DISSERTATION

VIRAL SHEDDING AND ANTIBODY RESPONSE OF
MALLARD DUCKS TO AVIAN INFLUENZA VIRUSES

Submitted by

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ABSTRACT

VIRAL SHEDDING AND ANTIBODY RESPONSE OF MALLARD DUCKS TO AVIAN INFLUENZA VIRUSES

Wild ducks are a key reservoir for avian influenza (AI) viruses. Their long distance migrations, coupled to frequent contact with domestic poultry enhances risk for spread of highly pathogenic avian influenza (HPAI) viruses. Despite years of study, our understanding of how AI viruses are maintained and transmitted in nature remains poorly understood. The work described here examines several aspects of avian influenza virus infections that play a role in perpetuation and spread of this disease, including persistence of virus in duck feces, effect of prior exposure to AI viruses on subsequent infections and the passage of maternal antibodies between hen and duckling.

In recent years, the emergence of H5N1 HPAI virus stimulated establishment of massive international surveillance programs to detect that virus in wild waterfowl. One deficit in these efforts was a lack of data on the stability of AI virus and AI virus RNA in bird feces under different environmental conditions. Consequently, an experiment was designed to address this knowledge gap. Feces were collected from mallards infected with a low pathogenic avian influenza (LPAI) virus (H5N2) on days 3 and 4 post infection and kept in environmental chambers for 21 days under the following conditions: 32°C/20% relative humidity (RH), 32°C/50%RH, 32°C/90%RH, 4.5°C/50%RH, 4.5°C/90%RH, and 0°C/50%RH. Sensitivity of detection of infectious virus in fresh fecal material was equivalent to that from cloacal swab samples, while time and environmental conditions did not significantly affect detection of AI virus RNA by PCR.
Infectious virus was isolated from feces for considerably shorter intervals than RNA could be detected and was isolated for longer periods of time when feces were maintained under cold conditions. High relative humidity also had a negative effect on virus isolation at 4.5°C. Use of quantitative reverse transcriptase PCR to detect AI virus in fecal samples is as a valuable tool in limiting the labor involved in surveying wild ducks for AI virus.

Few prior studies have examined virus shedding over the course of short interval, sequential infections of ducks with LPAI viruses, as likely occurs in natural settings such as breeding grounds. We characterized such infections by sequential inoculation of ducks with homosubtypic versus heterosubtypic with H5N2 and H3N8 LPAI viruses. We found that prior infection with either virus reduced the duration of viral shedding during a subsequent infection initiated 14 or 28 days later. Further, shedding was significantly shorter when the secondary infection occurred 28 days following the initial infection compared to 14 days. No difference in rate of shedding for the secondary infection were noted based on the viral subtype causing the initial infection, suggesting induction of some degree of heterosubtypic immunity. As reported from previous studies, some ducks shed virus but did not develop detectable antibody titers. There was no evidence of subtype cross-reactivity by antibodies as demonstrated by hemagglutination inhibition testing. The antibody response to a heterosubtypic virus was not improved by a prior infection while a second infection with the same virus was capable of boosting the antibody response to that virus. This information should be useful in parameterizing models examining the ecology of avian influenza infection.
Another factor of significance in understanding transmission of AI viruses among wild ducks is the influence of passive immunity. A third study was performed to evaluate the magnitude of passive transfer of anti-influenza virus antibodies in mallard ducks and to determine their rate of decay in ducklings. Since not all ducks develop antibodies following natural infection with AI virus and the antibody titers are typically low, a vaccine was used to induce consistent seroconversion. Four, 11 month-old mallard hens were inoculated with a recombinant H5 protein in adjuvant. Specifically, hens received a single injection of 20 µg of hemagglutinin protein derived from A/Vietnam/1203/2004 emulsified in Freund's incomplete adjuvant. Beginning two weeks post-vaccination, eggs were collected daily. Yolk was harvested from eggs laid at one-week intervals and the remainder of the eggs incubated for hatching. All hens developed detectable antibody titers with an average log 2 hemagglutination inhibition titer (HI) of 6.4. Maternal-origin antibodies were detected in the yolk of eggs laid by all hens. Antibody titers peaked in yolks three weeks post vaccination for two hens and were still rising four weeks post vaccination for the other two hens. The highest yolk HI antibody titer was 32. Serum samples from the ducklings hatched from vaccinated hens were collected between days 0 and 22 post-hatch. The calculated mean half-life of maternal antibody in ducklings was 2.3 days with a range of 1.6 to 4.0 days. The short duration of passive immunity in ducks is similar to what has been reported for other species of birds and suggests that maternal antibodies may not play a major role in modulating protection against AI virus infection in natural populations. The strong immune response elicited by the H5 protein suggested that further evaluation should be performed to determine the viability of this vaccine for ducks.
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CHAPTER I
LITERATURE REVIEW

INTRODUCTION

Influenza A viruses are an important disease-causing agent in humans, several species of mammals and birds worldwide. Viruses that have undergone gradual antigenic changes as a result of genetic drift cause seasonal human influenza epidemics. Sporadic pandemic strains arise from genetic shifts due to reassortment of different influenza viruses, which can yield viruses that induce significant morbidity and mortality. Recent research stimulated by the threat posed by highly pathogenic avian influenza (HPAI) H5N1 virus has suggested that pandemic strains may also result from avian influenza (AI) viruses adapting directly to humans. The genetic unpredictability of influenza viruses requires that vaccines against seasonal influenza viruses be manufactured each year based on predictions of which will be the predominant circulating strains. Additionally, vaccines to protect against future pandemic viruses are difficult to produce because of the random nature in which they are generated. To better prepare for pandemic influenza viruses, it is essential to enhance our understanding of the potential reservoir for these viruses. It is believed that avian influenza viruses serve as a genetic pool from which all other influenza viruses have originated. Research is needed to determine how the wide varieties of influenza viruses are maintained in wild bird populations. This dissertation examines the longevity and the ability to detect AI viruses in mallard (Anas platyrhynchos) feces, the effects of a pervious AI virus infection on subsequent infections in mallards and the transmission of maternal AI antibodies in mallards.
HISTORY OF AVIAN INFLUENZA

The name influenza comes from the Italian word influentia – to influence, and was originally used to describe epidemic diseases with unknown origins (1). The Italian, Perroncito, recorded the initial description of chickens infected with HPAI in 1878 using the term fowl plague (2). AI was demonstrated to be caused by a filterable agent by Centanni and Savunozzi in 1901 (3). Human influenza virus was first demonstrated to be a filterable agent in 1933 by Wilson Smith et al. (4), who was also the first person to grow the disease agent for AI in the lab (5). Schafer finally determined that the etiologic agent of fowl plague was an influenza A virus in 1955 (6). During the 1960s, low pathogenic avian influenza (LPAI) and moderately pathogenic avian influenza viruses were first described (2). Numerous names have been used to describe AI including: fowl pest, peste aviarie, Geflügelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease and fowl/bird grippe (2). At The First International Symposium on Avian Influenza in 1981, the term highly pathogenic avian influenza was adopted as the official disease name instead of fowl plague (2).

The initial isolation of an influenza virus from deceased wild birds was from an epizootic of common terns (Sterna hirundo) during 1961 in South Africa (7). The epizootic resulted in the death of over 1300 terns. The authors made the earliest suggestion that migratory birds might spread influenza between domesticated poultry. Further evidence of the involvement of wild birds in the ecology of influenza viruses was the discovery in 1970 of antibodies to the virus in wedge-tailed shearwaters (Puffinus pacificus) and noddy terns (Anous minutus) in Australia (8). Viruses were eventually isolated from healthy wild birds including a wedge-tailed shearwater in Australia in 1971.
and migrating California ducks in 1972 (9-11). The connection to human influenza and waterfowl was suggested in the 1970s when Robert Webster and Graeme Laver showed similar antigenicity between the H3 protein of the 1968 Hong Kong influenza outbreak and that of a duck AI virus (1). Today HPAI is a listed disease by the World Organization for Animal Health (Office International des Epizooties – OIE) and a reportable disease to the United States Department of Agriculture (USDA).

**Ecology of Avian Influenza**

The wildlife reservoirs for AI viruses include waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes and Laridae*) (12, 13). Except for the recently described H17 influenza subtype found only in bats, all 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes have been isolated out of wild birds and in almost all combinations (14, 15). Across multiple surveys in North America, AI viruses are most frequently isolated from mallards (*Anas platyrhynchos*) (14). Therefore, the studies presented here focus on infections of Mallard ducks. Poultry are not considered reservoirs for influenza viruses, but are infected when the virus is transmitted to them from wild birds (16). Recently it was suggested that passerines may play a role in the passage of influenza virus to domestic birds, but a prevalence of AI virus of only 0.89% was detected in 4,341 samples (17). The avirulent infections caused by AI in waterfowl and shorebirds, which indicates a balanced host virus relationship, lends supports to these animals being a reservoir host (12). Additionally, the evolutionary rate of AI viruses in ducks is slow, while when introduced into poultry, the rate is high. This is likely attributable to adaptation to its new host, similar to what is seen in mammals such as humans and swine (18, 19). The genes
of AI viruses found in ducks are conserved, while the genes of influenza viruses isolated from humans show considerable antigenic drift (20).

While humans and other mammals transmit and are infected by influenza viruses via a respiratory route, birds can be infected by influenza viruses by the fecal-oral route in addition to respiratory transmission (21). The fecal-oral route of transmission for wild birds was first suggested in 1976 (22). LPAI influenza viruses replicate in the intestinal mucosa of ducks (23-26) resulting in the shedding of LPAI viruses in large quantities in the feces (25). Transmission occurs between wild birds via the fecal-oral route, most likely from contaminated water (25, 27, 28). LPAI viruses remain infective and can be detected in feces from the environment as well as under laboratory conditions for up to 30 days at 4°C (25, 27). Previous work on the influence of environmental temperature on viability has been reported only for an H7 avian influenza virus, but without examining the effect of relative humidity (25).

AI viruses are most frequently detected during the southern migration of waterfowl and the northern migration of shorebirds (29). The percentage of birds where AI viruses are isolated is highest in breeding grounds when juvenile birds gather prior to migration (14). The frequency with which AI viruses are isolated decreases as birds progress south during migration. A study in Louisiana has shown that AI viruses can be isolated in wintering waterfowl, but at a very low prevalence (1986 + 1987 avg. 3.1%) (30). The most frequently isolated subtypes are H3, H4 and H6 (14). Viruses of the H5 and H7 subtype, which have potential to become highly pathogenic to poultry, are recovered at a significantly lower rate, ranging from 0.25% to 2% of isolates (14). There
is a periodicity to the isolation of different subtypes from ducks, possibly mirroring turnover in previously exposed individuals of the population (29).

It was originally believed that ducks and shorebirds had separate lineages of influenza viruses, but more recent research has shown the viruses from these groups of birds are related (31-34). A survey of viruses infecting wild birds did not note stable constellations of genes (35). In general, it is believed that lineages of avian influenza viruses are geographically restricted (33, 34, 36-47). This includes the hemagglutinin gene for LPAI H5 and H7 viruses in North America and the HPAI H5N1 virus in Asia (48, 49). The exchange of genetic material between the Eurasian and North American lineages of avian influenza is infrequent, but has been previously documented (32, 33, 35, 40, 47, 50-53). The movement of a complete virus genome between Eurasia and North America has not been documented (47).

Waterfowl are able to asymptomatically carry HPAI H5N1 virus infections under non-migratory conditions in the wild and in the laboratory (54-56). Migrating birds stay as close to wintering grounds as open water permits in order to conserve energy. As a result, outbreaks of HPAI H5N1 virus in wild birds is associated with temperatures approximate to 0°C (57). The HPAI H5N1 virus is now endemic in poultry in Southeast Asia, representing a constant source for possible transmission to migratory waterfowl (56, 58). A study in Thailand showed a strong association between HPAI H5N1 outbreaks in chickens and the presence of free-grazing domestic ducks (59). This suggests that the free-grazing ducks may be a reservoir for HPAI H5N1 or may allow the passage of the virus between wild ducks and chickens. Long-distance migrants have been shown to have a higher prevalence of infection by LPAI viruses most likely due to
an increase in exposure to susceptible individuals during migration allowing for the perpetuation of the infection throughout a population (60, 61). The presence of a constant source of exposure for migrating waterfowl and the known role of waterfowl in the spread of LPAI viruses indicates that there is a possibility that wild birds will spread HPAI viruses. Regardless of the current biology of HPAI H5N1, there is a constant risk for the adaptation of the virus making it more likely to spread to migratory birds. The lack of certainty about the risk of spread of HPAI H5N1 in migratory waterfowl and the known ability of these birds to asymptotically carry the virus makes it prudent to monitor these species for early detection of the virus in new regions.

**Seasonal Human Influenza**

Seasonal epidemics in humans occur due to influenza A and B viruses that circulate during the winter months of temperate climate zones (62). In tropical areas these viruses usually occur without seasonality, but in some regions they occur primarily during rainy periods (62). Seasonal influenza epidemics are the result of antigenic drift, point mutations in viral RNA resulting in amino acid changes in hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (63). The change in the structures of the HA and NA proteins causes altered antigenicity of the virus and the immune system selects for viruses that have undergone antigenic drift because the surface glycoproteins are the predominant target for antibody production (3). This change in immunogenicity allows for seasonal epidemic viruses to circulate in the population even though people have been previously exposed to the same subtype (64).

Seasonal epidemic strains of influenza virus are the descendants of the most recent pandemic strain (12). The strain responsible for each new pandemic usually
causes the current circulating seasonal influenza strain to be displaced (12). The exception is the 1977 H1N1 pandemic virus, which continued to co-circulate with the H3N2 virus derived from the 1968 pandemic (12). Seasonal influenza epidemics result in over 30,000 deaths a year in the United States (65). The majority of fatalities are people older than 65 years of age (62). Young children are also at significant risk for more severe illness (62). In comparison, the pandemic viruses of 1957 and 1968 resulted in hundreds of thousands of deaths worldwide (65).

**Pandemics**

Only a limited set of viral subtypes have resulted in pandemic strains of influenza viruses including HA subtypes H1, H2, H3 and NA subtypes N1 and N2 (13). The origin of these pandemic viruses has been postulated to be a result of recombination of gene segments, genetic shift, or direct adaptation of an AI virus. In the previous century, pandemics included H1N1 1918 (Spanish flu), H2N2 1957 (Asian flu), H3N2 1968 (Hong Kong flu) and H1N1 1977 (Russian flu) (5). The 1977 H1N1 virus was genetically similar to H1N1 isolates from the 1950s and it has been postulated that it was a release from a laboratory (5, 66). The most recent influenza pandemic in 2009 resulted from an H1N1 virus with genetic segments from an Asian swine virus (NA and M), a classic swine virus (HA, NP and NS), a swine triple reassortant virus of avian origin (PB2 and PA) and a swine triple reassortant virus of human origin (PB1) (67).

The 1918 Spanish flu is believed to have infected one third of the world’s population at that time (500 million people) (68). The mortality rate was 2.5% resulting in 50 million deaths, which is exceptionally higher than the estimated 0.1% death rate for the 1957, 1968 and 1977 pandemics (68, 69). The Spanish flu pandemic was unique in
that it resulted in three waves of illness from 1918 to 1919 with each wave being more severe (68). Another difference from the 1957 and 1968 pandemics was that healthy young adults were overrepresented in the number of deaths from the 1918 Spanish flu (68). Paraffin samples of lung recovered from an Inuit woman buried in the Alaskan permafrost in 1918 allowed reconstruction of the 1918 virus (70), which showed high-virulence, including a lack of a need for trypsin to grow in cell culture (71). Genetic analysis of the genes of the 1918 pandemic strain suggest that the virus was directly transmitted from birds to humans or was only recently introduced to mammals before the pandemic (72). Supporting the close relationship of the 1918 H1N1 virus to avian strains is that an isolate A/New York/1/18 preferentially bound avian receptors (73). The crystal structure of the HA of the 1918 influenza virus closely resembles those of AI viruses (74, 75). Additionally, only a single amino acid change was required to change the specificity from the human preferred virus to avian preferred receptors (73). Phylogenetic analysis of PB1, PB2 and PA suggests the 1918 virus originated from an avian virus (76).

The H2N2 virus that emerged in 1957 resulted from a recombination event with the circulating H1N1 virus gaining three new genes (PB1, HA and NA) (77). Isolates of H2N2 virus have shown that the NA has adapted to cleaving human receptors over time (78, 79). The H3N2 virus that emerged in 1968 resulted in a recombination event with the circulating H2N2 virus and gained H3 and PB1 genes (77). The H3 gene was similar to one isolated from ducks suggesting an avian origin for this segment (77). Early isolates of H3N2 replicated in ciliated human airway epithelial cells in vitro similarly to avian viruses, while later isolates replicated in both ciliated and non-ciliated human airway epithelial cells (80). This furthers the assertion the HA gene segment originated
from a reassortment with an avian virus. Genetic analysis of the PB1 gene from the 1957 and 1968 pandemics indicated this gene had an avian origin (81). During the pandemic years of 1918, 1957 and 1968 the majority of deaths were in people less than 65 years of age, while the percentage of deaths this age group comprised in following years dramatically decreases (82).

A key question has been from where do pandemic influenza viruses arise? Pigs have receptors for both human and avian influenza viruses on their tracheal epithelial cells (83). After entering the swine population, avian HA have been shown to increase their binding to human receptors (83). Since pigs are permissive to infection with both avian and human influenza virus strains it has been postulated that they serve as a mixing vessel during co-infection allowing for the generation of new viruses through genetic shift (19, 84). This idea that pigs could serve as a mixing vessel for human and avian influenza viruses was initially proposed by Scholtissek et al. 1985 (85).

Experimentally, reassortant viruses have been generated by co-infecting pigs with avian and human influenza viruses (86). Currently there are no human-avian reassortant virus isolates from pigs that are direct precursor to viruses that have caused a human pandemic (87). Therefore, it cannot be determined that swine served as a mixing vessel for viruses that caused either the 1957 or 1968 pandemics. Phylogenetic evidence has suggested that avian and human viruses have re-assorted and circulated among pigs (88, 89). There have even been swine, human and avian triple reassortant viruses isolated from pigs (90, 91). Previously to the most recent H1N1 pandemic, triple reassortant swine, human and AI viruses have been identified in human infections (92). A child in
Italy was infected by an avian-human reassortant virus from pigs and there is serologic evidence for such events (89, 93).

Until recently, new pandemic and seasonal strains of influenza virus were thought to originate in China (62). It had been proposed that Southern China is an epicenter for the introduction of new influenza viruses into the human population because of the high population density and close interactions between people and farm animals (94). Additionally, chickens, ducks and pigs intermingle in the Chinese agricultural system (94). The presence of the HPAI H5N1 virus in Southeast Asia increased the concern that it could become a pandemic strain by adapting directly to humans or re-assorting with other viruses. There has been a paradigm shift when the initial human infections with the 2009 H1N1 pandemic virus occurred in Mexico, indicating that novel human influenza viruses could arise in places outside of Southeast Asia (67).

