EXAMINING THE ROLE OF ADAPTIVE HETEROSUBTYPIC IMMUNITY IN THE EPIDEMIOLOGY OF LOW PATHOGENIC AVIAN INFLUENZA VIRUSES IN MALLARDS

by

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(Under the Direction of Monique S. França)

ABSTRACT

Influenza A virus (IAV) is a highly contagious pathogen that represents one of the most serious threats to animals and humans worldwide. Wild waterfowl from the order Anseriformes and Charadriiformes are considered the natural reservoirs for all subtypes of IAV. Several factors such as seasonality, spatial dynamics, host density, and immunity contribute to the epidemiology of IAV in these host populations. The primary goal of this research was to provide a better understanding of the role of heterosubtypic immunity (HSI) in the epidemiology of IAV in mallards. Our first objective was to evaluate the protective effect induced by prior infection with H3N8 LPAIV inoculation against subsequent infections with closely and distantly related LPAIV subtypes at different time points. Also, we wanted to determine if subsequent inoculation of birds with different IAV subtypes has a boosting effect on induction of this cross-protective immunity. The results demonstrated that the duration and extent of viral shedding was reduced in birds that had previously been infected with H3N8 and these effects were most pronounced when challenged with IAV subtypes genetically related by the hemagglutinin (HA); also,

these effects were boosted with each subsequent infection. Our second objective was to determine if previous infection with H3N8 and resulting HSI increased the infective dose of closely (H4N6) and distantly (H6N2) related LPAIV during subsequent challenge in mallards. In both cases, the required infective dose was higher in birds previously infected with H3N8 as compared to naïve birds and this increase was most apparent with the more closely genetically related H4N6 virus. Our third objective was to determine the agreement between the microneutralization and hemagglutination inhibition assays for antibody detection in sera of mallards experimentally infected with the H3N8 virus. We found a slight to substantial agreement between the assays when samples were tested at different time-points. Overall, the host and viral factors investigated in this research demonstrated that HSI could be an important factor related to seasonal IAV prevalence and subtype diversity in waterfowl, and the potential for new viruses to successfully establish in mallards and other waterfowl in nature.

INDEX WORDS: Influenza A viruses, mallards, heterosubtypic immunity, adaptive immunity, Low Pathogenic Avian Influenza viruses, hemagglutination inhibition, microneutralization.

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DEDICATION

Dedicated to my family for their unconditional love and support through these years.

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CHAPTER 1

INTRODUCTION

Influenza A viruses (IAV) are classified according to the antigenic properties of two surface proteins the hemagglutinin (HA) and neuraminidase (NA). Sixteen subtypes of HA and nine subtypes of NA have been described in wild birds around the world [1-3]. Wild waterfowl of the order Anseriformes and Charadriiformes are the natural reservoirs for all subtypes of low pathogenic avian influenza viruses (LPAIV) and are usually asymptomatic [1, 4]. The HA is the major antigenic and virulence determinant of IAV and plays a critical role in conferring protective immunity [5, 6].

Mallards are considered the major natural reservoirs for most subtypes of IAV in nature [2, 7]. Seasonal patterns of infections caused by IAV have been reported [8-11]. Peaks of infection are observed following the congregation of wild birds in breeding grounds at the end of the summer and beginning of the fall in the Northern hemisphere [1, 2]. A reduced prevalence is observed in wintering grounds in southern parts of North America [1, 2]. Seasonal subtype variations have also been observed in wild bird populations through the years [12, 13]. It was previously suggested that homo- and heterosubtypic immunity might be responsible for driving this subtype diversity favoring antigenic dissimilarity [13]. Despite extensive surveillance studies trying to describe the epidemiology of IAV in wild birds, the effect of adaptive immunity on shaping the prevalence patterns of IAV in wild bird populations is poorly understood. A better

understanding of the factors involved with subtype fluctuations in mallard populations, such as heterosubtypic immunity, is needed. Our aim was to evaluate the potential role of adaptive heterosubtypic immunity in the epidemiology of low pathogenic avian influenza viruses in mallards.

1. **Is the magnitude of heterosubtypic immunity conferred by an H3N8 inoculation positively associated with the genetic relatedness of the hemagglutinin of the viruses encountered?**

   Seasonal patterns of IAV prevalence among wild birds have been described; however, factors and mechanisms that drive diversity and IAV subtype fluctuations, such as homo- and heterosubtypic immunity, are still not completely understood. Previous studies have demonstrated the induction of homosubtypic and partial heterosubtypic immunity in mallards [16–20], and this has been supported by field observations [21]. However, additional experimental studies assessing the effect of reinfections with closely and distantly related LPAIV in mallards were still warranted.

2. **Does the extent of heterosubtypic immunity conferred by infection with H3N8 LPAIV increase the infective dose required in a second challenge?**

   Patterns of infections by LPAIV in wild birds have been observed in nature where circulation of similar or closely related subtypes has been negatively affected across time probably as a result of homo- or heterosubtypic immunity, which favors the emergence of antigenically distant strains (15, 16). Additional

research investigating the magnitude of this induced cross-protection and its effect on the probability and outcome of subsequent infections were still needed.

3. **Is there agreement between the results obtained by microneutralization and hemagglutination inhibition assays in mallard serum samples?**

Microneutralization assays have been demonstrated to be more sensitive in measuring humoral immunity against IAV as compared to hemagglutination inhibition assays in different species [29, 30]. Also, previous studies have suggested that ducks, as compared to chickens, have a reduced humoral immune response after infections with IAV [14]. Comparison of MN and HI assays for assessment of humoral immunity is needed to both improve the tools for serosurveillance and provide a needed perspective for the interpretation of serologic data from wild birds.

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CHAPTER 2

LITERATURE REVIEW

Influenza viruses

Influenza viruses belong to the *Orthomyxoviridae* family, which are single-stranded, negative-sense, and segmented RNA viruses [1]. These viruses comprise seven genera: *Influenzavirus* A, *Influenzavirus* B, *Influenzavirus* C, *Thogotovirus*, *Isavirus*, *Quaranfilvirus* and the new genus *Influenzavirus* D [2-5]. Only Influenza A viruses (IAV) are capable of infecting a wide variety of species including humans, birds, swine, horses, and dogs [3]. Occasionally, IAV have been isolated from other species such as cats, Owston civets, whales, seals, leopards, tigers, stone martens, mink, and camels [6-12].

Viral structure

The morphology and size of influenza viral particles are variable and range from spherical (80 to 100 nm) to filamentous forms that can be longer than 300 nm in length [13, 14]. The genome of IAV consists of eight negative sense segments which encode for at least ten open reading frames (ORFs) [1]. Those proteins include three polymerases (polymerase basic protein 1 [PB1], polymerase basic protein 2 [PB2], and polymerase acidic subunits protein [PA]), nucleoprotein (NP), two matrix proteins (M1 and M2), two non-structural proteins (NS1 and NS2), and two surface proteins (hemagglutinin [HA]

and neuraminidase [NA]) [2, 15, 16]. However, further studies have identified other proteins such as PB1-F2, PB-N40, PA-X, PA-N155, PA-N182, M42, N53, and a hypothetical NEG8 ORF [17-24]. Due to the small size of the IAV genome, the virus increases the coding capacity of individual segments by alternative splicing of viral mRNAs, leaky ribosomal scanning, non-AUG initiation, re-initiation, and ribosomal frameshifting [25]. IAV have three membrane-associated proteins projecting from the host surface: HA, NA and a small amount of M2 [2]. The M1 protein lies underneath the envelope and interacts with the cytoplasmic domains of the surface glycoproteins and the viral ribonucleoprotein complexes (vRNPs) [26]. The vRNPs consists of the viral RNA segments bound to the RNA-dependent RNA polymerase proteins (PB1, PB2, and PA) and the NP. The NEP/NS2 is present in clarified viral preparations [1].

Classification of Influenza A viruses

IAV are classified according to the antigenic properties of two surface proteins, the HA and NA [1]. Currently, 16 subtypes of HA and nine subtypes of NA have been reported in wild birds [27]. More recently, two additional subtypes of HA (H17 and H18), and two subtypes of NA (N10 and N11) have been described in bats [2, 28, 29]. IAV are also classified based on pathogenicity in chickens and molecular features in high (HPAIV) or low (LPAIV) pathogenic avian influenza viruses. To date, only some strains of the H5 and H7 subtypes have been able to cause outbreaks of HPAIV [27]. Currently, the H5, H7, H9 subtypes are considered to be particular public health threats, as these viruses are widespread in birds and can infect humans directly [3].

Viral cycle

IAV attaches to the 5-N-acetylneuramic acid (sialic acid) on the surface of cells via the HA glycoprotein [30]. Internalization of the virus occurs mainly by clathrin-mediated endocytosis [31]. However, other mechanisms such as non-clathrin, non-caveolae-mediated internalization have been described [32]. The HA protein is synthesized as a precursor (HA0) that is cleaved to generate the active HA1 and HA2 subunits [33]. The low pH (5.0) within the endosome triggers conformational changes of the HA that allows exposure of the fusogenic domain at the N-terminus of HA2, inducing fusion of the endosome and viral membranes [26]. The vRNPs are then released into the cytoplasm of the cell and transported into the nucleus through nuclear localization signals (NLSs) [34].

Once the vRNPs are localized in the nucleus, the negative sense viral RNAs (vRNA) are transcribed into messenger RNA (mRNA) by a primer-dependent mechanism [35]. The endonuclease activity of the PA protein and the cap-binding function of the PB2 are required to generate a 5'-capped primer that is stolen from the host pre-mRNA transcripts via "cap-snatching", while the PB1 subunit is directly involved in RNA synthesis [36, 37]. Transcription is then initiated and elongation of the mRNA is continued until the vRNA polymerase complex reaches the poly-U sequence motif at the 5' end of the vRNA template, producing the addition of the poly(A) tail to the mRNA [38, 39]. Viral mRNA is then transported to the cytoplasm and translated by the host cellular ribosomes [36].

Replication of IAV occur in two steps; first, a full-length positive sense copy of the vRNA is synthesized (cRNA), this template is used to produce more negative sense

vRNA [40]. It has been suggested that accumulation of free NP protein induces the switch between mRNA and cRNA [41]. Newly synthesized NP, PB1, PB2, and PA are imported into the nucleus, where new vRNP complexes are assembled [36]. The M1 and NEP/NS2 proteins direct the nuclear export of the vRNP to the cytoplasm [42, 43]. After translation of the HA, NA, and M2 by the host machinery, the three integral membrane proteins enter the endoplasmic reticulum, where the HA and NA are folded and glycosylated [1]. Next, the proteins are transferred to the Golgi apparatus where the cysteine residues of the HA and M2 are palmitoylated in the cis-Golgi network [1]. Cleavage of the HA0 protein with a series of basic amino acid at the proteolytic cleavage site may occur in the trans-Golgi network by ubiquitous proteases such as furin-like proteases that recognize the multibasic motif R-X-K/R-R [44]. Subsequently, the HA, NA, and M2 proteins are mobilized for assembly to the apical plasma membrane by their apical sorting signals [3]. Two mechanisms of packaging of viral RNA segments have been described. The first model assumes that vRNPs are randomly incorporated into budding virions, with some virions possessing more than eight complexes [45]. The second model suggests that incorporation of each vRNA segment occurs by its unique packaging signals, which assures that each virion contains a full complement of the eight vRNP segments [46].

The budding process requires extrusion of the viral particle until the membranes are fused at the base of the bud, and the enveloped viral particle is released [47]. This process involves the interaction of cellular and viral components, but the exact mechanism is still unknown [1, 48]. The budding process is very inefficient as only around 10% of the viral particles are released [49]. The sialidase activity of the NA

protein is required for release of viral particles that are anchored to the sialic acid of the cell by its HA and to avoid viral particle aggregation [1]. Cleavage of HA0 holding a single arginine at the cleavage site occurs during viral budding or infection, by secreted or membrane-bound host trypsin-like proteases [44]. These enzymes are only found at specific locations in the body, usually on mucosal surfaces of the respiratory and gastrointestinal tracts [44].

Molecular determinants of pathogenicity

Hemagglutinin

The HA is a homotrimeric rod-shaped type I transmembrane glycoprotein [15]. Each monomer unit has a length of 540-550 amino acids that contains an N-terminal signal sequence and a C-terminal membrane anchor [50, 51]. HA monomers are synthesized as precursors (HA0) that undergo proteolytic cleavage to generate disulfide-bonded HA1 and HA2 polypeptide chains before activation [52, 53]. Most IAV contain a single basic amino acid residue (arginine, rarely lysine) at the cleavage site and are classified as LPAIV [1]. Some H5 and H7 subtypes possess multiple basic amino acids that are cleaved by ubiquitous proteases that recognize the multibasic motif and are classified as highly pathogenic avian influenza viruses (HPAIV) [1].

The HA0 of LPAIV is cleaved by trypsin-like enzymes at the cell surface or after the release of the virus from the cell [54]. Trypsin-like proteases are secreted by the epithelial cells lining the respiratory and digestive tract [51]. The HA of HPAIV is cleaved by ubiquitous proteases such as furin-like enzymes resulting in systemic infections [55, 56]. Several studies have shown that HPAIV emerge from LPAIV as a

result of modification in the amino acid composition at the cleavage site [51]. The mechanisms of cleavage site alteration include the acquisition of basic amino acids due to polymerase slippage, recombination of the HA gene with other viral segments or ribosomal RNAs, and insertions [56-58].

The pivotal roles of the HA are the attachment to the host cell receptor and fusion activities [59]. HA binds to the sialic acid (SA) present on the surface of the host glycoproteins and glycolipids [60]. The head of the HA is entirely formed by HA1 residues and contains the receptor binding site (RBS) [44]. Each membrane-proximal "stem" region is assembled from the HA2 and part of the HA1 and holds the fusion machinery [59]. The conformation of the SA in the host cells determines the preference of the IAV binding; thus, avian and equine influenza viruses preferentially bind to SA attached to the penultimate galactose sugar by an α2,3 linkage (SAα2,3Gal), whereas human-adapted and swine viruses prefer SA with an α2,6 linkage (SAα2,6Gal) [60]. Differences in binding specificities between IAV can be matched with the glycan distribution on infection sites [51]. The SAα2,6 is abundantly present in the trachea, and bronchus of the human upper respiratory tract, and in the type I pneumocytes in the lower respiratory tract [61]. The alveolar type II pneumocytes express predominantly SAα2,3, limiting transmissibility of avian influenza viruses in humans [60, 62]. In contrast, the gut epithelial cells of ducks hold mostly SAα2,3; although, recent studies have shown the presence of SAα2,6 in ciliated cells of the trachea and in the colon [63, 64]. Chickens express SAα2,6Gal and SAα2,3Gal in the respiratory and intestinal tract [65]. Similarly, both SAα2,3Gal and SAα2,6Gal are displayed on tracheal and intestinal cells of quail, turkey, pheasant, and guinea fowl, and might play a role in the adaptation of avian

influenza viruses to mammalian species [63, 66]. The scarcity of receptors in the upper human respiratory tract for avian influenza viruses limits cross-species transmission [62]. However, the species barrier might be overcome when infection with high viral loads occurs [60]. Effective maintenance and airborne transmission of IAV in new hosts require alterations in the HA binding properties [62]. For instance, the HA of the H7N9 virus causing infections in humans was able to bind to both receptors (SAα2,3Gal and SAα2,6Gal) by glycan arrays [67]. Alterations in certain amino acid positions in the RBS are responsible for the shift of H2 and H3 preference from avian to human receptors, which includes Gln222Leu and Gly228Ser [68]. Also, Asn182Lys has been associated with the occurrence of H5N1 cases in humans [69].

The interaction between the HA with the host receptor is not only limited by the linkage between the SA and the penultimate sugar residue, but also by the structure of the glycans, the length of the carbohydrate chain, branching pattern, as well as sulfation and fucosylation [1]. Another mechanism of modulation of the HA function is through glycosylation [70]. The HA protein contains N-glycans that are synthesized by the host cellular machinery; as a result, variations of the carbohydrates in the HA are host dependent [71]. There are conserved N-glycosylation sites among various HAs, while the location of the other sites differs between viruses [72]. Conservation of the glycosylation sites in the stem regions of the HA suggests important functional roles such as stabilization of the HA before fusion activation and transport of the HA to the cell surface [73, 74]. Previous research showed that H5 and H7 HAs isolated from chickens showed increased glycosylation and a deletion in the NA stalk as compared to duck viruses, which did not have a deletion and were not glycosylated [75].

Neuraminidase

The NA is a type II integral membrane tetramer and the second major glycoprotein of IAV, each of the four identical subunits is synthesized as a polypeptide of 470 amino acids in length [76, 77]. The NA has a cytoplasmic conserved region and a hydrophobic transmembrane region, which holds the stalk and head domains [78]. The head of the NA is a homotetramer, and each monomer is composed of six identical sheets arranged in a propeller formation [79]. The globular head holds the enzyme active site (glycohydrolase) that is in charge of removing the sialic acid residues of the host cell surface and the newly formed viruses [72]. The process involves cleavage of the SAα2,3 and SAα2,6 ketosidic linkages, matching the specificity of the HA [80].

The polymerase complex

Analyses of the H5N1 HPAIV strains causing outbreaks in humans in 1997, showed evidence of the role of the polymerase complex in the interspecies transmission [81]. Transcription of IAV is primer-dependent and requires cleavage of the 5'cap end of the cellular mRNA [1]. The PB1 protein is in charge of RNA synthesis, and the PB2 subunit contains the cap binding region [35, 82]. The PB2 627-domain is a residue that is responsible for conferring host-specificity [83]. The 627 residue is commonly a Lysine (K) in viruses adapted to mammals, whereas a glutamic acid (E) is mainly found in avian viruses [84]. Some H5N1 and H7N9 isolated from human outbreaks did not contain the 627K but acquired an asparagine (N) at the position 701 instead [85]. Similarly, the most recent pandemic 2009 influenza strain H1N1 attained a different signature 590S/591R [86]. Also, alternative signatures of adaptation have been described in other proteins such as PB1, PA, NP and NEP [87].

