DRIVERS OF TYPE A INFLUENZA VIRUS DYNAMICS IN THE NON-ANSERIFORMES WILD BIRD RESERVOIR

by

CHARLIE BAHNSON

(Under the Direction of David Stallknecht)

ABSTRACT

Birds within the order Anseriformes are considered an important natural reservoir of Influenza A virus (IAV), yet other species likely contribute to the maintenance, distribution, and evolution of IAV. This work focused on drivers of IAV infection dynamics in species in other avian orders that have garnered less attention.

In the first study, we evaluated the importance of population immunity as a potential cause for which IAV subtypes are most commonly isolated from the IAV "hotspot" at Delaware Bay, New Jersey (DE Bay) in a given year. While we hypothesized that population immunity causes shifting IAV subtype dominance at DE Bay, our results suggested that IAV dynamics are subtype-dependent and population immunity has more of a bearing on which species is infected at DE Bay.

In the second study, we determined the susceptibility of Mallards (*Anas platyrhynchos*) and Laughing gulls (*Leucophaeus atricilla*) to IAVs isolated from Ruddy Turnstones (*Arenaria interpres morinella*) at DE Bay in order to gain insight into potential sources and host ranges of these viruses. We found that Mallards were far more permissive to infection with most viruses, suggesting that transmission between

Mallards and Ruddy Turnstones could occur. By contrast, host-adaptation of IAVs to Ruddy Turnstones may compromise the virus' ability to be transmitted back to gulls.

Finally, we evaluated the importance to IAV maintenance of a species not conventionally recognized as a significant host for IAV. The American White Ibis (*Eudocium albus*) is a wading bird that occupies aquatic habitats in the Southeastern United States. Through experimental challenge, and a multi-year, multi-season serosurvey, we determined that they are both susceptible and naturally exposed to IAV. Thus, they may represent a component of the IAV natural reservoir system.

Collectively, the findings reported in this dissertation provide focused, new insight into the well-studied IAV system at DE Bay, while also providing support for broadening our concept of how IAVs are maintained in multi-species, avian communities.

INDEX WORDS: Influenza A virus, Ruddy Turnstone, Red Knot, antibody, Laughing Gull, Mallard, Delaware Bay, Ibis, reservoir, serology, Florida

DRIVERS OF TYPE A INFLUENZA VIRUS DYNAMICS IN THE NON-ANSERIFORMES WILD BIRD RESERVOIR

by

CHARLIE BAHNSON

BA, Augustana University, 2009

DVM, Iowa State University College of Veterinary Medicine, 2015

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

DRIVERS OF TYPE A INFLUENZA VIRUS DYNAMICS IN THE NON-ANSERIFORMES WILD BIRD RESERVOIR

by

CHARLIE BAHNSON

Major Professor:	David Stallknecht
Committee:	Roy Berghaus
	Daniel Perez
	Mark Ruder
	Stephen M Tompkins

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2018

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

For most, the subject of “Influenza” is vast, ubiquitous, and complicated. We are bombarded every year with updates chronicling the flu season. There are reports of a virus in European birds. We learn of historical pandemics that killed millions and hear intermittent details of a smoldering “bird flu” in Asia. There is even news of a virus detection in United States poultry now and then. The significance and relatedness of these events are unclear and it is hard to put these things in context. However, when attempting to get a handle on these swirling details, a person is likely to repeatedly encounter a consistent theme: wild birds are the natural reservoir.

While this theme is simple enough on its surface, research efforts in the last several decades have uncovered what is, in fact, an exceedingly complicated disease system. Some major tenets are now well established, but there are still many details to fill in. The aim of this project is to add a few more small pieces to our understanding of how influenza is maintained in the natural reservoir. Influenza is likely to become only more relevant to human and domestic animal health in the coming years. Armed with a more complete understanding of how it exists in nature, we can more adequately address the emerging issues it presents. As Webster *et al.* rather dramatically stated 26 years ago:

> “The understanding of the ecology of influenza viruses in this benign reservoir of aquatic birds is imperative if we wish to find ways to intervene

and reduce or prevent the occasional catastrophic pandemics such as the one that decimated the human population of the world in 1918 after the appearance of 'Spanish' influenza' (1992)."

LITERATURE REVIEW

Classification

Influenza A virus (IAV) is a member of the Orthomyxoviridae family. This family includes seven genera: IAV, influenza B viruses, influenza C viruses, influenza D viruses, Isavirus, Thogotovirus, and Quaranfilvirus. Orthomyxoviruses are enveloped, singled stranded, helical, segmented, negative sense RNA viruses. They are relatively pleomorphic and range from spherical to filamentous (Wright *et al.* 2007).

Influenza A viruses consist of eight gene segments that encode ten viral proteins: hemagglutinin (HA), neuraminidase (NA), membrane ion channel proteins (M2), nucleoprotein (NP), matrix protein (M1), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), non-structural protein 1 (NS1), and non-structural protein 2 (NS2). Proteins HA, NA, and M2 are located on the surface of the virion while NP, M1, PB1, PB2, and PA are internal proteins. The latter three comprise the viral RNA-polymerase complex. Non-structural proteins 1 and 2 are non-structural, as their name implies (Palese and Shaw 2007; Suarez 2017).

Viral Replication

Viral replication begins with attachment to a host cell via the viral HA to host cell terminal sialic acid that is attached to oligosaccharides on the surface of epithelial cells, most commonly through α-2,3 or α-2,6 linkages. The distribution and availability of these two types of sialic acids varies by tissue and host species and is an important

determinant of host range (Rogers and Paulson 1983). Avian species predominantly express α-2,3 in the gastrointestinal tract while humans predominantly express α-2,6 in the respiratory tract and a given IAV tends to have strong biding affinity to one or the other (Suarez 2017).

Following attachment, the virus is brought into the cell via receptor-mediated endocytosis. Hydrogen ions from the host cytoplasm enter the endosome through the M2 protein, causing it to become acidified. This triggers a conformation change of the HA that activates its fusion domain, and leads to the formation of an open channel through the viral membrane and endosomal membrane, through which the viral ribonucleoprotein (RNP) is released into the cytoplasm (Xiong *et al.* 2014). Importantly, this conformational change in the HA can only occur if the HA has been previously cleaved into HA1 and HA2 subunits by host proteases. The ease in which this occurs for a given HA is a major determinant of viral pathogenicity (Chen et al 1998). The RNP consists of the viral RNA-polymerase complex, NP, and viral RNA segments. In response to signaling from the NP, the host cell actively transports the viral RNA-polymerase complex to its nucleus.

Once inside the cell nucleus, the RNA-polymerase complex transcribes the negative-sense viral RNA to positive-sense mRNA. This step also requires a host cell mRNA primer that is “snatched” from the host mRNA by the viral PB2 protein. The positive-sense viral mRNA is then transported back to the cytoplasm where it serves as a template for host machinery to translate new viral proteins. Meanwhile, the RNA-polymerase complex transcribes negative-sense viral RNA to “complementary RNA” in the nucleus. This serves as the template for replication and thus, production of new,

negative sense, viral RNA. Assembly of new virions begins in the host endoplasmic reticulum and is completed at the apical plasma membrane. It is largely mediated by the M1 protein. This process is prone to error, and many progeny virions are defective because they do not include all eight gene segments or the necessary viral proteins. Assembly of a new virion is completed at the host cell membrane where it buds from the apical plasma membrane. After budding is complete, the virion is still anchored to the host cell by HA-sialic acid binding. These anchors are cleaved by the NA protein, ultimately releasing the new virion from the host cell (Palese and Shaw, 2007).

Replication of RNA viruses is notoriously error-prone as compared to DNA viruses. This is particularly relevant for IAVs. As a result, point mutations regularly occur. This, paired with constant immunologic, selective pressure, drives a continuous "genetic drift." Older literature often states that IAVs in wild birds are in a state of "evolutionary stasis." However, more recent work illustrates that drift occurs in wild birds as well (Spackman 2005).

The Nature of the Virus

Influenza A viruses have a wide host range that includes numerous species of birds and mammals. They can be characterized based on at least four schemes. The first relates to the surface proteins, HA and NA. To date, 18 HAs and 11 NAs have been described. It was only recently that H17, H18, N10 and N11 were detected in bats in Central and South America (Tong 2012, Tong 2013). The degree to which our understanding of IAVs can be applied to these new subtypes is unclear.

A second, perhaps more colloquial classification scheme involves designating IAVs by which class of species they infect. Although, it is widely regarded that all IAVs

originated in wild birds, which are sometimes referred to as the “primordial reservoir” (Webster 1992), lineages of IAV endemic to humans, swine, and equine influenzas have been in circulation for decades. Only a limited number of HA and NA subtypes are represented in non-avian species. What leads a given IAV to become adapted to non-avian species is not completely understood, but is largely governed by a change in HA - host receptor binding preference (Xiong *et al.* 2014). Proteins NA, PB2, and non-structural protein 1 also contribute to host range but generally garner less focus than HA (Cauldwell *et al.* 2014)

A third classification system for IAVs involves the clinical disease they produce in domestic poultry. When first introduced into domestic poultry, most IAVs infections are asymptomatic or cause moderate to severe respiratory disease. These are termed “low pathogenicity avian influenza viruses” (LPAIVs). Occasionally, a LPAIV adopts a highly pathogenic phenotype, most commonly through the insertion of multiple basic amino acids at the HA cleavage site that makes it more easily cleaved by proteases throughout the body (reviewed in Suarez 2017). IAVs with this phenotype cause systemic disease and high mortality and are termed high pathogenicity avian influenza (HPAIV). To date, all known HPAIVs have had the H5 or H7 subtype. According to the World Organization for Animal Health (OIE), an IAV is considered an HPAIV if it causes death in six or more in a group of eight 4-8 week old chickens within 10 days of intravenous inoculation, has an intravenous pathogenicity index greater than 1.2 in 6 week old chickens, or is an H5 or H7 IAV with an amino acid sequence at the HA cleavage site that is similar to what has been previously documented in HPAIV isolates

(Swayne and Brown, 2012). The OIE also indicates that low pathogenic AIVs that are H1-4, H6, and H8-H16 are *not* avian influenzas.

In contrast to poultry, the pathogenicity of IAVs in wild birds is less established. For the majority of IAVs in the majority of wild bird species, disease ranges from unapparent to subtle and short-lived with minimal evidence of clinical signs or lesions (Franca and Brown, 2014). A few studies in free-ranging wild birds have suggested that infection may compromise weight gain or influence feeding and migratory behavior (van Gils *et al.* 2007, Latorre-Margalef *et al.* 2009). However, many other studies have failed to corroborate this theme. There is an inherent complexity in studying physiological parameters of wild birds in a natural setting, but currently there is insufficient evidence to believe North American IAVs have a meaningfully adverse effect on wild birds.

A major caveat to this statement involves a fourth characterization scheme of IAVs. Influenza viruses in wild birds can be clearly separated into Eurasian vs North American phylogenetic clades. Viruses with Eurasian genes are sometimes isolated in North America but this is rare, suggesting that unknown mechanisms that likely include geographic barriers limit the amount of viral exchange between the continents (Krauss *et al.* 2007). However, in the fall of 2014, a massive HPAIV outbreak occurred in North American poultry that lasted into the early summer of 2015 and affected 50 million poultry. The outbreak resulted from the introduction of a Eurasian-origin HPAIV H5 virus of the A/Goose/Guangdong/1/1996 lineage. Viruses of this lineage have persisted, likely involving wild birds, in Eurasia ever since they were first isolated in 1996 (Sims and Brown 2017). The introduced clade 2.3.4.4 HPAIV H5N8 reassorted with North American viruses and these were associated with mortality in a number of wild bird

species. The event challenged many of our conceptions of how IAVs are maintained in North America and reinvigorated the need to understand how IAVs work in the natural reservoir system (Ramey *et al.* 2018).

Immunology

An immune response is categorized into the innate and adaptive responses. The innate immune response is critical in early IAV infection and represents the first line of defense. There are numerous components in the innate response, but one important feature in IAV infections is the interferon (IFN) response. Pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) which initiates a cascade of cellular signals, ultimately resulting in a change in gene expression resulting in acute inflammation, apoptosis of infected cells, antiviral state in neighboring, susceptible cells, and stimulation of the adaptive immune response (Vervelde and Kapczynski, 2016). An important avian pathway for this is via the recognition of double stranded RNA by-products of IAV replication by retinoic acid-inducible gene-I (RIG-I). This is a critical component in early, rapid defense to infection with HPAIV that is largely protective in ducks. However, chickens appear to have lost this functional RIG-1 which may be a key to their susceptibility to HPAIV (Barber *et al.* 2010).

The adaptive immune system is more targeted to specific insults and can mount a more efficacious, tailored response when it reencounters a particular antigen. However, the adaptive immune system requires more time to respond to an insult than the innate system. Therefore, it may be of limited use in naïve poultry in a rapid HPAIV infection but is critical to long-lived wild birds exposed to numerous IAVs. The adaptive

response can be divided into cellular immune response and humoral immune response. Humoral immunity consists of antibodies produced by B-lymphocytes. These can target numerous epitopes on an IAV, but most emphasis is placed on antibodies targeting the IAV hemagglutinin. These prevent attachment and entry of IAVs into the host cell and are thus considered “neutralizing.” Cell mediated immunity consists of T and B lymphocytes. Cytotoxic T lymphocytes (CD8s) recognize short cytosolic antigen peptides presented on major histocompatibility complex 1 (MHC 1) molecules expressed on all nucleated cells. They effectively kill IAV-infected cells. Antigen presenting cells (APCs), including macrophages, dendritic cells, and B lymphocytes, display extra cellular antigen on MHC-II molecules which are recognized by T helper lymphocytes (CD4+). Activated T helper cells signal to macrophages to enhance phagocytic activity and also stimulate B cells to produce antibodies (Abbas *et al.* 2015). Interestingly, CD8+ CTLs from chickens previously vaccinated with H9N2 were injected into naïve chickens and were partially protective to H5N1 HPAIV challenge (Seo and Webster 2001, Seo *et al.* 2002). This implied that some CTLs were targeted toward more conserved internal proteins of IAV.

As in mammals, the first serum antibodies to be produced in response to IAV infection in a naïve duck are immunoglobulin M (IgM). Through class switching, this soon shifts to the production IgY, the avian analog of mammalian IgG. The classic functions of this antibody isotype include opsonization, complement activation and antibody dependent cellular cytotoxicity (Abbas *et al.* 2015). However, a significant portion (approximately 60%) of IgY in ducks have truncated Fc receptors and this portion appears to increase with age and secondary exposures (Magor 2011). The

significance of this is unclear. As Magor (2011) explains, although truncated IgY is unable to perform most functions listed above, it is still able to neutralize HA. These truncated IgYs may also be able to prevent membrane fusion within the endosome by crosslinking hemagglutinins- a function dependent on the Fab portion of the antibody (Barbey-Martin *et al.* 2002). Furthermore, in some cases, IAV may be able to bind to the Fc-receptor of IgY and use APCs to disseminate throughout a host. Therefore, limiting the population of full length IgY may be a protective adaptation. In practical terms, hemagglutinin inhibition assays likely fail to detect truncated IgYs in ducks while microneutralization assays should be able to detect either type.

Natural Reservoir; Anseriformes

Birds within the orders Anseriformes (ducks and geese) and Charadriiformes (gulls and shorebirds) are natural reservoirs of IAVs (Webster 1992, Krauss 2004, Olsen 2006). A robust body of literature has emerged in the last few decades describing the infection dynamics of IAVs in Anseriformes. Annual enzootics occur in dabbling ducks in northern latitudes in late summer during staging and early migration. At this time, approximately 10-33.4% of dabbling ducks are infected with IAV with much higher prevalence in young-of-year individuals (Hinshaw *et al.* 1985, Sharp *et al.* 1993, Hanson *et al.* 2003, Krauss *et al.* 2004, Wilcox *et al.* 2011, Ramey *et al.* 2014, Nallar *et al.* 2015, Papp *et al.* 2017). The prevalence detected in ducks further south and later in the year drops markedly (Stallknecht *et al.* 1990, Ramey *et al.* 2014, Nallar *et al.* 2015). This annual trend is probably driven by the presence of annual immunologically naive individuals that clear the infection with age and dispersal, as migration occurs. A high annual population turnover (≥50%) occurs in most of these species and prevents the

long-term buildup of population immunity. Mallards, blue winged teal, and northern pintails are the most represented species in the literature (Wallensten *et al.* 2007). The subtype of IAV that dominates infections appears to shift throughout the year. In North American blue winged teal and mallards, subtypes H3, H4, and H6 are the most prevalent in the fall (Sharp *et al.* 1997, Hanson *et al.* 2003, Krauss *et al.* 2004, Wilcox *et al.* 2011, Ramey *et al.* 2014). Surveillance at other times of the year is less common but H7 is the most common in blue winged teal in the winter and spring (Ramey *et al.* 2014). A similar pattern of annual endemics has been described in ducks in Europe (Munster 2007, Wallensten 2007).

Adaptive immunity at an individual and population level is likely a key driver in IAV dynamics in the natural reservoir. A number of experimental studies in Mallards have broadened our knowledge of the concept of homo and heterosubtypic immunity. Varying experimental designs involving multiple LPAIV subtypes and even some HPAIVs in Mallards have established that prior exposure to an IAV lessens the length and amount of viral shedding following reinfection with IAVs (Fereidouni *et al.* 2009, Costa *et al.* 2010, Jourdain *et al.* 2010, Pepin *et al.* 2012, Ferreira *et al.* 2015, Latorre-Margalef *et al.* 2017, Segovia *et al.* 2017). The strength of the effect is directly tied to the phylogenetic relatedness of the HA subtype, and the effect is strengthened with third and fourth challenges (Segovia *et al.* 2017). In addition to viral shedding, this “homo and heterosubtypic immunity” increases the challenge dose required for subsequent infections (Segovia *et al.* 2018). The effect can last for at least 15 weeks (Latorre-Margalef *et al.* 2017).

Projecting information derived from experimental studies to what occurs in nature always comes with uncertainty. For instance, mounting an immune system is energetically costly (Buehler et al 2010). Stress and nutritional limitations in wild birds may influence their immune response to IAV in a way that is poorly replicated in most experimental settings (van Gils *et al.* 2007). Illustrating our poor understanding of this, one study found that Mallards in poor nutritional condition due to limited food availability were actually *less* susceptible to infection and shed less virus for shorter periods of time (Arsnoe *et al.* 2011). There are also age, seasonal, and interspecies dynamics that aren't captured in experimental studies. Furthermore, even in experimental studies, a wide degree of individual variation can be observed (Pepin *et al.* 2012) and this may be often underrepresented, given that birds in experimental studies often come from single sources and are likely to be genetically similar.

Despite these caveats, some field work has corroborated the concept of homo and heterosubtypic immunity. In Ottenby, Sweden, a large stopover site for migrating mallards, the likelihood of reinfection with the same or phylogenetically related HA subtypes were decreased (Latorre-Margalef *et al.* 2013). Interestingly, this was time dependent. It took approximately seven days for "immunity" to develop which may relate to the time required for adaptive immunity to mount.

It is widely assumed that the major route of viral exposure in Anseriformes is through fecal/oral transmission in surface water. This may be a key to why dabbling duck species that feed in shallow water are involved in IAV cycles. As reviewed in Stallknecht *et al.* 2010, IAVs have been isolated from surface water in numerous aquatic habitats. Through laboratory-based experiments, it was established that IAVS

can remain infective for months to years at ideal, stable conditions: low temperatures (approximately 4° C), slightly basic pH, and low to moderate salinity. With this in mind, it is often suggested that environmental persistence may contribute to annual IAV maintenance (Stallknecht and Brown, 2017). However, most of our knowledge of environmental persistence has been conducted in a laboratory setting. Verifying this information in a field setting warrants further investigation.

Our knowledge of IAV dynamics in Anseriformes is partly a result of the ease in which samples can be acquired from pre-fledged ducks during the summer and hunter harvested birds in the fall and winter, as well as the reliability of how many of these samples are typically positive. Surveillance and research in other birds is more difficult, particularly if it is dependent on detection of virus which is typically shed for only a brief window in most species. Until recently, serosurveys in wild birds had limited utility. The agar gel immunodiffusion (AGID) test has been used in poultry for some time but has variable sensitivity in wild birds. Another common assay, hemagglutinin inhibition, is subtype specific and not useful for surveillance aimed at detecting exposure to a broad array of subtypes. In 2009, a commercially available blocking enzyme-linked immunosorbent assay (bELISA) that uses a mouse monoclonal antibody was validated as a sensitive test for numerous wild avian species in experimental settings (Brown *et al.* 2009). The following year, its utility for screening sera collected from many avian species in the field was also confirmed (Brown *et al.* 2010). This assay detects antibodies to the nucleoprotein, shared by all IAVs, requires a relatively small volume of sera, and has a high throughput.

Subsequent field studies revealed that antibody prevalence in many aquatic and semi-aquatic avian species can often be relatively high (≥60%) despite low (≤ 1-2%) rates of IAV detection (Hall *et al.* 2014, Johnson *et al.* 2014, Hall *et al.* 2015, Kistler *et al.* 2015, Wong *et al.* 2016, Samuel *et al.* 2015). A general conclusion from these studies is that exposure may be more common in such species than previously realized. However, these often tend to be longer-lived species and interpretation of such data is hampered by a poor grasp of how long antibodies persist in most of these species.