**Human Infection with Avian Influenza Viruses**

The first reported isolation of an AI virus from a human was a patient with hepatitis in 1959 (95, 96). The virus was of H7N1 subtype and was not initially suspected of causing his illness. A laboratory worker in 1976 developed keratoconjunctivitis after accidentally exposing her eye to allantoic fluid containing a chicken-derived H7N7 virus (97). In 1980, a marine biologist performing a necropsy on an influenza virus-infected harbor seal (*Phoca vitulina*) developed conjunctivitis from an H7N7 virus of avian origin (98, 99). Human volunteers in a 1990 study were experimentally infected with avian H4N8, H6N1 and H10N7 viruses resulting in mild respiratory illness (100). Although the test subjects shed virus, not all of them seroconverted, which suggests infection with AI viruses may be more common than has
been documented. In 1996, an Irish woman with conjunctivitis, who had contact with waterfowl, was shown to be infected with an AI H7N7 virus (101, 102). An avian H9N2 virus was isolated out of 2 children with influenza-like illness (gastro-intestinal illness and respiratory illness) in Hong Kong in 1999 (103, 104). Antibodies against the H9N2 virus were found in the serum of blood donors in Hong Kong suggesting the exposure to this virus was more than the two children showing illness (103). The internal genes of the H9N2 virus are closely related to HPAI H5N1 virus that has infected people (105). Genetic analysis indicated that the H9N2 virus was solely of avian origin (105). An H9N2 virus isolated from viruses circulating in poultry sampled from a Hong Kong live bird market in the late 90s had specificity to the human receptor (106). During the 2003 HPAI H7N7 virus outbreak in poultry in the Netherlands, 89 people were confirmed to have been infected (by virus isolation) including three individuals exposed by contact to infected poultry workers (107). The symptoms ranged from conjunctivitis to influenza-like illness including one fatality (108). The total number of infected people is believed to be greater than those from whom virus was isolated. Serology indicated that 49% of 508 exposed poultry workers and 64% of 63 contact individuals were positive for H7 antibodies by hemagglutination inhibition testing (109). Some estimates went as far as saying 1000 people had been infected (110). In 2003, a man in New York City was infected with an H7N2 virus of unknown origins resulting in respiratory illness (111). Additionally, a child with flu-like symptoms in 2003 in Hong Kong was infected with a H9N2 virus of unknown origins, but was similar to those found circulating in live poultry markets (111, 112). Two human patients became infected with a HPAI H7N3 avian
influenza virus from an outbreak in chickens in British Columbia in 2004 (113-115). The symptoms included conjunctivitis and “mild influenza-like illness.”

Further evidence of the risk to humans from infection with avian influenza viruses has been demonstrated by serologic and ferret model studies. In a 1985 study, neuraminidase inhibition assays found serologic evidence of infection with AI viral subtypes N4, N5, N6, N7 and N9 in residents of Milan, Italy (116). Analysis of serum collected from 1992-1993 found neuraminidase inhibitory antibodies to N4 in Chinese people with contact to ducks (117). A serological survey of poultry workers in Italy found seven workers with serologic evidence of infection with H7N3 during a time period of sporadic LPAI and HPAI H7N3 virus outbreaks in Italy (1999-2003) (118). A 1999 study in China found that 19% of people tested had antibodies to an H9N2 AI virus (119). HI is not as sensitive for detecting antigen towards avian influenza viruses in human patients as combining microneutralization assays with Western blot, or ELISA and Western blot (120). Therefore, evidence of other previous exposures may have been missed. Ferrets (a model for human infection) have been successfully infected by LPAI H6, LPAI H7 and HPAI H7 influenza viruses (121-123). Additionally, a mallard H7N3 virus could infect and be transmitted between ferrets without adaptation (124).

**Highly Pathogenic H5N1 Avian Influenza Virus**

A fatally ill 3-year old boy was the first recorded human case of HPAI H5N1, which occurred in Hong Kong SAR, China in May 1997 (125). Genetic analysis showed this virus was fully of avian origin (126, 127). The 1997 HPAI H5N1 virus isolated from humans retained its pathogenicity for chickens (128). The origin of this virus is unknown, but the internal genes for the HPAI H5N1 virus are postulated to have...
Between May and December of 1997, 17 more people were confirmed infected with HPAI H5N1 resulting in five deaths (132, 133). This outbreak was curtailed by mass culling of poultry in Hong Kong (134). All human cases were believed to be due to direct contact with poultry without human-to-human transmission, although there was serologic evidence of exposure in contact individuals (135-137). The HPAI H5N1 virus retained its α2,3SA receptor preference, which may have prevented transmission between humans (138). Most of these cases involved healthy individuals without underlying disease (132). Disease was characterized by viral pneumonia sometimes followed by acute respiratory distress, fever and lymphopenia (132). Some patients also had conjunctivitis, gastro-intestinal disease, liver disease and kidney failure (132, 139). Individuals who died had multiple organ failure (132). HPAI H5N1 viruses from the same lineage continued to circulate in southern China from 1997 to 2002 (140-142). The viruses were initially classified into five genotypes (A-E) in 2001, but by 2002 eight different genotypes were exclusively detected (V, W, X1, X2, X3, Y, Z and Z+) (141, 143).

Additional epizootic outbreaks of HPAI H5N1 occurred in several Southeast Asian countries in poultry in 2003-2004 (143, 144). The index re-emergence case in humans was a family in Hong Kong (145). Additional human cases occurred in Southeast Asia in 2003-2004 (146, 147). Unlike the initial outbreak of HPAI H5N1 where the virus was mainly restricted to the respiratory tract, cases of infections following 2003 resulted in virus disseminating to organs outside the respiratory system (148, 149). Human infections following 2003 primarily mirrored those of the earlier
1997 outbreak being characterized by viral pneumonia with complications including acute respiratory distress and multiple organ failure (145, 146). There is little evidence of human-to-human transmission except for a few cases where there is circumstantial evidence such as lack of contact of infected individuals to poultry (146, 150). Some HPAI H5N1 isolates from humans have adapted to bind both human and avian receptors (151).

In 2002, a strain emerged that was highly pathogenic to waterfowl resulting in the deaths of numerous wild birds in Penfold and Kowloon Parks in Hong Kong (152, 153). It was recognized that the HPAI H5N1 virus had become established in wild birds from an outbreak that resulted in the deaths of a large number of bar-headed geese (Anser indicus), brown-headed gulls (Larus brunnicephalus), great black-headed gulls (Larus ichthyaetus) and great cormorants (Phalacrocorax carbo) in April 2005 at Quinghai Lake, China (154-156). The same virus was isolated later that year in Mongolia and Russia (156). The HPAI H5N1 virus has since spread from Asia to the Middle East, Europe, and Africa potentially by migrating waterfowl (54, 56, 60, 80, 157-166). Other means including the transport of poultry may have been responsible for the spread of the HPAI H5N1 virus (167-171).

THE VIRUS

Classification

Influenza A viruses belong to the family Orthomyxoviridae, which has not been assigned an order. Viruses in the family Orthomyxoviridae have a genome consisting of 6-8 single stranded negative sense RNA segments. The virions are enveloped and either spherical or filamentous. Orthomyxoviridae contains 6 genera including: Influenzavirus
Influenza A, B, C, Isavirus, Thogotovirus and a recently described but unnamed genera of viruses (172). The genus Thogotovirus is comprised of tick-born viruses while Isavirus virus causes infectious salmon anemia.

Influenza A, B and C viruses are differentiated by the antigenicity of the NP and M1 proteins (66). Influenza A viruses are further divided by the antigenicity of their HA (H1-17) and NA (N1-9) glycoproteins (15, 66). The genome of influenza A and B viruses consists of 8 negative stranded segments, while influenza C has 7 segments (173). All three types can infect humans, with infections by type A generally being most severe and type C being the least. Seasonal epidemics are caused by both influenza A and B viruses (174). Unlike influenza A viruses, influenza B and C viruses do not have an animal reservoir and circulate exclusively in people with sporadic isolation in other mammals (174). As a result, only influenza A viruses cause pandemics due to the introduction of novel genes or whole viruses into the virus population that is circulating among humans. Influenza A viruses are the only viruses in the order Orthomyxoviridae that naturally cause infection in birds (175).

Influenza viruses are also identified by the host species which they infect. Influenza A viruses are known to infect a wide variety of birds, humans, swine, horses, sporadically sea mammals and recently, domestic dogs (12, 176, 177). In 2004 an H3N8 influenza virus believed to have originated from an equine virus began circulating in dogs (177). Phylogenetically, based on the nonstructural (NS) gene, virus isolates can be grouped into human-swine, American avian-equine, equine avian-swine, gull group and a group with one equine isolate (178).
Genome

The Influenza A virus genome consists of eight negative strand RNA segments ranging in size from 890-2341 nucleotides coding for 10 to 12 proteins (179-181). The segments are ordered based on size and code for the following proteins: segment 1: PB1 and sometimes PB1-F2 and/or N40; segment 2: PB2; segment 3: PA; segment 4: HA; segment 5: NP; segment 6: NA; segment 7: M1 and M2; and segment 8 NS1 and NS2 (179-181). Transcription of M2 and NS2 require splicing of the viral mRNA (12). The complimentary 3’ and 5’ ends of each strand form base pairs resulting in a panhandle shaped structure (182, 183). There is conservation of the 12 terminal nucleotides at the 3’ end and the 13 nucleotides at the 5’ end, which are complimentary to each other (184-186). These conserved terminal residues at both the 3’ and 5’ ends serve as a promoter and there is a binding site for RNA-polymerase at residues 9 to 12 on the 3’ end (187-190). A stretch of uridine residues is present 17-22 bases from the 3’ end of each segment (184, 191).

The combination of a high rate of mutation due to misinsertion errors and a fast rate of replication in RNA viruses allows for rapid evolution and antigenic drift (192, 193). Misinsertion errors occur due to a lack of proof reading ability in the RNA-dependent RNA-polymerase. The high mutation rate allows for plasticity in the genome of RNA viruses, permitting these viruses to change host species (192). The high mutation rate also allows for a quick increase in fitness in the new host (192). Additionally, the presence of these viruses in quasispecies allows for a dynamic pool of viruses with differing genetic characteristics improving the virus’ adaptability (194).
Structure

Influenza A viruses consist of spherical, pleomorphic or filamentous enveloped particles containing 8 ribonucleoproteins (12, 180, 181, 195). The virion ranges in size from 80-120nm (179). The envelope is derived from the host membrane with three viral proteins embedded (HA, NA and M2) (12). Underneath the envelope there is a protein shell consisting of the structural protein M1 (196-198). The genome is packaged as eight separate viral ribonucleoprotein (vRNP) complexes consisting of the RNA segments coated with NP and an attached RNA-polymerase (56, 60, 161, 199-202). This vRNP is maintained in a circular supercoiled form (203). The attached RNA-polymerase is a heterotrimer consisting of PB1, PB2 and PA (201, 204).

Polymerase Proteins: PB1, PB2 and PA

The three proteins of the viral RNA-polymerase, PB1, PB2 and PA, are encoded on separate gene segments (12). The polymerase proteins are named as such because PB1 and PB2 are basic proteins with PB1 being larger (179). PA is the only acid protein of the polymerase complex. The viral RNA polymerase transcribes the viral messenger RNA (mRNA), complimentary RNA (cRNA) and genomic viral RNA (vRNA). The viral RNA-polymerase is packaged into virions bound both to the 3’ and 5’ end of each genomic vRNA segment (161, 205). PB1 initiates the process of transcription of RNA segments (206). PB1 binds both the 5’ and 3’ end of the vRNA prior to initiating transcription (207). PB1 is also responsible for elongating the growing RNA chain by addition of nucleotides (208, 209). PB2 is responsible for binding the cap of host RNA (206, 208, 210-215). Co-immunoprecipitation experiments show that PB2 interacts with NP and several host proteins (216). Although initially attributed to PB1, the PA subunit
has the endonuclease activity responsible for cleaving the 5’ cap from cellular mRNA (214, 217, 218). PA plays a role in stabilizing the polymerase complex, cap binding activity as well as binding of the polymerase complex to the promoter region of vRNAs (219, 220). Mutations in PA affect packaging of all genomic vRNA gene segments into the virion (221).

**Hemagglutinin**

HA is a glycoprotein embedded in the viral envelope (12). It forms a homotrimer and is a major determinant of antigenicity (12). HA concentrates in lipid rafts on the virion to allow for improved viral fusion with the cell compared to a diffuse distribution (222). HA is cleaved by host trypsin-like proteases into HA$_1$ and HA$_2$ connected by a disulfide bond (12, 223). HA$_1$ forms a globular head and there is a stalk consisting of HA$_2$ and a small portion of HA$_1$ (12, 223). Cleavage of HA is a required step allowing the viral membrane to fuse with the host cell membrane. In fact, the cleavability of HA is a critical component determining the pathogenicity of AI viruses (224). HA$_1$ binds to the viral receptor, sialic acid residues on host glycoproteins and glycolipids (223). The virus enters the cytoplasm through fusion of the viral and host membranes via a conformation change of the HA$_2$ due to low pH in the endosome (223, 225, 226)

**Nucleoprotein**

The influenza virus NP coats vRNA by binding to the phosphate sugar backbone of the vRNA and thus is not sequence specific (182). NP forms a homo-oligomer and has a nuclear localization signal (227, 228). NP has been shown to directly interact with PB1 and PB2 proteins of the RNA polymerase and it has been suggested that this interaction results in switching the polymerase from transcription to replication (229). The NP
interaction with the viral RNA polymerase to allow for unprimed initiation of RNA
transcription to form cRNA and genomic vRNA (230). Besides binding to PB1 and PB2,
NP has been shown to bind M1 and several host proteins (231).

**Neuraminidase**

NA is an integral membrane protein that cleaves sialic acid at its α-ketosidic
linkage to adjacent sugar residues (232). Besides HA, NA is the other major antigenic
molecule located on the surface of the influenza virion (12). NA is a homotetramer with
an enzymatic head and a stalk, which inserts within the viral envelope (232). NA is
thought to cleave sialic acid in mucins allowing the virus to penetrate through mucus
secretions to reach target cells (232). Additionally, NA is thought to cleave sialic acids
on infected cells to prevent aggregation of virus and allow for viral release (189, 233,
234). The sialic acid specificity of NA generally matches that of the HA (78, 79). The
tail of the NA is involved in the morphology of influenza viruses, with mutations in NA
containing no tails resulting in a higher percentage of viruses in the filamentous shape
(235). The NA inhibitors oseltamivir (Tamiflu®) and zanamivir (Relenza®) bind NA
preventing the release of the virus from the host cell (236).

**Matrix 1 Protein**

M1 is the most abundant protein in the virion and is the primary protein
responsible for virus particle formation and budding (237, 238). M1 is encoded by
segment 7 of the influenza genome, which also encodes the M2 protein (12). M1 is
transcribed from unspliced viral mRNA (12). It is found in an ordered helix shell
underneath the viral envelope surrounding the vRNPs (196-198). M1 creates the
structure of the virion by forming a shell beneath the membrane by oligomerization (227,
Matrix 2 Protein

M2 is a transmembrane protein embedded in the virion envelope and is coded from the same segment as M1, sharing only 8 N-terminal residues (12, 252, 253). The M2 protein is transcribed from spliced viral mRNA from segment 7 of the influenza virus genome (12). M2 is a homotetramer held together by disulfide bonds (254, 255). M2 forms an ion channel that allows protons to flow into the virion when in the endosome during viral entry to the cell (256, 257). The protons acidify the inside of the virion...
causing a conformational change in M1 allowing for the release of vRNPs into the cytoplasm (160, 258). M2 is the target of the antiviral drugs amantadine and rimantadine, which block the acidification of the virion and subsequent release of vRNPs.

**Nonstructural Protein 1**

NS1 is important in suppressing the host antiviral response. Segment 8 of the influenza virus genome encodes for both NS1 and NS2 (12). NS1 has been shown to inhibit cytokine response to infection in cell culture (259, 260). NS1 prevents the interferon response of the host as demonstrated by the ability of viruses with deletion mutations of the NS1 gene capable of replicating in STAT1 deficient mice, but not in wild-type mice (261). NS1 binds to double stranded RNA preventing activation of the dsRNA-dependent protein kinase (PKR), which results in an interferon response (262-265). Additionally, NS1 directly inhibits the activities of PKR by binding to the protein (266). Deletion mutants of NS1 in a swine influenza virus demonstrated that NS1 was responsible for preventing production of IFN-α/β (267). NS1 inhibits the activation of NF-κB, which prevents the production of IFN-α/β (268).

In addition to inhibiting the host antiviral response, NS1 modulates host cell physiology and viral RNA synthesis. NS1 inhibits polyadenylation of host mRNA by binding to cleavage and polyadenylation specificity factor, as well as poly(A)-binding protein II (269-272). This has the added benefit of preventing the production of IFN-β mRNA; thus further limiting the cellular interferon response (272). The host machinery is not needed for providing a poly(A) tail on viral mRNA because the viral polymerase makes the poly (A) tail by copying a repeated uridine track (273). Binding of NS1 to cellular mRNA also prevents already polyadenylated cellular RNA from being exported.
from the nucleus (270). By binding the spliceosome, NS1 prevents the splicing of cellular pre-mRNA (274-276). NS1 stimulates the translation of viral proteins by recruiting eukaryotic translation initiation factor 4GI to viral mRNA (277-279). Additionally, NS1 has been shown to be involved in the induction of apoptosis in infected cells (280).

**Nonstructural protein 2/Nuclear Export Protein**

NS2 was renamed nuclear export protein (NEP) when its presence was demonstrated in the virion (248, 281, 282). NEP is translated from an mRNA derived from the eighth segment of the genome from splicing of the NS1 gene (283). Accumulation of NEP triggers the switch from producing mRNA products to transcribing genomic vRNA (284). NEP has a nuclear export system and has been shown to play a role in the export of vRNPs (282).

