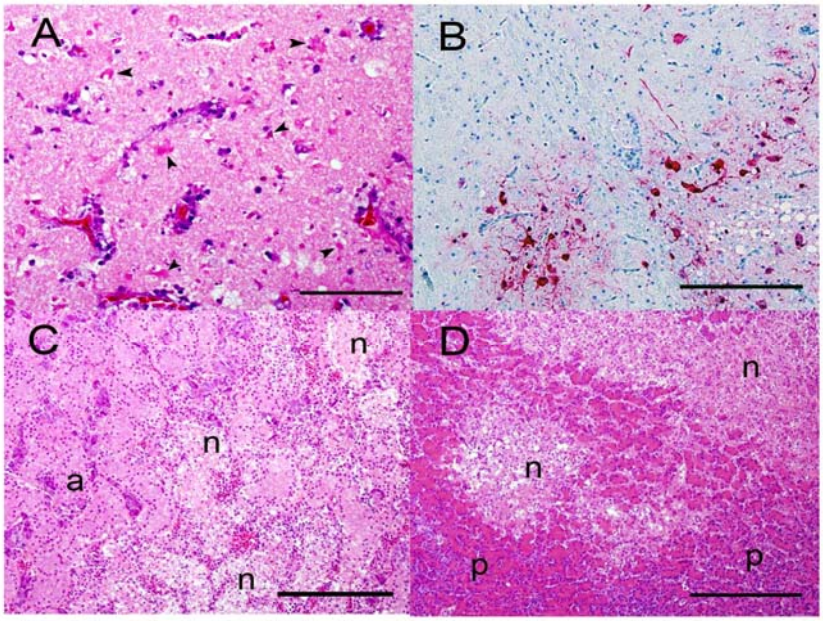
	AGP Serology		HI Serology	
Host*	**Pre-challenge (positives/total)**	**Post-challenge (positives/total)**	**Pre-challenge (positives/total)**	**Post-challenge (positives/total)**
Mongolia/05				
BWT	0/3	3/3	0/3	3/3 (13)†
RD	0/3	3/3	0/3	3/3 (26)
WD	0/3	1/1	1/3 (8)†	1/1 (128)
MD	0/3	0/3	0/3	1/3 (64)
NP	0/3	0/3	0/3	0/3
LG	0/3	1/1	1/3 (8)	1/1 (64)
Anyang/01				
BWT	0/3	0/3	0/3	3/3 (10)
RD	0/3	1/3	0/3	3/3 (20)
WD	0/3	2/2	0/3	2/2 (64)
MD	0/3	0/3	0/3	2/3 (16)
NP	0/3	0/3	0/3	2/3 (8)
LG	0/3	1/1	0/3	1/1 (32)

*BWT=blue-winged teal, RD=redhead, WD=wood duck, MD=mallard, NP=northern pintail, LG=laughing gull, Mongolia/05=A/Whooper Swan/Mongolia/244/05, Anyang/01=A/Duck Meat/Anyang/01

†geometric mean titer

Figure 4.1. A female wood duck with severe neurologic clinical signs of disease after intranasal inoculation with an Asian strain of highly pathogenic avian influenza H5N1 virus.

Figure 4.2. Photomicrographs of visceral organs from a wood duck that died after intranasal inoculation with a highly pathogenic avian influenza H5N1 virus. **A.** Brain with severe, multifocal to coalescing neuronal necrosis. Note the numerous necrotic neurons (arrowheads). Hematoxylin and eosin (HE) stain. Bar = 100 μm. **B.** Brain. Note the viral antigen (red) detected in the nucleus of several neurons. The unaffected brain tissue is blue. Immunohistochemical stain with hematoxylin counterstain. Bar = 200 μm. **C.** Adrenal with necrotizing adrenalitis. Note the multiple foci of necrosis (n) surrounded by normal adrenal parenchyma (a). HE. Bar = 200 μm. **D.** Pancreas with necrotizing pancreatitis. Note the two well-demarcated areas of necrosis (n) within the normal pancreatic tissue (p).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CHAPTER 5

SUSCEPTIBILITY OF WOOD DUCKS TO H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS[1]

[1] Brown, J.D., D.E. Stallknecht, S. Valeika, and D.E. Swayne. Submitted to the *Journal of Wildlife Diseases* (accepted, in press). Reprinted here with permission of publisher.

ABSTRACT

Since 2002, H5N1 highly pathogenic avian influenza (HPAI) viruses have caused mortality in numerous species of wild birds; this is atypical for avian influenza virus (AIV) infections in these avian species, especially for species within the Order Anseriformes. Although these infections document the susceptibility of wild birds to H5N1 HPAI viruses and the spillover of these viruses from infected domestic birds to wild birds, it is unknown if H5N1 HPAI viruses can persist in free-living avian populations. In a previous study, we established that wood ducks (Aix sponsa) are highly susceptible to infection with H5N1 HPAI viruses. In order to quantify this susceptibility and further evaluate the likelihood of H5N1 HPAI viral maintenance in a wild bird population, we determined the concentration of virus required to produce infection in wood ducks. To accomplish this, 25 wood ducks were inoculated intranasally at 12- to 16-weeks of age with decreasing concentrations of a H5N1 HPAI virus (A/Whooper Swan/Mongolia/244/05 [H5N1]). The median infectious dose and lethal dose of H5N1 HPAI virus in wood ducks were very low ($10^{0.95}$and $10^{1.71}$ median embryo infectious dose [EID_{50}], respectively) and less than that of chickens ($10^{2.80}$ and $10^{2.80}$ EID_{50}). These results confirm that wood ducks are highly susceptible to infection with H5N1 HPAI virus. The data from this study, combined with what is known experimentally about H5N1 HPAI virus infection in wood ducks and viral persistence in aquatic environments, suggest that the wood duck would represent a sensitive indicator species for H5N1 HPAI. Results also suggest that the potential for decreased transmission efficiency associated with reduced viral shedding (especially from the cloaca) and a loss of environmental fitness (in water), may be offset by the ability of this virus to be transmitted through a very low infectious dose.

KEY WORDS: Aix sponsa, avian influenza virus, $BID_{50,}$ BLD_{50}, H5N1, highly pathogenic avian influenza, infectious dose, susceptibility, wood duck.

INTRODUCTION

Wild aquatic birds in the Orders Anseriformes and Charadriiformes are the natural reservoirs for avian influenza viruses (AIV) (Hinshaw and Webster, 1982; Stallknecht and Shane, 1988). Traditionally, viral infection in anseriforms or charadriiforms have not been associated with morbidity or mortality (Webster et al., 1992) and AIV that are highly pathogenic to domestic poultry are not found in these wild aquatic bird populations (Suarez, 2000). However, in 2002, mortality associated with H5N1 highly pathogenic avian influenza (HPAI) virus infection was reported in wild and captive aquatic birds in two waterfowl parks in Hong Kong (Ellis et al., 2004). Since these outbreaks, H5N1 HPAI viruses have continued to cause mortality in wild birds in Asia (USGS, 2006). In 2005, H5N1 HPAI viruses spread into Europe and Africa, and epidemiologic observations and genetic studies suggest that this geographic dissemination of virus may have occurred through migratory waterfowl (Sabirovic et al., 2006). While field data from the epidemics in Eurasia confirm that H5N1 HPAI viruses can cause mortality in wild birds and suggest that migratory waterfowl play a role in the epidemiology of these viruses, it is unknown whether H5N1 HPAI viruses can persist in wild avian populations.