Natural Reservoir; Charadriiformes

While the role of Anseriformes as a reservoir for IAVs has been long established, the importance of a second order of birds, Charadriiformes, was firmly solidified only a few decades ago. In a thorough surveillance effort, Kawaoka *et al.* documented a focal, seasonal increase in IAV infections in shorebirds and gulls in the Delmarva Peninsula (1988). They then challenged chickens and Pekin ducks with several of these IAVs. Because many of the viruses did not appear to infect either species, they concluded that a separate “gene pool” of IAVs reside in gulls and shorebirds.

Since that study, our understanding of IAV dynamics at Delaware Bay, New Jersey (DB) has expanded considerably. Presently, DB is widely regarded as the only place where IAVs are consistently isolated from shorebirds (Hanson 2008, Krauss 2010). Published surveillance efforts in shorebirds at other times and locations have generally yielded few detections (Kawaoka *et al.* 1988; Krauss *et al.* 2004; D’Amico *et al.* 2007; Hanson *et al.* 2008, Winker *et al.* 2008, de Araugo *et al.* 2014, Johnson 2014). At DB each spring, migrating shorebirds stop for two to three weeks to feed on recently deposited eggs of horseshoe crabs (*Limulus polyphemus*) (Clark 1993). The four major

species of shorebirds include Ruddy Turnstones (*Arenaria interpres,* RUTUs), Red Knots (*Calidris canutus,* REKNs), Sanderlings (*Calidiris albo*), and Semipalmated Sandpipers (*Calidiris pusilla*) (Clark 1993). All four species have extremely long migrations from their wintering areas in the Southern United States, Central and South America, to their sub-arctic and arctic breeding grounds (Nettleship 2000, Macwhirther 2002, Hicklin 2010, Baker 2013). This comes at a substantial metabolic cost and Delaware Bay is a vital stopping point to replenish energy reserves. Birds regain 55-70% of their arrival weight in a few weeks (Robinson 2003).

Ruddy turnstones appear to be most important to the IAV disease system at DB. Infection rates in RUTUs average 12-14.1%; the highest of any species at DB (Krauss *et al.* 2004; Maxted *et al.* 2012). In contrast, IAV infection is detected in less than 2% of red knots (Calidris canutus rufa, REKNs), a bird that feeds alongside RUTUs at DB (Maxted *et al.* 2012a). Although IAV hemagglutinin (H) subtypes H1-H13 and H16 have been isolated from RUTUs at DB, annual IAV infections are dominated by a single HA subtype that reoccurs in periodic, erratic cycles (Krauss *et al.* 2004; Stallknecht *et al.* 2012). Subtype diversity in a given year does not differ between RUTUs and REKNs and in most years, all of the IAV subtype diversity can be detected in the RUTU population (Stallknecht *et al.* 2012).

Reasons for this shifting IAV subtype dominance remain enigmatic but individual and population immunity may be a driver. In contrast to Anseriformes described above, shorebirds are relatively long lived with less annual population turnover (Nettleship 2000; Baker *et al.* 2013). They also largely remain at their wintering grounds for the first

year. As a result, the population of shorebirds at DB consists of older individuals that are likely to have been previously exposed to one or more IAV subtypes.

Serologic data supports this by revealing that many RUTUs at DB have an IAV exposure history. It also suggests a potential role of a second shorebird species in addition to RUTUs. Despite a difference in infection rates, a comparable number of REKNs at DB have detectable antibodies to IAV. Antibodies to the IAV nucleoprotein (NP) have been detected in 55-65% and 53.6-86% of RUTUs and REKNs (Brown *et al.* 2010; Maxted *et al.* 2012a; Stallknecht *et al.* 2012) at DB. Exposure and seroconversion does not appear to occur at the same time for both species, however. By using date and body weight as a proxy for time spent at DB, Maxted *et al.* (2012) determined that antibody prevalence in RUTUs increases from less than 40% at arrival to over 95% at departure, implying that over half the population seroconverts while at DB. In contrast, the antibody prevalence in REKNs was 82% at arrival and slowly declined throughout the remainder of the season. This provides valuable insight into the DB system, but, because NP is conserved between IAV subtypes, these antibody patterns are of limited use when considering virus-host interactions at a subtype-specific level.

The importance of individual and population immunity as a driver of IAV dynamics at DB is, as of yet, unsubstantiated. Compared to dabbling ducks, the immune response of shorebirds to single or multiple IAV infections is poorly understood (Curran *et al.* 2013, Hall *et al.* 2013). It is also likely that the particularly severe physiologic demands of long-distance migration involves some cost to the immune system (reviewed in Buehler *et al.* 2010) which has the potential to further obfuscate the matter. In the first study (Chapter 2), we sought to investigate a potential immunological

basis for the phenomenon of shifting IAV subtype dominance at DB while also further exploring the role of REKNs.

A second major question surrounding IAV dynamics at DB concerns the source of viruses. There is currently little evidence to support the notion that IAVs are maintained within RUTUs at low levels of infection throughout the year, as surveillance efforts in shorebirds at other times and locations are generally unfruitful (Kawaoka *et al.* 1988; Krauss *et al.* 2004; D'Amico *et al.* 2007; Hanson *et al.* 2008, Winker *et al.* 2008, de Araugo *et al.* 2014, Hall *et al.* 2014, Johnson 2014). A second possibility is environmental persistence at DB. Influenza A viruses have been successfully isolated from sand cores at DB during annual IAV cycles (Poulson *et al.* 2017). However, the limited number of HA subtypes isolated corresponded to the viruses circulating in birds during that sample year. Therefore, environmental persistence of IAVs in this system may help to amplify outbreaks but is less likely to serve as a repository for the diversity of viruses observed at DB.

Another potential source of IAVs may come from migrating and resident ducks and gulls that cohabit the feeding and roosting areas at DB (Krause *et al.* 2004; Hanson *et al.* 2008; Guinn *et al.* 2016). Maxted *et al.* (2016) demonstrated that RUTUs roost in expansive salt marshes that are also used by resident ducks and a large number of Laughing gulls (LAGUs; *Larus atricilla*). The detection of IAVs of varying hemagglutinin subtypes is documented in both species from the area in the Influenza Research Database (IRD; Zhang *et al.* 2017). Hemagglutinin subtypes H1-7, 9-13, and 16 IAVs have been isolated from LAGUs at DB during the stopover and the prevalence of antibodies to NP as determined by blocking enzyme linked immunosorbent assay

(bELISA) was as high as 72% (Guinn *et al.* 2016). Detection of H1, 3, 4, 6, 11 IAVs in Mallards is also documented on the IRD but nearly all sampling in the area occurred in the fall, well after the shorebird stopover period in May. However, there is a resident population of dabbling ducks that breed in the area (Nichols and Jones, 2015).

Determining host range of a given species-adapted virus is difficult. Phylogenetic mapping of wild bird isolates tends to be of limited use for estimating species host-range or assigning isolates to a particular location (Spackman *et al.* 2005). It is also biased by the vastly limited number of sequences isolated compared to the number circulating in nature. Gambaryan *et al.* examined the affinity of numerous viruses isolated from ducks and gulls and concluded that as duck viruses circulate in, and become "gull-adapted," they acquire a higher affinity for sialic acid receptors that are bound to a penultimate glycine by 1-4 bond and that were also linked to a fucose sugar molecule (2005). By contrast, "duck-adapted" viruses preferentially bind to greatest affinity for a 1-3 bond between Neu5Acα2-3Gal and GalNacα. Franca *et al.* used lectin binding to assess the distribution of these isoforms in numerous species of wild birds (2013). While they found strong expression of "duck-virus SA receptors" in mallards, they found strong expression of both types of SA receptors in laughing gulls as well as numerous other wild bird species. The general conclusion is that our current understanding of receptor distribution in tissues is of limited use for determining species susceptibility.

By contrast, susceptibility of a species as determined by the mean infectious dose required to infect a given species, is an indicator of how host-adapted a particular IAV is to a species (Swayne and Slemons 2008). Paired with field surveillance data, experimental susceptibility studies can provide concrete evidence of host range. As

detailed above, only a limited number of shorebird isolates replicated in chickens and Pekin ducks, even at a challenge dose of 10^7 EID_{50} (Kawaoka *et al.* 1988). Both ducks and gulls may contribute to IAV dynamics during the stopover by serving as a source of IAVs, amplifying IAVs, or contributing to subtype reassortment. However, it remains unknown to what degree IAVs can be readily transmitted between these species. The conservation status of RUTUs and logistical concerns precludes an experimental challenge of RUTUs with gull and duck-origin IAVs. However, the ability of RUTU-origin IAVs to be transmitted back to ducks or gulls provides an indication of these species' importance to IAV dynamics at DE Bay. With this in mind, we challenged Mallards and LAGUs with RUTU-origin IAVs to assess the degree to which adaptation of IAVs to RUTUs permits or precludes subsequent infections in ducks and gulls (Chapter 3).

While the results in Mallards were not overly surprising, we did expect to find a higher level of susceptibility of LAGUs to some of the challenge viruses, especially the RUTU H13N6. This subtype, along with H16, is almost exclusively maintained in gull populations and widely regarded as a "gull virus" (Arnal *et al.* 2014). Annual cycles of H13 infections are driven by young, naïve birds (Verhagen *et al.* 2015) as infections with H13 IAVs spread rapidly through gull chicks in dense nesting colonies (Velarde *et al.* 2010). Adults at nesting colonies have high rates of antibody prevalence but low rates of infection (Velarde *et al.* 2010). The timing of these enzootics may depend on age of the gull: as maternal antibodies wane with age, fledglings become more mobile, which theoretically increases contact rates (Verhagen *et al.* 2014). The one to five-day old gull chicks we collected were among the first to hatch in that nesting colony (presumably prior to IAV circulation). Furthermore, they were raised to six weeks of age, past the

point of detectable maternal antibody level. Field investigations would suggest that 3 and 5 week old chicks are susceptible to H13 infection, even in a population where adults were 98% seropositive (Velarde *et al.* 2010). For these reasons, our failure to infect LAGUs with the RUTU H13N6 is puzzling.

These results indicate that if Laughing gulls contribute to IAV dynamics at DB, behavioral factors within this species (feeding strategies, high density roosting, etc.) and interspecies interactions must overcome a substantially higher threshold of exposure dose as compared to Mallards. As a final piece of the study, we phylogenetically compared the HA sequence of each challenge virus to other available sequences. We also examined the amino acid at position 222 (H3 numbering system). Recent work has suggested that adaptation of an IAV from ducks to gulls is accompanied by a substitution to a less bulky amino acid (Gambaryan *et al.* 2018). Collectively, this work demonstrated the presence of host adaptation of IAVs at DB that may influence the importance of gulls and ducks.

Natural Reservoir System

A central paradigm in our understanding of IAV concerns the concept of the natural reservoir. For several decades, research efforts have been informed by the notion that Anseriformes and Charadriiformes are the natural reservoir of IAVs. This is well supported by surveillance and experimental data, however, the complexity of the concept of “natural reservoir” is probably overlooked. A simplified concept of a natural reservoir places “wild birds” within a single reservoir category which serves as a single source for the target population of interest, whether that be humans, poultry, or something else. A more accurate framework, particularly when talking about IAV,

regards the natural reservoir as a complex system of maintenance and non-maintenance hosts that are epidemiologically connected (Haydon *et al.* 2002). Within this framework, the natural reservoir system is composed of numerous species and environmental sources. The collective interactions of these across a spatial and temporal scale comprise a continual source of IAVs to the target population, whichever that may be.

Unfortunately, the simplified IAV central paradigm concerning the natural reservoir has largely directed our surveillance efforts in wild birds and resulted in what is probably a biased understanding. If infection prevalence alone is considered the sole indicator of a reservoir species, eight bird orders have a global prevalence higher than Charadriiformes (Caron *et al.* 2017). However, as we know from Anseriformes, viral shedding is typically brief (< eight days) and highly temporally and spatially-dependent. Therefore, relying on surveillance of IAV detections as the sole indicator of inclusion or exclusion in the natural reservoir system results in an incomplete framework. Stallknecht and Brown (2017) argue: “documentation of both infection with and susceptibility to AIV does not determine whether a species is important as a reservoir.” Rather, stronger conclusions can be drawn by a multifaceted approach that also incorporates field serology, phylogenetic studies, and ecological plausibility in addition to species susceptibility.

White Ibis

As an exercise in challenging the paradigm, we looked to a species of wading bird, the American White Ibis (WHIB; *Eudocimus albus;* Chapter 4). White Ibis are common to many coastal habitats in the southeastern United States (Heath *et al.* 2009).

In south Florida, a large number have become highly urbanized and forage in neighborhood parks, artificial wetlands, backyards, and golf courses. However; the majority continue to nest and roost in natural wetlands in dense colonies (Hernandez *et al.* 2016). The species is largely nomadic and individuals and adults will travel up to 1600km to reach other breeding colonies in the Southeast United States and Caribbean (Frederick *et al.* 1996). As a result, WHIBS come into frequent contact with humans, waterfowl, domestic poultry, and other urbanized and non-urbanized avian species throughout their range. Therefore, numerous potential routes of IAV transmission exist, and a strong argument can be made that WHIBs are an ecologically plausible component to the natural reservoir system.

The importance of WHIBs to IAV dynamics has yet to be thoroughly explored. In 2010, SCWDS failed to isolate any IAVs from 60 samples collected from WHIBs in March of that year. However, infection rates approach 0% in Anseriformes outside of the fall months and additional evidence is needed to make an assessment regarding WHIBs (Stallknecht *et al.* 1990, Olsen *et al.* 2006). Furthermore, around that same time, SCWDS tested approximately 10 -20 WHIB serum samples collected in Florida and detected antibodies to numerous HA subtypes. Further research investigating the importance of WHIBs was justified.

CONCLUSIONS

In summary, these projects will serve to expand our understanding of the drivers of IAV dynamics in the natural reservoir system. In the first project, we assessed the importance of population immunity as a driver of infection rates and dominant hemagglutinin IAV subtypes at Delaware Bay. The second project focused on the

susceptibility of two species that may serve as sources of IAV at Delaware Bay. In the final project, we consider what constitutes the notion of the IAV “natural reservoir system” and explore the fringes of this idea by looking to an unstudied candidate, the White Ibis.

REFERENCES

Abbas AK, Lichtman AH, Pillai S. 2015. Major histocompatibility complex molecules and antigen presentation to T lymphocytes. In: Cellular and Molecular Immunology. Elsevier Saunders. Philadelphia, PA. 107-135.

Arnal A, Vittecoq M, Pearce-Duvet J, Gauthier-Clerc M, Boulinier T, Jourdain E. 2014. Laridae: a neglected reservoir that could play a major role in avian influenza virus epidemiological dynamics. *Crit Rev Microbiol* 41:508-519.

Arsne DM, Ip HS, Owen JC. 2011. Influence of body condition on influenza a virus infection in mallard ducks: experimental infection data. PLoS ONE 6:e22633.

Bahl AK, Pomeroy BS. 1977. Experimental exposure of Franklins' gulls (*Larvus pipixcan*) and Mallards (*Anas platrhynchos*) to a turkey influenza a virus A/Turkey/Minn?BF/72 (Hav6Neq2). Journal of Wildlife Diseases 13:420-426.

Baker A, Gonzalez P, Morrison RIG, Harrington BA. 2013. Red knot (*Calidris canutus*). In: The Birds of North America. PG Rodewell, Ed. Ithica: Cornell Lab of Ornithology; Retrieved from the Birds of North America: https://birdsna.org/Species-Account/bna/species/redkno DOI: 10.2173/bna.563 Accessed March 2017.

Barber MRW, Aldridge JR, Webster RG, Magor KE. 2010. Association of RIG-I with innate immunity of ducks to influenza. PNAS 107:5913-5918.

Barbey-Martin C, Gigant B, Bizebard T, Calder LJ, Wharton SA, Skehel JJ, KNossow M. 2002. An antibody that prevents the hemagglutinin low pH fusogenic transition. Virology 294:70-74.

Blanchfield K, Kamal RP, Tzeng WP, Music N, Wilson JR, Stevens J, Lipatov AS, Katz JM, York IA. 2014. Recombinant influenza H7 hemagglutinins induce lower neutralizing antibody titers in mice than do seasonal hemagglutinins. *Influenza Other Respir Viruses* 8:628-635.

Brown JD, Stallknecht DE, Beck JR, Suarez DL, Swayne DE. 2006. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. Emerging Infectious Diseases 12:1663-1670.

Brown JD, Stallknecht DE, Berghaus RD, Luttrell MP, Velek K, Kistler W, Costa T, Yabsley MJ, Swayne D. 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clinical and Vaccine Immunology. 16:824-829.

Brown JD, Luttrell MP, Berghaus RD, Kistler W, Keeler SP, Howey A, Wilcox B, Hall J, Niles L, Dey A, Knutsen G, Fritz K, Stallknecht DE. 2010. Prevalence of antibodies to type a influenza virus in wild avian species using two serologic assays. *J Wildl Dis* 46:896-911.

Brown J, Poulson R, Carter D, Lebarbenchon C, Pantin-Jackwood M, Spackman E, Shepherd E, Killian M, Stallknecht D. 2012. Susceptibility of avian species to North American H13 low pathogenic avian influenza viruses. Avian Diseases 56:969-975.

Buehler DM, Tieleman BI, Piersma T. 2010. How do migratory species stay healthy over the annual cycle? A conceptual model for immune function and for resistance to disease. Integrative and Comparative Biology 50:346-357.

Caron A, Cappelle J, Gaidet N. 2017. Challenging the conceptual framework of maintenance hosts for influenza A viruses in wild birds. *Journal of Applied Ecology* 54:681-690.

Cauldwell AV, Long JS, Moncorge O, Barclay WS. 2014. Viral determinants of influenza A virus host rage. Journal of General Virology 95:1193-1210.

Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wildey DC. 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. 95:409-417.

Clark KE, Niles LJ, Burger J. 1993. Abundance and distribution of migrant shorebirds in Delaware Bay. *Condor* 95:694-705.

Costa TP, Brown JD, Howerth EW, Stallknecht DE. 2010. Effect of a prior exposure of a low pathogenic avian influenza virus in the outcome of a heterosubtypic low pathogenic avian influenza infection in Mallards (*Anas platyrhynchos*). Avian Diseases 54:1286-1291

Costa TP, Brown JD, Howerth EW, Stallknecht DE. 2011. Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. *Avian Pathology* 40:119-124.

Curran JM, Robertson ID, Ellis TM, Selleck PW, O'Dea MA. 2013. Variation in the responses of wild species of duck, gull, and wader to inoculation with a wild-bird-origin H6N2 low pathogenicity avian influenza virus. Avian Diseases 57: 581-586.

D'Amico V, Bertellotti M, Baker AJ, Diaz LA. 2007. Exposure of red knots (Calidris canutus rufa) to select avian pathogens; Patagonia, Argentina. J Wildl Dis 43:794-797.

De Araugo J, de Azevedo Junior SM, Gaidet N, Hurtado RF, Walker D, Thomazelli LM, Ometto T, Seixas MMM, Rodrigues R, Galindo DB, da Silva ACS, Rodrigues AMM, Bomfim LL, Mota MM, Larrazabal ME, Branco JO, Serafini P, Neto IS, Franks J, Webby RJ, Webster RG, Durigon EL. 2014. Avian influenza virus (H11N9) in migrating shorebirds wintering in the Amazon region, Brazil. PLoS One 9(10): e110141. Doi:10.1371/journal.pone.0110141

Fereidouni SR, Grund C, Hauslaigner R, Lange E, Wilking H, Harder TC, Beer M, Starick E. 2010. Dynamics of specific antibody responses induced in mallards after infection by or immunization with low pathogenicity avian influenza viruses. Avian Dis 54:79-85.

Fereidouni SR, Starick E, Beer M, Wilking H, Kalthoff D, Grund C, Hauslaigner R, Breithaupt A, Lange E, Harder TC. 2009. Highly pathogenic avian influenza virus infection of mallards with homo and heterosubtypic immunity induced by low pathogenic avian influenza viruses. PLoS One 4(8): e6706. Doi:10.1371/journal.pone.006706.

Ferreira HL, Vangeluwe D, Borm SV, Poncin O, Dumont N, Ozhelvaci O, Munir M, van den Berg T, Lambrecht B. 2015. Differential viral fitness between H1N1 and H3N8 avian influenza viruses isolated from mallards (*Anas platyrhynchos*). Avian Diseases 59:498-507.

Franca M, Stallknecht DE, Howerth EW. 2013. Expression and distribution of sialic acid influenza virus receptors in wild birds. *Avian Path*. 42:60-71.

Franca MS, Brown JD. 2014. Influenza pathobiology and pathogenesis in avian species. *Current Topics in Microbiology and Immunology.* 385:221-242.

Frederick PC, Bildstein KL, Fleury B, Ogden J. 1996. Conservation of large, nomadic populations of white ibises (*Eudocimus albus*) in the United States. Conservation Biology. 10:203-216.

Gambaryan A, Yamnikova S, Lvov D, Tuzikov A, Chinarev A, Pazynina G, Webster R, Matrosovich M, Bovin N. 2005. Receptor specificity of influenza viruses from birds and mammals: New data on involvement of the inner fragments of the carbohydrate chain. *Virology* 334:276-283.

Gambaryan AS, Matrosovich TY, Boravleva EY, Lomakina NF, Yamnikova SS, Tuzikov AB, Pazynina GV, Bovin NV, Fouchier RAM, Klenk HD, Matrosovich MN. 2018. Receptor-binding properties of influenza viruses isolated from gulls. *Virology* 522:37-45.