**Recently Described Proteins PB1-F2 and N40**

PB1-F2 is a protein expressed from a +1 open reading frame from the PB1 gene and was first described in 2001 (180). PB1 has been found to localize to the mitochondria, but is also found in the cytoplasm and nucleus of host cells (180, 285-288). PB1-F2 contributes to the pathogenesis of some influenza viruses in the mouse model as well as HPAI H5N1 in mallard ducks (289-293). It has been suggested that the inclusion of avian origin PB1 genes in the H2N2 outbreak of 1957 and the H3N2 outbreak of 1968 contributed to the virulence of these viruses in the human population (81, 180). Although the complete mechanism has not been elucidated, PB1-F2 promotes apoptosis by altering mitochondrial function and permeabilizing the mitochondrial membrane possibly by pore formation (285, 286, 294-296). It has been suggested that this apoptotic effect, including
the targeting of monocytes, is important in increased pathogenesis (180). Later research has shown this effect of PB1-F2 to be strain dependent (288, 291). Increased virulence has been further suggested to be due to immunopathology due to increased expression of cytokines and recruitment of immune cells to the lungs (290-292). It has also been shown that PB1-F2 can bind PB1 and increase viral RNA-dependent RNA-polymerase activity in a tissue and strain dependent manner (288, 293, 297, 298). Intact sequence for PB1-F2 has been found in a high prevalence of avian influenza viruses, 96% of 861 isolates examined (287). N40 was first described in 2009 as a protein translated from a separate AUG at PB1 gene segment codon 40 (181). Little is currently known about N40. It appears to have a roll in replication in some isolates, but it is not necessary for viral replication nor is it expressed by all viruses.

**Influenza Virus Receptors**

AI viruses preferentially bind to α2,3SA-gal while human influenza virus isolates preferentially bind to α2,6SA-gal (58, 61, 168, 171, 299-302). The difference in the receptors is due to the linkage of the sialic acid to the penultimate sugar of glycolipids or glycoprotein to the third or the sixth carbon for α2,3SA-gal or α2,6SA-gal respectively (162). The presence of α2,6SA-gal linkages in mucin prevents viruses with this specificity from binding to human airway epithelial cells by binding free virus (303). Additionally, neuraminidase from avian isolates primarily recognize and cleave avian receptors, while human and swine isolates primarily recognized both α2,3SA-gal and α2,6SA-gal receptors (79).

The small and large intestinal epithelial cells in domestic ducks and turkeys have α2,3SA-gal linkages but not α2,6 SA-gal linkages (83, 164, 304, 305). Supporting the
lack of α2,6SA-gal receptors, experiments with human influenza viruses have not resulted in infection of duck intestinal epithelial cells (304). In contrast, chicken and quail small and large intestinal epithelial cells have both α2,3SA-gal linkages and α2,6 SA-gal linkages (164, 306). Both α2,3SA-gal and α2,6SA-gal linkages are present on the epithelial cells of the trachea of chickens, domestic ducks, turkey and quail (164, 307). The presence of influenza virus receptors has not been demonstrated in the lungs of chickens, ducks or turkeys, although lung tissue has been demonstrated to be infected by AI viruses (164, 308-310). The tubular epithelial cells of the kidney and mucosal epithelial cells of the esophagus in chickens, ducks and turkeys have both α2,3SA-gal linkages and α2,6 SA-gal linkages (164). The epithelial cells lining the oviduct of chickens, ducks and turkeys have only α2,3SA-gal linkages (164). Receptors have not been detected in the brain, breast muscle, bursa, spleen or cecal tonsils of birds (164). This in contrast to studies showing the presence of viral antigen in brain, skeletal muscle, spleen and bursa (64, 153, 308, 310, 311). The presence of both receptors on some avian cells suggests that human or avian viruses could reassort within birds. Additionally, the HA of an avian virus could adapt to binding α2,6 SA-gal linkages within their avian host.

There is a risk that humans co-infected with influenza viruses of avian and human origin may result in reassortment because numerous cell types have both receptors. Ciliated epithelial cells of the nasal mucosa, pharynx, trachea, bronchi and bronchioles have sialic acid residues of both α2,3SA-gal and α2,6SA-gal linkages (28, 80, 157, 167, 303, 312, 313). The non-ciliated epithelial (goblet) cells have both linkages, but α2,6SA-gal linkages predominate (80, 157, 167, 303, 312). Cells with α2,3SA-gal linkages occur sparsely in the upper respiratory tract, but are common in the lower respiratory tract
Although both linkages have been found to be present on non-ciliated epithelial cells, in studies AI virus replicated solely in the ciliated epithelial cells (80, 167). Seasonal human influenza viruses infect both ciliated and non-ciliated airway epithelial cells (80, 167). Within the lungs, α2,3SA-gal linkages are found on type II pneumocytes while α2,6SA-gal linkages are found on both type I and type II pneumocytes (157, 313).

Outside of the human respiratory system, α2,3SA-gal linkages are found on the neurons in the brain and intestine, T cells of the spleen, ocular and lacrimal duct epithelial cells as well as endothelial cells throughout the respiratory tract, brain, placenta, liver, heart, intestine and interstitial and glomerular tissue of the kidney (157, 165, 314). Additionally, α2,6SA-gal linkages are found on Hofbauer cells of the placenta, bile duct epithelial cells, Kupffer cells, hepatocytes, distal tubule epithelial cells and B cells of the spleen (157, 165). The distribution of α2,6SA-gal linkages on endothelial cells is similar to that of α2,3SA-gal linkages (157). Although α2,6SA-gal linkages have not been detected in the human intestine there are conflicting reports to its presence (157). It has been reported that α2,3SA-gal linkages are and are not present on intestinal epithelial cells, although human intestinal mucosa has been reported to be infected by the HPAI H5N1 virus (157, 315, 316).

**Replication**

The initial step of infection involves attachment of the virion to the host cell receptor through binding of the viral HA to host sialic acid residues (317). For successful viral replication there needs to be a balance between both HA and NA specificity and activity (318-320). The influenza virus then undergoes receptor-mediated endocytosis into cells via coated endosomal vesicles (317, 321). Proton pumps in the vesicle
membrane acidify the endosome causing a conformational change in HA (226). This conformational change results in HA$_2$ fusing the viral and endosomal membranes (225, 226). Concurrently, within the acidified endosome, the M2 ion channel allows proteins to flow into and acidify the virion (12, 317). The M1 protein disassociates from the vRNPs due to the decreased pH within the virion (160). Following membrane fusion and disassociation of M1, vRNPs are released into the cell’s cytoplasm (317). Finally, vRNPs are then actively transported into the nucleus through nuclear pores (163).

It is within the nucleus that viral mRNA and genomic vRNA is transcribed (322). The RNA-polymerase is already attached to the vRNAs when they enter the nucleus (271). Viral RNA-dependent RNA-Polymerase initially copies the vRNA into mRNA (12). Without a primer, the viral genome is then copied into the uncapped intermediate cRNA, which is used as a template to make new genomic vRNA. The vRNA is then used to increase the number of copies of mRNA and for packaging into new virions. The viral RNA-dependent RNA-polymerase binds both the 5’ and 3’ terminal sequences of vRNA, which activates the cap binding and endonuclease activity of the RNA-polymerase (190, 207, 323). It has been shown that binding of only the 5’ end of the vRNA is required for cap-snatching (324). Viral mRNA polymerization is primed with a 5’ cap of 10 to 13 bases long that are cleaved from host RNA by the endonuclease activity of the PA subunit of the viral RNA-polymerase, while the template for genomic RNA is synthesized without the cap and is not polyadenylated (214, 217, 218, 325-328). Approximately 17-22 bases from the 3’ end of the segment, a stretch of 4-7 uridine residues are present (184, 191, 326). At this stretch of uridine bases, the viral RNA polymerase creates a poly-A tail on the mRNA through reiterative copying. The creation
of cRNA requires the transcript to be initiated without a 5’cap (329). The NP protein is further needed to block the viral RNA-polymerase from creating a poly-A tail and creating a full transcript (329). Cellular enzymes are used to splice the mRNAs for M1 and M2 as well as NS1 and NS2 (271). As the M1 protein accumulates in the nucleus it interacts with genomic vRNA and inhibits transcription (250).

Host cellular machinery is used to translate viral proteins from viral mRNA in the cytoplasm (204). HA, NA and M2 proteins are transported to the cell surface while PB2, PB1, PA, NP, NS1, NS2 and M1 are actively transported to the nucleus of cells using a nuclear localization signal through nuclear pore complexes (317, 330, 331). The newly formed vRNPs are exported to the cellular membrane with M1 and NS2 (317). The M1 and NS2 proteins promote the export of vRNPs from the nucleus for viral assembly (160, 332). The cytoplasmic tails of NA and HA have a redundant function in interacting with M1 in virus assembly and are required for proper virion shape and genomic packaging (243, 333, 334). It is believed that the eight vRNPs are incorporated into each virion by a controlled process that requires specific coding signals within each segment (335-343). Influenza viruses assemble at and bud from areas of cellular membrane rich in lipid rafts (344). The replication cycle eventually results in lysis or apoptosis of the host cell (317, 345, 346).

INFECTION

High Pathogenicity versus Low Pathogenicity

LPAI viruses cause localized infections, while HPAI is characterized as a systemic disease causing high mortality (2, 347). AI viruses only of the H5 and H7 subtype have been documented to have the potential of becoming highly pathogenic to
poultry (175, 348). A HPAI virus is defined as having an intravenous pathogenicity index $\geq 1.2$ or causes mortality in at least 6 of 8 experimentally inoculated chickens (349). Additionally, it can be defined as having multiple basic amino acids at the HA cleavage site, where as LPAI viruses have only one (350-352). The ability of HA to be cleaved is a critical component of what determines the pathogenicity of AI viruses (224). The cleavability of the HA is also affected by glycosylation and therefore glycosylation affects the virulence (353). It has been shown through recombinant viruses that amino acid residues outside of the cleavage site, as well as within the cleavage site, play a role in the pathogenicity of the HPAI H5N1 virus for chickens (354). Recent outbreaks of H5 and H7 HPAI viruses have not adhered to the generalization of the association of basic amino acids in the cleavage site and pathogenicity (355). LPAI viruses can become pathogenic through genetic drift, as was shown to have happened in 1994 with a LPAI H5N2 virus in Mexico (356). As a result, all H5 and H7 avian influenza outbreaks are reportable to the OIE because of the unpredictable risk of the viruses changing to a highly pathogenic form.

Surveys of AI viruses noted that the cleavability of HA is a determinant of pathogenicity in chickens (224). This has been experimentally demonstrated by the production of viruses with varying degrees of HA cleavability from a known HPAI H5N9 virus via a reverse genetic technique (357). Viruses with a greater HA cleavability resulted in increased pathogenicity in chickens. The HA of HPAI viruses can be cleaved by ubiquitous endoproteases including furin and PC6 (358-360). This allows HPAI viruses to form plaques in cell culture without the addition of trypsin (350).
LPAI viruses have restricted tissue tropism because the cleavage of the HA can only be accomplished by trypsin-like proteases present in specific tissues (361). The HA of LPAI viruses has been shown to be cleaved by plasmin in cell culture, blood-clotting factor x-like protease in chick embryos and tryptase Clara in rat bronchiolar epithelial cells (362-364). Commensal organisms have also been demonstrated to be involved with cleavage of HA through proteases produced by Aerococcus viridans and Staphylococcus aureus as well as the stimulation of plasmin production by Streptococci sp. and Staphylococci sp. (365).

**Virulence Factors**

Experiments utilizing recombinant viruses have demonstrated that the HA, NP, PB2 and M2 proteins and the NS gene are involved with virulence of the HPAI H5N1 virus in chickens (259, 366-368). Additionally, a glycosylation site within the NA also contributes to the pathogenicity of HPAI H5N1 virus for chickens (354). The increased pathogenicity due to the NS1 gene has been attributed to inhibiting the interferon response (259). Conversely, the increased pathogenicity due to the NP gene has been attributed to an increase in IFN-α, IFN-γ, Mx1 and iNOS (367). Similarly, H5N1 viruses isolated out of swine were used to show by means of reverse genetics that NS1 contributes to virulence of influenza viruses in chickens (369). Besides individual genes, pathogenicity has been linked to certain constellations of genes. Pathogenicity of HPAI viruses in chickens has been correlated to an appropriate combination of polymerase genes (370). The polymerase proteins and NP have been shown to be an important factor in determining pathogenicity due to increased replication efficiency of AI H7N7 viruses in chick embryos (371). A study examining HPAI H5N1 virulence in ducks
demonstrated that the PA gene was a determinant (372). Reverse genetics studies showed that changes in the polymerase PA and PB1 genes resulted in increased pathogenicity for mallards in a 2004 HPAI H5N1 virus (373).

Studies have been performed in mice examining the virulence of both human and avian origin influenza viruses resulting in some similar conclusions to the responsible genes. For a mouse adapted strain of H1N1 it was shown that mutations in NA, PB1, PB2, HA and NA had roles in virulence (374). In a mouse model, mutations in PA, PB1 and PB2 of a seasonal human H1N1 virus contributed to virulence by enhancing the ability of the virus to replicate (375). Another study using a variant of the same strain of H1N1 virus in mice indicated that virulence was due to the HA, NA and polymerase genes and it was attributed to the viruses ability to replicate faster than the innate antiviral response could be formed (376). Studies with the 1918 pandemic H1N1 virus in mice indicated that the PB1, NS1, HA and NA proteins are responsible for virulence when recombinant viruses were made with a seasonal H1N1 virus (377-379). NS1 and PB1-F2 have been demonstrated to contribute to increased replication and virulence of specific isolates of the 2009 H1N1 pandemic virus in mice (380). Neurovirulence has been attributed to mutations in the NA, M, NS and HA genes through studies in mouse models (381).

Similar to results seen in chickens, an increased ability to replicate due to mutations in the polymerase genes PB1, PB2 and PA through adaptation of an HPAI H7N7 virus to mice was associated with increased virulence (382). Therefore, virulence in a new host may be associated with adaptation and optimization of replication within the host. Mutations in HA and PB2 have been shown to contribute to the virulence in
mice of the HPAI H7N7 virus from the outbreak in the Netherlands that resulted in human deaths (383). PA contributed to the pathogenicity of some mouse-adapted strains of an LPAI H5N2 virus in mice (384). Using reverse genetics to create chimeric viruses of the HPAI H5N1 virus of low and high pathogenicity to mice were used to demonstrate that virulence was polygenetic and influenced by the combined effects of mutations in the HA, NA and PB2 genes in mice (385). The virulence of HPAI H5N1 in mice was associated with the increased cleavability of HA (159). A similar approach demonstrated minor amino acid differences of M1, PB1, PB2 and NS1 can alter the pathogenicity of HPAI H5N1 in mice (159, 386-388). The increased pathogenicity due to NS1 was associated with an enhanced ability to antagonize IFN-α/β and inhibit the dsRNA activation of NF-κβ and IRF-3 (387).

Studies using other animal models have also been used to explore the genetic basis for increased virulence of influenza strains. Exchanging the polymerase genes of the HPAI H5N1 virus with that of a less virulent H5 virus resulted in attenuation of the pathogenicity in ferrets and mice suggesting that the polymerase genes are important in contributing to the virulence of influenza viruses (389). By examining unique residues in PB2 and NS1 proteins of an HPAI H5N1 virus, ferret studies suggested that these proteins are involved in pathogenicity (390). NS1 has been shown to be a virulence factor in swine influenza viruses by inhibiting IFN-α/β (267). In comparison, genetic characterization of H7N7 and H5N1 HPAI viruses infecting humans suggests that mutations in PB2 are important in the pathogenicity of avian influenza viruses in humans (108, 147).
Clinical Disease in Birds

Ducks infected with LPAI are asymptomatic and virus replication is primarily restricted to the epithelium of the enteric tract (24-26, 391-393). Ducks shed virus for approximately 3 to 8 days (394). It has been shown in wild mallard populations infected with LPAI viruses that the level of virus shedding is inversely correlated with body mass, suggesting natural infections affect the health of wild birds (394). The authors described the lower weights as being mild losses, but the overall health effects are unknown.

Ducks infected with HPAI generally have either subclinical or mild symptoms (395-398). Although the infection is considered subclinical, decreased feeding by mallards has been observed (397). Splenomegaly, decreased lucency of the air sacs and bursal atrophy have been noted on gross examination (396). Lesions have been described in subclinically infected ducks throughout the respiratory system. These lesions include: necrotizing/heterophilic rhinitis, sinusitis, laryngitis, tracheitis, airsacculitis and interstitial pneumonia (310, 396, 397). Additional lesions have been described in other organs: lymphoplasmacytic perivascular cuffing of cerebral blood vessels and gliosis in the brain as well as hyalinization and necrosis of skeletal muscle (310, 396). Although ducks may be asymptomatic and lacking significant gross pathology to HPAI, there still can be systemic spread of the virus (310, 396). Virus has been detected in the epithelium of the airways, lungs, ependymal cells of the brain, kidneys, periosseous mesenchymal cells of the skull, and skeletal muscle (310, 396, 397).

Occasionally, HPAI causes severe disease in ducks similar to what is seen in poultry. An outbreak of an HPAI H7N1 virus from 1999-2000 in Italy resulted in the death of Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var.)
domestica) (399). This outbreak caused neurologic disease in the Muscovy ducks, which exhibited tremors. Gross pathology included hardening and discoloration of the pancreas and hemorrhagic duodenitis. Histopathologic examination demonstrated foci of necrosis of the acinar cells in the pancreas. Additionally, lymphocytic encephalitis and perivascular cuffing in the brain was observed. Virus was demonstrated via immunohistochemistry from the acinar cells of the pancreas and astrocytes of the central nervous system, neurons and glia.

Since 2002, some strains of HPAI H5N1 have resulted in high morbidity and mortality of domestic and wild ducks (64, 152, 153, 373, 400-406). Ducks infected with HPAI H5N1 viruses shed the virus both orally and cloacally (406). The course of disease has been described using experiments with both domesticated ducks and wood ducks (Aix sponsa) (153, 393, 400, 403, 406, 407). Clinical signs include cloudy eyes, ruffled feathers, weight loss, respiratory signs, diarrhea and weakness (153, 393, 400, 406). Neurologic signs include depression, blindness, loss of balance, ataxia, tremors and paralysis (153, 393, 400, 407). Death most frequently occurs from between 3 and 7 days post infection (64, 403). Higher mortality due to infection with HPAI H5N1 virus is seen experimentally in 2-week old ducks compared to older ducks (402, 403). Gross examination reveals lung hemorrhage, mottled pancreas, petechiation of the pancreas, splenomegaly and necrosis of cecal tonsils (152, 400). Histopathology reveals similar lesions to asymptomatic ducks including: sinusitis, rhinitis and airsacculitis (401). More severe central nervous lesions are observed: encephalitis, hemorrhagic meningitis, perivascular cuffing, gliosis and nerve cell necrosis within the brain (153, 393, 400, 407). Further pathology includes pancreatic necrosis, pancreatitis, splenic vasculitis and
necrosis, hemorrhagic splenitis, adrenalitis, necrosis of adrenal corticotrophic cells, myocarditis, degeneration and necrosis of the myocardia and myocytes (153, 393, 400, 403, 406, 407). Virus disseminates systemically and is located in respiratory epithelial cells, air sac epithelium, lung, parasympathetic, small intestine, ganglia of the small intestine, cerebellar neurons, pancreatic acinar cells, spleen, liver, kidney, adrenal corticotrophic cells and medullary cells, cardiac myocytes, testicles, bursa, skeletal muscle, tissue macrophages and endothelial cells of numerous tissues (153, 393, 400, 401, 403, 408).

