INVESTIGATION OF HOST FACTORS THAT INFLUENCE THE INFECTIVITY OF AVIAN INFLUENZA VIRUSES TO WILD BIRDS

by

TAIANA PEREIRA DA COSTA

(Under the Direction of Elizabeth W. Howerth)

ABSTRACT

Avian influenza (AI) was first recognized as an avian pathogen in 1878, and since then, AI in poultry and wild birds has been extensively investigated. Despite the many studies that have been done to clarify the susceptibility and pathology of wild birds to H5N1 highly pathogenic avian influenza (HPAI) viruses, research addressing the susceptibility and transmissibility of low pathogenic avian influenza (LPAI) viruses in their natural host is still scarce. Information regarding inter-species differences in virus prevalence, as well as the distribution, ecology and life history of susceptible host species, is important in understanding AI virus epidemiology within the wild-bird reservoir system.

The overall goal of this study was to provide a better understanding of host factors that affect the susceptibility of wild birds to AI viruses. Specifically, experimental trials were designed to evaluate if age-at-infection or host species affect the outcome of a LPAI virus infection in wild birds. In addition, the effect of a pre-exposure to a LPAI virus on subsequent LPAI or HPAI virus infections was evaluated.

The results of these trials indicate that the susceptibility of wild birds to AI virus infection is complex and dependent on multiple factors, including AI virus isolate, age-at-

infection, bird species, and pre-existing immunity to LPAI virus. The susceptibility and viral shedding pattern data of this investigation can be used as a baseline for future experimental trials with wild birds, and help to interpret data acquired in surveillance and field studies. Furthermore, this research contributes to the comprehension of the natural history of wild type avian influenza, and helps us define and understand the epidemiology of AI viruses in wild avian population.

INDEX WORDS: Age-at-infection, Avian influenza virus, Ducks, Gulls, Host factors, HPAI, LPAI, Order Anseriformes, Order Charadriiformes, Prior exposure, Species, Susceptibility, Waterfowl, Wild birds.

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DVM, São Paulo State University, Brazil, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

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July 2010

ACKNOWLEDGEMENTS

I am heartily thankful to my graduate committee, Dr. Elizabeth Howerth, Dr. David Stallknecht, Dr. Susan Williams, and Dr. David Swayne, whose guidance, support, and help were essential for the development and completion of this project. I especially thank Buffy and Dave for the opportunity they gave me to pursue graduate studies in the USA; they offered invaluable assistance, encouragement, and mentorship throughout this investigation. I also specially thank Susan for being always supportive, since the day I first came to UGA as a visiting veterinarian at the Poultry Diagnostic Research Center.

I would like to thank the United States Department of Agriculture and the National Institutes of Health for providing financial support for this investigation.

I owe my deepest gratitude to Dr. Justin Brown for the friendship and unlimited help on designing and conducting the experimental trials of this study, and for the great feedback on the prospectus, manuscripts, and chapters of this dissertation.

I appreciate the faculty, staff, and fellow graduate students at SCWDS and at the Department of Pathology. In particular, I would like to thank Ginger Goekjian, Jennifer Smith, and Rebecca Poulson for their immense help on processing samples.

I am grateful to the staff of the Southeast Poultry Research Laboratory, especially Joan Beck, Kira Moresco and James Doster, for the great help on bird handling and sample testing; to Drs. Rachel Rech, Daniel Rissi, and Renata Casagrande, and to all the SCWDS externs and summer students who helped me to sample and handle birds, and perform necropsies; to the

Animal Resources of the College of Veterinary Medicine at UGA, for providing housing and animal care assistance for the birds used in this study.

I am thankful to everyone whose friendship and help made the adaptation to the life in the USA a lot easier, especially Ginger, Jennifer, Danny, Nanda, Shamus, and Corrie.

I wish to express my love and gratitude to my family, whose endless support allowed me to pursue my dreams, and who, despite being over 4,600 miles away, has been always there for me. And to my David, thanks for your unconditional love and support.

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CHAPTER 1

INTRODUCTION

Influenza virus type A of bird origin, also called avian influenza (AI) virus, was first described as an avian pathogen in 1878 (Perroncito, 1878) and, since then, has been responsible for endemic infections and outbreaks in poultry and wild birds, human infections and fatalities, and massive economic losses (Claas et al., 1998; Capua and Alexander, 2002; McLeod, 2008; Kalthoff et al., 2009; Lupiani and Reddy, 2009; Malik Peiris, 2009). The first reported isolation of an AI virus from a wild bird was in 1961, during a mortality event in common terns (*Sterna hirundo*) in South Africa (Becker, 1966). To date, surveillance studies have reported AI viruses from more than 100 species in 13 avian orders, most of them associated with aquatic habitats (Stallknecht et al., 2007). The two avian groups considered to be the most important reservoirs for AI viruses are the Anseriformes and Charadriiformes (Stallknecht, 2003; Stallknecht et al., 2007).

Avian influenza viruses are highly host adapted, producing subclinical infections in their native species. Occasionally, these viruses are transmitted to other hosts, including mammals and domestic poultry, producing subclinical infections. Nevertheless, when AI viruses adapt to a new host species, such as gallinaceous poultry, they can establish infection, producing clinical diseases and death. Recurring disease problems can occur if the viruses become endemic within a population (Swayne and Pantin-Jackwood, 2008).

The prevalence of AI infection is highly variable between avian taxa (Olsen et al., 2006) and is associated with host ecology (Stallknecht and Shane, 1988; Alexander, 2000; Stallknecht, 2003) such as population density, preference for wetland and aquatic environment (Webster et al., 1978; Webster et al., 1992), migration (Krauss et al., 2004; Tracey et al., 2004; Olsen et al., 2006), and feeding strategy (Olsen et al., 2006). As mentioned by Swayne (2008) "free-living birds should not be viewed as a single entity of 'wild birds' occupying one ecosystem with equal risk for low pathogenic AI (LPAI) virus infection". Understanding the link between inter-species differences in virus prevalence and the distribution, ecology and life history of susceptible host species is essential for a deep comprehension of AI epidemiology (Garamszegi and Moller, 2007).

Several factors influence susceptibility of birds to infection with highly pathogenic avian influenza (HPAI) and LPAI viruses, including species, virus isolate (Brown et al., 2006; Boon et al., 2007; Brown et al., 2007; Brown et al., 2008b; Keawcharoen et al., 2008), age at infection (Winkler et al., 1972; Lavoie et al., 2007; Pantin-Jackwood et al., 2007), route of infection (Brown et al., 2008a), and immune status (Pasick et al., 2007). Although extensive work on susceptibility of wild birds to H5N1 HPAI viruses and the pathologic findings have been done (Perkins and Swayne, 2002b, a, 2003b, a; Brown et al., 2006; Pasick et al., 2007), studies on the transmissibility and replication of wild type AI viruses in their natural host are scarce. A deep understanding of AI virus biology, prevalence, and epidemiology within the wild-bird reservoir system is needed in order to minimize the risk of transmission to domestic poultry and threat to human and minimize the threat to human and wildlife health, as recently seen with H5N1 HPAI outbreaks in Eurasia (Webster et al., 1992; Suarez, 2000; Webby and Webster, 2003; Capua and Alexander, 2004; Tracey et al., 2004; Clark and Hall, 2006; Olsen et al., 2006).

This research contributes to a better understanding of AI virus natural history and establishes baseline data and standards by which to evaluate atypical events, such as the HPAI outbreak in wild birds. The overall goal was to provide a better understanding of host factors that affect the susceptibility of wild birds to AI viruses. Specifically, the first objective of this investigation was to determine if age-at-infection and host species influence the susceptibility of wild birds to LPAI virus infections. This objective provides detailed information on the effect of age at infection and the host species on susceptibility and extent of viral shedding associated with LPAI infections in Anseriformes and Charadriiformes, the two recognized avian influenza virus reservoirs. Besides providing a baseline to interpret field data and AI epidemiology, the data acquired in this experiment provide insight into possible variation of viral shedding within closely related species within Anseriformes, clarify the effect of age at infection in previous experimental studies, and can be beneficial for future experimental trials by indicating the most appropriate age to perform experimental studies.

The second specific objective of this study was to determine if protective immunity related to infection with either a homologous or a heterologous subtype LPAI virus exists in wild birds subsequently exposed to a LPAI or a H5N1 HPAI virus. This objective provides detailed information about the outcome of consecutive infections with AI viruses in wild birds. It also presents insight into possible protective immunity related to hemagglutinin and/or neuraminidase glycoproteins, and helps clarify H5N1 HPAI epidemiology and risk of infection in different wild avian species. It also has application to understanding evolution of different AI viruses in the wild bird reservoir system.

By evaluating host factors that affect the infectivity of AI, this study helps us define and understand the epidemiology of avian influenza viruses, and provides much more detailed

information about the role of wild birds as AI reservoirs. Furthermore, this research contributes to the comprehension of the natural history of wild type avian influenza, helping prevent and control eventual enzootic infections, epidemics or pandemics. This investigation provides baseline data that can benefit future experimental trials with wild birds, and helps us interpret data acquired in surveillance and field studies.

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CHAPTER 2

LITERATURE REVIEW

Avian Influenza Virus

Avian influenza (AI) is caused by an influenza virus type A, which is enveloped, has a segmented, negative sense, single strand RNA genome, and belongs to the *Orthomyxoviridae* family, which consists of five genera: *Influenza virus type A*, *Influenza virus type B*, *Influenza virus type C*, *Thogotovirus* and *Isavirus* (Alexander, 2007a; Bouvier and Palese, 2008). A standardized international nomenclature for influenza viruses was established in 1980 (WHO, 1980) and consists of: antigenic type (A, B, or C), animal species from which it was isolated (this maybe omitted for human isolates); geographic origin of the isolate; reference identification number for each isolate; and year of isolation. Influenza virus type A, including AI viruses, are further classified into subtypes based on two surface glycoproteins used for host-cell entry by the virus during replication, the hemagglutinin (HA) and the neuraminidase (NA), which is often included in parenthesis (Bouvier and Palese, 2008; Suarez, 2008). For example, the A/mallard/MN/355779/00 (H5N2) was isolate number 355779, recovered from a Mallard (*Anas platyrhynchos*) in Minnesota in 2000, and it has an HA subtype 5 and an NA subtype 2. Currently, 16 HA and 9 NA distinct subtypes have been identified (Suarez, 2008).

For the purposes of international trade, the World Organization for Animal Health classifies AI viruses as follows (OIE, 2009): highly pathogenic notifiable avian influenza (HPNAI) viruses have an intravenous pathogenicity index (IVPI) greater than 1.2 or, as an alternative, cause at least 75% mortality in 4- to 8-week-old chickens infected intravenously -

IVPI is the mean score per bird per daily observation over ten days of 10 six-week-old chickens, inoculated intravenously with the virus under test; the birds are scored as follows: 0 = normal, 1 = sick, 2 = very sick or paralysed, 3 = dead (Alexander and Brown, 2009). H5 and H7 viruses which do not have an IVPI of greater than 1.2 or cause less than 75% mortality in an intravenous lethality test should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the hemagglutinin molecule (HA0); if the amino acid motif is similar to that observed for other HPNAI isolates, the isolate being tested should be considered as HPNAI; low pathogenicity notifiable avian influenza (LPNAI) viruses are all influenza A viruses of H5 and H7 subtype that are not HPNAI viruses. In general terms, high pathogenic avian influenza (HPAI) viruses are synonymous with HPNAI viruses by definition, while low pathogenic avian influenza (LPAI) viruses include all the AI viruses that are not HPNAI viruses using the above classification (Swayne, 2008). HPAI viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes are of the highly pathogenic pathotype.

The influenza types A and B virus genomes each comprise eight negative-sense, single-stranded viral RNA segments (while influenza C virus has a seven-segment genome) (Bouvier and Palese, 2008), each of which encodes at least one viral protein. The two smallest genomic RNA segments code for two proteins each (7^{th} segment: matrix [M] – M1 and M2; 8^{th} segment: nonstructural protein [NS] – NS1 and NS2). The 10 viral proteins of influenza viruses can be divided into three main categories: the surface proteins, the internal proteins, and nonstructural proteins that are not packaged in the virus particle.

- *Surface proteins*: The viral particle contains three surface proteins: the HA, NA, and matrix 2 (M2) proteins. The HA protein (divided into two domains — HA1 and HA2) has the two main functions of being the virus receptor binding site and containing the fusion domain

necessary for the viral RNA to be released into the host cell. The HA protein is a glycosylated integral membrane protein that forms a homotrimer on the surface of the virus (Suarez and Schultz-Cherry, 2000). The NA protein is an enzymatically active protein that is thought to be important in cleaving sialic acid allowing the virus to be released from the infected host cells. It also prevents aggregation of newly formed viral particles by removing sialic acid residues from the virus envelope itself, and allows penetration in mucus and respiratory tract by breaking down the mucins in respiratory tract secretions. The M2 protein is an integral membrane protein that functions as an ion channel for the virus particle. It plays a key role on endosome acidification and triggers fusion activity of the HA protein (Suarez and Schultz-Cherry, 2000; Pinto and Lamb, 2007).

- *Internal proteins*: The internal proteins include the three polymerases proteins, acidic protein (PA), basic polymerase 1 (PB1), and basic polymerase 2 (BP2), the nucleoprotein, and the matrix protein 1 (M1). PB2 controls the recognition of host-cell RNA, while PB1 catalyses nucleotide addition. PA might possess a transcriptase protease activity. The M1 encodes the main component of the viral capsid, and is believed to be critical in bridging the surface integral membrane proteins and the ribonucleoproteins complex and each of the eight viral segments before the virion is complete (Suarez and Schultz-Cherry, 2000; Suarez, 2008).

- *Nonstructural proteins*: The NS1 is the only protein that is not packaged into the virus particle, although it is produced in large quantities in infected cells. NS1 is associated with RNA transport, translation and splicing. NS2 is a minor component of the virion, the function of which is still unknown (Suarez and Schultz-Cherry, 2000).

Virus Life Cycle. The initial step in viral infection is the attachment of the viral HA glycoprotein to a sialic acid receptor at the host cell. Sialic acid is a general term for the terminal sugars found

in N- ad O-linked glycoproteins that can be made of many derivatives of neuraminic acid (Suarez, 2008). Molecules of sialic acid are classified based on their link to the underlying sugars by the α-2 carbon; the most common linkages are the α-2,3 and α-2,6 linkage (Suzuki, 2005). These different linkages result in different conformations of the sialic acid receptor, and affects virus binding. The HA protein has strong specificity for either the α-2,3 or α-2,6 linkage, and most viruses can only infect animals that express their preference of sialic acid (Suarez, 2008). Avian influenza virus strains bind preferentially to sialic acid attached to galactose with an α-2,3 linkage (Gambaryan et al., 1997; Matrosovich et al., 1997), while human influenza virus strains preferentially bind to sialic acid attached to galactose with an α-2,6 linkage (Rogers and D'Souza, 1989; Connor et al., 1994; Matrosovich et al., 2000).

Once attached, the virus enters the cell by the clathrin-dependent receptor-mediated endocytic pathway (Matrosovich et al., 2009). The viral membrane fuses with the membrane of the vesicle, releasing the viral nucleocapsids into the cytoplasm (Steinhauer, 1999). Upon acidification of the late endosome (approximately pH 5.0), the HA precursor (HA0) undergoes an acid-catalyze conformational rearrangement and is cleaved into HA1 and HA2, exposing a fusion peptide that mediates the merging of the viral envelope with the endosomal membrane. Hydrogen ions from the endosome are pumped into the virus particle via the M2 ion channel and disrupt internal protein–protein interactions, allowing viral ribonucleoproteins (vRNP) to be released from the viral matrix into the cellular cytoplasm (Steinhauer, 1999).

The fusion of the viral membrane and the endosomal membrane allows the viral RNA-polymerase complex to be released into the cytoplasm. These are then transported into the host cell nucleus by means of viral proteins' nuclear localization signals (O'Neill et al., 1995). Viral transcription, replication and progeny vRNP assembly occur in the nucleus (Krug, 1981). Using

the negative-sense genomic RNA (vRNA) as a template, the viral RNA-dependent RNA polymerase transcribes full-length positive-sense complimentary RNAs. These provide the template for the progeny negative-sense RNA genome. mRNA transcripts are also produced using the vRNA as a template (Whittaker et al., 1996). The negative-sense viral RNA is copied by virion RNA polymerase into positive-sense mRNA, using the capped 5'ends of host pre-mRNAs (or mRNAs) as primers to initiate synthesis, a process known as 'cap-snatching' (Krug, 1981). Some of the newly synthesized negative-sense RNAs enter the pathway for mRNAs synthesis. The M1 and NS1 proteins are transported into the nucleus. Binding of the M1 protein to the newly synthesized negative-sense RNAs shuts down viral mRNA synthesis and, in conjunction with the NEP protein, induces export of progeny nucleocapsids to the cytoplasm (Suarez and Schultz-Cherry, 2000). The viral assembly process includes the HA, NA, and small amounts of M2 proteins entering the endoplasmic reticulum, where they are folded and glycosylated before moving to the apical plasma membrane (Barman et al., 2001). The virion nucleocapsids, associated with the M1 and the NEP proteins, are transported to the cell surface and aligned with regions of the plasma membrane that contain the HA and NA proteins. Assembly of the virions is completed at this location by budding from the cellular membrane. The efficient budding requires the enzymatic activity of the NA protein to remove sialic acid from the surface glycoproteins, specifically the HA protein. This prevents self-binding of the protein and the aggregation of the virus at the cell surface (Seto and Rott, 1966; Flint et al., 2004; Matrosovich et al., 2004; Bouvier and Palese, 2008).

Historic Background of Avian Influenza

Edoardo Perroncito, in northern Italy, first described AI in 1878 as a disease capable of causing extremely high mortality in infected fowls (Perroncito, 1878). The disease, termed 'fowl plague', was initially confused with the acute septicemic form of fowl cholera. However, in 1880 Rivolta and Delprato showed that fowl plague was different from fowl cholera, based on clinical and pathological properties (Lupiani and Reddy, 2009). In 1901, it was determined that the causative agent of fowl plague was a virus (Centanni and Savonuzzi, 1901), but it was not until 1955 that the classic fowl plague virus was shown to be a type A influenza virus, based on the presence of type A influenza virus type-specific nucleoprotein. In 1981, the term fowl plague was substituted by the more appropriate term HPAI at the First International Symposium on Avian Influenza, Maryland, USA. It is believed that HPAI outbreaks occurred in Italy and other European countries prior to the first description by Perroncito. In the mid 1900s, less virulent forms of AI viruses (later classified as LPAI viruses) were isolated for the first time. The virus was known as "N" virus, and was first isolated from a dead chicken in Germany (A/chicken/Germany/49 (H10N7)) (Dinter and Bakos, 1950). From the 1960s onwards, several LPAI viruses of different subtypes were isolated from chickens, turkeys, quails, pheasants, partridges, and ducks with mild respiratory and reproductive disease, providing insight of the variability among AI viruses (Lupiani and Reddy, 2009).

The first confirmed HPAI outbreak was reported in Scotland in 1959 (Becker and Uys, 1967). Since then, more than 28 outbreaks have been recorded worldwide (Lupiani and Reddy, 2009). The majority of HPAI outbreaks have shown limited geographical spread, and even self-limited to a single flock of birds. However, during the HPAI outbreaks of 1983 in the USA (Eckroade and Bachin, 2003), 1994 in Mexico (Villareal and Flores, 2003), 1994 in Pakistan

(Naeem, 2003), 1997 in Hong Kong, 1999 in Italy (Mutinelli et al., 2003; Zanella, 2003), 2002 in Chile (Rojas et al., 2002), 2003 in The Netherlands (Stegeman et al., 2004), 2004 in Canada (Hirst et al., 2004), and 2003-present in Asia, Europe and Africa, the potential impact of HPAI was demonstrated when the disease became widespread, causing enormous economic losses.

The first isolation of avian influenza from a free-living bird was the H5N3 HPAI virus subtype obtained from a Common Tern (*Sterna hirundo*) in South Africa in 1961, causing a large mortality event (Becker, 1966). It was also the first isolation of HPAI virus that was not associated with an outbreak in domestic poultry. It was not until mid-1970s, however, that a systematic investigation of influenza in wild birds was undertaken (Alexander, 2000; Alexander and Brown, 2009). These investigations revealed the enormous pools of influenza viruses now known to be present in the wild bird population. Serologic surveys following the outbreak in common terns detected AI antibodies in several species of free-living birds, confirming that they are frequently exposed to these viruses and demonstrating that this potential reservoir involved a wide diversity of wild avian species (Easterday et al., 1968; Asplin, 1970; Laver and Webster, 1972; Winkler et al., 1972). AI viruses have been reported from wild birds representing more than 105 avian species in 26 different families and 12 orders, primarily birds in the orders Anseriformes and Charadriiformes, and all the known HA (H1-H16) and NA (N1-N9) subtypes have been isolated from wild birds (Stallknecht and Shane, 1988; Olsen et al., 2006; Stallknecht and Brown, 2008).

