PERSISTENCE, TRANSMISSION, AND MOVEMENT OF INFLUENZA A VIRUSES: A HOST AND ENVIRONMENTAL PERSPECTIVE

by

REBECCA LYNNE POULSON

(Under the Direction of David E. Stallknecht)

ABSTRACT

Transmission and maintenance of influenza A viruses (IAVs) between and across species are dependent on viruses remaining infectious while associated with both host and environmental components of the maintenance cycle. Transmission and maintenance of IAVs in wild aquatic avian reservoirs, primarily associated with the orders Anseriformes (ducks, geese) and Charadriiformes (gulls, shorebirds), are also dependent on short and long-distance bird movements which serve to link susceptible hosts. The first research objective addressed the potential role of environment in cross species transmission, specifically by characterizing the effects of temperature, pH, and salinity on the persistence and infectivity of swine and pandemic human IAVs in a distilled water model. The viruses did not differ significantly in their responses to these variables, indicating no apparent environmental adaptation as related to the movement of IAV into different host systems. The second and third objectives were aimed at understanding environmental persistence, transmission, and maintenance of IAV in a unique natural system: shorebirds at Delaware Bay. The stabilities of two of 19 IAV isolated from environmental sources and one Ruddy Turnstone (*Arenaria interpres*) at Delaware Bay were assessed under temperature, pH, salinity, and substrate conditions comparable to those found in the local

microenvironment. All viruses remained infectious in a sand milieu for seven days, indicating that the beach environment can support short-term maintenance of IAV. Finally, the genomes of IAV isolated from shorebirds at overwintering sites in the southeastern United States were compared to those isolated from shorebirds at Delaware Bay several weeks to months later. A high degree of genetic similarity was detected for all eight gene segments, though the transfer of a homologous genome from winter to spring sites was not identified. This indicates that migrating Ruddy Turnstones are one source for introducing IAV into the Delaware Bay ecosystem. Once introduced, these viruses reassort with and are assimilated into the larger IAV gene pool at this unique site. Results of these projects allowed for the deconstruction of the factors responsible for persistence and movement of virus, and the subsequent reconstruction of the dynamics of these mechanisms at an ecological hotspot for IAV – Delaware Bay.

INDEX WORDS: *Arenaria interpres*, Delaware Bay, Environment, Host, Influenza A virus, Migration, Pandemic influenza, Persistence, Ruddy Turnstone, Swine influenza

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DEDICATION

This work is dedicated to the memory of my grandparents, whose steadfast and enduring love will never be forgotten.

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CHAPTER 1

INTRODUCTION

Wild birds in the orders Anseriformes and Charadriformes are recognized as the primordial reservoirs of all influenza A viruses (IAVs); these viruses not only can infect birds but also humans, swine, horses, and a suite of other mammalian hosts. The ability of these viruses to persist, remain infectious, and be transmitted represents a complex interaction of host, pathogen, and environmental factors. Currently such interactions are poorly understood.

It is well-established, based on results from laboratory experiments, that avian IAV can remain infective for days to months, and sometimes even years, depending upon the environmental milieu in which they exist (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). Although wild bird reservoirs represent the historic source of IAVs that infect domestic animals and humans, the transmission and adaptation mechanisms that facilitate efficient cross species transmission and establishment in new host populations are not well defined. Predominant routes of transmission vary between host populations. Likewise, the extent of IAV environmental stability needed for efficient transmission within these varied populations also may differ.

The potential for viruses such as pandemic H1N1 to emerge as a result of reassortment between human, swine, and avian viruses involves unlikely transmission events. With birds, transmission of IAV occurs primarily via a fecal-oral route (Ito and Kawaoka, 2000; Webster et al., 1977) in which water plays a large role. While the main mode(s) of transmission of influenza between humans is believed to be via an aerosol, droplet, or direct contact route (Brankston et

al., 2007; Olsen, 2002), indirect transmission through contaminated surfaces may also contribute to viral spread. Likewise, swine influenza viruses are primarily transmitted via direct contact or through aerosols or droplets (Olsen et al., 2006), but indirect contact through fomites cannot be discounted, especially with regard to movement within or between production facilities. The inter- and intra-species transmission and maintenance of IAV is dependent upon factors at the host, viral, and environmental levels, and regardless of transmission route, IAV must remain infectious in the environment that separates individual hosts.