HPAI virus infections causing high morbidity and mortality in other waterfowl that are usually asymptomatic have been described. Infections in four species of geese, Bar-headed (Anser indicus), Cackling goose (Branta buccinator), Canada goose (Branta Canadensis) and domestic geese (Anser anser domesticus), have been described (152, 395, 396, 405, 407). Infection in geese can include ruffled feathers, cloudy eyes, diarrhea, depression, listlessness, seizures, tremors, torticollis, ataxia and death. On examination, geese have conjunctivitis, rhinitis, tracheitis, airsacculitis, lung edema, hemorrhage into the lungs, thinning of the intestinal wall, cerebral malacia, gliosis, lymphoplasmacytic perivascular cuffing, encephalitis, splenic congestion, pancreatitis, hepatitis and adenitis, thymic and bursal atrophy. Areas of necrosis are also seen within the brain, glia, ependymal cells, pancreas, liver and adrenals. Viral antigen has been demonstrated within neurons of brain, pancreatic acinar epithelium, cardiomyocytes, hepatocytes, Kupffer cells, biliary epithelial cells, and adrenal glands.

Clinical signs and pathology in swans including black swan (Cygnus atratus), Coscoroba swan (Coscoroba coscoroba), trumpeter swan (Cygnus buccinators), mute
swan (*Cygnus olor*) and whooper swan (*Cygnus cygnus* trumpeter) have been described (152, 405). During infection swans can present with seizures, tremors and ataxia and even acute death without clinical signs. Necrotic foci are seen in the liver, spleen, kidney, intestines, proventriculus, cecal tonsils, ovaries and oviduct. Virus has been demonstrated in trachea, air sacs, lung, gastrointestinal tract, intestinal parasympathetic ganglia, astrocytes, parenchyma of the brain, pancreas, spleen, liver, kidney, adrenal gland and endothelial cells in numerous organs.

Laughing gulls (*Larus atricilla*), shorebirds also considered to be a wildlife reservoir for AI, are asymptomatic for infection with a HPAI H5N3 virus and early isolates of the HPAI H5N1 virus (309, 409). Pathology still occurs in laughing gulls without clinical signs resulting in decreased lucency of the air sacs, splenomegaly, pancreatic mottling and conjunctival edema. Histologically, heterophilic to lymphoplasmacytic airsacculitis, interstitial pneumonia, necrotizing pancreatitis and hepatitis are seen. Virus can be isolated from the lung and kidney and viral antigens have been demonstrated in the liver and pancreas. Some more recent HPAI H5N1 isolates cause high morbidity and mortality in laughing gulls similar to affected ducks (400). Clinical signs include ruffled feathers, cloudy eyes, weakness, ataxia and torticollis. Petechial hemorrhage is seen in the ventriculus, heart, cerebrum and pancreas. Histopathology shows necrotizing pancreatitis, neural necrosis of the brain, lymphoplasmacytic perivascular encephalitis, heterophilic pancreatitis and adenitis. Virus was located in air sacs, lungs, small intestine, eye, neurons, glial cells, ependymal cells, pancreatic acinar cells, cortical and medullary cells of the adrenal gland, kidney, heart, thymus and endothelial cells.
While AI infection in ducks is mainly an enteric disease, respiratory signs primarily characterize LPAI infection in poultry. LPAI infections in turkeys is similar to chickens (398). The virus is capable of replicating in and being excreted from both the respiratory and intestinal tracts (308, 410). Clinical signs of LPAI in chickens can include: rales, coughing, conjunctivitis and airsacculitis (411). There may be a decrease in egg production with the presence of misshapen eggs (347). Lesions restricted to the respiratory tract, including tracheitis, bronchitis, airsacculitis and pneumonia (308, 412, 413). Histologic signs of LPAI in chickens and turkeys include: loss of cilia, heterophilic infiltrate and luminal exudate in the trachea as well as bronchitis and interstitial pneumonia (411). Some isolates of LPAI have been shown to result in renal tubule necrosis, interstitial nephritis, lymphocyte necrosis and depletion in the cloacal bursa, spleen and thymus in addition to respiratory pathology (414). Virus can be isolated from lung and has demonstrated in the air sac epithelium (413). Virus can also be isolated from the oviduct, ovary and tubular epithelium of the kidney of LAPI infected chickens although there is no pathology (413, 414). Experimental systemic infections induced by intravenous inoculation of chickens with LPAI virus has been shown to lead to virus isolation frequently in the kidney tubule epithelial cells leading to tubule necrosis and nephritis (412). Chickens that succumb to LPAI infections die from respiratory failure and in some cases, renal failure (412).

Infection in chickens with HPAI H5N1 is similar to infection with other HPAI viruses (415). Described clinical signs include anorexia, ruffled feathers, swollen hemorrhagic necrotic wattle and comb, congested legs, cyanosis, dermal hemorrhage, hematochezia, coma and can even include acute death without clinical signs (414, 416,
Gross lesions include subcutaneous edema, mottled pancreas, petechial hemorrhage on the surface of serosa, splenomegaly, renomegaly, systemic congestion/hemorrhage, pulmonary congestion, pulmonary hemorrhage and edema/consolidation, conjunctival hyperemia/edema and hemorrhage of the enteric tract (415, 416). Histologic lesions that are observed include loss of cilia of the respiratory tract, interstitial pneumonia, perivascular cuffing of the brain, gliosis, lymphocytic meningitis, meningoencephalitis, nephrosis, nephritis, adrenalitis, myocarditis, myositis, depletion and necrosis of lymphocytes in the cloacal bursa, spleen, thymus and cecal tonsils (308, 310, 407, 414-417). Necrosis is observed in the intestinal epithelial, pancreas, spleen, adrenal glands, collecting duct, proximal and distal tubules of the kidney, heart, bursa and skeletal muscle (308, 310, 407, 414-417). HPAI virus infection results in a systemic infection with detectable virus in inflammatory cells and endothelium of the lung, smooth muscle of small intestine, brain neurons, glia, ependymal cells, choroid epithelium, pancreatic acinar epithelium, islet cells, hepatocytes, kidney tubular epithelial cells, adrenal corticotropic cells, thymic epithelium, cardiac myocytes, skeletal muscle, theca cells of the ovary, interstitial cells of the testicle, feather follicular cells, osteoclasts, erythroid and myeloid precursors in bone marrow, tissue macrophages and endothelium (308, 310, 407, 414, 415, 417, 418).

Similar clinical signs, pathology and presence of virus in tissues are seen in other gallinaceous species including: turkeys (*Meleagris gallopavo*), Japanese quail (*Coturnix coturnix japonicus*), bobwhite quail (*Colinus virginianus*), Pearl guinea fowl (*Numida meleagris*), ring-necked pheasants (*Phasianus colchicus*) and Chukar partridge (*Alectoris chukar*) (398, 401, 409, 415).
Investigators have looked at the pathogenicity of the H5N1 virus in other families of birds. Passerines may serve as an intermediate host allowing for transmission of HPAI viruses between wildlife reservoirs and/or to poultry. Experimental infection of zebra finches (*Taeniopygia guttata*), house finches (*Carpodacus mexicanus*) and house sparrows (*Carpodacus mexicanus*) with the HPAI H5N1 virus found that these birds had a disease course similar to chickens although they had varying morbidity and mortality (311, 419). On the other hand, European starlings (*Sturnus vulgaris*) shed virus but remained healthy throughout the experimental infection and lacked pathologic lesions (311). Budgerigars (*Melopsittacus undulatus*), psittacines, are kept as pets and may transmit HPAI H5N1 virus to humans. HPAI infection in budgerigars is also similar to chickens, but generally results in less mortality (311). Ratites, which are farmed and therefore have economic importance and close contact with humans have also been experimentally infected with HPAI. Emus (*Dramaius novaehollandiae*) and ostriches (*Struthio camelus*) exhibit clinical signs and pathology similar to chickens (328, 396, 409).

**Clinical Disease in Humans**

Humans infected with seasonal influenza viruses display a disease course that is best characterized by cough, fever and nasal congestion, but may also include: headache, sore throat, muscle soreness, weakness and loss of appetite (420). Human infection with the HPAI H5N1 virus ranges from subclinical to death (421). Infections do occur in previously healthy individuals without any pre-existing conditions (146). The disease is characterized by lower respiratory tract clinical signs with or without upper respiratory tract disease, intestinal disease, bleeding from the nose/gums and even encephalopathy.
 Conjunctivitis has also been described in humans infected with HPAI H5N1 (139). Human cases of HPAI H5N1 have lead to hemorrhagic and consolidated lungs (148). Also seen in the lungs is alveolar damage with lymphoplasmacytic infiltrates (148). Furthermore, central lobular necrosis of the liver as well as tubular necrosis of the kidneys is seen (148). Additionally, brain edema also is observed (148).

Matching the location of the receptor in humans, HPAI H5N1 virus can be found in the type II pneumocytes, ciliated and non-ciliated epithelial cells of the respiratory tract, neurons in the brain, T-cells, intestinal mucosa and fetal tissues (315, 422). HPAI H5N1 can also infect cultured cells of the nasopharynx, adenoids and tonsils (28). Although numerous cell types are permissive for replication of AI viruses, HPAI H5N1 replicates primarily in cells in the lower airway (423). In the lower respiratory tract, human influenza viruses primarily attach to ciliated epithelial cells in the trachea and bronchi and type I pneumocytes in the alveolus, while AI viruses bind primarily to non-ciliated epithelial cells and type II pneumocytes (HPAI H5N1 also infects alveolar macrophages) (28, 166, 169, 423, 424). Experimental studies have confirmed that HPAI H5N1 can infect human pulmonary endothelial cells resulting in productive replication and induction of unusually high levels of inflammatory cytokines (425). Confirming the presence of avian receptors in eyes, AI viruses of subtypes HPAI H5N1, HPAI H7N7, LPAI H7N7 and HPAI H7N3 have resulted in conjunctivitis from infecting ocular tissues (97-99, 101, 102, 108, 113-115, 139).

**Adaptation**

Adaptation of HA to bind α2,6 sialic acid receptor improves transmission of influenza viruses among humans (170, 426, 427). Although the difference in receptors
for human and avian hosts is a significant barrier, the HA of the 1918 H1N1 virus requires only one amino acid substitution to change preference for the avian receptor (73). One of the initial adaptations of pandemic influenza viruses is to bind $\alpha$2,6 sialic acid linkages, which can require minimal amino acid changes (168, 428). A single amino acid changed the receptor binding specificity of a human H3N2 virus from being able to bind both to only the human receptor (429). Mutation in only two amino acids in the HA of HPAI H5N1 changed the receptor specificity to the human receptor (430). The change in two amino acids in the HA of a human H3 virus can change receptor specificity to $\alpha$2,3 sialic acid linkages (431). Changes in two amino acids in the HA of human H1 allowed for replication in ducks (432). The HA gene has been implicating in the virulence of and in adapting seasonal human influenza viruses to mice (433). In the laboratory, receptor preference of influenza viruses can be changed by passage in a new host. Egg adapted strains of human influenza viruses demonstrate an increased binding affinity for $\alpha$2,3sialic acid linkages (302). Studies of H3N2 viruses show that the antigenicity of virus is closer to human isolates grown in MDCK cells as compared to egg grown virus (434). An important step may be balancing of the HA and NA activities of the virus, which is required for efficient viral replication (435). NA from avian isolates primarily recognizes avian receptors, while human and swine isolates primarily recognize both $\alpha$2,6SA-gal and $\alpha$2,6SA-gal receptors (79). The difference in recognition between receptors is as a little as two amino acids (79).

The ability to cross the species barrier appears to be a polygenic trait requiring compatibility between different genes (3). Adaptation to the mouse host of an H1N1 virus, mutations in NA, PB1, PB2, HA and NA have been identified (374). The
polymerase genes play an important role in the adaptation of H7N7 AI virus to mice (371, 382).

The roll of PB2 appears to be especially important. Mutations in PB2 have been shown to be important in the adaptation of influenza viruses to humans as well as mice (123, 436). PB2 has been shown to be a determinant of whether influenza virus can efficiently adapt to replicate in either human or avian cells (216, 437, 438). In studies of human and avian cell lines, adaptations to new hosts rely heavily on NP and PB2, which are important components allowing the proteins to enter the nucleus of the infected cell (439). One study suggested that cellular proteins regulating the interaction of NP and the viral RNA-polymerase restrict adaptation of avian influenza viruses to humans (440). The importance of NP is furthered by an experiment with an AI virus with a temperature sensitive mutant of the NP gene, which could not be rescued by duel infection with human isolates in chick embryo cell culture (85).

**Host Immune Response**

The immune response to AI viruses in birds is poorly understood compared to infections of HPAI viruses and human influenza viruses in mammals. Antibodies to HA are protective and block the HA binding site for its receptor preventing attachment of the virus to the cell (441, 442). Additionally, antibodies against the other major surface glycoprotein NA can also be protective (443). Vaccine producers have focused on the surface glycoprotein because of the protective role of antibodies that recognize these molecules.

Not all vaccines produce detectable hemagglutination inhibition (HI) antibody titers, but can still prevent disease in ducks. Vaccination with an inactivated oil emulsion
vaccine against HPAI H5N1 from recombinant virus resulted in complete protection, but some ducks did not have detectable HI antibody or virus neutralization titers (158). Ducks were protected from infection with a HPAI H5N1 virus after receiving a bivalent H5N9/H7N1 inactivated virus oil emulsion vaccine that was boosted 3 weeks later, although they did not have detectable HI titers to the HPAI H5N1 virus (444). This vaccine prevented illness but did not prevent shedding in all birds. A recombinant duck enteritis virus vaccine expressing H5 resulted in prevention of clinical signs, although one duck shed virus (445). This vaccine resulted in detectable HI titers in five out of six ducks at two weeks post vaccination.

Some vaccines result in low antibody titers that only rise to high levels following a booster. In one study, a group of 30 day old Pekin ducks inoculated with an inactivated oil emulsion vaccine created using a LPAI H5N2 virus developed an antibody titer of log2 GMT 2.69 (446). Only after a second vaccination the titer reached 7.69 log2 GMT. This vaccination prevented both illness and viral shedding. These results were mirrored in another study using Muscovy ducks where initial vaccination with an inactivated oil emulsion vaccine resulted in an HI titer of 5.4 log2 that was boosted to a titer of 7.5 log2 (447). In comparison a fowlpox-vectored recombinant vaccine expressing the H5 of an HPAI H5N1 isolate produced an initial antibody titer of 2.5 log2, which was boosted to 3.4 log2. Both vaccines prevented illness, but did not prevent shedding of virus. A reverse genetic derived inactivated H5N3 oil emulsion vaccine created with the H5 from a HPAI H5N1 that was boosted 3 weeks following the initial administration of the vaccine resulted in titers that ranged from 8 to 64, with the majority of ducks having a titer of 32 (444). This vaccine prevented illness and shedding in all birds.
In another study the kinetics of HI titers following vaccination in ducks with an inactivated oil emulsion vaccine containing an H5N1 virus created by reverse genetics was examined (448). Antibodies were detectable at 1 week following vaccination and were 3 log2. The peak HI antibody titer was at 4 weeks following vaccination and was 8 log2. The antibodies declined to 4 log2 14 weeks following vaccination. A booster vaccination increased titers to 10 log2. This vaccine prevented illness and shedding of virus. The variable response of ducks to vaccination against HPAI H5N1 indicates further research needs to be performed to create a vaccine that reliably produces a high antibody response and that is protective. A recombinant subunit vaccine in Chapter IV, which is used as a means of assessing maternal antibody transfer, may prove through further research to be a reliable duck vaccine for HPAI.

*In vitro* studies suggest that influenza viruses are capable of replicating in avian macrophages and disrupting their function (449, 450). CD8+ T lymphocytes from chickens infected with LPAI H9N2 virus provided a degree of cross-protection to infection by HPAI H5N1 virus (451, 452). Changes in NP have been shown to increase the pathogenicity of HPAI H5N1 in chickens and correlates with an increase in alpha-interferon, gamma-interferon, Mx1 and iNOS (367). In comparing two H5N1 viruses and their ability to cause disease in chickens it has been demonstrated that residue Ala149 in NS1 resulted in high pathogenicity and caused an inhibition of interferon response in cell culture (259). Interferon also restricts the replication of avian influenza viruses in chicken embryo culture cells (453). RIG-I has been proposed as a key molecule in innate immunity that allows HPAI infections in ducks to be asymptomatic, while the absence of RIG-I in chickens results in high pathogenicity (454). RT-PCR of cytokine gene
expression in mononuclear cells of chickens and ducks indicates a difference in response to LPAI. The chicken response was characterized by increased levels of IL-1β, IL-6 and IFN-β. (455). Levels of these cytokines were nearly unchanged in ducks while IL-2 was strongly increased. There was an increase in both IFN-α and IFN-γ in both chickens and ducks, but chickens had a greater increase in IFN-γ.

Similar to what has been observed with birds, antibodies to HA and NA are an important component of the immune response to influenza viruses in humans (12). Cytotoxic T lymphocytes (CTL) have been shown to recognize and lyse cells containing the influenza internal proteins PB1, PB2, PA and NS1 (456). Mice studies have shown that the NP is the major target molecule recognized by cytotoxic T cells, although CTL can also recognize HA (457, 458). Influenza infection in mammalian cells has been shown to induce the IFN-α/β response (459). Interferon can also protect mammalian cells from influenza infection (459). Different NS1 genes have been shown to result in different induction of host interferon responses in lung epithelial cells (460). The increased pathogenicity due to NS1 is associated with an enhanced ability to antagonize IFN-α/β and inhibit the dsRNA activation of NF-κβ and IRF-3 (386). The Mx1 protein of mice inhibits transcription of influenza viral mRNA, while MxA protein of humans prevents viral mRNA from being transcribed (461). Mx appears to have little to no effect on AI replication in duck and chicken studies (462).