Based on our current knowledge of AIV in wild birds, three factors are important for viral persistence in waterfowl populations: 1) the ability to remain infective for long durations in aquatic habitats; 2) highly concentrated and prolonged viral shedding by birds in these populations; and 3) an ample supply of susceptible birds. The low pathogenic avian influenza (LPAI) viruses that naturally circulate in waterfowl populations, referred to herewithin as "wild-type AIV", have evolved over time into the perfect host-parasite relationship (Webster et al.,

1978), satisfying all three of these factors for viral maintenance. These LPAI viruses can persist for long durations in water (Stallknecht et al., 1990; Brown et al., 2007) and experimentally infected ducks shed high concentrations of virus for a prolonged duration via the fecal route (Webster et al., 1978). Susceptible birds and a high prevalence of AIV infections occur annually during the fall when juvenile ducks congregate at marshalling sites prior to fall migration (Halvorson et al., 1985). Compared to these wild-type AIV, H5N1 HPAI viruses do not appear to be as well-adapted to fulfill all of these prerequisites for maintenance in a wild bird population. Experimentally, H5N1 HPAI viruses remain infective in water for a shorter duration than wild-type viruses, suggesting the H5N1 HPAI viruses may not be as environmentally fit as the wild-type viruses (Brown et al., 2007). In addition, four out of five North American duck species experimentally infected with H5N1 HPAI viruses excreted low viral titers for short durations, and in all species, shedding was primarily associated with oropharyngeal (OP) rather than a cloacal route (Brown et al., 2006). Potential losses in environmental fitness in aquatic habitats, and the decreased viral shedding associated with H5N1 HPAI virus infections in most species of ducks, may greatly reduce the viral burden in the aquatic environment. This implies that transmission must be very efficient in at least some aquatic bird species for these H5N1 HPAI viruses to be transmitted and potentially persist in waterfowl populations. This increased transmission efficiency may be provided by the ability of these H5N1 HPAI viruses to infect a susceptible bird at a very low dose. Currently, there is no available information on infective dose for any H5N1 HPAI or wild-type AIV in any wild duck species.

Mortality reports from the ongoing H5N1 HPAI virus epidemics in Eurasia suggest that these viruses are especially virulent for anseriform species (Sabirovic et al., 2006; USGS, 2006). In particular, field and experimental data indicate that wood ducks are highly susceptible to

H5N1 HPAI virus infection (Ellis et al., 2004; Brown et al., 2006). However, all experimental infections which have evaluated H5N1 HPAI viruses in wood ducks and other duck species, to date, have been conducted with high viral inoculation doses, and the concentration of virus required to produce infection in this or any other wild anseriform species is currently unknown. Without this information it is impossible to fully understand the sensitivity of wood ducks or other susceptible waterfowl species to infection with H5N1 HPAI viruses.

The objectives of this study were: 1) to determine and evaluate the concentration of H5N1 HPAI virus required to produce infection or death in wood ducks; 2) to compare the median infectious and lethal dose in wood ducks to white-leghorn chickens; and 3) to evaluate the effect that H5N1 HPAI viral dose has on morbidity, mortality, and viral shedding.

MATERIALS AND METHODS

The virus

The H5N1 HPAI virus (A/Whooper Swan/Mongolia/244/05 [H5N1]; Mongolia/05) used in this study was obtained from the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, Georgia, USA. This virus was originally isolated from a dead whooper swan (Cygnus cygnus) in Mongolia during a 2005 outbreak of H5N1 HPAI virus in waterfowl (OIE Disease Information, 2005; Brown et al., 2006). The Mongolia/05 strain is in the Goose/Guandong/96 lineage and phylogenetically is included in clade 2 (World Health Organization Global Influenza Program Surveillance Network, 2005). Mongolia/05 was selected for use in this study because it is representative of the H5N1 HPAI viruses that have been reported from wild birds in Asia, Europe, and Africa (Brown et al., 2006).

Virus was propagated by second passage in 9- to 11-day old specific pathogen free (SPF) embryonated chicken eggs. Allantoic fluid from the inoculated eggs was diluted in brain-heart infusion (BHI) medium to yield the final titers of $10^{1.5}$, $10^{3.0}$, $10^{4.5}$, and $10^{6.0}$ median embryo infectious doses (EID_{50}) per 0.1 ml (single bird inoculum) for the wood duck trial and $10^{1.0}$, $10^{3.0}$, and $10^{5.0}$ EID_{50}/0.1 ml for the white-leghorn chicken trial. Back-titers on these doses were determined in 9- to 11-day old SPF chicken eggs and were $10^{1.9}$, $10^{3.1}$, $10^{4.9}$, $10^{6.1}$ EID_{50}/0.1ml for the wood duck trial and $10^{0.9}$, $10^{3.1}$, $10^{5.1}$ EID_{50}/0.1ml for the chicken trial. A sham-inoculum was prepared by diluting sterile allantoic fluid 1:30 in BHI.

Animals

Twenty five captive-bred wood ducks were acquired from a private breeder at 12- to 16-weeks of age (Chenoa Farms, Martin, Tennessee, USA). This age was selected because it corresponds to the time in nature when North American ducks would most likely be infected; the peak prevalence of AIV in wild waterfowl is associated with pre-migration staging in the late summer/early fall (Halvorson et al., 1985). Both male and female ducks were included in approximately equal numbers. Ducks were housed in groups of five in self-contained isolation units which were ventilated under negative pressure with high efficiency particulate air (HEPA)-filtered air. The birds were maintained under continuous lighting and food and water were provided *ad libitum*.

Fifteen white-leghorn (WL) chickens (Gallus gallus domesticus) were acquired at 3-weeks of age from a SPF flock maintained at SEPRL. The chickens were housed in groups of five in negative pressure HEPA ventilated stainless steel isolation cabinets units. The birds were maintained under continuous lighting and food and water were provided *ad libitum*.

General care was provided in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Craig et al., 1995) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at both SEPRL and the University of Georgia (UGA). All experiments were performed in the USDA-certified BSL 3-Ag facility at SEPRL (Barbeito et al., 1999).

Virus isolation and serologic testing

Cloacal and OP swabs were stored at -70º C until virus isolations and titrations were performed. Standard procedures were used for virus isolation from cloacal and OP swabs using SPF embryonated chicken eggs (Swayne et al., 1998). Viral titers for positive samples were determined by microtiter endpoint titration (Reed and Muench, 1938) using primary cultures of chicken embryo fibroblasts (CEFs). In this protocol, sequential ten-fold dilutions (10^{-1} to 10^{-8}) were made of the clarified swab suspension in serum-free Eagle's minimum essential medium (MEM) supplemented with antibiotics (100 u penicillin G with 100 µg streptomycin sulfate/ml). Each well of a 96-well microtiter plate received 100 µl of cell suspension and 50 µl of the appropriate virus dilution. Primary cultures of CEFs from 9- to 11-day-old SPF chicken embryos were used for these cell suspensions. Final cell suspensions consisted of $3x10^6$ CEF/ml suspended in serum-free MEM supplemented with antibiotics. Supplemental trypsin was not added to these assays. The covered plates were incubated at 37º C under 5% CO_2 for 96 hr. Examination for cytopathic effects was performed with light microscopy and plates were then stained with 1% crystal violet in 10% neutral buffered formalin for further confirmatory examination. Endpoints were recorded as 100% monolayer destruction. Viral titers were expressed as median tissue culture infectious dose ($TCID_{50}$)/ml. The minimal detectable limit of

this assay is $10^{1.96}$ $TCID_{50}$/ml. Serologic testing was performed via the agar gel precipitin (AGP) test using standard procedures (Swayne et al., 1998).

Experimental design

Wood ducks were evenly divided into five treatment groups with five birds in each group. Each group was intranasally (IN) inoculated with either a sham-inoculum or one of four different viral doses of the Mongolia/05; doses consisted of $10^{1.5}$, $10^{3.0}$, $10^{4.5}$, or $10^{6.0}$ EID_{50} in a volume of 0.1 ml per bird. Prior to inoculation, blood was collected from all ducks for serologic testing via the AGP test to ensure that birds did not possess antibodies to AIV. In addition, cloacal and OP swabs were collected from each bird for virus isolation prior to inoculation to verify that the ducks were not shedding AIV at the start of the study. After inoculation, birds were observed daily for morbidity and mortality. Morbidity was defined as any clinical abnormality observed after inoculation with virus, including weakness, cloudy eyes, respiratory difficulty, shivering, crowding, ruffled feathers, hemorrhage on the unfeathered skin, or neurologic signs. Cloacal and OP swabs were collected from all ducks on 0, 2, 4, 6, and 14 days post-inoculation (DPI). In addition, cloacal and OP swabs were collected from all ducks that were found dead or were euthanized due to severe neurologic clinical signs. At 14 DPI, blood was collected from the surviving birds for serologic testing and all remaining ducks were euthanized with intravenous (IV) administration of sodium pentobarbital (100 mg/kg). The mean death time (MDT) was determined for each group and expressed as DPI. The median bird infectious dose (BID_{50}) and median bird lethal dose (BLD_{50}) were calculated for each of the four viral-inoculated groups using the Spearman and Karber method (Finney, 1964) and expressed as EID_{50}. This method was performed with the assumption that no birds became infected or died with a viral dose of 10^0 EID_{50}. Infection for BID_{50} calculation in wood ducks was determined based on virus isolation

(in birds that died) and serologic testing (in surviving birds). Lethality for the BLD_{50} calculation in wood ducks was determined based on mortality.