Guinn K, Fojtik A, Davis-Fields N, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2016. Antibodies to influenza A viruses in gulls at Delaware Bay, USA. Avian Dis 60:341-345.

Gulyaeva MA, Sharshov KA, Zaykovskaia AV, Shestopalova LV, Shestopalov AM. 2016. Experimental infection and pathology of clade 2.2 H5N1 virus in gulls. Journal of Veterinary Science. 17:179-188.

Guo L, Zhang X, Ren L, Yu X, Chen L, Zhou H, Gao X, Teng Z, Li J, Hu J, Wu C, Xiao X, Zhu Y, Wang Q, Pang X, Jin Q, Wu F, Wang J. 2014. Human antibody

response to avian influenza A (H7N9) virus, 2013. *Emerging Infect Dis* 20:192-200.

Hall JS, Krauss S, Franson JC, TeSlaa JL, Nashold SW, Stallknecht DE, Webby RJ, Webster RG. 2013. Avian influenza in shorebirds: experimental infection of ruddy turnstones (Arenaria interpres) with avian influenza virus. Influenza Other Respir Viruses 7:85-92.

Hall JS, Hallgrimsson GT, Suwannanarn K, Sreevatsen S, Ip HS, Magnusdottir E, TeSlaa JL, Nashold SW, Dusek RJ. 2014. Avian influenza virus ecology in Iceland shorebirds: intercontinental reassortment and movement. Infection, Genetics and Evolution 28:130-136.

Hall JS, Russell RE, Franson JC, Soos C, Dusek RJ, Allen RB, Nashold SW, TeSlaa JL, Jonsson JE, Ballard JR, Harms NJ, Brown JD. 2015. Avian influenza ecology in North Atlantic sea ducks: not all ducks are created equal. 10(12) e0144524.

Hanson BA, Luttrell MP, Goekjian VH, Niles L, Swayne DE, Senne DA, Stallknecht DE. 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? J Wildl Dis 44:351-361.

Hanson BA, Stallknecht DE, Swayne DE, Lewis LA, Senne DA. 2003. Avian influenza viruses in Minnesota ducks during 1998-2000. Avian Diseases 47:P867-871.

Haydon DT, Cleaveland S, Taylor LH, Laurenson MK. 2002. Identifying reservoirs of infection: a conceptual and practical challenge. Emerging Infectious Diseases 8:1468-1473.

Heath JA, Frederick PC, Kushlan JA, Bildstein KL. 2009. White Ibis (*Eudocimus albus*), version 2.0. In The birds of North America (P.G. Rodewald, editor). Cornell Lab of Ornithology, Ithaca, New York, USA

Hernandez SM, Wlech CN, Peters VE, Lipp EK, Curry S *et al.* 1996. Urbanized white ibises (*Eudocimus albus*) as carriers of *Salmonella enterica* of significance to public health and wildlife. PLoS ONE 11:e0164402.

Hicklin P, Gratto-Trevor CL. 2010. Semipalmated Sandpiper (Calidris pusilla), In: The Birds of North America P. G. Rodewald, Ed. Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America: https://birdsna.org/Species-Account/bna/species/semsan DOI: 10.2173/bna.6 Accessed March 2017.

Hinshaw VS, Wood JM, Webster RG, Deibel R, Turner B. 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. *Bulletin of the World Health Organization* 63: 711-719.

Johnson JA, DeCicco LH, Ruthrauff DR, Krauss S, Hall JS. 2014. Avian influenza virus antibodies in pacific coast red knots (*Calidris canutus roselaari*). J Wildl Dis 50:671-675.

Jourdain E, Gunnarsson G, Wahlgren J, Latorre-Margalef N, Brojer C, Sahlin S, vensson L, Waldenstrom J, Lundkvist A, Olsen B. 2010. Influenza virus in a natural host, the mallard: experimental infection data. PLoS One 5:e8935.

Kawaoka Y, Chambers TM, Sladen WL, Webster RG. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163:247-250.

Kistler WM, Stallknecht DE, Lebarbenchon C, Pedersen K, Marks dR, Mickley R, DeeLiberto TJ, Yabsley MJ. 2015. Influenza A virus H5-specific antibodies in mute swans (*Cygnus olor*) in the USA. Journal of Wildlife Diseases 51:523-526.

Knossow M, Guadier M, Douglas A, Barrere B, Bizebard T, Barbey C, Gigant B, Skehelt JJ. 2002. Mechanism of neutralization of influenza virus infectivity by antibodies. Virology 302: 294-298.

Krauss S, Obert CA, Franks J, Walker D, Jones K, *et al.* 2007. Influenza in migratory birds and evidence of limited intercontinental virus exchange. PLoS Pathogens 3(11):e167

Krauss S, Stallknecht DE, Negovetich NJ, Niles LJ, Webby RJ, Webster RG. 2010. Coincident ruddy turnstone migration and horseshoe crab spawning creates an ecological 'hot spot' for influenza viruses. *P Roy Soc Lond B Biol Sci* 277:3373-3379.

Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. 2004. Influenza A viruses of migrating wild aquatic birds in North America. *Vector-borne and Zoonotic Dis* 4:177-189.

Latorre-Margalef N, Brown JD, Fojtik A, Poulson RL, Carter D, Franca M, Stallknecht DE. 2017. Competition between influenza A virus subtypes through heterosubtypic immunity modulates re-infection and antibody dynamics in the mallard duck. PLoS Pathog. 13:e1006419.

Latorre-Margalef N, Grosbois V, Wahlgren J, Munster VJ, Tolf C, Fouchier RAM, Osterhaus ADME, Olsen B, Waldenstrom J. 2013. Heterosubtypic immunity to

influenza A virus infections in mallards may explain existence of multiple virus subtypes. PLoS Pathog 9(6): e1003443. Doi:10.1371/journal.ppat.1003443.

Latorre-Margalef N, Gunnarsson G, Munster VJ, *et al.* 2009. Effects of influenza a virus infection on migrating mallard ducks. Proc. R. Soc. B 276: 1029-1036.

Macwhirther RB, Austin-Smith P, Kroodsma DE. 2002. Sanderling (*Calidris alba*). In: The Birds of North America. PG Rodewell, Ed. Ithica: Cornell Lab of Ornithology; Retrieved from the birds of North America: https://birdsna.org/Species-Account/bna/species/sander DOI: 10.2173/bna.653 Accessed March 2017.

Magor KE. 2011. Immunoglobulin genetics and antibody responses to influenza in ducks. Developmental and Comparative Immunology 35:1008-1017.

Maxted AG, Luttrell MP, Goekjian VH, Brown JD, Niles LJ, Dey AD, Kalasz KS, Swayne DE, Stallknecht DE. 2012a. Avian influenza virus infection dynamics in shorebird hosts. *J Wildl Dis* 48:322-334.

Maxted AG, Porter RR, Luttrell MP, Goekjian VH, Dey AD, Kalasz KS, Niles LJ, Stallknecht DE. 2012b. Annual survival of ruddy turnstones is not affected by natural infection with low pathogenicity avian influenza viruses. *Avian Diseases* 56:567-573.

Maxted AM, Sitters HP, Luttrell MP, Dey AD, Kalasz KS, Niles LJ, Stallknecht DE. 2016. Spring migration stopover ecology of avian influenza virus shorebird hosts at Delaware Bay. *Avian Dis* 60:394-405.

Munster VJ, Baas C, Lexmond P, et al. 2007. Spatial, temporal and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS 3: e61. Doi:10.1371/journal.ppat.0030061.

Nallar R, Papp Z, Epp T, et al. 2015. Demographic and spatiotemporal patterns of avian influenza infection at the continental scale, and in relation to annual life cycle of a migratory host. PLoS ONE 10(6): e0130662. Doi:10.1371/journal.pone.0130662

Nichols TC, Jones OE. 2015. Population trends of breeding waterfowl in New Jersey, 1993-2012. Bull. N.M. Acad. Sci. 60:7-13.

Nettleship, DN. 2000. Ruddy turnstone (*Arenaria interpres*), In: The Birds of North America. PG Rodewald, Ed. Ithica: Cornell Lab of Ornithology; Retrieved from the Birds of North America: https://birdsna.org/Species-Account/bna/species/rudtur DOI: 10.2173/bna.537 Accessed March 2017.

Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus ADME, Fouchier RAM. 2006. Global patterns of influenza A virus in wild birds. *Science* 312:384-388.

Palese P, Shaw ML. 2007 Orthomyxoviridae: the viruses and their replication. In: *Fields Virology,* Knipe DM and Howley PM Editors. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania. Pp. 1647-1689.

Papp Z, Clark RG, Parmley EJ, Leighton FA, Waldner C, Soos C. 2017. The ecology of avian influenza viruses in wild dabbling ducks (*Anas* spp.) in Canada. PLoS One 12:e0176297.

Pepin KM, VanDalen KK, Mooers NL, Ellis JW, Sullivan HJ, Root JJ, Webb CT, Fraklin AB, Shriner SA. 2012. Quantification of heterosubtypic immunity between avian influenza subtypes H3N8 and H4N6 in multiple avian host species. *Journal of General Virology* 93:2575-2583.

Perkins LEL and Swayne DE. 2002. Susceptibility of laughing gulls (*Larus atricilla*) to H5N1 and H5N3 highly pathogenic avian influenza viruses. *Avian Diseases* 46:877-885.

Poulson RL, Luttrell PM, Slusher MJ, Wilcox BR, Niles LJ, Dey AD, Berghaus RD, Krauss S, Webster RG, Stallknecht DE. 2017. Influenza A virus: sampling of the unique shorebird habitat at Delaware Bay, USA. Royal Soc. Open sci. 4:171420.

Puzelli S, Di Trani L, Fabiani C, Compitelli L, De Marco MA, Capua I, Aguilera JF, Zombon M, Donatelli I. 2005. Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. *J Infect Dis* 192:1318-1322.

Ramey AM, Poulson RL, Gonzalez-Reiche AS, et al. 2014. Evidence for seasonal patterns in the relative abundance of avian influenza virus subtypes in blue-winged teal (*Anas discors*). Journal of Wildlife Diseases 50: 916-922.

Ramey AM, DeLiberto TJ, Berhane Y, Swayne DE, Stallknecht DE. 2018. Lessons learned from research and surveillance directed at highly pathogenic influenza A viruses in wild birds inhabiting North America. Virology. 518:55-63.

Robinson RA, Atkinson PW, Clark NA. 2003. Arrival and weight gain of red knot *Calidris canutus,* ruddy turnstone *Arenaria interpres* and sanderling *Calidris alba* staging in Delaware Bay in spring. British Trust for Ornithology Research Report No. 307.

Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127:361-373.

Samuel MD, Hall JS, Brown JD, Goldberg DR, Ip H, Baranyuk VV. 2015. The dynamics of avian influenza in lesser snow geese: implications for annual and migratory infection patterns. 25:1851-1859.

Segovia KM, Stallknecht DE, Kapczynski DR, Stabler L, Berghaus RD, Fotjik A, Latorre-Margalef N, Franca MS. 2017. Adaptive heterosubtypic immunity to low pathogenic avian influenza viruses in experimentally infected mallards. PLoS ONE 12(1): e0170335

Segovia KM, Franca MS, Leyson CL, Kapczynski DR, Chrzastek K, Bahnson CS, Stallknecht DE. 2018. Heterosubtypic immunity increases infectious dose required to infect Mallard ducks with Influenza A virus. PLoS ONE 13(4): e0196394

Seo SH, Peiris M, Webster RG. 2002. Protective cross-reactive cellular immunity to lethal A/Goose/Guangong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD*+ T cells expressing gamma interferon. Journal of Virology. 76:4886-4890.

Seo SH, Webster RG. 2001. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. Journal of Virology 75:2516-2525.

Sharp GB, Kawaoka Y, Jones DJ, Bean WJ, Pryor SP, Hinshaw V, Webster R. 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. Journal of Virology 71:6128-6135.

Sharp GB, Kawaoka Y, Wright SM, Turner B, Hinshaw V, Webster RG. 1993. Wild ducks are the reservoir for only a limited number of influenza A subtypes. Epidemiol. Infect. 110:161-176.

Sims LD, Brown IH. 2017. Multi-continental panzootic of H5 highly pathogenic avian influenza (1996-2015). In: *Animal Influenza,* Swayne DE, editor. John Wiley & Sons, Ames Iowa 2nd Edition John Wiley & Sons, Ames, Iowa. Pp. 202-247.

Skowronski DM, Li Y, Tweed SA, Tam TWS, Petric M, David ST, Marra F, Bastien N, Lee SW, Krajden M, Brunham RC. 2007. Protective measures and human antibody response during an avian influenza H7N3 outbreak in poultry in British Columbia, Canada. *Can Med Assoc J* 176:47-53.

Spackman E, Stallknecht DE, Slemons RD, Winker K, Suarez DL, Scott M, Swayne DE. 2005. Phylogenetic analysis of type A influenza genes in natural reservoir species in North America reveals genetic variation. Virus Research 114: 89-100.

Stallknecht DE, Brown JD. 2017. Wild bird infections and the ecology of avian influenza viruses In: *Animal Influenza,* Swayne DE, editor. John Wiley & Sons, Ames Iowa 2nd Edition John Wiley & Sons, Ames, Iowa. Pp. 153-176.

Stallknecht DE, Goekjian VH, Wilcox BR, Poulson RL, Brown JD. 2010. Avian influenza virus in aquatic habitats: what do we need to learn? Avian Diseases 54:461-465.

Stallknecht DE, Luttrell MP, Poulson R, Goekjian V, Niles L, Dey A, Krauss S, Webster RG. 2012. Detection of avian influenza viruses from shorebirds: evaluation of surveillance and testing approaches. *J Wildl Dis* 48:382-393.

Stallknecht DE, Shane SM, Swank PJ, Senne DA, Kearney MT. 1990. Avian influenza viruses from migratory and resident ducks of coastal Louisiana. Avian Diseases 34: 398-405.

Suarez DL 2017 Influenza A Virus In: *Animal Influenza,* Swayne DE, editor. John Wiley & Sons, Ames Iowa 2nd Edition John Wiley & Sons, Ames, Iowa. Pp. 3-30.

Swayne DE, Slemons RD. 2008. Using mean infectious dose of high- and low-pathogenicity avian influenza viruses originating from wild duck and poultry as one measure of infectivity and adaptation to poultry. Avian Diseases 52:455-460.

Swayne D, Brown I. 2012 Avian influenza (Infection with Avian Influenza Viruses) In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* 7th Edition OIE

Tong S, Li Y, Rivailler P *et al.* 2012. A distinct lineage of influenza A virus from bats. PNAS 109: 4269-4274.

Tong S, Zhu X, Li Y. *et al.* 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog 9: e1003657. Doi:10.1371/journal.ppat.1003657.

Van Gils JA, Munster VJ, Radersma R, Liefhebber D, Fouchier RAM, Klaassen M. 2007. Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. PLoS ONE 2(1). Doi:10.1371/journal.pone.0000184

Velarde R, Calvin SE, Ojkic D, Barker IK, Nagy E. 2010. Avian influenza virus H13 circulating in ring-billed gulls (*Larus delawarensis*) in Southern Ontario, Canada. Avian Diseases 54:411-419.

Verhagen JH, Hofle U, van Amerognen G, van de Bildt M, Majoor F, Fouchier RAM, Kuiken T. 2015. Long-term effect of serial infections with H13 and H16 low-

pathogenic avian influenza viruses in black-headed gulls. Journal of Virology 89:11507-11522.

Verhagen JH, Majoor F, Lexmond P, Vuolng O, Kasemir G, Lutterop D, Osterhaus ADME, Fouchier RAM, Kuiken T. 2014. Epidemiology of influenza a virus among black headed gulls, the Netherlands 2006-2010. Emerging Infectious Diseases 20:138-141.

Vervelde L, Kapczynski DR. 2016. The innate and adaptive immune response to avian influenza virus. In: *Animal Influenza*. Swayne DE Ed. John Wiley & Sons, Inc. Ames, IA. 135-152.

Viana M, Mancy R, Biek R, Cleaveland S, Cross PC, Lloyd-Smith JO, Haydon DT. 2014. Assembling evidence for identifying reservoirs of infection. *Trends in Ecology and Evolution.* 29:270-279.

Wallensten A, Munster VJ, Latorre-Margalef N, *et al.* 2007. Surveillance of influenza a virus in migratory waterfowl in northern Europe. Emerging Infection Diseases 13:404-411.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza a viruses. Microbiological Reviews 56:152-179.

Wilcox BR, Knutsen GA, Berdeen J, Goekjian V, Poulson R, Goyal S, Sreevatsan S, Cardona C, Berghaus RD, Swayne DE, Yabsley MJ, Stallknecht DE. 2011. Influenza-A viruses in ducks in northwestern Minnesota: fine scale spatial and temporal variation in prevalence and subtype diversity. PLoS One 6:e24010

Winker K, Spackman E, Swayne DE. 2008. Rarity of influenza A virus in spring shorebirds, Southern Alaska. Emerging Infectious Diseases 14:1314-1316.

Wong JK, Wilcox BR, Fojtik A, Poulson RL, Stallknecht DE. 2016. Antibodies to influenza A viruses in wintering snow geese (*Chen caerulescens*) in Texas. *Avian Diseases* 60:337-340.

Wright PF, Neumann G, Kawaoka Y. 2007 Orthomyxoviruses. . In: *Fields Virology,* Knipe DM and Howley PM Editors. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania. Pp. 1647-1689.

Xiong X, McCauley JW, Steinhauer DA. 2014. Receptor binding properties of the influenza virus hemagglutinin as a determinant of host range. Current Topics in Microbiology and Immunology 385:63-91.

Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z, He S Kumar S, Larsen CN, Lee AJ, *et. al.* 2017. Influenza research database: an integrated bioinformatics resource for influenza virus research. *Nucleic Acids Res.* 45:D466-474.

CHAPTER 2

NEUTRALIZING ANTIBODIES TO TYPE A INFLUENZA VIRUSES IN SHOREBIRDS AT DELAWARE BAY, NEW JERSEY[1]

[1]Bahnson CS, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2018. *Journal of Wildlife Diseases* 54:708-715. Reprinted here with permission of publisher.

ABSTRACT

Influenza A virus (IAV) infections in shorebirds at Delaware Bay, New Jersey, US, have historically included avian hemagglutinin (HA) subtypes H1-13, and H16. In a given year, infections are characterized by a limited number of HA and neuraminidase subtypes and a dominant HA subtype that often represents >50% of all isolates. Predominant HA subtypes shift between consecutive years. In addition, infection prevalence is consistently higher in ruddy turnstones (*Arenaria interpres morinella,* RUTU) compared to red knots (*Calidris canutus rufa,* REKN) despite comparable rates of exposure. To investigate a potential immunological basis for this phenomenon, a virus microneutralization (MN) assay was used to detect subtype-specific, neutralizing antibodies to H1-H12 in sera collected from RUTUs from 2012-2016 and REKNs in 2012, 2013, and 2016. Neutralizing antibodies to one or more subtypes were detected in 36% (222/611) of RUTUs. Prevalence of antibodies to subtypes H6 and H11 remained high throughout the study and these virus subtypes were isolated every year, suggesting a continual source of exposure. Antibody prevalence was intermediate for most IAV subtypes that were isolated in two to three out of 5 yr (H1, H3, H5, H9, H10, and H12) but was low for H7 viruses, despite the isolation of this virus subtype in three of 5 yr. This suggests a reduced antigenicity of H7 IAVs compared to other subtypes. Antibody prevalence was low for H4 virus that was isolated once and H2 and H8 viruses that were never isolated. Neutralizing antibodies were detected in 66% (169/257) of REKNs and subtype specific antibody prevalences were higher in REKNs than RUTUs with few exceptions. The results suggest that population immunity influences which

species is infected at Delaware Bay, indicate that IAV dynamics are subtype-dependent, and demonstrate the utility of the MN assay as a supportive tool for field research.

INTRODUCTION

The natural reservoirs of influenza A virus (IAV) are birds of the orders Anseriformes (ducks and geese) and Charadriiformes (gulls and shorebirds; Webster et al. 1992; Krauss et al. 2004; Olsen et al. 2006). While IAV is routinely isolated from ducks, geese, and gull populations throughout the world, this virus is consistently isolated from shorebirds only at Delaware Bay (DB) in the northeastern United States during spring migration when shorebirds stop to refuel on eggs of spawning horseshoe crabs (Hanson et al. 2008; Krauss et al. 2010).

Ruddy turnstones (*Arenaria interpres morinella,* RUTUs), red knots (*Calidris canutus rufa,* REKNs), semipalmated sandpipers (*Calidris pusilla*), and sanderlings (*Calidris alba*) are the predominant species of shorebirds using beach habitats at DB (Clark et al. 1993). Of these, RUTUs appear to be most important in this local IAV system. Infection rates in RUTUs average 12-14%, the highest of any species at DB (Krauss et al. 2004; Maxted et al. 2012). In contrast, IAV infection rates in any of the other shorebird species feeding alongside RUTUs at DB is less than 2% (Hanson et al. 2008; Maxted et al. 2012). Although IAV hemagglutinin (HA) subtypes H1-H13 and H16 have been variously isolated from RUTUs at DB, annual IAV infections are typically dominated by a single HA subtype that reoccurs in periodic, erratic cycles (Krauss et al. 2004; Stallknecht et al. 2012). Subtype diversity in a given year does not differ between RUTUs and REKNs and, in most years, all of the IAV subtype diversity present at DB can be detected in the RUTU population (Stallknecht et al. 2012).