Emergence of H5N1 HPAI Virus in Asia

In 1996, a H5N1 HPAI virus was recovered from a sick goose in Guandong province in southern China (Xu et al., 1999; Chen et al., 2004; Sims et al., 2005). In 1997, H5N1 HPAI virus

outbreaks occurred in Hong Kong on three chicken farms (Shortridge et al., 1998), causing serious disease in poultry and humans (Claas et al., 1998; Sims et al., 2003). Six out of the 18 human cases detected by the end of that year had been fatal (Chan, 2002). This was the first indication of the most devastating HPAI panzootic yet to occur, with an estimate of hundreds of millions of poultry involved (Alexander, 2007a; Sims and Brown, 2008; Alexander and Brown, 2009). The causal H5N1 HPAI viruses were reassortants with an HA gene derived from its precursor H5N1 isolated in 1996 from a goose in Guangdong, and the other seven genes derived from other non-H5 AI viruses (Webster et al., 2002). Multiple genotypes of H5N1 HPAI viruses continued to circulate in the subsequent years in both aquatic and terrestrial birds in Asia (Sims and Brown, 2008).

In 2002, H5N1 HPAI virus infection caused significant mortality of captive and wild aquatic birds in two waterfowl parks in Hong Kong (Ellis et al., 2004; Sturm-Ramirez et al., 2004). Mortality associated with these outbreaks was also reported in free-living Grey Heron (*Ardea cinerea*) and Black-headed Gulls (*Chroicocephalus ridibundus*). Since 2002, the Asian lineage of H5N1 HPAI viruses have caused morbidity and mortality in a variety of wild aquatic and terrestrial avian species (Ellis et al., 2004; Li et al., 2004; Swayne and Pantin-Jackwood, 2008; Desvaux et al., 2009).

In 2005, H5N1 HPAI virus was responsible for a large mortality event in wild birds at Qinghai Lake (Qinghai province, western China), an important breeding location for migratory birds that overwinter in Southeast Asia, Tibet, and India. Several species were affected, including Bar-headed Goose (*Anser indicus*), Great Black-headed Gull (*Ichthyaeus ichthyaeus*), and Brown-headed Gull (*Chroicocephalus brunnicephalus*) (Chen et al., 2005; Liu et al., 2005).

Altogether, over 6,300 birds from different species died in the subsequent weeks following this outbreak (WHO, 2010) .

In 2006, H5N1 HPAI viruses extended their range through Africa, Western Europe, west Asia, and the Middle East, affecting wild birds, poultry, humans and other mammals (Alexander, 2007b; Sims and Brown, 2008; OIE, 2010), and it has been responsible for mortalities in over 75 wild bird species in 38 countries (FAO, 2007). Migratory birds as well as trade involving live poultry and poultry products have been suggested as the most likely causes of dispersal of the virus (Liu et al., 2005; Gilbert et al., 2006; Kilpatrick et al., 2006; Feare, 2007; Munster and Fouchier, 2009; Feare, 2010).

Reservoir Hosts

A reservoir is defined as "any animate or inanimate object or any combination of these serving as a habitat of a pathogen that reproduces itself in such a way as to be transmitted to a susceptible host" (Toma et al., 1999). Most of the wild bird species from which AI viruses have been isolated belong to two broad taxonomic groups, the Anseriformes (duck, geese, and swans) and Charadriiformes (gulls, shorebirds, and terns) which are known to be the natural reservoirs for AI viruses (Stallknecht and Brown, 2008). Many of the species in these two orders have worldwide distributions, are associated with long-range migration patterns, and utilize a diversity of aquatic habitats ranging from inland fresh water to pelagic (Stallknecht, 2003). Nevertheless, not every avian species in these two orders contributes equally to maintaining AI viruses. For example, within Anseriformes, the majority of AI viruses have been isolated from ducks (Subfamily *Anatinae*), particularly from dabbling ducks of the Genus *Anas*, probably because their feeding behavior favors ingestion of viral particles (Olsen et al., 2006; Munster et al., 2007;

Jourdain et al., 2010). Furthermore, more isolations have been reported from Mallards than any other duck species making them an important component of the wild duck AI virus reservoir worldwide (Hinshaw et al., 1980b; Stallknecht et al., 1990b; Krauss et al., 2004; Munster et al., 2005; Olsen et al., 2006; Stallknecht and Brown, 2007; Hesterberg et al., 2009). Within Charadriiformes, the majority of AI virus have been isolated from species in the families Scolopacidae and Laridae, and a marked variation in AI prevalence between species has been observed (Stallknecht and Brown, 2008). For instance, while a very low prevalence of infection in Scolopacidae has been documented globally (Olsen et al., 2006), most AI virus isolations have been associated with one particular species, the Ruddy Turnstone (*Arenaria interpres*), at one geographic location (Delaware Bay, USA) in May/June (Hanson et al., 2008).

Subtype Diversity and Prevalence in Wild Avian Populations

Avian influenza virus subtypes do not circulate equally among wild bird populations and variation can occur between host species, geographic location, and years (Stallknecht and Brown, 2008). Among North American ducks, H3, H4 and H6 are the most common subtypes isolated (Stallknecht et al., 1990b; Sharp et al., 1993; Hanson et al., 2003; Krauss et al., 2004), while the H5, H7, H8 and H9 are generally isolated at a lower rate (Stallknecht and Shane, 1988; Krauss et al., 2004). In Europe, however, the predominant HA subtypes represented among wild duck populations are: H1, H2, and H4 in Germany (Süss et al., 1994); and H4, H6, and H7 in Sweden (Wallensten et al., 2007). Subtype diversity is not well understood for shorebirds, but a clear difference exists between AI viruses observed from Charadriiformes compared to ducks. This is particular evident for the H13 and H16 subtypes, which appear to be associated with infection in gulls (Hinshaw et al., 1982; Hinshaw et al., 1983; Fouchier et al., 2005; Munster et

al., 2007). Concurrent or successive infections with different low pathogenic avian influenza (LPAI) subtypes have been reported in wild bird populations and in poultry (Hinshaw et al., 1980a; Sharp et al., 1997; Jonassen and Handeland, 2007; Dugan et al., 2008).

In North America, the prevalence of AI viruses in duck populations peaks in late summer/early fall (August-September) and is associated with the high concentration of susceptible hatching-year birds during premigration staging (Hinshaw et al., 1985), when AI virus infection rates can exceed 30% in this age group. The prevalence rapidly decreases during migration, and on wintering areas AI virus prevalence is lower than 1 or 2% (Stallknecht and Shane, 1988; Stallknecht et al., 1990b). In North Europe, however, studies have reported a prevalence peak of AI virus in duck in the fall (September-November) (Globig et al., 2006; Munster et al., 2007; Wallensten et al., 2007; Hesterberg et al., 2009). Species-related differences in biology, distribution, habitat utilization, migration behavior, and susceptibility to infection potentially influence a species' role in AI virus epidemiology and may explain trends in prevalence of AI virus in duck populations (Stallknecht and Brown, 2007).

Spatial and temporal patterns of AI prevalence in Charadriiformes are less understood. Within Scolopacidae, a strong spatiotemporal relationship has been observed in Ruddy Turnstones during spring migration stopovers at Delaware Bay (Kawaoka et al., 1988; Krauss et al., 2004; Hanson et al., 2008), with a peak of AI prevalence occurring in the spring, and a lesser peak in the fall (Kawaoka et al., 1988). However, this seasonality is largely based on observation from Ruddy Turnstones at Delaware Bay – the only site, worldwide, where consistent AI virus isolations from shorebirds have been reported (Hanson et al., 2008; Stallknecht and Brown, 2008). Within Laridae, a temporal and spatial relationship is less clear, despite often detection of AI viruses in gulls. A high prevalence of H13N6 LPAI viruses has recently been reported in

nestling Ring-billed Gulls (*Larus delawarensis*) in Canada (Velarde et al., 2010). In Europe, a high prevalence of AI infection in gulls was observed in the late summer and early fall; presumably corresponding to the arrival of juvenile gulls (Fouchier et al., 2005). A summer peak has also been reported in Ringed-billed Gulls in Baltimore, Maryland, USA (Graves, 1992). Avian influenza of H13 and H16 subtypes are almost exclusively maintained in gull and tern populations, and rarely detected in other wild avian taxa (Fouchier et al., 2005; Fouchier et al., 2007; Velarde et al., 2010).

Maintenance and Transmission Cycle

Avian influenza viruses are transmitted in wild bird populations through an indirect fecal-oral route, involving fecal-contaminated water on shared aquatic habitats (Webster et al., 1978; Hinshaw et al., 1979; Sinnecker et al., 1983; Hinshaw, 1986; Sandhu and Hinshaw, 2003). In ducks, avian influenza virus replicates primarily in the epithelial cells of the intestinal tract and is excreted in high concentrations in feces, contaminating the environment, which can serve as a common source of infection for susceptible animals (Webster et al., 1978; Kida et al., 1980; Roche et al., 2009). Experimentally infected Muscovy Ducks (*Cairina moschata*) can shed approximately 10^{10} mean embryo infectious dose (EID_{50}) of AI virus in fecal material in a 24-hour period (Webster et al., 1978). Cloacal viral shedding in Mallards has been detected as early as one hour after oral inoculation (Webster et al., 1978), and can persist for over 28 days in Pekin Ducks (Hinshaw et al., 1980b). Avian influenza viruses have been recovered from surface lake and pond water, lake sediments, and frozen water (Hinshaw et al., 1980b; Markwell and Shortridge, 1982; Halvorson et al., 1983; Ito et al., 1995; Zhang et al., 2006; Lang et al., 2008; Stallknecht et al., 2010). These observations suggest that AI viruses can persist in lake water,

sediments, and ice, and be a source of infection for susceptible birds in subsequent years (Webster et al., 1992; Lang et al., 2008). Experimental studies demonstrated that AI viruses could remain infective in water for long periods of time, supporting an environmental component to AI transmission (Stallknecht et al., 1990a; Brown et al., 2007b; Brown et al., 2008a; Domanska-Blicharz et al., 2010; Nazir et al., 2010).

Pathobiology of Avian Influenza Viruses

As previously mentioned, the HA cleavage into HA1 and HA2 is necessary for successful virus entry into the host cell (Steinhauer, 1999). For LPAI viruses, the HA cleavage is mediated by trypsin-like enzymes located within specific epithelial cells, present in respiratory secretions, or produced by some types of bacteria. On the contrary, HA of HPAI viruses can be cleaved either by trypsin-like enzymes as for LPAI viruses or by ubiquitous proteases of the furin enzyme family that are contained in most cell types (Swayne and Pantin-Jackwood, 2008). This difference in HA cleavage between LPAI and HPAI viruses is responsible for variations in replication sites and lesion produced by these viruses. Replication patterns of AI virus in birds may also vary according to the host species, and the ability of the host to respond to the infection (Kida et al., 1980; Suarez and Schultz-Cherry, 2000). Differences are also apparent when comparing the immune responses of different species to avian influenza virus infections (Kida et al., 1980; Suarez and Schultz-Cherry, 2000).

LPAI and H5N1 HPAI Viruses in Poultry. In poultry infected with LPAI viruses, high morbidity (>50%) and low mortality rates (<5%) are typical, but mortality rates can be higher in young birds, or in the presence of secondary infections (Bano et al., 2003; Swayne and Pantin-Jackwood, 2008). In gallinaceous poultry, clinical signs caused by LPAI infection reflect

changes in respiratory (coughing, sneezing, rales, rattles, and excessive lacrimation), reproductive (increased broodiness and decreased egg production), digestive, and urinary systems (Swayne and Halvorson, 2008). Rarely LPAI have caused systemic infection in chicken infected experimentally, causing damage in kidney tubules, pancreatic acinar epithelium, and other organs which contain epithelial cells with trypsin-like enzymes (Bano et al., 2003). In domestic ducks and geese, LPAI infection has varied in clinical outcome from asymptomatic infections to respiratory disease, the latter usually associated with secondary bacterial infection (Swayne and Pantin-Jackwood, 2008).

Gross lesions caused by LPAI viruses are variable depending on the virus strain, phase of infection, host species, and presence of secondary pathogens (Swayne and Halvorson, 2008). Frequent lesions include: fibrinous to fibrinopurulent rhinitis, sinusitis, bronchopneumonia, air sacculitis, and coelomitis. In turkeys, catarrhal to fibrinous enteritis, typhlitis and proctitis may be observed, as well as mottled pale discoloration and hemorrhage of pancreas (Swayne and Pantin-Jackwood, 2008). Histological lesions associated with LPAI virus infection in poultry include: heterophilic to lymphocytic tracheitis and bronchitis, lymphocytic pneumonia, pancreatic acinar necrosis and pancreatitis, and lymphocyte depletion and necrosis in cloacal bursa, thymus, and spleen. Nephrosis and nephritis have been rarely observed (Mo et al., 1997; Swayne et al., 1997; Swayne and Pantin-Jackwood, 2006; Pantin-Jackwood and Swayne, 2009).

HPAI viruses cause severe systemic disease with very high mortality in gallinaceous poultry, but typically do not cause morbidity or mortality in domestic ducks and geese (Swayne and Pantin-Jackwood, 2008). However, since 1996, the panzootic H5N1 HPAI viruses have been responsible for clinical disease and death in both gallinaceous and non-gallinaceous poultry, as well as a variety of captive and wild bird species (Perkins and Swayne, 2003a; Elbers et al.,

2004; Pantin-Jackwood and Swayne, 2007). Birds can be found dead prior to the appearance of clinical signs, or, in peracute cases, nervous disorders such as tremors of head and neck, incoordination, inability to stand, torticollis, paresis and paralysis can be observed (Swayne and Pantin-Jackwood, 2008). However, the appearance of clinical signs will vary with the virus strain, host species, age at infection, sex, presence of co-infections, acquired immunity and environmental factors (Pantin-Jackwood et al., 2007; OIE, 2008; Swayne and Pantin-Jackwood, 2008).

In gallinaceous poultry, gross lesions caused by HPAI viruses are the result of either direct virus replication, indirect effects by cellular mediators, or ischemia from vascular thrombosis, and are characterized by edema, hemorrhage, and necrosis in multiple visceral organs, cardiovascular, integumentary and nervous systems, and vary according to the phase of infection. Turkeys and chickens HPAI viruses are shed in high titers in the respiratory secretions and, in a lesser extent, feces (Swayne and Pantin-Jackwood, 2006, 2008). In general, no gross lesions are observed in the peracute phase (1 to 2 days after inoculation). In the acute phase (2 to 5 days after inoculation), subcutaneous edema of the head, face, upper neck, leg shanks, and feet may be observed, associated or not with subcutaneous petechial or ecchymotic hemorrhages. Cyanosis, resulting from ischemic necrosis, may be observed in combs, wattles and snood, associated with hemorrhage and necrosis. Internal lesions are characterized by hemorrhage on serosal and mucosal surfaces and parenchymal necrosis of multiple visceral organs, and include: hemorrhage in epicardium and coronary fat, pectoral muscles, serosa and mucosa of proventriculus and ventriculus, and Peyer's patches of small intestine; edema and hemorrhage in the lungs; red to light orange to brown mottling discoloration of pancreas (Swayne and Pantin-Jackwood, 2008).

Classic histological lesions consist of necrosis and/or inflammation in multiple organs, including skin, brain, heart, pancreas, adrenal glands, lungs, and lymphoid organs (Hooper and Selleck, 2003). In the first two to five days of infection, the predominant lesion is necrosis and, to a lesser extent, apoptotic cell death associated with inflammation, hemorrhage and edema. The longer the bird survives, the more prominent is the inflammation and the less prominent are the necrosis and apoptosis (Swayne and Pantin-Jackwood, 2008). Two forms of neural pathogenesis have been described for HPAI viruses: an early form within 3 days after inoculation, characterized by vascular endothelial disease, microgliosis and necrosis of brain parenchyma; and a later form, observed from 4 to 7 days after inoculation, and characterized by ventriculitis and periventricular necrosis and inflammation (Hooper and Selleck, 2003). Histological lesions commonly observed include: lymphocytic meningoencephalitis with neuronal necrosis, neuronophagia, and gliosis; focal degeneration to multifocal coagulative necrosis of cardiac myocytes, associated with lymphohistiocytic inflammation; necrosis of skeletal myofibers, renal tubules, vascular endothelial cells, corticotrophic cells of adrenal glands, and pancreatic acinar epithelium; and microthrombosis of unfeathered skin with vasculitis, perivascular to generalized edema, and epidermal vesicle formation progressing to full thickness necrosis (Perkins and Swayne, 2001; Swayne and Pantin-Jackwood, 2006, 2008).

LPAI Viruses in Wild Birds. Natural LPAI virus infection in wild aquatic birds is not well studied as it is in poultry. LPAI viruses typically produce asymptomatic infections in wild birds, and are passed between and within species of birds that occupy the same ecosystem (Swayne and Halvorson, 2008). These viruses preferentially infect intestinal epithelial cells of wild birds and are excreted in feces (Webster et al., 1978; Kida et al., 1980; Wallensten et al., 2006; Fouchier and Munster, 2009; Pantin-Jackwood and Swayne, 2009). The specific site of LPAI replication is

thought to be the crypts of Lieberkühn in the large intestine (Kida et al., 1980). The respiratory tract is also a potential target organ for LPAI viruses in ducks, as observed in an experimental trial of Mallards where there was immunostaining of respiratory epithelial cells for influenza virus and mild pneumonia, with lymphocyte and macrophage infiltration (Cooley et al., 1989). A transient decrease in egg production has also been observed in Mallards experimentally infected with LPAI viruses (Laudert et al., 1993). Recent field data suggest that LPAI infection in wild waterfowl causes subclinical changes, negatively affecting gain weight in Mallards (Latorre-Margalef et al., 2009) and migration and forging behavior in Bewick's Swans (*Cygnus columbianus bewickii*) (van Gils et al., 2007).

Although multiple experimental trials have been conducted with LPAI viruses in wild birds, most of them used Mallards or Pekin Ducks (*Anas platyrhynchos domesticus*), a Mallard-type domestic duck, as experimental models (Bahl and Pomeroy, 1977; Kida et al., 1980; Cooley et al., 1989; Mundt et al., 2009; Jourdain et al., 2010). Species-related variation is known to occur and it should be kept in mind not to extrapolate the results of these trials to all anseriform and charadriiform species. Therefore, the current understanding of susceptibility and viral shedding of AI viruses in their natural host is still very narrow. One experimental study reported the existence of a partial immune protection against LPAI virus re-infection in Pekin Ducks (Kida et al., 1980) and Mallards (Jourdain et al., 2010).

H5N1 HPAI Viruses in Wild Birds. In contrast to LPAI viruses, HPAI viruses have been less frequently isolated from wild birds (United States Geological Survey, 2010). It is documented that HPAI viruses arise from LPAI viruses after circulation in gallinaceous poultry and are the result of mutations at the proteolytic cleavage site of the HA glycoprotein (Swayne and Suarez, 2000). The existence of a wild bird reservoir has not been demonstrated (Rohm et

al., 1995), and domestic ducks in Asia and Africa are believed to be the major reservoir and propagators of H5N1 HPAI virus within these regions (Hulse-Post et al., 2005; Sturm-Ramirez et al., 2005). Since 2002, H5N1 HPAI viruses have caused severe illness and fatal infections in many avian orders, including Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Gruiformes, Passeriformes, Pelecaniformes, Phoenicopteriformes, Podicipediformes, and Strigiformes (Ellis et al., 2004; Chen et al., 2005; Kwon et al., 2005a; Olsen et al., 2006; Ducatez et al., 2007; United States Geological Survey, 2010). The lesions caused by H5N1 HPAI infection in wild water birds resemble those observed in chickens (Ellis et al., 2004).

Due to the economic importance and zoonotic potential of H5N1 HPAI viruses, many experimental studies have been done with these highly pathogenic strains. Studies addressing the susceptibility of wild birds to H5N1 HPAI viruses have clearly shown that there is a significant variation in pathogenicity of the these viruses for different species of birds, including species within the same order (Perkins and Swayne, 2003b; Brown et al., 2006; Brown et al., 2008b). For example, Mallard, Redhead (*Aythya americana*), Bar-headed Goose, Cackling Goose (*Branta hutchiinsii*), Northern Pintail (*Anas acuta*), Blue-wing Teal (*A. discors*), Rock Pigeon (*Columba livia*), House Sparrow (*Passer domesticus*), and European Starling (*Sternus vulgaris*) have shown a relative resistance to H5N1 HPAI virus infection; while Wood Duck (*Aix sponsa*), Whooper Swan (*Cygnus cygnus*), Black Swan (*C. atratus*), Trumpeter Swan (*C. buccinator*), Laughing Gull (*Leucophaeus atricilla*), Zebra Finch (*Taeniopygia guttata*), House Finch (*Carpodacus mexicanus*), and Budgerigar (*Melopsittacus undulatus*) showed moderate to severe disease and/or died after infection (Perkins and Swayne, 2003b; Brown et al., 2006; Brown et al., 2007a; Brown et al., 2008b; Brown et al., 2009). In one experimental study with Laughing Gull,

however, neither morbidity nor mortality was observed after inoculation with H5N1 or H5N3 HPAI viruses (Perkins and Swayne, 2002).