Potential mechanisms for the enhanced polymerase activity of the avian strains adapted to mammalian hosts have been suggested [88]. The first suggests that the replication of avian viruses requires higher temperatures (40 °C) in comparison to human viruses that prefer lower temperatures for replication (33-37 °C) [89]. However, the precise mechanism by which modifications at the 627 site would enhance viral replication at low temperature still needs to be elucidated. There is sustainable evidence of the requirement of alterations in the PB2 for inter-species transmission [90]. Modifications in PB2 E627K or D701N are important for transmission between mammalian hosts. However, these signatures alone are not sufficient for sustained airborne transmission in mammals and should be paired with changes in the HA as previously described [91].

Determinants of emergence and evolution of Influenza A virus strains

The genetic evolution of influenza viruses occurs by two different mechanisms: random mutations in the genome that occurs during the transcription process and genetic reassortment due to the segmented nature of the genome of IAV [26].

Random mutations

RNA viruses have a high mutation rate due to the lack of proofreading activity of the RNA-dependent RNA polymerase (RdRP) during viral replication [92]. The mutation rate of IAV is approximately 2.0×10^{-6} mutations per site per infectious cycle [93]. As a result, the subpopulations of RNA virus harbored by an infected host are genetically diverse "quasispecies theory" [94]. This mechanism allows the virus the opportunity to most readily adapt to changing environments and overcome selective pressures [95].

Selective evolutionary pressures include adaptation to new hosts or immune pressure that might select high fitness variants [86]. The rate of antigenic variation among the genes of IAV is variable, probably as a result of the differences in selective pressure that provide an advantage in the new host [3, 96].

High mutation rates are observed among the two major antigenic determinants such as the HA and NA and in the NS1 segment which works as an antagonist of the type I interferon pathway [97]. The HA has the highest mutation rate amongst the eight IAV segments due to the high selective pressure that is exerted on this protein [98]. The accumulation of mutations is responsible for the epidemics caused by IAV variants that prevail every 1 to 5 years in humans [1]. It was previously suggested that the HA of viruses isolated from wild aquatic birds evolve slower than those from terrestrial species such as poultry, swine, and humans [96, 99]. This variation might be associated with the differences in selective pressures among viral strains [99]. Another hypothesis suggests that environmental transmission might play a role in the slower evolutionary rates in wild birds by reducing the number of replications per unit time [96].

Genetic reassortment

Coinfection of one single cell by two or more different IAV strains might give origin to reassorted progenies that contain gene segments of both parental viruses resulting in "antigenic shift" [100]. The newly introduced proteins are antigenically distinct from the previous circulating strains and result in high infection rates of the novel virus in the immunologically naive population, resulting in pandemics [101]. There are at least 256 possible combinations that can be originated from reassortment between two different viruses, but not all combinations might be compatible in nature [1]. Three out of

four viruses causing human influenza pandemics in the last century have originated by reassortment of avian, swine and human viruses [102]. The importance of swine as "mixed vessels" for the emergence of avian influenza viruses have been extensively described [103].

Recombination

While homologous recombination is controversial for IAV, recombination by template switching has been demonstrated to play a significant role in altering the virulence or fitness of some IAV [1]. Outbreaks of HPAIV in poultry associated with recombination events have been previously reported [58]. LPAIV have turned into HPAIV following insertion of nucleotides from other gene segments [15]. For instance, the HPAIV strain causing the outbreak in Chile in 2002 originated from the insertion of 10 nucleotides in the cleavage site as a result of recombination between the HA and NP genes [58].

All of these mechanisms allow IAV to generate viral subpopulations with significant genetic diversity that might be able to cross the species barrier, escape neutralizing antibodies or confer antiviral resistance [16].

Antigenicity of Influenza A viruses

During influenza infections, antibodies to all the main viral proteins including surface glycoproteins and internal proteins are produced, indicating that these antigens are exposed to the humoral immune system [104]. As the major determinant of antigenicity, with a proportion of four HA to one NA, the HA induce a vigorous adaptive immune response which usually results in the formation of neutralizing antibodies [105].

The production of neutralizing antibodies exerts immune pressure and lead to the selection of "antibody escape" variants [106]. Most of the mutations occur in the HA1 region (near the receptor-binding site) and accumulate over time [107].

Antibodies against the stem region of the HA have been recognized to be extremely broad [108]. Those antibodies were able to neutralize all subtypes of HA within group 1 and/or group 2 [109, 110]. The most likely process by which these cross-reactive anti-stem antibodies neutralize influenza viruses is by blocking conformational rearrangements associated with fusion of the virus to the endosome [111]. A broadly neutralizing human monoclonal antibody that recognizes the highly conserved sialic acid binding site of H1 HAs has also been described [112]. The use of these conserved epitopes as immunogens with the possibility of developing universal influenza virus vaccines was expected [113]. However, induction of high levels of these stem-directed antibodies by vaccination continues to be a challenge, either because of poor immunogenicity, the method of immunization, or the more limited access to the HA stem [114].

NA molecules are also antigenic determinants under selective pressure in natural infections [115]. In contrast to the HA, antibodies produced against the NA do not seem to neutralize viral infectivity but block the release of the virus [116]. Reduction of general illness and viral loads after re-infection with viruses containing homologous NA proteins have been observed [104, 117]. Other structural and non-structural proteins such as the polymerase complex, matrix and nucleoprotein are also immunogenic; however, they do not seem to play a significant role in conferring protective immunity [104, 117, 118].

Ecology of Influenza in wild birds

Influenza A viruses have been described in at least 105 species of birds around the world [119]. All subtypes of HA and NA have been associated with waterfowl (ducks, geese, and swans) except H13 and H16 (which are related to shorebirds and gulls), and constitute the main reservoirs [119, 120]. The prevalence of IAV in waterfowl is changeable and varies depending on species, age and season [121]. In North America, IAV reaches peak prevalence at the end of the summer and the beginning of the fall (August-September), following the breeding season and it is associated with aggregation of young birds [120, 122, 123]. The IAV prevalence declines during the fall migration, and it is reduced when birds reach the wintering grounds [15]. Similar trends have been observed in Northern Europe; however, high prevalence from August to December with peaks occurring during late fall (October-November) have been observed [122]. Prevalence of IAV is higher in young birds as compared to adults which reflect their lack of immunity [124-127]. Among the dabbling ducks of the *Anas* genus, mallard (*Anas platyrhynchos*) is the most frequent species found to be infected with IAV [15, 119]. The highest prevalence rate in mallards in comparison to other bird species might be attributed to behavior, population size, or increased surveillance directed at this species [120].

In a long-term study conducted in Canada, the most common HA subtypes associated with wild ducks were H3, H4, and H6; while N2, N6, and N8 the most common NA subtypes found. Subtypes H1, H2, H7, H10, and H11, were less frequently isolated; and H5, H8, H9, and H12 only sporadically found [126]. Moreover, the presence of the H14 subtype was not demonstrated before 2014 in wild birds in North America

[128]. Although the H4 and H6 subtypes were also frequent in surveillance studies in Europe, the frequency of other subtypes was not significantly different [122, 129]. Thus, the prevalence of IAV in general, as well as the particular distribution of subtypes may differ among the different surveillance studies depending on species, time, and place [122]. Cycling patterns of viral subtypes from year to year have been observed, perhaps as a result of herd immunity to viruses of the respective subtype [123, 130, 131].

The H13 and H16 subtypes of LPAIV are primarily detected in gulls [120, 132, 133]. Experimental studies on different gull species propose that IAV is mainly or equally shed through the oropharynx [134, 135]. The H13 and H16 viruses have segments that are genetically separated from those of IAV found in Anseriformes, suggesting a recent genetic divergence from other LPAIV [136, 137]. There is no evidence of seasonality patterns caused by IAV infections in Laridae and prevalence rates reported are lower in comparison to Anatids [138]. Extended lifespan and acquisition of immunity may explain the differences in prevalence observed [138]. Long distances traveled by Laridae species including migration from Eurasia and North America might account for the intercontinental exchange of IAV [139, 140].

Waders in the Charadriidae and Scolopacidae families are adjusted to aquatic areas and often share habitats with ducks [141]. Long-term surveillance studies in waders are still warranted, but data obtained in North America propose a distinct role of these birds in the perpetuation of certain virus subtypes [101]. Seasonal patterns of IAV in waders appear to be reversed, with increased virus prevalence rates during spring migration [123]. These findings suggest a role for waders in the perpetuation of IAV, as carriers of viruses to the breeding grounds of ducks in the north during the spring [101].

Despite intensive surveillance programs, HPAIV H5N1 viruses have predominantly been found in dead wild birds [142]. However, wild waterfowl may have involved in the spreading of the HPAIV H5N1 from Asia to Europe, the Middle East, and Africa, as ducks have asymptomatic infections and can fly long distances [143]. In 2014, a novel HPAIV (H5N8) and its reassortant H5N2 isolated from wild birds caused outbreaks in poultry flocks in North America. Genetic analyses showed genetic reassortment of the viruses with Eurasian (EA) and North American strains [144]. The viruses were detected in live and some dead wild birds, and cause a high mortality rate in turkeys and chickens [145].

Pathology of IAV in Anseriformes

Low Pathogenicity Avian influenza

LPAIV replicate mainly in the epithelium of the bursa and lower intestine (ileum, ceca, and colon) [107-109]. Replication of IAV is associated with high viral loads in the cells and contents of the intestine ($10^{7.8}$ EID50/ml) during the first days of infection [110]. The IAV nucleoprotein antigen has been detected in a few epithelial cells of larynx and trachea at day one post-inoculation in mallards inoculated with an H3N8 LPAIV, and viral antigen was associated with inflammatory cells in both tissues [146]. The viral antigen has also detected in macrophages lining the lamina propia of these sites, without significant microscopic lesions [146]. Viral shedding decreases and clearing of the virus starts on day 5-6 post-inoculation [147, 148]. The effects of natural LPAIV infections in mallards is still controversial [149, 150].

High Pathogenicity Avian Influenza

Infections by HPAIV in Anseriformes are not commonly associated with symptomatology. Exceptions were observed during the HPAIV H7N1 outbreak in 2000 that was pathogenic for Muscovy ducks and domestic geese in Italy [151]. Ducks that were experimentally infected with H5 and H7 HPAIV isolated prior to 2002 mainly developed subclinical to mild disease with a few exceptions [152-154]. This trend changed with the emergence of the Asian HPAIV H5N1 in 2002. Mortality of exotic wild birds such as tufted ducks, flamingos, geese, swans, and other species was observed in two public parks in Hong Kong [155]. Experimental infections of Pekin ducks with the HPAIV H5N1 Asian strains in 2002 showed variations in lesions and lethality. Replication of the virus was associated with high viral loads and pathology was observed in multiple organs, especially in the brain [156]. Histological lesions were observed in the upper respiratory tract, brain, heart, pancreas, and adrenal glands. Severe lymphocyte infiltration was observed in the brain and heart and was associated with more severe lesion scores [156]. Age of the ducks at the time of infection was a factor determining the outcome and severity the disease [157]. Similar findings were observed when mallards were inoculated with an Asian H5N1 HPAIV. Ducks showed depression, mild diarrhea, decreased food intake and neurological signs. Recovery of the birds was slow and mortality was observed in one out of seven ducks [158]. In chickens and other species of Galliformes infected with specific strains of HPAIVs, the viruses preferably multiplied in the vascular and capillary endothelial cells [55, 159]. Endothelial tropism appears to be absent in ducks and wild birds except in black swans [156].

Transmission and persistence of IAV in wild birds

Transmission of LPAIV occurs primarily through the fecal-oral route by ingestion of contaminated water on shared aquatic habitats [148, 160] . Another route of transmission is cloacal drinking, or the intake of fluids through the cloaca, which may play a role in the infection of the cloacal bursa of young birds [150, 161]. The LPAIV replicate mainly in the gastrointestinal tract of wild and domestic ducks [148, 162, 163]. As a result, high viral loads are shed in the feces with an estimate of $10^{8.7}$ mean egg infectious doses (EID) per gram of feces [15, 164]. Different studies conducted in Canada, Minnesota and Alaska were able to isolate the virus from environmental samples [121, 165-167]. Experimental studies showed that IAV particles might remain infectious in water for prolonged periods of time [148, 168, 169]. As a result, environmental persistence has been suggested to play a role in the endemicity of avian influenza viruses in wild birds [15].

Different mechanisms of environmental persistence of IAV viruses from year to year have been suggested. The first one requires enough number of susceptible individuals infected at any given time to transmit the strain to new individuals to allow continuous circulation of IAV within reservoirs species [120]. A second mechanism suggests that IAV persists for long periods in frozen environments and infect individuals on their way back from the wintering grounds [160, 163]. The third mechanism suggests that interaction between different species that act as reservoirs for IAV such as waterfowl and waders allows transmission of the virus in either way [123].

Migrating behavior

In order to find more suitable conditions to elute changing environmental conditions, birds have adapted to migrate [170]. Every fall season an approximate of five billion birds start moving from North America to Central and South America [141]. Equivalent patterns are observed between Eastern Europe and Africa [171]. Short distance migrants barely travel few hundred miles from their breeding to their wintering grounds. In some cases, migration of these birds is more associated with changes in altitude rather than horizontal movement [172]. Certain species of birds can travel hundreds to thousands of miles to reach wintering sites. However, long-distance migrants need to equilibrate the costs and benefits of migration and replenish fat resources in places known as "stopovers" [173]. Birds spend a greater amount of time at stopovers resting and replenishing energy reserves [171]. Stopovers are crucial for the epidemiology of infectious diseases [174, 175]. These staging areas provide the opportunity for mixing and contact of birds traveling from different places that otherwise would be widely separated during the majority of the year [172]. Long-distance migrants from the orders Anseriformes and Charadriiformes such as ducks and waders prefer wetlands or tidal mudflats which can be limited [176]. Food availability and nutrients stored at spring stopovers are elemental to ducks, not only for body subsistence and survival but also for reproductive success [177].

Several mallard populations are migratory and depend on stopovers to complete their annual migrations [178, 179]. In the Americas, mallard ducks winter in areas south to Mexico, but also regularly deviate into Central America and the Caribbean between September and May. In Europe, mallards winter from Denmark to Northern France and

can be long-distance migrants [180]. During migration, mallards follow coastal lines and may exhibit leapfrog migration patterns [181]. Survival rates have reported being higher for adults than young birds, probably as a result of a more experienced behavior to avoid threats [179].

Mallards (*Anas platyrhynchos*) distribution and behavior

Mallard is the most numerous and widespread wild duck species around the Northern hemisphere [178]. Its breeding sites correspond to temperate and tropical regions of the Northern Hemisphere, including Alaska and southern Green land, and have been introduced into other regions in the southern hemisphere [178]. Mallards are dabbling ducks, which are characterized by surface feeding and drinking behavior [178]. Mallards are omnivorous and feed dabbling or grazing [141]. Duck diet consists of small invertebrates during breeding seasons, and vegetative elements during non-breeding seasons [182, 183]. The estimated mallard (*Anas platyrhynchos*) populations in North America in 2015 was around 11.6 million birds. Large populations are presumably more able of maintaining a great diversity of IAV subtypes, as recognized in dabbling ducks [130].

Mallards display a strong sex dimorphism, while females are covered by a brown feathered coat, males acquire their breeding plumage (metallic-green head) and mate in autumn [184]. Normally, individuals find new pairs each year, although re-mating might occur [178, 185]. Copulating males stay close to the female until eggs are hatched to avoid extra-copulations, once the eggs are hatched after two weeks, females take care of the ducklings until they are 50-60 days old [141].

Duck immunology against influenza infections

Innate immunity

Low and highly pathogenic influenza viruses initially infect cells lining the digestive or respiratory route in ducks respectively [146, 156]. Once these cells are infected, infection of macrophages and dendritic cells might occur [186]. Innate immune response is initiated when pathogen recognition patterns (PRRs) of the host senses the presence of viral RNA containing triphosphate groups at their 5' ends [187]. PRRs include Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I) and nucleotide-binding oligomerization domain (NOD)-like receptors [188]. TLRs recognize sense viral RNA presented at the cell surface or within the endosomal compartment [189]. Each of these TLRs has distinct ligand specificity, for instance, TLR3 recognize dsRNA, whereas TLR7/8 recognize ssRNA in mammals [190]. Similar functions to mammalian TLR were found in chicken and duck immune cells for ChTLR3 and ChTLR7, supporting the presence of and functional downstream signaling pathways [191, 192]. Although, TLR8 is disrupted and fragmented in chickens and ducks [192, 193]. TLR7 activation through MYD88 led to secretion of nuclear factor-kB (NF-kB) or interferon regulatory factor 7 (IRF7) and final stimulation of secretion of pro-inflammatory cytokines and type I IFNs respectively [194].

Another PRRs that play a major role in recognition of viral RNA are RIG-like receptors (RLRs) [195]. The RLRs family includes three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I preferentially recognizes dsRNA structures and short, blunt-ended 5'-triphosphorylated (5'ppp) dsRNA generated by viral RNA polymerases [196]. In contrast

to RIG-I, MDA-5 recognizes either a viral RNA 5' end carrying structures that are distinct from 5'ppp or longer structured RNA that would be generated during the viral life cycle [197]. RIG-I and MDA5 contain an N-terminal region consisting of two tandem caspase activation and recruitment domains (CARD1, and CARD2) which, upon virus sensing initiate downstream signaling via interaction with the mitochondrial antiviral signal adaptor protein (MAVS) [198]. This interplay initiates a signal cascade leading to activation and nuclear translocation of the transcription factors IRF-3 and NF-kB, which are needed to initiate transcription of messenger IFN-β [190, 194].

NOD-like receptors family include the NLRP3 or cryopyrin and have been well described. NLRP3 recruits apoptosis-associated speck-like protein (ASC) which contains a domain that activates pro-caspase-1 [199]. Caspase-1 activation leads to the processing and maturation of cytokines IL-1b and IL-18 [200]. Viral proteins of IAV such as M2 and PB1-F2 were found to induce NLRP3 inflammasome activation [199]. NLRC5 gene was found to be up-regulated in chicken lung tissues infected with after infection with HPAI H5N1 [201]. However, the role of avian NLRs in influenza viral immunity is largely unknown.