This shifting infection pattern may have an immunologic basis. Natural and experimental studies in Mallards (*Anas platyrhynchos*) have suggested that homo- and hetero-subtypic immunity induced through infection with IAV may drive population infection dynamics (Fereidouni et al. 2010; Latorre-Margalef et al. 2013; Segovia et al. 2017). The immune response to single or multiple IAV infections is not as well understood in shorebirds. Ruddy turnstones and REKNs are relatively long lived and individuals remain at wintering grounds for their first year (Nettleship 2000; Baker et al. 2013). As a result, the population of shorebirds at DB consists of older individuals that are likely to have been previously exposed to one or more IAV subtypes.

Previous serologic investigations at DB have reported that the prevalences of IAV antibodies in RUTUs and REKNs are comparable. Antibodies to the IAV nucleoprotein (NP) have been detected in 55-65% and 54-86% of RUTUs and REKNs at DB, respectively (Brown et al. 2010; Maxted et al. 2012; Stallknecht et al. 2012). Exposure and seroconversion do not appear to occur at the same time for both species, however. By using date and body weight as a proxy for time spent at DB, Maxted et al. (2012) determined that IAV antibody prevalence in RUTUs increased from less than 40% at arrival to over 95% at departure, implying that over half the population seroconverts while at DB. In contrast, the antibody prevalence in REKNs was 82% at arrival and slowly declined throughout the remainder of the season, a period that lasts from mid-May to early June. This provides valuable insight into species-specific IAV exposures within the DB system, but because NP is conserved between IAV subtypes, these antibody patterns are of limited use when considering virus-host interactions at a subtype-specific level. In this study, we sought to broaden our understanding of IAV

infection dynamics at DB by employing a microneutralization (MN) assay to detect HA-specific neutralizing antibodies.

We hypothesized that subtype specific antibody patterns, as determined by MN, could be used to explain annually shifting IAV infection patterns. Our objectives were to: 1) compare IAV antibody patterns in RUTUs to REKNs; 2) determine if annual subtype-specific IAV antibody patterns in RUTUs reflect the observed IAV subtype diversity at DB; and 3) demonstrate the utility of the MN assay as a supportive tool for field research.

MATERIALS AND METHODS

Sample Collection

Fieldwork was conducted during 16-29 May 2012, 15-29 May 2013, 14-27 May 2014, 12-28 May 2015, and 16-22 May 2016, at DB, New Jersey under federal scientific collection permit number MB779238 and New Jersey scientific collection permit numbers 2012029, 2013037, 2014057, 2015003, and 2016006. Ruddy turnstones and REKNs were captured with cannon nets as part of a long-term population study. Blood samples were collected by jugular venipuncture at a total volume less than or equal to 1% of the bird's body mass. Blood samples were not collected from REKNs in 2014 and 2015. Samples were kept on ice until they could be centrifuged the same day of collection. The serum fraction was aliquoted and stored at -20 C until testing by blocking enzyme-linked immunosorbent assay (bELISA), and then stored at -20 C until testing by MN. Research was approved by the University of Georgia Animal Care and Use Committee AUP numbers A2010 06-101, A2013 05-021, and A2016 05-020. The number of serum samples tested was: 128 RUTUs and 102 REKNs in 2012; 116

RUTUs and 104 REKNs in 2013; 115 RUTUs in 2014; 112 RUTUs in 2015; and 140 RUTUs and 49 REKNs in 2016.

bELISA

All sera were tested by bELISA (IDEXX Laboratories, Westbrook, Maine, USA) for antibodies to the NP (Brown et al. 2010). Sera were considered positive if the serum-sample-to-negative-control absorbance value was less than 0.50. The negative control was provided by the manufacturer and consisted of dilute chicken (*Gallus gallus domesticus*) serum that was not reactive to IAV.

Microneutralization

Serum samples were tested for antibodies against H1-H12 by virus MN (Wong et al. 2016), with the exception that serum and antigen were allowed to incubate at room temperature for 1.5 h rather than 2 h. Viruses used as antigens included A/mallard/NJ/AI12-4823/2012 (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-00796/2008 (H6N1), A/mallard/MN/AI08-3770/2009 (H7N9), A/mallard/MN/SG-01048/2008 (H8N4), A/RUTU/DE/AI11-809/2011 (H9N2), A/mallard/MN/SG-00999/2008 (H10N7), A/mallard/MN/SG-00930/2008 (H11N9), and A/mallard/MN/AI07-3285/2007 (H12N5). Subtypes H13 and H16 were not included in testing because adequate viral titers could not be achieved in Madin-Darby canine kidney cells (American Type Culture Collection, Manassas, Virginia, USA) through conventional methods and neither has been a dominant IAV subtype in RUTUs in previous years (Stallknecht et al. 2012).

Virus Isolation

Virus isolation data were provided by St. Jude Children's Research Hospital (SJCRH) and the University of Georgia (UGA). Samples were collected at DB in May 2012-2016 and were tested for IAVs by virus isolation (Stallknecht et al. 2012). The HA of each isolated IAV was determined by hemagglutination inhibition (Hanson et al. 2008). Samples tested by SJCRH consisted of shorebird fecal swabs collected on the beaches of DB and totaled 610 in 2012, 600 in 2013, 600 in 2014, 600 in 2015, and 672 in 2016. Samples tested by UGA consisted of oropharyngeal and cloacal swabs collected from RUTUs at the time of capture, as well as RUTU fecal swabs collected from the beaches of DB. The number of samples collected and tested each year by UGA was 1087 in 2012, 1002 in 2013, 823 in 2014, 978 in 2015, and 697 in 2016. The prevalence of IAVs was calculated from the UGA dataset. The proportion of H1-H12 viral isolates was calculated from the combined datasets of SJCRH and UGA.

Statistical Analysis

The mean prevalences of antibodies and IAVs were calculated for 2012-2016 and 2012, 2013, and 2016 by dividing the total number of positive samples from that time period by the number of samples tested. Ninety-five percent confidence intervals for prevalences were calculated using the Wilson method. A Fisher's exact test was used to compare the prevalences of neutralizing antibodies to one or more HA subtypes, neutralizing antibodies to two or more HA subtypes, and antibodies to NP between RUTUs and REKNs. Mean, subtype-specific prevalences were organized into three class intervals designated "highest," "intermediate," and "lowest." based on the range of prevalences recorded for each species. Comparisons were made between

species for individual years (2012, 2013, 2016) when sera were available for both species as well as the mean prevalence for these three years. A Fisher's exact test was also used to compare the annual change in prevalence of neutralizing antibodies for each HA subtype in RUTUs. The difference between antibody prevalence was considered significant if $P<0.05$. The proportion of viral isolates in a given year was calculated by dividing the number of IAVs of each HA isolated by the total number of H1-H12 IAVs isolated that year. Calculations were performed in Stata 13.1 (StataCorp LP, College Station, Texas, USA).

RESULTS

The prevalence of IAVs in RUTU samples at DB from 2012-2016 was 14% (642/4587; 95% CI: 13-15%) and ranged from 9% in 2014 (74/823; 95% CI: 7-11%) to 20% in 2016 (139/697; 95% CI: 17-23%, Table 2.1). The mean prevalence of antibodies to the NP, as determined by bELISA, in 2012, 2013, and 2016, for RUTUs and REKNs was 62% (238/383; 95% CI: 57-67%) and 69% (172/251; 95% CI: 63-74%), respectively, and was not statistically different between species ($P=0.107$). Neutralizing antibodies to one or more subtype of IAV were detected by MN in 36% (222/611; 95% CI: 33-40%) of 611 RUTUs sampled from 2012-2016 and in 66% (169/257; 95% CI: 60-72%) of 255 REKNs sampled in 2012, 2013, and 2016. For the same years, neutralizing antibodies to two or more subtypes were detected by MN in 18% (108/611; 95% CI: 15-21%) of RUTUs and 49% (126/257; 95% CI: 43-55%) of REKNs. The mean MN prevalence across 2012, 2013, and 2016 was significantly different by species for both ≥1 and ≥2 subtypes ($P<0.001$).

Over the five years surveyed, HA-specific antibodies to H1-12 were detected in RUTUs with H6, H9, and H11 detected in the highest prevalence (12, 11, 10%, respectively), followed by H1, H3, H5, H10, and H12 at an intermediate prevalence (6-8%), and H2, H4, H7, and H8 at the lowest prevalence (less than 2%). Prevalence varied by year and subtype with the highest single-year prevalence of seropositive RUTUs recorded in 2016 for H1 (19%, Fig.2.1), while no antibodies were detected for H4 or H8 in multiple years.

The degree to which annual RUTU serologic results corresponded to IAV isolation data varied by IAV subtype (Fig. 2.1). While they were never dominant subtypes, H6 and H11 IAVs were isolated every year surveyed. Antibodies to these subtypes, along with H9, were detected at the highest mean rates. Subtypes H1, H10, and H12 were each dominant or codominant IAVs in 2/5 yr of sampling. The annual prevalence of antibodies to these subtypes varied but the mean prevalence over the study period was intermediate compared to other subtypes. Subtypes H3, H5, and H9 IAVs were each isolated in two of five years but these were never dominant subtypes. However; the mean antibody prevalence to these subtypes was also intermediate (H3 and H5), or high (H9) compared to other subtypes. The prevalence of antibodies to H7 fit isolation data the least well. Despite the repeated isolation of H7, which was also the dominant subtype in 2015, antibody levels remained low (less than 2%) throughout the study. One H4 IAV was isolated in 2014 and subtypes H2 and H8 were never isolated. Prevalence of antibodies to these subtypes was also low (mean less than 2%). A significant increase in neutralizing antibodies was detected for H1 from 2015 to 2016 (P=0.003) and corresponded with the detection of H1 IAVs in 2015. The number of

neutralizing antibodies to H3 increased significantly from 2013 to 2014 (*P*=0.006) and viruses of this subtype were isolated in 2014. The prevalence of neutralizing antibodies to H10 increased significantly from 2015 to 2016 (*P*=0.005) and this was a dominant IAV subtype in 2016. A significant decrease in neutralizing antibodies was detected for H9 from 2012 to 2013 (*P*=0.018). Subtype H9 IAVs were detected in 2012, but not again until 2016. No other single-year change in antibody prevalence was found to be significant at a level of 5%.

In the three years that REKNs were surveyed, HA-specific antibodies to H1-H12 were detected with H6, H9, H11, and H1 detected in the highest prevalence (36, 35, 32, 28%). Antibodies to H5, H12, H2, and H10 were detected at an intermediate rate (23, 22, 15, and 15%). The prevalence of antibodies to the H7 was among the lowest (11%), as were the prevalences of H3, H4, and H8 (less than 2%). The highest antibody prevalence recorded for a single year was H9 in 2016 (43%, Fig. 2.1).

Because serological data from 2014 and 2015 are unavailable for REKNs, pairing antibody prevalences in REKNs to virus isolation data is challenging. The subtype specific antibody patterns in REKNs mirrored that described for RUTUs with two exceptions. While an intermediate prevalence of neutralizing antibodies to H3 was detected in RUTUs, only 1% of REKNs had antibodies to H3 despite this subtype being isolated in 2016. In contrast, the prevalence of antibodies to H2 was intermediate in REKNs but low in RUTUs. No H2 IAVs were ever isolated.

For the three years when data were available for both species, the mean antibody prevalence was significantly different by species for every subtype with the exception of H4 (*P*=0.219). For individual years, antibody prevalence was significantly

higher in REKNs for most subtypes and most years (Fig. 2.1). Exceptions include H3, which was significantly higher in RUTUs in 2016 but not different in 2012 or 2013 (P=0.104, P=0.626, respectively); H4, which was not significantly different in 2012 (P=0.262) and wasn't detected in either species in 2013 and 2016; H5, which was not significantly different in 2016 (P=0.090); H8, which was not detected in either species in 2013 and not significantly different in 2016 (P=0.090); and H10, which was not significantly different in 2012 or 2016 (P=0.085, P=0.343, respectively).

DISCUSSION

Of the several shorebird species that feed alongside one another at DB, RUTUs are the only species where IAV is consistently isolated at high rates (Kawaoka et al. 1988; Hanson et al. 2008). Previous authors have suggested this may be attributed to subtle behavioral differences such as feeding strategies or roost-site selection that lead to higher rates of exposure (Hanson et al. 2008; Brown et al. 2010; Maxted et al. 2012). The comparatively high rate of neutralizing antibodies in REKNs versus RUTUs supports our hypothesis that population immunity may be an additional factor in determining which species is infected at DB.

The extent of immunity that develops as a result of infections at DB versus infections prior to arrival is unclear. Both species are long-distance migrants that could potentially be infected at diverse sites and at different times in North and South America. Unfortunately, the limited surveillance efforts in shorebirds and the few IAV detections reported from these species outside of DB provide an inadequate basis to determine where or how population immunity develops or persists in them (Kawaoka et al. 1988; Krauss et al. 2004; Hanson et al. 2008). Regardless, our data suggest that, as

compared to RUTUs, REKNs at DB have antibody profiles that are more robust in neutralizing antibody subtype prevalence and diversity.

We expected to detect a prodigious population of subtype-specific, neutralizing IAV antibodies within the sampled RUTUs that would partially account for the shifting IAV infection patterns. While it is unknown what antibody prevalence would be necessary to drive the shift in annual dominant virus subtype, our data do not seem to sufficiently account for this phenomenon. Nevertheless, they do reveal heretofore uncharacterized patterns that may be related to the combined effects of IAV exposure rates and antibody response. With regard to exposure as determined by the annual IAV isolation results, several patterns were evident: rarely detected viral isolates with low antibody prevalence (H2, H4, H8), occasionally detected viral subtypes with intermediate prevalence (H1, H3, H5, H9, H10, H12), and routinely detected viral isolates with a high antibody prevalence (H6, H11).

The source of the IAVs that contribute to annual infections at DB remains unknown. Some subtypes may be maintained in the population at low prevalence and occasionally emerge as a dominant subtype before receding to low, maintenance levels of infection. This is possible with the majority of subtypes, particularly those within the first two categories: rarely detected viral isolates with intermediate prevalence and occasionally detected viral subtypes with intermediate prevalence. By contrast, many have suggested migrating and resident ducks and gulls serve as a continual source of IAV infection (Hanson et al. 2008; Guinn et al. 2016; Maxted et al. 2016). This is plausible with subtypes that are represented nearly every year in low isolation prevalence and high antibody prevalence (i.e., H6 and H11). In fact, Guinn et al. (2016)

detected the highest prevalence of antibodies to H6 and H11 in gulls at DB, although the directionality of viral transmission cannot be determined.

A final pattern observed was with neutralizing antibodies to H7. Viruses of this subtype were isolated at DB in three of the five years and in 54% of viruses isolated in 2015. Despite this, the annual prevalence of neutralizing antibodies to H7 in RUTUs was never above 2%. In recent years, H7 subtype viruses have received attention within human health research because they appear to generate an antibody response that is either reduced or not detectable by conventional hemagglutination inhibition (HI) or MN assays, despite confirmed or suspected exposures of and infections in humans (Puzelli et al. 2005; Skowronski et al. 2007; Guo et al. 2014). Humanized mice injected with recombinant hemagglutinin from H7 viruses generated an antibody response that was similar by bELISA, but significantly lower as measured by HI and MN compared to mice injected with hemagglutinin from H1 and H3 viruses (Blanchfield et al. 2014). Infections with H7 IAVs in shorebirds at DB may also generate fewer neutralizing antibodies or antibodies that were less detectable by our MN assay as compared to other subtypes.

In a disease system involving long-lived hosts that are exposed to multiple IAVs, the potential exists for a manifestation of ‘original antigenic sin’ (OAS) to be influencing antibody patterns. Under this framework, an imprinting occurs in a host upon first exposure to an IAV and subsequent exposures generate a humoral response more tailored to the original IAV than the challenge IAV (Fazekas de St. Groth and Webster, 1966). Subtle evidence of OAS was recently observed in a Mallard challenge study (Latorre-Margalef et al. 2017). Currently, an effect of OAS on wild bird infection dynamics, including those at DB, is plausible, but its magnitude remains unknown.

Finally, our study demonstrates the importance to field research of assessing subtype-specific antibody patterns. As demonstrated here, the infection status of one species within an IAV system may reveal an incomplete story that is likely obscured by heterosubtypic immunity. For example, viral shedding was abrogated, reduced, or shortened in previously infected Mallards challenged with other subtypes of IAV while antibodies to the challenge IAV were often still detected by MN (Segovia et al. 2017). Extrapolating this to shorebirds at DB validates MN and other serologic tools as a valuable component of IAV surveillance. In this study, it revealed unique patterns related to the potential contribution of specific species and non-dominant viral subtypes to IAV dynamics that would not be captured with infection data alone.

Interpretation of serologic data comes with limitations and is particularly complex in relation to IAV in wild birds where multiple IAV subtypes annually infect numerous avian species. The extent of cross reactive HA antibodies related to repeated infections with different subtypes, the potential effects of antibodies to neuraminidase subtypes on test specificity, and the duration of the detectable immune response are not well established. In addition, many of these questions cannot be addressed in exhaustive challenge studies. In its current form, serologic testing cannot replace virus detection, but with technical refinement and improved interpretive guidelines, serologic data has the potential to provide an additional and valuable perspective in our efforts to unravel the epidemiology of IAV in wild bird populations.

ACKNOWLEDGEMENTS

We thank Larry Niles, Amanda Dey, and the numerous volunteers for capturing the shorebirds in this study and for their ongoing conservation efforts at Delaware Bay.

We thank Deborah Carter, Nicholas Davis-Fields, Alinde Fojtik, Laura Hollander, and Clara Kienzle for sample collection and processing. We thank Roy Berghaus for help with statistics. We thank personnel at St. Jude Children's Research Hospital, Memphis, Tennessee for collection and sample processing. This project was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, department of Health and Human Services, under contract HHSN272201400006C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

LITERATURE CITED

Baker A, Gonzalez P, Morrison RIG, Harrington BA. 2013. Red knot (*Calidris canutus*). In: *The Birds of North America*. Rodewell PG, editor. Cornell Lab of Ornithology; Ithaca, New York. https://birdsna.org/Species-Account/bna/species/redkno DOI: 10.2173/bna.563.Accessed March 2017.

Blanchfield K, Kamal RP, Tzeng WP, Music N, Wilson JR, Stevens J, Lipatov AS, Katz JM, York IA. 2014. Recombinant influenza H7 hemagglutinins induce lower neutralizing antibody titers in mice than do seasonal hemagglutinins. *Influenza Other Respir Viruses* 8:628-635.

Brown JD, Luttrell MP, Berghaus RD, Kistler W, Keeler SP, Howey A, Wilcox B, Hall J, Niles L, Dey A, et al. 2010. Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. *J Wildl Dis* 46:896-911.

Clark KE, Niles LJ, Burger J. 1993. Abundance and distribution of migrant shorebirds in Delaware Bay. *Condor* 95:694-705.

Fazekas de St. Groth S and Webster RG. 1966. Disquisitions on original antigenic sin. II. Proof in lower creatures. *J Exp Med* 124:347-361.

Fereidouni SR, Grund C, Häuslaigner R, Lange E, Wilking H, Harder TC, Beer M, Starick E. 2010. Dynamics of specific antibody responses induced in mallards after infection by or immunization with low pathogenicity avian influenza viruses. *Avian Dis* 54:79-85.

Guinn K, Fojtik A, Davis-Fields N, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2016. Antibodies to influenza A viruses in gulls at Delaware Bay, USA. *Avian Dis* 60:341-345.

Guo L, Zhang X, Ren L, Yu X, Chen L, Zhou H, Gao X, Teng Z, Li J, Hu J, Wu C, Xiao X, Zhu Y, Wang Q, Pang X, Jin Q, Wu F, Wang J. 2014. Human antibody response to avian influenza A (H7N9) virus, 2013. *Emerging Infect Dis* 20:192-200.

Hanson BA, Luttrell MP, Goekjian VH, Niles L, Swayne DE, Senne DA, Stallknecht DE. 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? *J Wildl Dis* 44:351-361.

Kawaoka Y, Chambers TM, Sladen WL, Webster RG. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology* 163:247-250.

Krauss S, Stallknecht DE, Negovetich NJ, Niles LJ, Webby RJ, Webster RG. 2010. Coincident ruddy turnstone migration and horseshoe crab spawning creates an ecological ‘hot spot’ for influenza viruses. *P Roy Soc Lond B Biol Sci* 277:3373-3379.

Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. 2004. Influenza A viruses of migrating wild aquatic birds in North America. *Vector-Borne Zoonot Dis* 4:177-189.

Latorre-Margalef N, Grosbois V, Wahlgren J, Munster VJ, Tolf C, Fouchier RAM, Osterhaus ADME, Olsen B, Waldenström J. 2013. Heterosubtypic immunity to influenza A virus infections in mallards may explain existence of multiple virus subtypes. *PLoS Pathog* 9: e1003443. Doi:10.1371/journal.ppat.1003443.

Maxted AM, Luttrell MP, Goekjian VH, Brown JD, Niles LJ, Dey AD, Kalasz KS, Swayne DE, Stallknecht DE. 2012. Avian influenza virus infection dynamics in shorebird hosts. *J Wildl Dis* 48:322-334.

Maxted AM, Sitters HP, Luttrell MP, Dey AD, Kalasz KS, Niles LJ, Stallknecht DE. 2016. Spring migration stopover ecology of avian influenza virus shorebird hosts at Delaware Bay. *Avian Dis* 60:394-405.