H5N1 HPAI virus infection seems also to vary between age groups, as observed in a natural outbreak of HPAI H5N1 influenza virus in commercial ducks in South Korea. Morbidity and up to 12% mortality were observed in 14-day-old meat ducks, while decreased egg production and feed consumption, but no mortality, were observed in adult ducks (Kwon et al., 2005b). Another study showed that 5-week-old Pekin Ducks experimentally inoculated with different strains of HPAI H5N1 virus had moderate mortality and less severe neurologic signs that 2-week-old Pekin Ducks, although the proposed mechanism for this age-related protection is still unclear (Pantin-Jackwood et al., 2007). However, in gallinaceous domestic poultry it was observed that infection with HPAI viruses produces high morbidity and mortality regardless of age at infection (Swayne and Pantin-Jackwood, 2006; Swayne and Halvorson, 2008).

Few studies have investigated the effect of a previous exposure to a LPAI virus on the outcome of a HPAI challenge in wild birds. A recent study with juvenile Canada Geese demonstrated that a re-exposure to LPAI viruses has a partial protective effect against a lethal H5N1 HPAI virus challenge (Pasick et al., 2007; Berhane et al., 2010). Similar experimental trials have been done in Mute Swans (*Cygnus olor*) (Kalthoff et al., 2008), Mallards (Fereidouni et al., 2009) and chickens (Seo and Webster, 2001; Seo et al., 2002; van der Goot et al., 2003). This protective immunity has been proposed as an explanation for reduced morbidity observed in a naturally occurring H5N1 HPAI outbreak in poultry on Hong Kong (Webster et al., 2006), and in wild birds in Germany, despite the presence of several hundred thousand birds in the affected area (Globig et al., 2009). The mechanisms of this protective effect, however, are still poorly defined.

Conclusions

Many host, viral, and environmental factors can influence the outcome of AI virus infection in birds, and although ducks are a very well studied group with regards to the epidemiology of AI virus, there are many species, genera, and populations worldwide for which little information is available. Furthermore, while significant work has been done in the past several years to improve our understanding of H5N1 HPAI infection in domestic poultry and wild birds, much of the ecology of LPAI viruses in their natural reservoir remains unknown. Consequently, additional studies are needed to characterize AI infection and clarify the epidemiology of AI viruses, particularly LPAI viruses, in the wild avian reservoir system.

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CHAPTER 3

THE EFFECT OF AGE ON AVIAN INFLUENZA VIRAL SHEDDING IN MALLARDS (*Anas platyrhynchos*)[1]

[1] Costa, T.P., J. D. Brown, E.W. Howerth, D.E. Stallknecht. 2010. *Avian Diseases* 54: 581-585. Reprinted here with permission of publisher.

Abstract

Avian influenza virus (AIV) prevalence in wild aquatic bird populations varies with season, geographic location, host species, and age. It is not clear how age at infection affects the extent of viral shedding. To better understand the influence of age at infection on viral shedding of wild bird-origin low pathogenicity avian influenza (LPAI) viruses, Mallards (*Anas platyrhynchos)* of increasing age (2-week, 1-month, 2-month, 3-month, and 4-month-old) were experimentally inoculated via choanal cleft with a 10^6 median embryo infectious dose (EID_{50}) of either A/Mallard/MN/355779/00 (H5N2) or A/Mallard/MN/199106/99 (H3N8). Birds in all five age groups were infected with both AIV isolates and excreted virus via the oropharynx and cloaca. The 1-month and older groups consistently shed virus from 1 to 4 days post inoculation (dpi), whereas, viral shedding was delayed by one day in the 2-week-old group. Past 4 dpi, viral shedding in all groups varied between individual birds, but virus was isolated from some birds in each group up to 21 dpi when the trial was terminated. The 1-month-old group had the most productive shedding with a higher number of cloacal swabs that tested positive for virus over the study period and lower cycle threshold (Ct) values on rRT-PCR. The viral shedding pattern observed in this study suggests that, although Mallards from different age groups become infected and shed LPAI viruses, age at time of infection might have an effect on extent of viral shedding and thereby impact transmission of LPAI viruses within the wild bird reservoir system. This information may help us better understand the natural history of these viruses, interpret field and experimental data, and plan future experimental trials.

Key words: Anas platyrhynchos, avian influenza virus, mallard, age, viral shedding.

Wild aquatic birds, especially those from the orders Anseriformes and Charadriiformes, are believed to be the natural reservoir for avian influenza virus (AIV) (Stallknecht, 2003; Swayne and Halvorson, 2008). Within this reservoir system, AIV prevalence varies with season, geographic location, and host species (Stallknecht and Brown, 2007). Movement and age of birds appear to be important in the natural history of AIV and are correlated with seasonal effects (Hinshaw et al., 1985). In wild duck populations, peak AIV prevalence occurs in the late summer and early fall, when high concentrations of susceptible juvenile ducks (approximately 2- to 3-month-old) congregate at pre-migrational staging areas (Deibel et al., 1985; Halvorson et al., 1985; Hinshaw et al., 1985). Age can affect the outcome of infection with avian reovirus in chickens (Montgomery et al., 1986; Roessler and Rosenberger, 1989; Pantin-Jackwood et al., 2007), Hepatitis B virus (Jilbert et al., 1998), and highly pathogenic avian influenza (HPAI) viruses (Pantin-Jackwood et al., 2007) in Pekin ducks (*Anas platyrhynchos domesticus*). These effects have been attributed to both increasing maturation of the immune system with age and a link between host cell maturation and the capacity to support viral replication (Jilbert et al., 1998). Adult Japanese Quail (*Coturnix coturnix japonica*) experimentally inoculated with H9N2 AIV developed a more protective immunity than juvenile and aged individuals as measured by clinical and serologic responses (Lavoie et al., 2007). At present there is little information on the potential effects of the age at infection on extent of viral shedding associated with a low pathogenic avian influenza (LPAI) virus infection. Such information is needed to fully evaluate experimental and field data often derived from birds of varying ages. The objective of this study was to investigate the viral shedding pattern of different age classes of Mallards (*Anas platyrhynchos*) inoculated with two LPAI viruses.

Materials and Methods

Viruses. LPAI viruses A/Mallard/MN/355779/00 (H5N2) and A/Mallard/MN/199106/99 (H3N8) were used in this study. These viruses were originally isolated in specific pathogen free (SPF) embryonated chicken eggs (ECE) from cloacal swab material. Virus stocks were propagated by second passage in 9- to 11-day-old SPF ECE. A sham inoculum was prepared using uninfected brain heart infusion solution. Serial titrations were performed in SPF ECE and median embryo infectious dose (EID_{50}) titers were determined by testing hemagglutination activity as previously described (Swayne et al., 1998).

Animals. One-day-old Mallards were purchased from a commercial source (Murray McMurray Hatchery, Webster City, Iowa, USA) and raised under confined conditions until they were utilized in this study. Both males and females were included in approximately equal numbers. In order to acclimatize the birds, three days before inoculation the birds were housed in groups of five in self-contained isolation units ventilated under negative pressure with high-efficiency particulate air (HEPA)-filtered air. Food and water were provided *ad libitum*. All birds were negative for antibodies to type A influenza virus by agar gel precipitation (AGP) test and negative for AIV by virus isolation (VI) and real-time reverse-transcription polymerase chain reaction (rRT- PCR), on cloacal and oropharyngeal (OP) swabs, tested on 0 days post-inoculation (dpi). General animal care was provided under an animal use protocol approved by the Institutional Animal Care and Use Committee at The University of Georgia.

Experimental Design. Five age classes of Mallards were used: 2-week-old, and 1-, 2-, 3-, and 4-month-old. For each age class, 15 Mallards were evenly divided between two treatments and one negative control group, with five birds in each group. Treatment groups were inoculated via choanal cleft with a volume of 0.1 ml containing an infectious titer of 10^6 EID_{50} of one of the

two LPAI viruses. Back titers varied from $10^{5.27}$ to $10^{6.36}$ EID_{50}. Control groups were sham inoculated with 0.1 ml of brain heart infusion solution via choanal cleft. Animals were evaluated twice daily. Oropharyngeal and cloacal swabs were collected on 0, 1, 2, 4, 7, 9, 11, 14, 16, and 21 dpi and blood samples were collected on 0, 14, and 21 dpi. At 21 dpi, birds were humanely euthanized by CO_2 inhalation. Experimental infections were performed in a BSL-Ag2+ facility at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia.

Virus Isolation. Cloacal and OP swabs were stored at -70°C until analyses were performed. Previously described procedures were used to test the swabs by VI in embryonating chicken eggs (Swayne et al., 1998).

RNA Extraction. RNA was extracted from cloacal and oropharyngeal swab material by using the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambiron, Austin, TX) using the Thermo Electron KingFisher magnetic particle processor (Thermo Electron Corporation, Waltham, MA).

Real time RT-PCR. RNA was tested for AIV by quantitative rRT-PCR targeted to the influenza matrix gene as previously described (Spackman and Suarez, 2008). Samples with a cycle threshold (Ct) value equal or less than 40.00 were considered positive on rRT-PCR.

Serology. Blood samples were collected from the right jugular vein, and serum samples were stored at -20°C until they were tested using AGP as previously described (Swayne et al., 1998). The serum samples of the 21 dpi also were tested by the multiS-screen enzyme-linked immunosorbent assay (bELISA) AIV antibody test kit (IDEXX laboratories, Westbrook, ME) according to the manufacturer's instructions.

Statistical Analysis. The number of virus isolation per each bird for each age class was analyzed using the Kruskal-Wallis nonparametric one-way analysis of variance, followed by nonparametric Bonferroni multiple comparisons, using α=0.10 overall comparisons.

Results and Discussion

All the age classes were infected with both A/Mallard/MN/355779/00 (H5N2) (Table 3.1) and A/Mallard/MN/199106/99 (H3N8) (Table 3.2) as shown by viral shedding and seroconversion (Table 3.3) after inoculation, confirming the susceptibility of all the age classes to infection with these LPAI viruses. All treatment birds of the 2-, 3-, and 4-month-old groups inoculated with either AIV isolate were seropositive on both 14 and 21 dpi (Table 3.3). Although all treatment birds in the 2-week and 1-month-old groups were seropositive on 14 dpi, two birds in the 2-week-old group and one bird in the 1-month-old group that were inoculated with A/Mallard/MN/199106/99 (H3N8) tested negative by AGP; these three birds tested positive by bELISA on 21 dpi. The bELISA has been reported to be more sensitive than AGP for detecting AIV infection in wild birds (Brown et al., 2009).

With the exception of the 2-week-old group, birds in all treatment groups consistently excreted virus via oropharynx and cloaca from 1 to 4 dpi, as detected on VI and rRT-PCR (Tables 3.1 and 3.2). Beyond 4 dpi viral shedding varied between individual birds, even within the same treatment group, with birds shedding virus via oropharynx up to 21 dpi. Viral shedding in the 2-week-old group was delayed 1 day, with consistent shedding starting on 2 dpi and intermittent shedding by individual birds up to 16 dpi. This observed delay in young birds might be related to host cell maturation and the capacity to support viral replication, as previously observed for Hepatitis B virus (Jilbert et al., 1998) and avian reovirus (Montgomery et al., 1986).

To ensure accuracy, all the rRT-PCR tests were run with negative and positive controls, for both the RNA extraction and the rRT-PCR. The possible presence of PCR inhibitors in the swab samples might explain the apparent lack of sensitivity observed of the rRT-PCR compared to VI (Tables 3.1 and 3.2). However, further tests to investigate the presence of PCR inhibitors were not performed. The 1-month-old group had the highest number of virus isolations from cloaca compared to the other age groups (Figure 3.1); this was consistent with both viruses. However, this difference was not statistically significant for the H5N2 group, and was only statistically significant for the H3N8 group in the comparison between the 1-month-old and the 4-month-old groups (Kruskal-Wallis test P=0.063, pairwise comparisons performed using a non-parametric Bonferroni test with α=0.10 overall comparisons). Results were not as clear with virus isolations from the OP swabs, but, with both viruses, the highest number of virus isolations was observed in the 2-month-old group, which was significantly higher than the number of virus isolations in the 2-week-old group infected with H5N2 using the same statistical tests (Kruskal-Wallis test for overall group comparisons, P=0.006, and non -parametric Bonferroni test with α=0.10 for pairwise comparison). The difference in virus isolation from oropharynx observed in the H3N8 group was statistically significant between age classes (P=0.057); however the Bonferroni paiwise test found no significant difference between groups. To estimate the relative amount of virus in cloacal and OP swabs, mean Ct values (combined from both viruses, n=10) were compared between groups on 2 and 4 dpi (Figure 3.2). On both days, the lowest mean Ct values were observed in cloacal swabs from the 1-month-old birds; this is consistent with virus isolation results. The average Ct values from OP swabs were always higher than that observed from cloacal swabs (Figure 3.2) supporting replication of the viruses in the gastrointestinal tract, as previously reported for LPAI viruses (Webster et al., 1978). Our results suggest that, although

age does not affect susceptibility to infection with LPAI virus, it does influence the extent of viral shedding. The higher prevalence of infection observed in juvenile ducks under field situations (Deibel et al., 1985; Halvorson et al., 1985; Hinshaw et al., 1985) has generally been attributed to acquisition of population immunity to these viruses. It is further apparent from our results that even minor differences in age can influence experimental outcomes; and, in order to compare such results, it would be beneficial to utilize birds of similar age.

Acknowledgement

Funding for this work was provided by the ARS CRIS project #6612-32000-048-00D and the Specific Cooperative Agreement #58-6612-2-0220 between the Southeast Poultry Research Laboratory and the Southeastern Cooperative Wildlife Disease Study. Additionally, this work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN266200700007C. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Table 3.1. Virus shedding pattern as determined by virus isolation (VI) and real time RT-PCR (rRT-PCR)[a] of Mallards (*Anas platyrhynchos*) of different age classes inoculated[b] with A/Mallard/MN/355779/00 (H5N2).

Age Class	Swab[c]	Days post inoculation; number of positives (n=5)																			
		0		1		2		4		7		9		11		14		16		21	
		VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR
2 weeks	OP	0	0	1	3	5	1	5	2	3	1	0	1	1	1	0	1	0	1	0	0
	CLO	0	0	1	0	5	1	5	5	5	3	2	2	2	3	0	0	0	0	0	0
1 month	OP	0	0	5	5	5	5	5	5	0	1	1	0	3	0	3	0	3	0	1	0
	CLO	0	0	4	2	5	5	5	5	3	2	2	2	2	2	2	0	1	1	0	0
2 months	OP	0	0	5	5	5	5	5	5	2	3	2	2	2	0	2	0	3	0	0	0
	CLO	0	0	5	5	5	5	5	5	1	1	0	1	2	0	4	0	1	0	0	0
3 months	OP	0	0	5	5	5	5	5	5	0	0	0	0	0	0	0	0	2	0	1	0
	CLO	0	0	5	4	5	5	5	5	1	0	0	0	1	0	1	0	1	0	0	0
4 months	OP	0	0	5	0	5	4	5	0	3	0	3	0	2	0	2	0	0	0	0	0
	CLO	0	0	5	3	5	5	5	4	1	0	1	0	0	0	1	0	0	0	0	0

[a] rRT-PCR - cut off: Ct 40.00.
[b] Ducks were inoculated via choanal cleft with a volume of 0.1 ml containing an infectious titer of 10^6 EID_{50}.
[c] OP = oropharyngeal swab; CLO = cloacal swab.

Table 3.2. Virus shedding pattern as determined by virus isolation (VI) and real time RT-PCR (rRT-PCR)[a] of Mallards (*Anas platyrhynchos*) of different age classes inoculated[b] with A/Mallard/MN/199106/99 (H3N8).

Age Class	Swab[c]	Days post inoculation; number of positives (n=5)																			
		0		1		2		4		7		9		11		14		16		21	
		VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR	VI	rRT-PCR
2 weeks	OP	0	0	4	0	5	0	5	5	5	2	0	0	4	2	0	0	0	0	0	0
	CLO	0	0	0	0	5	2	5	5	5	4	3	3	4	3	2	0	1	0	0	0
1 month	OP	0	0	5	5	5	5	5	5	2	4	2	1	3	1	1	0	0	0	1	0
	CLO	0	0	5	5	5	5	5	5	5	4	5	4	4	2	1	1	0	0	0	0
2 months	OP	0	0	5	4	5	5	5	5	1	2	1	1	3	0	2	0	4	1	2	1
	CLO	0	0	4	1	5	5	5	5	3	1	3	2	2	2	2	0	0	0	0	0
3 months	OP	0	0	5	5	5	5	5	5	2	4	0	0	0	0	1	0	0	0	0	0
	CLO	0	0	5	3	5	5	5	5	2	1	0	1	2	1	0	1	1	0	0	0
4 months	OP	0	0	5	3	5	5	5	2	0	0	0	0	1	0	0	0	0	0	2	0
	CLO	0	0	5	4	5	5	5	5	2	0	1	0	0	0	1	0	0	0	0	0

[a] rRT-PCR: real time RT-PCR - cut off: Ct 40.00.
[b] Ducks were inoculated via choanal cleft with a volume of 0.1 ml containing an infectious titer of 10^6 EID_{50}.
[c] OP = oropharyngeal swab; CLO = cloacal swab.

Table 3.3. Seroconversion[a] in Mallards (*Anas platyrhynchos*) of different age classes inoculated[b] with low pathogenic avian influenza (LPAI) viruses[c].

		Age Class				
Virus[c]	Dpi[d]	2 weeks	1 month	2 months	3 months	4 months
H5N2	14	5/5[e]	5/5	5/5	5/5	5/5
	21	5/5	5/5	5/5	5/5	5/5
H3N8	14	5/5	5/5	5/5	5/5	5/5
	21	5/5[f]	5/5 [f]	5/5	5/5	5/5

[a] As detected by the agar gel precipitation (AGP) test.
[b] Ducks were inoculated via choanal cleft with a volume of 0.1 ml containing an infectious titer of 10^6 EID_{50} of one of the two LPAI viruses.
[c] H5N2: A/Mallard/MN/355779/00 (H5N2); A/Mallard/MN/199106/99 (H3N8).
[d] dpi: days post-inoculation.
[e] Number of birds that seroconverted/Total number of inoculated ducks.
[f] Samples of 21 dpi were also tested by bELISA. For the H3N8 treatment group, two birds in the 2-week-old group and one bird in the 1-month-old group tested negative by AGP but tested positive by bELISA.

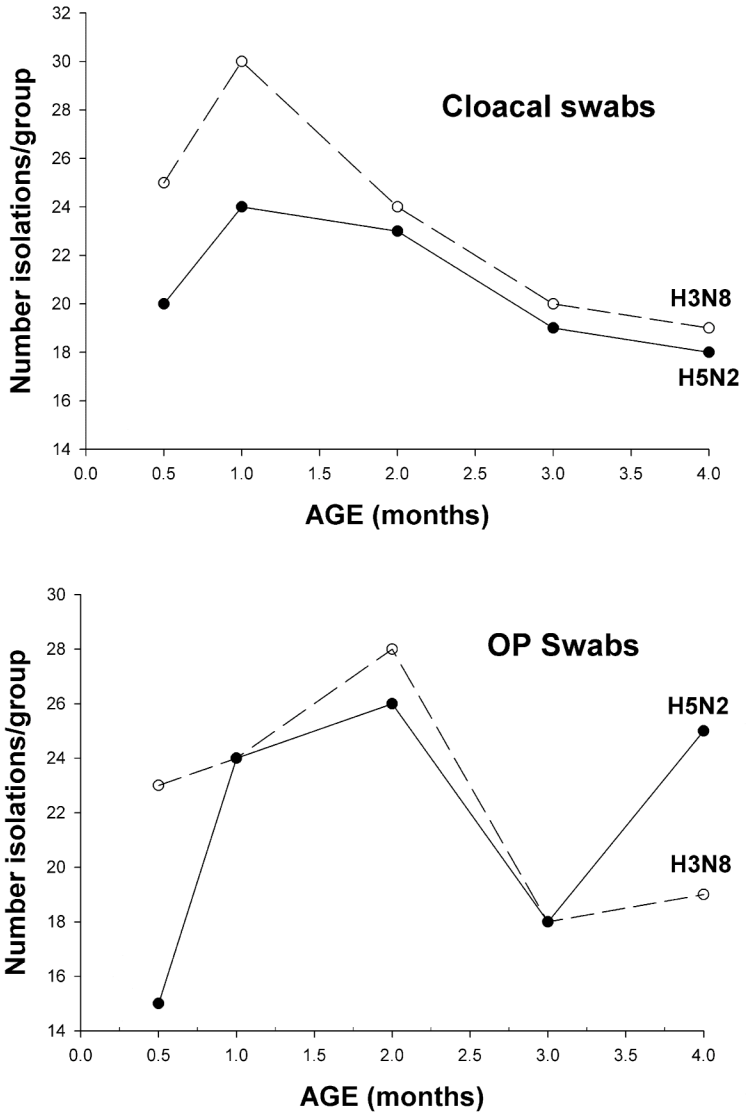

Figure 3.1. Number of virus isolations from cloacal and oropharyngeal (OP) swabs of Mallards (*Anas platyrhynchos*) of different age classes inoculated with low pathogenic avian influenza (LPAI) viruses - A/Mallard/MN/355779/00 (H5N2), and A/Mallard/MN/199106/99 (H3N8).