Type I and II interferon bind to their corresponding cell surface receptors: Interferon alpha receptor 1 and 2 (IFNAR1/IFNAR2) and interferon gamma receptor 1 and 2 (IFNGR1/IFNGR2) [202]. The receptor subunits dimerize and activate the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway [190, 194, 203]. Activation of Jak-STAT signaling pathways results in the downstream induction of a variety of interferon-stimulated genes (ISGs) through the generation of a nuclear IFN-stimulated gene factor-3 transcription factor complex in response to IAV infection [204].

Observed ISG following IAV infection in avian species include myxovirus resistance gene A (MxA), oligoadenylate synthetase-like (OASL), interferon transmembrane proteins 5/6 (IFITM5, IFITM3), interferon-induced protein (IFIT5), protein kinase R (PKR), transforming growth factor beta 1 (TGFB1), guanylate binding protein 1/7 (GBP1/GBP7), and the radical S-adenosyl methionine domain containing protein 2 (RSAD2) [204].

Chemokines expression, including CCL3, CCL4, CCL5, CCL10, CCL19, CCL21, CXCR4, CXCL10, CXCL9, CXCL11, and CXCL12, has been observed in chickens and ducks infected with influenza viruses [186, 188, 205, 206]. Chemokines control lymphocyte trafficking and are critical for T cell priming and initiation of the adaptive immune response [207]. Recruitment and aggregation of leukocytes at the site of infection in response to CCL19 and CCL21 chemokines expression has been demonstrated in ducks infected following HPAIV H5N1 infection [208].

Ducks are often asymptomatic to infection with IAV which can cause mortality in chickens [152]. Previous studies have suggested that the absence of RIG-1 in chickens might offer an explanation to their high susceptibility to influenza as compared to ducks [186, 209]. Recently, the increased induction of apoptosis in duck cells as compared to chicken cells was suggested as a mechanism of difference in pathogenicity after infection with HPAIV [210]. Rapid induction of IL-1b, IL6, and iNOS mRNA was observed in ducks at 8 hours post infection, whereas this response was delayed in chickens at one-day post-inoculation [186]. Also, supporting information about faster induction of apoptosis in the lungs of ducks at early times after infections as compared to chickens was observed [186]. Overall these differences during early stages of the innate immune response can

contribute to the differences in the outcome of infection between chickens and ducks. Dysregulation of cytokines and chemokines response may be a critical determinant of the severity and the outcome of influenza infection [188]. Cytokine storm has been reported in chickens and ducks after HPAIV infection [156, 211]. However, more information is warranted about dysregulation of cytokines and chemokines in birds after HPAIV or LPAIV infection.

Adaptive immunity

Neutralizing antibodies, as well as specific T-cell responses, are important for clearance from IAV infections [106]. Antigen presenting cells such as dendritic cells and macrophages have an important role in driving adaptive immune response. CD8 precursors T cells recognize antigens presented in MHC I and mature in cytotoxic T lymphocytes (CTL) [212]. Antigens presented by effector MHC I molecules are recognized by CTL that release cytolytic granules and kill infected cells [213]. CD4 precursors T cells recognize IAV antigens presented by MHC II molecules and mature in Th1 and Th2 cells [214]. Th1 cells secrete IFN- and IL-2, the latter is required for the proliferation of virus-specific CTL [187]. Secretion of IL-10 during early immune response induce differentiation into Th2 cells that secrete IL-4, IL-5, and IL-13 and aid in the induction of antibodies plasma secreting cells [106]. Antibodies target a variety of proteins but the ones directed against the trimeric globular head of the HA can provide sterilizing immunity[113]. NA-specific antibodies are less important than HA because they interfere with the last phase of viral but do not block infection [215]. Antibodies targeting the M2 protein do not afford complete protection but lessens the amount of virus that is shed and provide some protection from disease [216].

Cellular immunity following IAV infection induce memory CD4+ T cells that contribute to a faster control of subsequent IAV infections by direct effector mechanisms or helper functions [217]. CTLs cells target mainly conserved internal viral proteins such as M1, NP, PA, and PB2 [218]. Activation of memory CTLs cells results in lytic responses and release of perforin and granzymes causing apoptosis of infected cells [212].

The humoral response against IAV in poultry includes induction of systemic and mucosal antibodies [219]. Ig M is detected around five days after infection in turkeys and chickens [219]. Ig Y, the avian equivalent of Ig G but with four constant region domains, is detected shortly after [219]. Comparable to chickens, ducks produce three isotypes of heavy chains [220]. Ig M is the predominant B cell surface Ig and the antigen-specific antibody following antigen exposure that is replaced by Ig Y on time [220, 221]. Ig A is abundantly secreted in bile and mucosas and can neutralize IAV and inhibit virus hemagglutination [222, 223].

Immunoglobulins present in the serum prevent systemic dissemination of the IAV following mucosal infection [220]. Ig Y is secreted in two forms in ducks (7.8 S and 5.7 S sedimentation coefficient) [221, 224]. The first secreted Ig Y form have four domains in the constant region of the heavy chain. The second version of duck Ig Y has only two domains in the same region, and it is designated as IgYΔFc [220]. The proportion of IgY/IgYΔFc is around 3:5 but might be variable, and the latter predominates in late immune response [225]. The IgYΔFc antibodies are thought to be faulty in processes such as antigen internalization, complement fixation, opsonization, antibody dependent cellular cytotoxicity (ADCC), precipitation reactions or hemagglutination inhibition (HI)

[220]. Although IgYΔFc retains specificity for the antigen, viral opsonization by this truncate version of IgY would not facilitate viral clearance; however, this has not been formally tested [226]. Early experimental infections of Pekin ducks and pintails showed productive infection of ducks with LPAIV with scarce induction of HI antibodies [162].

Maternal antibodies, mainly full-length Ig Y, are passed to ducklings through the yolk [220]. The suggested mechanisms of protection by maternal antibodies is through the formation of antigen-antibody complexes and the subsequent reduction of antigen density [227]. The mucosal surfaces of the respiratory and intestinal tract of ducks are not protected by IgA through the first two weeks of age, this time corresponds to the period where ducklings are certainly exposed to influenza viruses in pond water [220].

Heterosubtypic immunity

Infection with one subtype of IAV typically confers protective immunity against reinfection by the homologous strain, and it is referred as "homosubtypic immunity." Also, previous studies in mice, ferrets, pigs and cotton rats have demonstrated the existence of "heterosubtypic immunity" [147, 228-231]. Heterosubtypic immunity (HSI) refers to the cross-protective immunity induced by infection with one subtype against a different subtype of IAV, this effect is particular for IAV reinfections, as re-infections with an Influenzavirus B is not affected [228].

Heterosubtypic immunity in other species

It has been known for decades that infection by IAV in animals confers some degree of protection against viruses bearing a different HA and NA subtype [228]. Antibodies against the HA can be divided into two categories: those reactive against the

globular head of the HA and those directed against the conserved stalk domain [113]. Antibodies directed against the globular head can block viral entry and fail to neutralize influenza virions when drift mutations occur [1]. Similarly to mammals, the most efficacious vaccines in chickens are the ones that closely match the head domain of the HA of the circulating strain which is highly variable [219]. Five distinct antigenic sites surrounding the receptor binding site have been identified in the head of the HA [1]. Those predicted sites are designated as Sa, Sb, Ca1, Ca2, and Cb in the H1 and A, B, C, D, and E in the H3 [44]. Recently some broad spectrum monoclonal antibodies targeting the RBS along with adjacent regions in the globular head of the HA have been identified [232]. However, neutralizing activity against different subtypes of IAV viruses is scattered in both groups [233]. Also, some antibodies targeting conserved epitopes surrounding the RBS across H3 strains [232].

Certain antibodies against the stalk domain that confer cross-protective immunity between different HA subtypes within or between groups have been described [234-237]. Anti-stem antibodies block the fusion of the viral membrane in endosomes, prevent cleavage of the HA0 by extracellular proteases, prevent viral egress or engage in Fc-Fc receptor interactions [234]. Indirect mechanisms include antibody-dependent cellular cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) [113]. However, HA stalk-antibodies are less frequent due to poor immunogenicity of the domain [238].

Besides the HA, the NA is also a target for conferring cross-protective immunity. Recently, a universal epitope in NA conserved among all influenza A and B viruses has been described [215]. M2 is a highly conserved protein between different subtypes of

IAV, transfer of monoclonal antibodies targeting the M2 protein confers protection in mice following infection with IAV [216]. Antibodies directed against another conserved protein the NP might contribute to cross-protective immunity [239].

Both antibodies and activated T-lymphocytes are produced in response to viral infection [106]. However, unlike antibody responses cellular immunity targets viral proteins that are more likely to be shared between different virus strains and subtypes, thereby allowing a greater breadth of protection [240]. Unlike the mouse model of IAV infection, few research has focused on the role of helper (CD4+) and cytotoxic (CD8+) T lymphocytes in IAV pathogenesis [219].

Cellular immunity also plays an important role in cross-protection and mainly targets internal proteins such as NP and other internal antigens such as M1 or polymerases [213, 241]. Previous studies in knockout mice demonstrate that even in the absence of antibody, a protective immune response is generated where CD8+ CTLs cells appear more effective than CD4+ [228]. Transfer of activated CD4+ CD8+ cells after infection with an H9N2 virus to naive chickens induced protection against infection by a H5N1 HPAIV; whereas, transfer of serum or antibodies did not have any protection effect [242]. Furthermore, H9N2 primed lymphocytes showed proliferation as compared to control cells when exposed to an heterosubtypic virus such as H7N2 [243].

Heterosubtypic immunity in ducks

Evidence of occurrence of heterosubtypic immunity in mallards was found in nature in a surveillance study in Sweden. Analysis of the patterns of infections showed a strong evidence of adaptive HSI preventing re-infections by IAV that shared the same HA clade [130].

Induction of HSI has also been experimentally assessed. Inoculation of mallards with the H3N8 followed by the H5N2 virus or vice-versa in 21 days intervals; showed partial cross-protective immunity. A stronger effect was observed in the group that was first inoculated with the H3N8 virus [244]. Fereidouni et al., inoculated two groups of 13-week old mallards with two distantly related LPAIV (H5N2 or H4N6), birds were then challenged with an HPAIV H5N1 when they were 20 weeks old. Clinical signs were not observed in the group primed with the H5N2 virus and were slightly reduced in the H4N6 group [158]. A different study attempted to simulate the natural transmission within seasons of IAV in wild birds, three-month-old mallards were infected with an H7N7 LPAIV and sequentially challenged with a homologous (H7N7) and heterologous (H5N2) LPAIV after 21 and 35 days, respectively. Homologous reinfection was limited, while heterologous challenge produced active infection in only one out of 6 ducks [147].

The overall goal of this research is to experimentally examine the role of adaptive heterosubtypic immunity in the epidemiology of low pathogenic avian influenza viruses in mallards (1) To assess the protective effect induced by prior infection with H3N8 LPAIV inoculation against subsequent infections with H4N6, H10N7 or H14N5 LPAIV and the cumulative effect of H3N8xH4N6 and H3N8xH4N6xH10N7 infections against subsequent challenges with H10N7 and H14N5, respectively. (2) To determine if cross-protective immunity conferred by H3N8 infection increases the required infective dose during re-infection with closely (H4N6) and distantly (H6N2) related low pathogenic avian influenza viruses in mallards. Additionally, we will try (3) To compare the agreement between hemagglutination inhibition (HI), and microneutralization assays (MN) assays in serum samples of experimentally infected mallards.

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CHAPTER 3

ADAPTIVE HETEROSUBTYPIC IMMUNITY TO LOW PATHOGENIC AVIAN INFLUENZA VIRUSES IN EXPERIMENTALLY INFECTED MALLARDS[1]

[1] Segovia KM, Stallknecht DE, Kapczynski DR, Stabler L, Berghaus RD, et al. (2017) Adaptive Heterosubtypic Immunity to Low Pathogenic Avian Influenza Viruses in Experimentally Infected Mallards. PLOS ONE 12(1): e0170335. Reprinted here with permission of publisher.

Abstract

Mallards are widely recognized as reservoirs for Influenza A viruses (IAV); however, host factors that might prompt seasonality and trends in subtype diversity of IAV such as adaptive heterosubtypic immunity (HSI) are not well understood. To investigate this, we inoculated mallards with a prevailing H3N8 low pathogenic avian influenza virus (LPAIV) subtype in waterfowl to determine if prior infection with this virus would be protective against heterosubtypic infections with the H4N6, H10N7 and H14N5 LPAIV subtypes after one, two and three months, respectively. Also, we investigated the effect of cumulative immunity after sequential inoculation of mallards with these viruses in one-month intervals. Humoral immunity was assessed by microneutralization assays using a subset of representative LPAIV subtypes as antigens. Our results indicate that prior inoculation with the H3N8 virus confers partial protective immunity against subsequent heterosubtypic infections with the robustness of HSI related to the phylogenetic similarity of the HA protein of the strains used. Furthermore, induced HSI was boosted and followed by repeated exposure to more than one LPAIV subtype. Our findings provide further information on the contributions of HSI and its role in the dynamics of IAV subtype diversity in mallards.

Keywords: mallards, Influenza A virus, heterosubtypic immunity.

Introduction

Wild aquatic birds from the order Anseriformes and Charadriiformes are the major reservoir of Influenza A viruses (IAV) [1, 2]. Mallard (*Anas platyrhynchos*) is the most common dabbling duck species in North America and Europe and is an important species in the ecology of avian influenza [3, 4]. Seasonal patterns of IAV infections in waterfowl have been described in the Northern Hemisphere (North America, Europe, and Asia) [1, 5-7] where IAV prevalence increases at the end of the summer and peaks in early fall as a result of the congregation of adult and immunologically naïve young birds in breeding grounds prior to Southern migration [2, 3]. Detection rates drop during winters with slight increases during spring migrations [5, 8]. Prevalence of IAV is higher among juvenile ducks as compared to adults, which is probably a result of immunity induced by previous IAV infections in adult birds [5, 9, 10]. In North America, the more common HA subtypes of IAV reported in ducks are H3 and H4, followed by H1, H2, H6, H7, H10, and H11, while the H14 subtype has rarely been detected in surveillance studies [3, 5, 11, 12]. Although the H4 and H6 subtypes were also frequent in surveillance studies in Europe, recurrent detection of other subtypes was not significantly different [5, 13]. Seasonal patterns of IAV prevalence among wild birds have been described; however, factors and mechanisms that drive diversity and prevalence of IAV subtypes such as the effects of homo- and heterosubtypic immunity remain unclear [14, 15].

Previous studies have demonstrated the induction of homosubtypic and partial heterosubtypic immunity in mallards [16-20], and this has been supported further by field observations [21]. At the same time, additional studies are needed to understand better

the effect of reinfections with common and less frequently detected subtypes of LPAIV on the ecology of influenza in the wild bird reservoir.

The objectives of this research were to investigate *i)* the protective effect induced by prior infection with H3N8 LPAIV inoculation against subsequent infections with H4N6, H10N7 or H14N5 LPAIV after 1, 2 or 3 months, respectively and *ii)* the cumulative effect of H3N8xH4N6 and H3N8xH4N6xH10N7 infections against subsequent challenges with H10N7 and H14N5, respectively. All HA subtypes used in this study are classified into Group 2 HAs with H3, H4 and H14 subtypes clustering together in the H3 clade and the H10 subtype clustering in the H7 clade [21-23]. The neuraminidase (NA) subtypes N5 and N8 are classified into Group 1 within clade N8; whereas N6 and N7 into Group 2 within the N7 clade [24]. We hypothesized that prior LPAIV infections in mallards would induce cross-protective immunity that would reduce viral shedding of subsequent inoculations with phylogenetically closely related LPAIV subtypes.

Materials and Methods

Ethics Statement

General care and handling of birds were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC), as outlined in the Guide for the care and Use of Agricultural Animals in Agricultural Research and Teaching and under an animal use protocol approved by the IACUC at the University of Georgia (UGA; AUP# A2012 09-019-Y3-A3).

Animals

Thirty-five one-day-old mallards were purchased from a commercial waterfowl supplier (eFowl Hatchery, Denver, Colorado, USA). Ducks were raised under approved conditions in the Animal Resources building at The University of Georgia until they were a month old. All ducks were observed twice daily for evidence of clinical disease signs. Three days before each inoculation, the ducks were transferred to high-efficiency particulate (HEPA) filter isolators located in an animal biosafety level 2 (ABSL-2) room for acclimatization.

Viruses

Four wild bird-origin LPAIV A/mallard/MN/Sg-000169/2007 (H3N8), A/mallard/MN/AI11-4979/2011 (H4N6), A/mallard/MN/AI11-4412/2011 (H10N7) and A/blue winged-teal/TX/AI13-1028/2013(H14N5) were used in this study. Inocula were generated by a second passage of viral stocks in 9- to 11-day old specific pathogen free (SPF) embryonated chicken eggs (ECEs). The viruses were titrated in ECEs, and the 50% embryo infectious dose (EID_{50}) was calculated by the Reed and Muench method [25]. Back titrations were performed on the day of the challenge to confirm the titer of inoculated viruses.

Study design

Two groups of fifteen one-month-old mallards were inoculated with 10^6 EID_{50}/0.1 ml of the H3N8 virus or the same volume of mock inoculum via the choanal cleft, respectively. One month later, at two months of age, birds were divided into six groups of five ducks each. Groups 1, 2 and 3 (G1, G2, and G3) consisted of birds previously inoculated with H3N8 virus, while groups 4, 5 and 6 (G4, G5, and G6) consisted of

mock-inoculated ducks and served as naïve birds in following challenges. One month after H3N8 inoculation, G1 and the naïve ducks in G4 were challenged with 10^6 EID_{50}/0.1 ml of the H4N6 virus. Two months after H3N8 inoculation, G1, G2 and the naïve ducks in G5 were challenged with 10^6 EID_{50}/0.1 ml of H10N7. Finally, G1, G3 and the naïve ducks in G6 were challenged with 10^6 EID_{50}/0.1 ml of H14N5, three months after H3N8 inoculation. The experimental design is schematically shown in Figure 3.1.