Nettleship, DN. 2000. Ruddy turnstone (*Arenaria interpres*), In: *The Birds of North America*. Rodewald PG, editor. Cornell Lab of Ornithology, Ithaca New York; https://birdsna.org/Species-Account/bna/species/rudtur Doi:10.2173/bna.537 Accessed March 2017.

Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus ADME, Fouchier RAM. 2006. Global patterns of influenza A virus in wild birds. *Science* 312:384-388.

Puzelli S, Di Trani L, Fabiani C, Compitelli L, De Marco MA, Capua I, Aguilera JF, Zombon M, Donatelli I. 2005. Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. *J Infect Dis* 192:1318-1322.

Segovia KM, Stallknecht DE, Kapczynski DR, Stabler L, Berghaus RD, Fotjik A, Latorre-Margalef N, Franca MS. 2017. Adaptive heterosubtypic immunity to low pathogenic avian influenza viruses in experimentally infected mallards. PLoS One 12: e0170335. Doi:10.1371/journal.pone.0170335

Skowronski DM, Li Y, Tweed SA, Tam TWS, Petric M, David ST, Marra F, Bastien N, Lee SW, Krajden M, Brunham RC. 2007. Protective measures and human antibody response during an avian influenza H7N3 outbreak in poultry in British Columbia, Canada. *Can Med Assoc J* 176:47-53.

Stallknecht DE, Luttrell MP, Poulson R, Goekjian V, Niles L, Dey A, Krauss S, Webster RG. 2012. Detection of avian influenza viruses from shorebirds: evaluation of surveillance and testing approaches. *J Wildl Dis* 48:382-393.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* 56:152-179.

Wong JK, Wilcox BR, Fojtik A, Poulson RL, Stallknecht DE. 2016. Antibodies to influenza A viruses in wintering snow geese (*Chen caerulescens*) in Texas. *Avian Diseases* 60:337-340.

Table 2.1: The prevalence of hemagglutinin (HA) subtype 1-12 influenza A viruses (IAVs) isolated from ruddy turnstones (*Arenaria interpres morinella*, RUTU) and RUTU feces, and percentage of RUTU and red knot (*Calidris canutus rufa*, REKN) sera that were antibody positive by bELISA or microneutralization (MN) positive for neutralizing antibodies to one or more IAV HA subtypes at Delaware Bay, New Jersey, US from 2012-2016.

Year	Virus Isolation	bELISA			MN		
		RUTU	REKN	*P*-value[a]	RUTU	REKN	*P*-value[a]
	Percent positive (n)				Percent positive (n)		
2012	10 (1087)	69 (128)	65 (102)	0.573	36 (128)	61 (102)	<0.001
2013	15 (1002)	60 (115)	73 (104)	0.046	31 (116)	66 (106)	<0.001
2014	9 (823)	57 (115)	-	-	28 (115)	-	-
2015	17 (978)	68 (111)	-	-	36 (112)	-	-
2016	20 (697)	58 (140)	67 (45)	0.382	49 (140)	78 (49)	<0.001
Sub-Total[b]	14 (2786)	62 (383)	69 (251)	0.100	39 (384)	66 (257)	<0.001
Total[c]	14 (4587)	62 (609)	-	-	36 (611)	-	-

[a] *P*-value for difference in percent positive between each species for given assay and year.
[b] Mean percent positive from 2012, 2013, and 2016.
[c] Mean percent positive for 2012-2016.

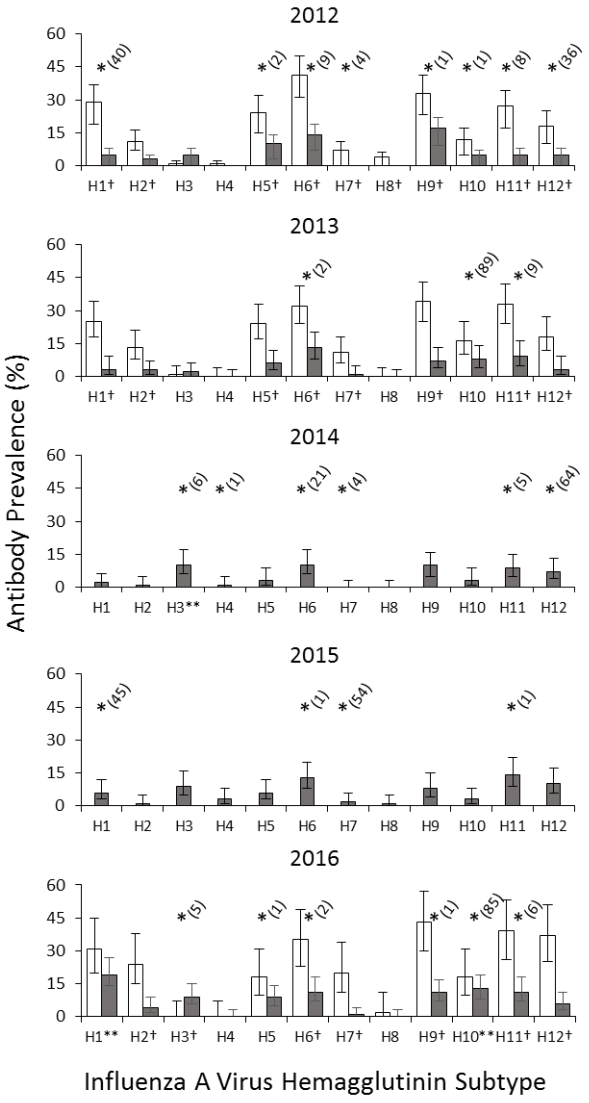

Figure 2.1. The prevalence of antibodies to influenza A viruses (IAVs) in ruddy turnstones (*Arenaria interpres morinella*, RUTU; gray bars) and red knots (*Calidris canutus rufa,* REKN; white bars) as determined by microneutralization assay and distribution of hemagglutinin (HA) subtype IAVs isolated from RUTUs and shorebird fecal samples (asterisk) from 2012-2016 in Delaware Bay, New Jersey, US. The number adjacent to asterisks is the percentage of IAVs of a given HA subtype, calculated by dividing the number of isolated viruses of a given HA subtype by the total number of IAVs isolated that year. Any number between 0 and 1% was rounded up to 1%. Subtypes marked with a dagger (†) were significantly different between species ($P \leq 0.05$) for that year. Subtypes marked with a double asterisk (**) were significantly higher in RUTUs compared to the previous year. Error bars represent 95% confidence limits.

CHAPTER 3

SUSCEPTIBILITY OF LAUGHING GULLS AND MALLARDS TO RUDDY TURNSTONE-ORIGIN TYPE A INFLUENZA VIRUSES[1]

[1]Bahnson CS, Poulson RL, Hollander L, Crum JA, Stallknecht DE. Prepared for submission to the *Journal of Wildlife Diseases.*

ABSTRACT

Delaware Bay is the only documented location where influenza A virus (IAV) is consistently detected in a shorebird species, the Ruddy Turnstone (RUTU; *Arenaria interpres morinella*). Although this disease system has been well studied over the past few decades, the importance of other species and the annual source of IAVs that infect RUTUs each spring remains unclear. We determined the susceptibility of Mallards (*Anas platyrhynchos*) and Laughing gulls (*Leucophaeus atricilla*), to IAV isolated from RUTUs in order to gain insight into potential sources and host range of these viruses. Captive-reared gulls were challenged with RUTU-origin H6N1, H10N7, H11N9, H12N4, and H13N6 IAV; as well as Mallard-origin H6N1 and H11N9. We challenged captive-reared Mallards with the same viruses, with the exception of H13N6. At a biologically plausible challenge dose (10^4 EID_{50}/0.1ml), one of five gulls challenged with both H6N1 IAVs briefly shed virus. The remaining gulls were resistant to infection with all viruses. In contrast, all Mallards shed virus, with the exception of the H12N4 challenge group, in which no birds were infected. These results demonstrate that mallards are permissive to infection with viruses originating from a shorebird host and that interspecies transmission could occur. However, based on these results we cannot identify either a duck or gull source for these shorebird viruses as it is possible that host-adaptation of IAVs to RUTUs may compromise their ability to be transmitted back to gulls.

INTRODUCTION

Delaware Bay, New Jersey (DE Bay) remains the only location where a high prevalence of influenza A virus (IAV) is consistently detected in a shorebird species, the Ruddy Turnstone (*Arenaria interpres morinella*; Hanson et al. 2008). This disease

system has been well studied over the past few decades, yet the annual source of IAVs that infect Ruddy Turnstones (RUTUs) at DE Bay and the necessity of a multispecies avian community to maintain these viruses in shorebird populations remains unclear. Some have suggested these IAVs originate from gulls that cohabit DE Bay (Hanson *et al*. 2008, Guinn *et al.* 2016). Laughing gulls (LAGU; *Leucophaeus atricilla*) are commonly observed feeding alongside RUTU flocks during the stopover. Alternatively, as RUTUs utilize marsh habitats that are also used by dabbling ducks such as Mallards (*Anas platyrhynchos*), a waterfowl origin for these IAV cannot be discounted (Brown et al. 2012; Maxted *et al* 2016).

Infections with IAVs have been documented in ducks and LAGUs in the area. By searching surveillance data in the Influenza Research Database (IRD; Zhang *et al.* 2017) from Delaware, New Jersey, and New York, Guinn *et al.* estimated an IAV prevalence of 3.1% in LAGUs from 1986-2014 (2016). This included IAV hemagglutinin (HA) subtypes H1-4, 6, 7, 9-12, 13, and 16. Furthermore, antibodies to IAV were detected in 61% of the 199 LAGUs sampled during the RUTU stopover from 2010-2014 and neutralizing antibodies to H1-H12 were detected (Guinn *et al.* 2016). Influenza A virus was detected in 7.7% of 1,001 Mallards sampled in these same states from 2008-2016 (Zhang *et al.* 2017). Hemagglutinin subtypes 1, 3, 4, 6, and 11 were detected. Although sampling of Mallards occurred in September through December and likely included a large portion of migratory birds, salt marshes at DE Bay are used by resident, breeding Mallards and other dabbling duck species during the spring (Nichols and Jones, 2015).

Both ducks and gulls may contribute to IAV dynamics during the stopover by serving as a source of IAVs, amplifying IAVs, or contributing to subtype reassortment. However, it remains unknown to what degree IAVs can be readily transmitted between these species. The conservation status of RUTUs and logistical concerns precludes an experimental challenge of RUTUs with gull and duck-origin IAVs. However, the ability of RUTU-adapted IAVs to be transmitted back to ducks or gulls provides an indication of the potential for these viruses to be shared among these birds. In this study, we challenged Mallards and LAGUs with RUTU-origin IAVs to assess the degree to which adaptation of IAVs to RUTUs permits or precludes subsequent infections in ducks and gulls.

MATERIALS AND METHODS

Viruses

The seven, wild-bird origin, low pathogenic IAVs included: A/ruddy turnstone/NJ/UGAI14-1984/2014 (H6N1, “RUTU H6N1”), A/mallard/MN/AI09-4345/2009 (H6N1, “MALL H6N1”), A/ruddy turnstone/NJ/AI09-036/2009 (H10N7, “RUTU H10N7”), A/ruddy turnstone/NJ/AI09-1164/2009 (H11N9, “RUTU H11N9”), A/mallard/MN/AI08-3267/2008 (H11N9, “MALL H11N9”), A/ruddy turnstone/NJ/UGAI14-1995/2014 (H12N4, “RUTU H12N4”), A/ruddy turnstone/NJ/UGAI14-1436/2014 (H13N6, “RUTU H13N6”). Viruses were propagated by second passage in 9- to 11-day old specific pathogen free, embryonated chicken eggs (ECEs). Viruses were titrated in ECEs and the 50% embryo infectious dose was calculated using the Reed and Muench Method (Reed and Muench 1938). On day 0, inocula were diluted in brain-heart infusion (BHI) media to the desired 50% embryo infectious dose (EID_{50}) and back titrations were performed in ECEs to

confirm the titer. The calculated titers per 0.1mL were 10^4 EID_{50} for RUTU H6N1, $10^{5.17}$ EID_{50} for MALL H6N1, $10^{3.5}$ EID_{50} for RUTU H10N7, $10^{4.17}$ EID_{50} for RUTU H11N9, $10^{4.17}$ EID_{50} for MALL H11N9, $10^{3.83}$ EID_{50} for RUTU H12N4, and $10^{3.5}$ EID_{50} for RUTU H13N6. We chose this viral challenge dose (10^4 EID_{50}/0.1ml) because previous work suggests that a challenge dose of 10^6 EID_{50}/0.1ml of H5N2, H3N8, or H7N3 was sufficient to infect all LAGUs (Costa et al. 2011), and 10^4 EID_{50}/0.2ml of H13N9 was sufficient to infect 7/8 Ring-billed gulls (Brown et al. 2012).

Animals

All husbandry, procedures, and methods used in this study complied with the animal care and use proposal approved by the Institutional Animal Care and Use Committee at the University of Georgia (AUP # A201609-012-A1). Laughing gulls were acquired under appropriate federal and state permits (MB779238-2 and SC-17-2017) through the Southeastern Cooperative Wildlife Disease Study, University of Georgia. One to five-day-old chicks were hand-caught in Charleston County, South Carolina. They were raised in confinement at UGA to six weeks of age, then transferred to a BSL-2Ag+ facility where they were randomly assigned to groups of five individuals and placed in poultry isolators. One-day-old Mallards were purchased from a commercial supplier (Metzer Farms, Gonzales, CA). They were raised in confinement at UGA to 4-5 weeks of age, then transferred to the BSL-2Ag+ facility where they were randomly assigned to groups of five individuals and placed in poultry isolators. On the day they were transferred to the new facility, all birds tested negative for antibodies to the IAV nucleoprotein (NP) by blocking enzyme-linked immunosorbent assay (bELISA;IDEXX Laboratories, Westbrooke, ME;Brown et al. 2010).

All birds were allowed to acclimate in the BSL-2-Ag+ facility for 72 hours. On day 0, all five gulls in each isolator were inoculated via the choanal cleft with 0.1mL of inoculum containing one of the seven viruses listed. Mallards were inoculated in the same manner with the same viruses, with the exception of RUTU H13N6.

Birds were monitored twice daily for evidence of clinical signs. Cloacal and oropharyngeal swabs were collected at 0, 2, 4, 6, and 14 days post inoculation (DPI). Swabs were placed in separate tubes containing 2 ml of BHI media supplemented with antimicrobials and were kept cold until long-term storage at -80C. Before they were frozen, virus isolation was attempted from all swabs collected at 4 DPI (Webster *et al.* 2002). All swabs were thawed once and extraction of RNA and molecular detection via quantitative real-time reverse transcriptase PCR (qRT-PCR) was attempted (Brown *et al.* 2013). Cycle threshold (CT) values below 40 were considered positive. Serum was collected prior to inoculation and at 14 DPI. All birds were humanely euthanized at 14 DPI via CO_2 inhalation followed by cervical dislocation.

All sera were tested for antibodies to the NP by blocking enzyme-linked immunosorbent assay bELISA. In addition, the 14 DPI sera from all birds excluding the H13 group were tested for neutralizing antibodies to the hemagglutinin subtype that the bird was challenged with (Wong *et al.* 2016). Viruses used as antigens included A/mallard/MN/Sg-00796/2008 (H6N1), A/mallard/MN/SG-00999/2008 (H10N7), A/mallard/MN/SG-00930/2008 (H11N9), and A/mallard/MN/AI07-3285/2007 (H12N5). The H13 group was excluded from microneutralization (MN) testing because adequate viral titers of H13 IAV cannot be achieved in Madin-Darby canine kidney cells (American Type Culture Collection, Manassas, Virginia) through conventional methods.

Challenge Virus Sequence Analysis and Phylogenetics

Complete sequences were generated for all hemagglutinin genes from the seven IAVs used as inocula and assembled as described (Mena *et al.* 2016). Nucleotide sequences (1737-1760 bp) for HA gene segments were compared to sequences on NCBI GenBank using the nucleotide Blast function (accessed 26 September 2018). The relationships between HA genes within subtype were assessed through phylogenetic analysis by comparing nucleotide sequences for HA genes sequenced as part of this study with those available on the NCBI GenBank Influenza Virus Resource databased (accessed 18 July 2018). Sequences were aligned and cropped to a common length and maximum-likelihood trees were constructed with MEGA version 6.0 (Tamura, 2013)

Based on results from Gambaryon *et al.* 2018 that reported differences in amino acids at position 222 (H3 numbering system) in viruses from ducks, shorebirds, and gulls, the amino acid at this position was determined for each challenge virus. Hemagglutinin protein sequences of each isolate were converted to H3 numbering using the cross-subtype numbering method (Burke and Smith, 2014).

Statistics

The durations of shedding and mean CT values were compared for mallards challenged with RUTU H6N1 versus MALL H6N1 and Mallards challenged with RUTU H11N9 versus MALL H11N9. The duration of shedding was assessed by using a non-parametric test (Wilcoxon rank-sum test). The CT-values were compared for days 2, 4, and 6 using linear mixed models with bird as a random effect. Negative rt qRT-PCR results were assigned a value of 45. All calculations were performed in STATA 15.1 (StataCorp LP, College Station, Texas).

RESULTS

No birds displayed clinical signs during the study. One LAGU from both groups challenged with an H6N1 virus briefly shed virus via the oropharyngeal route (Table 1). With the exception of the group challenged with RUTU H12N4, all Mallards shed virus through the cloacal and oropharyngeal route beginning at 2 DPI. Shedding from both routes continued for most Mallards through 6 DPI. With the exception of two Mallards challenged with MALL H11N9, viral shedding was no longer detected by day 14. Virus isolation from swabs collected at 4 DPI confirmed qRT-PCR data: viable IAVs were isolated from all birds that were positive by qRT-PCR.

The mean duration of oropharyngeal shedding was longer for Mallards infected with MALL H6N1 compared to RUTU H6N1 (4 versus 2 days; P=0.009). A significant difference was not detected in the median duration of cloacal shedding between these groups, nor was a significant difference detected for the median duration of cloacal or orphorpharyngeal shedding between Mallards challenged with MALL H11N9 compared to Mallards challenged with RUTU H11N9. The mean CT value of oropharyngeal shedding for Mallards challenged with RUTU H6N1 was significantly lower than the MALL H6N1 group at 2 DPI (27.7 versus 31.8; P=0.001) and 4 DPI (29.8 versus 27.7; P=0.02; Figure 1). At 6 DPI, the mean CT value for oropharyngeal shedding was lower for the MALL H6N1 group (35 versus 45; P<0.001). The mean CT value for cloacal shedding was not significantly different between the two H6N1 groups at 2 and 4 DPI but it was significantly lower for the MALL H6N1 group at 6 DPI (29.5 versus 36.3; P<0.001). There was no significant difference detected between the mean CT value of

oropharyngeal or cloacal shedding for the Mallard groups challenged with MALL H11N9 versus RUTU H11N9.

All five Mallards challenged with MALL H6N1, RUTU H10N7, and MALL H11N9 seroconverted as determined by MN (Table 1). Four Mallards in the RUTU H6N1 group and one Mallard in the RUTU H11N9 group also seroconverted by MN. Results of bELISA testing mirrored MN results for these groups except for three Mallards in the RUTU H6N1 and one Mallard in the MALL H11N9 group that were only seropositive by MN. No Mallards in the RUTU H12N4 seroconverted by bELISA or MN. One LAGU challenged with MALL H6N1 seroconverted as determined by bELISA but no LAGUS were positive by MN.

The HA genes for five of the IAV isolates used in this study shared high identity (≥98%) with isolates derived from wild ducks in North America. The RUTU H6 from 2014 was 98% identical at the nucleotide level to a RUTU H6 isolated in 2012, and the RUTU H13 was closely related (99%) to a ring-billed gull H13, also detected two years prior to 2014. Inferred phylogenetic relationships for RUTU H6N1 and MALL H6N1 are shown in Figure 2. RUTU H6N1 is closely related to Anseriformes H6 viruses detected in the same and previous years. Similar phylogenetic relationships were inferred from the H10, H11, and H12 IAVs used in this study with RUTU isolates clustering with duck isolates (data not shown).

At consensus position 222 on the HA, RUTU H6N1 harbored a proline; MALL H6N1 harbored an alanine; RUTU H10N7 harbored a leucine; RUTU H11N9 harbored an arginine; Mall H11N9 harbored a lysine; RUTU H12N5 harbored a valine; and RUTU H13N6 harbored a glycine.

DISCUSSION

In a previous study of IAV host range for IAV isolated from shorebirds, Kawaoka *et al.* (1988) challenged leghorn chickens (*Gallus gallus domesticus*) and Pekin ducks (*Anas platyrhynchos domesticus*) with several IAVs isolated from DE Bay. Because many of the viruses did not infect either species, Kawaoka *et al.* concluded that a separate "gene pool" of IAVs resides in gulls and shorebirds. In the present study, Mallards were susceptible to three of the four RUTU-origin IAVs. By comparison, at what we argue is a biologically plausible challenge dose (10^4 EID_{50}), LAGUs were far more resistant to several IAVs of varying subtypes. Phylogenetic mapping of the HA genes suggests that all seven challenge viruses were good representatives of viruses in circulation in North American Anseriformes. HA genome sequences closely matched other viruses isolated close to or during the same year at similar locations, in similar shorebird species, and ducks. This lends strength to our overall conclusion: Mallards are more susceptible than LAGUs to RUTU-adapted viruses of several hemagglutinin subtypes. However, exceptions to this theme were observed in three of the five HA subtypes: H6, which replicated poorly in gulls; H12, which did not replicate in either species; and H13, which did not replicate in gulls but was not used to challenge Mallards.