The pathogenicity attributed to H5N1 in human cases is believed to be caused in part by a proinflammatory cytokine induction resulting in a cytokine storm. It has been shown that HPAI H5N1 induces a strong TNF-α and IFN-β response in human macrophages (360). As compared to a seasonal H1N1 virus isolate, HPAI H5N1 virus
infections produced a more potent response of pro-inflammatory cytokines as measured by IP-10, IFN-β, RANTES and IL-6 in pulmonary epithelial cells in culture (463). In human cases it has been shown there are increases in serum cytokines (145). Even though the cytokine response to the HPAI H5N1 virus has been suggested to be responsible for the morbidity and mortality, mice deficient in IL-6, TNF-α or CC chemokine ligand 2 or who have their cytokine response suppressed with corticosteroids are not protected from death (464). Recombinant swine IFN-α, IFN-γ TNF-α do not alter viral infection in swine lung epithelial cell culture (260).
CHAPTER II
DETECTION OF H5N2 AVIAN INFLUENZA VIRUS IN MALLARD FECES UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

INTRODUCTION

It has been postulated that some of the spread of the highly pathogenic avian influenza (HPAI) H5N1 virus from Asia to the Middle East, Europe, and Africa occurred due to migrating waterfowl (34, 154, 155, 465-475). Others have argued that waterfowl have a lesser role in the spread of the HPAI H5N1 virus (476-480). At the moment, there is not enough information to determine the role of migration in the spread of the HPAI H5N1 virus, but a strong argument can be made for a sensible research approach to examining the possibility (480-483). Waterfowl are able to asymptotically carry the infection under non-migratory conditions in the wild and in the laboratory (55, 467, 475). The HPAI H5N1 virus is now endemic in poultry in Southeast Asia, representing a constant source for possible transmission to migratory waterfowl (143, 467). Long-distance migrants have been shown to have a higher prevalence of infection by low pathogenic avian influenza (LPAI) viruses most likely due to an increase in exposure to susceptible individuals during migration allowing for the perpetuation of the infection throughout a population (34, 484). The presence of a constant source of exposure for migrating waterfowl and the known role waterfowl play in the spread of LPAI viruses indicates that there is a possibility that wild birds will spread HPAI viruses. Regardless of the current biology of HPAI H5N1 virus infection, there is a constant risk for the adaptation of the virus making it more likely to spread to migratory birds. The lack of certainty about the risk of spread of the HPAI H5N1 virus in migratory waterfowl and the
known ability of these birds to asymptotically carry the virus makes it prudent to
monitor these species for early detection of the virus in new regions.

The pattern of infection of LPAI viruses in North American waterfowl peaks just
prior to fall migration and decreases as the birds head south (419, 485-488). Of concern,
is the scenario that birds migrating from Asia may transmit the HPAI H5N1 virus to North
American waterfowl species in the Arctic. The virus would then spread along fall
migration routes. To confront this risk, the United State Department of Agriculture
(USDA), Animal Plant Health Inspection Service (APHIS), Wildlife Services has
partaken in a strategic plan for the early detection of HPAI H5N1 virus focusing on
migrating waterfowl and shorebirds (489). From April 2006 through March 2009, this
surveillance program collected 261,946 samples from wild birds and 101,457 fecal
samples, representing a large commitment in resources by the USDA and other agencies
(490). Collecting fecal samples is more appealing than capturing wild birds because it
potentially requires fewer resources and avoids the stress of handling the birds.
Similarly, the need for the ease of environmental sampling has resulted in the
development of a method to detect HPAI H5N1 virus by concentrating the virus from
surface water on erythrocytes before isolation in embryonated chicken eggs (491).

LPAI viruses replicate in the intestinal mucosa of ducks (23-25). This results in
the shedding of LPAI viruses in large quantities in the feces (25). Transmission occurs
between wild birds via the fecal oral route, most likely from contaminated water (25, 27,
492). LPAI viruses remain infective and can be detected in feces from the environment
as well as under laboratory conditions for up to 30 days at 4°C (25, 27). Previous work
has been done only with an H7 AI virus to examine the viability at different temperatures,
but without examining the relative humidity (25). From 2006 and into early 2009, the USDA limited their fecal sampling only to fresh feces. The USDA stopped collecting fecal samples as of April 2, 2009 because the value of environmental sampling by fecal swabs under different conditions compared to cloacal swabs of captured waterfowl is unknown. The results of this study suggest environmental sampling of fecal material may be a valuable tool in surveying migratory birds for avian influenza viruses.

AIMS: This study was undertaken to establish the efficiency of detecting avian influenza viruses by swabbing feces under differing environmental conditions. The first aim was to demonstrate that detection of an LPAI virus in fresh fecal samples reflected that of cloacal swabs at days 3 and 4 post infection. Secondly the study sought to compare efficiency of detection of virus from feces by real-time RT-PCR and virus isolation under differing environmental conditions of temperature and relative humidity.

HYPOTHESES: Real-time RT-PCR detection of LPAI virus in fecal material will accurately predict infection in mallards at days 3 and 4 post infection. Post-defecation time, ambient temperature and relative humidity will not affect the detection of LPAI virus by real-time RT-PCR in fecal material. In contrast, post-defecation time, ambient temperature and relative humidity will affect the detection of LPAI virus by virus isolation from fecal material.

MATERIALS AND METHODS

Animals, Housing and Infection

A mixture of male and female juvenile mallards (n=15) were kept under animal biosafety level 2 conditions. Five groups of three ducks were housed uncaged in a room with ad libitum feed and water. Ducks were labeled 1-15 by placing colored zip-ties
loosely around one leg. All ducks were humanely euthanized following the experiment. The ducks were infected with 1 ml of brain-heart infusion broth (BHI) containing $1 \times 10^6$ pfu/ml of A/Mallard/MN/346250/00 LPAI H5N2, which was administered by distributing the inoculum ocularly, intranasally and orally.

Collection of Samples

Feces were collected on days 3 and 4 post infection, with that timing based on preliminary data indicating that mallards would reliably shed virus on those days (data not shown). On the night prior to days 3 and 4 post infection, feed was removed from the duck rooms. In the morning feed was placed back in with the ducks for half an hour and the ducks were then placed individually in cages and checked every 15 minutes for defecation. Feces were collected from underneath the cages on wax paper and split into two samples in a weigh boat. Although the fecal matter was split, it was not mixed in order to maintain a sample similar to what might be encountered in the environment.

Immediately after collection, the fresh feces were placed in weigh boats and cloacal swabs were obtained from each duck. The weigh boats with fecal material were placed in environmental chambers under one of the following conditions: 32˚C/90%RH, 32˚C/50%RH, 32˚C/20%RH, 4.5˚C/50%RH, 4.5˚C/20%RH and 0˚C/50%RH. Temperatures were chosen to reflect conditions during migration where waterfowl move South before freezing temperatures. The high temperature of 32˚C was chosen to represent the conditions the birds may encounter at their warm wintering grounds. Feces were swabbed at 12h, 24h, 48h, 72h, 5d, 7d, 14d and 21d, or until all feces were removed due to sampling. Swabs were placed in 2ml of brain heart infusion broth containing 50,000 U/ml penicillin, 2.5 µg/ml streptomycin, 100,000 U/ml polymyxin B, 2,500 U/ml
nystatin and 100 µg/ml gentamicin. The swabs were removed and the broth was split into 2 cryovials for either virus isolation or real-time RT-PCR and stored frozen at -80°C.

**Assays for AI virus**

The presence of infectious virus from cloacal and fecal swab samples was assayed by inoculation of embryonating chicken eggs. Each sample had been slowly thawed at room temperature and centrifuged at 400 x g for 5 minutes to remove particulate matter from the solution. Fertile specific pathogen-free chicken eggs (Sunrise Farms, Inc, Catskill, NY) were incubated at 36-39°C with 80% relative humidity for 9-11 days in GQF 1502 Digital Sportsman Cabinet Style Incubator (GQF Manufacturing Company, Savannah, GE) and candled to determine their viability; infertile or dead eggs were discarded. Immediately prior to inoculation, the air pocket of the egg was located and marked, and a small hole was drilled into the shell through which 100 µl of swab sample fluid was inoculated into the allantoic fluid using a 25 gauge needle. The hole in the shell was sealed using Elmer’s glue. Three eggs were inoculated with each sample to detect infectious AI virus. The eggs were then incubated for 3 days after inoculation, chilled overnight and allantoic fluid was harvested and tested by hemagglutination.

Chicken erythrocytes suspended in Alsever’s solution were purchased (Lampire, Biologic Products, Pipersville, PA), washed by repeated centrifugation and resuspension in PBS, and then diluted in PBS to make a 0.5% solution. A screening hemagglutination assay was performed by placing 50 µl of allantoic fluid and 50 µl of 0.5% chicken erythrocyte solution into each well of a 96-well V-bottom microtiter plate. The plates were allowed to incubate at room temperature for 30 minutes and wells were examined for the presence of hemagglutination. A lack of hemagglutination was confirmed by
tilting the plates and observing a “tear drop”, the running of the erythrocyte pellet along the bottom of the well. Each sample was assayed in duplicate and a sample with stock virus and allantoic fluid from a non-inoculated egg were included in each assay as positive and negative controls.

In order to detect the presence of viral RNA in fecal samples, real-time, RT-PCR was performed. Viral RNA was purified from fecal swab fluid using MagMax-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using a matrix specific primer and probe set (493). The detection limit of the assay has been determined by Spackman et al. to be 1,000 copies of RNA or 0.150% egg infectious dose of AI virus. All real-time RT-PCR analysis was performed at the Colorado State University Veterinary Diagnostic Laboratory. Samples were considered positive if the Ct value was less than 35.

RESULTS

Comparison of Cloacal Swab with Fresh Fecal Swabs

Cloacal swabs of all ducks were positive via virus isolation and real-time RT-PCR on days 3 and 4 post infection. Additionally, all swabs of fresh feces on days 3 and 4 post infection were positive for virus isolation and real-time RT-PCR. This indicated an association between the detection of virus from cloacal swabs and fresh feces.

Isolation of Virus from Feces Under Different Environmental Conditions

A summary of the virus isolation results is presented in Figure 2-4. The limited amount of feces prevented sampling at some later time points. Figure 2-1 depicts the proportion of positive samples for the relative humidity settings at 32°C. The proportion of positive samples decreased at a more rapid rate than for the colder temperature settings.
(Figures 2-1, 2-2). The longest time period at which a sample was positive for virus isolation was day 21 for Mallard 6, day 4 collection at 0°C/50%RH. The harshest condition was 32°C/50%RH where only one sample tested positive at 12 hours.

Figure 2-1. The proportion of fecal samples testing positive for virus isolation at 32°C with differing relative humidity levels. The presence of infectious virus was tested by swabbing feces and injecting 100 µl of swab fluid into embryonated chicken eggs and the resulting allantoic fluids were tested for hemagglutination.
Figure 2-2. The proportion of fecal samples testing positive for virus isolation at 4.5°C and 0°C with differing relative humidity levels. The presence of infectious virus was tested by swabbing feces and injecting 100 µl of swab fluid into embryonated chicken eggs and the resulting allantoic fluids were tested for hemagglutination.

The difference in average length of incubation time for positive samples between 32°C and the colder 4.5°C and 0°C conditions was statistically significant by analysis of variance using Tukey’s HSD ($p<0.05$, Figure 2-3). When calculating the average length of time of virus presence, for colder temperatures only samples where the virus was at high enough concentrations to be detected at 12 hours were used. There was also a significant difference between 4.5°C/50%RH and 4.5°C/90%RH. The difference between the two relative humidities maybe also due to the growth of bacteria and enzymatic degradation of the viral particles. Colder and dryer temperatures preserve the virus in feces over longer periods of time allowing for viral isolation.
Figure 2-3. The average duration for virus isolation from fecal samples at different temperature relative humidity combinations. Means with different letters are significant at p < .05, accounting for multiple tests using Tuckey’s HSD.

Detection of Virus by Real-Time RT-PCR

Real-time RT-PCR was performed on all fecal samples and cloacal swab samples on collection days. The following samples did not test positive for the presence of viral RNA: Mallard 3 collection day 4 32°C/50%RH days 7, 14 and 21 as well as Mallard 4 collection day 4 0°C/90%RH day 21 (Figure 2-4). Detection of virus by real-time RT-PCR does not indicate the presence of infectious material, but only the presence of viral RNA. Although in old samples of feces infectious virus may no longer be present, they may remain a useful tool for surveying waterfowl populations for influenza infections.
### 32°C/20%RH

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<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>5d</th>
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Figure 2-4. Detection of LPAI H5N2 virus in fecal samples by virus isolation/RT-PCR. NS indicates no sample for that time point.
### DISCUSSION

The results of this study demonstrate that real-time RT-PCR can accurately detect shedding of LPAI virus in fresh feces and fecal matter stored for up to 21 days old.

Environmental conditions greatly affected virus isolation over time, but not the detection...
rate of real-time RT-PCR. The LPAI H5N2 virus remained viable for longer periods of
time under colder conditions. The proportion of positive fecal swabs that tested positive
fluctuated up and down for 32°C/20%H and 0°C/50%H. This is most likely because fecal
matter is not a homogenous mixture. Depending on where the swab was placed may
influence the probability of detecting the virus. Regardless of the individual samples; on
the whole, the presence of infectious virus dissipated at a quicker rate at higher
temperatures.

At 32°C virus could be isolated for shorter periods of time at 90%RH compared
to 50%RH. One may have predicted that high relative humidity would have prevented
the feces from desiccating as quickly as other samples at 32°C, thus preserving the
integrity of the virus. Fecal material consists of a heterogeneous mixture of enzymes,
chemicals and bacteria. Moisture may allow enzymes to retain their function, bacteria to
propagate and the overall sample to mix. An H7N2 LPAI virus mixed into chicken
manure lost infectivity 5-10x more quickly than virus maintained in allantoic fluid under
the same conditions (494). The virus lost infectivity as assayed by inoculation into
embryonated chicken eggs when mixed within manure under a week at temperature
conditions ranging from 15-20°C. This suggests that there are inactivating properties in
bird feces.

The use of mallards for this study was particularly pertinent because of their role
in the biology of influenza viruses and their susceptibility to the HPAI H5N1 virus.
Mallards are species of duck most frequently studied and most often detected in the wild
to be infected with influenza compared to other waterfowl, except the closely related
American black duck (34, 486). An interagency strategic surveillance plan of shorebirds
and waterfowl over 2006-2008 in the United States found that the highest frequency of detection of H5 viruses was in mallard (495). The most frequently detected H5 virus over this period was of the subtype H5N2, which is the same subtype used in these experiments (495, 496). Additionally, nearly all subtypes of influenza virus have been isolated from mallards (497). In Europe, LPAI viruses closely related to recent outbreaks of HPAI circulate in the wild in mallards (497). Laboratory passage of the HPAI H5N1 virus in domestic mallards results in the selection of viruses that are not pathogenic to ducks, but remains pathogenic to chickens (402). This suggests that domestic ducks and possibly their closely related wild counterparts could maintain the HPAI H5N1 virus in a form that remains pathogenic to other species. Domestic mallards are capable of shedding virus for prolonged periods of time; in one study they shed virus up to 17 days (402). Mallards experimentally infected with HPAI H5N1 virus excrete high quantities of virus with few clinical signs (55, 475). This information suggests that mallards may have the ability to spread HPAI H5N1 over their long distance fall migration.

This study demonstrated that infectious virus can be maintained in feces at different temperature relative humidity combinations for periods of days. Feces may be an important environmental source for the virus. Contamination on shoes or other equipment may spread the virus. Therefore, care should be taken to prevent transfer of fecal material when at a study site. Additionally, influenza viruses may remain viable in feces until rains or floods result in fecal contamination of water bodies. Environmental contamination is recognized as an important part of the biological cycle of AI infections. Modeling predicts that waterborne transmission of low pathogenic influenza viruses is critical in explaining the incidence and periodicity of infection as well as persistence of
influenza viruses within a population (498-500). Avian influenza viruses have been shown to persist for extended periods of time under different environmental conditions in water (temperature, salinity, pH) with longer persistence at colder temperatures (27, 501-505). In a previous study it was shown that there is little difference in viability over time between LPAI H5 and H7 influenza viruses compared to other isolates, but the HPAI H5N1 viruses persist for shorter periods of time (504). It is important to note the lack of difference in the persistence of H5 and H7 viruses because only AI viruses of the H5 and H7 subtype have been documented to have the potential of becoming highly pathogenic to poultry (175, 348). Influenza viruses may persist in their avian reservoir by being maintained in the water environment (52). This may be applicable in the difference in the prevalence of certain AI viruses in different species, as surface feeders like mallards have a higher prevalence of infection (16, 34, 506).

Waterborne transmission has been identified as the cause of infection in farmed domestic ducks (507). In some cases LPAI viruses have been isolated from surface water (392, 508, 509). It is thought that in Alaska AI viruses can persist in nature in both water and ice over winter while migratory ducks are absent and infect ducks upon their return (503). Sentinel ducks have become infected when placed in pens that allowed for contact with wild birds and local pond water (487, 508). LPAI outbreaks in poultry have correlated with the presence of the virus in water (507, 509).

Water is not the only environmental source for influenza viruses. In the instance of an outbreak of H13N2 virus in 1991, it was suggested that turkeys contracted the virus directly from fecal material excreted by gulls or a contaminated water source (510). Another research group demonstrated that a 1997 isolate of HPAI H5N1 virus remains
infective longer under moist and cooler conditions (395). HPAI H5N1 fecal material maintained in phosphate buffered saline at 4°C remained infective for 40 days. Comparatively, feces allowed to desiccate at room temperature 25°C lost infectivity by 24 hours. Feces maintained in PBS at room temperature remained infective for 4 days. Feces kept at 35°C in PBS lost infectivity after 48 hours. HPAI H5N1 RNA has been detected by real-time RT-PCR in the hot humid climate of Cambodia up to 12 days after an influenza outbreak in environmental samples including: mud, pond water, water plants and soil swabs (511). The virus could not be isolated from any of these samples further demonstrating the difference between the presence of infectious material and viral RNA.

One limitation of this study was that, in contrast to LPAI viruses, HPAI H5N1 virus appears to be primarily a respiratory tract infection in waterfowl (55, 152, 400, 402, 475). As a result, the fecal-oral route of contamination may not be the primary mode of spread. This would make it more difficult to detect the virus in feces. Additionally, it would be important to perform oral swab on ducks to detect HPAI H5N1 virus. This does not prevent fecal swabs from being useful. Adaptation of HPAI H5N1 virus may result in a tropism in ducks for the intestinal tract, thus making fecal swabs a good method for detection. Fecal swabs can be pooled allowing for surveying larger numbers of waterfowl increasing the opportunity to detect the virus. Another limitation was that the effects of ultraviolet light were not examined. All samples were kept in the dark inside the environmental chambers. This study does provide an initial estimate on the longevity of viral genetic material and viability that would be useful during overcast weather when ultraviolet light would have a minimal effect.
The results from this study also suggest that assay of fecal material can be a useful method for surveying wild duck populations for the presence of influenza viruses without stressing birds during capture. Fresh fecal material is representative of infection by a LPAI H5 virus when compared to fecal swabs at 3 and 4 days post infection. It would be more useful to take fecal samples when environmental conditions are known to be colder. Although infective virus cannot be isolated from all samples, real-time RT-PCR can detect the presence of viral material in older samples. This methodology would be useful not only for the detection of HPAI H5N1 virus from Asia, but detect possible future HPAI viruses and gain a better understanding of the prevalence of LPAI viruses in their natural reservoir.