After completing the experiments in groups G4 and G5, birds were euthanized with CO_2 and cervical dislocation at 3 and 4 months of age respectively. All remaining ducks, which tested negative for IAV at five months of age by virus isolation and RT-PCR, were subsequently transferred to the Southeast Poultry Research Laboratory, U.S. National Poultry Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA, USA for a follow-up study.

Oropharyngeal (OP) and cloacal (CL) swabs were collected once daily on days 0-8, then on 10, 12 and 14 days post-inoculation (dpi). Swabs were placed into 2 ml of sterile brain-heart-infusion (BHI) supplemented with antibiotics (penicillin 1 000 units/ml, streptomycin 1 mg/ml, amphotericin B 25 µg/ml, gentamycin 250 µg/ml and kanamycin 500 µg/ml). Individual blood samples were obtained from the brachial or jugular vein on 0 and 14 dpi and processed 1-2 hours after sampling; tubes were centrifuged at 1500 *g* for 10 min, and sera was separated and stored at -20 C until testing.

Virus Isolation

Virus isolation from OP and CL swabs was attempted through inoculation of 9- to 11- day-old SPF chicken embryos as previously described [25]. Allantoic fluid from each

inoculated egg was screened for virus by hemagglutination (HA) assay using 0.5% chicken red blood cells [25].

RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Viral RNA extraction was carried out from 50 µl of the OP and CL swab supernatants with the MagMAX-96 AI-ND viral RNA isolation kit (Ambion, Austin, TX, USA) using the automated KingFisher™ magnetic particle processor (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's specifications slightly modified [26]. RNA was eluted with 50 µl of elution buffer and stored at -80 °C until testing.

Previously described primers and probe targeting the Influenza A virus matrix gene [27] were used to perform the qRT-PCR reaction by using the One-Step RT-PCR Kit (QIAGEN, Valencia, CA, USA) on a StepOne Real-time PCR System (Applied Biosystems, Darmstadt, Germany). Briefly, one µl of kit-supplied enzyme mixture, 0.4 µM of each primer, 0.12 µM probe, 320 µM of each dNTP, 3.75 mM $MgCl_2$ and 13 U of RNase inhibitor (Promega, Madison, WI, USA) were used in a 25 µl reaction. Cycling conditions consisted of reverse transcription for 30 mins at 50°C and denaturation for 15 mins at 94°C, followed by 45 cycles of 0 s at 95°C and 1 min at 60°C. Samples with Ct values ≤ 40 were considered positive for Influenza A virus. We arbitrarily assigned a Ct value of 45 to samples showing undetermined RT-PCR results for statistical analyses. All titrated viral stocks were serially diluted for RNA extraction and RT-PCR. Standard curves were generated to convert the experimental Ct values to viral titers in EID_{50}/ml equivalents.

Subtype-specific RT-PCR

To confirm that ducks were effectively infected and shedding the virus inoculated at each time point, subtype-specific RT-PCRs were performed on the viruses isolated from OP and CL swab samples collected on day three post-inoculation. Previously described primers for H3 [28], H4 [29] , H10 [30] and H14 [31] were used for subtype identification.

Serology

All serum samples were analyzed using the AI MultiS-Screen Ab ELISA kit (Idexx, Westbrook, ME, USA) according to the manufacturer's protocol. Samples with signal-to-noise ratio values ≤0.5 were considered positive to Influenza A virus nucleoprotein (NP). Sera also were tested by microneutralization (MN) as previously described [32]. Viruses used as antigens for MN assays included A/mallard/NJ/AI10-4263/2010 (H1N1), A/mallard/MN/AI08-2755/2008 (H2N3), A/mallard/MN/AI10-2593/2010 (H3N8), A/mallard/MN/AI10-3208/2010 (H4N6), A/mallard/MN/AI11-3933/2011 (H5N1), A/mallard/MN/SG-01048/2008 (H6N1), A/mallard/MN/AI09-3770/2009 (H7N9), A/mallard/MN/AI08 2721/2008 (H8N4), A/RUTU/NJ/AI07-293/2007 (H9N1), A/RUTU/DE/AI11-809/2011 (H9N2), A/mallard/MN/SG-00999/2008 (H10N7), A/mallard/MN/SG-00930/2008 (H11N9), A/mallard/MN/SG-3285/2007 (H12N5), A/blue-winged teal/TX/AI13-1028/2013 (H14N5), and A/wedge-tailed shearwater/Western Australia/2327/1983 (H15N6). Antibody titers were converted to $\log_2$ for all analyses.

Statistical analysis

Comparisons of the duration of viral excretion and neutralizing antibody titers between groups were performed by using non-parametric tests (Kruskal-Wallis or Mann-Whitney U). Ct values between groups over time were compared using linear mixed models with bird as a random effect. Post hoc multiple pairwise comparisons were conducted using the Bonferroni adjustment to limit the type I error rate to 5%. Hypothesis test assumed a two-sided alternative hypothesis, and $P < 0.050$ was considered statistically significant. All statistical tests were conducted using a commercially available software (Stata version 14.0, StataCorp LP, College Station, TX) and graphs were generated using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Overt clinical signs were not observed in any of the experimental groups, and all OP and CL samples collected immediately before each inoculation tested negative for IAV by virus isolation and qRT-PCR. Serum samples from all ducks collected before IAV inoculation tested negative by both ELISA and MN. Back titration of the inocula determined a titer of $10^{6.2}$ EID_{50}/0.1 ml for H3N8, $10^{6.0}$ EID_{50}/0.1 ml for H4N6, $10^{6.3}$ EID_{50}/0.1 ml for H10N7 and $10^{5.7}$ EID_{50}/0.1 ml for H14N5. Also, subtyping by RT-PCR of the viruses isolated after each infection confirmed that birds were excreting only the subtype of virus inoculated at each time point (data not shown).

Primary H3N8 inoculation in 1-month-old ducks

Fifteen one-month-old mallards were inoculated with the H3N8 virus to investigate whether this virus would induce cross-protective immunity against heterosubtypic infections. Viral shedding started at 1 dpi in OP and CL swabs, and the duration of viral excretion of H3N8 was not significantly different among groups (G1, G2 and G3), for OP ($P > 0.999$) or CL ($P = 0.853$) swabs (Figure 3.2a). The peak of H3N8 viral RNA excretion detected by qRT-PCR was at 2 and 3 dpi for OP swabs and 2 dpi for CL swabs. Intermittent viral RNA shedding continued for 8 dpi in all birds, with a general range of 8 to 14 days in OP swabs and 10 to 14 days in CL swabs. The Ct values for OP ($P = 0.065$) and CL ($P = 0.673$) swabs did not show significant differences between groups (G1, G2 and G3) over time (Figure 3.3a,b). All mock-inoculated ducks (G4, G5, G6) tested negative for IAV by virus isolation and RT-PCR (data not shown).

H4N6 inoculation in 2-month-old ducks

To investigate whether the previous infection of ducks with H3N8 would confer heterosubtypic immunity against H4N6, 2-month-old ducks from the groups G1 (H3N8 primed) and G4 (mock-inoculated) were challenged with the H4N6 virus. The results showed statistically significant differences in the duration of viral excretion between groups for OP ($P = 0.040$) but not for CL ($P = 0.127$) swabs (Figure 3.2b). However, significant differences in Ct values between groups at 3, 4 and 5 dpi in CL swabs (Figure 3.3d) but not in OP swabs at any time point were observed (Figure 3.3c). Overall, we observed a reduction in the duration of the excretion of viable virus and viral RNA after challenge with the H4N6 in the group previously inoculated with the H3N8 virus, correlated with the induction of partial level of cross-protective immunity.

H10N7 inoculation in 3-month-old ducks

To investigate whether previous infection with H3N8 or H3N8xH4N6 induced cross-protective immunity against H10N7, the experimental groups G1 (H3N8xH4N6 primed), G2 (H3N8 primed) and G5 (mock-inoculated) were challenged with the H10N7 virus when ducks were 3-months-old. All ducks in G5, which served as the control group in the infection with this virus, started viral shedding at 1 dpi in both OP and CL swabs. In G2, OP and CL viral shedding was delayed by 1 or 2 days, respectively. In G1, viral excretion was delayed by 1 day in 1/5 birds and not detected by virus isolation in 3/5 birds throughout the study period. Statistically significant differences in duration of OP (P = 0.012) and CL (P = 0.016) viral shedding were detected only among groups G1 and G5 (Figure 3.2c). There were statistically significant differences in the amount of viral RNA excretion between groups G1 and G5 from 1 to 10 dpi in OP swabs, and at 1, 2, 3, 5, 7 and 12 dpi in CL swabs (Figure 3.3e,f). Hence, these results show that previous infection of ducks with two LPAIV subtypes (H3N8XH4N6), confers a stronger cross-protective immunity against H10N7 as compared to previous infection with only one virus subtype (H3N8).

H14N5 inoculation in 4-month-old ducks

To investigate whether previous infection with H3N8 or H3N8XH4N6XH10N7 viruses induced cross-protective immunity against H14N5 virus, the experimental groups G1 (H3N8xH4N6xH10N7 primed), G3 (H3N8 primed) and G6 (mock-inoculated) were challenged with the H14N5 virus when ducks were 4 months old. Ducks from the G6 group started OP and CL viral shedding at 1 dpi, the OP swab of only one bird in G3 tested positive at 1 dpi, and CL viral excretion was not detected by virus isolation in this

group. OP and CL viral shedding were completely abrogated in G1, which was demonstrated by virus isolation and qRT-PCR results. There were statistically significant differences in the duration of viral excretion between groups G1 and G6 ($P = 0.004$) and between groups G3 and G6 ($P = 0.014$) (Figure 3.2d). No statistically significant differences in the duration of viral shedding were found between groups G1 and G3 ($P = 1.00$) (Figure 3.2d). Ct values in groups G1 and G3 were significantly higher than G6 from 2 to 8 dpi in OP swabs, and from 2 to 6 dpi in CL swabs (Figure 3.3g,h). These results show that previous inoculation with one (H3N8) or three AIV subtypes (H3N8XH4N6XH10N7) confers a strong cross-protective immunity against H14N5 virus infection in ducks.

Serology

A blocking ELISA assay was performed to confirm seroconversion after inoculation with each IAV used in this study. All ducks inoculated with IAV showed seroconversion at 14 days post-challenges (data not shown).

Microneutralization (MN) assay: MN assays were performed using a panel of different subtypes of IAV as antigens to determine if serum samples collected at 14 dpi contained homo- or heterosubtypic neutralizing antibodies. The MN assay was used because of its higher sensitivity as compared with hemagglutination inhibition test [33].

Post H3N8 inoculation: Fourteen out of fifteen birds tested positive for the homologous H3N8 virus on 14 dpi. Also, priming with H3N8 induced cross-reactive antibodies against H14N5 subtype in 2/15 serum samples ($\log_2$ 4.32, 4.32). Cross-reactive antibodies against the other IAV subtypes tested were not detected (Table 3.1).

Post H4N6 inoculation: Four out of five birds in the groups G1 (H3N8xH4N6) and 3/5 in G4 (H4N6) tested positive for the homologous H4N6 virus. Antibodies to H14N5 were detected in 1/5 serum samples (log_2 5.32) from the G1 and 2/5 (log_2 4.32, 4.32) samples from the G4 group. Cross-reactive antibodies against the other subtypes of IAV tested were not detected after H4N6 inoculation (Table 3.1).

Post H10N7 inoculation: All birds from G1 (H3N8xH4N6xH10N7), G5 (H10N7) and 4/5 from the G2 (H3N8xH10N7) groups tested positive for the homologous H10N7 virus. Interestingly, 3/5 birds from the G1 group had cross-reactive antibodies against an H9N2 virus (log_2 4.32, 4.32, 4.32). Furthermore, 1/5 serum samples from each G2 and G5 groups had cross-reactive antibodies against an H12N5 (log_2 4.32) virus (Table 3.1).

Post H14N5 inoculation: Three out of five serum samples from G1 (H3N8xH4N6xH10N7xH14N5), 4/5 from G3 (H3N8xH14N5) and 5/5 from G5 (H14N5) groups tested positive for the homologous H14N5 IAV. Interestingly, 2/5 samples from G3 group had cross-reactive antibodies against H4N6 (log_2 5.32, 6.32). Cross-reactive antibodies against the other IAV tested were not detected after H14N5 inoculation (Table 3.1).

Evaluation of boost in the humoral immune response against H3N8 virus after heterosubtypic challenges: We tested all serum samples from the H3N8 primed birds (G1, G2, and G3) collected prior and after each heterosubtypic inoculation (0 and 14 dpi) by MN assay. We observed an increase in the MN titers against H3N8 virus in the group G1 after H4N6 virus inoculation and in group G3 after H14N5 inoculation. However, no statistically significant differences were detected within groups at any time point (Figure 3.4).

Discussion

Previous infection with the H3N8 did not prevent reinfection with the H4N6, H10N7, and H14N5 LPAIVs after one, two and three months, respectively; but induced different levels of protective immunity as evidenced by a decrease in the duration and amount of viral shedding after heterosubtypic infections. The induced protective immunity was boosted following exposure to more than one virus subtype, with complete abrogation of viral shedding upon inoculation with H14N5 in birds previously exposed to three different subtypes (H3N8xH4N6xH10N7). Moreover, three out of five ducks primed with H3N8 were protected against secondary infection with the HA and NA clade-related H14N5 virus three months later, as none of them had detectable levels of viral excretion by virus isolation or RT-PCR after 1 dpi. Shedding of H10N7 was delayed in some birds primed with H3N8 or H3N8xH4N6, and the viral shedding was lower in ducks previously exposed to H3N8xH4N6 viruses.

Based on MN results, there was an induction of cross-reactive antibodies to H14N5 in ducks primed with H3N8, H3N8xH4N6, and H3N8xH4N6xH10N7 viruses; these findings were correlated with a reduction or abrogation in H14N5 virus shedding in groups previously inoculated with H3N8 and with H3N8xH4N6xH10N7 viruses, respectively. Also, infection with H4N6 induced cross-reactive antibodies in some ducks against the HA clade-related H14N5 virus. This finding is in agreement with previous hemagglutination inhibition (HI) results that showed cross-reactive antibodies induced by H4 against H14 subtype but not vice versa [34]. It is generally accepted that virus-specific antibodies neutralize viruses through interaction with the variable regions of the viral HA which prevents its attachment to the host cell receptor; the observed cross-

protection may be explained by the affinity of antibodies toward the phylogenetically related HA strains [21]. However, we cannot rule out that antibodies to other major surface proteins such as NA, M2, and NP or other HA epitopes (stem region) may also be playing a role in the cross-protective immunity [35, 36].

Our findings are in agreement with previous experimental studies that reported partial cross-protective immunity conferred by primary H3N8 inoculation against H4N6 virus challenge in mallards [20]. Our data also support results from a longitudinal field study where re-infections with phylogenetically related HA subtypes were rare in mallards, and cross-protection between subtypes within the H1 and H3 clades lasted for at least 30 days [21]. In the present study, we demonstrate that HSI to LPAIV subtypes within the same HA and NA clade can last for at least three months. Seasonal trends of coexistence of IAV and subtype diversity are influenced by the relative host lifespan, population density, and population immunity [1, 14, 37]. The induction of HSI alters the host immune profile and as described here affects the outcome of future infections with IAV in mallards. However, infection with a given IAV in wild birds may induce various levels of protective immunity against subsequent infections with homologous and heterologous subtypes. This variable response could transiently influence the seasonal prevalence of specific viruses [14, 21]. A previous study in Blue-winged Teal (*Anas discors*), an abundant long distance migrant dabbling duck between North America and South America, described H3/H4 and N6/N8 as the most common combination subtypes during the summer and fall [14]. Conversely, H14 and H15 subtypes are absent from most of the surveillance studies, and the former was not reported in North America until 2010 [3, 5, 10, 11]. Hence, our results may add some possible explanations to the cycling

patterns in naturally exposed birds, as priming with H3N8 virus induced complete abrogation of H14N5 viral shedding after three months in 3 out of 5 birds.

Although the HI assay is a generally accepted method for assessing the induction of humoral immunity to influenza viruses, we performed the MN assay because of its higher sensitivity, capacity to detect neutralizing antibodies, and its potential to detect cross-neutralizing antibodies [33]. Furthermore, the presence of the truncated form of IgY in ducks, which lacks HI activity, may contribute to neutralizing activity [38]. Additional tests such as HI and neuraminidase inhibition (NI) tests may add further information about the specific cross-reactivity among strains that are phylogenetically related by its HA and/or NA proteins, respectively [34]. None of the serum samples of the G6 (H14N5) tested positive for the HA clade-related H3N8 or H4N6 viruses by MN. It is suggested that these IAV subtypes (H3N8/H4N6), in addition to the H6N2 subtype, are best adapted to wild ducks (mallards, pintails, and Blue-winged Teals) [39]. This fitness might be correlated with a more rapid replication and greater immunogenicity [39, 40] and it is possible that viruses that exhibit greater fitness induce cross-protective immunity against antigenically related and less adapted viruses but, not necessarily the other way [21]. However, additional experimental studies are necessary to test this hypothesis. The detection of low cross-reactive antibody titers to Group 1 IAV (H9N2 and H12N5) in birds in groups G1 (H3N8xH4N6xH10N7) and G5 (H10N7) cannot be explained at this time but may have resulted from NA-related similarities [41]. These observations might also be explained by the existence of broadly neutralizing antibodies targeting the stem region of the HA protein that might confer cross-protective immunity between groups [36].