That some LAGUs and all Mallards were susceptible to both H6 IAVs is not surprising. This was the most common HA subtype isolated during 26 years of surveillance in Canadian wild ducks, and it appears to have the largest host range (Krauss *et al.* 2004, Munster *et al.* 2007). At DE Bay, the subtype was well represented in LAGU isolation and serology data (Guinn *et al.* 2016). Additionally, Franklin's gulls

were readily infected with $10^{3.49}$ EID_{50} of a domestic turkey origin H6N2 (Bahl and Pomeroy, 1977).

The failure to detect virus in gulls challenged with H12, as well as H10, is consistent with field observations from DE Bay that have found low antibody prevalence in gulls despite the common occurrence of these subtypes in shorebirds (Guinn *et al.* 2016). However, there is no field data to suggest that Mallards would be resistant to RUTU H12N4. As it turns out, the results of this study may reveal more about the challenge virus than the potential host. In a previous study, $10^{6.5}$ EID_{50} of Mallard-origin H12N5 was insufficient to re-infect Mallards previously challenged with Mallard-origin H3N8, but this dose was sufficient to infect Mallards with Mallard-origin H4N5, H10N7, and H6N2 (Latorre-Margalef *et al.* 2017). A recombinant virus expressing H12 replicated to much higher titers in embryonated chicken eggs as compared to MDCK cells ($10^{7.0}$ vs $10^{4.4}$; Keawcharoen *et al.* 2010), which may be a result of the lower cleavage activity trypsin has on the H12 protein compared to other hemagglutinin subtypes (Galloway *et al.* 2013). As a result, the *embryo* infectious dose 50 may be a less reliable correlate to *bird* infectious dose 50 for H12 as compared to other subtypes.

Our H13 results were perhaps the most unexpected, as this is considered a gull-adapted subtype (Arnal et al. 2014). Annual cycles of H13 infections in gull nesting colonies are driven by young, naïve birds (Verhagen *et al.* 2015). As fledglings age, they become more mobile within a nesting colony, theoretically leading to an increase in contact between individuals which coincides with waning yolk-derived antibodies (Verhagen *et al.* 2014). This is typically followed by a rapid spread of H13 IAVs through the nesting colony which may occur in chicks as young as three weeks of age (Velarde

et al. 2010). The one to five-day old gull chicks we collected were among the first to hatch in that nesting colony and were presumably removed prior to IAV circulation. Furthermore, they were raised to six weeks of age, past the point of detectable yolk-derived antibody level. It therefore seems unlikely that previous exposure history or yolk-derived immunity reduced the susceptibility of LAGUs in this study. Alternatively, these results may provide further evidence that RUTU-adaptation of an IAV comes at the expense of fitness in LAGU. Further studies involving multiple H13 IAV isolates would be required to assess this possibility.

This study demonstrated a resistance of LAGUs to infection with RUTU and Mallard-adapted IAVs, but the underlying determinants of this observed host restriction are not fully understood. However, one key adaptation of a duck virus to gulls may be a shift in preferential affinity for sialic acids bound to a penultimate galactose that is fucosylated (Gambaryan *et al.* 2005). For at least some IAV subtypes, the ability to bind to “gull” sialic acid receptors is obtained through a substitution to a less bulky amino acid at position 222 in the HA (H3 numbering system; Gambaryan *et al.* 2018). This provides one possible mechanism by which our challenge viruses failed to replicate in gulls. For example, RUTU H11N9 had an arginine at position 222 that is even larger than lysine observed at this position in most H11 IAVs isolated from Anseriformes, including MALL H11N9. This same substitution was observed in a portion of 94 shorebird isolates, but none of 12 gull isolates (Gambaryan *et al.* 2018). Consequently, the “bulkier” amino acid in RUTU H11N9 may have compromised its ability to bind to fucosylated sialic acid receptors in gulls. The importance of amino acids at 222 was not evaluated for H10 and H12 viruses, and substitutions at other positions, such as 227,

appear to be important for enabling H6 and H13 proteins to bind to fucosylated receptors (Gambaryan *et al.* 2018). Infection dynamics at DE Bay may provide selective pressure for IAVs that result in a loss of affinity for gull fucosylated receptors. This is an area of future research that may be aided by our results, including sequence data from our challenge viruses, as well as those from field isolates.

The impetus for this study was the desire to determine the source of IAVs infecting RUTUs at DE Bay each year. We approached the question by evaluating the degree to which RUTU-adaptation permitted or precluded subsequent infection in LAGUs and Mallards. Susceptibility of a species, as determined by the mean infectious dose required to infect a given species, is an indicator of how host-adapted a particular IAV is to a species (Swayne and Slemons 2008). Thus, the overall pattern of our findings demonstrated that RUTU-origin IAVs are poorly host-adapted to LAGUs, yet readily infect ducks. While either ducks or gulls may be a source of IAVs for RUTUs at DE Bay, host-adaptation to RUTUs may compromise the ability of these viruses to be transmitted back to gulls, whereas, IAVs may be readily exchanged between RUTUs and ducks.

ACKNOWLEDGMENTS

We thank Felicia Sanders and South Carolina Department of Natural Resources for providing LAGU chicks. We thank Deborah Carter, Nicholas Davis-Fields, Alinde Fojtik, and Clara Kienzle for technical help. This project was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, department of Health and Human Services, under contract HHSN272201400006C. The content is

solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

LITERATURE CITED

Arnal A, Vittecoq M, Pearce-Duvet J, Gauthier-Clerc M, Boulinier T, Jourdain E. 2014. Laridae: A neglected reservoir that could play a major role in avian influenza virus epidemiological dynamics. *Crit Rev Microbiol*. 41:508-519.

Bahnson CS, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2018. Neutralizing antibodies to type A influenza viruses in shorebirds at Delaware Bay, New Jersey. *J Wild Dis* 54:708-715.

Brown J, Poulson R, Carter D, Lebarbenchon C, Patin-Jackwood M, Spackman E, Shepherd E, Killian M, Stallknecht D. 2012. Susceptibility of avian species to North American H13 low pathogenic avian influenza viruses. *Avian Dis* 56:969-975.

Brown JD, Luttrell MP, Berghaus RD, Kistler W, Keeler SP, Howey A, Wilcox B, Hall J, Niles L, Dey A, Knutsen G, Fritz K, Stallknecht DE. 2010. Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. *J Wildl Dis* 46:896-911.

Brown JD, Poulson R, Carter DL, Lebarbenchon C, Stallknecht DE. 2013. Infectivity of avian influenza virus-positive field samples for mallards: what do our diagnostic results mean? *J Wildl Dis* 49:180-185.

Brown VL, Drake JM, Stallknecht DE, Brown JD, Pedersen K, Rohani P. 2012. Dissecting a wildlife disease hotspot: the impact of multiple host species,

environmental transmission and seasonality in migration, breeding and mortality. J R Soc Interface 10:20120804

Burke DF, Smith DJ. 2014. A recommended numbering scheme for influenza a HA subtypes. PLoS ONE 9(11): e112302.

Costa TP, Brown JD, Howerth EW, Stallknecht DE. Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. *Avian Path* 40:119-124.

Franca M, Stallknecht DE, Howerth EW. 2013. Expression and distribution of sialic acid influenza virus receptors in wild birds. *Avian Path*. 42:60-71.

Galloway SE, Reed ML, Russell CJ, Steinhauer DA. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. PLoS Pathog 9(2): e1003151.

Gambaryan A, Yamnikova S, Lvov D, Tuzikov A, Chinarev A, Pazynina G, Webster R, Matrosovich M, Bovin N. 2005. Receptor specificity of influenza viruses from birds and mammals: New data on involvement of the inner fragments of the carbohydrate chain. *Virology* 334:276-283.

Gambaryan AS, Matrosovich TY, Boravleva EY, Lomakina NF, Yamnikova SS, Tuzikov AB, Pazynina GV, Bovin NV, Fouchier RAM, Klenk HD, Matrosovich MN. 2018. Receptor-binding properties of influenza viruses isolated from gulls. *Virology* 522:37-45.

Guinn K, Fojtik A, Davis-Fields N, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2016. Antibodies to influenza A viruses in gulls at Delaware Bay, USA. *Avian Dis* 60:341-345.

Hanson BA, Luttrell MP, Goekjian VH, Niles L, Swayne DE, Senne DA, Stallknecht DE. 2008. Is the occurrence of avian influenza virus in charadriiformes species and location dependent? *J Wildl Dis* 44:351-361.

Kawaoka Y, Chambers TM, Sladen WL, Webster RG. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163:247-250.

Keawcharoen J, Spronken MIJ, Vuong O, Bestebroer TM, Munster VJ, Osterhaus ADME, Rimmelzwaan GF, Fouchier RAM. 2010. Repository of Eurasian influenza virus hemagglutinin and neuraminidase reverse genetics vectors and recombinant viruses. Vaccine 28:5803-5809.

Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. 2004. Influenza A viruses of migrating wild aquatic birds in North America. Vector-Borne and Zoonotic Diseases. 4:177-189.

Latorre-Margalef N, Brwn JD, Fojtik A, Poulson RL, Carter D, Franca M, Stallknecht DE. 2017. Competition between influenza A virus subtypes through heterosubtypic immunity modulates re-infection and antibody dynamics in the mallard duck. PLoS Pathog 13(6): e1006419

Maxted AM, Sitters HP, Luttrell MP, Dey AD, Kalasz KS, Niles LJ, Stallknecht DE. 2016. Spring migration stopover ecology of avian influenza virus shorebird hosts at Delaware Bay. *Avian Dis* 60:394-405.

Mena I, Nelson MI, Quezada-Monroy F, Dutta J, Cortes-Fernandez R, Lara-Puente JH, Castro-Peralta F, Cunha LF, Trovao NS, Lozano-Dubernard B, Manbaut A, van

Bakel H, Garcia-Sastre A. 2016. Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. *eLife* 5:e16777. 107554/eLife.16777.

Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WEP, Schutten M, Olsen B, Osterhaus ADME, Fouchier RAM. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *Plos Pathog* 3(5):e61. Doi:10.1371/journal.ppat.0030061

Nichols TC, Jones OE. 2015. Population trends of breeding waterfowl in New Jersey, 1993-2012. Bull. N.M. Acad. Sci. 60:7-13.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol.* 27:493-497.

Stallknecht DE, Brown JD. 2017. Wild bird infections and the ecology of avian influenza viruses In: *Animal Influenza,* Swayne DE, editor. John Wiley & Sons, Ames Iowa 2nd Edition John Wiley & Sons, Ames, Iowa. Pp. 153-176.

Swayne DE, Slemons RD. 2008. Using mean infectious dose of high- and low-pathogenicity avian influenza viruses originating from wild duck and poultry as one measure of infectivity and adaptation to poultry. Avian Diseases 52:455-460.

Tamura K, Stecher, G, Peterson, D, Filipski, A, Kumar, S. 2013. Mega6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725-2729.

Webster R, Cox N, Storh K. WHO Manual on Animal Influenza Diagnosis and Surveillance. WHO: WHO Global Influenza Programme. 2002.

Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z, He S Kumar S, Larsen CN, Lee AJ, *et. al.* 2017. Influenza research database: an integrated

bioinformatics resource for influenza virus research. *Nucleic Acids Res.* 45:D466-474.

Table 3.1. Summary of testing results for Laughing Gulls (*Leucophaeus atricilla*) and Mallards (*Anas platyrhynchos*) challenged with a given strain of influenza A virus. Oropharyngeal (OP) and cloacal (CL) swabs were collected at days 0, 2, 4, 6, and 14 days post-inoculation and later tested by real-time reverse-transcription PCR (qRT-PCR). Serum was collected at 14 days post-challenge and tested by enzyme linked immunosorbent assay and microneutralization.

Species	Virus[a]	Day 0		Day 2		Day 4		Day 6		Day 14		Seroconversion
		OP	CL	OP	CL	OP	CL	OP	CL	OP	CL	
Laughing Gull	RUTU H6N1	0/5[b]	0/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
	MALL H6N1	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5
	RUTU H10N7	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	RUTU H11N9	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	MALL H11N9	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	RUTU H12N4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	RUTU H13N6	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Mallard	RUTU H6N1	0/5	0/5	5/5	5/5	5/5	5/5	0/5	3/5	0/5	0/5	4/5
	MALL H6N1	0/5	0/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5	0/5	5/5
	RUTU H10N7	0/5	0/5	5/5	5/5	5/5	5/5	2/5	5/5	0/5	0/5	5/5
	RUTU H11N9	0/5	0/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5	0/5	1/5
	MALL H11N9	0/5	0/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5	2/5	5/5
	RUTU H12N4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] Challenge viruses: A/ruddy turnstone/NJ/UGAI14-1984/2014 (H6N1, “RUTU H6N1”), A/mallard/MN/AI09-4345/2009 (H6N1, “MALL H6N1”), A/ruddy turnstone/NJ/AI09-036/2009 (H10N7, “RUTU H10N7”), A/ruddy turnstone/NJ/AI09-1164/2009 (H11N9, “RUTU H11N9”), A/mallard/MN/AI08-3267/2008 (H11N9, “MALL H11N9”), A/ruddy turnstone/NJ/UGAI14-1995/2014(H12N4, “RUTU H12N4”), A/ruddy turnstone/NJ/UGAI14-1436/2014 (H13N6, “RUTU H13N6”)

[b] Number positive/number challenged

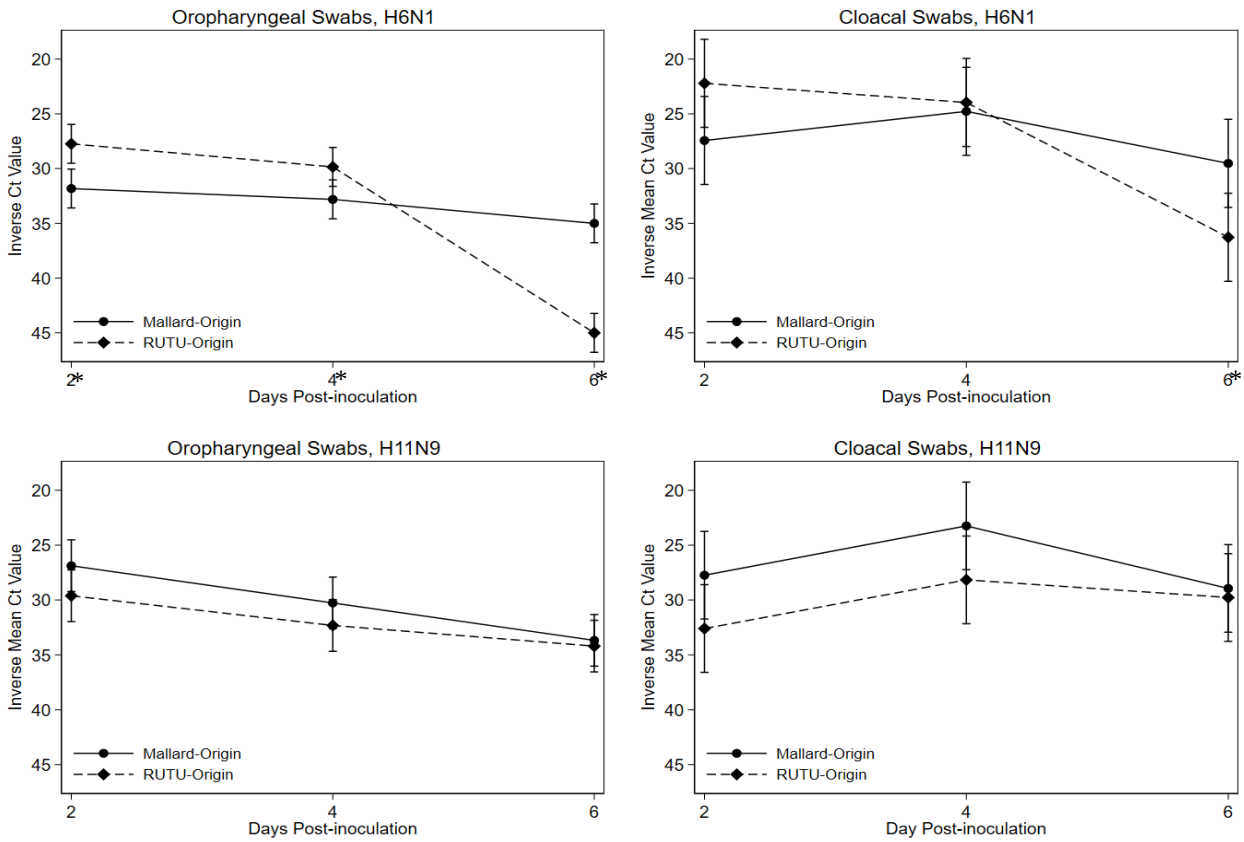

Figure 3.1. Mean cycle threshold (CT) values of oropharyngeal and cloacal swabs collected from Mallards (*Anas platyrhynchos*) 2, 4, and 6 days after inoculation. Five Mallards were challenged with one of the following viruses: Mallard-origin H6N1, RUTU-origin H6N1, Mallard-origin H11N9, and RUTU-origin H11N9. Negative results were assigned a CT value of 45. Days when a significant difference in mean CT values was detected between challenge groups are designated with an asterisk (*). Error bars represent 95% confidence intervals.

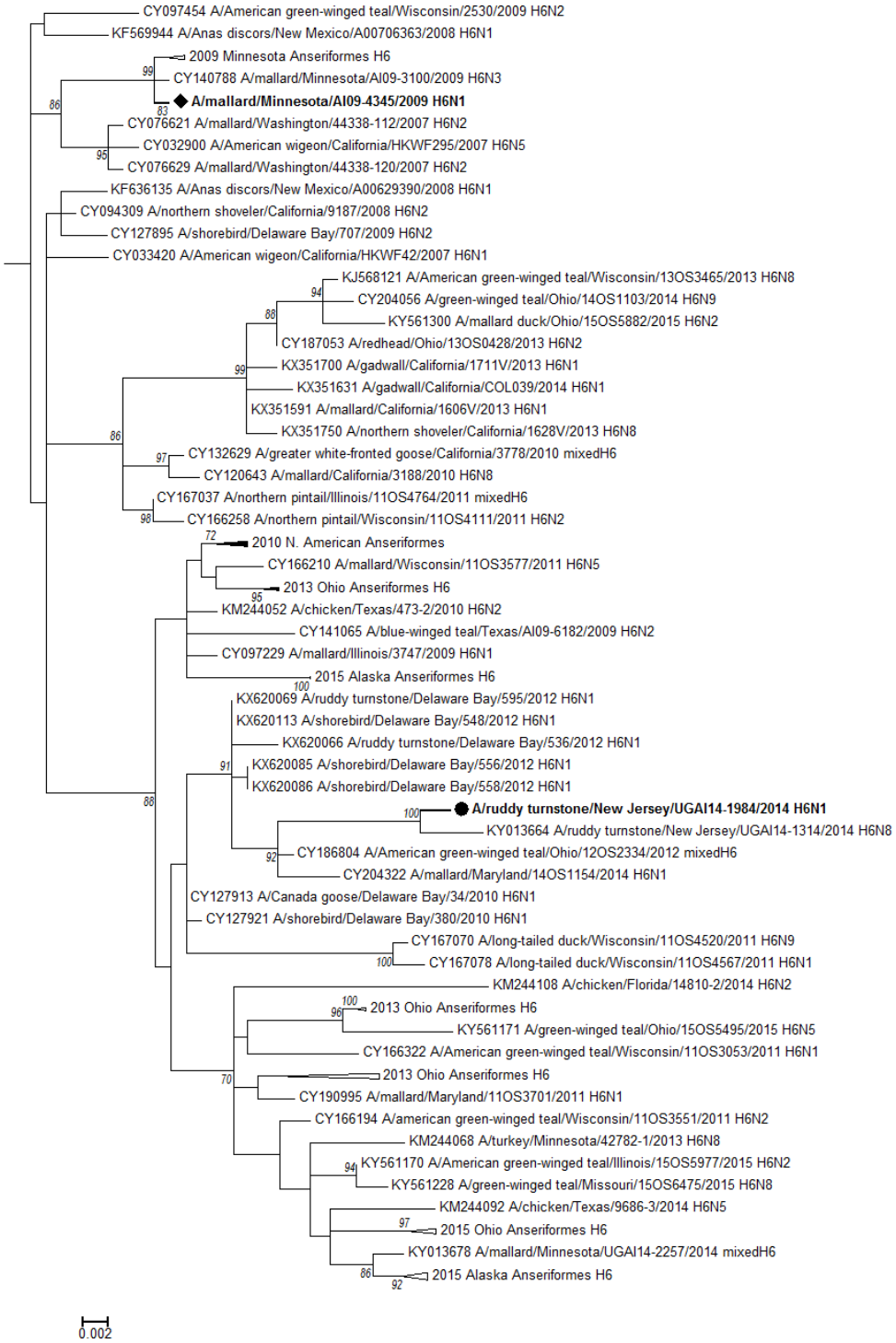

Figure 3.2. Maximum likelihood phylogenetic subtree showing inferred relationship among nucleotide sequences for the hemagglutinin gene of influenza A viruses of the H6 subtype. Bootstrap values less than 70 are omitted. Isolates characterized as part of this study are identified with a filled diamond (Mall H6N1) or a filled circle (RUTU H6N1).