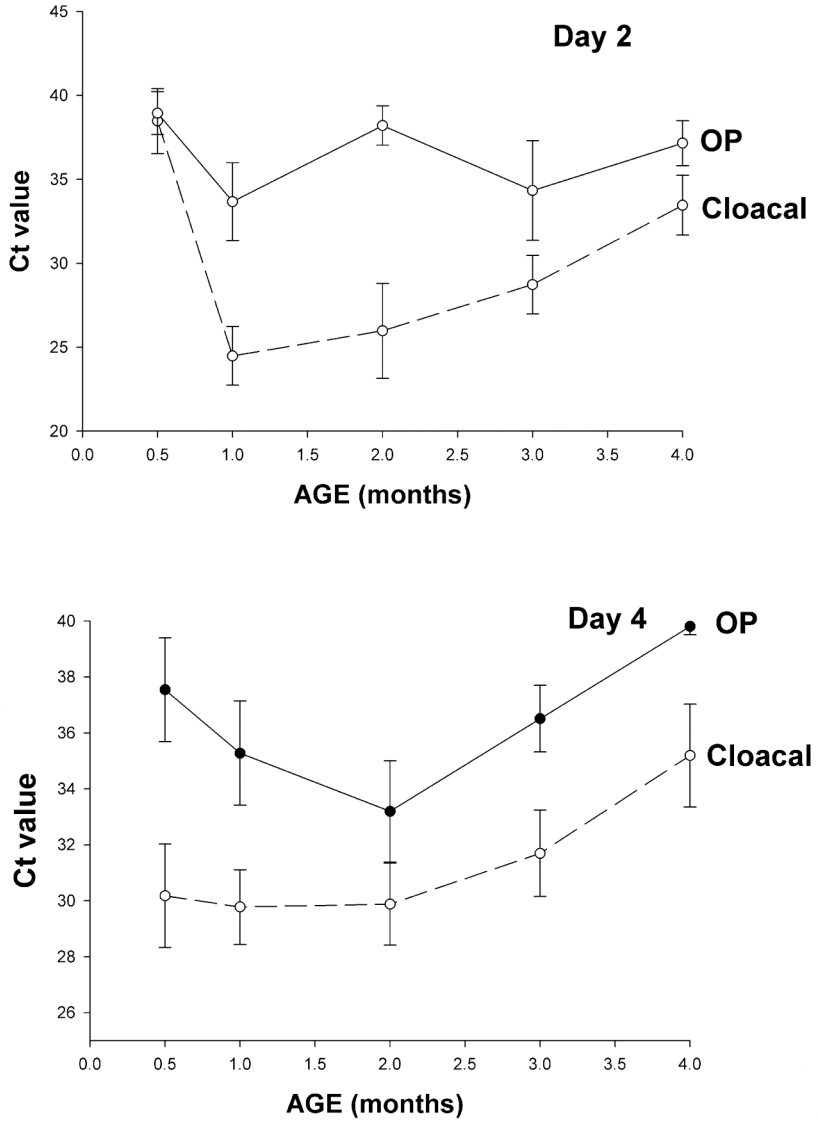

Figure 3.2. Mean Ct values (combined from both viruses, n=10) from cloacal and oropharyngeal (OP) swabs of Mallards (*Anas platyrhynchos*) of different age classes inoculated with low pathogenic avian influenza (LPAI) viruses - A/Mallard/MN/355779/00 (H5N2), and A/Mallard/MN/199106/99 (H3N8), on days 2 and 4 after inoculation.

CHAPTER 4

VARIATION IN VIRAL SHEDDING PATTERNS BETWEEN DIFFERENT WILD BIRD SPECIES EXPERIMENTALLY INFECTED WITH WILD BIRD-ORIGIN LOW PATHOGENICITY AVIAN INFLUENZA VIRUSES[2]

[2] Costa, T.P., J.D. Brown, E.W. Howerth, and D.E. Stallknecht. Submitted to *Avian Pathology*.

Abstract

Prevalence of infection with avian influenza (AI) virus varies significantly between taxonomic Orders and even between species within the same Order. The current understanding of AI infection and viral shedding parameters in wild birds is limited and largely based on trials conducted in Mallards (*Anas platyrhynchos*). The objective of this study was to provide experimental data to examine species-related differences in susceptibility and viral shedding associated with wild bird-origin LPAI viruses in multiple duck species and gulls. Mallards, Redheads (*Aythya americana*), Wood Ducks (*Aix sponsa*), and Laughing Gulls (*Leucophaeus atricilla*) were experimentally inoculated with three wild bird-origin LPAI viruses representing multiple subtypes. Variation in susceptibility and patterns of viral shedding associated with LPAI virus infection were evident between the duck and gull species. Consistent with the literature, Mallards excreted virus predominately via the gastrointestinal tract. In Wood Ducks, Redheads, and Laughing Gulls, AI virus was detected more often in oropharyngeal swabs than cloacal swabs. The results of this study suggest that LPAI shedding varies between taxonomically related avian species. Such differences may be important for understanding the potential role of individual species in the transmission and maintenance of LPAI viruses and may have implications for improving sampling strategies for LPAI detection. Additional comparative studies are necessary to further characterize LPAI infection in wild avian species other than Mallards and provide a mechanism to explain these differences in viral excretion.

Key words: avian influenza, ducks, gull, LPAI, species, viral shedding

Although avian influenza (AI) viruses have been reported from over 100 wild avian species (Stallknecht and Shane, 1988; Olsen et al., 2006; Stallknecht and Brown, 2008), the vast majority of virus isolations have been associated with species utilizing wetlands and aquatic environments (Webster et al., 1992; Stallknecht and Brown, 2007). To date, most AI virus isolations have been reported from Anseriform and Charadriiform species (Stallknecht, 2003; Stallknecht and Brown, 2008), which together are considered to form the primordial reservoir for AI virus. Several decades of surveillance indicate that the prevalence of AI virus infection varies significantly between different genera and species within these two taxonomic orders (Stallknecht, 2003). Within Anseriformes, the majority of AI virus isolations are from dabbling ducks in the Genus *Anas* (reference) and more isolations have been reported from Mallards (*Anas platyrhynchos*) than any other avian species (Hinshaw et al., 1980; Stallknecht and Shane, 1988; Stallknecht et al., 1990; Krauss et al., 2004; Munster et al., 2005; Olsen et al., 2006; Stallknecht and Brown, 2007). AI virus is frequently isolated from other genera of ducks, geese and swans, but at a lower prevalence and with much less consistency (Stallknecht et al., 1990; Olsen et al., 2006; Stallknecht et al., 2007). The epidemiology and host range of AI in the Order Charadriiformes is much less understood than for the Anseriformes. Within Charadriiformes, most AI viruses have been isolated from species in two families, Scolopacidae and Laridae (Stallknecht and Brown, 2007). Within Scolopacidae, one species, Ruddy Turnstone (*Arenaria interpres*), accounts for the vast majority of AI isolations (Hanson et al., 2008). AI virus is rarely detected in other Scolopacidae species, including species that sympatric with infected Ruddy Turnstones (Hanson et al., 2008). Since the first isolation of AI virus from a wild bird in 1961 during an outbreak of highly pathogenic avian influenza virus of the H5N3 subtype in Common Terns (*Sterna hirundo*) (Becker, 1966), AI viruses have been routinely isolated from

gulls and less frequently from terns (Olsen et al., 2006; Stallknecht and Brown, 2008). Avian influenza of H13 and H16 subtypes are almost exclusively maintained in gull and tern populations, and rarely detected in other wild avian taxa (Fouchier et al., 2005; Fouchier et al., 2007; Velarde et al., 2010).

This variation in prevalence may be explained by species-related differences in habitat utilization, behavior, or biology, which collectively affect the likelihood of exposure to AI virus. Alternatively, observed differences in AI virus prevalence between groups of wild birds could relate to differences in AI virus infection between avian taxa, including permissiveness and patterns of viral excretion.

Although multiple experimental trials have been conducted with LPAI viruses in wild birds, most of them used Mallards or Pekin Ducks (*Anas platyrhynchos domesticus*), a Mallard-type domestic duck, as experimental models (Bahl and Pomeroy, 1977; Kida et al., 1980; Cooley et al., 1989; Mundt et al., 2009; Jourdain et al., 2010). Species-related variation is known to occur (Bahl and Pomeroy, 1977; Morales et al., 2009; Mundt et al., 2009) and, therefore, results obtained from Mallards cannot be extrapolated to all anseriform and charadriiform species. In this manner, the current knowledge susceptibility and viral shedding of AI viruses in their natural host is still very narrow. A deeper understanding of AI virus transmission and maintenance in the wild bird reservoir system is needed to help interpret field surveillance results, guide future AI virus sampling efforts, and define risks of virus spilling over into domestic birds.

The objective of this study was to evaluate susceptibility and patterns of viral shedding in multiple duck and gull species experimentally inoculated with wild bird-origin LPAI viruses. Four indigenous North American species were included in this study: Mallards, Redheads (*Aythya americana*), Wood Ducks (*Aix sponsa*), and Laughing Gulls (*Leucophaeus atricilla*).

Materials and Methods

Animals. Three Anseriform and one Charadriiform species were included in this study. Ten to 16-week-old Mallards, Redheads, and Wood Ducks were purchased from a commercial breeder (Chenoa Waterfowl, Martin, Tennessee, USA). Nestling Laughing Gulls were hand-caught in McIntosh County, Georgia at approximately 7 to 10 days of age, by personnel of Georgia Department of Natural Resources. The gulls were raised under confined conditions at the College of Veterinary Medicine, The University of Georgia (UGA), until approximately 12 weeks of age. Gulls used in this investigation were acquired through the Southeastern Cooperative Wildlife Disease Study (SCWDS), UGA, under federal permit. For each species, 20 birds were included in the experimental trial, and both males and females were represented in approximately equal numbers.

Viruses. Three wild bird-origin LPAI viruses representing different hemagglutinin (HA) subtypes were used in this study: A/mallard/MN/355779/00 (H5N2), A/mallard/MN/182761/98 (H7N3), and A/mallard/MN/199106/99 (H3N8). All viruses were originally isolated in specific pathogen free (SPF) embryonated chicken eggs (ECE) from cloacal swabs collected from Mallards. Viral stocks were propagated by second passage in 9- to 11-day-old SPF ECE, and infectious titer of the stock was calculated by determining the EID_{50} (Reed and Muench, 1938; Woolcock, 2008). To prepare the viral inoculums, infective allantoic fluid was diluted in sterile brain-heart-infusion (BHI) medium to yield a final titer of 10^6 EID_{50} per 0.1 mL (single-bird inoculum). The back titers were determined by titration in SPF ECE after the ducks and gulls were inoculated and varied from $10^{5.27}$ to $10^{6.27}$ EID_{50}/0.1 mL. A 0.1 mL of sterile BHI medium was used as a sham inoculum.

Experimental Design. For each of the four species, 20 birds were randomly divided in three treatment and one negative control groups, with five birds in each group. The groups were housed separately in biocontainment isolation units ventilated under negative pressure with a high efficiency particulate air filters on intake and exhaust. Food and water were provided *ad libitum*. After a 4-day acclimation period, treatment or negative control groups were inoculated via choanal cleft with one of the three LPAI viruses or sterile BHI medium, respectively. Birds were observed two times a day for behavioral change or clinical signs. Cloacal and oropharyngeal (OP) swabs were collected from all birds at 0, 1, 2, 3, 4, 5, 8, 12, 16, and 21 days post inoculation (dpi). Blood samples for serologic testing were collected from the right jugular vein at 0, 12, and 21 dpi. The trial was terminated at 21 dpi, when birds were euthanized by CO_2 inhalation, and a full necropsy was performed. Experimental trials were performed in a BSL-Ag2+ facility at the Poultry Diagnostic and Research Center, UGA. General animal care was provided and experimental sampling was performed according to an animal care and use protocol approved by the Institutional Animal Care and Use Committee at UGA.

Virus Isolation. Cloacal and OP swabs were collected in vials containing 2 mL of sterile BHI medium with antimicrobial drugs (100 μg/mL gentamicin, 100 units/mL penicillin, and 5 μg/mL amphotericin B) and were stored at -70°C until further testing. Virus isolations were performed in 9 to 11-day-old SPF ECE and, after incubation, amnioallantoic fluid (AAF) was tested for the presence of hemagglutination activity using standard protocols (Killian, 2008; Woolcock, 2008).

RNA extraction and real time RT-PCR. RNA was extracted from cloacal and OP swab material and from all the AAF samples that tested positive for the presence of hemagglutination activity after virus isolation. The MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambiron, Austin, TX) and the Thermo Electron KingFisher magnetic particle processor (Thermo Electron

Corporation, Waltham, MA) were used for the RNA extraction. RNA was tested for AI virus by quantitative real time reverse transcription-PCR (rRT-PCR) targeted to the influenza matrix gene as previously described (Spackman and Suarez, 2008). Samples with a cycle threshold (Ct) value equal or less than 40.00 on rRT-PCR were considered positive.

Serology. Serum samples were stored at -20°C until tested for the presence of influenza A antibodies using agar gel immunodiffusion (AGID) test (Swayne et al., 1998), and a commercial blocking enzyme-linked immunosorbent assay (bELISA) AI virus antibody test kit (FlockChek AI MultiS-Screen antibody test kit; Idexx Laboratories, Westbrook, Maine). The AGID test was performed using previously reported procedures (Swayne et al., 1998). The commercial bELISA was performed following the manufacturer's instructions.

Histopathological Analyses. Samples of cerebrum, cerebellum, heart, lung, trachea, liver, spleen, esophagus, proventriculus, ventriculus, small intestine, large intestine, pancreas, adrenal gland, ovaries/testis, kidney, bursa, pectoral muscle, nasal turbinates and sinus were collected from all the animals during necropsy, and fixed in 10% neutral buffered formalin for histopathological examination. After fixation, the tissues were processed and embedded in paraffin, and 5μm sections were stained with hematoxylin and eosin using standard histopathology protocols. Turbinates were decalcified with Kristensen's decalcifying solution (Kristensen, 1948) before being processed for histopathologic examination as described for the other tissues.

Statistical Analyses. Kappa coefficient with 95% confidence interval was used to compare agreement between virus isolation and rRT-PCR results. Interpretation of κ was performed based on Landis and Koch (Landis and Koch, 1977).

Results

None of the birds had preexisting antibodies to AI virus or were excreting virus at the start of the experiment, as demonstrated by negative serological and virus isolation results for samples collected at 0 dpi. The birds in the negative control group remained seronegative throughout the trial and virus was not detected in swab samples collected at any time point from this group. Morbidity or mortality was not observed in any of the three species of ducks in any of the treatment groups. However, three Laughing Gulls, one in each treatment group, died during the trial. Based on post-mortem examination, the mortality in these three gulls was related to complications due to aspergillosis, pododermatitis, and septic arthritis. As a result of these deaths, each of the three gull treatment groups had a total of four birds. The results and analysis presented below reflect this change in sample size.

Viral Shedding. The pattern of viral shedding, including route and duration, varied between duck and gull species and individual virus isolates. Based on virologic (Table 4.1, Figure 4.1) and/or serologic (Table 4.2) results, all three LPAI virus strains replicated in each of the four species included in this study. However not all birds were infected by the H3N8 (Wood Ducks and Redheads) or the H7N3 (all species). The agreement rate between the results obtained with rRT-PCR and those observed on virus isolation was 91% (kappa of 0.81), indicating a high association between these two tests.

With infected Mallards, VI and rRT-PCR primarily detected AI virus in cloacal swabs. The H7N3, however, was unique in this species in that the virus detection was restricted to OP swabs. With all other species (Wood Duck, Redhead, and Laughing Gull) positive VI or rRT-PCR results were predominantly associated with OP swabs. This was especially pronounced in Wood Ducks where cloacal shedding was limited to only one bird in each treatment group.

The duration of viral shedding, as detected by VI and rRT-PCR also varied between species and, with the exception of the H7N3 virus, was detected the longest in Mallards; positive cloacal swabs could be detected for 21 and 12 days for H5N2 and H3N8, respectively. Shedding was detected up to 12 days for Wood Ducks (H7N3, OP swab) and Redheads (H5N2, cloacal swab), and 16 days in Laughing Gulls (H3N8, OP swab).

Serology. The frequency of seroconversion varied between virus isolates. All birds in each of the four species, seroconverted after exposure to the H5N2 virus. Seroconversion rates for the H3N8 and H7N3 treatment groups were 74 and 63%, respectively (Table 4.2). The agreement between the results of the AGID test and the bELISA was 88% (κ of 0.77), indicating a substantial association between these two tests.

Necropsy and Histopathological Analysis. Gross or histopathological lesions associated with AI virus infection were not observed in any of the treatment or negative control groups.

Discussion

In this study, the frequency of infection and patterns of viral shedding varied between LPAI viruses and duck and gull species. In general, Mallards and Laughing Gulls had higher frequencies of infection than Wood Ducks and Redheads, suggesting they may be more permissive hosts for wild bird origin LPAI viruses. This was most apparent for the H5N2 and H3N8 LPAI virus treatment groups. The H7N3 virus was the least infective of the viruses for all four species. Based on these results, the H7N3 LPAI virus appears to be a less fit virus for replication in wild avian reservoir hosts; a surprising observation considering the H7N3 LPAI virus was originally isolated from a Mallard. The reason for the poor infectivity of the H7N3 LPAI virus is not known; however, several potential explanations include adaptation of the virus

to eggs during the propagation of stock, poor adaptation to the new host species, and/or differences in host susceptibility.

Seroconversion was directly related with the ability of the viruses to replicate. The higher rate of seroconversion was observed in the H5N2 groups, which also had a higher rate of viral shedding. Although a substantial association was observed between the results of the AGID test and the bELISA, lack of sensitivity of AGID test for waterfowl has been reported (Slemons and Easterday, 1972; Cattoli and Capua, 2007; Swayne and Halvorson, 2008).

In this study, Mallards had the most extensive viral shedding based on numbers of bird shedding the virus and the duration of shedding. This observation is consistent with historic surveillance results and supports the important role that Mallards play in the natural history of AI. The Mallard is distributed worldwide and, as previously mentioned, more AI viruses have been isolated from this species than any other wild avian species (Hinshaw et al., 1980; Stallknecht et al., 1990; Krauss et al., 2004; Munster et al., 2005; Olsen et al., 2006; Stallknecht and Brown, 2007; Hesterberg et al., 2009). Mallards in the H5N2 and H3N8 treatment groups had an extended period of viral shedding, primarily via the cloaca. This is consistent with previously reported experimental and field results from this species (Ellström et al., 2008; Costa et al., 2010). Interestingly, as opposed to Mallards, viral shedding in Wood Ducks, Redheads, and Laughing Gulls was predominately via the oropharynx, suggesting the importance of respiratory tract replication of LPAI viruses in these species. Another study with experimental AI virus infection reported viral shedding up to 23 days via the oropharynx in Franklin's Gulls (*Leucophaeus pipixcan*) (Bahl and Pomeroy, 1977). These results differ from the traditional paradigm that the replication of LPAI viruses in waterfowl occurs primarily in cells lining the lower intestinal tract (Slemons and Easterday, 1977; Webster et al., 1978; Kida et al., 1980), but

corroborate the opinion that the replication site of AI viruses vary according to the host species and virus strain (Kida et al., 1980).

Based on these observations, cloacal swabs would represent the ideal sampling method for surveillance studies of Mallards, as previously reported (Ellström et al., 2008) and may have relevance to other closely related species in the genus *Anas*. However, for avian species other than dabbling ducks a combination of OP and cloacal swabs might be more appropriate and increase the likelihood of detection in waterfowl.

Interestingly, the difference in tissue tropism (respiratory versus intestinal tract) of LPAI viruses observed in the present study may be in some way related to the previously reported variation on susceptibility to Eurasian H5N1 HPAI virus among these species. For instance, high susceptibility to Eurasian H5N1 HPAI virus has been reported in Wood Ducks and Laughing Gulls, and moderate susceptibility has been observed in Redheads (Brown et al., 2006; Brown et al., 2007; Keawcharoen et al., 2008). In this experimental trial, Wood Ducks, Laughing Gull and Redheads had predominantly oropharyngeal shedding of AI viruses. Mallards, however, are less susceptible to a Eurasian H5N1 HPAI infection (Brown et al., 2006) and, in this study, primarily cloacal viral shedding was observed in Mallards. Although AI virus receptor distribution, among other factors, may contribute to this host species-variation, additional studies are required to elucidate possible mechanisms involved in this process.