Fifteen chickens were evenly divided into three groups and birds in each group were IN inoculated with one of three different doses of the Mongolia/05 virus, including $10^{1.0}$, $10^{3.0}$, and $10^{5.0}$ EID_{50} in a 0.1 ml volume per bird. Prior to inoculation, serum was collected from each bird to ensure that they were serologically naïve to AIV. After inoculation, the birds were monitored daily for morbidity and mortality. Morbidity was defined using the same standards as described for the wood ducks. Cloacal and OP swabs were not collected from chickens in this study. At 10 DPI, blood was collected from the surviving chickens for serologic testing via the AGP test. The surviving chickens were then euthanized by IV administration of sodium pentobarbital (100 mg/kg). The MDT, BID_{50} and BLD_{50} were determined as described for the wood duck experiment. Infection for BID_{50} calculation in chickens was determined based on mortality and serologic testing. Lethality for the BLD_{50} calculation in chickens was determined based on mortality.

RESULTS

Morbidity, mortality, and serologic testing

Morbidity and mortality data are summarized in Table 1. None of the sham-inoculated wood ducks exhibited morbidity or mortality. All five wood ducks inoculated with $10^{4.5}$ and $10^{6.0}$ EID_{50} of the Mongolia/05 virus died and the MDT in these groups was 6.8 and 5.2 DPI, respectively. One of the wood ducks in the $10^{4.5}$ EID_{50} group died on 14 DPI, which was eight to 10 days after the other four ducks in this group. All cloacal and OP swabs collected from this bird after 4 DPI were negative for AIV on virus isolation, including swabs collected at the time of death. These results suggest that this wood duck did not die as a direct result of H5N1 HPAI

viral infection, but rather most likely succumbed to secondary infections or lesions associated with the viral infection. Discounting this wood duck, the corrected MDT for wood ducks that died from H5N1 HPAI infection in this group was 5.0 DPI. Four of the five wood ducks in each group inoculated with the $10^{1.5}$ and $10^{3.0}$ EID_{50} viral doses died and the MDT were 6.3 and 5.5 DPI, respectively. The two wood ducks that survived did not exhibit clinical signs of disease at any time during the trial, but both ducks developed antibodies to AIV as detected via the AGP test. We were unable to determine a low viral dose that did not cause any mortality or seroconversion in wood ducks, which prohibited calculating a specific median dose for infectivity or lethality. Alternatively, we calculated the BID_{50} and BLD_{50} based on the data at the lowest inoculation dose with the assumption that the 10^{0} EID_{50} dose caused no morbidity or seroconversion, and reported these values. The results of this study indicate that wood ducks have a BID_{50} titer of $10^{0.95}$ EID_{50} and a BLD_{50} titer of $10^{1.71}$ EID_{50}.

All five of the WL chickens inoculated with the $10^{5.0}$ EID_{50} dose died and the MDT was 2 DPI. Three of the five chickens died at the $10^{3.0}$ EID_{50} dose and the MDT in this group was 4.3 DPI. None of the chickens died that were inoculated with $10^{1.0}$ EID_{50} of the Mongolia/05 virus. None of the surviving chickens at any viral dose produced antibodies to AIV so the BID_{50} and BLD_{50} titers in this species were equal and calculated to be $10^{2.80}$ EID_{50} (Table 1).

Virus isolation

Virus isolation results from the wood ducks are summarized in Table 1. None of the sham-inoculated ducks excreted virus or developed antibodies to AIV. Viral shedding was detected in all wood ducks in each of the four viral-inoculated groups on 2 DPI. In ducks that died, viral shedding was detected at all other sampling points in OP swabs and generally viral titers increased from 2 DPI until death. There were exceptions, in which viral titers in some ducks

remained constant or slightly decreased just prior to death. The average OP titer in the wood ducks that died with evidence of active viral infection (discounting the one wood duck in the $10^{4.5}$ EID_{50} group mentioned above) was $10^{4.7}$ $TCID_{50}$/ml (range = $10^{3.3}$ to $10^{5.8}$ $TCID_{50}$/ml). Viral titers in all antemortem and postmortem swabs were higher in OP swabs than cloacal swabs in each of the 20 wood ducks infected in this study, and cloacal shedding did not occur at all time points in every duck.

Viral shedding in the two wood ducks that survived was either at a very low titer or for a brief duration. The surviving wood duck in the $10^{1.5}$ group excreted moderately high titers (average OP titer =$10^{4.3}$ $TCID_{50}$/ml), but virus was not detected after 4 DPI in OP swabs or 2 DPI in cloacal swabs. The surviving wood duck in the $10^{3.0}$ group shed virus for 6 DPI in OP swabs and 4 DPI in cloacal swabs, but titers were very low (average OP and cloacal titer was less than $10^{1.96}$ $TCID_{50}$/ml).

DISCUSSION

The results of this study indicate that wood ducks are highly susceptible to infection with H5N1 HPAI viruses and relatively small concentrations of these viruses are required to produce infection and death in this species. As a comparative measure of wood duck susceptibility, lower concentrations of H5N1 HPAI virus are required to produce infection and death in wood ducks than in domestic chickens, which are one of the most susceptible avian species to H5N1 HPAI virus (Perkins and Swayne, 2003).

Because only two ducks survived in this study, accurate conclusions cannot be made on the risk of surviving birds transmitting or geographically disseminating H5N1 HPAI virus during an outbreak in waterfowl. However, as opposed to chickens, in which there was no serologic evidence of infection in surviving birds, wood ducks that survived did seroconvert and shed virus suggesting

that asymptomatically infected wood ducks could contribute to the transmission and spread of virus during an outbreak, though viral shedding is at a lower titer (average maximum OP titer for both survivors = $10^{3.13}$ $TCID_{50}$/ml; average OP titer for all non-survivors = $10^{5.11}$ $TCID_{50}$/ml) and for a shorter duration than birds that eventually succumbed to infection.

Ducks and chickens in this experiment were housed in groups making it impossible to determine whether viral infection in each bird occurred from the initial viral inoculum or transmission from another infected bird within the same housing unit. Consequently, the MDT may be overestimated in this study. However, housing chickens individually did not affect the BID_{50} (D. Swayne personal communication) and the comparable susceptibility of wood ducks would suggest that similar results would be expected. In addition all inoculated birds were shedding virus at 2 DPI suggesting that these infections resulted from the original inoculum. After correcting for the one outlying duck in the $10^{4.5}$ group that died late in the study without viral shedding, the MDT appears to be negatively associated with viral dose. However, differences between dose groups were minor and not statistically significant suggesting that this dose-related variation would have little if any impact on the extent of environmental viral contamination or potential H5N1 HPAI viral spread via infected birds.

Experimental studies evaluating the transmission of H5N1 HPAI virus in wood ducks indicate the following: 1) H5N1 HPAI viruses are virulent in wood ducks causing high morbidity and mortality; 2) infected wood ducks shed virus for 4 to 7 days which is primarily in respiratory secretions and less in the feces (Brown et al., 2006); and 3) very small concentrations of virus produce infection and death in wood ducks. Thus, the low BID_{50} titer of wood ducks may compensate for the more rapid viral degradation of H5N1 HPAI viruses in aquatic habitats. Taken together, these data suggest that the wood duck would represent a very effective indicator

species for H5N1 HPAI virus. Although the information provides some insight related to the potential for H5N1 HPAI viruses to be maintained in waterfowl populations, these wood duck data are not sufficient to indicate or suggest that wood ducks or other wild birds could maintain these viruses in nature. Under natural conditions the biology of wood ducks or other duck species may be much more important in understanding reservoir potential than susceptibility. In a previous study, five other species of North American ducks shed low viral titers for short durations, even after inoculation with high concentrations of H5N1 HPAI virus (Brown et al., 2006). It is possible that H5N1 HPAI virus epidemics in wild waterfowl are dependent on the presence of highly susceptible species like wood ducks, which may represent a small component of the avian community; such epidemics may represent short-term spill-over events that are driven by species composition and the specific ecological, climatic, or environmental conditions that may influence avian distribution and behavior. This theory is consistent with field data from the H5N1 HPAI outbreaks in Europe during 2005-2006, in which the majority of mortality involved limited anseriform species such as mute swans (Cygnus olor) and tufted ducks (Aythya fuligula) (Sabirovic et al., 2006). It is believed that severe weather in Eastern Europe during the winter of 2005-2006 disrupted the migration patterns of waterfowl and resulted in the congregation of high concentrations of these susceptible species, in which the H5N1 HPAI virus outbreaks could occur (Sabirovic et al., 2006).