**SUMMARY**

The influence of environmental temperature and humidity on detection of a LPAI H5N2 virus in mallard feces was evaluated. Feces was collected from LPAI H5N2 virus infected mallards on days 3 and 4 post infection and kept in environmental chambers for 21 days under the following conditions: 32°C/20%RH, 32°C/50%RH, 32°C/90%RH, 4.5°C/50%RH, 4.5°C/90%RH, and 0°C/50%RH. Detection of virus in fresh fecal material was equivalent to cloacal swabs for days 3 and 4 post infection. Time of incubation did not affect detection by real-time RT-PCR. In contrast, virus could be isolated for longer periods of time under cold conditions. High relative humidity also had a negative effect on virus isolation at 4.5°C, but not at warmer temperatures. Environmental conditions did not affect detection by real-time RT-PCR.
INTRODUCTION

One of the hallmarks of influenza A viruses is their antigenic diversity. Currently, there are 17 defined hemagglutinin (HA) and 9 neuraminidase (NA) types which, when shuffled via reassortment of gene segments can potentially lead to a large number of viral subtypes. Except for the H17 HA recently discovered in bats (15), all other 16 HA types have been identified in waterfowl and shorebirds, both of which are considered to be the primary reservoir for all influenza A viruses (12). Understanding the ecology of avian influenza (AI) viruses and how so many subtypes are maintained in waterfowl populations present critical challenges to understanding these pathogens. There is year-to-year variation in which subtypes circulate in waterfowl (469) and some subtypes are detected only infrequently (29). Isolation of AI viruses from waterfowl is typically common prior to the beginning of migration in the fall, but the rate of isolation decreases as the birds head south (30). AI viruses are infrequently isolated from waterfowl on their wintering grounds (30). It is unknown how the viruses are maintained in the population to re-infect birds the following fall. Understanding this seasonal cycle would be especially valuable if highly pathogenic (HP) AI virus H5N1 were to appear and potentially become established in North American waterfowl.

Additionally, the viruses circulating through waterfowl constitutes a pool of potential viruses that can infect and cause disease in poultry. Viruses of both H5 and H7 low pathogenic (LP) AI subtypes are also frequently found in ducks (469). As presently known, only viruses of the H5 and H7 subtypes have the potential to become highly pathogenic.
pathogenic in poultry and cause high morbidity, mortality and economic loss. These viruses can also potentially be transmitted directly to other hosts or contribute genetics to influenza viruses present in other species.

One feature of AI virus ecology in waterfowl that has received scant attention, but may be of great importance, is cross-subtype immunity. With few exceptions, the protective effect engendered by infection with one subtype on infection with a different subtype is unknown. One relevant study reported sequential infections of ducks with AI viruses using subtypes infrequently found in wild populations of ducks (H7N7 and H5N2) (512). In this experiment, ducks were also re-infected with the primary virus before infecting them with the secondary subtype (512). Additional studies have examined re-infection with the same virus (26) or focused on the effects of a prior homosubtypic and heterosubtypic LPAI infection on a HPAI H5N1 infection (513, 514). The studies presented here were designed to determine how successive infection of mallard ducks with two different subtypes of LPAI virus altered virus shedding and whether or not demonstrable cross protection was induced between the two viruses. In contrast to previous studies, a LPAI H3N8 virus was chosen because previous surveys identified H3N8 as one of the most prevalent subtypes in North American waterfowl. In one study it was determined that H3N8 viruses accounted for 22.8% of AI virus isolates from duck samples collected between 1976 and 2001 in North America (29). Interestingly, H3N8 and other common subtype viruses have been observed only infrequently in co-infections (515), suggesting perhaps that they induce a broadly cross-protective immunity against AI viruses of other subtypes.
Conversely, viral subtypes that are detected infrequently have been identified more commonly as co-infections (515). It has been postulated this occurs because these viruses are less well adapted to their avian host and therefore gain an advantage when co-infecting a duck. Although AI viruses of the H5N2 subtype are rarely detected, H5N2 viruses have not been observed causing a co-infection (29, 515). A survey of samples taken over 15 years in Alberta Canada, revealed that co-infections are a relative rare event (515). Therefore, sequential infections may be a more common means of perpetuating the variety of subtypes of avian influenza observed in nature. An H5N2 virus was chosen for this study because of the potential for these viruses to convert to a HP phenotype. The mallard was chosen for these experiments because they are the most abundant duck present in North America, Europe and Asia (469, 516). Additionally, within North America, avian influenza viruses are most frequently isolated from mallards (14).

AIMS: There were three main aims of this project to characterize how mallard ducks respond to sequential influenza virus infections during the same season. The first was to determine whether pre-infection with an H3N8 LPAI virus limited shedding after infection with an H5N2 LPAI virus and vice versa. A second aim was to characterize the antibody response to sequential infections with H3N8 and H5N2 viruses and determine whether cross-subtype humoral immunity was induced that could be detected using a standard hemagglutination inhibition assay. Finally, these experiments sought to determine whether there was a temporal difference in protection to AI infection between 14 and 28 days following the initial AI infection. Day 14 was chosen because it
represents the earliest time point for a subsequent infection when the ducks have likely cleared the initial virus, while day 28 was chosen as a later time point for comparison.

HYPOTHESES: We hypothesized that prior infection with a heterosubtypic virus would result in decreased shedding of the second infection, but to a lesser degree than prior infection with a homologous virus. Additionally, it was speculated that prior infection with both the homologous and heterosubtypic viruses would boost the antibody response to both the initial and secondary viral subtypes. Finally, we hypothesized that secondary infection at the earlier time point (14 days) would result in less viral shedding than when secondary viral infection was induced at a later time point (28 days).

MATERIALS AND METHODS

Virus Strains

Both viruses used in these studies were kindly provided by Dr. David Stallknecht, College of Veterinary Medicine, University of Georgia, Athens, GA. The H3N8 subtype virus was A/Ring-Necked Duck/Minnesota/SG-00066/2007 and the H5N2 subtype virus was A/Mallard/MN/346250/00. Stocks of both viruses were obtained by inoculation of 10 day old specific pathogen free chicken eggs and harvest of allantoic fluid following 3 days of incubation. Allantoic fluids were frozen in multiple aliquots and titrated by plaque assay using Madin Darby Canine Kidney cells for the H5N2 virus and egg infectious dose (EID_{50}) for the H3N8 virus.

Animals

A mixture of male and female juvenile mallards (n=25) were kept under animal biosafety level 3 conditions. Ducks were purchased from a local game farm and were 12 weeks of age at the time of purchase. The ducks were allowed to acclimate to their new...
surroundings for a period of seven days. Prior to use, all ducks were tested negative for antibodies to H3 and H5 AI viruses via hemagglutination inhibition assay (described below). Ducks were labeled 1-25 and were identified by colored zip ties placed comfortably around their legs. At the end of the study, ducks were humanely euthanized with an intravenous overdose of pentobarbital and carcasses were incinerated. All experiments were approved the Institutional Animal Care and Use Committee at Colorado State University.

**Challenge and Collection of Samples**

Two experiments were conducted using a study design depicted in Figure 3-1. In both cases, ducks inoculated with different viruses were housed in separate rooms. Ducks were infected by inoculation of $10^6$ to $10^7$ pfu of A/Mallard/MN/346250/00 H5N2 or $10^{6.5}$ EID$_{50}$ Duck/Minnesota/SG-00066/2007 H3N8 viruses. The inoculum was distributed intranasally, conjunctivally and orally.

Blood was collected weekly via the brachial vein starting at day 0 and extending through four weeks following the second challenge. Blood was allowed to clot and centrifuged at 2000 x g for 10 minutes, and serum was decanted from the blood clot and frozen at -20°C.

Cloacal swab samples were collected to evaluate viral shedding. All ducks were swabbed daily from day 0 through 7 following the first inoculation, then twice a week for another seven days post infection. Swabs were twirled into 2 ml of brain heart infusion broth with antibiotics as previously described (Chapter II). The swabs were removed and the broth was split into 2 cryovials and frozen at -80°C until assay.
Study 1

Ducks 1-7
H3N8 Inoculation

Ducks 5-7
H3N8 Inoculation

Ducks 8-10
H5N2 Inoculation

Ducks 11-13
H3N8 Inoculation

Primary Infection

Secondary Infection

-7 Acclimation 0 14 28 Secondary Infection 56 Days

Ducks 14-16
H5N2 Inoculation

Ducks 17-19
H3N8 Inoculation

Study 2

Ducks 14-19
H3N8 Inoculation

Ducks 20-25
H5N2 Inoculation

Ducks 14-16
H5N2 Inoculation

Ducks 17-19
H3N8 Inoculation

Ducks 20-22
H5N2 Inoculation

Ducks 23-25
H3N8 Inoculation

Figure 3-1. Experimental Design
Assay for AI Virus

AI viruses from cloacal swab samples were assayed by inoculation of embryonating chicken eggs as described in Chapter II. Fertile specific pathogen-free chicken eggs (Sunrise Farms, Inc, Catskill, NY) were incubated at 36-39°C with 80% relative humidity for 9-11 days in GQF 1502 Digital Sportsman Cabinet Style Incubator (GQF Manufacturing Company, Savannah, GE). Three eggs were inoculated with 100 µl of swab sample fluid into the allantoic fluid using a 25 gauge needle; the hole in the shell was sealed using Elmer’s glue. The eggs were then incubated for 3 days after inoculation, chilled overnight and allantoic fluid was harvested and tested by hemagglutination.

A screening hemagglutination assay was performed in duplicate by placing 50 µl of allantoic fluid and 50 µl of 0.5% chicken erythrocyte solution into each well of a 96-well V-bottom microtiter plate. The plates were allowed to incubate at room temperature for 30 minutes and wells were examined for the presence of hemagglutination or a pellet of erythrocytes at the bottom of the well. A lack of hemagglutination was confirmed by tilting the plates and observing a “tear drop” running of the erythrocyte pellet along the bottom of the well. A sample with stock virus and allantoic fluid from a non-inoculated egg were included in each assay as positive and negative controls.

Hemagglutination Inhibition Assay

Humoral immune responses to AI virus infections were assessed using a hemagglutination inhibition assay as described previously (349, 517). Chicken erythrocytes were prepared as described in Chapter II for the hemagglutination assay. Briefly, serial two-fold dilutions of duck serum were prepared in a 96 well V-bottom
microtiter plate, leaving a volume of 25 µl in each well. The duck serum was not pre-treated with receptor destroying enzyme because no non-specific inhibition was detected. In these wells 25 µl containing 4 hemagglutinating units of either the H5N2 or H3N8 virus was pipetted. Each assay contained a virus back titration, and positive and negative control sera. The virus and serum samples were allowed to incubate at room temperature for 30 minutes, then 50 µl of 0.5% chicken erythrocytes were added. The samples were gently mixed and were allowed to incubate again for 30 minutes at room temperature. The hemagglutination inhibition (HI) titer is defined as “the highest dilution of serum causing complete inhibition of hemagglutination of 4 HAU of antigen” (517).

Hemagglutination inhibition was determined by the presence of a button of red blood cells and a “tear drop” of erythrocytes running when the plate was tilted. All samples were assayed in duplicate and antibody titer was defined as the reciprocal of the highest dilution of serum in which hemagglutination was not observed.

**Statistical Analyses**

The major parameter evaluated in this study was the duration of virus shedding. In order to determine if prior infection with a heterosubtypic virus provided some immunity to subsequent infections, comparisons were made between the mean duration of shedding. Analysis was conducted using SAS version 9.2. Repeated measures were analyzed using Proc Mixed. The response variable was duration of shedding. The model included fixed effects corresponding to virus combination (H3xH5, H3xH3, H5xH5, H5xH3), secondary inoculation date (14 or 28 days), primary or secondary shed information plus all two and three way interaction effects. The model also included a random duck effect to account for repeated measures (primary and secondary shedding.
was measured on each duck). Ducks are nested within the combination of virus combination and secondary inoculation date. Contrasts were used to estimate and test various mean differences in duration of shed (Table 3-1). Least squares means were used to estimate mean shedding duration for various groups when comparisons were made using repeated measures ANOVA.

Direct comparisons between two means were based on Student’s two-sample or paired t-tests (one-tailed). A paired t-test was used for the comparison of primary versus secondary mean shed duration for ducks initially infected with either the H3 or H5 virus. Two-sample t-tests were performed to compare mean duration of secondary shed for homologous and heterosubtypic infections. A two sample t-test was also used to compare secondary shedding for ducks either initially infected with the H3 or H5 virus.

RESULTS

Virus Shedding During Primary and Secondary Infections with AI virus

In experiment 1, all ducks inoculated initially with either H3N8 or H5N2 viruses had detectable shedding of virus, indicating an active infection (Figure 3-2). Four ducks infected with H3N8 virus shed for up to 10 days post infection, while 2 ducks infected with H5N2 shed virus up to 10 days. The earliest day that virus was detected from cloacal swabs was day 1 post-inoculation for both H5N2 and H3N8 virus infected ducks. The range of time for which ducks shed virus was 3 to 10 days for those inoculated with H3N8 virus and was 3 to 9 days for those inoculated with the H5N2 virus.

Ducks initially infected with H3N8 virus and then challenged 14 days later with the heterosubtypic H5N2 virus, shed virus 1 to 4 days following secondary infection. For the duck that shed for only a single day, the inoculum passing through the intestinal tract
may have been detected because virus was only isolated 24 hours following the inoculation. The ducks initially infected with H3N8 virus and challenged with the homologous virus all shed virus for 4 days.

Ducks initially infected with H5N2 virus and re-challenged with the same H5N2 virus 14 days later shed virus for a range of 1 to 6 days. In these birds, virus was detected 24 hours after the infection in all three birds, but not detected again until 5 and 6 days later in two birds. Therefore, the initial virus detection may be a result of infectious virus passing through the gastro-intestinal tract. The birds may in fact have only shed for 0-1 days. In contrast, when ducks previously infected with H5N2 were subsequently infected with the heterosubtypic virus H3N8, shedding of that virus was detected from 3 to 7 days.
Figure 3-2. Proportion of duck shedding virus after primary infection on day 0 and secondary infection on day 14. (a) Primary infection with H3N8 virus, (b) primary infection with H5N2 virus.
In experiment 2, the initial inoculation of either an H3N8 or H5N2 virus was followed by shedding of virus in all ducks, confirming infection (Figure 3-3). Ducks infected with the H3N8 virus shed virus for 4 to 7 days post-inoculation, where as ducks infected with the H5N2 virus had detectable shedding for a range of 2 to 8 days, similar to what had been observed in Experiment 1. Prior infection with H3N8 virus followed by infection 28 days later with the heterosubtypic H5N2 virus resulted in a range of shedding from 1 to 4 days. In contrast, secondary infection with the homologous H3N8 virus resulted in shedding from only one of three ducks and lasted for 2 days. In comparison, ducks in study 1 that were re-infected with the H3N8 virus at 14 days post initial challenge all shed virus.

Similar results were observed with ducks initially infected with the H5N2 virus and then re-challenged 28 days later. When challenged with the heterosubtypic H3N8 virus, all 3 ducks shed virus for 2 to 7 days and when challenged with the homologous H5N2 virus only 1 of 3 ducks shedding virus for a single day.
Figure 3-3. Proportion of duck shedding virus after primary infection on day 0 and secondary infection on day 28. (a) Primary infection with H3N8 virus, (b) primary infection with H5N2 virus.
No statistical difference was noted for the shedding duration of either the H3N8 or H5N2 viruses during the primary infection (statistical analysis is presented in Table 3-1). Therefore, it was assumed that infection with these two different viruses were comparable. We hypothesized that a prior infection with heterosubtypic virus would provide a degree of cross-protection for subsequent infections. We found that there was a statistical difference between the shedding duration for the primary infection in comparison with the shedding duration for secondary infections with both the heterosubtypic and homologous viruses. This indicated that a prior infection with a heterosubtypic virus provided a degree of immunity to the subsequent infections. A difference was also noted between the heterosubtypic and homologous virus infections at day 28. This suggested that over time an increase in adaptive immune responses to the previous homologous virus increased creating greater immunity for when the virus was encountered at a later time point. Whether a mallard was initially infected with an H3 or H5 virus did not make a difference in the duration of shedding for the secondary infection.

We also hypothesized that there would be decreased shedding of virus at the earlier time point (14 days) in comparison to an infection at 28 days. We had believed that non-specific immunity would be active when the ducks were challenged with the secondary virus at an earlier time point. A statistical difference was encountered when comparing the primary to the secondary shedding durations at both 14 and 28 days indicating that a prior infection provided a degree of immunity to subsequent infections. Contrary to our hypothesis, the duration of virus shedding for ducks infected at day 28 was significantly shorter than the duration of shedding for ducks infected at 14 days.
When examining the data by comparing specific primary and secondary virus combinations for 14 and 28 days the only statistical difference was noted for ducks challenged with the homologous virus. This suggested that subtype specific antibodies may have been responsible for this increased level of protection at the later time point. The longer time period may have allowed the ducks to develop memory B cells that are ready for the second encounter of the homologous virus.
Table 3-1. Statistical analysis examining the difference in mean duration of shedding following either primary or secondary infection with either the LPAI H5N2 or H3N8 viruses. T-tests were performed when simple, direct comparisons were made, while repeated measures ANOVA were used in other cases. Sequential infections are represented as primary virus x secondary virus. SE indicates the standard error. Statistically significant comparisons are shaded grey (p<0.05).