The observations in this study suggest that different avian species can have dissimilar roles on the transmission of LPAI viruses, and may explain the variation in prevalence observed in wild bird populations (Stallknecht and Shane, 1988; Olsen et al., 2006). Further comparative studies are necessary to explain these variations and to elucidate the reservoir potential of

different anseriform and charadriiform species for LPAI viruses, as well as to provide a better understanding of the evolution of LPAI viruses in their natural hosts.

Acknowledgements

We thank the personnel of the Southeast Cooperative Wildlife Disease Study for their technical assistance, especially Ginger Goekjian, Rebecca Poulson, and Jennifer Smith, and the Georgia Department of Natural Resources personnel for collecting the gulls. We deeply appreciate Dr. Renata Casagrande for her help on bird handling and sample collection. Funding for this work was provided through a cooperative agreement between the Southeast Poultry Research Laboratory and the Southeastern Cooperative Wildlife Disease Study, and through the continued sponsorship of SCWDS member state and federal agencies.

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Table 4.1. Virus isolation and rRT-PCR results of oropharyngeal and cloacal swabs from four species of wild birds experimentally inoculated[a] with three different low pathogenic avian influenza (LPAI) viruses.

SPECIES		LPAI viruses[b]					
		H5N2		H3N8		H7N3	
		Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
Mallard	rRT-PCR[c]	5/5 (4-5; 4.6)[d]	5/5 (5-12; 9)	5/5 (5; 5)	5/5 (5-12; 7.6)	3/5 (1-3; 1.7)	0/5
	VI	5/5 (2-5; 3.8)	5/5 (5-21; 8.8)	5/5 (4-5; 4.4)	5/5 (5-12; 8.2)	3/5 (1-4; 3)	0/5
Wood Duck	rRT-PCR	5/5 (1-5; 2.6)	1/5 (1)	4/5 (1-5; 3.5)	0/5	5/5 (1-3; 1.4)	0/5
	VI	5/5 (1-5; 3)	1/5 (1)	4/5 (1-5; 3.5)	1/5 (5)	4/5 (1-12; 4.2)	1/5 (1)
Redhead	rRT-PCR	5/5 (3-5; 4)	1/5 (4)	2/5 (3-4; 3.5)	0/5	4/5 (1-2; 1.5)	0/5
	VI	5/5 (4-8; 6.6)	3/5 (5-12; 8.3)	5/5 (2-8; 4.6)	0/5	5/5 (2-8; 3.6)	0/5
Laughing Gull	rRT-PCR	5/5 (4-8; 5.6)	4/5 (5-8; 5.8)	4/5 (5-8; 5.8)	2/5 (3; 3)	5/5 (1-3; 1.4)	0/5
	VI	5/5 (4-8; 5.4)	3/5 (3-4; 3.7)	5/5 (5; 5)	2/5 (5-16; 10.5)	5/5 (1-4; 1.8)	0/5

[a] Ducks were inoculated via choanal cleft with 0.1mL and an infectious dose of $10^6 EID_{50}$.
[b] H5N2: A/mallard/MN/355779/00 (H5N2); H3N8: A/mallard/MN/199106/99 (H3N8); H7N3: A/mallard/MN/182761/98 (H7N3).
[c] rRT-PCR: real time RT-PCR (cut off: Ct 40.00); VI: virus isolation.
[d] Positive/total no. birds (range in days; average in days).

Table 4.2. Seroconversion as of 12 or 21 days post infection from four species of wild birds experimentally inoculated[a] with three different low pathogenic avian influenza (LPAI) viruses.

LPAI viruses[b]	SPECIES							
	Mallard		Wood Duck		Redhead		Laughing Gull	
	AGID[c]	bELISA	AGID	bELISA	AGID	bELISA	AGID	bELISA
H5N2	5/5[d]	5/5	5/5	5/5	5/5	5/5	4/4	4/4
H3N8	5/5	5/5	2/5	3/5	1/5	2/5	4/4	4/4
H7N3	2/5	2/5	2/5	3/5	1/5	4/5	2/4	3/4

[a] Ducks were inoculated via choanal cleft with 0.1mL and an infectious dose of $10^6 EID_{50}$.
[b] H5N2: A/mallard/MN/355779/00 (H5N2); H3N8: A/mallard/MN/199106/99 (H3N8); H7N3: A/mallard/MN/182761/98 (H7N3).
[c] AGID: agar gel immunodiffusion; bELISA: blocking ELISA (multiS-screen ELISA AI virus antibody test kit, IDEXX laboratories, Westbrook, ME).
[d] Positive/total number of birds.

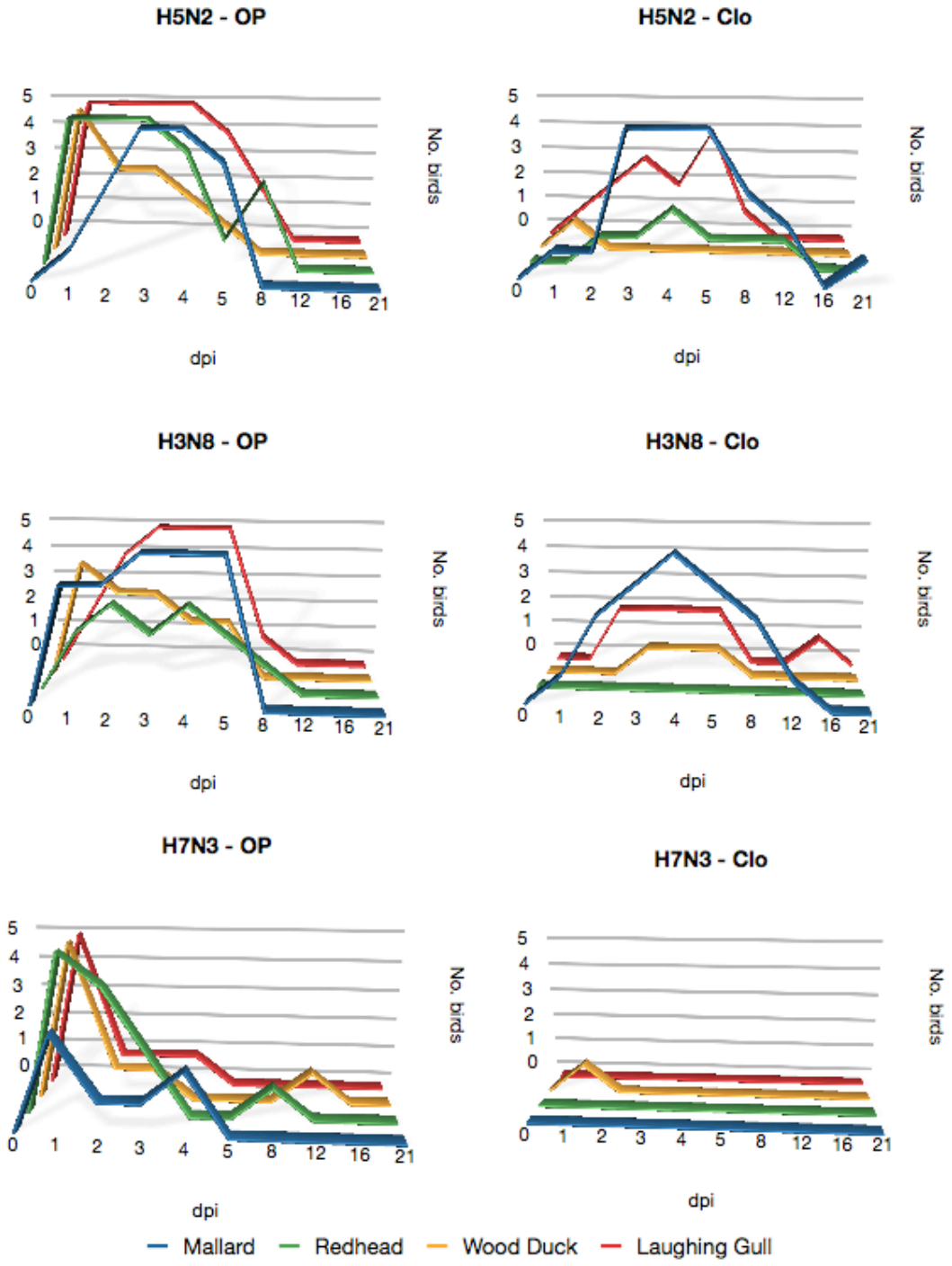

Figure 4.1. Viral shedding pattern of four different wild bird species, as determined by either virus isolation or real time RT-PCR of oropharyngeal (OP) and cloacal (Clo) swabs. Birds were inoculated via choanal cleft with an infectious dose of $10^6 EID_{50}$ (0.1 mL) of either one of the LPAI viruses: A/mallard/MN/355779/00 (H5N2); A/mallard/MN/199106/99 (H3N8); A/mallard/MN/182761/98 (H7N3). dpi: days post inoculation.

CHAPTER 5

EFFECT OF A PRIOR EXPOSURE TO A LOW PATHOGENIC AVIAN INFLUENZA VIRUS ON THE OUTCOME OF A HETEROSUBTYPIC LOW PATHOGENIC AVIAN INFLUENZA VIRUS INFECTION IN MALLARDS (*Anas platyrhynchos*)[3]

[3] Costa, T.P., J.D. Brown, E.W. Howerth, and D.E. Stallknecht. To be submitted to *Avian Diseases*.

Abstract

Wild birds, particularly Anseriformes and Charadriiformes, are considered the natural reservoir of low pathogenic avian influenza (LPAI) viruses, and the high prevalence and subtype diversity of avian influenza (AI) viruses at staging areas provide the perfect opportunity for multiple exposures to different LPAI virus subtypes. Natural consecutive and concurrent infections of sentinel ducks with different LPAI virus subtypes have been reported. The protective immune response from different infections is not understood nor is the effect of such repeated exposures. This study experimentally evaluated the effect of a prior exposure to a LPAI virus on the outcome of a heterosubtypic LPAI virus infection in Mallards (*Anas platyrhynchos*). The results of this investigation suggest that recent prior exposure to a LPAI virus may negatively affect the outcome of a subsequent heterosubtypic LPAI infection in Mallards, by reducing the duration of cloacal and oropharyngeal viral shedding and also the viral load excreted via the cloaca. Wild Mallards are likely exposed to multiple subtypes of LPAI virus during the periods of peak viral circulation and the results of this study suggest that the duration of viral shedding of subsequent exposure might be reduced. The results of this study are significant for understanding the ecology of these viruses in wild avian populations.

Keywords: *Anas platyrhynchos*, heterosubtypic infection, low pathogenic avian influenza, Mallard, prior exposure.

Wild birds, particularly Anseriformes and Charadiiformes, are known to be the natural reservoirs of low pathogenic avian influenza (LPAI) viruses. Within Anseriformes, the prevalence of natural infections is particularly high in members of the Subfamily Anatinae (dabbling and diving ducks), and the vast majority of the isolations have been from Mallards (*Anas platyrhynchos*) (Stallknecht and Brown, 2008). In Mallards, LPAI viruses replicate in epithelial cells lining the intestinal tract (Kida et al., 1980), and are excreted in high concentration in feces (Webster et al., 1978; Hinshaw et al., 1982). Viral transmission in waterfowl is dependent on a fecal/oral route (Webster et al., 1992). The prevalence of LPAI in wild duck populations in North America peaks in late summer/early fall, when susceptible hatch-year birds and adult birds congregate prior to south migration (Hinshaw et al., 1985). AI virus subtypes do not circulate equally among wild bird populations and variation can occur between host species, geographic location, and years (Stallknecht and Brown, 2008). Among North American ducks, H3, H4 and H6 are the most common subtypes isolated (Stallknecht et al., 1990; Sharp et al., 1993; Hanson et al., 2003; Krauss et al., 2004), while the H5, H7, H8 and H9 are generally isolated at a lower rate (Stallknecht and Shane, 1988; Krauss et al., 2004).

The high prevalence and subtype diversity of AI viruses in staging areas provides the perfect opportunity for multiple exposures to different LPAI virus subtypes. Natural consecutive infections of sentinel ducks with different LPAI virus subtypes have been reported, providing evidence that a prior exposure does not protect against a subsequent AI virus infection (Sinnecker et al., 1982; Süss et al., 1994). Few studies have experimentally addressed the outcome of concurrent or subsequent LPAI infections in its natural host. It has been observed that viral replication and viral shedding is suppressed in Pekin ducks followed by a second exposure to the same LPAI isolate, and that a secondary immune response is mounted if the

challenge occurs 46 days after the primary inoculation or later (Kida et al., 1980). A recent study concluded that infection by a LPAI virus in Mallard is limited by prior infection with a homosubtypic (HA homologous) strain, and it may be prevented by prior infection with a heterosubtypic (HA heterologous) strain (Jourdain et al., 2010). The co-circulation of many subtypes and the possibility of concurrent and subsequent infection of wild ducks with different subtypes are evidence of the complex natural history of AI (Dugan et al., 2008). A prior infection with a LPAI virus could potentially affect the outcome of a subsequent exposure to a LPAI virus, by influencing susceptibility, viral shedding, and/or immune response.

The goal of this study was to experimentally evaluate the outcome of a LPAI challenge on viral shedding and antibody response in Mallard pre-exposed to a heterosubtypic LPAI virus. Mallard was chosen as the experimental species due its worldwide distribution and importance as a LPAI reservoir (Krauss et al., 2004; Wallensten et al., 2007).

Materials and Methods

Viruses. LPAI viruses A/Mallard/MN/355779/00 (H5N2) and A/Mallard/MN/199106/99 (H3N8) were used in this study. The viruses were originally isolated in specific pathogen free (SPF) embryonated chicken eggs (ECE) from cloacal swab material collected from wild Mallards. Viral stocks were propagated by second passage in 9- to 11-day-old SFP ECE and titrated using described techniques (Reed and Muench, 1938; Woolcock, 2008). Infective allantoic fluid was diluted in sterile brain-heart-infusion (BHI) medium to yield 10^6 median egg infectious dose (EID_{50}) per 0.1 mL (single-bird inoculum). Back-titers varied from $10^{5.36}$ to $10^{6.27}$ EID_{50}/0.1 mL. A sham inoculum was prepared using uninfected sterile BHI medium.

Animals. One-day-old Mallards were purchased from a commercial source (Murray McMurray Hatchery, Webster City, Iowa, USA) and raised under confined conditions until they were 16 weeks of age. Both males and females were included in approximately equal numbers. Birds were housed in groups of five in self-contained isolation units ventilated under negative pressure with high-efficiency particulate air filters. Food and water were provided *ad libitum*. General animal care was provided under an animal use protocol approved by the Institutional Animal Care and Use Committee at The University of Georgia, Athens, Georgia, USA.

Experimental Design. Twenty-five Mallards were evenly divided in four treatments and one negative control group. Each bird in the negative control group was inoculated intrachoanally with 0.1 mL of sham inoculum. The birds in the two heterosubtypic challenge groups were inoculated sequentially with both of the two LPAI strains, allowing for 21-day interval between inoculations (consecutive infection groups). Therefore, one group was first inoculated with A/Mallard/MN/355779/00 (H5N2) (primary inoculation) at 0 days post primary inoculation (dpi) and challenged with A/Mallard/MN/199106/99 (H3N8) (heterosubtypic challenge) at 21 dpi (H5N2xH3N8 group); the other group was initially inoculated with A/Mallard/MN/199106/99 (H3N8) at 0 dpi and challenged with A/Mallard/MN/355779/00 (H5N2) at 21 dpi (H3N8xH5N2 group).

Birds in the two single LPAI virus challenge groups (single-infection groups) were inoculated with only one of the two LPAI viruses used in this study, either A/Mallard/MN/355779/00 (H5N2) (H5N2 group) or A/Mallard/MN/199106/99 (H3N8) (H3N8 group). These groups served as positive controls. In order to allow data comparison and ensure that all the treatment groups were at the same age, the birds in the single-infection groups were inoculated at the same time point that the consecutive infection groups were challenged with the

heterosubtypic LPAI virus; therefore, the trials for the two single-infection groups started 21 days after the trials for the consecutive infection groups.

All birds were evaluated twice daily for behavioral changes and clinical signs. Oropharyngeal (OP) and cloacal swabs were collected on 0, 1, 2, 4, 7, 9, 11, 14, 16, and 21 dpi, and at the same time interval of days post heterosubtypic challenge (dpc). Blood samples were collected on 0, 14, and 21 dpi and on 14 and 21 dpc. At 21 dpc, all birds were humanely euthanized by CO_2 inhalation and a full necropsy was performed. Experimental infections were performed in a BSL-Ag2+ facility at the Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia, Athens, Georgia, USA.

Virus Isolation. Cloacal and OP swabs were collected in vials containing 2 mL of sterile BHI medium with antimicrobial drugs (100 μg/mL gentamicin, 100 units/mL penicillin, and 5 μg/mL amphotericin B) and were stored at -70°C until further testing. Virus isolations were performed in 9- to 11-day-old SPF ECE using standard protocols (Swayne et al., 2008). Positive cloacal swabs samples collected at 2 dpc were also titrated in 9- to 11-day old SPF ECE by determining the EID_{50}/mL (Reed and Muench, 1938), after two cycles of free-thaw.

Subtyping. Cloacal samples from the consecutive infection groups collected at 2 dpc were further tested for HA subtype. RNA was extracted from cloacal swabs by using the QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), and tested for AI virus by standard one-step RT-PCR targeted to the influenza A matrix gene. The RT-PCR mixture for each reaction contained 1X Green GoTaq™ Buffer (Promega, Madison, Wisconsin, USA), 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 200μM of forward primer, 200μM of reverse primer, 2.5 units of AMV Reverse Transcripase (Promega, Madison, Wisconsin, USA), 1.25 units Taq DNA Polymerase (Promega, Madison, Wisconsin, USA), and nuclease-free water to a final volume of 50 μl. The H3 and H5

primers used have been previously described (Tsukamoto et al., 2008). PCR amplification was performed as follows: reverse transcription for 30 minutes at 42°C; denaturation for 2 min at 94°C; followed by 39 cycles of PCR amplification, with each cycle consisting of 40 seconds of denaturation at 94°C, 60 seconds of annealing at 50°C, and 60 seconds of elongation at 72°C; and one final cycle of elongation for 10 min at 72°C. The amplified PCR products were analyzed on a 2% agarose gel electrophoresis.

Serologic Assay. Blood samples were collected from the right jugular vein, and serum samples were stored at -20°C until they were tested. Serologic testing was performed on all samples via the hemagglutination inhibition (HI) test by using standard procedures (Pedersen, 2008). Reference antigens were prepared using A/Mallard/MN/355779/00 (H5N2) and A/Mallard/MN/199106/99 (H3N8) LPAI viruses. Samples with HI titer ≥ 8 were considered positive.

Microscopic Analyses. Samples of cerebrum, cerebellum, heart, lung, trachea, liver, spleen, esophagus, proventriculus, ventriculus, small intestine, large intestine, pancreas, adrenal gland, ovaries/testis, kidney, bursa, pectoral muscle, nasal turbinates and sinus were collected from all the animals during necropsy, and fixed in 10% neutral buffered formalin for microscopic examination. After fixation, the tissues were processed and embedded in paraffin, and 5μm sections were stained with hematoxylin and eosin using standard histopathology protocols. Turbinates were decalcified with Kristensen's decalcifying solution (Kristensen, 1948) before being processed for microscopic examination as described for the other tissues.

Results

Behavioral changes or clinical signs were not observed in any of the birds for the entire length of the trial. Neither seroconversion nor virus isolation were detected in any of the birds in the negative control group.

Antibodies against AI viruses were detected in all the birds in the treatment groups at 14 days after inoculation (Table 5.1). The HI titers against the A/Mallard/MN/199106/99 (H3N8) ranged from 8 to 16 (mean 10). All the birds in the H3N8xH5N3 and the H3N8 groups seroconverted after the primary H3N8 exposure, but only 4/5 birds in the H5N2xH3N8 group developed H3 antibodies after being challenged with H3N8 virus (Table 5.1). In addition, a loss of antibodies against H3 after the heterosubtypic challenge was observed in both consecutive infection groups, as only 2/5 birds at the H5N2xH3N8 group (birds 1 and 5) and 3/5 birds at the H3N8xH5N2 group (birds 6, 8, and 9) had detectable HI titers against H3 at 21 dpc. Antibody titers against the A/Mallard/MN/355779/00 (H5N2) ranged from 8 to 128 (mean 25), and seemed to last longer when compared with antibodies against the H3. For example, all the birds in the H5N2xH3N8 group had detectable levels of antibodies until 42 days after primary inoculation (21 dpc), when the trial was terminated.