Previous studies have shown that reinfection with the same virus subtype is capable of boosting homosubtypic immunity as observed in a previous study in mallards inoculated with H3N8 virus [42]. In the present study, we assessed the effect of subsequent heterosubtypic challenges in the induction of neutralizing antibodies against H3N8 virus. We detected an increase in neutralizing antibody titers against the H3N8 virus after challenge with the clade-related HA subtypes H4N6 and H14N5 virus, but there were no statistically significant differences between the groups. We were not able to assess the role of cell-mediated immunity in conferring cross-protective immunity in mallards because lymphocyte proliferation assays used in other species were not replicable in our hands. However, it would be important to determine if cross-reactive, cell-mediated immunity in response to internal proteins as demonstrated for other species is induced when mallards are challenged with different IAV subtypes [43], and if it is influenced by the phylogenetic relatedness of the surface glycoproteins such as HA and NA. Additional studies to assess the role of mucosal immunity in homosubtypic and heterosubtypic infections are also needed, as LPAIV predominantly replicate in the intestinal tract in ducks.

In summary, we showed that single or multiple infections with an LPAIV subtype can induce partial or complete HSI against subsequent challenges in mallards and this cross-protective immunity lasts for at least three months. The robustness and duration of HSI might have important implications in the dynamics of IAVs transmission as well as in the circulation of certain virus subtypes and strains, such as the highly pathogenic H5Nx clade 2.3.4.4 viruses, which were introduced in North America in 2014 by wild

birds. The information obtained from this study improves our understanding of the effect of HSI on the ecology of avian influenza in mallards.

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Table 3.1. Microneutralization titers against homo- and heterosubtypic LPAIV in mallards. The table shows microneutralization (MN) titers expressed in log_2 (median and range) detected in samples collected at 14 days post inoculation with H3N8, H4N6, H10N7 and H14N5 LPAIV and at 1, 2, 3 and 4 months of age, respectively.

Age	Group	Primed with / Inoculum	Group 1								Group 2			
			H3 Clade						H7 Clade		H9 Clade			
			H3N8		H4N6		H14N5		H10N7		H9N2		H12N5	
			Positive samples	Median log_2 MN titers (range)	Positive samples	Median log_2 MN titers (range)	Positive samples	Median log_2 MN titers (range)	Positive samples	Median log_2 MN titers (range)	Positive samples	Median log_2 MN titers (range)	Positive samples	Median log_2 MN titers (range)
1 month	G4,G5,G6	Naïve **Mock-**	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
	G1,G2,G3	Naïve **H3N8**	14/15	5.32 (0 - 8.32)	0/15	0	2/15	0 (0 - 4.32)	0/15	0	0/15	0	0/15	0
2 months	G1	H3N8x **H4N6**	5/5	8.32 (6.32 - 8.32)	4/5	6.32 (0 - 8.32)	1/5	0 (0 - 5.32)	0/5	0	0/5	0	0/5	0
	G4	Mock- **H4N6**	0/5	0	3/5	4.32 (0 - 7.32)	2/5	0 (0 - 4.32)	0/5	0	0/5	0	0/5	0
3 months	G1	H3N8xH4N6x **H10N7**	5/5	7.32 (6.32 - 8.32)	5/5	5.32 (4.32 - 7.32)	2/5	0 (0 - 5.32)	5/5	4.32 (4.32 - 6.32)	3/5	4.32 (0 - 4.32)	0/5	0
	G2	H3N8x **H10N7**	5/5	6.32 (4.32 - 7.32)	0/5	0	1/5	0 (0 - 4.32)	4/5	7.32 (7.32 - 8.32)	0/5	0	1/5	0 (0 - 4.32)
	G5	Mock- **H10N7**	0/5	0	0/5	0	0/5	0	5/5	6.32 (6.32 - 8.32)	0/5	0	1/5	0 (0 - 4.32)
4 months	G1	H3N8xH4N6xH10N7x**H14N5**	5/5	7.32 (6.32 - 8.32)	5/5	6.32 (5.32 - 8.32)	3/5	5.32 (0 - 6.32)	4/5	6.32 (0 - 7.32)	0/5	0	0/5	0
	G3	H3N8x **H14N5**	5/5	7.32 (6.32 - 8.32)	2/5	0 (0 - 6.32)	4/5	6.32 (0 - 8.32)	0/5	0	0/5	0	0/5	0
	G6	Mock **H14N5**	0/5	0	0/5	0	5/5	8.32 (7.32 - 8.32)	0/5	0	0/5	0	0/5	0

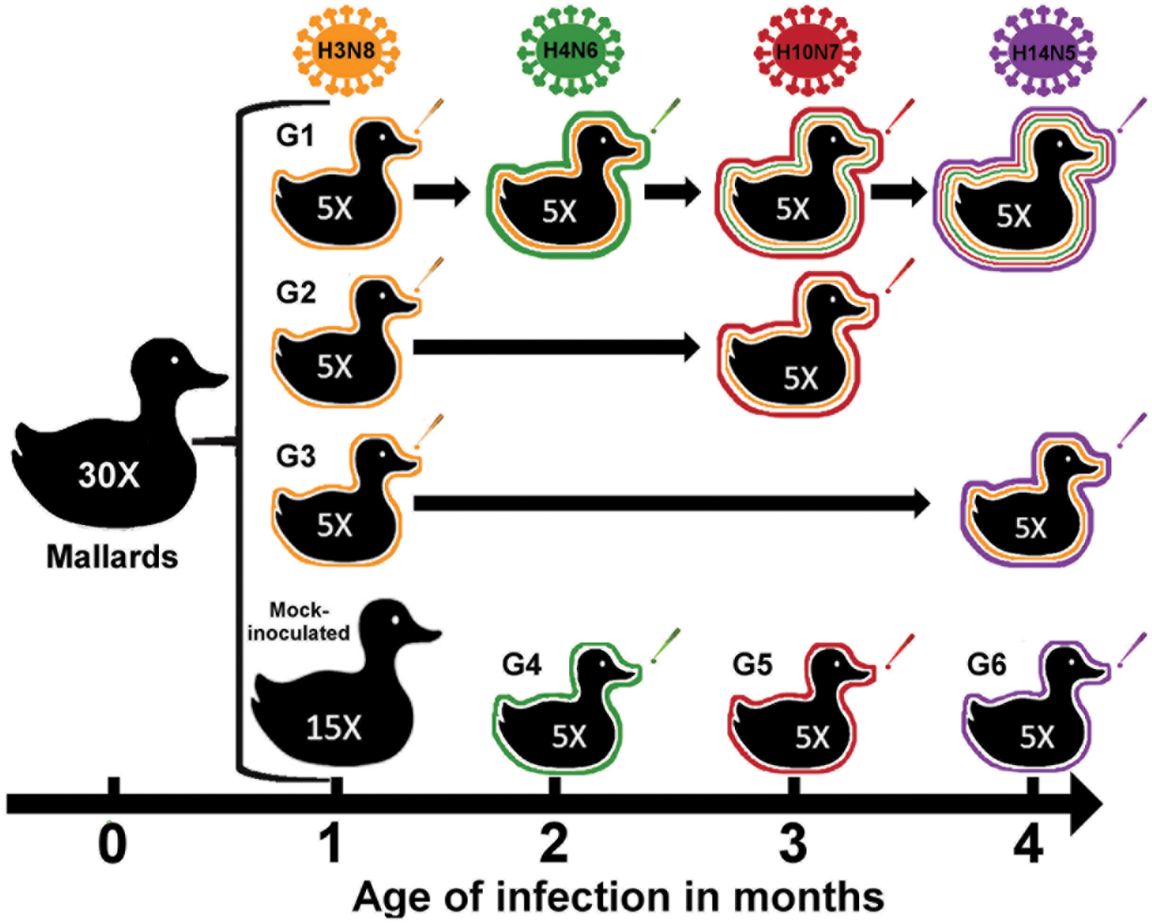

Figure 3.1. Design of the experimental infections of mallards with different subtypes of LPAIV. Fifteen 1-month-old mallards were inoculated with the H3N8 LPAIV via choanal cleft and randomly divided into three challenge groups with five ducks each. Group 1 was sequentially challenged with H4N6, H10N7 and H14N5 LPAIV at 1-month interval each. Group 2 was challenged with H10N7 two months after H3N8 inoculation, while group 3 was challenged with H14N5 after three months. Single infection and age-matched control ducks were included for all inoculations.

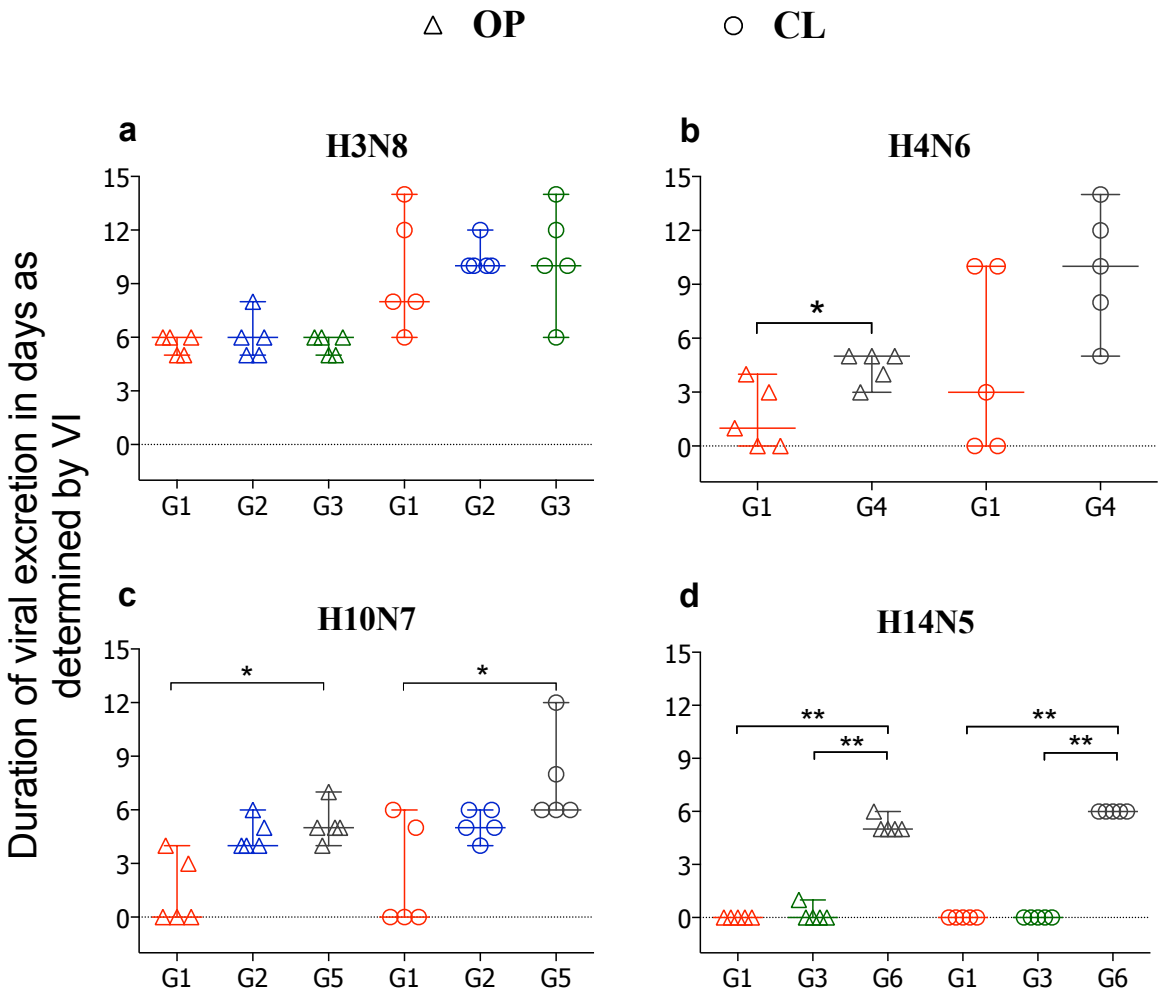

Figure 3.2. Duration of viral shedding after LPAIV inoculation. Graphs compare duration of virus shedding (median and range) after LPAIV inoculation as demonstrated by VI in oropharyngeal (OP) and cloacal (CL) swabs a) H3N8 inoculation in 1-month-old naïve birds (G1, G2 and G3); b) H4N6 inoculation in 2-month-old birds (G1: H3N8 primed and G4: mock-inoculated) c) H10N7 inoculation in 3-month-old birds (G1: H3N8XH4N6XH10N7-, G2:H3N8 primed, G5: mock-inoculated) d) H14N5 inoculation (G1: H3N8XH4N6XH10N7-, G3:H3N8 primed and G6: mock-inoculated). * and ** denotes significant difference $P < 0.05$ and $P < 0.01$, respectively.

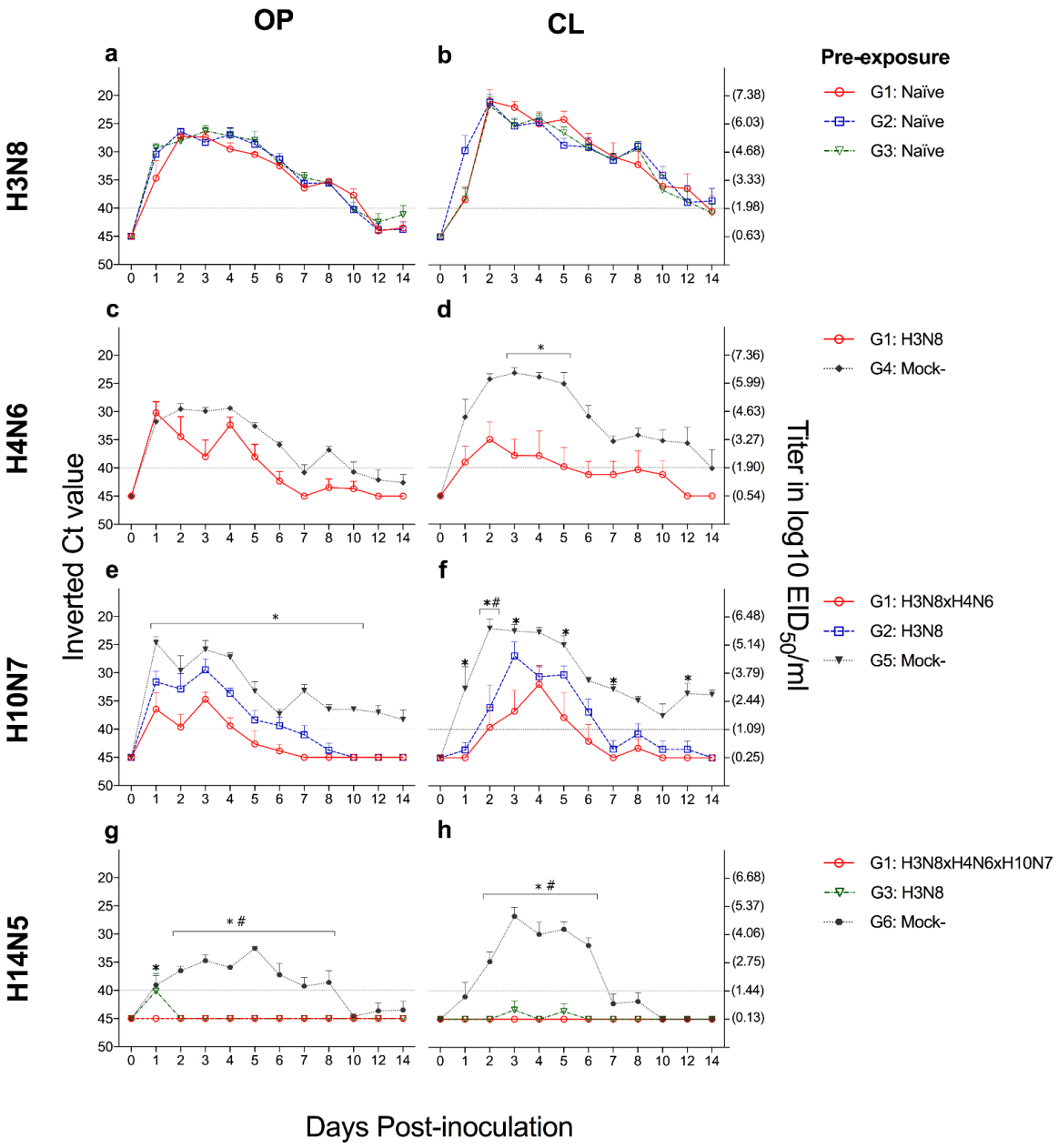

Figure 3.3. Ct values of RNA viral shedding after LPAIV inoculation.
Graphs compare the mean Ct values detected over 14 days in oropharyngeal (OP, left) and cloacal (CL, right) swabs after a,b) H3N8 c,d) H4N6 e,f) H10N7 and g,h) H14N5 inoculation. * denotes significant differences ($P < 0.05$) between G1 and naïve birds after infection with the respective LPAIV, while # between G2 or G3 with the same virus.

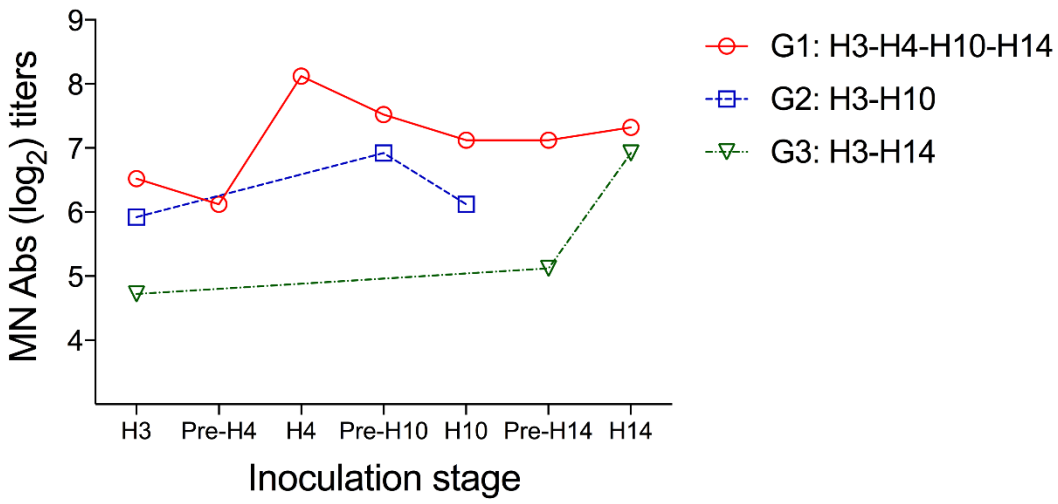

Figure 3.4. Microneutralization titers against the H3N8 virus after heterosubtypic LPAIV inoculations. The graph compares variation in the mean $\log_2$ titers against the H3N8 virus in serum samples collected on days 0 and 14 post each heterosubtypic inoculation.