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 1 Mean ± SE</th>
<th>Group 2 Mean ± SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary infection: H3 (n=13)</td>
<td>Primary infection: H5 (n=12)</td>
<td>6.2 ± 0.6</td>
<td>5.2 ± 0.6</td>
<td>0.250^A</td>
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<tr>
<td>Primary infection: H3 or H5 (n=25)</td>
<td>Secondary infection: H3 or H5, 14 or 28 days post-primary infection (n=25)</td>
<td>5.7 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>&lt;0.001^A</td>
</tr>
<tr>
<td>Primary infection: H3 or H5 (n=13)</td>
<td>Secondary infection: H3 or H5, 14 days post-primary infection (n=13)</td>
<td>6 ± 0.8</td>
<td>3.8 ± 0.4</td>
<td>0.034^T</td>
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<td>Primary infection: H3 or H5 (n=12)</td>
<td>Secondary infection: H3 or H5, 28 days post-primary infection (n=12)</td>
<td>5.4 ± 0.5</td>
<td>1.8 ± 0.6</td>
<td>0.028^A</td>
</tr>
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<td>Secondary infection: H3 or H5, 14 days post-primary infection (n=13)</td>
<td>Secondary infection: H3 or H5, 28 days post-primary infection (n=12)</td>
<td>3.8 ± 0.4</td>
<td>1.8 ± 0.6</td>
<td>0.007^A</td>
</tr>
<tr>
<td>Primary infection: H3 or H5 (n=25)</td>
<td>Secondary Homologous Infections: H3 or H5, 14 or 28 days post primary infection (n=12)</td>
<td>5.7 ± 0.4</td>
<td>2.3 ± 0.6</td>
<td>&lt;0.001^T</td>
</tr>
<tr>
<td>Primary infection: H3 or H5 (n=25)</td>
<td>Secondary Heterosubtypic Infections: H3 or H5, 14 or 28 days post primary infection (n=13)</td>
<td>5.7 ± 0.4</td>
<td>3.4 ± 0.5</td>
<td>0.001^A</td>
</tr>
<tr>
<td>Secondary Heterosubtypic Infections: H3 or H5, 14 or 28 days post primary infection (n=13)</td>
<td>Secondary Homologous Infections: H3 or H5, 14 or 28 days post primary infection (n=12)</td>
<td>3.4 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>0.080^A</td>
</tr>
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<td>Secondary Heterosubtypic Infections: H3 or H5, 14 day post primary infection (n=7)</td>
<td>Secondary Homologous Infections: H3 or H5, 14 day post primary infection (n=12)</td>
<td>3.7 ± 0.6</td>
<td>4.0 ± 0.7</td>
<td>0.380^A</td>
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<tr>
<td>Secondary Heterosubtypic Infections: H3 or H5, 28 days post primary infection (n=6)</td>
<td>Secondary Homologous Infections: H3 or H5, 28 days post primary infection (n=12)</td>
<td>3.0 ± 0.9</td>
<td>0.5 ± 0.3</td>
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<tr>
<td>Secondary infection, H3 or H5 following primary infection with H3 (n=13)</td>
<td>Secondary infection H3 or H5 following primary infection with H5 (n=12)</td>
<td>2.5 ± 0.4</td>
<td>3.3 ± 0.7</td>
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<td>Secondary infection: H3xH5, Day 28 (n=3)</td>
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<td>2.0 ± 0.6</td>
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<td>Secondary infection: H3xH3, Day 28 (n=3)</td>
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<td>Secondary infection: H5xH3 Day 28 (n=3)</td>
<td>4.7 ± 0.9</td>
<td>4.0 ± 1.5</td>
<td>0.664^A</td>
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</tbody>
</table>

^A – repeated measures ANOVA. ^T – t-test.
Serologic Responses of Ducks Following Primary and Secondary Infection with AI Virus

The antibody response to the initial infection to the H3N8 virus did not reach what is considered by the World Organization for Animal Health (OIE) to be a positive serum HI titer of 16 (517) in 5 of the 7 ducks by day 14 (Table 3-2). An additional duck had a titer of 16 by 21 days post primary infection, but that was 7 days following the secondary challenge with the H5N2 virus. It is unlikely that the H5N2 virus stimulated the increase in the titer because antibodies to the H5N2 virus were not detected until 14 days following the secondary infection. The two ducks with a positive titer only had a titer of 16. The HI titer of ducks initially infected with the H5N2 virus was only marginally higher. Four ducks had an HI titer of 16 and one had a titer of 32. Infection with the homologous virus at 14 days boostered the titer of ducks that were challenged with either the H3N8 or H5N2 viruses. The HI titers started to slowly wane by 28 days post infection. None of the ducks produced a positive antibody titer to the heterosubtypic infection.

Only four ducks in study 2 produced positive antibody titers to the virus they were initially challenged with prior to the secondary challenge (Table 3-3). Challenge with the homologous virus at 28 days post infection resulted in boosting of the HI antibodies. Antibody titers peaked at 128 for H3N8 virus and 256 for H5N2 virus, which is higher than that seen in study 1. Similar to study 1, none of the ducks developed positive HI antibody titers following challenge to the secondary heterosubtypic virus.
Table 3-2. Hemagglutination inhibition testing for mallards in study 1 for both H3N8 and H5N2. Primary infection occurred on Day 0; secondary infection occurred on Day 14. The detection limit is an HI titer of 4. – Indicates an HI titer <4, which is below the limit of detection.

<table>
<thead>
<tr>
<th>Duck Infection(^1)</th>
<th>HI Test</th>
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<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
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\(^1\) Primary infection X secondary infection
Table 3-3. Hemagglutination inhibition testing for mallards in study 2 for both H3N8 and H5N2. Primary infection occurred on Day 0; secondary infection occurred on Day 28. The detection limit is an HI titer of 4. – Indicates an HI titer < 4, which is below the limit of detection.

<table>
<thead>
<tr>
<th>Duck Infection¹</th>
<th>HI Test</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
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¹ Primary infection X secondary infection

**DISCUSSION**

Prior infection with a LPAI virus decreased the length of shedding of a subsequent infection by a second LPAI virus. The duration of shedding for the primary infection in this study was similar to previous reports that ducks regularly shed virus in feces up to 7 days post infection (26). Ducks can infrequently and sporadically shed virus after the 7 days post infection (26). The length of shedding for a secondary infection was shorter than a primary infection when data from both subtypes were combined. This is in agreement with our hypothesis that a prior heterosubtypic infection
would result in partial immunity to a secondary challenge resulting in decreased shedding. We believed that a non-specific immune response as well as some antibody cross-reactivity would result in this protection. The lack of antibodies that reacted across subtype suggests that a non-specific immune response plays the primary role in the decreased shedding duration.

The results from our experiments are similar to a previous study, which reported decreased levels of shedding of virus following both a secondary (21 days post primary) homologous and tertiary heterosubtypic infection (14 days post secondary) (512). The results presented here and previous studies suggest that there may be some heterosubtypic immunity provided by a previous AI infection. The study by Jourdain et al. (2010) assessed shedding by RT-PCR, but not by examining the presence of infectious virus (512). This study may have found even shorter shedding periods if only infectious virus was examined since RT-PCR detects viral RNA rather than intact infectious virions. The tertiary infection with the H5N2 virus resulted in detectable shedding in only 4 out of 6 birds (512). This is in contrast to the present study where all ducks shed virus during the secondary heterosubtypic challenge. There may have been a benefit of exposing birds to the same virus twice causing an overall increase in immune system activity.

We found most ducks re-infected with the same virus shed virus post-challenge, regardless of whether ducks were re-infected at 14 days or 28 days post primary infection and regardless of subtype. Some ducks that were infected with the homologous virus at the later time point did not shed virus, while all ducks at the earlier time point did shed virus. This is in contrast to previous experiments where none of these birds shed virus in their feces following re-inoculation with a LPAI H7N7 (26). Birds were protected up to
96 days post infection from re-infection in that study. The research presented here suggests shedding of virus from ducks infected following secondary exposure to the same LPAI virus may be strain dependent.

Since birds are not fully protected from a secondary heterosubtypic AI infection at short time periods, this may help to explain perpetuation of AI viruses in nature. Ducks congregate in northern nesting areas prior to migration where isolation of AI viruses is highest (12). This congregation may result in birds being exposed to numerous subtypes of AI viruses over a short period. As a result, the birds may shed different viruses for short periods of time following an initial infection allowing for multiple viruses to be maintained. As the birds migrate south the detection of AI decreases. The birds may continue to be exposed and shed virus, but due to prior infections they would only shed for a short duration. These short time periods may make it difficult to detect all of the circulating viruses in a population. These short durations of shedding may explain why some viruses are isolated more frequently, why there seems to be a yearly periodicity to the subtype isolated and the lack of isolation in southern migratory locations.

Ducks challenged with a secondary infection at 28 days following the primary infection shed virus for a shorter period of time than ducks challenged at 14 days. We initially hypothesized that shedding would be shorter for the secondary infection at the 14 day time point in comparison to the 28 day time point, believing that activation of the non-specific immune response would curb the secondary challenge at the earlier time point. At the later time point we believed that this response would have waned. Instead, the duration of shedding was significantly shorter for ducks challenged at 28 days. The shorter duration of shedding at the 14 day time point, which was not statistically different
between homologous and heterosubtypic infections suggests that non-specific immunity plays a role in partially protecting ducks from sequential infections at short time intervals. At the 28 day time point, the homologous infection was significantly shorter than the 14 day infection, suggesting that the ducks had time to develop a humoral immune response that played a greater role in protecting the duck at longer time points.

Other studies focused on the difference in morbidity and mortality of ducks previously infected by a LPAI virus then challenged with an HPAI H5N1 virus. One study examined wood ducks (Aix sponsa) previously exposed with homosubtypic LPAI H5N1 and H5N2 viruses and heterosubtypic LPAI H1N1 virus 21 days prior to HPAI H5N1 virus exposure (513). The study found that there was a significant level of protection by prior exposure to homosubtypic H5 virus infection. Protection was afforded to the 5 birds infected with the LPAI H5N1 virus that did not develop a detectable humoral immune response by HI. The infection resulted in an increase in HI titer to the initial virus. In comparison, there was not a significant decrease in mortality by prior exposure to the H1N1 virus. In the ducks previously infected with the H1N1 virus, mortality was a moderate 60% in comparison to the naive ducks, which was 100%. Further demonstrating that there may be some cross protective effects by prior infection with a heterosubtypic virus, wood ducks that sero-converted to the initial H1N1 virus, only one third died. In another paper, mallards were infected with LPAI H4N6 or H5N2 viruses and 7 weeks later were inoculated with an HPAI H5N1 virus (514). Prior infection with heterosubtypic H4N6 virus resulted in decreased morbidity and mortality. Prior infection with an LPAI H5N2 virus resulted in no clinical signs when ducks were later infected with the HPAI H5N1 virus. The morbidity/mortality results were mirrored
by the results of viral shedding. There was decreased viral shedding in the group that received the H4N6 virus, while only a few ducks in the study that were previously infected with the H5N2 virus shed. Cross-protection from a LPAI virus to HPAI H5N1 has also been noted in chickens (518). These findings strengthen the role of prior infection with a heterosubtypic AI providing a degree of protection to ducks later infected with another AI virus.

Infection did not induce antibodies that cross-reacted between H3 and H5 subtype viruses. Although antibodies were not detected in many ducks in study 2 following the initial challenge, there most likely was an immune response that resulted in activation of B cells with antibodies to the HA. Ducks challenged with the homologous virus developed a strong immune response as determined by HI antibodies only seven days following the secondary infection. None of the primary infections resulted in this robust of an antibody response. Therefore, these results suggest the presence of memory B cells that responded to the secondary infection. Potentially, the AI virus can be cleared from the GI tract before a detectable humoral immune response is necessary. As a result, detectable HI titers do not occur.

Other previous studies noted a lack of antibody response in ducks to LPAI. In one study, primary infection in ducks with an H7N7 virus did not result in an HI antibody titers greater than 20, while secondary infection resulted in antibody titers greater than 20 in only four out of 6 birds (512). In the same study these birds received a tertiary challenge with an H5N2 virus, resulting in H5 specific antibodies detected by ELISA in only 1 out 6 birds and none of the six birds had HI H5 titers greater than 20.
An unknown factor in this study is whether or not a cell mediated immune response is involved in cross-subtype protection. Humans have CD8+ T cells that may provide a degree of cross protection by recognizing M1 and NP, which are more highly conserved than the surface glycoproteins HA and NA (519-522). It has been suggested that multiple infections of influenza viruses of different antigenicity results in selection for cross protective CD8+ T cells (523). The presence of CD4+ T cells that respond to M1, NP and NA proteins of HPAI H5N1 virus have been demonstrated in humans that have only been exposed to seasonal influenza viruses (520, 521, 524). Whether or not these immune cells provide a degree of cross-protection to humans infected with HPAI H5N1 virus is unknown. The difference in rate of symptomatic infections in adults versus children during the 1957 H2N2 influenza pandemic suggests that a degree of cross-reactive immunity from previous infection with seasonal H1N1 may have existed in adults (525). In chickens, CD8+ T lymphocytes provided a degree of cross-protection to infection by HPAI H5N1 virus, but this may not hold true for ducks (451, 452). Mitogen/antigen stimulation assays have suggested that infection with a LPAI virus results in suppression of duck T-cells (526). Therefore, the cellular mediated arm of the immune system may play less of a roll in influenza infections in ducks compared to mammals. The initial study to look at re-infection of ducks with a LPAI H7N7 virus found a similar weak humoral immune responses (26). In that study two out of three duck exhibited antibody titers of 1:16 at time points 14 (1 duck) and 21/28dpi (both ducks). An additional 10 ducks were re-infected with the same virus at intervals from 21-84 days (initially very little antibody response). Only one duck re-inoculated before 28 days exhibited an HI titer of 16 at 7 days post infection. Duck re-infected at 46 and 56
days post initial had peak antibody titers of 256. This mirrors findings from the current study where the HI antibody titers were frequently low or undetectable.

The research presented here suggests that sequential infections of ducks with AI viruses may result in shedding of the secondary virus for only short durations. Although the longer the period of time between infections results in a shorter duration of shedding due to re-infection with homologous virus, this does not hold true for heterosubtypic viruses. Therefore, the short period of time the ducks may shed virus during secondary infections may interfere with surveillance of waterfowl by mitigating detection of AI viruses in ducks as they migrate south. The lack of complete immunity of ducks to a secondary infection with a heterosubtypic viruses resulting in a short shed duration may allow for their continued circulation through overwintering populations without detection. The lack of cross-subtype antibodies suggests that this partial immunity is mediated by an innate immune response. Modeling for the potential introduction of the HPAI H5N1 virus in North American waterfowl should take into account the short duration of virus shedding caused by prior infection with other AI virus subtypes.

**SUMMARY**

Prior infection with a LPAI virus altered the length of shedding of virus in the feces. Shedding was significantly shorter for ducks infected with either a homologous or heterosubtypic virus. Shedding was also significantly shorter when the secondary infection occurred 28 days following the initial infection compared to 14 days. No difference in shedding rates for the secondary infection were noted based on the viral subtype causing the initial infection. As seen in previous studies, low to no HI antibody titers were noted following the initial LPAI infection. A cross-reactive antibody response
was not detected following the secondary infection. Prior infection with a heterosubtypic virus did not appear to improve the antibody response to the second virus. A second infection with the homologous virus was capable of boosting the antibody response. Therefore, the innate immune response may be responsible for the shorter duration of viral shed in ducks infected with the heterosubtypic virus. The short duration of viral shedding following a secondary infection may allow for AI viruses to be maintained overwinter in populations of waterfowl without being detected. When developing models of AI in nature, consideration must be given to the probable effects of sequential infections and how they alter viral shedding in subsequent infections.
CHAPTER IV
TRANSFER OF MATERNAL ANTIBODIES TO HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA FROM MALLARD HENS TO DUCKLINGS

INTRODUCTION

At the time of birth or hatch, neonates from a majority of species lack antibodies but receive passive immunity from their dam via maternal antibodies (MAB). MABs aid in protecting neonates from pathogens experienced by their dam, which greatly assists in protecting them until they start producing their own adaptive immune responses (527). While MAB for several pathogens have been demonstrated to be passed through yolk to chicks in a variety of birds, currently there is no information on MAB against avian influenza virus in mallard ducks (*Anas platyrhynchos*). The research described here demonstrates the passage of MAB against highly pathogenic avian influenza virus (HPAI) from vaccinated mallard hens to ducklings.

Passive transfer of MAB has been demonstrated to be important for the survival of offspring from a variety of avian species (527). This phenomenon has been studied most extensively in chickens, where IgY, comparable to IgG in mammals, is transferred from the dam to the offspring through yolk (528). A survey of eggs at a broiler farm found transfer of MABs for the following pathogens: avian encephalomyelitis virus, avian influenza virus, chicken anemia virus, infectious bronchitis virus, Newcastle disease virus, infectious bursal disease virus, laryngotracheitis virus, *Mycoplasma gallisepticum, Mycoplasma synoviae* and reovirus (529). MABs were transferred to chicks following infection of hens with adenovirus BC14, a causative agent of egg drop syndrome (530) and MAB was demonstrated in yolks of experimental inoculation of hens with an H7N2, H6N2 and H7N2 avian influenza (AI) viruses (531, 532). MAB transfer
following vaccination of hens has been demonstrated for infectious bronchitis virus, infectious bursal disease, Newcastle disease, avian leukosis virus/Rous associate virus and avian reovirus (533-552). Turkey poult receive protective levels of MAB against hemorrhagic enteritis and MABs that are not protective to rhinotracheitis (553-555). Vaccination of Pekin ducks (Anas domesticus) with a DNA based hepadnavirus vaccine resulted in the passage of MAB in yolk (556). A common finding from many of these studies was a direct correlation between the level of antibody in the dam’s serum to that in yolk and in the chick’s serum (528, 539), although the relative efficiency of transfer of MAB varies greatly by pathogen (529). It is therefore important to examine each disease agent and avian species to characterize the ability of a dam to pass MAB in the yolk to her offspring.

Among wild birds, passive transfer of MAB has been most extensively studied for Flaviviruses. Wild bird chicks and eggs have been found to have MABs to Eastern equine encephalitis (EEEV), Western equine encephalitis (WEEV), St. Louis encephalitis (SLEV), Japanese Encephalitis (JEV), Murray Valley encephalitis and West Nile (WNV) viruses (557-564). Field studies indicated the presence of MABs against WNV in a wide range of species representing numerous families of birds. Birds for which nestlings have been demonstrated to have MABs to WNV include Cooper’s hawks (Accipiter cooperii), red-tailed hawks (Bubo virginanus), great horned owls (Bubo virginanus), black-crowned night herons (Nycticorax nycticorax), cattle egrets (Bubulcus ibis), double-crested cormorants (Phalacrocorax auritus), great egrets (Ardea alba), yellow-legged gulls (Larus cachinnans), greater flamingos (Phoenicoperus ruber) and glossy ibis (Plegadis falcinellus) (562-564). The presence of MAB to WNV in chicks has also been
demonstrated in naturally infected captive populations of rock pigeon (*Columba livia*) and Eastern screech owls (*Megascops asio*) (565, 566). Additionally, experimental infection of chickens with WEEV, SLEV, JEV and WNV, as well as doves with WEEV, SLEV and EEEV resulted in demonstrable transfer of maternal antibodies to chicks (558-560, 567, 568). House sparrows have been shown to pass MABs to chicks against SLEV and WNV (569, 570).

Little research has been conducted to examine the importance of MAB in ducks and particularly in mallards. Similar to chickens, IgY is the primary antibody passed from mother to duckling through the yolk sac (571). Considering the important role of passive immunity in protecting young birds from diseases experienced by adults, it is important to characterize the dynamics of passive transfer of antibodies to influenza viruses in mallards. There are currently many questions surrounding the ecology of AI viruses in the wild and a better understanding of the role of MAB in protecting ducklings from these viruses is important in modeling and predicting future outbreaks of AI. Similarly, an enhanced understanding of passive immunity to AI in ducks may be critical to developing more effective vaccination strategies, particularly for domestic ducks in countries where highly pathogenic avian influenza (HPAI) virus is endemic. Finally, devising vaccination programs for ducks requires a more precise knowledge of the decay of passive immunity in order to better recommend vaccination times that avoid interference via the presence of passive antibody.