Viral shedding via oropharynx and cloaca was consistent during the first four days after the primary inoculation (in both single-infection and consecutive infection groups). After that, viral shedding was intermittent and virus was recovered from OP swabs up to 21 dpi (H3N8xH5N2 group, oropharyngeal swab) (Figure 5.1). Both groups pre-exposed to a LPAI virus had reduced duration of viral shedding via respiratory and intestinal tracts after challenge with a heterosubtypic LPAI virus, when compared to single-inoculation control groups (Table 5.2, Figure 5.1). The mean viral shedding via OP and cloaca for the consecutive infection groups

after heterosubtypic challenge was 4.2 and 4.2 days for the H5N3xH3N8 and 3.8 and 2.4 days for the H3N8xH5N2 groups, respectively. In comparison, the mean viral shedding via OP and cloaca for the single infection groups was 6.4 and 6.0 days for the H5N3 group, and 9.2 and 4.4 days for the H3N8 group, respectively (Table 5.2).

Cloacal viral shedding was completely suppressed in one bird (H3N8xH5N2 group, bird 9). Viral titrations of positive cloacal swabs collected at 2 dpc were performed after two freeze-thaws cycles, and mean viral titers were: $10^{1.6}$ EID_{50}/mL for the H5N2xH3N8 group; $10^{1.5}$ EID_{50}/mL for the H3N8xH5N2 group; $10^{2.9}$ EID_{50}/mL for the H5N2 group; and $10^{2.7}$ EID_{50}/mL for the H3N8 group. The viral subtyping of cloacal swab samples collected at 2 dpc confirmed that the virus being shed was of the same HA subtype as the viruses used for the heterosubtypic challenge.

Gross or histopathological lesions associated with AI virus infection were not observed in any of the treatment or negative control groups

Discussion

A primary exposure to a LPAI virus did not protect Mallards against a subsequent infection with a heterosubtypic LPAI virus, as observed by seroconversion and viral shedding after the heterosubtypic challenge (Table 5.1, Figure 5.1). Nevertheless, based on duration of viral shedding and viral titers in cloacal swabs after heterosubtypic challenge, the two consecutive infection groups had reduced viral shedding both in the respiratory and enteric tracts when compared to the single inoculation control groups. The reduction in duration of viral shedding varied between treatment groups and route of viral shedding, being more evident for OP shedding in the H3N8xH5N2 group (Figure 5.1). Cloacal viral shedding post heterosubtypic

challenge was completely suppressed in one bird in the H3N8xH5N2 group (bird 9), although seroconversion was detected (HI titer of 8 against the H5 virus at 14 and 21 dpc) (Table 5.1).

At 2 dpc, a one-log reduction in viral titer was observed between cloacal swab samples collected from the single-infection and consecutive infection groups. The viral titrations were performed after the samples underwent two freeze-thaws cycles, which is known to negatively affect the influenza viral load of a sample (Greiff et al., 1954; Jackson and Muldoon, 1975; Quinlivan et al., 2004). Consequently, the viral titers obtained do not likely reflect the actual viral load being shed by the birds at 2 dpc, but the reduction in viral titers was consistent among birds in the consecutive infection groups. These observations suggest that the viral load shed via cloaca after a LPAI infection may be reduced by prior infection with a heterosubtypic LPAI strain in Mallards.

A difference in immunogenicity was observed between the two LPAI viruses used in this experimental trial, as demonstrated by higher HI titers produced against the H5N2 virus (8 to 128, mean 25) than HI titers against the H3N8 virus (8 to 16, mean 10). A loss of antibodies against H3 at 21 dpc was observed in two birds in the H3N8xH5N2 group (birds 7 and 10), which had an HI titer of 8 until 21 dpc, when a negative HI result was obtained. Furthermore, based on HI results of the H5N2xH3N8 group (Table 5.1), the production of antibodies against H3 was negatively affected by the pre-existing heterosubtypic immunity, as only 4/5 birds had detectable HI titers against H3 at 14 dpc (birds 1, 3, 4, and 5), and only 2/5 at 21 dpc (birds 1 and 5). Although no antibodies against H3 were detected in bird 2 (Table 5.1), cloacal viral shedding was observed from 1 to 4 dpc.

The observations of this study suggest that a previous exposure to a heterosubtypic LPAI virus may negatively affect the outcome of a second LPAI exposure in Mallards, by reducing the

duration of cloacal and oropharyngeal viral shedding, and also the viral load shed via cloaca. These results are in accordance with previous experimental cross-protective studies in Mallards (Jourdain et al., 2010).

This heterosubtypic LPAI virus-induced partial protection might be of significant importance for the ecology of these viruses in wild avian populations, as an infected duck with heterosubtypic immunity may shed the LPAI virus at lower concentrations in feces and for a shorter period of time; as a result, environmental contamination would be reduced. These observations, however, should be interpreted with caution, as the heterosubtypic LPAI virus-induced protective effect might decrease if the heterosubtypic challenge occurs after the first three weeks of primary infection. In addition, based on the constant circulation of different LPAI virus subtypes in wild duck populations, it is improbable that a total protective immunity is mounted. In conclusion, the protective effects of subsequent heterosubtypic LPAI virus infections may limit the pattern of AI infection in wild birds, but likely do not completely suppress it.

Acknowledgement

We would like to express our gratitude to the personnel of the Southeast Cooperative Wildlife Disease Study for their technical assistance, especially Ginger Goekjian, Rebecca Poulson, Jennifer Smith, and Deb Carter. Funding for this work was provided through a cooperative agreement between the Southeast Poultry Research Laboratory and the Southeastern Cooperative Wildlife Disease Study, and through the continued sponsorship of SCWDS member state and federal agencies.

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Table 5.1. Serological status, as determined by hemagglutination inhibition, of Mallards (*Anas platyrhynchos*) at 14 and 21 days post primary inoculation (dpi) and at 14 and 21 days post heterosubtypic challenge (dpc) with low pathogenicity avian influenza viruses[a].

Group[b], bird ID	Hemagglutination Inhibition Result[c]							
	14 dpi[d]		21 dpi		14 dpc		21 dpc	
	H5N2	H3N8	H5N2	H3N8	H5N2	H3N8	H5N2	H3N8
H5N2xH3N8								
1	128	-	64	-	32	16	16	8
2	32	-	32	-	16	-	8	-
3	16	-	16	-	16	8	16	-
4	16	-	16	-	8	8	8	-
5	32	-	32	-	16	8	32	8
H3N8xH5N2								
6	-	16	-	8	16	16	16	8
7	-	8	-	8	16	8	16	-
8	-	16	-	16	16	8	32	8
9	-	16	-	16	8	16	8	16
10	-	8	-	8	16	8	16	-
H5N2 control								
11	NA	NA	-	-	16	-	16	-
12	NA	NA	-	-	32	-	16	-
13	NA	NA	-	-	32	-	16	-
14	NA	NA	-	-	32	-	32	-
15	NA	NA	-	-	32	-	64	-
H3N8 control								
16	NA	NA	-	-	-	16	-	16
17	NA	NA	-	-	-	16	-	16
18	NA	NA	-	-	-	16	-	16
19	NA	NA	-	-	-	16	-	16
20	NA	NA	-	-	-	16	-	16

[a] Birds were inoculated via choanal cleft with a dose of $10^6 EID_{50}$ of either A/Mallard/MN/355779/00 (H5N2) and/or A/Mallard/MN/199106/99 (H3N8). Serologic data of the negative control group were omitted.

[b] H5N2xH3N8 group: exposed to H5N2 at 0 dpi, and subsequently challenged with H3N8 at 21 dpi; H3N8xH5N2: exposed to H3N8 at 0 dpi, and subsequently challenged with H5N2 at 21 dpi; H5N2 control group: exposed to H5N2 only; H3N8 control group: exposed to H3N8 only.

[c] Hemagglutination inhibition test using antigen against A/Mallard/MN/355779/00 (H5N2) or A/Mallard/MN/199106/99 (H3N8). Samples with HI titer ≥ 8 were considered positive.

[d] Abbreviations: dpi = days post primary inoculation; dpc = days post heterosubtypic challenge; NA = non applicable; - = negative.

Table 5.2. Oropharyngeal and cloacal viral shedding pattern of Mallards (*Anas platyrhynchos*) after challenge with heterosubtypic low pathogenicity avian influenza viruses[a].

Groups[b]	Viral Shedding, range (mean), days	
	Oropharyngeal	Cloacal
H5N2xH3N8	2-11 (4.2)	2-7 (4.2)
H3N8xH5N2	2-11 (3.8)	0-4 (2.4)
H5N2	4-16 (6.4)	4-14 (6.0)
H3N8	4-11 (9.2)	4-16 (4.4)

[a] Birds were inoculated via choanal cleft with a dose of $10^6 EID_{50}$ of either A/Mallard/MN/355779/00 (H5N2) and/or A/Mallard/MN/199106/99 (H3N8).

[b] H5N2xH3N8 group: exposed to H5N2 at 0 days post primary inoculation (dpi), and subsequently challenged with H3N8 at 21 dpi; H3N8xH5N2: exposed to H3N8 at 0 dpi, and subsequently challenged with H5N2 at 21 dpi; H5N2 control group: exposed to H5N2 only; H3N8 control group: exposed to H3N8 only.

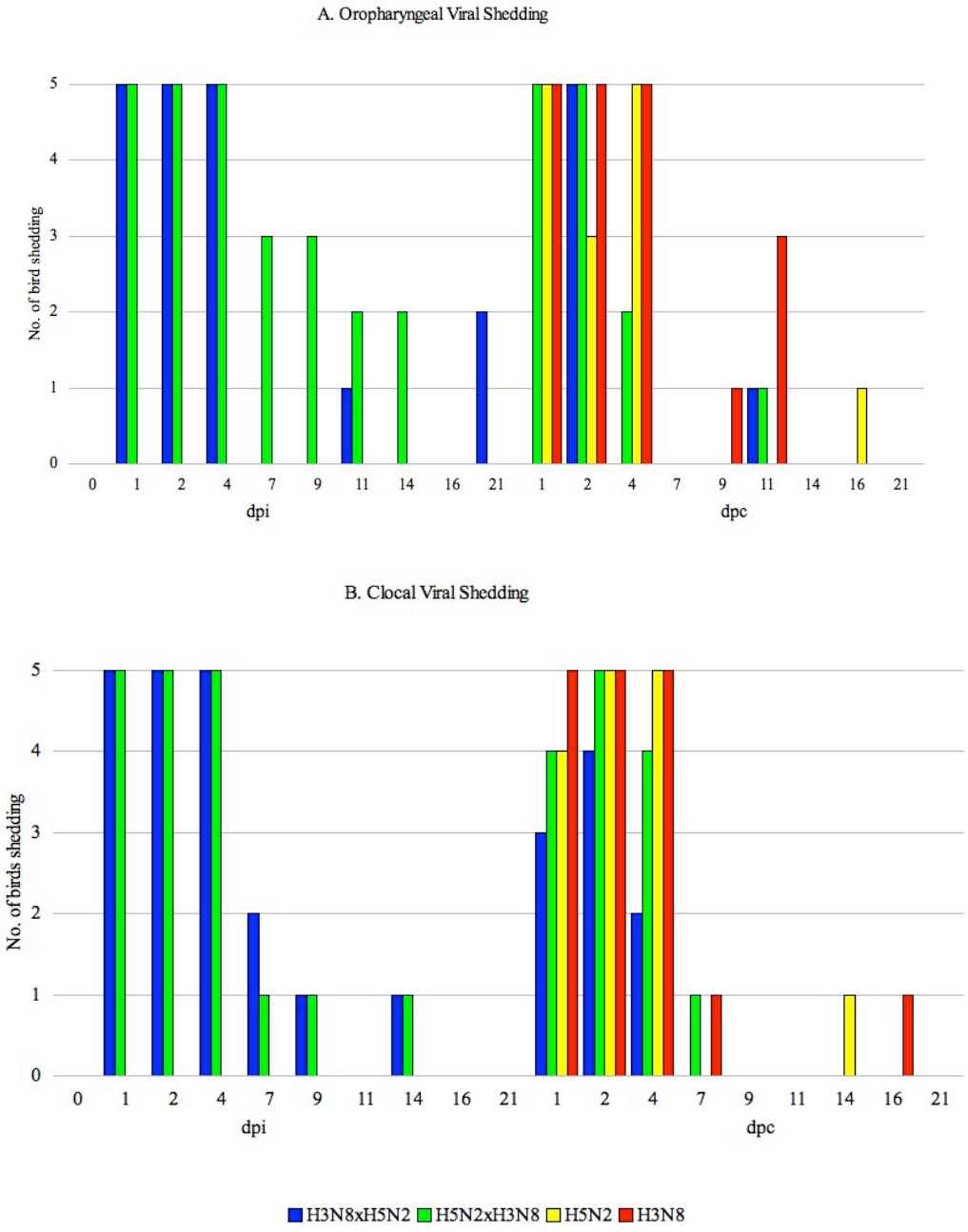

Figure 5.1. Oropharyngeal (A) and cloacal (B) viral shedding of mallards (*Anas platyrhynchos*) experimentally inoculated with 10^6 EID_{50} of either A/Mallard/MN/355779/00 (H5N2) and/or A/Mallard/MN/199106/99 (H3N8), at 21 days post primary inoculation (dpi) and 21 days post heterosubtypic challenge (dpc). Co-infection groups: H5N2xH3N8 - exposed to H5N2 at 0 dpi, and subsequently challenged to H3N8 at 21 dpi; H3N8xH5N2 - exposed to H3N8 at 0 dpi, and subsequently challenged to H5N2 at 21 dpi. Single inoculation groups: H5N2 - exposed to H5N2 only; H3N8 - exposed to H3N8 only (these single inoculation groups were included in the trial at 21 dpi).

CHAPTER 6

EFFECT OF HOMOSUBTYPIC AND HETEROSUBTYPIC LOW PATHOGENIC AVIAN INFLUENZA EXPOSURE ON H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS INFECTION IN WOOD DUCKS (*Aix sponsa*)[4]

[4] Costa, T.P., J.D. Brown, E.W. Howerth, D.E. Stallknecht, and D.E. Swayne. To be submitted to *Veterinary Pathology*.

Abstract

Wild birds in the Orders Anseriformes and Charadriiformes are the natural reservoirs for avian influenza (AI) viruses. Although they are often infected with multiple AI viruses, the significance and extent of acquired immunity in these populations is not understood. Pre-existing immunity to AI virus has been shown to modulate the outcome of a highly pathogenic avian influenza (HPAI) virus infection in multiple domestic avian species, but few studies have addressed this effect in wild birds. In this study, the effect of pre-exposure to homosubtypic (hemagglutinin homologous) and heterosubtypic (hemagglutinin heterologous) low pathogenic avian influenza (LPAI) viruses on the outcome of a H5N1 HPAI virus infection in Wood Ducks (*Aix sponsa*) was evaluated. A LPAI virus pre-exposure did not prevent Wood Ducks from infection with and shedding of the H5N1 HPAI virus, but decreased the mortality rate and increased the mean time to death after HPAI challenge. The magnitude of this effect on the outcome of the HPAI virus infection varied between LPAI viruses, and was associated with efficient LPAI viral replication and the development of a humoral immune response. These observations suggest that in natural occurring HPAI outbreaks, birds with pre-existing immunity to AI with homologous hemagglutinin or neuraminidase may either survive the HPAI infection or live longer than naïve birds, potentially posing a greater risk for contributing to viral transmission and dissemination. The mechanisms responsible for this protection and the duration of this protective immunity remain unknown. The results of this study are important for surveillance efforts and help clarify epidemiological data from outbreaks in wild bird populations.

Keywords: *Aix sponsa*, avian influenza virus, challenge, experimental infection, H5N1, HPAI, immunity, LPAI.

Wild birds, especially those in the Orders Anseriformes and Charadriiformes, are the natural reservoirs for all known hemagglutinin (HA) (H1-H16) and neuraminidase (NA) (N1-N9) subtypes of avian influenza (AI) viruses (Hinshaw et al., 1980b; Stallknecht and Shane, 1988; Webster et al., 1992; Süss et al., 1994; Olsen et al., 2006). It has been further suggested that wild birds might be involved in the spread, transmission to humans, and persistence of H5N1 highly pathogenic avian influenza (HPAI) virus (Kilpatrick et al., 2006; Gaidet et al., 2008; Keawcharoen et al., 2008).

Concurrent or successive infections with different low pathogenic avian influenza (LPAI) subtypes have been reported in wild bird populations and in poultry (Hinshaw et al., 1980a; Sharp et al., 1997; Jonassen and Handeland, 2007; Dugan et al., 2008), and an experimental infection trial with Pekin Ducks (*Anas platyrhynchos domesticus*) has shown that a primary inoculation with a LPAI induces partial protection against a secondary inoculation with the same virus (Kida et al., 1980). Pre-exposure to LPAI viruses has a partial protective effect on H5N1 HPAI virus infection in Canada Geese (*Branta canadensis*) (Pasick et al., 2007; Berhane et al., 2010), Mute Swans (*Cygnus olor*) (Kalthoff et al., 2008), Mallards (*Anas platyrhynchos*) (Fereidouni et al., 2009), and chickens (Seo and Webster, 2001; Seo et al., 2002; van der Goot et al., 2003). Such field immunity is one explanation for reduced morbidity observed in a naturally occurring H5N1 HPAI outbreak in poultry on Hong Kong (Webster et al., 2006), and in wild birds in Germany, despite the presence of several hundred thousand birds in the affected area (Globig et al., 2009). The mechanisms of this protective effect, however, are still poorly defined.

To better understand the epidemiology of H5N1 HPAI virus infections in wild bird populations, we experimentally investigated the effects that pre-exposure with homosubtypic (HA homologous) (H5N1, H5N2) and heterosubtypic (HA heterologous) (H1N1) LPAI viruses

had on the outcome of a Eurasian H5N1 HPAI virus challenge in Wood Ducks (*Aix sponsa*). The LPAI viruses used in this study belong to North American and Eurasian lineages. The Wood Duck was chosen as the experimental animal model due to the availability of data on the susceptibility, viral shedding patterns, and pathobiology associated with H5N1 HPAI virus infection in this highly susceptible wild avian species (Brown et al., 2006; Brown et al., 2007).

Materials and Methods

Animals. Twenty-five four-month-old Wood Ducks were purchased from a commercial source (Chenoa Waterfowl, Martin, Tennessee, USA). Both males and females were included in approximately equal numbers. Food and water were provided *ad libitum*. General animal care was provided under an animal use protocol approved by the Institutional Animal Care and Use Committee at both The University of Georgia and the Southeast Poultry Research Laboratory (SEPRL), United States Department of Agriculture, Agricultural Research Service, Athens, GA, USA.

Viruses. Three North American and one Eurasian wild bird-origin LPAI viruses were included in this study and used for pre-exposure inoculation. The LPAI virus strains included: A/mallard/MN/355779/00 (H5N2) and A/blue-winged teal/LA/B228/86 (H1N1) (Southeastern Cooperative Wildlife Disease Study [SCWDS], Athens, Georgia), A/mute swan/MI/451072-2/06 (H5N1) (SEPRL, Athens, Georgia), and A/mallard/Netherlands/2/05 (H5N2) (Ron Fouchier, Erasmus University, Rotterdam, The Netherlands). The H5N1 HPAI isolate A/whooper swan/Mongolia/244/05 (H5N1) (Mongolia/05), obtained from the SEPRL, was used for challenge virus. The Mongolia/05, which appears to be a genetically representative isolate from the wild bird HPAI virus (H5N1) (clade 2.2) that has been reported in Asia, Europe, and Africa

(Brown et al., 2006), was originally isolated from a dead whooper swan during a large die-off of waterfowl, and it was chosen for use in this study because of its established lethality in wild waterfowl, particularly Wood Ducks, under natural and experimental conditions (Ellis et al., 2004; Brown et al., 2006; Brown et al., 2007).

Individual stocks of the LPAI and the HPAI viruses used in this study were produced by second passage in 9-day-old specific pathogen free (SPF) embryonating chicken eggs (ECE). Serial titrations were performed in SPF ECE and median embryo infectious dose (EID_{50}) titers were determined by testing hemagglutination activity as previously described (Killian, 2008). Allantoic fluid from the inoculated eggs was diluted in sterile brain-heart-infusion (BHI) medium to yield a final titer of 10^6 EID_{50} per 0.1 mL for LPAI viruses and 10^4 per 0.1 mL for the HPAI (single bird inoculum).