Maintenance of IAVs in a given system is related to not only their ability to persist and remain viable in a given environment, but also to be present in a manner by which they are readily available to susceptible hosts. The unique foraging strategies of Ruddy Turnstones (*Arenaria interpres*) coupled with their propensity to amplify IAV provides an opportunity to explore the interplay where the natural environmental persistence of influenza, host behavior, and viral transmission intersect. These shorebirds utilize a foraging behavior that involves probing and, as their name implies, "turning stones" over in an effort to locate invertebrates, and when available, as is the case on the shores of Delaware Bay, USA during the month of May, energy-packed horseshoe crab (*Limulus polyphemus*) eggs (Tsipoura and Burger, 1999). They probe as deep as several centimeters into sand and cobble (Colwell and Landrum, 1993). It is conceivable that burial of virus, either by wave and wind action, or in horseshoe crab depressions at even a shallow depth in surrounding sand and substrate could protect it from the harsh effects of ultraviolet (UV) light, salt water, and waves. Additionally, a protective matrix of surrounding substrate may potentially stabilize IAV at a temperature cooler than that found at the sand-air interface. Recovery of IAV from sand cores during routine surveillance for influenza at Delaware Bay over several collection seasons allowed us an opportunity to examine this idea in a

laboratory setting. Coupled with real time temperature profiles from sand at several Delaware Bay coastal locations, we examined the persistence profiles of these viruses within a relevant sand substrate in the laboratory. If these viruses are capable of even short-term environmental persistence, it is conceivable that this tenacity could play a role in the transmission and maintenance cycle of IAV in the Delaware Bay ecosystem.

Birds migrate; pathogens do too. If we explore the viral diversity of IAV along a migratory pathway, will similar viruses or viral gene segments be identified at points separated spatially and temporally? Delaware Bay represents a unique system in which large numbers of shorebirds and gulls congregate, in densities approaching 210 birds per m^2 (Clark et al., 1993; Gillings et al., 2007), for the purpose of replenishing energy stores during their migration from wintering grounds in South America and the southern United States to their Arctic breeding grounds. Delaware Bay (in New Jersey and Delaware) is an ecological hotspot for IAV (Krauss et al., 2010), where shorebirds, especially Ruddy Turnstones (*Arenaria interpres*), serve to amplify and maintain the viruses for the several weeks during migration. Despite extensive surveillance for IAV at this site since 1985 (Krauss et al., 2010; Stallknecht et al., 2012), it remains unknown how the viruses become so rampant in the system in such a short window of time or diversify into dozens of different hemagglutinin (HA) and neuraminidase (NA) combinations. The source of these viruses also is unknown, and it is still to be determined whether migrating shorebirds migrate to Delaware Bay with these viruses, are infected from a local source, or both.

This dissertation research has both laboratory and field components, and is aimed at looking at individual pieces of this puzzle to better understand (1) the ability of influenza viruses of avian, swine, and human origin to remain viable in the environment; (2) the role the natural environment may have in maintaining and facilitating transmission of IAV; and (3) the potential

migration of viruses with their avian shorebird hosts. **Chapter 1** provides a brief introduction to the research projects reported herein, and a comprehensive Literature Review is provided in **Chapter 2** to provide a framework for research details presented in **Chapters 3 – 5**, described below. **Chapter 6** serves as a summary of dissertation results.

The specific aims of this body of work are to:

1. Evaluate the environmental tolerances – temperature, pH, and salinity - for IAV from swine and human hosts using a model distilled water system. These variables have been shown to modulate the duration of infectivity of IAV in water. The working hypothesis is that there will be differences in the environmental tolerances of IAV derived from different host species as it is expected that some degree of environmental adaptation is required to adapt to unique environments or transmission routes associated with these varied hosts. This work is presented in **Chapter 3** of this dissertation;
2. Evaluate the maintenance and transmission of avian IAV in shorebirds in their natural environment. The working hypothesis is that viruses are able to persist on local beach environments and would be readily available for transmission to foraging shorebirds. This work is presented in **Chapter 4** of this dissertation;
3. Investigate the genetic relatedness of viruses isolated at different points in space and time along a migratory flyway. The working hypothesis is that there is a low-level circulation of IAV in shorebirds during winter months; these viruses can move with birds during spring migration and become available to large numbers of susceptible birds when population density

dramatically increases at the Delaware Bay IAV ecological “hotspot”. This work is presented in **Chapter 5** of this dissertation.