CHAPTER 4

HETEROSUBTYPIC IMMUNITY CAN INCREASE THE INFECTIOUS DOSE REQUIRED TO INFECT MALLARDS IN SUBSEQUENT INFLUENZA A VIRUS INFECTIONS[2]

[2] Segovia KM, França MS, Leyson CL, Kapczynski DR. Chrzastek K, Bahnson CS, Stallknecht DE. Heterosubtypic immunity can increase the Infectious dose required to infect Mallards in subsequent Influenza A virus infections. Manuscript prepared to be submitted to Molecular Ecology

Abstract

Previous field and experimental studies have demonstrated that heterosubtypic immunity (HSI) as a potential driver of Influenza A virus (IAV) prevalence and subtype diversity in mallards. Prior infection with IAV can reduce viral shedding during secondary infections with IAV holding hemagglutinin (HA) proteins that are antigenically related. In this experiment, we evaluated the effect of HSI conferred by an H3N8 IAV infection against increasing challenge doses of closely (H4N6) and distantly (H6N2) related IAV subtypes in mallards. Thirty one-month-old mallards were inoculated with $10^{5.9}$ median egg infective dose (EID50) of H3N8 virus. One month later, groups of five birds each were challenged with increasing doses (approximately 10^2, 10^3 and 10^4 EID50/0.1ml) of H4N6 or H6N2 viruses. Control birds were infected after inoculation with low (~10^3) and medium (~10^4) EID50 doses, but not with very low (~10^2) EID50 dose of either IAV. In contrast, with birds previously infected with H3N8, none of the ducks challenged with H4N6 were infected, and with H6N2, only birds challenged with the median dose were infected. Viral shedding in these H6N2 infected ducks was also reduced on days 2 and 3 post-inoculation. In order to explain the varied responses between H3 and H6 challenged ducks, we mapped the amino acid sequence changes between H6 and H3 on predicted three-dimensional structures. Most of the sequence differences between H3 and H6 occurred at known antigenic sites of H3. Our findings demonstrate that the required infectious dose to infect mallards with IAV can increase as a result of HSI induced by previous infections and that this effect is most pronounced when the HA of the viruses are genetically related.

Keywords: mallards, Influenza A virus, heterosubtypic immunity, doses-reponse.

Introduction

Wild birds are the natural reservoir for all the 16 subtypes of hemagglutinin (HA) and nine subtypes of neuraminidase (NA) of Influenza A viruses (IAV) [1-3]. Wild ducks, especially mallards (*Anas platyrhynchos*) are the primary reservoir for most strains, except the H13 and H16 subtypes [3-8]. Some subtypes occur consistently in ducks such as the H3, H4, and H6 in North America and Europe, while the H14 is sporadically reported [4]. Several factors influence the dynamics of IAV in waterfowl such as seasonality, spatial dynamics, and host density [2, 6, 9-11] and it has been suggested that host immunity might drive antigenic diversity [12, 13]. In support of this, patterns of infections have been observed in nature where circulation of similar or closely related subtypes has been negatively affected due to homo- or heterosubtypic immunity; favoring the emergence of antigenically distant strains [14, 15]. Those findings have been supported by experimental homo- and heterosubtypic infections in mallards [16-20]. Previously, we demonstrated that cross-protective immunity between IAV subtypes is positively associated with the phylogenetic relatedness of their HAs in mallards [21]. However, more information about the effect of this induced cross-protection against variant challenge doses that may better represent those encountered during natural exposure was warranted. To address this question, we designed a dose-response experiment in which one-month-old mallards were infected with a single high dose of the H3N8 IAV followed by very low (10^2), low (10^3), and medium (10^4) egg-infectious doses (EID50) of H4N6 or H6N2 IAV. In addition, we wanted to compare HA amino acid sequence and map the amino acid sequence changes between H3 and H4 or H6 on predicted three-dimensional structures. Our aim was to determine if HSI induced by an

H3N8 infection would increase the infectious dose required to infect mallards in subsequent challenges with H4N6 or H6N2 IAV.

Materials and Methods

Animals and husbandry.

Sixty one-day-old mallards were obtained from a commercial waterfowl supplier (McMurray Hatchery, Webster City, IA, USA). All work was done in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC) of The University of Georgia under an approved animal use protocol (AUP# A2015 12-002-Y1-A0). All experimental and laboratory work was conducted at biosafety level 2 (ABSL2) facility. Ducks that were relocated to high-efficiency particulate (HEPA) filter isolators were acclimatized for a week before secondary virus inoculations. Animals were provided with food and water ad libitum and monitored twice a day throughout the duration of the study. Once the study was completed, surviving animals were humanely euthanized by carbon dioxide followed by cervical dislocation.

Inocula

All three IAV isolates used in this study were obtained from ducks during wild bird surveillance studies in Minnesota, USA; A/Mallard/MN/AI07-4724/2007(H3N8), A/Mallard/MN/AI11-4979/2011(H4N6), and A/Mallard/MN/AI11-4982/2011(H6N2). The viruses had undergone two passages in 9- to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) before their use in this experiment. Viral stocks stored at -80°C were thawed and diluted to obtain a 10^6 50% embryo infectious dose (EID50) per 0.1 ml of H3N8 virus and 10^2, 10^3 and 10^4 EID50/0.1 ml of H4N6 and H6N2 viruses.

Back titrations of the inocula were performed in SPF ECE on the inoculation day and the EID50 of H3N8 ($10^{5.9}$), H4N6 ($10^{1.8}$, 10^{3}, and $10^{4.1}$ EID50/0.1ml), and H6N2 ($10^{1.9}$, 10^{3}, and $10^{3.9}$ EID/0.1ml) was calculated by the Reed and Muench method. The 50% mean bird infectious doses (BID_{50}) were calculated by same method. A mock-inoculum consisting of viral transport media was used as previously described [21].

Viral inoculations

Two groups of thirty one-month-old ducks were inoculated with either $10^{5.9}$ EID50 of an H3N8 IAV or a mock-inoculum in a total volume of 0.1 ml via the choanal cleft. At two months of age these birds were challenged with either an H4N6 or H6N2 viruses. Six groups of five birds each from the H3N8 infected or from the mock-inoculated groups were challenged with very low (~10^{2}), low (~10^{3}), and medium (~10^{4}) doses of H4N6 or H6N2 viruses.

Sample collection

Oropharyngeal (OP) and cloacal (CL) swabs were collected in viral transport media before each inoculation to confirm absence of IAV shedding. OP and CL swabs were collected at 3 and 28 days post-inoculation (DPI) with H3N8 IAV to confirm infection and absence of viral shedding, respectively. Swab samplings of the ducks after challenge with the second virus (H4N6 or H6N2) were done at 0, 2 to 5, 7, and 14 DPI and samples were stored at -80°C prior to processing. Individual blood samples were obtained from the jugular vein at 0 and 14 DPI and stored at -20 C prior to serological testing.

Virus isolation and RT-PCR

Virus isolation and real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) was performed after the first thawing of the samples. Details of the methodology for virus isolation (VI), RNA extraction, and rRT-PCR have been described elsewhere [22-24].

Serology

All serum samples were analyzed using the AI MultiS-Screen Ab ELISA kit (cELISA, Idexx, Westbrook, ME, USA) according to manufacturer's recommendations. Samples were also tested by microneutralization (MN) assays as previously described [25]; the viruses A/mallard/MN/AI10-2593/2010 (H3N8), A/mallard/MN/AI10-3208/2010 (H4N6), and A/mallard/MN/SG-01048/2008 (H6N1) were used as antigens.

Statistical analysis

Comparison of Ct values between groups over time to assess differences in viral RNA loads was done using linear mixed models with repeated measurements. Samples with negative Ct values were assigned values of 45 for the purpose of statistical analyses. The Bonferroni correction was used to limit the type I error rate to 5%. Differences in the MN antibody titers among experimental groups were done by using non-parametric tests (Kruskal-Wallis or Mann-Whitney U). The STATA software version 14.0 (StataCorp LP, College Station, TX) was used for statistical analyses, and the graphs were generated with GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Sequence alignment

Sequences of the HA and NA of the viruses used for the challenges were downloaded from the GenBank database (accession numbers KX814374, KX814375, KX814369, KX814370). Multiple sequence alignment of the HA and NA were constructed with MAFFT, and an amino acid distance matrix was obtained by using the Genious version 8.1.9.

Prediction of hemagglutinin molecular structure

Amino acid sequences obtained from GenBank were subjected to homology modeling using I-TASSER [26]. The signal peptide (amino acid positions 1-18) were removed from the sequences submitted to the I-TASSER server. PyMoL was then used to visualize molecular structures [27]. Additional image processing was also performed using Adobe Photoshop CS6 (Adobe Systems Inc.).

Results

Viral shedding and BID_{50}

Ducks remained healthy during the duration of the experiment. All thirty one-month-old mallards inoculated with $10^{5.9}$ EID50/0.1 ml of H3N8 IAV were effectively infected, as all birds were positive at 3 DPI by VI and RT-PCR in OP and CL swabs. Viral shedding was cleared by 28 DPI and mock-inoculated birds remained negative for any IAV infection during this part of the experiment.

Control and H3N8-primed ducks were not susceptible to infection with very low doses (~10^2) of the H4N6 or H6N2 IAV at two months of age, as none of them had detectable virus in OP or CL swabs by VI and rRT-PCR (Table 4.1). Also, all ducks inoculated with H3N8 IAV, followed by low (10^3) and medium ($10^{4.1}$) doses of H4N6

virus were negative for IAV by VI (Table 4.1). Only one OP swab of the group inoculated with the medium virus dose was positive by rRT-PCR at 2 DPI. Significant differences in Ct values among H3N8-primed and control groups were observed from 2 to 7 DPI for OP and CL swabs in both groups (Fig 4.1).

Ducks primed with H3N8 did not shed viable virus in OP or CL swabs after inoculation with low dose (10^3) of the H6N2 virus as ducks were negative for IAV by VI; however, two out of five birds shed viral RNA and had positive Ct values in OP swabs at 2 DPI. Viral excretion was delayed by 2 days in all five birds inoculated with the medium virus dose as detected by VI in OP and CL swabs. Significant differences in Ct values between control and H3N8-primed groups in OP and CL swabs were observed from 2 to 5 DPI in the low dose group, and at 2 DPI in the medium dose group (Fig 4.1). The calculated BID_{50} for both H4N6 and H6N2 viruses in naïve two-month-old mallards was 10^3 EID50. For mallards previously infected with H3N8 the BID_{50} increased to 10^4 for H6N2 virus and was higher than 10^4 for the H4N6 virus.

Serology

All birds inoculated with H3N8 virus seroconverted by 14 DPI as determined by cELISA. Consistent with virus isolation results, none of the control birds inoculated with very low doses (~10^2) of either viruses (H4N6 or H6N2) seroconverted by cELISA. All naïve birds inoculated with low and medium doses of H4N6 and H6N2 seroconverted by cELISA at 14 DPI (data not shown).

Neutralizing antibodies against the homologous antigen (H4N6 or H6N2) were detected in naïve birds challenged with the low and medium doses of the respective virus (Table 4.2). Among the H3N8-primed groups, neutralizing antibodies were only detected

in birds challenged with the medium dose of the H6N2 virus. Heterosubtypic inoculations did not have a boosting effect in the MN antibodies previously induced by the H3N8 virus as differences in the geometric mean titers were not significantly different in samples collected pre- and post-heterosubtypic challenges (p>0.05) (Table 4.2).

Sequence alignment and predicted hemagglutinin molecular structure

The HA amino acid sequence of the H3N8 virus was 68.3% similar to the H4N6 virus and 41.9% similar to the H6N2 virus, while the NA sequences of these viruses were 43.5% and 45.3% similar, respectively. Also, most of the differences between subtypes were found in the HA1 region of IAV strains with 57.1% similarity between the H3 and H4 and 37.5% between H3 and H6. We used I-TASSER to generate a predicted molecular model for the hemagglutinin (HA0) of the three viruses used in this study (Fig 4.2). On the predicted H3 model, we marked the amino acid positions that contained a residue that was not identical to that found in either the H4 or H6 sequences. Additionally, we mapped antigenic sites that were previously described for avian influenza viruses bearing H3 [28]. As expected, we observed that there are more sequence differences in H6 (Fig 4.2, A and B). Many of these sequence changes are found in the HA1 region, particularly in antigenic sites A, B, and D (Fig 4.2, A-C). Notably, these antigenic sites are found at the globular head domain where the receptor binding and vestigial esterase regions are found [29].

Discussion

We have previously demonstrated that prior infection of mallards with an IAV can result in reduced viral shedding after subsequent inoculations with a different IAV

subtype. Also, the magnitude of this effect increased with the genetic relatedness of the HAs the HA of the viruses. In this study, the same effect was observed with infectious dose. Prior infection with the H3N8 virus in mallards, resulted in an increase in the minimum dose required to produce subsequent infection with the H4N6 or H6N2 viruses. Also, the extent of this effect was enhanced if the HA of the viruses were closely related. Results were consistent as determined by virus isolation and RT-PCR, and the detection of neutralizing antibodies.

In a previous study using the same H3N8 and H4N6 viruses, we showed that three out of five ducks that had been primed with H3N8 virus were infected after challenge with a high dose (10^6) of the H4N6 virus. The present study shows that the 50% bird infectious doses (BID) in mallards previously infected with an H3N8 is higher than 10^4 EID50 for the same H4N6 strain. This might suggest that the BID in H3N8-primed birds is between 10^5-10^6 EID for the H4N6 virus, and 10^4 EID for the H6N2 virus used in this study; while, the BID doses was lower (10^3 EID50) for naïve mallards for both viruses.

None of the ducks receiving a very low dose of either virus were infected. Similar results were reported for Pekin ducks that were inoculated via the intranasal route with two H4N8 IAV strains, where the BID50 ranged from $10^{3.1}$-$10^{3.3}$ EID50. In contrast, lower doses ($10^{1.9}$ EID50) were required when H5N1 LPAIV were used as inocula [30]. The IAV used in the present study had undergone two passages in SPF ECE prior to duck inoculations. Since passage in ECE can produce adjustment or adaptation of the virus to the new host, it is possible that this procedure might have affected the ability of the viruses to produce infection when very low doses were used [31].

The probability of isolation of LPAIV from juvenile birds are higher than adult birds regardless of the time of collection. It was suggested that those findings are related to the acquired immunity of adults birds due to of exposure to IAV [32]. Also, trends of subtype diversity have been observed in mallards in the Northern hemisphere; where IAV within the H3 class tend to appear at the end of the summer and beginning of the fall, whereas H1 class viruses are more commonly isolated at the end of the season, favoring antigenic dissimilarity [14]. Our results provide support to these findings as the doses required to produce secondary infection with a different virus subtype was increased in birds previously infected with the H3N8 virus. Furthermore, the extent of this cross-protective effect was more evident when birds were re-challenged with a virus genetically related to the first one by the HA.

Heterosubtypic immunity is related to induction of memory cells generated following influenza infection [33]. The targets of these cross-protective immunity conferring cells are conserved regions within all of the major viral proteins including the HA [34]. Antibodies against conserved epitopes in the stem regions of the HA and common epitopes in the globular head have been previously described [35, 36]. Indeed, examination of sequence changes observed between the HA protein genes of H3N8 and H4N6 or H6N2 viruses showed that many of the sequence changes between H3N8 and other viruses are found in the antigenic regions close to the globular head of HA site and more sequence differences were observed between the HAs of the H3N8 and H6N2 viruses.

Heterosubtypic immunity in our study occurred in absence of cross-neutralizing antibodies in serum against the viruses used for the secondary challenges (H4N6 or

H6N2). Our findings are correlated with previous observations of HSI in mice where cross-neutralizing antibodies were not detected [37-39]. Heterosubtypic inoculation of H3N8-primed ducks did not induce specific MN antibodies against the secondary virus when birds were challenged with low and medium doses of the H4N6 and with low doses of the H6N2 virus. Those findings might be associated to the rapid clearance and decrease in the abundance of new viral antigens, leading to the reduced recruitment and expansion of antibody secreting cells [40, 41]. Also, neutralizing antibodies were not detected in some birds where infection was confirmed by virus isolation. Overall, we demonstrated that the required infectious dose to infect mallards with IAV can increase as a result of HIS induced by previous infections and that this effect is most pronounced when the HA of these viruses are more genetically related. Our findings suggest that the effect of the doses in the dynamics of LPAIV possibly influences the probability of transmission of IAV genetically related by the HA in mallards.

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Table 4.1 Viral shedding in H3N8-primed and control birds with increasing doses of two LPAIV. The table shows the proportion of positive samples by VI in OP and CL swabs on 0, 2-5, 7 and 14 DPI after inoculation with increasing doses (EID50) of two LPAIV.

	0 DPI		2 DPI		3 DPI		4 DPI		5 DPI		7 DPI		14 DPI	
	OP	**CL**	**OP**	**CL**	**OP**	**CL**	**OP**	**CL**	**OP**	**CL**	**OP**	**CL**	**OP**	**CL**
H3N8XH4N6(10^2)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H4N6(10^2)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H3N8XH4N6(10^3)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H4N6(10^3)	0/5	0/5	3/5	2/5	5/5	5/5	5/5	5/5	4/5	5/5	1/5	1/5	0/5	0/5
H3N8XH4N6(10^4)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H4N6(10^4)	0/5	0/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	1/5	2/5	0/5	0/5
H3N8XH6N2(10^2)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H6N2(10^2)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H3N8XH6N2(10^3)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
H6N2(10^3)	0/5	0/5	4/5	5/5	1/5	5/5	5/5	5/5	3/5	5/5	0/5	1/5	0/5	0/5
H3N8XH6N2(10^4)	0/5	0/5	0/5	2/5	2/5	2/5	1/5	5/5	1/5	4/5	1/5	2/5	0/5	0/5
H6N2(10^4)	0/5	0/5	4/5	5/5	2/5	5/5	5/5	5/5	3/5	5/5	0/5	2/5	0/5	0/5

Table 4.2. Microneutralization titers after heterosubtypic LPAIV inoculation in H3N8-primed mallards. The table shows the proportion of positive samples and log2 microneutralization (MN) titers (media and range) induced 0 and 14 DPI after inoculation with increasing doses of two LPAIV.