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TABLE 5.1. Morbidity, mortality, and virus isolation data from wood duck and chickens inoculated intranasally with different doses of the A/Whooper Swan/Mongolia/244/05 (H5N1) highly pathogenic avian influenza virus.

Group ID	**Morbidity** (sick/total)	**Mortality** (dead/total)	**MDT**[a] (days)	**AMT**[b] **(oropharygeal/cloacal)** (log_{10} $TCID_{50}$/ml)	**BID_{50}/BLD_{50}**[c] (log_{10} EID_{50})
Wood Duck					0.95 / 1.71[e]
$10^{1.5}$ EID_{50}[d]	4/5	4/5	6.3	5.01 / 2.74	
$10^{3.0}$ EID_{50}	4/5	4/5	5.5	4.38 / 1.96	
$10^{4.5}$ EID_{50}	5/5	5/5	6.8 (5.0)[f]	5.14 / 2.91	
$10^{6.0}$ EID_{50}	5/5	5/5	5.2	4.43 / 3.74	
White Leghorn Chicken					2.80 / 2.80
$10^{1.0}$ EID_{50}	0/5	0/5	---	NP[g]	
$10^{3.0}$ EID_{50}	3/5	3/5	4.3	NP	
$10^{5.0}$ EID_{50}	5/5	5/5	2.0	NP	

[a] Mean death time

[b] Average mean titer in oropharyngeal and cloacal swabs collected from birds that died

[c] BID_{50} = Median bird infectious dose, BLD_{50} = Median bird lethal dose

[d] Dose of virus inoculated IN to each of the groups of wood ducks and chickens

[e] These values were calculated with the assumption that an inoculation dose of 10^0 EID_{50} resulted in no morbidity, mortality, or seroconversion

[f] MDT for this group after disregarding a single outlying value of one wood duck that died on 14 DPI

[g] NP = not performed

CHAPTER 6

EXPERIMENTAL INFECTIONS OF SWANS AND GEESE WITH AN ASIAN LINEAGE OF H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS[1]

[1] Brown, J.D., D.E. Stallknecht, and D.E. Swayne. Submitted to *Emerging Infectious Diseases* (accepted, in press). Reprinted here with permission of publisher.

ABSTRACT

The role of wild birds in the epidemiology of the Asian-lineage H5N1 highly pathogenic avian influenza (HPAI) virus epizootic and their contribution to the spread of the responsible H5N1 viruses in Eurasia and Africa is unclear. To better understand the potential role of swans and geese in the epidemiology of this virus, we infected four species of swans and two species of geese with an Asian lineage, highly pathogenic type A influenza virus recovered from a whooper swan in Mongolia in 2005, A/whooper swan/Mongolia/244/05 (H5N1), to evaluate their susceptibility and viral shedding. The highest mortality rates were observed in swans and species-related differences in clinical illness and viral shedding were evident. These results suggest that the potential for H5N1 HPAI viral shedding and the movement of infected birds may be species-dependent and provides an explanation for observed mortality that has been associated with H5N1 HPAI-related mortality in anseriforms in Eurasia.

INTRODUCTION

The first indication of wild bird involvement in the Asian-lineage H5N1 HPAI epizootic occurred late in 2002 and 2003 when H5N1 HPAI viruses were isolated from captive and wild birds in Hong Kong (1). Since these initial outbreaks, H5N1 HPAI viruses have continued to cause morbidity and mortality in a variety of wild birds in Asia (2), and in 2005 the virus was recovered from migratory waterfowl during a mortality event involving primarily bar-headed geese (*Anser indicus*) at Qinghai Lake, China (3). Though several thousand birds died in this outbreak (4), it was undetermined how many birds, including other species, were infected and dispersed from Qinghai Lake. In the late summer to fall of 2005, Asian lineage H5N1 HPAI virus was first detected in Europe, where it was isolated from dead wild waterfowl in several European Union member states and neighboring countries (5). The majority of these H5N1

HPAI virus isolates were recovered from a limited number of species in the Order Anseriformes, including mute swans (*Cygnus olor*), whooper swans (*Cygnus cygnus*), tufted ducks (*Aythya fuligula*), and Canada geese (*Branta canadensis*) (5, 6).

Although the ability of Asian lineage H5N1 HPAI viruses to infect and cause mortality in wild birds has been documented, the epidemiology of these viruses in free-ranging avian populations is unclear. Wild avian species infected by these viruses in Asia have been taxonomically diverse, while most mortality, and H5N1 HPAI virus isolations, in Europe have been limited to only a few species of geese and swans. A growing body of genetic and epidemiologic evidence suggests that, in 2005, migratory waterfowl played a role in the geographic spread of Asian lineage H5N1 HPAI virus to Europe (5, 7). However, viruses have not been detected in clinically healthy wild birds in Europe that were not associated with ongoing mortality events (8) and there is no clear evidence that the virus is maintained or geographically spread by infected asymptomatic wild birds. A reliance on dead bird surveillance makes the evaluation of H5N1 HPAI virus in wild waterfowl difficult and has left several gaps in our understanding (9). It is unknown whether Asian lineage H5N1 HPAI viruses that spilled-over from domestic poultry to migratory waterfowl have or can be maintained in wild avian populations or whether similar outbreaks will reoccur.

The objective of this study was to evaluate the susceptibility and viral shedding patterns in four swan and two geese species experimentally infected with a H5N1 HPAI virus, and to potentially extrapolate on each species ability to geographically spread the virus. Susceptibility was determined based on prevalence and onset of morbidity and mortality and distribution of microscopic lesions and viral antigen. Viral shedding patterns were determined based on duration, route, and concentration of viral excretion. The potential ability for a given species to

geographically move the virus was evaluated based on the duration and viral titers associated with asymptomatic shedding.

METHODS

Animals

Four species of swans and two species of geese were used in this study, whooper swan, black swan (*Cygnus atratus*), trumpeter swan (*Cygnus buccinator*), mute swan, bar-headed goose, and cackling goose (*Branta hutchinsii*). All of the birds used in this study were captive-bred and purchased from commercial breeders in the United States of America. The swans were 5- to 6-weeks of age at the time of the experiment. This age was chosen based on availability of birds and size restrictions imposed by the isolation units. Geese were approximately 12-weeks of age at the time of the experiment, which corresponds to the age of juvenile waterfowl during the peak prevalence of AIV in wild waterfowl in North America (10). Both male and female birds were included in each species and were approximately equal in representation. Infected birds for each species were housed separately in groups of four or five (inoculated and contact birds) in self-contained isolation units which were ventilated under negative pressure with high efficiency particulate air (HEPA)-filtered air. Sham-inoculated birds were maintained in separate units from the infected birds and grouped by individual species. The birds were maintained under continuous lighting and food and water were provided *ad libitum*.

All birds used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (11) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at both Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States

Department of Agriculture (USDA), Athens, Georgia, USA and the University of Georgia (UGA). All experiments were performed in the USDA-certified BSL 3-Ag facility at SEPRL (12).

Viruses

A stock of the A/whooper swan/Mongolia/244/05 (H5N1) (Mongolia/05) HPAI virus was produced by second passage in 9-day-old embryonated chicken eggs. Allantoic fluid from the inoculated eggs was diluted in brain-heart infusion (BHI) medium to yield a final titer of 10^6 median embryo infectious doses (EID_{50}) per 0.1 ml (single-bird inoculum) as determined by standard procedures (13). A sham-inoculum was prepared by diluting sterile allantoic fluid 1:100 in BHI. The Mongolia/05 virus was originally isolated from a dead whooper swan during a large mortality event involving waterfowl (14) and the isolate was chosen for use in this study because of its known lethality in wild waterfowl under natural, and experimental (15) conditions. In addition, this strain genetically appears to be a representative isolate from the wild bird H5N1 HPAI viruses (clade 2.2) that have been reported in Asia, Europe, and Africa (15). Extrapolations from our data herein were made with the assumption that the Mongolia/05 virus is representative of the 2005-2006 Asian lineage H5N1 HPAI viruses that were isolated from dead wild birds in Eurasia.