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CHAPTER 2

LITERATURE REVIEW

INFLUENZA A VIRUS

Influenza viruses A, B, and C, together with the tick-borne *Thogotovirus*, salmonid pathogen *Isavirus* (infectious salmon anemia virus), and recently proposed *Quarjavirus* (Presti et al., 2009) and influenza virus D (Hause et al., 2013; Hause et al., 2014) represent the seven genera of the viral family Orthomyxoviridae. Influenza A viruses (IAVs) are distinguished from types B, C, and D based upon antigenic and genetic differences in their matrix (M) and nucleocapsid proteins (NP). These four influenza genera differ in host range and pathogenicity, likely diverging from one another thousands of years ago (Taubenberger and Kash, 2010). While type A viruses can infect a wide variety of animals including swine, horses, humans, seals, and birds, types B and C are adapted primarily to humans, with a few exceptions; influenza B viruses have been isolated from seals (Osterhaus et al., 2000), and influenza C viruses from dogs and swine (Wright et al., 2007). Influenza D viruses have recently been described in cattle and swine (Jiang et al., 2014; Collin et al., 2015; Ducatez et al., 2015).

IAVs are enveloped, single-stranded, negative sense ribonucleic acid (RNA) viruses with a host cell-derived lipid membrane. The IAV genome is made up of eight independent segments, is approximately 14kb, and codes for 10-11 different proteins. It is pleomorphic; shapes range from small and spherical with a diameter of 80-120nm, to long and filamentous, reaching lengths greater than 300nm depending upon viral genetics and the cell type in which the virus is grown

or host from which it is isolated. IAVs are classified based upon serologic reactions to two predominant antigenic glycoproteins: hemagglutinin (HA) and neuraminidase (NA), which are attached to the surface of the viral envelope in a layer of nearly 500 morphological spikes in an HA:NA ratio of four or five to one (Lamb and Krug, 2001). Other structural proteins include the nucleocapsid protein (NP), matrix protein 1 (M1), the membrane ion channel protein (M2) embedded in the viral envelope, and the proteins that form the polymerase complex: polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA); there are also two nonstructural proteins, virulence factor NS1 (host interferon antagonist) and NS2 (also known as the nuclear export protein, NEP) (Palese and Shaw, 2007).

The HA is a rod-shaped trimer that mediates binding to the host cell receptor and entry of the virus into host cells. It is synthesized as a precursor polypeptide (HA_0) (Chen et al., 1998) which must be enzymatically cleaved by host cell proteases into subunits HA1 and HA2 in order to activate membrane fusion and infectivity. The NA, a mushroom-shaped tetramer, is responsible for release of virus from the host cell upon completion of replication through cleavage of a terminal sialic acid residue on the host cell. The third of the surface proteins, M2, mediates viral uncoating after entry into the host cell. The lipid membrane together with these three integral proteins overlay the inner M1 shell, which is the most abundant protein in influenza viruses (Lamb and Krug, 2001). M1 encloses the viral core and gives rigidity and strength to the viral envelope while also having regulatory function during viral replication. Internal to M1 is the ribonucleoprotein (RNP) complex. The RNP is the minimal functional unit of replication (Bouvier and Palese, 2008; Lee and Saif, 2009), and consists of the viral RNA segments coated with NP as well as the RNA-dependent RNA polymerase, made up of PA, PB1, and PB2. The NS1 and NS2 proteins, produced by influenza viruses, have cell cycle regulatory

functions. Some IAV produce an eleventh protein, PB1-F2, which serves as a proapoptotic accessory protein to enhance virus-induced cell death in the host (Chen et al., 2001; Gibbs et al., 2003).