Experimental Design. Wood ducks were randomly assembled in groups of five and each group was housed separately in biocontainment isolation units ventilated under negative pressure with a high efficiency particulate air filters on intake and exhaust. After a 4-day acclimation period, the four LPAI-exposure groups were inoculated via choanal cleft into the middle nasal cavity with 0.1 mL of one of the four LPAI viruses and designated accordingly: A/mallard/Netherlands/2/05 (H5N2) group; A/blue-winged teal/LA/B228/86 (H1N1) group; A/mute swan/MI/451072-2/06 (H5N1) group; and A/mallard/MN/355779/00 (H5N2) group. One group was not experimentally exposed to a LPAI virus and served as a negative control (naïve group). Prior to the exposure to the LPAI virus, blood was collected from all birds to test whether any of the Wood Ducks possessed antibodies to avian influenza virus. In addition, cloacal and oropharyngeal (OP) swabs were collected from each bird for virus isolation prior to LPAI pre-exposure to verify that no birds were shedding virus at the start of the study. After inoculation, birds were visually

evaluated daily by observation for behavior changes and/or the development of any clinical signs. Cloacal and OP swabs were collected from all birds at 0, 2, 4, 7 and 21 days post-LPAI virus exposure (LPAIV-dpe), and blood samples were collected at 0 and 21 LPAIV-dpe. At 21 LPAIV-dpe, the four treatment groups and the naïve group were challenged via choanal cleft with 0.1 mL of Mongolia/05. Cloacal and OP swabs were collected from all the birds at 0, 1, 2, 4, 8, and 10 days post-HPAI virus challenge (HPAIV-dpc), and from birds that were found dead or were euthanized due the severity of their clinical condition. Blood samples were collected at 10 HPAIV-dpc. Birds were evaluated daily as described above. Wood Ducks exhibiting severe clinical signs (severe paresis or paralysis and/or inability to eat or drink) were euthanized with intravenous administration of sodium pentobarbital (100 mg/kg), and a full necropsy was performed. The experiment was terminated at 10 HPAIV-dpc at which time all the remaining birds were euthanized as described above and full necropsies were performed. Experimental infections were performed in a Biosafety Level-3-Enhanced facility at the SEPRL.

Serology. Blood samples were collected from the right jugular vein at three time points: 0 and 21 LPAIV-dpe, and 10 HPAIV-dpc. Serum samples were stored at -20°C until tested for the presence of influenza A antibodies. Samples were tested using the multiS-screen blocking enzyme-linked immunosorbent assay (bELISA) AIV antibody test kit (IDEXX laboratories, Westbrook, Maine) according to the manufacturer's instructions. All samples were further analyzed with the hemagglutination inhibition (HI) test following procedures previously described (Pedersen, 2008), using a 0.5% suspension of chicken erythrocytes in phosphate-buffered saline. The HI test was performed with antisera specific for the LPAI viruses used for pre-exposure and the Mongolia/05 used for challenge. The serum was pre-treated with chicken

red blood cells to neutralize any naturally occurring serum hemagglutinins and the first dilution on the test plate was 1:2. All HI titers ≥ 8 were considered positive.

Virus Isolation. Cloacal and OP swabs were collected in brain-heart-infusion (BHI) medium with antimicrobial drugs (100 μg/mL gentamicin, 100 units/mL penicillin, and 5 μg/mL amphotericin B). Samples were stored at -70°C until testing was performed. Isolation of virus from the swabs was performed in the allantoic sac of 9-day-old SPF ECE using standard procedures, and allantoic fluid was harvested and tested for the presence of hemagglutinating activity (Woolcock, 2008).

Histopathology and Immunohistochemistry. Samples of cerebrum, cerebellum, heart, lung, trachea, liver, spleen, esophagus, proventriculus, ventriculus, small intestine, large intestine, pancreas, adrenal, ovaries/testis, kidney, cloacal bursa, pectoral muscle, femur, turbinates and sinus were collected from all the animals and were fixed in 10% buffered formalin for histopathology and immunohistochemistry (IHC) analysis. After fixation, the tissues were processed and embedded in paraffin, and 5μm sections were stained with hematoxylin and eosin using standard histopathology protocols. Femur and turbinates were decalcified with Kristensen's decalcifying solution before being processed. Duplicate sections were immunohistochemically stained by using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen as the primary antibody (SEPRL, Athens, Georgia, USA). Procedures used to perform the immunohistochemical testing have been previously described (Pantin-Jackwood, 2008). Fast red was used as the substrate chromogen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromogen deposition in the nucleus, with or without chromogen deposition in the cytoplasm.

Statistical Analysis. Chi-square (χ^2) distribution with a level of significance (α) of 0.05 was employed to analyze the relation between survival rate following H5N1 HPAI challenge and the serological status upon arrival.

Results

Status Upon Arrival

Twelve of the 24 Wood Ducks tested positive for influenza A virus nucleoprotein antibodies with the bELISA upon arrival (0 LPAIV-dpe) (Table 6.1). These birds were distributed among the treatment groups. Serum samples were further analyzed with a HI test using antisera specific for the LPAI viruses used for pre-exposure and for the Mongolia/05 used for challenge. All the birds were negative on HI upon arrival, indicating the absence of pre-existing antibodies against the homosubtypic LPAI and Mongolia/05 virus. Cloacal and OP swab specimens collected at 0 LPAIV-dpe were negative on virus isolation, indicating that the birds were not infected upon arrival.

LPAI Pre-exposure

All Wood Ducks in each of the four LPAI virus exposure groups remained clinically and behaviorally normal, and had an average weight gain of 8.9% (2.0 - 15.3%) during the 21 days following the LPAI virus pre-exposure.

Detection of viral shedding (Table 6.1) and seroconversion, as determined by HI (Tables 6.1 and 6.2), varied between treatment groups. A/mallard/Netherlands/2/05 (H5N2) did not replicate in any of the inoculated Wood Ducks, as demonstrated by lack of viral shedding and seroconversion (by both bELISA and HI) in this treatment group. The A/mute swan/MI/451072-2/06 (H5N1) was recovered from only two Wood Ducks (birds number 9 and 10) in OP swabs

collected at 2 LPAIV-dpe. Although both of these birds were seronegative at 21 LPAIV-dpe by H5 HI (Table 6.2), antibodies were detected by bELISA in one of them (bird number 9).

Viral shedding was detected in four Wood Ducks (birds number 11, 12, 13, and 14) inoculated with A/blue-winged teal/LA/B228/86 (H1N1) from 4 to 7 LPAIV-dpe (mean 4.75 days), and did not differ between birds with and without pre-existing antibodies upon arrival (as determined by bELISA). Three out of the four birds that shed the A/blue-winged teal/LA/B228/86 (H1N1) virus (birds number 12, 13, and 14) seroconverted, as detected by H1 specific HI. The A/mallard/MN/355779/00 (H5N2) virus efficiently replicated in all the five inoculated Wood Ducks independent of bELISA antibody status upon arrival, and the duration of viral shedding ranged from 2 to 4 LPAIV-dpe (mean 2.4 days). Four birds in this group seroconverted, as detected by bELISA and H5 HI.

A/whooper swan/Mongolia/244/05 (H5N1) Challenge

Serological Status. Serological status as determined by HI at 21 LPAIV-dpe (against the homosubtypic LPAI viruses used for pre-exposure) and at 10 HPAIV-dpc (against Mongolia/05) is shown in Table 6.2. Wood ducks that survived HPAI virus infection developed HI titers against the challenge virus ranging from 8 to 512 at 10 HPAIV-dpc. Serological data of the naïve group were omitted from Table 6.3 due the 100% mortality observed in this group after Mongolia/05 challenge. Data of the A/mallard/Netherlands/2/05 (H5N2) group also were omitted as none of these birds seroconverted after the LPAI virus pre-exposure, and four of five died. The only Wood Duck in this group that survived the Mongolia/05 challenge had an HI titer of 16 at 10 HPAIV-dpc. The highest HI titers against Mongolia/05 were observed in the A/mute swan/MI/451072-2/06 (H5N1) treatment group.

Following the Mongolia/05 challenge (at 10 HPAIV-dpc), all of the surviving birds had an increase in the HI titer against the LPAI virus used for pre-exposure; this was particularly evident within the A/mute swan/MI/451072-2/06 (H5N1) and A/mallard/MN/355779/00 (H5N2) groups. Three of four birds that succumbed to HPAI virus infection (two birds in the A/blue-winged teal/LA/B228/86 (H1N1) group and one bird in the A/mute swan/MI/451072-2/06 (H5N1) group) had negative HI titers (< 8) against the homosubtypic LPAI virus used for pre-exposure (Table 6.2).

Morbidity and Mortality. At 21 LPAIV-dpe, Wood Ducks in the four LPAI pre-exposure groups and the naïve group (not pre-exposed to a LPAI virus) were challenged with 10^6 EID_{50} of Mongolia/05 HPAI virus. Wood ducks that succumbed to HPAI virus challenge (n=13) progressively lost weight, while the ones that survived (n=12) showed an initial weight loss during the first days after HPAI virus infection, followed by a progressive weight gain throughout the remainder of the trial. All Wood Ducks that succumbed to infection either exhibited premonitory neurologic signs or were found dead. The onset of morbidity or death ranged from 4 to 8 HPAIV-dpc. Clinical signs varied from mild to severe and included lethargy, incoordination, paresis, walking in circles, and head circling and tremors. One Wood Duck in the naïve group showed a unilateral cloudy blue eye one day before it was found dead at 5 HPAIV-dpc.

Mortality rates varied greatly among groups, ranging from 0 to 100% (Table 6.1). All five Wood Ducks in the naïve group, which were not pre-exposed to any of the LPAI viruses, died, with a mean death time (MDT) of 5 days (range 4-6 days). Among the LPAI virus pre-exposure groups, the observed mortality rates were 80% (4/5) in the A/mallard/Netherlands/2/05 (H5N2) group (MDT of 6 days; range 6 days), 60% (3/5) in the A/blue-winged teal/LA/B228/86

(H1N1) group (MDT of 6 days; range 5-7 days), and 20% (1/5) in the A/mute swan/MI/451072-2/06 (H5N1) group (MDT of 8 days). Neither morbidity nor mortality was observed in the group pre-exposed to the A/mallard/MN/355779/00 (H5N2). The serological status of the Wood Ducks upon arrival had minimal if any effect on the outcome of H5N1 HPAI virus infection, as demonstrated by similar mortality rates for birds within each group (Table 6.1); statistical analyses revealed that the survival rate following H5N1 HPAI challenge was not associated with the serological status upon arrival ($\chi^2 = 0.987$, $P>0.05$).

Viral Shedding. H5N1 HPAI virus was isolated from every bird after challenge. In general, viral shedding via oropharynx was greater than via cloaca, as demonstrated by a higher number of isolations from OP than cloacal swabs (Table 6.3). Viral shedding via oropharynx was consistent from 1 to 4 HPAIV-dpc and intermittent up to 10 HPAIV-dpc. Cloacal shedding was less consistent, and was detected from 1 to 10 HPAIV-dpc, with the highest number of virus isolations at 4 HPAIV-dpc. Furthermore, cloacal shedding was detected more frequently in birds that succumbed to HPAI infection than in birds that survived.

Necropsy Findings. Gross lesions were not detected in any of the Wood Ducks that survived the HPAI virus challenge and necropsied at 10 HPAIV-dpe. All the Wood Ducks that succumbed to HPAI virus infection had one or more of the following gross lesions: multifocal to coalescing areas (1-7mm in diameter) of red mottling in pancreas (10/13), congestion of meningeal vessels of cerebrum and cerebellum (7/13), splenomegaly (5/13), multiple red foci in adrenal gland (4/13), and a small tan spleen (2/13).

Histopathology and Immunohistochemistry. The histopathologic lesions observed in Wood Ducks that succumbed to infection and those that survived the Mongolia/05 challenge are described on Table 6.4 and illustrated on Figures 6.1-6.5. Among the 13 Wood Ducks that

succumbed, the most consistent microscopic lesions were observed in the central nervous system (CNS), pancreas, liver, and adrenal gland. Lesions of the CNS were characterized by moderate to severe, multifocal to coalescing, nonsuppurative encephalitis and neuronal necrosis. Moderate to severe choroiditis (Figure 6.1a), meningitis, gliosis, vacuolar degeneration (associated or not with lymphoplasmacytic infiltrate), and perivascular cuffing were also observed in the CNS. Pancreatic lesions were characterized by severe multifocal to coalescing necrosis of acinar cells (Figure 6.2). Hepatic changes included multifocal hepatocellular necrosis and portal/perivascular hepatitis. Severe multifocal to coalescing necrosis of corticotrophic and chromaffin cells was seen in adrenal glands. Other variably detected microscopic lesions included: neuritis and necrosis of myenteric plexus of small intestine, shortening and fusion of intestinal villi, orchitis, oophoritis, pulmonary edema, tracheitis, sinusitis, necrosis of follicles in cloacal bursa, and degeneration of sheathed capillaries, lymphoid necrosis, and subcapsular hemorrhage in spleen (Table 6.4).

Positive IHC staining in association with histological lesions was consistently observed in nervous, digestive, and endocrine systems (Table 6.5, Figure 6.1) of the Wood Ducks that succumbed to Mongolia/05 virus infection, and included: neurons and glial cells of cerebrum (Figure 6.3), cerebellum, and brain stem, epithelial cells of choroid plexus (Figure 6.1b), neurons of myenteric and submucosal plexus of small and large intestines (Figure 6.4), Kupffer cells, hepatocytes, acinar pancreatic cells, and corticotrophic and chromaffin cells of adrenal gland, epithelial cells of trachea, epithelial cells of the seminiferous tubules, and granulosa cell layer of ovarian follicle. Positive immunohistochemical staining in the absence of microscopic lesions was observed in cerebrum (4/13), cerebellum (3/13), brain stem (4/13), submucosal (8/13) and myenteric (5/13) plexus of small and large intestines, and occasionally observed in meninges

(1/13), salivary gland (1/13), cloacal bursa (1/13), testis (1/13), heart (2/13), nasal sinus (1/13), globet cells of trachea (1/13), lung (1/13) and skin (2/13) of the Wood Ducks that succumbed to Mongolia/05 virus infection (Table 6.5).

Among the 12 Wood Ducks that survived virus challenge and were necropsied at 10 HPAIV-dpc, the microscopic lesions included portal/perivascular lymphoplasmacytic hepatitis, splenic white pulp hyperplasia, severe lymphoplasmacytic encephalitis characterized by marked perivascular cuffing (associated or not with meningitis) (Figure 6.5), neuritis of myenteric plexus of small intestine, pulmonary edema, and splenic amyloidosis and subcapsular hemorrhage (Table 6.4). Severe lymphoplasmacytic encephalitis associated with scattered positive IHC staining in neurons and glial cells of cerebrum was observed on two birds that survived the Mongolia/05 challenge.

Discussion

Wood ducks used in this study were acquired from a duck farm where several species of Anseriform and Galliform birds were raised outdoors and had direct contact with wild birds. The rearing of poultry outdoors is a major risk factor for avian influenza virus introduction (Swayne, 2008), and explains the fact that 12 out of 25 Wood Ducks used in this study tested positive for the presence of influenza A type-specific antibodies upon arrival. Further testing revealed that all the birds were seronegative on the HI assay for the presence of pre-existing antibodies directed against the HA of the LPAI (H1 and H5) and the Mongolia/05 (H5) viruses used in this experiment. Although unexpected, the presence of individual birds with pre-existing influenza A antibodies to nucleoprotein, which are non-neutralizing and non-protective, did not preclude us from acquiring useful data from this study. As shown on Table 6.1, in each of the LPAI virus-

exposure groups, the difference in the mortality rate observed between Wood Ducks that tested positive for nucleoprotein antibodies upon arrival and those that tested negative was minimal (more or less one bird). This was confirmed by statistical analyses, which revealed that the survival rate following H5N1 HPAI challenge was not influenced by the serological status upon arrival.

The influence of pre-exposure to a LPAI virus on the outcome of a HPAI challenge varied from total protection (0% morbidity/mortality) to no protection (100% mortality) (Table 6.1). The magnitude of this protection varied among the LPAI viruses used for pre-exposure, and was strongly related with their ability to replicate and the development of a humoral immune response. For instance, all the Wood Ducks in the naïve group died, confirming the high susceptibility of this bird species to H5N1 HPAI virus observed in previous studies (Brown et al., 2006; Brown et al., 2007). The North American A/mallard/MN/355779/00 (H5N2) and A/mute swan/MI/451072-2/06 (H5N1) viruses (both homosubtypic to the Mongolia/05 challenge virus) induced the highest protection among the groups as demonstrated by the lowest mortality rates (0 and 20%, respectively) after HPAI virus challenge (Table 6.1). Alternatively, the Eurasian isolate A/mallard/Netherlands/2/05 (H5N2) virus (also homosubtypic to the Mongolia/05 challenge virus) did not replicate in the Wood Ducks and, consequently, provided no protective immunity. This was reflected in the high mortality rate (80%) of this pre-exposure group. The A/blue-winged teal/LA/B228/86 (H1N1) isolate (heterosubtypic to the Mongolia/05 challenge virus) provided partial protection against the Mongolia/05 virus, as evidenced by comparatively moderate mortality (60%). These observations suggest that although humoral immunity against HA, and possible NA in one group, seems to play an important role, as observed in previous studies (Chambers et al., 1988; Webster et al., 1991; Swayne and

Kapczynski, 2008), an efficient replication of the LPAI virus is necessary to induce protection against the H5N1 HPAI virus, and the role of cell mediate immunity (CMI) may play an additional protective role, as evident by one H5 HI negative duck surviving challenge. Further investigation is needed to clarify the role of humoral immunity against NA and CMI in the development of protection against the HPAI challenge.

An adequate humoral immune response to HA seems to be fundamental for the protection against the challenge virus in multiple avian species (Swayne and Kapczynski, 2008). In the current study, three out of four Wood Ducks that succumbed to H5N1 HPAI virus infection (two birds in the A/blue-winged teal/LA/B228/86 [H1N1] group and one bird in the A/mute swan/MI/451072-2/06 [H5N1] group) had a negative HI titer against the homologous HA at 21 LPAIV-dpe when they were subsequently challenged with the Mongolia/05 virus (Table 6.2). Moreover, a higher HI titer against the homologous LPAI isolate at 10 HPAIV-dpc was observed, particularly in the surviving birds in the groups A/mute swan/MI/451072-2/06 (H5N1) and A/mallard/MN/355779/00 (H5N2) (Table 6.2). This increase in titer may be either an effect of the H5N1 HPAI virus challenge or related to the timing of the original immune response (31 after the LPAI exposure).

The A/whooper swan/Mongolia/244/05 (H5N1) virus replicated systemically in Wood Ducks and had a strong tropism for the CNS, pancreas, and adrenal gland, and failure and/or dysfunction of one of multiple of these organs was likely the cause of death. Cloudy eye, as observed in one Wood Duck in the naïve group, has been previously reported as a clinical sign of HPAI virus infection in birds (Sturm-Ramirez et al., 2004; Sturm-Ramirez et al., 2005; Brown et al., 2007; Brown et al., 2008b, a; Kim et al., 2008; Fereidouni et al., 2009). Although no histopathological analyses of the eye of the Wood Duck in this study was performed, the

cloudiness in the eye is probably the deposition of fibrin or white blood cells in the anterior chamber or corneal edema, both of which can be features of uveitis.

Oropharyngeal shedding was more pronounced than cloacal shedding (Table 6.3). The viral tropism and shedding pattern aforementioned have been observed in other experimental studies in birds inoculated with H5N1 HPAI viruses (Sturm-Ramirez et al., 2005; Brown et al., 2006; Brown et al., 2007; Brown et al., 2008a; Fereidouni et al., 2009). In this study, viral shedding was not completely suppressed in any of the groups of Wood Ducks pre-exposed to a LPAI virus, contrary to what was observed in a previous experimental investigation with Mallards (Fereidouni et al., 2009). Although Wood Ducks that succumbed to infection died between 4 and 8 HPAIV-dpc, the cloacal viral shedding observed in these birds was more prominent than in birds that survived the HPAI virus challenge (Table 6.3). This is the result of widespread viral replication, supported by the presence of viral antigen in adrenal glands and gastrointestinal (pancreas, liver, and myenteric and submucosal plexus of small and large intestines) and reproductive (ovaries and testicle) tracts (Tables 6.4 and 6.5, Figure 6.1), both of which contribute to fecal excretion of the virus. Therefore, Wood Ducks that succumb to HPAI virus infection would potentially play an important role in environmental contamination and subsequent transmission.

With regard to the potential movement of HPAI virus with migrating infected birds, three observations from this study suggest that previous exposure to an LPAI virus may affect this potential. First, previous exposure prevented disease but not infection with or viral shedding of HPAI H5N1. Second, a clear increase of the MDT was observed among the treatment groups (Table 6.1), suggesting that, upon a H5N1 HPAI challenge, birds with pre-existing immunity to a LPAI virus, live longer than naïve birds and, therefore, the duration of viral shedding is

increased. Third, a pre-exposure to a LPAI virus decreases mortality rate after a H5N1 HPAI virus challenge, and, as observed in this study, birds that survive may shed the virus via cloaca and oropharynx up to 10 HPAIV-dpc (Table 6.3) without showing any clinical signs of HPAI infection, further contributing to virus transmission and dispersal.

This study demonstrates that the degree of protection against the H5N1 HPAI virus varies in response to the LPAI virus subtype that the bird was previously infected with. The mechanisms responsible for this protection, as well as the extent (between different subtypes) and duration of this protective immunity still need to be elucidated in order to understand the epidemiology of AI viruses in waterfowl reservoirs and to better define the potential risks of new viruses, such as H5N1 HPAI virus, to infect, move with, or become established in wild bird populations.