Experimental Design

Pre-inoculation serum was collected from each bird to confirm they were serologically negative to influenza A type specific antigens by the agar gel precipitin (AGP) test and the H5 hemagglutinin subtype by the hemagglutinin inhibition (HI) test. In addition, oropharyngeal and cloacal swabs were collected from each bird to ensure they were not actively infected and shedding AIV at the start of the study. Two (whooper swan and cackling goose) or three

(trumpeter swan, black swan, mute swan, and bar-headed goose) birds from each species were inoculated intranasally (IN) with a 0.1 ml volume of the Mongolia/05 virus solution. Twenty four hours later, two additional birds from each species were placed in the housing unit with the inoculated birds. All birds were monitored daily for morbidity or mortality for a period of 14 days. Morbidity was defined as any clinical abnormality detectable upon physical examination after inoculation with, or contact exposure to, the H5N1 HPAI virus, including weakness, cloudy eyes, respiratory difficulty, shivering, crowding, ruffled feathers, hemorrhage on the unfeathered skin, and neurologic signs, such as tremors, seizures, severe incoordination, and torticollis. Oropharyngeal and cloacal swabs were collected and then placed in BHI with antibiotics (400 μg/ml gentamicin, 4000 units/ml penicillin, and 5 μg/ml amphotericin B) from all birds at 1, 2, 3, 4, 5, 7, 10, and 14 days post-exposure (DPE). At 14 DPE, blood was collected from the surviving birds for serologic testing and the birds were humanely euthanized via intravenous sodium pentobarbital (100 mg/kg body weight). Blood was not collected from birds that died during the course of the study. Necropsies were performed on all birds and routine tissues were collected for histopathologic and immunohistochemical evaluation. In addition, oropharyngeal and cloacal swabs were collected from all birds that died and were stored in BHI with antibiotics for virus isolation. In reporting the temporal data, 0 days post-contact (when the contact birds were placed into the cage with the inoculated birds) was assumed to be equivalent to 0 days post-inoculation.

One bird from each species was IN inoculated with a sham solution and housed in a separate unit from the viral-exposed birds. Oropharyngeal and cloacal swabs and pre-inoculation serum were collected from these birds prior to inoculation to confirm that they were not actively infected with and were negative for serum antibodies to the type specific A antigen and the H5 hemagglutinin. These birds were monitored for morbidity and mortality for the 14 day trial. At

14 DPE, serum was collected from each sham-inoculated bird for serologic testing and they were euthanized as described above. A necropsy was performed on each bird and samples were collected for histopathologic and immunohistochemical evaluation.

Histopathology and Immunohistochemistry

Tissues samples collected at necropsy were preserved in 10% neutral buffered formalin. After fixation, the tissues were routinely processed and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. Duplicate sections were cut and immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein (NP) antigen as the primary antibody (SEPRL, Athens, GA). The procedures used to perform the immunohistochemistry (IHC) followed those previously described (16). Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen deposition in the nucleus, with or without chromagen deposition in the cytoplasm.

Virus Isolation

Oropharyngeal and cloacal swabs collected at necropsy were stored at -70°C until virus isolations and titrations were performed. Isolation of virus from the swabs was performed in 9- to 11-day-old embryonated chicken eggs using standard procedures (13). Positive samples were also titrated in 9- to 11-day-old embryonated chicken eggs by determining the EID_{50}. The minimal detectable titer from the swabs was $10^{0.97}$ EID_{50}/ml.

Serologic Assays

Serologic testing was performed on the pre- and post-inoculation serum via the AGP and HI tests using standard procedures (17). The HI tests were performed using a 0.5 % suspension of chicken erythrocytes in phosphate-buffered saline. Serum was pre-treated with chicken

erythrocytes to neutralize any naturally occurring serum hemagglutinins and the first dilution on the test plate was 1:8. All HI titers $\geq$ 8 were considered positive.

RESULTS

Morbidity, mortality, viral distribution, and pathology differed between the swans and geese infected with the Mongolia/05 virus (Table 1). All species of swans experienced 100% mortality after infection with the Mongolia/05 virus, including all birds that were directly inoculated with the virus and those that acquired the infection through contact exposure. Viral shedding was detected in each of the IN-inoculated birds (including all swan and goose species) at 1 DPE (average oropharyngeal titer at 1 day post inoculation: black swans = $10^{4.30}$ EID_{50}/ml; mute swans = $10^{3.23}$ EID_{50}/ml; trumpeter swans = $10^{4.17}$ EID_{50}/ml; whooper swans = $10^{4.10}$ EID_{50}/ml; bar-headed geese = $10^{3.83}$ EID_{50}/ml; cackling geese = $10^{3.50}$ EID_{50}/ml). Challenge virus was detected in oropharyngeal and cloacal swabs in every IN inoculated and contact bird in each species during the study except one bar-headed goose, which is described later. Viral shedding was detected in each of the contact swans by 1 DPE but was delayed in contact geese; virus was not detected until 3 DPE in the cackling geese and 2 DPE in the bar-headed geese. Similarly, there was no difference between the onset of detectable clinical signs and death in swans that were inoculated with virus and those that were exposed through contact. Contact geese, however, had a delayed onset of detectable clinical signs (cackling geese = 6.5 days, bar-headed geese = 6.5 days) and death (cackling geese = 7.5 days, bar-headed geese = 8.0 days) compared to IN inoculated geese (clinical signs: cackling geese = 3.5 days, bar-headed geese = 3.3 days; death: cackling geese = 4.0 days, bar-headed geese = 6.0 days)

Black swans were the most susceptible species examined in this study with 100% mortality occurring within two to three DPE. Most black swans were found dead without

exhibiting any clinical signs of disease. When morbidity was observed, it lasted for less than 24 hours and clinical signs included severe listlessness and neurologic dysfunction consisting of seizures, tremors, and marked incoordination. Influenza viral antigen was detected primarily in endothelial cells lining the blood vessels throughout most visceral organs and the brain (Figure 1A). Microscopically, all black swans that died had widespread multi-organ necrosis with mild acute inflammation which was strongly correlated to the distribution of the virus. All of the black swans shed virus prior to death and, as with all birds in this study, titers were higher in respiratory secretions than in the feces (Table 2). Consistent among birds that died from any of the waterfowl species, viral shedding, in respiratory secretions and feces, generally increased with time and reached a maximum within 24 to 48 hours of death.

Susceptibility was similar in the remaining three species of swans. Morbidity and mortality occurred later in these species and the duration of morbidity, with one exception, was longer. Clinical signs consisted of mild to moderate listlessness which progressively worsened to severe listlessness with neurologic signs similar to those observed with the black swans. Viral antigen was detected in the neurons (Figure 1B), astrocytes, and other parenchymal cells of the brain and most of the examined visceral organs, as opposed to the vasculotropic distribution in black swans. Microscopic lesions were strongly associated with the anatomic location of detectable viral antigen and consisted of multifocal to coalescing necrosis with mild to moderate acute inflammation. Within this category, the Mongolia/05 virus infection in mute swans was unique. Mute swans had a delayed onset of morbidity (5-7 DPE), which was later than any of the species examined in this study. The duration of morbidity in this species was extremely short (less than 24 hours) and comparable to the black swans. The clinical signs observed in mute swans were similar to those in the whooper and trumpeter swans. Birds in all three of these

species shed high concentrations of virus in respiratory secretions with maximum titers approximating those of the black swans.

The two species of geese differed in their susceptibility to the Mongolia/05 virus and were less susceptible than the swan species. All of the cackling geese became sick after inoculation with the Mongolia/05virus, but only three of the four birds died and the remaining bird slowly recovered until clinical signs of disease were no longer apparent upon physical examination. The cackling geese that died exhibited severe listlessness and marked neurologic signs similar to those observed in the swans. The single goose that survived became moderately listless with ruffled feathers and cloudy eyes prior to resolution of clinical signs, but did not exhibit neurologic clinical signs during the study. This goose produced post-exposure antibodies to AIV that were detected by the AGP and HI tests. Cackling geese that died had a short duration of morbidity (average duration = 1.67 days) as opposed to the goose that survived, which exhibited detectable clinical signs for 9 days prior to resolution. Viral antigen in the three geese that died was restricted to the brain, pancreas, liver, and adrenal gland. Microscopic lesions primarily involved these organs and included multiple foci of necrosis with moderate subacute inflammation. The one goose that survived had minimal amounts of viral antigen in the neurons of the brain and mild perivascular encephalitis. The surviving goose shed lower concentrations of virus (maximum oropharyngeal titer = $10^{3.9}$ EID_{50}/ml) than the three geese that died (average maximum oropharyngeal titer (AMOT) = $10^{5.7}$ EID_{50}/ml), but had an approximately similar duration of shedding in both oropharyngeal and cloacal swabs.