Historically, IAVs have been differentiated into subtypes through serologic reactions to the surface HA and NA proteins; subtyping can now be accomplished via genetic characterization. To date, 16 different HA (H1 – H16) and nine different NA (N1 – N9) subtypes have been described in wild birds (Ottis and Bachmann, 1983; Kawaoka et al., 1990; Fouchier et al., 2005; Alexander, 2007; Munster et al., 2007). Recently, two additional subtype combinations were reported from bats (H17N10 and H18N11) (Tong et al., 2012; Tong et al., 2013). Hypothetically, the HA and NA subtypes circulating in wild birds can interact in any combination with the potential for 144 possible permutations; over 110 such possible HA/NA combinations have been described in wild birds (Alexander, 2007; Munster et al., 2007; Olson et al., 2014). IAV can be further characterized based on the cleavage structure of the HA precursor (HA_0) (Steinhauer, 1999). Low pathogenicity (LP) IAV have a single arginine residue at the precursor cleavage site, while high pathogenicity (HP) viruses have a multibasic cleavage site. Categorization into the LP or HP pathotype is further based upon the severity of the illness the virus causes in poultry. The World Organization for Animal Health (OIE) defines HP IAVs as having an intravenous pathogenicity index in six-week old chickens greater than 1.2, or causing mortality in $\geq$ 75% of four- to eight-week old chickens infected intravenously within 10 days (Swayne and Suarez, 2000). Of the 16 different HA subtypes, only H5 and H7 are known to have the HP IAV phenotype (Suarez and Swayne, 2008).

IAVs are highly mutable, can evade host immune defenses, and are also successful at invading new host species (Vandegrift et al., 2010). The RNA-dependent RNA polymerase is

error-prone, lacks exonuclease proofreading ability (Holland et al., 1982) and has an inherently high error rate, resulting in as many as 10^{-4} mutations per site (Steinhauer and Holland, 1987). The potential for this dynamic evolutionary change is supported by substitution rates ranging from $1.8 – 8.4 \times 10^{-3}$ substitutions per nucleotide site per year (Chen and Holmes, 2006); this mutation rate varies by gene segment, and is typically highest for the surface proteins, HA and NA (Saitou and Nei, 1986), due largely to host immune selection pressure (Webster et al., 1992). Any errors made during nucleotide polymerization cannot be corrected, and can become incorporated into the newly formed RNA. While most mutations at this level will be silent or generate stop codons, some will, by chance, result in amino acid changes in the translated proteins. Mutations that affect amino acids in the antigenic portions of the HA and NA may provide a selective advantage by allowing the virus to modify antigenicity (Das et al., 2011) and evade the host immune response. Over time, the gradual accumulation of such changes can lead to antigenic drift, altering the immunogenic epitope of the surface proteins. IAV can also undergo more sudden and disruptive changes, known as antigenic shift, through reassortment, direct transmission of a virus from one host species to another, or reintroduction of an older influenza strain into a naïve population. The segmented nature of the genome allows for exchange of large genomic segments between viruses infecting the same cell at the same time (reassortment). Theoretically, 256 (2^8) possible combinations of the eight individual gene segments are possible from the reassortment of two parental viruses. Evolution is most apparent in the HA and NA surface glycoproteins of IAV, but all gene segments are prone to mutation and subjected to evolutionary pressures.

AVIAN INFLUENZA AND TRANSMISSION

Though IAV can infect a variety of animals, wild birds, primarily those of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, shorebirds, and terns), are considered the most important avian groups in the ecology and epidemiology of IAV. Worldwide, IAV has been detected in more than 105 wild bird species from 26 different families (Stallknecht and Shane, 1988; Olsen et al., 2006; Stallknecht and Brown, 2009). LP IAV does not cause overt disease in waterfowl though mallards naturally infected with IAV were shown to have reduced body mass (Latorre-Margalef et al., 2009). There also were no long-term effects of infection with LP IAV on the annual survival of Ruddy Turnstones at Delaware Bay (Maxted et al., 2012b). In experimental settings most infections remain asymptomatic (Kida et al., 1980; Kawaoka et al., 1988). This seemingly avirulent relationship between wild birds and IAV has likely evolved from years of viral/host adaptation which has served to facilitate the perpetuation of the virus in the reservoir (Bean et al., 1992; Webster, 1998).