Acknowledgement

We thank the personnel of the Southeast Poultry Research Laboratory for their technical assistance, especially J. Beck, J. Doster, and K. Moresco. We deeply appreciate Dr. Fouchier for providing the Asian H5N1 HPAI virus. Funding for this work was provided through specific Cooperative Agreement #58-6612-2-0220 between the Southeast Poultry Research Laboratory and the Southeastern Cooperative Wildlife Disease Study, through the continued sponsorship of SCWDS member state and federal agencies, and Current Research Information System project 6612-32000-048-00D.

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Table 6.1. Serological status before and after the experimental low pathogenicity avian influenza (LPAI) virus exposure, virus isolation data, and mortality rate of Wood Ducks (*Aix sponsa*) experimentally inoculated[a] with different subtypes of LPAI viruses and subsequently challenged with the highly pathogenic avian influenza (HPAI) isolate A/whooper swan/Mongolia/244/05 (H5N1)[b].

GROUPS[c]	Serological status upon arrival	LPAI virus Pre-exposure		HPAI virus Challenge			
		Virus Isolation	Serology	Virus Isolation	Mortality		
	bELISA result (n) [e,f]	Prevalence, no. positive/total	HI[g] no. positive/total	Prevalence, no. positive/total	no. deaths/total (%)	% (n=5)	MDT (d) (n=5)
Naïve[d]	+ (1)	NA	NA	1/1	1/1 (100)	100	5
	- (4)	NA	NA	4/4	4/4 (100)		
A/mallard/Netherlands/2/05 (H5N2)	+ (2)	0/2	0/2	2/2	2/2 (100)	80	6
	- (3)	0/3	0/3	3/3	2/3 (66)		
A/blue-winged teal/LA/B228/86 (H1N1)	+ (2)	2/2	2/2	2/2	1/2 (50)	60	6
	- (3)	2/3	1/3	3/3	2/3 (66)		
A/mute swan/MI/451072-2/06 (H5N1)	+ (3)	0/3	0/3	3/3	1/3 (33)	20	8
	- (2)	2/2	0/2	2/2	0/2 (0)		
A/mallard/MN/355779/00 (H5N2)	+ (4)	4/4	3/4	4/4	0/4 (0)	0	NA
	- (1)	1/1	1/1	1/1	0/1 (0)		

[a] Birds were pre-exposed via choanal cleft with a dose of $10^6 EID_{50}$

[b] Birds were challenged via choanal cleft with a dose of $10^4 EID_{50}$ 21 days after experimental pre-exposure to LPAI viruses.

[c] Groups of Wood Ducks (n=5) experimentally pre-exposed to different LPAI viruses.

[d] The naïve group was not experimentally pre-exposed to LPAI virus.

[e] Abbreviations: bELISA = blocking ELISA; HI = hemagglutination inhibition; MDT = mean death time (days); + = positive; - = negative; NA = non applicable.

[f] bELISA result (number of birds). Twelve out of 25 birds had avian influenza nucleoprotein antibodies at the beginning of the trial, before experimental exposure to a LPAI virus.

[g] HI using antigen against homosubtypic LPAI virus, either H5N2, H1N1, or H5N1.

Table 6.2. Serological status, as determined by hemagglutination inhibition, of Wood Ducks (*Aix sponsa*) 21 days after experimental pre-exposure to low pathogenicity avian influenza (LPAI) viruses[a], and 10 days after challenge with the highly pathogenic avian influenza (HPAI) isolate A/whooper swan/Mongolia/244/05 (H5N1)[b].

Sera from exposed birds by group, bird ID	Hemagglutination Inhibition Result[c]		
	Homosubtypic LPAI virus antigen[d]		A/whooper swan/244/ 05 antigen[e]
	21 LPAIV-dpe[f]	10 HPAIV-dpc	10 HPAIV-dpc
A/blue-winged teal/LA/B228/86 (H1N1)			
11	-	†	†
12	8	8	-
13	8	†	†
14	16	16	-
15	-	†	†
A/mute swan/MI/451072-2/06 (H5N1)			
6	-	128	16
7	-	†	†
8	-	256	128
9	-	128	32
10	-	256	512
A/mallard/MN/355779/00 (H5N2)			
16	8	128	8
17	-	256	-
18	16	64	-
19	16	1024	32
20	8	32	-

[a] Birds were inoculated via choanal cleft with a dose of $10^6 EID_{50}$ of different LPAI viruses. Serologic data of the naïve group were omitted due the 100% mortality observed in this group, and data of the A/mallard/Netherlands/2/05 (H5N2) group were omitted due lack of seroconversion.

[b] Birds were challenged via choanal cleft with a dose of $10^4 EID_{50}$ of A/whooper swan/Mongolia/244/05 (H5N1), 21 days after experimental pre-exposure to different subtypes of LPAI viruses.

[c] Samples with HI titer ≥ 8 were considered positive.

[d] HI using antigen against homosubtypic LPAI virus, either H1N1, H5N1, or H5N2.

[e] HI using antigen against A/whooper swan/Mongolia/244/05 (H5N1).

[f] Abbreviations: LPAIV-dpe = days after LPAI pre-exposure; HPAIV-dpc = days after H5N1 HPAI challenge; † = succumbed to HPAI H5N1 infection; - = negative.

Table 6.3. Clinical outcome and virus isolation data of Wood Ducks (*Aix sponsa*) challenged[a] with A/whooper swan/Mongolia/244/05 (H5N1).

Clinical Outcome[b]	Virus isolation, HPAIV-dpc[c,d]													
	1		2		4		5[e]		6[e]		7		10	
	OP	CLO	OP	CLO	OP	CLO	OP	CLO	OP	CLO	OP	CLO	OP	CLO
Succumbed	12/13	1/13	13/13	4/13	13/13	11/13	4/12	4/12	4/8	4/8	0/2	0/2	NA	NA
Survived	10/12	0/12	10/12	2/12	9/12	1/12	*	*	*	*	1/12	0/12	1/12	3/12

[a] Birds were challenged via choanal cleft with a dose of $10^4 EID_{50}$ of A/whooper swan/Mongolia/244/05 (H5N1), 21 days after experimental pre-exposure to different subtypes of LPAI viruses.
[b] Five out of 13 birds that succumbed and six out of 12 birds that survived had pre-existing antibodies against avian influenza.
[c] Abbreviation: HPAIV-dpc = days post H5N1 HPAI challenge; OP = oropharyngeal swab; CLO = cloacal swab; NA = non applicable; * = not tested.
[d] no. of birds that shed the virus/total.
[e] Only birds that died were tested. Therefore, 4/12 birds were tested at 5 HPAIV-dpc, and 7/8 were tested at 6 HPAIV-dpc.

Table 6.4. Histopathology lesions observed in Wood Ducks (*Aix sponsa*) that succumbed or survived after challenge with the highly pathogenic avian influenza virus A/whooper swan/Mongolia/244/05 (H5N1)[a].

System/Tissue	Lesion	Succumbed (n=13)	Survived (n=12)
Nervous			
Cerebrum	Neuronal degeneration, gliosis, perivascular cuffing, vacuolar degeneration	12	2
	Choroid plexus, necrosis, inflammation	7	0
Cerebellum	Purkinje cell necrosis, gliosis, perivascular cuffing, status spongiosus, gitter cells	10	0
	Meninges, inflammation, necrosis	2	1
Brain Stem	Vacuolation, gliosis, perivascular cuffing, central chromatolysis	10	1
Endocrine			
Pancreas	Acinar cells, necrosis	8	0
Adrenal Gland	Corticotrophic and chromaffin cells, necrosis	3	0
Digestive			
Liver	Necrosis, multifocal	10	0
	Perivascular/portal hepatitis	5	10
Small Intestine	Myenteric plexus, neuronal necrosis	2	0
	Myenteric plexus, neuritis	0	2
	Villi, shortening and fusion	1	0
Large Intestine	Villi, shortening and fusion	1	0
Reproductive			
Testis	Seminiferous tubules, necrosis	1	0
Ovary	Immature follicle, necrosis, inflammation	1	0
Respiratory			
Lung	Edema	4	2
Trachea	Tracheitis	1	0
Nasal Sinus	Sinusitis	1	1
Lymphoid			
Cloacal Bursa	Necrosis, follicle	1	0
Spleen	Sheathed capillaries, degeneration	1	0
	Lymphoid necrosis	1	0
	Subcapsular hemorrhage	1	1
	White pulp, hyperplasia	0	4
	Amyloidosis	0	1

[a] Wood Ducks were challenged via choanal cleft with a dose of $10^4 EID_{50}$ of A/whooper swan/Mongolia/244/05 (H5N1) 21 days after experimental pre-exposure to different subtypes of low pathogenic avian influenza viruses.

Table 6.5. Immunohistochemical analysis for nucleoprotein of avian influenza virus of wood ducks (*Aix sponsa*) that succumbed to infection (n=13) with the highly pathogenic avian influenza virus A/whooper swan/Mongolia/244/05 (H5N1)[a].

System/Tissue	Cell Type	No. of wood ducks	No. of positive cells[b]
Nervous			
Cerebrum	Neuron	8*	+++
	Epithelial cell of choroid plexus	8	+++
	Glial cells	6*	+++
	Ependymal cells	3	+++
	Endothelium of blood vessels	2	+
	Meninges	1**	++
Cerebellum	Purkinje cells	8	+++
	Neurons	5*	++
	Glial cells	7*	+++
Brain Stem	Epithelial cell of choroid plexus	6	+++
	Neurons	6*	+++
	Glial cells	6*	+++
	Endothelium	1	+
Digestive			
Salivary Gland	Epithelium	1**	+
Esophagus	Endothelium of submucosa	1	+
Intestines	Myenteric plexus	8*	++
	Submucosal plexus	9*	++
Liver	Kupffer cells	8	++
	Hepatocytes	1	++
Endocrine			
Pancreas	Acinar cells	6	+++
Adrenal Gland	Corticotrophic and chromaffin cells	4	+++
Respiratory			
Nasal Sinus	Epithelial cells	1**	+
	Perivascular staining	1**	+
Trachea	Epithelial cells	1	+
	Globet cells	1**	+
Lung	Epithelial cells	1**	+
	Secondary bronchus, submucosa	1**	+
Integumentary			
Skin	Epidermis, basal layer	2**	+
	Endothelial cells	1**	+
Cardiovascular			
Heart	Myocytes	2**	+
Reproductive			
Ovary	Follicle, granulosa cell layer	1	+
Testis	Seminiferous tubules	1	+
	Fibrovascular stroma	1**	+
Lymphoid			
Cloacal Bursa	Follicle, epithelial tuft	2	+
	Follicle, medulla	1**	+

[a] Wood ducks were challenged via choanal cleft with a dose of $10^4 EID_{50}$ of A/whooper swan/Mongolia/244/05 (H5N1) 21 days after experimental pre-exposure to different subtypes of low pathogenic avian influenza viruses.
[b] Numbers of immunohistochemically positive cells: + = few; ++ = moderate; +++ = numerous.
* Positive immunohistochemical staining associated or not with microscopic lesions.
** Positive immunohistochemical staining not associated with microscopic lesions.

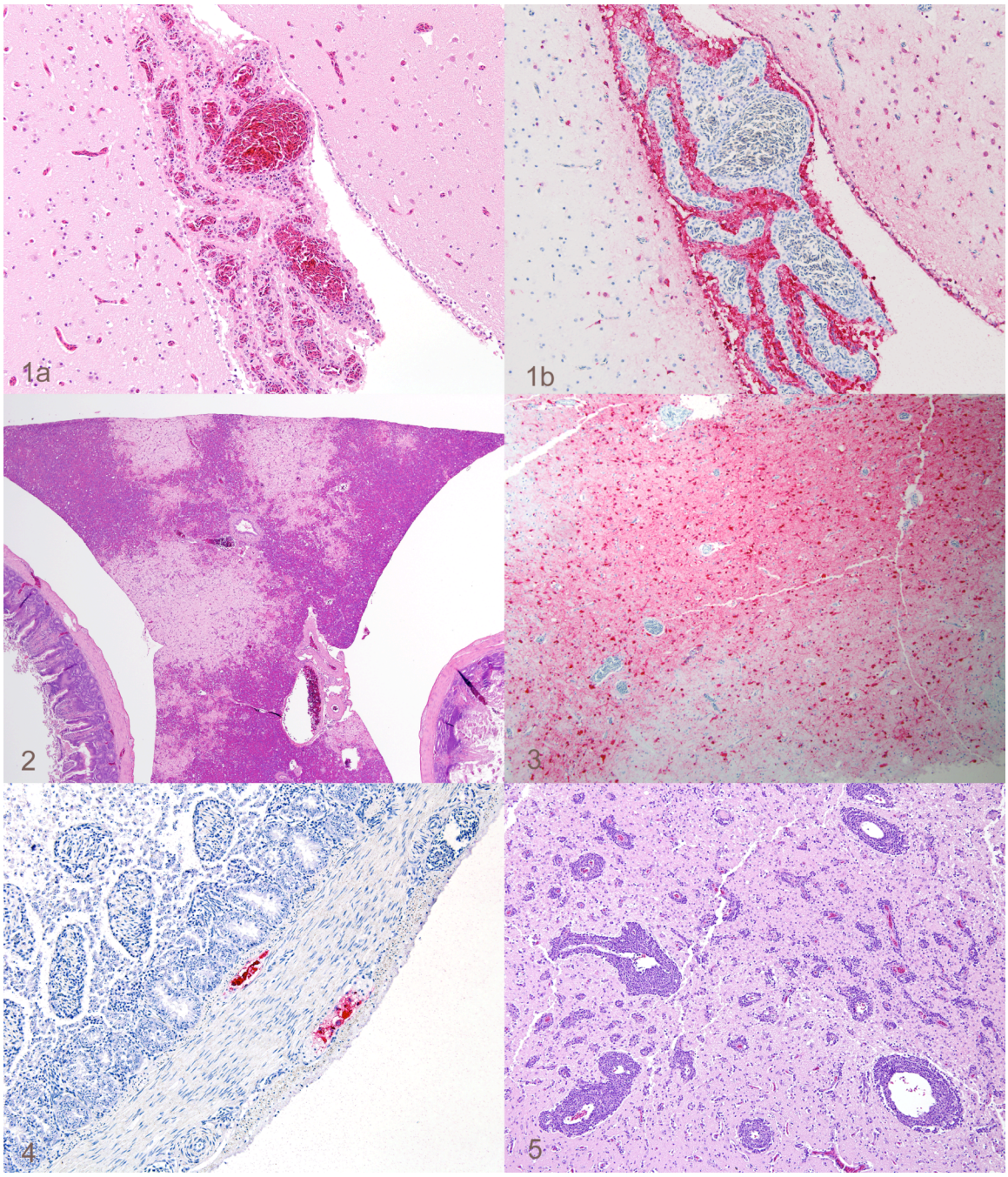

Figure 6.1a. Choroid plexus; Wood Duck that succumbed to Mongolia/05 infection. Severe choroiditis characterized by necrosis of epithelial cells of choroid plexus, vasculitis and heterophilic infiltrate. HE. Figure 6.1b. Positive staining in epithelial cells of choroid plexus. Immunohistochemical staining: mouse-derived monoclonal antibody (P13C11), biotin-streptavidin alkaline-phosphatase detection method, Fast Red substrate-chromogen, hematoxylin Counterstain. Figure 6.2. Pancreas; Wood Duck that succumbed to Mongolia/05 infection.

Severe multifocal to coalescing necrosis of acinar cells. HE. Figure 6.3. Cerebrum; Wood Duck that succumbed to Mongolia/05 infection. Intense positive immunohistochemical staining of neurons and glial cells. Immunohistochemical staining: mouse-derived monoclonal antibody (P13C11), biotin-streptavidin alkaline-phosphatase detection method, Fast Red substrate-chromogen, hematoxylin counterstain. Figure 6.4. Small intestine; Wood Duck that succumbed to Mongolia/05 infection. Positive immunohistochemical staining in neurons of myenteric and submucosal plexus. Immunohistochemical staining: mouse-derived monoclonal antibody (P13C11), biotin-streptavidin alkaline-phosphatase detection method, Fast Red substrate-chromogen, hematoxylin counterstain. Figure 6.5. Cerebrum; Wood Duck that survived the Mongolia/05 challenge. Severe lymphoplasmacytic encephalitis characterized by marked perivascular cuffing. HE.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Over the past decade, the susceptibility and pathology of wild birds to H5N1 highly pathogenic avian influenza (HPAI) viruses have been extensively investigated, partially due to the high zoonotic potential of this virus and the extreme negative economic impact of H5N1 HPAI outbreaks in poultry. The susceptibility and transmissibility of wild type avian influenza (AI) viruses in their natural host, however, have received much less attention, and studies addressing these effect are still scarce. Understanding the link between inter-species differences in virus prevalence, as well as the distribution, ecology and life history of susceptible host species, is necessary for a full comprehension of AI virus epidemiology within the wild-bird reservoir system. The overall goal of this study was to provide a better understanding of host factors that influence the susceptibility of wild birds to AI viruses.

The effect of age at infection on the outcome of a LPAI virus infection was evaluated in five different age groups using Mallards (*Anas platyrhynchos*) as the experimental model (Chapter 3). The age groups in this experimental trial were 2-week-old and 1-, 2-, 3-, and 4-month-old, and the viruses used were two LPAI viruses, H5N2 and H3N8. All the age groups were infected with both LPAI viruses. Oropharyngeal and cloacal viral shedding were consistently detected in the first four days after inoculation and intermittently after that; oropharyngeal viral shedding was detected up to 21 days. The 1-month-old group had the highest

number of virus isolations from cloaca compared to the other age groups, while the 2-month-old group had the highest number of virus isolations from OP swabs. The results of this trial indicated that although age does not affect susceptibility to infection with LPAI virus, it does influence the extent of viral shedding, and even minor differences in age at infection can influence experimental outcomes.

Possible variation in LPAI viral shedding pattern between four wild bird species was investigated (Chapter 4). Four avian species (Mallards, Wood Ducks [*Aix sponsa*], Redheads [*Aythya americana*], and Laughing Gulls [*Leucophaeus atricilla*]) and three LPAI virus isolates (H5N2, H3N8, and H7N3) were used in this trial. The pattern of viral shedding, including route and duration, varied between duck and gull species and individual virus isolates. For instance, Mallards in the H5N2 and H3N8 treatment groups had an extended period of viral shedding, primarily via the cloaca; while Wood Ducks, Redheads, and Laughing Gulls had viral shedding predominately via the oropharynx, suggesting the importance of respiratory tract replication of LPAI viruses in these species. Viral shedding was detected up to 21 days for Mallards (H5N2, cloacal swab), 16 days in Laughing Gulls (H3N8, oropharyngeal swab), and 12 days for Wood Ducks (H7N3, oropharyngeal swab) and Redheads (H5N2, cloacal swab). Taken together, the observations of this trial suggest that different avian species can have dissimilar roles in the transmission of LPAI viruses, and this may explain the variation in prevalence observed in surveillance studies of wild bird populations.

Two co-infection trials were conducted in order to investigate the effect of a previous infection to LPAI virus in a subsequent challenge with a LPAI or a H5N1 HPAI virus. The first co-infection trial evaluated the outcome of a LPAI challenge on viral shedding and antibody response in Mallard pre-exposed to a heterosubtypic LPAI virus (Chapter 5). The results of this

experiment indicated that although a primary exposure to a LPAI virus does not protect Mallards against a subsequent infection with a heterosubtypic LPAI virus, it does reduce the duration of cloacal and oropharyngeal viral shedding and also the viral load shed via cloaca. The heterosubtypic LPAI virus-induced partial protection observed in this trial might be of significant importance for the ecology of these viruses in wild avian populations, an infected duck with heterosubtypic immunity may shed the LPAI virus for a shorter period of time.

The second co-infection trial investigated the effects that pre-exposure with homosubtypic (HA homologous) (H5N1, H5N2) and heterosubtypic (HA heterologous) (H1N1) LPAI viruses had on the outcome of a Eurasian H5N1 HPAI virus challenge in Wood Ducks (Chapter 6). The results of this trial indicate that a previous exposure to a LPAI virus may prevent disease but not infection with or viral shedding of HPAI H5N1 virus; and it also decreases mortality rate and increases mean time to death after a H5N1 HPAI virus challenge. Thus, birds with pre-existing immunity against a LPAI virus may live longer or even survive a HPAI H5N1 virus challenge, further contributing to virus transmission and dispersal. The magnitude of this protection, however, varied among the LPAI viruses used for pre-exposure, and was strongly related to their ability to replicate and the development of a humoral immune response. Further investigation is needed to clarify the mechanisms responsible for the protection observed in these two co-infection trials, as well as the extent (between different subtypes) and duration of this protective immunity.

Taken together, the results of these experimental trials indicate the susceptibility of wild birds to AI virus infection is complex and dependent on multiple factors, including AI virus isolate, age-at-infection, host species, and pre-existing immunity to LPAI virus. The susceptibility and viral shedding pattern data of this investigation can be used as a baseline for

future experimental trials with wild birds, and help to interpret data acquired in surveillance and field studies. Furthermore, this research contributes to the comprehension of the natural history of wild type avian influenza, and helps up define and understand the epidemiology of AI viruses in wild avian populations.