Bar-headed geese were the least susceptible of the six species examined in this study. All five of the geese infected with the Mongolia/05 virus exhibited clinical signs of infection; two of these birds died and the remaining three became sick but the clinical signs slowly resolved until

they were no longer apparent upon physical examination. The duration of clinical signs and onset of morbidity and mortality were similar to that of the cackling geese. The bar-headed geese that died exhibited severe depression and neurologic clinical signs. The three geese that survived became mildly depressed with transiently cloudy eyes, but did not exhibit neurologic clinical signs. The duration of morbidity was longer in the geese that survived (average duration = 5.33 days) than in the geese that died (average duration = 2.50 days). All three of these surviving geese produced antibodies to AIV which were detected by the AGP and HI tests. Viral antigen and microscopic lesions in bar-headed geese were primarily present in the brain. Viral antigen staining was more intense in the two geese that died than in the three that survived. Microscopic lesions consisted of moderate perivascular encephalitis and neuronal necrosis in geese that died and mild perivascular encephalitis in birds that survived. The concentration and duration of viral shedding was similar between bar-headed geese that died and those that survived. Cloacal shedding was detected in all of the bar-headed geese except one, which was one of the surviving birds.

DISCUSSION

During the Asian lineage H5N1 HPAI outbreaks in Europe in 2005/2006, certain duck and swan species were overrepresented in the mortality reports (5). While field data from the outbreaks indicated that these waterfowl species were susceptible, their contribution to the spread of H5N1 HPAI virus is not clear. In general, asymptomatic birds can shed virus prior to the onset of morbidity or after clinical signs have resolved. In this study, all six waterfowl species shed virus prior to the onset of clinical signs, though species-related differences were apparent (Figure 2). Some geese survived infection (in both species), but none of the surviving birds were actively shedding detectable virus after the resolution of clinical signs.

Black swans were the most susceptible species to H5N1 HPAI virus infection with morbidity, mortality, and viral distribution similar to gallinaceous poultry (16). While all of the black swans shed virus prior to dying, the asymptomatic viral titers were low and the rapid course of disease would most likely preclude geographic spread of virus by this species. The high susceptibility of black swans to infection, however, would make them a good sentinel species for detection of H5N1 HPAI virus in Australia and New Zealand, where this species is found naturally. In addition, the high concentrations of virus shed after the onset of clinical signs, but prior to death would allow this species to contribute locally to the viral transmission during an outbreak in waterfowl.

Morbidity and mortality occurred later in whooper swans, mute swans, and trumpeter swans, allowing actively infected, and shedding, birds in these species more potential time to spread virus during their movements. This is particularly true for mute swans which shed moderate to high concentrations of virus for several days without showing clinical signs of disease. The longer duration of asymptomatic viral shedding would allow this species ample time to travel and have contact with other wild birds and shared aquatic habitats. Mute swans do not migrate, however freezing temperatures may cause many populations to move during the winter season as waterways freeze; this has been suggested as a factor that contributed to the spread of H5N1 HPAI virus in Europe during 2005/2006 (6).

All of the swans used in this study were inoculated at 5- to 6-weeks of age and the high virulence observed in these species may be attributable to the young age of the birds. A negative association between age and mortality associated with H5N1 HPAI virus infection occurs in ducks up to 4 weeks but not in chickens (18). Whether or not similar age-related differences in susceptibility to H5N1 HPAI virus exist with swans is not known. However, the Mongolia/05

H5N1 HPAI virus was as lethal as or more lethal to the four species of swans in this study as other H5N1 HPAI viruses have been in a variety of age-matched or younger wild avian species, including gallinaceous birds, waterfowl, and gulls (19, 20). If age-related susceptibility does exist in swans, it is possible that older birds may be more likely to survive the infection.

The cackling geese were more susceptible to H5N1 HPAI virus than the bar-headed geese as evidenced by higher mortality and more systemic viral distribution, and both of these geese species were generally less susceptible than swans. The delayed viral shedding, morbidity, and mortality of contact geese compared to inoculated geese provides further support that geese are less susceptible than swans, and may suggest viral transmission within populations of these geese species would occur at a slower rate than in swans. While susceptibility varied between these two species, both had similar onsets of morbidity and mortality, duration and concentrations of viral shedding, and duration of asymptomatic shedding. Based on these parameters, bar-headed geese and cackling geese would be equally efficient disseminators of H5N1 HPAI virus. Cackling geese are closely related to Canada geese, which were affected in some European outbreaks of H5N1 HPAI virus (6). The lower susceptibility, relative to the other species examined in this study, of bar-headed geese was surprising considering the large number of birds that died at the Qinghai Lake outbreak in 2005. Based on the mortality in this study it is possible that many bar-headed geese were infected and survived, and our results support the possibility that this species played a role in the transmission H5N1 HPAI virus to waterfowl or other wild birds outside of Qinghai Lake.

Consistent with reported wild bird mortality data from previous outbreaks, this study identified species-related variability in susceptibility to H5N1 HPAI virus among wild species of waterfowl. Several important characteristics of H5N1 HPAI virus infection differ between

waterfowl species, including duration of asymptomatic shedding and duration and concentration of viral shedding. Based on these characteristics, mute swans, cackling geese, and bar-headed geese may be recognized as species which pose a greater risk for transmission and spread of H5N1 HPAI virus. It is possible that relatively few species, rather that anseriform species as a whole, contributed to most of the spread of H5N1 HPAI within Eurasia. This is consistent with observed mortality patterns during outbreaks and from the failure to detect H5N1 HPAI in clinically normal anseriform species despite intensive sampling. If this is indeed the case, it implies that the epidemiology of this particular lineage of AIV in waterfowl populations differs from that of LPAI viruses that naturally circulate in wild birds and that the establishment of a silent (without detectable morbidity and mortality) natural reservoir for Asian lineage H5N1 HPAI viruses within wild waterfowl populations may be unlikely.

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Table 6.1. Morbidity, mortality, and pathologic data from four species of swans and two species of geese exposed to H5N1 HPAI virus via IN inoculation and contact with infected birds*.

	Morbidity		Mortality	Viral distribution
ID[†]	Rate (days to onset)	Duration[‡]	Rate (days to death)	
BS	5/5 (1-2)	<1 (0-1)	5/5 (2-3)	Blood vessels
TS	5/5 (2)	4 (3-5)	5/5 (4-6)	Brain, skin, multiple organs [§]
WS	4/4 (2-4)	3 (1-5)	4/4 (4-6)	Brain, skin, multiple organs
MS	5/5 (5-7)	<1 (0-1)	5/5 (5-8)	Brain, skin, multiple organs
CG	4/4 (3-7)	3 (1-9)	3/4 (4-8)	Brain, pancreas, liver, adrenal gland
BHG	5/5 (3-7)	4 (1-8)	2/5 (6-7)	Brain

*Exposure date for each species was adjusted so that 0 days post-contact (when the contact birds were placed into the cage with the inoculated birds) was assumed to be equivalent to 0 days post-inoculation.

[†]BS=black swan, TS=trumpeter swan, WS=whooper swan, MS=mute swan, CG=cackling geese, BHG=bar-headed geese

[‡]Average duration of detectable clinical signs in days (range)

[§]Adrenal gland, pancreas, liver, lungs, heart, spleen, kidneys, air sacs, trachea, intestinal parasympathetic ganglia, and gastrointestinal tract

Table 6.2. Virus isolation (VI) data from four species of swans and two species of geese exposed to H5N1 HPAI virus via IN inoculation and contact with infected birds*..