**AIMS:** Determine the immunogenicity of a recombinant H5 hemagglutinin vaccine in adult ducks and characterize the transfer of maternal antibodies to this protein to both egg yolk and ducklings, as well as the decay of antibodies in ducklings over time.
HYPOTHESES: Mallards will develop a strong immune response as determined by a high antibody titer to vaccination with the H5 subunit vaccine. The rise of antibody titers in the mallard’s serum will mirror the detectable levels observed in the yolk. Maternal antibodies will be present in the ducklings’ serum and the half-life will be similar to that seen in other studies of passive immunity in birds.

MATERIALS AND METHODS

Animals

Four eleven month-old mallard hens and one drake were purchased from Field Trial Game Birds (Wellington, CO). The birds were identified by placing colored zip-ties loosely around one leg. On arrival, the hens were tested negative for antibodies to HPAI H5N1 by hemagglutination inhibition and were housed for the duration of the experiment under BSL-1 conditions in a horse stall. The birds were given straw hay for bedding, which also allowed them to build nests for laying eggs. They were fed a commercial wild game bird diet ad libitum and freely had access to water. In addition, the birds were given crushed shell for calcium supplementation once a week. The birds were placed under 16 hours of artificial lighting to stimulate egg laying. At weekly intervals, each hen’s cloaca was swabbed with a specific food coloring to determine which egg came from each bird. The eggs were dappled with the corresponding color to the hen when laid. Unfortunately, this could not be done daily because of concern over inducing excessive stress and affecting their laying rate.

Starting two weeks post-vaccination, eggs were collected every day and kept in a dark and dry location at room temperature. Eggs were placed into an egg-incubator twice
weekly until five weeks post vaccination. The eggs were incubated between 36-39°C at 80% relative humidity for 28 days in GQF 1502 Digital Sportsman Cabinet Style Incubator (GQF Manufacturing Company, Savannah, GE). Two days prior to hatching, eggs were moved from the rotating shelf to the bottom of the incubator. On the day of hatch the incubator was checked for ducklings every four hours. Although some eggs were labeled with the food coloring dye, the mobility of the precocious ducklings prevented determining with confidence which duckling hatched from which eggs. Following hatch, ducklings were transferred to rubber tubs with wood shavings for bedding and a heat lamp at one end. Ducklings were identified by colored zip-ties placed in combinations around each leg and had *ad libitum* access to gamebird feed and water. When the ducklings grew larger they were moved into a six foot diameter metal stock tank. At the end of the study, all birds were humanely euthanized using with an intravenous overdose of pentobarbital and carcasses were incinerated.

**Vaccine**

A recombinant H5 protein was provided by Dr. Joe Rininger of Protein Sciences, Inc. This protein was expressed from transformed Drosophila cells and was equivalent to the H5 protein from A/Vietnam/1203/2004 virus. Once the birds were laying eggs routinely, 18 days following being placed under artificial lighting, they were vaccinated a single time. Each hen was injected intramuscularly with a total of 20μg of the H5 protein in a volume of 200μl containing 50% Fruend's incomplete adjuvant (Sigma Chemical, Inc). The vaccine was administered as two 0.1 ml injections into each the left breast muscle and left thigh muscles.
Collection of Specimens

Blood from each mallard hen was drawn from the brachial vein at weekly intervals starting at day 0 through day 35 post-vaccination. Blood was drawn from the jugular vein of ducklings on the day of hatch and at 2, 4, 6, 10, 14, 18 and 22 days post-hatch. Blood samples were allowed to clot and centrifuged at 2000 x g for 10 minutes. Serum was decanted from the blood clot and frozen at -20°C until assayed.

Eggs were collected at weekly intervals until day 28 post-vaccination for examination of antibodies in the yolk. Since hens do not lay eggs on a daily basis, the egg laid nearest to the seven-day interval was collected and used for antibody analysis. Hens had stopped laying eggs regularly by day 35 and therefore collection of eggs for yolk analysis after that time point was not possible. The yolk was separated from the eggs and frozen at -20°C until assayed.

Hemagglutination Inhibition Assay

The hemagglutination inhibition assay was performed as described in chapter II. Briefly, two-fold dilutions of serum were prepared in a V-bottom 96 well and each well was then inoculated with 4 HAU of BPL-inactivated A/Vietnam/1203/2004 virus. For each assay, a column was used for back titration of the virus and for titration of a positive control antiserum. The virus and serum samples were allowed to incubate at room temperature for 30 minutes, followed by addition of 0.5% chicken erythrocytes. The samples were gently mixed and allowed to incubate for 30 minutes at room temperature. Hemagglutination inhibition was determined by the presence of a button of red blood cells and a “tear drop” running of erythrocytes when the plate was tilted. All samples are
assayed in duplicate and antibody titer was determined as the highest dilution serum to cause inhibition of hemagglutination.

To assay antibody in eggs, yolks were thawed and 0.5 ml aliquots were mixed with 0.5 ml of PBS in a 15ml centrifuge tube. The diluted yolk samples were sonified for one minute, then mixed vigorously with an equal volume of chloroform and centrifuged at 6000 x g for 15 minutes (572). The supernatant was decanted and tested by hemagglutination inhibition.

The half-life of anti-H5 MABs was calculated using ten ducklings with the highest antibody levels. The two time points used were two days post-hatching, to account for continued yolk absorption following hatching, and six days post-hatching since most ducklings still had detectable antibodies at this point. The half-life was calculated using the following equation that has been previously described for radioactive decay (573) (HI indicates hemagglutination inhibition antibody titer):

\[
\text{half-life} = \frac{(\text{days elapsed}) \times \log_{10} 2}{\log_{10} \left( \frac{\text{Initial HI}}{\text{Final HI}} \right)}
\]

**RESULTS**

All hens seroconverted by 14 days post-vaccination (Figure 4-1). Three mallard hens had detectable antibody levels by day 7 post vaccination with the highest HI antibody titer being 32. The titers for all four ducks peaked 14 days post vaccination then declined before leveling off at 28 days post vaccination. Hens 3 and 4 had the highest HI antibody titers of 124 at 14 days post vaccination. The HI antibody titers remained at 32 for hens 3 and 4, and 16 for hens 1 and 2 at 35 days post vaccination.
MAB was detected in the yolk of eggs that were collected from all of the mallard hens (Figure 4-2). Unfortunately, the ducks ceased laying eggs at five weeks post infection, most likely due to the stress of frequent handling for the project. Anti-H5 antibody could first be detected on day 9 post infection in an egg laid by hen 1. The results of the HI antibody titer for the egg yolks mirrored that of the hens. Mallard hens 3 and 4, which had the highest serum HI antibody titers, also had the highest levels of antibodies in the egg yolks. The highest antibody titer was recorded from Mallard 4 on day 28 post-vaccination. Egg yolk antibody titers for hens 1 and 2 peaked approximately three weeks post vaccination. Egg yolk antibody titers were highest on the last day measured, four weeks post vaccination.
Figure 4.2. Hemagglutination inhibition antibody titers against A/Vietnam/1203/2004 influenza virus using egg yolk from vaccinated mallard hens.

Eggs were collected for hatching once it was determined that hens had mounted a strong antibody response 14 days post vaccination. Ducklings were combined into three groups; those that were from eggs laid between 15 and 20 days post infection, from eggs laid between 22 and 27 days post-infection and those laid between 29 and 34 days post infection. The first group included 7 ducklings, the second group included 15 ducklings and the third group included 16 ducklings. Due to small veins, blood was unable to be drawn from one duckling in group 2 at day 0 and day 2 as well as one duckling in group 3 on day 2. A duckling in group 2 was found dead 12 days post hatching. Two ducklings lost their identification bands on day 10 post hatching in group 3 and one duckling in group 2, 15 day post hatching, and sera from these ducklings was not included in the analyses. Before the decision had been made to group sets of hatchling by the week they
were born, blood was not drawn on 3 ducklings in group 1 day 18 and 6 ducklings in group 2 day 18. All ducklings had MABs present in the serum (Figure 4-3).

The highest HI antibody titer was 128 in ducks laid in group 1 on the day of hatching. The lowest measured HI antibody titer on the day of hatching was 8 in a duckling from group 3. Antibodies were not detected in the majority of ducklings by 18 days post-hatch. The calculated half-life of the MABs for the ten ducklings with the highest HI antibody titer was 2.3 days. The range for the half-life was 1.6-4.0 days.

Figure 4-3. Mean hemagglutination inhibition antibody titers against A/Vietnam/1203/2004 influenza virus in the sera of ducklings from vaccinated hens. Group 1 includes ducklings from eggs laid 15-20 days post vaccination, group 2 from eggs laid 22-27 days post vaccination and group 3 from eggs laid 29-34 days post vaccination.
DISCUSSION

The Drosophila-derived H5 vaccine used in this study elicited antibody titers in mallard ducks similar to or greater than those seen in other vaccine studies (158, 444, 446-448). The HI titers in these previous studies ranged from ducks having no detectable titer to an average HI titer of 10 log2. These studies also indicate that HI antibody titers are not indicative of prevention of viral shedding. Therefore, it will be important to evaluate viral shedding of mallard hens vaccinated with the novel drosophila derived vaccine following challenge with HPAI H5N1 in future research. In this study, all hens developed detectable antibody titers following vaccination. The average HI antibody titer for the four mallard hens was 6.4 log2 from a single vaccination. This study found an antibody titer of 3.4 log2 one week post vaccination. Five days following vaccination, the average antibody titer was 4.6 log2, which is similar to the HI antibody titer of another study, 4 log2 14 weeks following vaccination (448). It would be important to determine how long antibodies would be present in ducks following vaccination with the novel drosophila derived vaccine. Additionally, it would be interesting to determine what the antibody response would be following a booster vaccination. Although this study was not designed as a vaccine trial, the strong immune response of the ducks suggests that the vaccine deserves further evaluation.

The presence of antibodies to HPAI H5 virus present in yolk is comparable to other species and other diseases. Passage of MAB has been observed in the yolk of WNV experimentally infected house sparrows (570). Chickens infected with WNV pass antibodies through the yolk to their offspring (568). This has also been previously observed with avian influenza antibodies. A field survey found MABs to AI virus in the
egg yolks of wild Yellow-legged gulls (*Larus michahellis*) (574). The passive transfer of MAB to AI has been examined in chickens inoculated with an H7N2 or H6N2 virus (531, 532).

Previous research demonstrated that detection of antibodies to an H7N2 LPAI virus was consistent in egg yolks 14 days post infection in chickens (531). Although the species are different, this is similar to the current study since antibodies were not consistently detectable until 14 days post vaccination. During an outbreak of H7N2 LPAI, sampling of egg yolks for maternal antibody was performed and it was concluded that it could be a helpful method for monitoring for AI (575). The data from this experiment suggests that egg yolks could be useful for monitoring AI infection in commercial and wild flocks of ducks. Egg yolks could also be used to monitor the efficacy of vaccination of ducks.

The peak antibody titer of yolks for two of the ducks was found a week following the peak antibody titer of the serum, which is similar to previous results for chickens infected with a LPAI H6N2 (532). Chickens, like the ducks in the current study, developed serum antibodies to AI virus earlier than detectable levels of antibodies in yolk. The serum titers in the infected chickens were also higher than that detected in egg yolk. One dissimilarity in the studies was the time difference in peak antibody levels. In chickens inoculated with LPAI H6N2 the peak antibody titer was 4 weeks post infections and the peak titers in the yolks was 5 weeks post infection. Following vaccination, the peak antibody titer in the mallard hens was 2 weeks post vaccination and the peak antibody titer in the yolks was 3 weeks post vaccination for two of the mallards. The difference in timing of peak antibody titer in the serum may be due to the difference in
continued antigenic stimulation as a result of an active infection versus vaccination. The HI titers in yolk were lower than those recorded for an Egyptian poultry farm that uses Yebio H5N1 Re-1 vaccine where HI titers of yolk from day old chicks ranged from 10-160 with a GMT 48 (576). The difference in HI antibody titers may be due to a difference in preparation of yolk since Kim et al. 2010 did not use a chloroform extraction and used horse red blood cells. The same samples were tested with chicken red blood cells and no detectable HI titer was recorded. Finally, these eggs were collected from a poultry farm rather than under strict experimental conditions. The hens may have received more than one vaccination or there may have been circulating AI viruses that increased the serum antibody titers of the hens.

In previous studies, the half-life of MABs to influenza virus and the rate of decay of passive immunity were not calculated. The half-life for MABs against WNV for house sparrows was slightly longer than that seen in this study (3 days versus 2.3) (570). The ranges for the individual birds were similar (1.4-5.9 days versus 1.6-4 days). This and other studies indicate that the half-life of MABs of birds is greatly shorter than that of mammals (527, 570). There are likely two important reasons for the quick decay in MABs in the duckling’s serum. First, mallard ducklings grow quickly resulting in a rapid increase in circulatory volume (577). This in turn causes a dilution effect on the concentration of antibodies in the serum. Secondly, studies in chickens suggest that the high metabolic rate of birds results in a catabolism of MAB present in the serum (578). One study indicated that MABs remain high from hatching until 5 days of age (571). We found that the majority of ducklings in groups 1 and 2 had HI antibody titers that remained at 16 or above by 6 days post-hatching. In several cases the individual antibody
titer for a duckling increased after birth and peaked at day 2. This has been previously described with duck antibodies peaking at 3-7 days of age due to continued absorption of the yolk after hatching (571). It has been demonstrated that ducklings absorb the majority of their yolk sac over the first 5 days of life (579). Transfer of MABs against AI viruses may be an important means of protection of young ducklings since de novo synthesis of antibodies by the ducklings were not detected until 20 days of age (571); this result is surprising and bears re-evaluation.

The presence of maternal antibodies in ducklings does not indicate their biological significance. In some instances, MABs may suppress responses to a pathogen. MABs against St. Louis encephalitis virus have been shown to be passed in house sparrows, but instead of protecting the chicks they appeared to result in viral enhancement (569). MABs may also lack efficacy in protection of young animals from disease. Vaccination of turkey hens with rhinotracheitis virus was associated with the transfer of maternal antibodies, but they were not protective (554, 555). The protective effects of maternal antibodies have been noted in some instances. Turkey poults were protected until 6 weeks of age by MAB against hemorrhagic enteritis (553). Vaccination of chickens with an inactivated reovirus vaccine resulted in the transfer of passive immunity in the yolk and protection of chicks from challenge (548-550). MAB has been shown to be protective in chicks and interfere with vaccination of chicks for infectious bronchitis virus and infectious bursal disease as well as in poults for hemorrhagic enteritis (533-540, 553). Maternal antibody to H5N1 HPAI virus in chicks from vaccinated farms in Egypt was believed to inhibit the immune response to vaccination (576).
Additional areas of research on this topic should include vaccinating ducklings that had MAB to determine if MABs to HPAI H5N1 prevents effective vaccination. The strong immune response produced by the hens to the vaccine used in this study may indicate that the vaccine may be able to overcome maternal protection. Additionally, vaccinated adults and ducklings with MABs should be challenged with HPAI H5N1 virus to determine the efficacy of the vaccine in ducks. Finally, to determine if MABs play a role in AI in nature, challenging ducks with a live virus and demonstrating transfer of MABs to ducklings would be useful. Additionally, harvesting eggs from nests of wild ducks to test for maternal antibodies in yolk could demonstrate their transfer to ducklings in nature.

**SUMMARY**

The focus of this study was to evaluate the magnitude of passive transfer of anti-influenza virus antibodies in ducks and to determine the rate of decay of such immunity after hatch. This is particularly important to know considering that ducks and other waterfowl are the natural reservoir host for this type of virus. The subunit H5 vaccine elicited antibody titers in adult mallard ducks similar to or greater than those seen in other vaccine trials. The antibody titer peaked two weeks following vaccination with the highest detected titer being 124. Although high antibody titers were detected, it is unknown if they would prevent disease or shedding. MABs against H5 HPAI can be detected in the egg yolk of vaccinated mallard hens. Yolk antibody titers peaked three weeks post vaccination for two hens, while the titers were still rising four weeks post vaccination for the other two hens. The highest yolk antibody titer recorded was 32. Testing duck egg yolks for AI antibodies may provide a means of testing for the presence
of circulating AI in flocks or the efficacy of vaccination programs. The high levels of MABs in recently vaccinated mallard hens may protect ducklings from infection, but may also interfere with vaccination.
PERSPECTIVE

One of the main mysteries about avian influenza virus is how it is maintained in nature, because of the difficulty in detecting virus in waterfowl outside of fall migration. As birds migrate south, the rate of detection of avian influenza decreases. Additionally, there is great diversity in the subtypes of avian influenza virus and only a few subtypes predominate in surveys. The chapters presented in this dissertation help to explain this ecological conundrum.

The results of Chapter II indicate that under cold conditions live virus can persist for up to two weeks in feces. Since ducks migrate in a pattern that follows near freezing conditions, virus may persist in the environment for up to two weeks. Virus can be detected even longer (up to three weeks) by real-time RT-PCR allowing for a less labor and time intensive method for surveying wild waterfowl for the presence of avian influenza. Further research should be pursued to determine if the virus that persists in feces can infect ducks in order to demonstrate that feces may pose a source of infectious virus in the nature.

Sequential infections with a heterosubtypic avian influenza viruses was shown to result in a shorter duration of viral shed during the second infection compared to the primary infection in Chapter III. No cross-reactive antibodies were detected by hemagglutination inhibition between the viral subtypes. As a result, the decreased viral shedding is believed to be due to the innate immune response. As ducks congregate and migrate south, they are increasingly exposed to different avian influenza subtypes. Surveys of wild ducks may not detect avian influenza virus due to the decreased duration of viral shed. Even though avian influenza virus is detected at a low rate in ducks on
wintering grounds, this may reflect the short duration during which the virus is shed rather than an absence of circulating virus.

An experimental HPAI H5 subunit vaccine was used in Chapter IV to test for transfer of maternal antibodies to ducklings. Although not designed as a vaccine trial, the strong antibody response elicited by the vaccine suggests that further evaluation for commercial use may be fruitful. Other vaccines used in ducks do not always elicit detectable antibodies although they are protective. A vaccine that can reliably stimulate the production of antibodies would be useful to show that the vaccine elicited an immune response and that there was not a vaccination failure. This study showed that it was possible for maternal antibodies to be transmitted between hen and duckling although there was rapid decay of the antibody titer in the ducklings. Further research should be pursued to determine if these antibodies are protective and whether or not they may interfere with vaccination. When models are created for the potential spread of the HPAI H5N1 virus in North American waterfowl, they should take into account the persistence of virus in feces, the short duration of virus shed during secondary infections and the presence of maternal antibodies in ducklings.
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