Transmission of IAV both among and between species is controlled by factors at the viral, host, and environmental levels. An indirect fecal-oral route involving fecal-contaminated water in shared aquatic habitats has long been considered the primary route of transmission for IAV in wild birds (Webster et al., 1992), and recent work highlights the important role of water-borne transmission in driving IAV infection dynamics at a waterfowl site in France (Roche et al., 2009). Preferential replication of avian IAV occurs in the cells in the lower gastrointestinal (GI) tract and bursa of wild birds; this is due to the binding specificity of the viruses for galactose a-2,3 sialic acid (a-2,3 SA) linkages found on epithelial cells of the avian GI tract (Suzuki et al., 2000). High concentrations of virus can be excreted in the feces (Webster et al., 1978), and though viral shedding typically lasts 3 – 12 days (Costa et al., 2010; Brown et al., 2012),

experimentally infected Pekin ducks (*Anas platyrhynchos*) shed infectious virus for up to 28 days (Hinshaw et al., 1980). As such, infected birds may contaminate nearby surface waters with IAV via their excreta; if viruses are able to survive in the conditions of the surrounding environmental background, they may be available in high enough concentration to infect susceptible birds upon subsequent drinking or foraging efforts (Delogu et al., 2003). This may be especially important in the transmission of IAV to dabbling ducks which feed on surface waters and have been implicated in the perpetuation of most HA subtypes (Webster et al., 1992; Krauss et al., 2007; Munster et al. 2007). "Cloacal-drinking" (uptake of water via the cloaca) has also been proposed as a means of fecal-fecal transmission of IAV (Daoust et al., 2011). The isolation of IAV from surface waters, ice, and aquatic substrate (Hinshaw et al., 1980; Halvorson et al., 1985; Sivanandan et al., 1991; Zhang et al., 2006; Lang et al., 2008; Stallknecht et al., 2010; Deboosere et al., 2011), the identification of nearly homologous H6N9 IAVs isolated over a year apart in the Czech Republic (Nagy et al., 2014), and the isolation of highly identical HA and NA H3N2 genes from a sentinel system in Germany more than three months apart (Globig et al., 2013) further support recent studies that suggest the ability of IAV to be maintained in the environment likely has a role in its transmission cycle (Breban et al., 2009; Rohani et al., 2009; Brown et al., 2013; Roche et al., 2014). Environmental durability of IAV may also serve to: increase genetic diversity with the environment serving as a source for multiple subtypes and strains (Roche and Rohani, 2010), preserve older strains for subsequent reintroduction to susceptible hosts (Nagy et al., 2014), and sustain transmission on a longer time-scale than is possible when IAV shedding birds are no longer present on the habitat (Roche and Rohani, 2010; Roche et al., 2014). However, the precise roles of the biogeochemical and physical environment in this process remain poorly understood.

In the laboratory, wild-bird origin IAV are capable of remaining infectious from days to years in model water systems depending upon simulated environmental conditions (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). It was first demonstrated that a single duck-origin IAV could persist in non-chlorinated river water for more than 30 days at 0°C (Webster et al., 1978). Since then, ample research in this area has aimed at elucidating the relationships between viral persistence and environmental variables, in both distilled (Brown et al. 2007; Brown et al., 2009; Negovetich and Webster, 2010; Lebarbenchon et al., 2011) and freshwater (Bradley et al., 2011; Keeler et al., 2013) models. In nature, avian IAV are likely to encounter temperatures ranging from 4°C – 37°C. Within this range, viruses persist longest at colder temperatures, displaying decreasing infectivity with increasing temperatures (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009; Bradley et al., 2011; Lebarbenchon et al., 2011). Infectivity of IAV tends to be most robust at a slightly basic pH (7.4 – 8.2); virus incubated in low pH environments undergoes a conformational change in the HA which causes a decrease in infectivity (Scholtissek, 1985; Junankar and Cherry, 1986; Reed et al., 2009). Most avian IAV assessed experimentally tend to persist for comparable amounts of time in fresh to slightly brackish water (0 – 20ppt), but increasing the salt concentration above 20ppt yields a considerable decline in viral tenacity. In distilled water systems, subtype and strain related variation in these general responses has been noted (Brown et al., 2007; Brown et al., 2009; Negovetich and Webster, 2010), and is most apparent at cold temperatures. It should also be noted that these are generalizations about persistence of viral populations in the presence of a singly manipulated laboratory variable in distilled water. With multiple variables, or when biotic components are introduced into the system, the relationship between persistence and the environment becomes more complicated

(Brown et al., 2009; Lebarbenchon et al., 2011; Keeler et al., 2013; Nielsen et al., 2013) and warrants further investigation.