	VI Oropharyngeal		VI Cloacal	
ID[†]	Average Duration[‡]	AMT[§] (EID_{50}/ml)	Average Duration	AMT (EID_{50}/ml)
BS	2 (2-3)	6.46	2 (1-2)	4.94
TS	5 (4-6)	6.14	4 (2-5)	3.18
WS	5 (4-6)	6.30	4 (3-5)	4.25
MS	5 (3-7)	5.58	4 (3-4)	4.46
CG	5 (4-6)	5.25	3 (2-5)	3.05
BHG	6 (5-8)	5.10	3 (0-7)	2.55[¶]

*Exposure date for each species was adjusted so that 0 days post-contact (when the contact birds were placed into the cage with the inoculated birds) was assumed to be equivalent to 0 days post-inoculation.

[†]BS=black swan, TS=trumpeter swan, WS=whooper swan, MS=mute swan, CG=cackling geese, BHG=bar-headed geese

[‡]Average duration of viral shedding in days (range)

[§]Average maximum titer for birds that shed virus. All of the contact and inoculated birds shed detectable concentrations of virus via the oropharyngeal and cloacal route with one exception (noted below)

[¶]One bar-headed goose did not excrete detectable virus via the cloacal route and the average maximum titer for cloacal shedding in this species was calculated based on the 4 birds with detectable cloacal shedding. If all five geese were included in this calculation, the average cloacal shedding would be $\log_{10}$ 2.04 EID_{50}/ml.

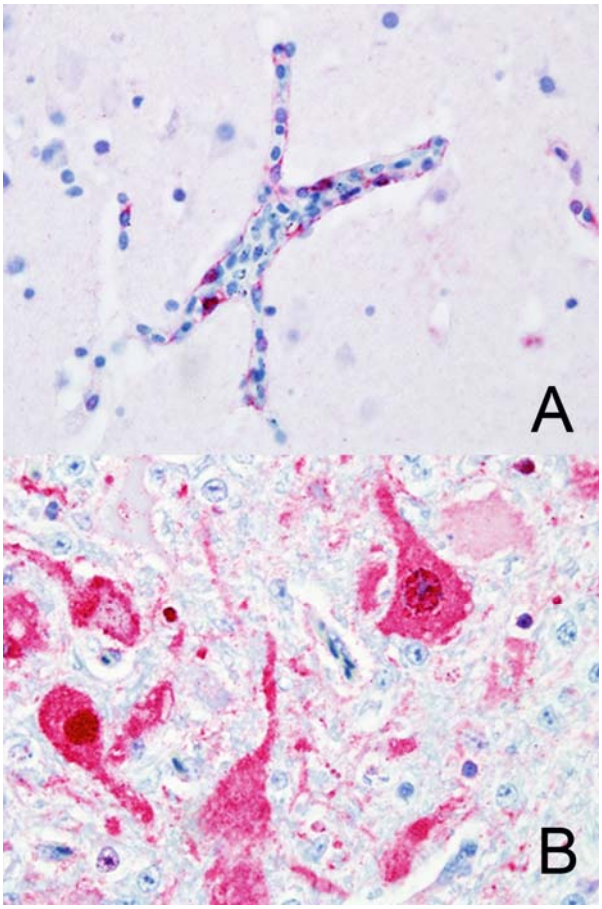
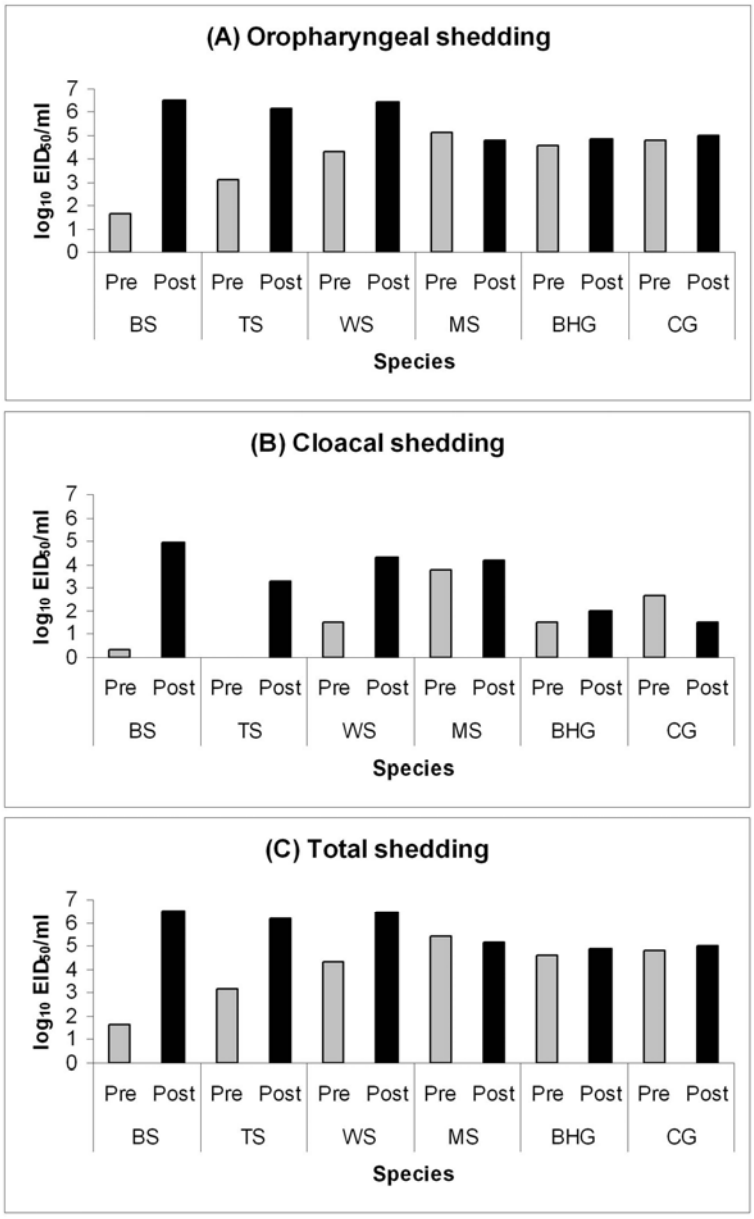


Figure 6.1. . Photomicrograph of viral antigen (red) in **A**. endothelial cells lining a blood vessel in the brain of a black swan and **B**. neurons in the brain of a mute swan, which both died after experimental infection with a H5N1 HPAI virus. Immunohistochemical stain with hematoxylin counterstain.

Figure 6.2. The average concentration of viral shedding in oropharyngeal (**A.**), cloacal (**B.**), and combined (**C**.) routes before (Pre) and after (Post) the onset of clinical signs in four species of swans and two species of geese exposed to H5N1 HPAI virus via IN inoculation or contact with infected birds. Viral concentrations were determined by adding viral titers before and after the onset of clinical signs for each individual bird and using these values to calculate a pre- and post-clinical average for each species. (BS=black swans, TS=trumpeter swans, WS=whooper swans, MS=mute swans, BHG=bar-headed geese, and CG=cackling geese). The one bar-headed goose that did not shed detectable concentrations of virus was included in the calculation of the averages for this species.


(A) Oropharyngeal shedding
log10 EID50/ml
0
1
2
3
4
5
6
7
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post
BS TS WS MS BHG CG
Species
(B) Cloacal shedding
log10 EID50/ml
0
1
2
3
4
5
6
7
Pre Post Pre Post Pre Post Pre Post Pre Post Pre Post
BS TS WS MS BHG CG
Species
(C) Total shedding
log10 EID50/ml
0
1
2
3
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CHAPTER 7

SUMMARY AND CONCLUSIONS

Over the last 10 years the H5N1 HPAI virus has continuously challenged what we consider to be the "traditional AIV paradigm" and displayed unique biologic traits. In particular, the broad avian and mammalian host range of H5N1 HPAI and the high virulence for certain wild bird species are unmatched among AIV. Since 2002, H5N1 HPAI virus has caused morbidity and mortality in wild birds in Southeast Asia, where the virus is endemic in domestic poultry (including domestic ducks). In 2005-2006 the virus spread to Central Asia, Europe, and Africa with wild bird mortality detected along the proposed route of dissemination. Significant research efforts have been devoted toward improving our understanding of this virus and the extent of its unique biology. However, the role that wild birds play in the epidemiology of the ongoing H5N1 HPAI virus epidemic remains poorly understood. The overall objective of this study was to evaluate the ecology of H5N1 HPAI virus in wild birds under experimental conditions in order to better understand the potential for this virus to be maintained or efficiently transmitted within free-living avian populations. Essentially we were attempting to answer the question of whether wild birds were potential reservoirs for H5N1 HPAI virus or simply dead-end hosts.