CHAPTER 4

EXPERIMENTAL INFECTIONS AND SEROLOGY PROVIDE EVIDENCE FOR INCLUDING WHITE IBIS (*EUDOCIUMS ALBUS*) AMONG THE TYPE A INFLUENZA VIRUS NATURAL RESERVOIR SYSTEM[1]

[1]Bahnson CS, Hernandez SM, Poulson RL, Cooper RE, Curry S, Ellison TJ, Stallknecht DE. Prepared for submission to *Journal of Wildlife Diseases.*

ABSTRACT

The American White Ibis (*Eudocimus albus*) is a nomadic wading bird common to many coastal habitats in the southeastern United States. In South Florida, habitat encroachment has driven a large number of Ibis to become highly urbanized. While they forage in neighborhood parks, artificial wetlands, backyards, and golf courses, the majority continue to nest and roost in natural wetlands, often in dense colonies. Adults and juveniles commonly disperse thousands of kilometers to other breeding colonies in the Southeast, resulting in potential close contact with humans, domestic animals and other wild bird species. Historically, wading birds were not considered significant hosts for influenza A virus (IAV), yet as Ibis regularly move among various human, domestic animal, and wildlife interfaces, their potential to be exposed or infected with IAV deserves attention. To investigate this: we experimentally challenged wild-caught, captive-reared Ibis with three low pathogenic IAVs; tested wild Ibis for IAV; and serologically tested wild Ibis for antibodies to IAV. Ibis were highly susceptible to experimental challenge with H6N1 and H11N9 IAVs with cloacal shedding lasting an average of six days. All thirteen infected birds seroconverted by 14 days post infection as determined by microneutralization. In contrast, no Ibis challenged with H3N8 were infected. We tested 118 swabs and 330 serum samples from Ibis captured in southeastern Florida for IAV infection and antibodies to IAV, respectively. Although no IAVs were isolated, 59% were antibody-positive by blocking enzyme-linked immunosorbent assay (bELISA). Neutralizing antibodies to H1-H12 were detected in 96% of bELISA positive birds and 81% tested antibody positive to two or more HA subtypes, indicating that exposure to multiple IAVs is common. These results provide

compelling evidence that Ibis are susceptible and naturally infected with IAV and may represent a component of the IAV natural reservoir system.

INTRODUCTION

Since the isolation of low pathogenic influenza A virus (IAV) in free ranging waterfowl in 1972, extensive research and surveillance efforts have firmly established that wild bird species of two orders, Anseriformes and Charadriiformes, are important for maintaining IAVs (Slemons et al. 1972, Webster et al. 1992, Olsen et al. 2006). Although our understanding of temporal and spatial patterns of infection in these orders has grown considerably, many questions pertaining to IAV maintenance and the potential contribution of other species within avian communities remain. To understand the full scale of the natural IAV reservoir, our research scope may need to be broadened to focus on the role of other species not conventionally recognized as IAV hosts (Caron *et al.* 2017*)*.

With this in mind, a starting point is to consider species with life histories compatible with established IAV ecology and that have contact with known IAV reservoirs. One such species is the American White Ibis (*Eudocimus albus*). Like dabbling ducks, gulls, and shorebirds, Ibis utilize aquatic habitats and nest and roost in dense colonies that can number as high as 100,000 pairs in a single colony (Frederick *et al.* 1996). Although not classically considered a migratory bird, Ibis cohabit areas utilized by overwintering Anseriforme and Charadriiforme species (Sauer *et al.* 2016). While all Ibis return to natural areas during the breeding season in the spring, a large portion of Ibis in southern Florida have become highly urbanized, resulting in close contact with humans, peridomestic waterfowl, backyard poultry, and other avian species

as they frequent parks, golf courses, backyards, and zoos where they forage (Hernandez *et al.* 2016, Murray *et al.* 2017). Furthermore, individuals may disperse as far as 1600 km to other populations throughout the southeastern United States (Frederick *et al.* 1996). Thus, Ibis may serve to maintain, amplify, or disseminate IAVs throughout the region while also presenting a potential threat to human and domestic animal health.

Ibis are members of the Order Pelicaniformes which also includes herons and egrets. Birds in this Order have rarely been a focus of IAV surveillance, yet viral detections or serologic evidence of infections have been reported from this group of birds on all continents excluding Antarctica (e.g. Pfitzer *et al.* 2000 (South Africa; Hadada Ibis); Ellis *et al.* 2004 (China; Egrets and Grey Herons); Epstein *et al.* 2007 (Australia; Australian White Ibis); Ghersi *et al.* 2009 (Peru; Peruvian pelican); Niqueux *et al.* 2010 (France; Sacred Ibis); Siembieda *et al.* 2010 (California, USA; Egrets and Herons)). Importantly, Grey Herons (*Ardea cinerea*) are one of species from which viruses have been commonly isolated during recent highly pathogenic IAV outbreaks in Eurasia (Lee *et al.* 2017; Pohlmann *et al.* 2017; Woo *et al.* 2017).

Given their life history, investigating the potential for Ibis to be exposed to and infected with IAVs was warranted. In this study, we hypothesized that Ibis are susceptible and naturally infected with IAV and that wading and related wading birds may be an important component of the IAV natural reservoir system. We assessed this by 1) experimentally challenging wild-caught, captive reared Ibis with three low pathogenic IAVs; 2) testing wild-caught Ibis for IAVs; and 3) testing sera from wild-caught Ibis for antibodies to IAV.

MATERIALS AND METHODS

Experimental Infection

All husbandry, procedures, and methods used in this study were approved by the University of Georgia Institutional Animal Care and Use Committee (UGIACUC; Animal Use Permit # A201609-012-A1). White Ibis were acquired under Florida scientific collection permit number LSSC-11-00119G and federal collection permit number MB779238-2 through the Southeastern Cooperative Wildlife Disease Study, University of Georgia. Twenty, approximately two- week-old nestlings were hand-caught in Broward County, Florida. They were raised in confinement at UGA to approximately seven months old, then transferred to a BSL-2Ag+ facility where they were assigned to one of three groups housed in separate rooms. The H3N8 and H6N1 groups each included five challenge birds with two control birds that had direct contact with challenge birds. The H11N9 group included four challenge birds and two control birds. Water was provided in 43.2 cm diameter, round rubber tubs. Four tubs were used in each room and filled to a depth of approximately 7.5 cm. These were cleaned and refilled with fresh water a minimum of twice daily. Birds were fed a mixture of a commercially-available pelleted diet (Mazuri flamingo breeder; PMI Nutrition International LLC, St. Louis, MO), seafood (smelt, shrimps), and supplements in platters twice daily. Platters were cleaned and sanitized twice daily.

The three, Mallard-origin, low pathogenic IAVs used in this study included: A/mallard/MN/AI07-4724/2007 (H3N8), A/mallard/MN/AI09-4345/2009 (H6N1), and A/mallard/MN/AI08-3267/2008 (H11N9). Viruses were propagated by second passage in 9- to 11-day old specific pathogen free, embryonated chicken eggs (ECEs). Viruses

were titrated in ECEs and the 50% embryo infectious dose was calculated using the Reed and Muench Method (Reed and Muench 1938). On day 0, inocula were diluted in brain-heart infusion (BHI) media to the desired EID 50 and back titrations were performed in ECEs to confirm the titer. The calculated titers were $10^{5.6}$ EID_{50}/0.1ml of H3N8, $10^{6.2}$ EID_{50}/0.1ml of H6N1, and $10^{6.2}$ EID_{50}/0.1ml of H11N9.

Birds were allowed to acclimate in the BSL-2AG+ rooms for ten days prior to inoculation. Three days prior to inoculation, blood was collected from each bird by jugular venipuncture at a total volume less than or equal to 1% of the individual's body mass. All birds tested negative for antibodies to the nucleoprotein (NP) by blocking enzyme-linked immunosorbent assay (bELISA) (IDEXX Laboratories, Westbrooke, ME) (Brown et al. 2010). On day 0, challenge birds were inoculated via the choanal cleft with 0.1mL of inoculum containing one of the three viruses listed. Control birds were inoculated via the choanal cleft with 0.1mL of BHI media.

Ibis were monitored a minimum of twice daily for evidence of clinical signs. Cloacal and oropharyngeal swabs were collected immediately prior to inoculation and at 2, 4, 6, 8, 10, and 14 days post inoculation (DPI). All swabs were placed in separate tubes containing 2 ml of BHI media supplemented with antimicrobials and were kept on ice packs until long-term storage at -80C. At 4DPI, water from each tub (four per room) was sampled by saturating a sterile cotton tipped applicator (Puritan Medical Products Company LLC, Guilford, ME) prior to the tub being cleaned. An additional serum sample was collected at 14 DPI, at which time all birds were humanely euthanized via CO_2 inhalation, followed by cervical dislocation.

Virus isolation from all swabs collected at 4, 10, and 14 DPI was attempted in 9-11 day old ECEs (Webster *et al.* 2002). Extraction of RNA and molecular detection via quantitative real-time reverse transcriptase PCR (qrtPCR) was attempted on all swabs (Latorre-Margalef et al. 2017). Cycle threshold values below 40 were considered positive. All sera were tested for antibodies to the NP by bELISA, and sera collected at 14 DPI were tested for neutralizing antibodies to H1-12 by microneutralization (MN; Wong et al. 2016). Viruses used as antigens included A/mallard/NJ/AI12-4823/2012 (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-00796/2008 (H6N1), A/mallard/MN/AI08-3770/2009 (H7N9), A/mallard/MN/SG-01048/2008 (H8N4), A/RUTU/DE/AI11-809/2011 (H9N2), A/mallard/MN/SG-00999/2008 (H10N7), A/mallard/MN/SG-00930/2008 (H11N9), and A/mallard/MN/AI07-3285/2007 (H12N5). Viral subtypes H13 and H16 were not included in testing because appropriate viral titers cannot be achieved in Madin-Darby canine kidney cells (American Type Culture Collection, Manassas, Virginia, USA) with conventional techniques.

Serologic testing of wild Ibis

White Ibis were live captured in Palm Beach and Martin Counties in Southern Florida between October 2015 and August 2017 under Florida scientific collection permit number LSSC1-11-100119G and federal collection permit number MB779238-2. Birds were captured using nylon slip-knot leg lassos, modified manually operated flip traps, and mist nets with decoys (Murray *et al.* 2018). Blood samples were collected by jugular venipuncture at a total volume less than or equal to 1% of the individual's body

mass. Samples were kept on ice until they could be centrifuged within two hours of collection. The plasma fraction was aliquoted and stored at -20 C until testing by bELISA, and then stored at -80 C until testing for neutralizing antibodies to H1-12 by MN as described above. Wild Ibis capture and handling was approved by the UGIACUC (Animal Use Permit number A2016 11-019-Y2-A1).

Virus Isolation from wild Ibis

White Ibis were live captured in Palm Beach County, Florida in March 2010 (n=60) and August 2013 (n=21) as part of a previous study (Hernandez *et al.* 2016). Thirty-seven birds were also captured in March 2014 under Florida scientific collection permit number LSSC-11-00119C, federal collection permit number MB779238-2, and UGIACUC AUP number A2013 01-005-R3. An oropharyngeal swab and either cloacal or fecal swab were collected from each bird and placed in a single vial containing 2 ml of BHI media supplemented with antimicrobials. These were kept on ice packs until placed in long-term storage at -80C. They were later thawed and virus isolation was attempted in 9-11 day old ECEs (Webster *et al.* 2002).

Statistical Analysis:

The average duration of cloacal and oropharyngeal shedding for each viral challenge group was calculated by taking the mean number of days positive qrtPCRs were collected from challenged birds. The mean CT-value was calculated for oropharyngeal and cloacal samples from challenged birds in each group each day. Negative qrt-PCR results were assigned a value of 45.

Wild Ibis plasma samples collected in February and March were designated “spring”; samples collected in June, July, and August were designated “summer”; and

samples collected in October and November were designated “fall.” The Kruskal-Wallis test was used to compare the number of bELISA positive samples between seasons. It was also performed comparing the prevalence of neutralizing antibodies for each subtype between seasons. All calculations were performed in STATA 15.1 (StataCorp LP, College Station, Texas, USA).

RESULTS

Experimental Challenge

No evidence of clinical disease was observed in any bird. All birds inoculated with H6N1 and H11N9 shed virus as detected by qrtPCR and virus isolation, while no virus was detected from the H3N8 group (Table 1). Cloacal shedding lasted longer than oropharyngeal shedding for both infected groups, averaging 6 days for the H6N1 group and 5 days for the H11N9 (Figure 1). For both groups, the inverse mean CT-value of oropharyngeal shedding peaked at 4 DPI while the value for cloacal shedding peaked at 6 DPI.

Both control birds in the H6N1 and H11N9 groups also became positive. Virus was detected by qrtPCR in all water samples collected from the H6N1 and H11N9 challenge rooms. It was isolated from three of four samples collected in the H6N1 room and two of four samples from the H11N9 room. Virus was not detected or isolated from the four water samples collected in the H3N8 challenge room.

By 14 DPI, three of five inoculated birds and one of two control birds in the H6N1 group seroconverted, as determined by bELISA (Table 1). Neutralizing antibodies to H6 were detected in all birds in this group Two birds also had neutralizing antibodies to the H1 antigen. All inoculated birds and one of two control birds in the H11N9 group

seroconverted, as determined by bELISA. Neutralizing antibodies to H11 were detected in all birds in this group. Antibodies to other HA subtypes were not detected. None of the birds in the H3N8 group seroconverted as detected by bELISA or MN.

Virus isolation and IAV antibodies in Wild Ibis

No viruses were isolated from the 118 samples collected from wild Ibis. Antibodies to the nucleoprotein (NP) were detected by bELISA in 58.6% of serum samples collected from wild birds (95% CI 53.3-63.9; Figure 2). The seasonal NP antibody prevalences for fall, spring, and summer were 58.9, 63.4, and 50.0%, respectively. These were not significantly different at a level of 5% over all three comparisons. In bELISA positive samples, neutralizing antibodies to one or more HA subtypes were detected in 95.9% (93.1-98.7) of 196 birds. Antibodies to H6, H12, H9, H5, and H1 were most commonly detected (39.4 – 61.6%). Antibodies to H3, H4, and H8 were rarely or never detected (≤ 2.0%). There was not a significant difference in the prevalence of neutralizing antibodies by season for any subtype (P>0.05).

DISCUSSION

A pathogen reservoir can consist of a system of interconnected maintenance and non-maintenance populations (Haydon *et al.* 2002). Species in the Anseriformes and Charadriiformes are well recognized components of the IAV reservoir system, yet we have a nascent understanding of how other species contribute to IAV maintenance dynamics. The unique life history of Ibis, particularly those that exploit urban environments during their non-breeding period, puts them in close contact with peridomestic and native waterfowl, gulls, and several other aquatic and semi-aquatic avian species, some of which they would never contact in natural settings. Our results

indicate that Ibis can be experimentally infected with IAV and that a high proportion have serologic evidence of previous natural infections. Given their shift from natural foraging to urban living, Ibis may become more significant to IAV epidemiology.

The relatively high antibody prevalence detected in wild Ibis is comparable with those detected in a number Anseriformes and Charadriiformes species (e.g. Maxted *et al.* 2012; Hall *et al.* 2014; Johnson *et al.* 2014; Hall *et al.* 2015; Kistler *et al.* 2015A; Samuel *et al.* 2015; Guinn *et al.* 2016; Wong *et al.* 2016). Interpreting serology in field investigations is often confounded by incomplete information about antibody dynamics in a given species. Knowing the length of antibody persistence following exposure would be helpful in determining if the antibody profile we detected in the Ibis population represents a continuous cycle of frequent exposures or if it reflects single, sporadic exposures that occur infrequently but remain detectable for many years. Regardless, our experimental challenge data suggests that every Ibis from which neutralizing antibodies were detected, underwent a productive infection that, in an experimental setting, was sufficient to infect other individuals in close proximity.

We suspect that mock-inoculated birds in the H6N1 and H11N9 group became infected through IAV-contaminated water, as we were able to isolate IAV in water samples at Day 4 and feces-contaminated water is considered a major route by which IAVs are transmitted in Anseriformes (Stallknecht *et al.* 2010). Shedding patterns in Ibis would have been best characterized by performing viral titrations on fecal samples. While we did not attempt this, the qrtPCR CT-values obtained from cloacal swabs were comparable and often much lower than values that were sufficient to infect Mallards (Brown *et al.* 2013). Furthermore, we observed no detectable change in behavior during

infection, suggesting that IAV-infected wild Ibis maintain the same movement, roosting, and foraging patterns, regardless of infection status.

Our failure to isolate IAV from free-ranging Ibis may seem to undermine our hypothesis. However, if infection dynamics within Ibis have a spatial and temporal aspect, as is well documented in Anseriformes and Charadriiformes (Krauss *et al.* 2004), our sampling effort was far from exhaustive. For example, *Stallknecht* et al. isolated only one IAV from 272 ducks sampled in December and January in coastal Louisiana (1990), and there are numerous examples of negative detections in Ruddy Turnstones outside the Delaware Bay “hot spot” where most viral isolations are reported in this species (Krauss *et al.* 2010). Therefore, additional sampling from Ibis and related species throughout the year in the southeastern United States is warranted.

A continued area of interest in IAV ecology is the role of avian communities on wintering grounds in maintaining IAVs and driving their evolution. In North American wintering grounds, this may be a function of interactions between migrating birds and resident avian species such as Mottled ducks (*Anas fulvigula*) in coastal Louisiana or egrets (*Egretta* sp.) and herons (Family Ardeidae) in California (Stallknecht *et al.* 1990; Siembieda *et al.* 2010). While the overall prevalence of infections at these areas is much lower than in northern latitudes, a higher diversity of subtypes is typically isolated, suggesting that wintering grounds are an important place for IAV reassortment and evolution (Hill *et al.* 2012). Our findings that Ibis are susceptible and have antibodies against various subtypes are consistent with this framework and provide a number of mechanisms by which Ibis and related wading birds may contribute to IAV dynamics on wintering grounds. At the very least, they may serve to maintain IAVs in the short-term

while also amplifying the viral load in aquatic environments. In addition, the diversity of neutralizing antibodies in wild Ibis indicates that coinfections are plausible which is the well-recognized mechanism for IAV reassortment. Finally; there was a marked paucity of neutralizing antibodies to H3, and H4, which are the most common subtypes isolated from ducks in northern latitudes (Wilcox *et al.* 2011). While this could be a function of Ibis' resistance to these subtypes, as demonstrated in our experimental challenge, it may also reveal a temporal mismatch. The vast majority of migrating waterfowl may clear infections with these subtypes by the time they reach wintering grounds, resulting in a void in which less common IAV subtypes can circulate in avian communities.

In the USA, agricultural and urban development has been disproportionately intense in wintering areas in the last century which may be resulting in a higher density of mixed species of avian communities in remaining aquatic habitats (Dahl *et al.* 2011; Hill *et al.* 2012). Wetlands in Florida have been especially affected, yet Ibis have been able to respond by utilizing urban areas to forage (Dahl 2005; Hernandez *et al.* 2016). Recent work demonstrated that a large portion of urbanized Ibis shed *Salmonella*, which may have important health consequences to humans and the numerous gull and duck species with which they have close contact (Hernandez *et al.* 2016). Our findings indicate that IAV is an additional concern. Consequently, the humans, domestic animals, and peridomestic species that Ibis are in direct contact with are now epidemiologically linked to IAV ecology in altered wintering areas and it is important to acknowledge the potential disease risk this presents.

There remains much to learn about what interacting forces and species maintain IAVs, drive their evolution, and pose risk to human and domestic animal health in

established and emerging systems (Stallknecht and Brown, 2017). The results presented here are a response to the call to “reconsider the role of other bird groups in the transmission, maintenance, and diversity of IAV (Caron *et al.* 2017).” By doing so, we provide footing for the next step forward in the conceptual knowledge of IAV.

ACKNOWLEDGEMENTS

We thank the numerous technicians, students, and volunteers that have helped with the ongoing fieldwork in Florida. We thank Laura Hollander and Alinde Fojtik for assistance with animal sampling. This project was partially funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, department of Health and Human Services, under contract HHSN272201400006C and the National Science Foundation Ecology and Evolution of Infectious Diseases (DEB-1518611). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

LITERATURE CITED

Arnal A, Vittecoq M, Pearce-Duvet J, Gauthier-Clerc M, Boulinier T, Jourdain E. 2014. Laridae: a neglected reservoir that could play a major role in avian influenza virus epidemiological dynamics. *Crit Rev Microbiol* 41:508-519.

Brown JD, Luttrell MP, Berghaus RD, Kistler W, Keeler SP, Howey A, Wilcox B, Hall J, Niles L, Dey A, et al. 2010. Prevalence of antibodies to type A influenza virus in wild avian species using two serologic assays. *J Wildl Dis* 46:896-911.

Brown JD, Stallknecht DE, Berghaus RD, Luttrell MP, Velek K, Kistler W, Costa T, Yabsley MJ, Swayne D. 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple

experimentally infected avian species. Clinical and Vaccine Immunology. 16:824-829.

Brown JD, Poulson R, Carter DL, Lebarbenchon C, Stallknecht DE. 2013. Infectivity of avian influenza virus-positive field samples for mallards: what do our diagnostic results mean? *Journal of Wildlife Diseases* 49:180-185.

Caron A, Cappelle J, Gaidet N. 2017. Challenging the conceptual framework of maintenance hosts for influenza A viruses in wild birds. *Journal of Applied Ecology* 54:681-690.

Dahl TE. Florida's wetlands: an update on status and trends 1985 to 1996. U.S. Department of Interior, Fish and Wildlife Service, Washington, D.C. 80 pp.