Experimental groups	**0 DPI**	**Median and range Log2 titers**	**14 DPI**	**Median and range Log2 titers**	**MN Antigen**
H3N8X**H4N6(10^2)**	0/5	0	0/5	0	H4N6
H4N6(10^2)	0/5	0	0/5	0	
H3N8X**H4N6(10^3)**	0/5	0	0/5	0	
H4N6(10^3)	0/5	0	4/5	6.32 (0-7.32)	
H3N8X**H4N6(10^4)**	0/5	0	0/5	0	
H4N6(10^4)	0/5	0	3/5	4.32 (0-5.32)	
H3N8X**H6N2(10^2)**	0/5	0	0/5	0	H6N2
H6N2(10^2)	0/5	0	0/5	0	
H3N8X**H6N2(10^3)**	0/5	0	0/5	0	
H6N2(10^3)	0/5	0	3/5	4.32 (0-6.32)	
H3N8X**H6N2(10^4)**	0/5	0	3/5	4.32 (0-7.32)	
H6N2(10^4)	0/5	0	5/5	5.32 (5.32-7.32)	

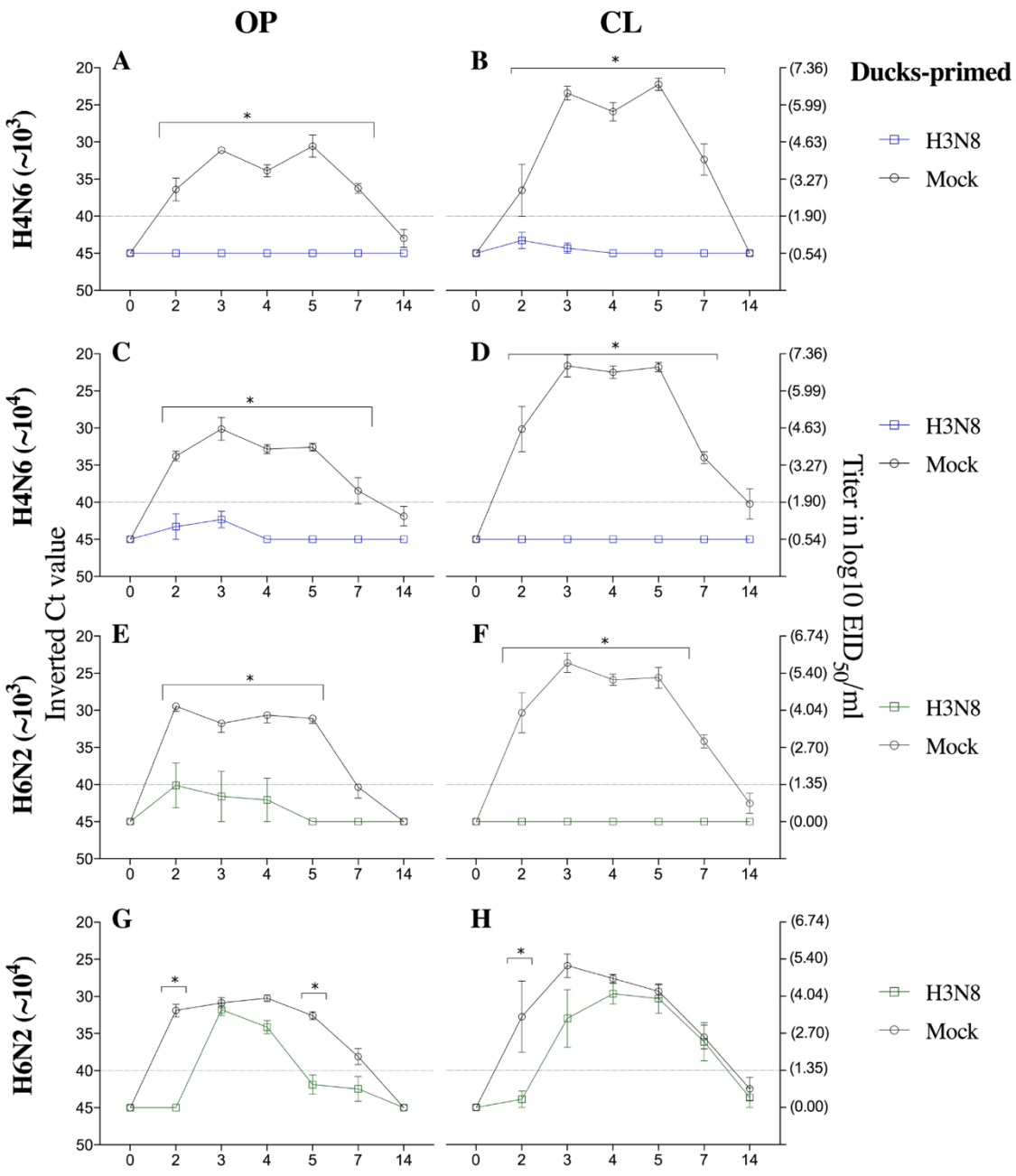

Figure 4.1 Ct values after inoculation of H3N8-primed and control mallards with increasing doses of two distinct LPAIV. Graphs compare the time course of the mean Ct values at 2-5, 7, and 14 DPI in OP (left) and CL (right) swabs after A,B) H4N6 (~10^3EID50); C,D) H4N6(~10^4EID50); E,F) H6N2(~10^3EID50) and G,H) H6N2(~10^4EID50) inoculation. * denotes significant differences ($P < 0.05$) between H3N8-primed and control birds.

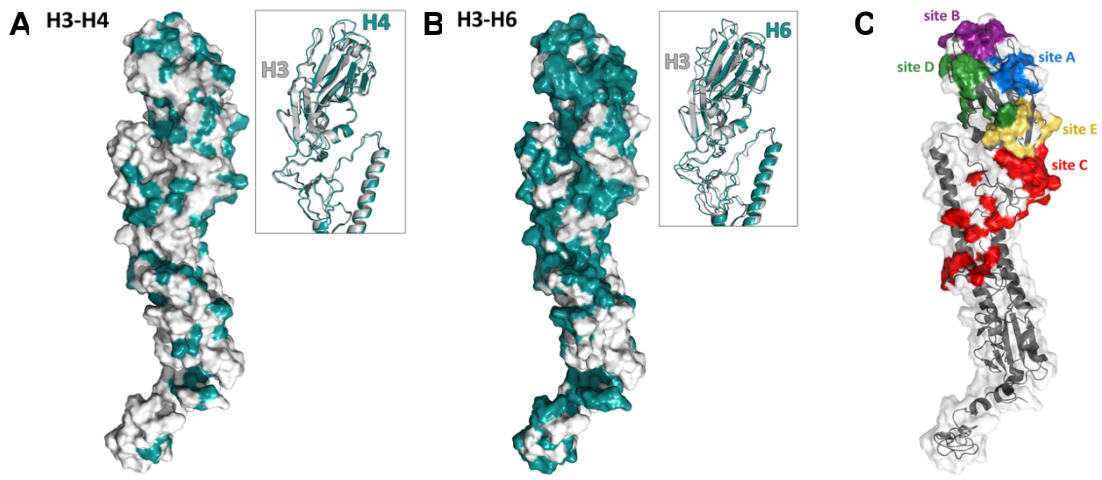

Figure 4.2. Predicted three-dimensional structures of the hemagglutinin protein (HA0) with I-TASSER. Differences in the amino acid sequence between H3 and H4 (A), and between H3 and H6 (B) are highlighted in teal. Insets show aligned peptide backbone of the indicated hemagglutinin subtypes. Known antigenic sites of H3 are mapped on the predicted structure of H3 hemagglutinin (C). More sequences differences are observed between H3 and H6 compared to those between H3 and H4 (A, B). Aligned peptide backbones also show that the overall topology is more different between H3 and H6 predicted structure (A, B insets). Additionally, many of the sequence differences between H3 and H6 are found on the globular head and on antigenic sites A, B, and D; suggesting that these sequence differences could have an impact on cross-protective immune responses (B, C).

CHAPTER 5

COMPARISON OF MICRONEUTRALIZATION AND HEMAGGLUTINATION INHIBITION ASSAYS RESULTS FROM EXPERIMENTALLY INFECTED MALLARDS [3]

[3] Segovia KM, França MS, Bahnson CS, LaTorre-Margalef, N, Stallknecht DE. Comparison of microneutralization and hemagglutination inhibition assays results from experimentally infected mallards. Manuscript prepared to be submitted to Avian Diseases

Abstract

Hemagglutination inhibition (HI) assays are commonly used to assess humoral immune response against avian influenza viruses. However, microneutralization (MN) assays have been reported to have higher sensitivity when testing human and other species sera. This study aimed to determine the agreement between MN and HI tests and compare the proportion of positive samples detected by both methods in sera of mallards primary infected with the H3N8 virus and re-infected with homologous or heterologous low pathogenic avian influenza viruses (LPAIV). Overall we found a slight agreement (PABAK= 0.15 - 0.20) between MN and HI assays in serum samples collected two weeks after H3N8 inoculation; this observed agreement was increased to substantial (PABAK= 0.66 - 0.67) in samples collected 4 to 5 weeks post-inoculation. A slightly higher proportion of positive samples was detected by MN as compared to HI assays. In addition, a boosting effect in MN and HI titers was observed when birds were subsequently inoculated with LPAIV within the same HA clade. This effect was not observed when birds were re-challenged with viruses from a different HA clade.

Keywords: Hemagglutination inhibition, microneutralization, mallards, serology, Influenza A virus.

Introduction

Influenza A viruses (IAV) are classified based on serological relatedness of two surface proteins, the hemagglutinin (HA) and neuraminidase (NA) [1]. To date, 16 subtypes of HA and nine subtypes of NA have been described in birds [2, 3]. Ducks, especially mallards, are considered the primary reservoirs for most IAV subtypes and play a major role in the epidemiology of these viruses [3]. Various serological methods have been used for assessing immunity in serum samples of wild birds during surveillance and experimental studies and new methods are being developed [4-7]. These methods include screening assays that measure antibody responses to conserved internal proteins such as blocking enzyme-linked immunoabsorbent assays (ELISAs) and subtype-specific tests such as hemagglutination-inhibition (HI) [8, 9]. HI is an easy technique that relies on the ability of antibodies to prevent binding of the HA to the sialic acids on red blood cells (RBCs) [10]. The microtiter neutralization (MN) assay is another method that assesses the ability of the antibodies to prevent infection of mammalian cells in vitro [11, 12]. HI assays have been widely used to evaluate humoral immunity against IAV, and are widely used for IAV subtyping [13]. However, lack of sensitivity was reported when testing duck serum samples and this resulted in underestimated IAV antibody prevalence [4]. MN assays have been shown to be more sensitive when testing human and other species serum samples [14]. Given the benefits of the MN assays and its ability to detect antibodies that lack HI activity, we wanted to determine the correspondence between the results of both assays when duck serum samples are tested. To understand this we performed HI and MN assays in serum samples of mallards

experimentally infected with H3N8 LPAIV and subsequently infected with homologous or heterologous IAV.

Methods

Serum samples

A total of 345 paired serum samples from 75 mallards collected at different time points were tested in this study. Samples were collected during three previous experimental infection studies of IAV conducted at the University of Georgia, Athens, GA, USA [15, 16]. In these studies, 1-month-old-ducks were inoculated with approximately 10^6 50% embryo infectious doses (EID_{50}) of H3N8 virus, A/mallard/MN/Sg-000169/2007 (H3N8) via the choanal cleft, followed by similar single or multiple challenges (~10^6 EID50) with homo- or heterologous LPAIV at different time intervals and with different doses (10^3 and 10^4) (Table 5.1).The strains used for the heterologous inoculations are listed as follows: A/Mallard/MN/AI11-4213/2011(H4N5), A/Mallard/MN/AI11-4982/2011(H6N2), A/Mallard/MN/AI11-4412/2011(H10N7), A/Mallard/MN/AI11-3866/2011(H12N5), A/mallard/MN/AI11-4979/2011 (H4N6), and A/blue winged-teal/TX/AI13-1028/2013(H14N5). All animals tested positive after inoculation with the H3N8 virus by RRT-PCR and virus isolation at 2 days post-inoculation (DPI) in oropharyngeal and cloacal swabs, while patterns of infection after homologous or heterologous challenges were variable. The experimental design varied between trials, but all serum samples were collected on 0 and 14 DPI from the brachial or jugular vein. Additional serum samples at intermediate time point between inoculations

were collected in the groups that received more than 2 challenges or that had an interval between inoculations larger than 5 months.

MN assays

A second passage in embryonated chicken eggs of the A/mallard/MN/AI10-2593/2010 (H3N8) was used as antigen for the MN assays in this study. Samples were tested by MN in Madin–Darby canine kidney cells as previously described [17]. Briefly, serum samples were diluted 1:10 in minimal essential media (MEM; Sigma-Aldrich, St. Louis MO) and heat inactivated at 57 °C for 30 min. Two-fold serial dilutions of sera were prepared in 96-well flat-bottomed plates (1:20-1:640). MEM and a positive chicken serum against the H3N8 virus were used as a negative and positive control respectively. An equal volume of virus dilution containing 10^2 tissue culture infective doses (TCID50) was added to each well containing sera. Plates were incubated at 37 °C, and the positive titer was recorded as the highest dilution at which no cytopathic effect was visualized at 48-72 hr. Serum samples with titers ≥1:20 were considered positive.

HI assays

The A/mallard/MN/Sg-000169/2007 (H3N8) and A/mallard/MN/AI10-2593/2010 (H3N8) were used as HI antigens in this study. The first antigen corresponded to the homologous antigen used for primary inoculation of the ducks at 4 weeks-of-age, and was denominated 2007 antigen for the purpose of the study. The second antigen corresponded to a heterologous virus of the same subtype used as antigen for the MN assays, and was denominated 2010 antigen. The HI assays were performed as described elsewhere [13]. Briefly, sera were treated with receptor destroying enzyme (RDE) derived from *Vibrio cholerae* for 18-20 hr at 37 °C followed by heat inactivation at 56 °C

for an hr. A complete package of turkey RBCs was used to adsorb non-specific agglutinins before HI testing. Two-fold serial dilution of serum samples (1:16-1:1024) were made in 96-well U-bottom microtiter plates. An equal volume (25 µl) of PBS containing four hemagglutinating units (HAU) of the virus was added to each well (1:1) and incubated for 1 hr at 4 °C. Finally, 50 ul of 0.5% turkey RBCs were added to the plate and incubated at 37 °C for 1 hr. All HI tests were conducted by using the two H3N8 antigens and in duplicate. The HI titer was defined as the reciprocal of the last dilution of serum that completely inhibits hemagglutination. The cut-off value for determining a sample to be positive was 1:16.

Statistical analyses

Serum samples detected negative at a dilution of 1:20 or 1:16 for MN and HI assays, respectively, were assigned values of 1:10. Titers obtained from both methods were transformed to $\log_2$ values for the purpose of statistical analyses. Measured agreement between assays was determined by using the Cohen's Kappa coefficient. Kappa values of <0 were considered of poor agreement, 0.01-0.20 slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement and 0.81-1.0 almost perfect agreement. Due to the presence of the bias and high prevalence, the adjusted kappa statistic (PABAK) was also calculated. The statistical comparison of titers at different time points within groups was performed using paired Student t-test on the $\log_2$ titers. All statistical tests were conducted using a commercially available software (Stata version 14.0, StataCorp LP, College Station, TX).

Results

Dynamics of MN and HI antibodies against H3N8 virus

All serum samples collected at four weeks-of-age and prior to inoculation with H3N8 LPAIV were serologically negative by MN and HI assays. The dynamics of antibodies against the H3N8 viruses detected by both methods were similar after infection. MN titers were higher at 2 WPI with the H3N8 virus as compared to titers at 5 WPI (p=0.02). HI titers were not significantly different when measured at 2 and 5 WPI with the H3N8 virus (p>0.05). Re-inoculation of H3N8-primed ducks after 11 or 15 weeks with the same virus induced a boosting effect in the MN and HI titers to this virus; however significant differences were only observed in the former (p<0.05). Similarly, secondary inoculation with phylogenetically related viruses belonging to the same HA clade (H4N5, H4N6 and H14N5) caused a boosting effect on serological titers to the H3N8 virus when samples were tested by MN and HI assays; however, the increase was not significantly different (p>0.05). Heterologous re-infection with the H6, H10, and H12 subtypes of LPAIV did not show evidence of a boosting effect in the antibody titers against H3N8 antigen (p>0.05) (Figure 5.1).

Agreement after single H3N8 inoculation

MN antibodies were detected in 92% (95% confidence interval [CI] 0.83-0.97) of the samples tested two WPI with the H3N8 virus. Similarly, 63% (CI 0.51-0.73) and 57% (CI 0.46-0.67) of the samples tested at this time point were positive by the HI assays using the 2007 and 2010 antigens, respectively. A slight agreement between the MN and both $HI_{2007,2010}$ assays was observed at this time point (PABAK=0.20 and 0.15, respectively). A lower proportion of serum samples positive to MN were detected at 4 to

5 weeks post-H3N8 inoculation (p=0.04) as compared to 2 WPI. On the other hand, a higher proportion of samples were positive when tested by the HI assays; however statistical significant differences were not found (p>0.05). Substantial agreement between the MN and $HI_{2007,2010}$ assays (PABAK=0.66 and 0.67) was observed in samples collected at 4 to 5 WPI. Perfect agreement between both assays was observed when samples were tested at 11 and 15 weeks after primary H3N8 inoculation (Table 5,2).