The ability of H5 and H7 AIV to persist in water was evaluated in a series of experiments using a previously validated distilled water model to determine the range of environmental fitness for viruses of these two agriculturally important subtypes (Chapter 3). Included in these viruses were four H5 LPAI viruses, four H7 LPAI viruses, and two H5N1 HPAI viruses. The results of the persistence trials indicated the following: 1) H5 and H7 LPAI viruses that naturally circulate in free-living anseriforms and charadriiforms can persist for extended periods of time in

water (months at 17º C and weeks at 28º C), with a duration of infectivity comparable to AIV of other hemagglutinin subtypes; 2) The temperature and salinity of the water, within ranges normally found in nature, have a negative effect on the persistence of H5 and H7 AIV; and 3) The two examined H5N1 HPAI viruses did not persist as long as the H5 or H7 LPAI viruses, suggesting that at some point in the evolution of H5N1 HPAI, these viruses lost some degree of environmental fitness. The effect that this loss of fitness has on the ability of H5N1 HPAI viruses to persist or transmit within free-living aquatic bird populations, however, is not known.

The second group of experiments (Chapter 4) evaluated the susceptibility and viral shedding of five duck species (mallard, Northern pintail, wood duck, blue-winged teal, and redhead) and laughing gulls infected with two H5N1 HPAI viruses. Both H5N1 HPAI viruses yielded similar results: 1) All six species were infected based on virus isolation data and seroconversion, but only the wood ducks and laughing gulls exhibited morbidity or mortality; 2) Clinical syndromes exhibited by the wood ducks and laughing gulls that eventually died were neurologic, consisting initially of weakness and lethargy, which progressed to seizures, torticollis, and severe imbalance. Individual birds that became sick and recovered were only mildly to moderately lethargic; 3) Viral antigen and associated microscopic lesions were only present in birds that exhibited morbidity and were most widespread and severe in birds that died. The virus had a predilection for particular organs, including the brain, pancreas, adrenal glands, and heart; and 4) In all six species, viral titers were higher in oropharyngeal swabs than cloacal swabs and fecal shedding did not occur in all birds. The concentration and duration of viral shedding was proportional to the severity of clinical signs. The concentration was higher and viral shedding was longer in the wood ducks and laughing gulls that died.

Results of this experiment indicate that species-related differences in susceptibility and viral shedding exist among birds in the Order Anseriformes and Charadriiformes, which have traditionally been considered the natural reservoirs for AIV. In addition, the results of this study suggest that H5N1 HPAI viruses are highly virulent for certain species in these two orders, and infection by these viruses are associated with neurologic clinical signs and high mortality. Within these species, morbidity characterized by neurologic clinical signs may be an indicator of fatal disease outcome. This high virulence for select aquatic bird species is atypical for HPAI viruses, which traditionally replicate inefficiently in wild avian species and rarely cause clinical signs of disease in these hosts. Viral shedding in ducks and other aquatic birds infected with H5N1 HPAI viruses has predominately, and consistently, been via respiratory secretions as opposed to the traditional fecal-oral transmission cycle of AIV in waterfowl. It is uncertain what impact this shedding pattern may have on the extent of environmental contamination, the persistence in aquatic habitats, or the transmission between birds (both wild and domestic). Transmission of H5N1 HPAI has been demonstrated experimentally between infected and contact mallards under penned conditions (Sturm-Ramirez, 2005) and obviously also occurs in man-made agricultural systems, including production of gallinaceous poultry and domestic ducks and live bird markets. It is unknown, however, if respiratory viral shedding can result in the same environmental contamination levels or transmission efficiency as fecal shedding in free-living avian population.

In order to further examine the susceptibility of a "highly susceptible" wild bird species, we determined the median bird infectious dose (BID_{50}) and lethal dose (BLD_{50}) of an H5N1 HPAI virus for wood ducks after IN inoculation (Chapter 5). The results of this study indicated that wood ducks have a low BID_{50} and BLD_{50} of $10^{0.95}$ EID_{50}/ml and $10^{1.71}$ EID_{50}/ml,

respectively. These infectious and lethal viral doses are less than those of domestic chickens, traditionally considered one of the most susceptible avian species to H5N1 HPAI virus. These results confirm that wood ducks are highly susceptible to H5N1 HPAI virus and suggest that wild avian species that appear to be "highly susceptible" to H5N1 HPAI virus based on field data or experimental infection trials, are truly sensitive to infection with this virus. It is possible that the low viral infectious doses of highly susceptible species such as wood ducks may compensate for the decreased environmental fitness of these viruses that we described above, and potentially enable H5N1 HPAI viruses to persist and circulate within a free-living waterfowl population for longer durations than we would expect based on our current knowledge of H5N1 HPAI ecology in wild birds. Furthermore, this ability to persist for longer durations in the free-living avian population may help explain the outbreaks of H5N1 HPAI virus in areas lacking domestic poultry as a source of virus spill-over, such as Mongolia.

In the next set of experiments (Chapter 6), we experimentally inoculated four species of swans (mute swans, black swans, trumpeter swans, and whooper swans) and two species of geese (bar-headed geese and cackler geese) with a H5N1 HPAI virus to evaluate species susceptibility and asymptomatic viral shedding patterns. For each species, additional non-infected birds were placed into the same housing unit as the inoculated birds after 24 hours. This study design was used to determine the species-related susceptibility of selected swan and geese (morbidity and mortality), transmission efficiency within these avian hosts (infection in contact birds), and viral shedding for each species. Furthermore, the duration and concentration of asymptomatic viral shedding was evaluated for each species to determine the potential for infected birds in each species to geographically spread the virus. The results of this study indicated the following:

1) All four species of swans were highly susceptible to H5N1 HPAI virus exhibited by 100% mortality, though the swan species varied in onset of clinical signs, duration of morbidity, and mean death times. The H5N1 HPAI virus was also virulent for both species of geese, but less than for the swans as evidenced by the lower mortality rates and a more restricted distribution of viral replication in the body.

2) Clinical signs in all birds that died in each species were neurologic. Birds that became sick but then recovered only exhibited mild to moderate weakness and lethargy.

3) Viral shedding and microscopic lesions were widespread in the highly susceptible swan species, but were more restricted in the geese and primarily confined to the brain, pancreas, adrenal glands, heart, and liver. The microscopic lesions were primarily necrotizing, with mild to moderate infiltrates of heterophils that increased with duration of individual bird survival.

4) Mute swans, cackler geese, and bar-headed geese had the longest duration of asymptomatic viral shedding and excreted the highest concentrations of virus prior to the onset of clinical signs. Based on these data, infected birds in these species would have the greatest potential to geographically spread H5N1 HPAI virus.

The results from the experimental infection trials indicate that H5N1 HPAI viruses are virulent for select wild avian species and this is consistent with field data from the outbreaks of H5N1 HPAI throughout Eurasia. Based on these studies, unless clinical disease is evident, viral titers are low and shedding is of short-duration. This is consistent with the Eurasian active field surveillance results thus far, in which isolation of H5N1 HPAI from clinically healthy wild birds has been extremely rare. Taken together, these experimental and field data suggest that the wild bird species that are affected clinically are the primary species involved in the transmission and spread of H5N1 HPAI virus in wild bird populations, as opposed to an asymptomatic avian host.

Furthermore, these data suggest that a wild bird reservoir for H5N1 HPAI virus may not exist and that epidemics in Europe during 2005-2006 likely represent spill-over events from domestic poultry into wild birds with limited persistence and transmission within the wild avian population.

The information herein provides insight related to the potential for H5N1 HPAI viruses to be transmitted, persist, and geographically spread in waterfowl populations. Under natural conditions, however, the characteristics of the H5N1 HPAI isolate, the biology of each individual wild bird species, the environmental conditions, and the species composition of the avian population must be considered when evaluating maintenance potential of wild birds. These ecologic factors are unique to each H5N1 HPAI outbreak and result in distinct host-pathogen-environment dynamics. Subsequently, these factors should be analyzed for each outbreak in order to understand the constantly changing epidemiology of H5N1 HPAI in wild birds.