Dahl TE. 2011. Status and trends of wetlands in the conterminous United States 2004 to 2009. U.S. Department of Interior; Fish and Wildlife Service, Washington, D.C. 108 pp.

Ellis TM, Bousfield RB, Bissett LA, Dyrting KC, Luk GSM, Tsim ST, Sturm-Ramirez K, Webster RG, Guan Y, Peiris JSM. 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathology* 33:492-505.

Epstein JH, McKee J, Shaw P, Hicks V, Micalizzi G, Daszak P, Kilpatrick AM, Kaufman G. 2007. The Australian white ibis (*Threskiornis Molucca*) as a reservoir of zoonotic and livestock pathogens. *EcoHealth* 3:290-298.

Frederick PC, Bildstein KL, Fleury B, Ogden J. 1996. Conservation of large, nomadic populations of white ibis (*Eudocimus albus*) in the United States. Conservation Biology 10:203-2016.

Ghersi BM, Blazes DL, Icochea E, Gonzalez RI, Kochel T, Tinoco Y, Sovero MM, Lindstrom S, Shu B, Klimov A, Gonzalez AE, Montgomery JM. 2009. Avian influenza in wild birds, central coast of Peru. *Emerging Infectious Diseases* 15:935-938.

Guinn K, Fojtik A, Davis-Fields N, Poulson RL, Krauss S, Webster RG, Stallknecht DE. 2016. Antibodies to influenza A viruses in gulls at Delaware Bay, USA. Avian Dis 60:341-345.

Hall JS, Hallgrimsson GT, Suwannanarn K, Sreevatsen S, Ip HS, Magnusdottir E, TeSlaa JL, Nashold SW, Dusek RJ. 2014. Avian influenza virus ecology in Iceland shorebirds: intercontinental reassortment and movement. Infection, Genetics and Evolution 28:130-136.

Hall JS, Russell RE, Franson JC, Soos C, Dusek RJ, Allen RB, Nashold SW, TeSlaa JL, Jonsson JE, Ballard JR, Harms NJ, Brown JD. 2015. Avian influenza ecology in North Atlantic sea ducks: not all ducks are created equal. 10(12) e0144524.

Haydon DT, Cleaveland S, Taylor LH, Laurenson MK. 2002. Identifying reservoirs of infection: a conceptual and practical challenge. *Emerging Infections Diseases* 12:1468-1473.

Hernandez SM *et al.* 2016. Urbanized White Ibis (*Eudocimus albus*) as carriers of *Salmonella enterica* of significance to public health and wildlife. *PloS ONE* 11:1-22.

Hill NJ, Takekawa JY, Cardona CJ, Meixell BW, Ackerman JT, Runstadler JA, Boyce WM. 2012. Cross-seasonal patterns of avian influenza virus in breeding and

wintering migratory birds: a flyway perspective. *Vector-borne and Zoonotic Diseases* 12:243-253.

Johnson JA, DeCicco LH, Ruthrauff DR, Krauss S, Hall JS. 2014. Avian influenza virus antibodies in pacific coast red knots (*Calidris canutus roselaari*). J Wildl Dis 50:671-675.

Kistler WM, Gibbs SEJ, Stallknecht DE, Yabsley MJ. 2015. Wood ducks (Aix sponsa) as potential reservoirs for avian influenza and avian paramyxoviruses. Avian Pathog 44:167-174.

Kistler WM, Stallknecht DE, Lebarbenchon C, Pedersen K, Marks DR, Mickley R, DeeLiberto TJ, Yabsley MJ. 2015. Influenza A virus H5-specific antibodies in mute swans (*Cygnus color*) in the USA. Journal of Wildlife Diseases 51:523-526.

Krauss S, Stallknecht DE, Negovetich NJ, Niles LJ, Webby RJ, Webster RG. 2010. Coincident ruddy turnstone migration and horseshoe crab spawning creates an ecological 'hot spot' for influenza viruses. *P Roy Soc Lond B Biol Sci* 277:3373-3379.

Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG. 2004. Influenza A viruses of migrating wild aquatic birds in North America. *Vector-borne and Zoonotic Dis* 4:177-189.

Latorre-Margalef N, Brown JD, Fojtik A, Poulson RL, Carter D, Franca M, Stallknecht DE. 2017. Competition between influenza A virus subtypes through heterosubtypic immunity modulates re-infection and antibody dynamics in the mallard duck. PLoS Pathog. 13:e1006419.

Lee DH, Sharshov K, Swayne DE, Kurskaya O, Sobolev I, Kabilov M, Alekseev A, Irza V, Shestopalov A. 2017. Novel reassortment clade 2.3.4.4 avian influenza A (H5N8) virus in wild aquatic birds, Russia, 2016. *Emerging Infectious Diseases* 23:358-360.

Maxted AG, Luttrell MP, Goekjian VH, Brown JD, Niles LJ, Dey AD, Kalasz KS, Swayne DE, Stallknecht DE. 2012a. Avian influenza virus infection dynamics in shorebird hosts. *J Wildl Dis* 48:322-334.

Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WEP, Schutten M, Olsen B, Osterhaus ADME, Rouchier RAM. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *Plos Pathog* 3(5):e61. Doi:10.1371/journal.ppat.0030061

Murray MH, Kidd AD, Curry SE, Hepinstall-Cymerman J, Yabsley MJ, Adams HC, Ellison T, Welch CN, Hernandez SM. 2018. From wetland specialists to hand-fed generalist: shifts in diet and condition with provisioning for a recently urbanized wading bird. Phil. Trans. R. Soc. B 373: 20170100.

Nallar R, Papp Z, Epp T, et al. 2015. Demographic and spatiotemporal patterns of avian influenza infection at the continental scale, and in relation to annual life cycle of a migratory host. PLoS ONE 10(6): e0130662. Doi:10.1371/journal.pone.0130662

Niqueux E, Guionie O, Schmitz A, Hars J, Jestin V. 2012. Presence of serum antibodies to influenza A subtypes H5 and N1 in swans and ibises in French wetlands,

irrespective of highly pathogenic H5N1 natural infection. *Avian Diseases* 54:502-508.

Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus ADME, Fouchier RAM. 2006. Global patterns of influenza A virus in wild birds. Science 312:384-388.

Perez-Ramirez E, Gerrikagoitia X, Barral M, Hofle U. 2010. Detection of low pathogenic avian influenza viurese in wild birds in Castilla-La Mancha (south central Spain). *Veterinary Microbiology* 146:200-208.

Pfitzer S, Verwoerd DJ, Gerdes GH, Labuschagne AE, Erasmus A, Manvell RJ, Grund C. 2000. Newcastle disease and avian influenza a virus in wild waterfowl in South Africa. *Avian Diseases* 44:655-660.

Pohlmann A, Starick E, Harder T, Grund C, Hoper D, Globig A, STaubach C, Dietze K, Strevelow G, Ulrich RG, Schinkothe J, Teifke JP, Conraths FJ, Mettenleiter TC, Beer M. 2017. Outbreaks among wild birds and domestic poultry caused by reasserted influenza A(H5N8) clade 2.3.4.4 viruses, Germany, 2016. *Emerging Infections Diseases* 23:633-636.

Ramey AM, Poulson RL, Gonzalez-Reiche AS, et al. 2014. Evidence for seasonal patterns in the relative abundance of avian influenza virus subtypes in blue-winged teal (*Anas discors*). Journal of Wildlife Diseases 50: 916-922.

Redig PT and Goyal SM. 2012. Serologic evidence of exposure of raptors to influenza A virus. *Avian Diseases* 56:411-413.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol.* 27:493-497.

Samuel MD, Hall JS, Brown JD, Goldberg DR, Ip H, Baranyuk VV. 2015. The dynamics of avian influenza in lesser snow geese: implications for annual and migratory infection patterns. 25:1851-1859.

Sauer JR, Hines JE, Fallon JE, Pardierck KL, Ziolkowski DJ, Link WA. 2016. The North American breeding bird survey, results and analysis 1966-2015, Version 01.30.2015. USGS Patuxent Wildlife Research Center, Laurel, MD.

Siembieda JL, Johnson CK, Cardona C, Anchell N, Dao N, Reisen W, Boyce W. 2010. Influenza A viruses in wild birds of the pacific flyway, 2005-2008. *Vector-borne and zoonotic diseases.* 10:793-800.

Slemons RD, Johnson DC, Osborn JS, Hayes F. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Diseases 18:119-124.

Slusher MJ, Wilcox BR, Lutrell MP, Poulson RL, Brown JD, Yabsley MJ, Stallkecnht DE. 2014. Are passerine birds reservoirs for influenza A viruses? *J Wild Dis* 50:792-809.

Stallknecht DE, Brown JD. 2017. Wild bird infections and the ecology of avian influenza viruses In: *Animal Influenza,* Swayne DE, editor. John Wiley & Sons, Ames Iowa 2nd Edition John Wiley & Sons, Ames, Iowa. Pp. 153-176.

Stallknecht DE, Goekjian VH, Wilcox BR, Poulson RL, Brown JD. 2010. Avian influenza virus in aquatic habitats: what do we need to learn? Avian Diseases 54:461-465.

Stallknecht DE, Shane SM, Swank PJ, Senne DA, Kearney MT. 1990. Avian influenza viruses from migratory and resident ducks of coastal Louisiana. Avian Diseases 34: 398-405.

Webster R, Cox N, Storh K. WHO Manual on Animal Influenza Diagnosis and Surveillance. WHO: WHO Global Influenza Programme. 2002.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev 56:152-179.

Wilcox BR, Knutsen GA, Berdeen J, Goekjian V, Poulson R, Goyal S, Sreevatsan S, Cardona C, Berghaus RD, Swayne DE, Yabsley MJ, Stallknecht DE. 2011. Influenza-A viruses in ducks in northwestern Minnesota: fine scale spatial and temporal variation in prevalence and subtype diversity. PLoS One 6:e24010

Wong JK, Wilcox BR, Fojtik A, Poulson RL, Stallknecht DE. 2016. Antibodies to influenza A viruses in wintering snow geese (*Chen caerulescens*) in Texas. *Avian Diseases* 60:337-340.

Woo C, Kwon JH, Lee DH, Kim Y, Lee K, Jo SD, Son K, Oem JK, Wang SJ, Kim Y, Shin J, Song CS, Jheong W, Jeong J. 2017. Novel reassortant clade 2.3.4.4 avian influenza A (H5N8) virus in a grey heron in South Korea in 2017. *Arch Virol* 162:3887-3891.

Table 4.1. Summary of infection results obtained by challenging White Ibis (*Eudocimus albus*) with one of three influenza A viruses.

Virus		Viral Shedding[b]		Serology[c]	
		RT-PCR	VI	bELISA	MN
H3N8	Mock-inoculated	0/2[a]	0/2	0/2	0/2
	Inoculated	0/5	0/5	0/5	0/5
H6N1	Mock-inoculated	2/2	2/2	1/2	2/2
	Inoculated	5/5	5/5	3/5	5/5
H11N9	Mock-inoculated	2/2	2/2	1/2	2/2
	Inoculated	4/4	3/4	4/4	4/4

[a] Number positive/number in group.

[b] Viral shedding as determined by quantitative real-time reverse transcriptase PCR (qRT-PCR) and virus isolation (VI). A bird was considered positive by qRT-PCR if a cycle threshold value less than 40 was detected from oropharyngeal (OP) or cloacal (CL) swabs collected at 0, 2, 4, 6, 8, 10, or 14 days post inoculation (DPI). A bird was considered positive by VI if virus was isolated from OP or CL swabs at days 4, 10, or 14.

[c] Serologic results from sera collected at 14 DPI as determined by blocking enzyme linked immunosorbent assay (bELISA) and microneutralization (MN).

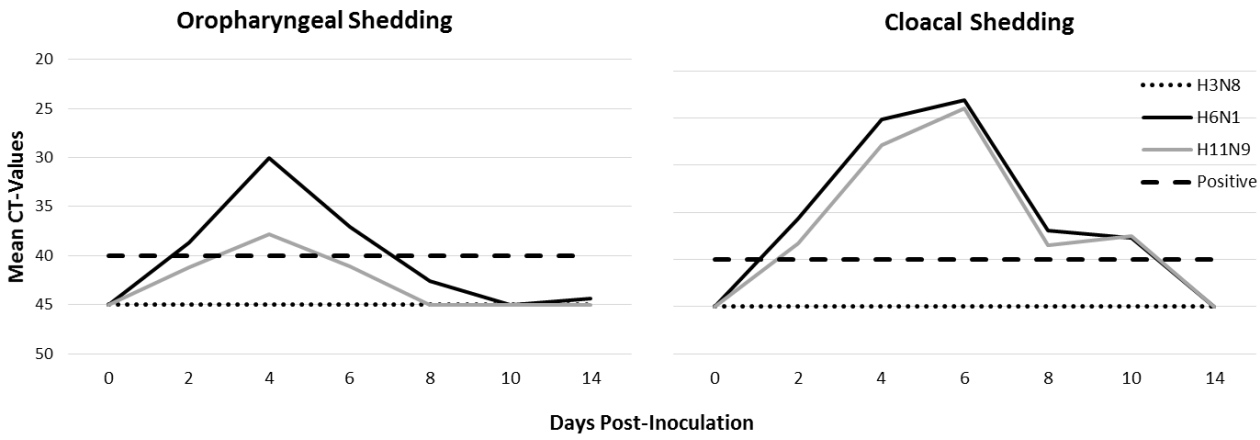

Figure 4.1: Mean quantitative real-time reverse transcriptase PCR cycle threshold (CT) values of oropharyngeal and cloacal swabs collected from American White Ibis (*Eudocimus albus*) experimentally challenged with H3N8 (n=5), H6N1 (n=5), and H11N9 (n=4). Values less than 40 are considered positive. Negative PCR results were assigned a value of 45.

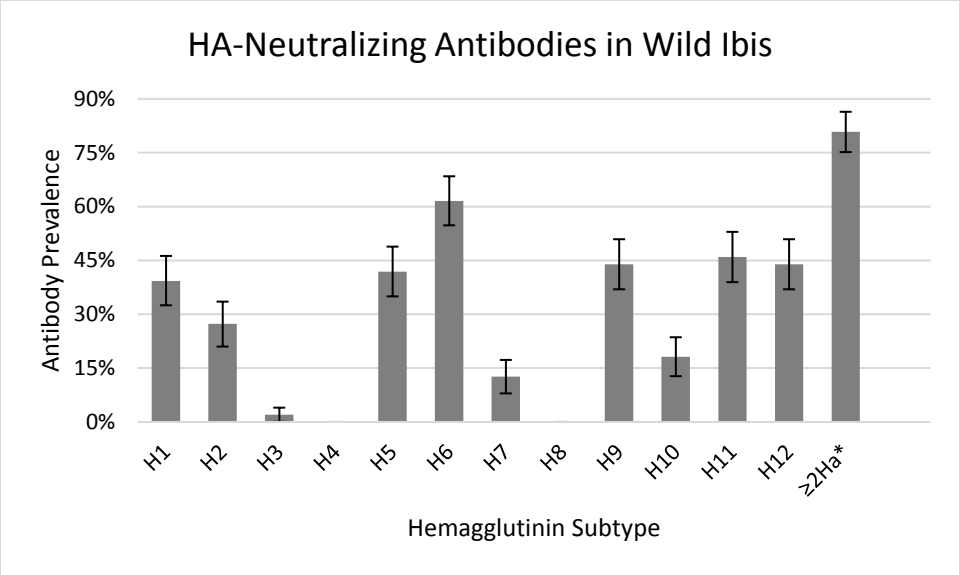

Figure 4.2: Prevalence of influenza A virus (IAV) neutralizing antibodies detected in serum samples from the 196 wild-caught American White Ibis (*Eudocimus albus*) that tested positive for antibodies by bELISA. The bar marked with an asterisk (*) represents the percentage of samples that neutralized more than one IAV subtype. Error bars represent 95% confidence limits.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Exactly one-hundred years ago, the world was in the throes of the worst pandemic in modern history. By the spring of 1919, at least 50 million people had died. It took another decade for researchers to conclude that the disease was caused by a virus (Smith *et al.* 1933). Thirty years later, a mortality event in terns in South Africa indicated that birds may carry IAVs (Becker 1966). This was confirmed, almost by chance, in 1974 (Slemons *et al.* 1974). Since then, there have been countless advances in our understanding of IAVs in wild birds, yet the threat of these viruses to human and domestic animal health is more imminent than ever and a comprehensive understanding of how IAVs exist within wild avian communities remains paramount. The objective of this project was to fill in a few knowledge gaps.

In the first study (Chapter 2), we sought to investigate a potential immunological basis for the phenomenon of shifting IAV subtype dominance observed at the well-established IAV hotspot, Delaware Bay (DE Bay). We hypothesized that the population of subtype-specific antibodies within the Ruddy Turnstone (RUTU) population would reflect the high rate of annual exposure and explain why a different HA subtype appears to dominate infections each year. We evaluated this by testing serum samples collected over five seasons for the presence of neutralizing antibodies to H1-H12 IAV. We then compared this to infection patterns over those same years. Finally, we compared the

antibody trends in RUTUs to Red Knots (REKNs), a bird that cohabits the beaches, yet is rarely found to be infected.

What was most noteworthy was the overall paucity of neutralizing antibodies detected in RUTUs. Only 36% (222/611) had neutralizing antibodies to H1-H12; whereas 66% (169/257) of REKNs had neutralizing antibodies to one or more subtypes. The pattern of antibody prevalence over the years was a poor correlate to infection patterns. However a general pattern did emerged. Subtypes that were isolated from birds in low prevalence every year (i.e. H6 and H11) had the highest prevalence of antibodies over the study period, while subtypes that were rarely or never isolated (i.e. H4 and H8) were not represented by neutralizing antibodies. Taken together, the results of this study suggest that population immunity influences which species is infected at Delaware Bay, indicate that IAV dynamics are subtype-dependent, and demonstrated the utility of the MN assay as a supportive tool for field research.

In the second study (Chapter 3), we attempted to address another large question surrounding the DE Bay phenomenon: the role of ducks and gull species that RUTUs come into contact with during the stopover. It has been widely speculated in the literature that ducks or gulls serve as a source of IAVs that cause annual endemics. At the very least, they may contribute to IAV dynamics by amplifying IAVs or contributing to subtype reassortment. However; the ease in which viruses can be readily transmitted among these species was unknown. That is to say, the degree to which host-adaptation permits or precludes infections in other hosts was unknown. The conservation status of RUTUs and logistical concerns prevented a challenge study involving RUTUs. Instead,

we challenged Mallards and Laughing Gulls (LAGUs) with RUTU-origin IAVs to assess this question.

Captive-reared gulls were challenged with RUTU-origin H6N1, H10N7, H11N9, H12N4, and H13N6 IAV; as well as Mallard-origin H6N1 and H11N9. We challenged captive-reared Mallards with the same viruses, with the exception of H13N6. At a biologically plausible challenge dose (10^4 EID_{50}/0.1ml), one of five gulls challenged with both H6N1 IAVs briefly shed virus. The remaining gulls were resistant to infection with all viruses. In contrast, all Mallards shed virus, with the exception of the H12N4 challenge group, in which no birds were infected. These results demonstrate that while either ducks or gulls may serve as a source of IAVs that infect RUTUs at Delaware Bay, host-adaptation of IAVs to RUTUs may compromise their ability to be transmitted back to gulls.

For the final study (Chapter 4), we examined the importance of an avian species that is not classically included within the IAV reservoir paradigm. The American White Ibis is a resident of coastal habitats in southeastern United States with a unique life history that results in regular contact with humans and a variety of wild aquatic, domestic, and peri-domestic avian species. Because of this, investigating the potential for Ibis to be exposed and infected with IAV was warranted.

To investigate this: we experimentally challenged wild-caught, captive-reared Ibis with three low pathogenic IAVs; tested wild Ibis for IAV; and serologically tested wild Ibis for antibodies to IAV. Ibis were highly susceptible to experimental challenge with H6N1 and H11N9 IAVs with cloacal shedding lasting an average of six days. All thirteen infected birds seroconverted by 14 days post infection as determined by

microneutralization. In contrast, no Ibis challenged with H3N8 were infected. We tested 118 swabs and 330 serum samples from Ibis captured in southeastern Florida for IAV infection and antibodies to IAV, respectively. Although no IAVs were isolated, 59% were antibody-positive by blocking enzyme-linked immunosorbent assay (bELISA). Neutralizing antibodies to H1-H12 were detected in 96% of bELISA positive birds and 81% tested antibody positive to two or more HA subtypes, indicating that exposure to multiple IAVs is common. These results provide compelling evidence that Ibis are susceptible and naturally infected with IAV and may represent a component of the IAV natural reservoir system.

Addressing the “Spanish flu” of last century required massive leaps in our understanding, some aspects of which took decades. Today, the magnitude of remaining questions may be comparatively small, but many persist and more continue to emerge. The overall objective of this project was to provide a few more details to our conceptual framework of IAVs in wild birds. By taking on these questions as they surface, we ensue that future challenges presented by IAV require small steps rather than large bounds.

REFERENCES

Becker WB. 1966. The isolation and classification of Tern virus: Influenza Virus A/Tern/South Africa/1961. *Journal of Hygiene* 64:309-320.

Slemons RD, Johnson DC, Osborn JS, Hayes F. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Diseases 18:119-124.

Smith W, Andrewes CH, Laidlaw PP. 1933. A virus obtained from influenza patients. *The Lancet* 222:66-68.