Agreement after homologous re-infection

The agreement between the MN and $HI_{2007, 2010}$ assays was perfect (PABAK=1) in samples collected after re-inoculation with the homologous H3N8 virus when birds were 15 and 19 weeks old (Table 5,2).

Agreement after heterologous re-infection

Substantial and perfect agreement between MN and $HI_{2007, 2010}$ assays was observed when birds were re-infected with the H4N5 or H4N6 viruses (PABAK=0.68, 0.76). Perfect agreement was observed in birds re-infected with the H14N5 virus (PABAK=1.0, 1.0). Agreement between both assays was variable when birds were re-infected with viruses outside the phylogenetic H3 clade (Table 5,2).

Discussion

In this study, we compared the proportion of positive samples detected with MN and HI tests and determined the agreement between these assays in mallards experimentally infected with LPAIV. Paired serum samples of experimentally infected mallards with the H3N8 virus and re-infected with homo- or heterosubtypic LPAIV were tested. Overall, we found a slight agreement between MN and HI assays in serum

samples collected two weeks after primary H3N8 infection; this observation was largely attributable to poor antibody detection by the HI assay. Agreement between MN and HI increased to substantial in samples collected 4 to 5 WPI.

MN assays are believed to be more sensitive than HI assays because of their capability to also detect neutralizing antibodies with no-HI activity such as the truncated version of the IgY secreted in ducks [18, 19]. In agreement with this, a slightly higher proportion of positive samples was detected by MN as compared to HI assays. Also, previous studies have shown the low sensitivity of the HI assay for detection of humoral response against IAV in ducks when chicken RBCs are used [4]. In our study, 57-63 % of the birds seroconverted by HI at 2 WPI with the H3N8 virus. The proportion of positive samples increased to 71-83% when samples were tested at 4-5 WPI and they were in substantial agreement with the MN results. This might suggest that our HI assays using turkey red blood cells have a good sensitivity for detecting antibodies after IAV infection in ducks that might be comparable to the MN results in samples collected 4-5 WPI.

Avian influenza viruses preferentially bind to SAα2,3Gal receptors. Turkey and chicken RBCs express a mix of essentially SAα2,3Gal and SAα2,6-Gal [20, 21]. Chicken RBCs mainly hold SAα2,3Gal [20]; however, opposite results about the relative proportion of SAa2,3Gal and SAa2,6Gal linkages on turkey RBCs have been reported [22, 23]. Our findings and another similar study have shown that a high proportion of samples was HI positive by the use of turkey RBCs [24]. Our study suggests a good performance of turkey RBCs for detection of HI antibodies against H3N8 virus of wild bird origin. However, more studies that compare performance of chicken versus turkey red blood cells when duck serum samples are tested are warranted.

Good agreement between MN and HI assays were found in serum samples from ducks infected with just one H3N8 virus until 11 weeks-post-inoculation. Re-inoculation of birds with the same virus after 11 or 15 weeks had a boosting effect in MN and HI titers. This pattern was not observed in birds re-infected after five weeks, probably as a result of rapid clearance of the virus and reduced activation of recall antibody response [25]. The observed agreement between MN and HI assays was variable following infection of H3N8-primed ducks with a different virus subtype. For instance, substantial to perfect agreement between MN and HI assays was observed when birds were re-infected with IAV within the H3 clade (H4N5, H4N6, and H14N5), while slight to substantial agreement was observed in serum samples of birds re-inoculated with IAV outside the H3 clade (H6N2, H10N7, and H12N5).

A boosting effect to H3N8 was observed when H3N8-primed birds were inoculated with IAV within the same HA clade (H4 or H14). Those findings might be explained by the "original antigenic sin" phenomenon (OAS). OAS suggests that if an individual is sequentially exposed to different viruses that are antigenically related, the immune system can identify the shared epitopes, and progress to a strong immune response against the first virus [25, 26]. On the other hand, subsequent infections with viruses from a different HA clade (H10, H12 or H6) did not show any boosting effect in the antibodies against H3N8 virus. Studies have shown that antigenically dissimilar or divergent strains of influenza viruses failed to induce OAS [27]. Inoculation of live viruses have been associated with induction of cross-neutralizing antibodies in some individuals, probably as a result of induction of a subset of B cells secreting antibodies targeting common HA antigens between different IAV subtypes [15, 28]. In addition,

MN assays also detect antibodies targeting the NA which have been demonstrated to confer partial level of protective immunity [29]. Those findings might suggest that the higher sensitivity of MN assays as compared to HI might be accompanied with a relative loss of specificity when testing serum samples positive to IAV antibodies. However, more studies that compare sensitivity and specificity of between methods by the use of different IAV subtypes as antigens are warranted.

In summary, our results showed that MN assay appeared to be the best method to detect humoral immunity against IAV as compared to HI in mallards. The agreement between assays varies depending on the time-point at which the sample was collected after infection. Also, re-inoculation of ducks with a homologous or heterologous strain might affect the observed agreement.

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Table 5.1 Description of groups of ducks experimentally inoculated with LPAIV from which sera was analyzed in this study. All serum samples of birds corresponded to birds primed with the H3N8 virus at 4 weeks of age. Groups 1, 2, and 3 corresponded to ducks re-challenged with the same virus after 5, 11, and 15 weeks. Groups 4, 5, 6, 7, and 8 were inoculated with heterosubtypic viruses after 5 months. Group 9 was re-inoculated with the H4N6xH10N7xH14N5 viruses in 4 week intervals. Groups 10 and 11 were challenged with the H10N7 and H14N5 viruses after 8 or 12 weeks respectively. Groups 12, 13, 14, and 15 were inoculated with different doses of the H6N2 or H4N6 viruses after 5 months.

Group	Description (Intervals between inoculations in weeks)		# birds	4	6	8	9	10	11	12	14	15	16	17	18	19	21
1	H3N8xH3N8	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
2	H3N8xH3N8	(11)	5	I^{S}	^{S}		^{S}		^{S}			II^{S}		^{S}			
3	H3N8xH3N8	(15)	5	I^{S}	^{S}		^{S}		^{S}			^{S}		^{S}		II	^{S}
4	H3N8xH4N6/H6N2	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
5	H3N8xH4N5	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
6	H3N8xH10N7	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
7	H3N8xH6N2	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
8	H3N8xH12N5	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
9	H3N8xH4N6xH10N7xH14N5	(4, 4, 4)	5	I^{S}	^{S}	II^{S}		^{S}		III^{S}	^{S}		IV^{S}		^{S}		
10	H3N8xH10N7	(8)	5	I^{S}	^{S}			^{S}		II^{S}	^{S}						
11	H3N8xH14N5	(12)	5	I^{S}	^{S}								II^{S}		^{S}		
12	H3N8xH6N2(10^3)	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
13	H3N8xH6N2(10^4)	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
14	H3N8xH4N6(10^3)	(5)	5	I^{S}	^{S}		II^{S}		^{S}								
15	H3N8xH4N6(10^4)	(5)	5	I^{S}	^{S}		II^{S}		^{S}								

I, II, III, IV, IV Primary, secondary, tertiary, and quaternary inoculation. ^{S} Serum sample collected

Table 5.2. Proportion of positive samples and agreement between HI and MN assays following exposure to LPAIV in mallards at different time-points. The table shows the proportion (P) of positive sample by microneutralization (MN) and hemagglutination assays (HI) by the use of the 2007* or 2010** antigens. The percentage of agreement and PABAK coefficient with 95% confidence intervals (CI) between MN and HI assays are displayed to the right.

Week of age	Virus exposed (weeks post-inoculation)	Proportion of positive samples						MN_{2010} vs HI_{2007}		MN_{2010} vs HI_{2010}		HI_{2007} vs HI_{2010}	
		MN_{2010}	P_{2010}	HI_{2007}	P_{2007}	HI_{2010}	P_{2010}	Agreement%	PABAK (CI)	Agreement%	PABAK (CI)	Agreement%	PABAK (CI)
4	Naïve	0/75	0	0/75	0	0/75	0	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
6	**H3N8 (2)**	69/75	0.92	47/75	0.63	43/75	0.57	60	0.20 (0.02-0.42)	57.33	0.15 (0.08-0.37)	86.67	0.73 (0.57-0.89)
8	**H3N8 (4)**	4/5	0.80	5/5	1	5/5	1	83.08	0.66 (0.48-0.84)	81.54	0.63 (0.44-0.83)	89.23	0.75 (0.58-0.92)
9	**H3N8 (5)**	46/60	0.77	45/55	0.83	38/55	0.71						
10	H3N8x**H4N6 (2)**	5/5	1	5/5	1	5/5	1						
11	H3N8x**H4N6/H6N2 (2)**	5/5	1	5/5	1	5/5	1						
	H3N8x**H4N5 (2)**	5/5	1	5/5	1	5/5	1	84	0.68 (0.39-0.96)	88	0.76 (0.51 - 1.00)	80	0.6 (0.29-0.91)
	H3N8x**H4N6 (10^3) (2)**	3/5	0.6	4/5	0.8	3/5	0.6						
	H3N8x**H4N6 (10^4) (2)**	3/5	0.6	4/5	0.8	2/5	0.4						
	H3N8x**H3N8 (2)**	5/5	1	4/5	0.8	4/5	0.8	80	0.6 (-0.1-1.30)	80	0.6 (-0.1-1.30)	80	1 (1-1)
	H3N8 (7)	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8 (7)	5/5	1	5/5	1	5/5	1						
	H3N8x**H6N2 (2)**	4/5	0.8	3/5	0.6	2/5	0.4						
	H3N8x**H6N2 (10^3) (2)**	3/5	0.6	4/5	0.8	3/5	0.6	73.33	0.47 (0.02-0.91)	66.67	0.33 (-0.14-0.81)	80	0.6 (0.19-1.00)
	H3N8x**H6N2 (10^4) (2)**	3/5	0.6	4/5	0.8	2/5	0.4						
	H3N8x**H10N7 (2)**	3/5	0.6	2/5	0.40	2/5	0.40	40	-0.2 (0.65-1.06)	40	0 (-0.98-0.98)	100	1 (1-1)
	H3N8x**H12N5 (2)**	3/5	0.60	1/5	0.20	1/5	0.20	60	0.2 (-0.65-1.06)	60	0.2 (-0.65-1.06)	100	1 (1-1)
12	H3N8x**H4N6 (4)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8 (8)	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
14	H3N8xH4N6x**H10N7 (2)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8X**H10N7 (2)**	5/5	1	5/5	1	5/5	1						
15	**H3N8 (11)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8 (11)	5/5	1	5/5	1	5/5	1						
16	H3N8xH4N6x**H10N7 (2)**	5/5	1	4/5	0.8	4/5	0.8	80	0.6 (-0.1-1.30)	80	0.6 (-0.1-1.30)	100	1 (1-1)
	H3N8x**H10N7 (2)**	5/5	1	3/5	0.6	3/5	0.6	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
17	H3N8x**H3N8 (2)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8 (11)	5/5	1	4/5	1	4/5	1						
18	H3xH4xH10x**H14 (2)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
	H3N8x**H14N5 (2)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
19	**H3N8 (15)**	4/5	0.8	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)
21	H3N8x**H3N8 (2)**	5/5	1	5/5	1	5/5	1	100	1 (1-1)	100	1 (1-1)	100	1 (1-1)

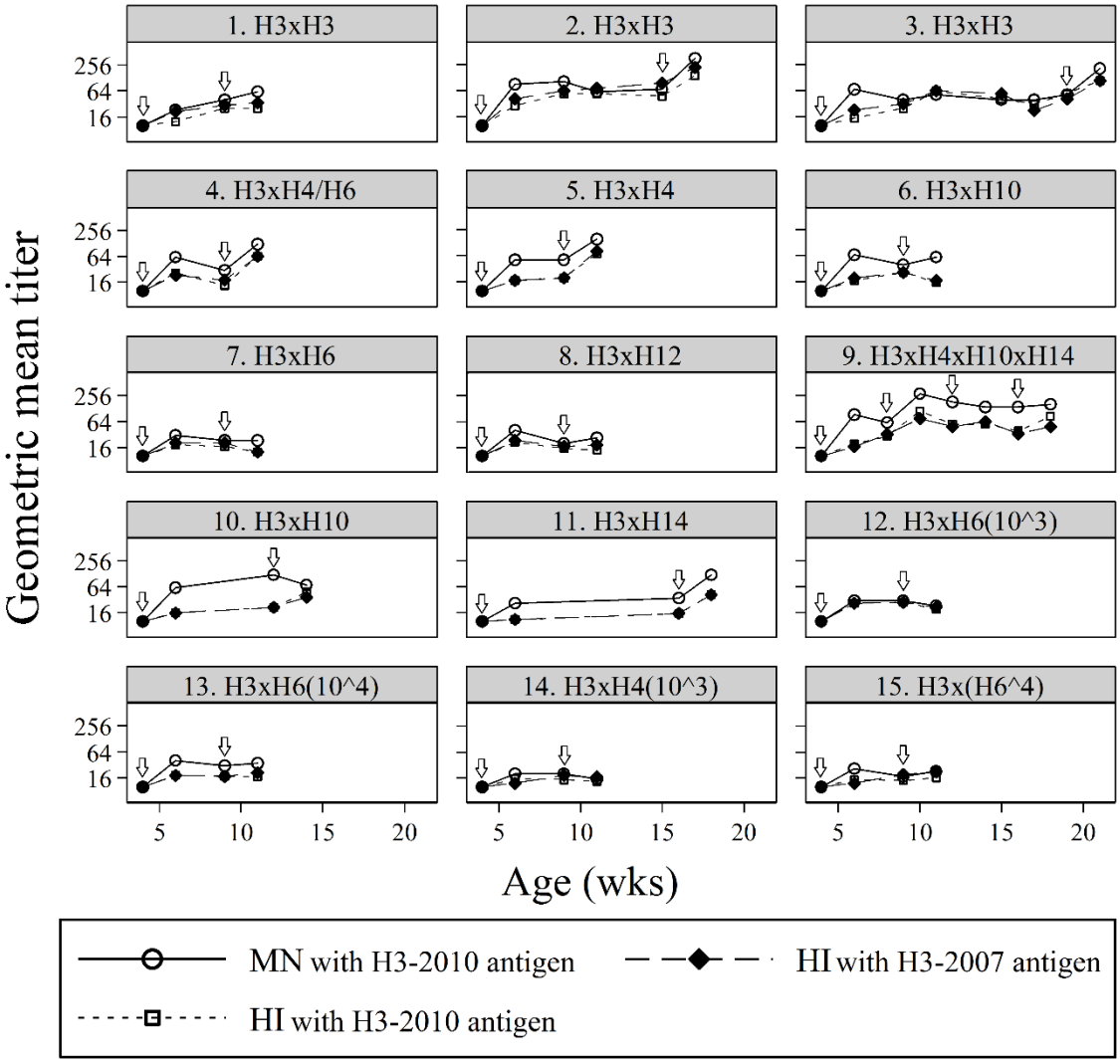

Figure 5.1 Dynamics of serological response by MN and HI assays. The graph shows variation of geometric mean antibody titers across time based on HI and MN assays in different experimental groups. Arrows indicate the time points of infections and circles, diamonds, and squares the time-points at which samples were tested by MN and HI assays.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Wild birds serve as the major reservoirs for all subtypes of Influenza A viruses (IAV) in nature. Developing a better understanding of the role of adaptive immunity in influencing the ecology of low pathogenic avian influenza viruses in one of the major reservoirs such as mallards (*Anas platyrhynchos*) needs to be continually explored. Results from these studies indicate that heterosubtypic immunity can reduce viral shedding and increase the infective dose required for subsequent IAV infections. These effects may be important factors that influence patterns of subtype prevalence and diversity in mallards in nature.

Our first experiment used the mallard animal model to experimentally assess if susceptibility to re-infections by heterosubtypic subtypes of IAV was determined by the phylogenetic relatedness of the hemagglutinin of the viruses encountered. Inoculation of mallards with a prevailing H3N8 LPAIV subtype in waterfowl induced different levels of protective immunity against heterosubtypic infections with the H4N6, H10N7, and H14N5 LPAIV subtypes after different time intervals. The degree of this cross-protective immunity was positively associated to the genetic relatedness of the HA of the viruses. Wild birds are frequently exposed to more than one virus subtype in nature. Therefore, we also investigated the effect of sequential exposure of mallards to different virus subtypes, and as expected our results showed a boosting effect on the degree of HSI

following exposure to more than one LPAIV subtype.

Once we determined that heterosubtypic immunity was associated with the genetic similarity of the HA of the viruses, we also wanted to determine if it may affect the median infectious dose of IAV required to infect mallards during subsequent infections. Experimental inoculation of H3N8-primed mallards with two increasing doses of one closely (H4N6) and one distantly (H6N2) related LPAIV determined that in addition to the phylogenetic similarity of the viruses, the dose of the virus upon secondary exposure determines the outcome of the heterosubtypic challenge. Lower doses of distantly related viruses were needed to overcome previous heterosubtypic immunity induced by inoculation with the H3N8 virus as compared to a closely related subtype.

Finally, we wanted to assess the agreement of a widely used assay for determining humoral immunity against IAV such as hemagglutination inhibition (HI) assays with a more sensitive method such as microneutralization (MN). We observed slight agreement when samples were tested 2 weeks after H3N8 inoculation, the observed agreement was increased when samples were tested 2 weeks later. As expected we observed MN assays detected a higher proportion of positive samples as compared to HI. In addition, challenge of birds previously exposed to the H3N8 virus with IAV within the same HA clade induced a boosting effect in the humoral response against the first virus. This effect was not observed in birds re-challenged with IAV of different HA clades.

Our findings provide further information and understanding on the contributions of HSI and its role in the dynamics of IAV subtype diversity in mallards. The information

obtained in this research and can be used to aid results obtained from surveillance studies. Additional and integrated research is necessary to further understand the host, viral and environmental interactions that determine effective transmission and maintenance of IAV viruses in wild bird populations.