Introduction of mechanical disruption of virus particles, as is the case with freeze-thaw cycles, has been shown to quickly reduce IAV infectivity (Stallknecht et al., 2010; Lebarbenchon et al., 2011) and may play a deleterious role in the ability of IAV to over-winter; this would have implications for long-term maintenance and transmission of IAV in natural open water systems that freeze during winter months. However, IAV of different subtypes and origins have been shown to remain infectious longer in lake sediments and sand (66 – 394 days at 0°C) (Nazir et al., 2011) than in lake water alone (31 – 35 days at 0°C) (Nazir et al., 2010). This association with substrate may help to stabilize viruses and make them more resistant to mechanical disruptive forces, but this remains an underexplored area of study. Biotic components in natural water systems also affect the availability and infectivity of IAV to susceptible hosts. In a laboratory setting, filter-feeding bivalves (clams; *Corbicula fluminea*) were shown to remove and reduce the titer of both LP and HP IAV (Faust et al., 2009) and the planktonic filter-feeding *Daphnia* greatly reduced the concentration of the genomic material of an avian-origin H3N8 IAV in water (Meixell et al., 2013).

While the mechanisms of IAV dispersal remain largely undefined, the movement of avian IAV can be linked, at least partially, to the movement of host species through intercontinental migration events (Ramey et al., 2010; Bahl et al., 2013; Ramey et al., 2015; Hill et al., 2016), or through movements on a smaller spatial scale (foraging, roosting, etc.). In a generic sense, migration connects many species across time and space, including birds and the pathogens that they may be harboring. It holds that the more negligible the effect a pathogen has on its host, (ex. host-pathogen combinations with low virulence and/or high host tolerance for the pathogen)

(Altizer et al., 2011), the further it can be carried, and potentially be introduced to different bird populations and multi-species assemblages. Additionally, the use of common migratory stopover sites may provide the potential for step-wise movement of IAV along a migratory pathway (Hill et al., 2016). In wild birds, IAVs circulate throughout the year (Stallknecht et al., 1990c, Hanson et al., 2005) with documented peaks and predictable patterns in prevalence and subtype diversity, usually occurring at staging locations. This is the case with dabbling ducks in the fall (Hinshaw et al., 1980; Webster et al., 1992; Hanson et al., 2003; Wallensten et al., 2007; Wilcox et al., 2011; Ramey et al., 2014) and shorebirds in the spring at Delaware Bay in the northeastern United States (Krauss et al., 2010).

SWINE INFLUENZA AND TRANSMISSION

While wild birds host a wide diversity of HA and NA IAV combinations, swine are host to a limited but dynamic group of H1N1, H1N2, and H3N2 IAV subtypes that circulate worldwide (Olsen, 2002). The first H1N1 swine influenza virus (SIV) was isolated from swine in 1930 (Shope, 1931), and is antigenically and genetically similar to the reconstructed 1918 H1N1 virus (Tumpey et al., 2004) that caused millions of human deaths. This viral lineage is referred to as "classical swine influenza" (cSIV), and has remained fairly antigenically stable for nearly 100 years (Vincent et al., 2006). Prior to 1998, only cSIV was known to circulate in the United States. However, around 1998, cSIV reassorted with a contemporary human H3N2 IAV, resulting in the isolation of a reassortant H3N2 (rH3N2) from swine in North Carolina that contained components of both swine (PB2, PA, NP, M, NS) and human (PB1, HA, NA) IAV (Zhou et al., 1999). Not long after the detection of rH3N2, a triple reassortant H3N2 (trH3N2) was isolated from swine herds that had internal PA and PB2 gene segments of avian origin (Zhou

et al., 1999). This trH3N2 stabilized, and continues to circulate in North American swine populations today (Zhou et al., 1999; Webby et al., 2000). Further genetic mixing of trH3N2 and cSIV H1N1 gave rise to additional triple reassortant swine H1N1 and H1N2 viruses. In 2009, novel swine-origin pandemic (pdm) H1N1 emerged and was determined to be a quadruple reassortant composed of gene segments not previously reported in swine or human IAV; it included Eurasian swine-origin NA and M genes (Garten et al., 2009; Smith et al., 2009b).

Compared to avian IAV which remain largely asymptomatic in the natural host, swine influenza is typically characterized by nearly 100% morbidity – symptoms include coughing, wheezing, lethargy, depressed appetite, and elevated temperatures in infected populations – and less than 1% mortality (Kothalawala et al., 2006; Vincent et al., 2008). It is both an economically and epidemiologically important acute respiratory disease of swine. Large amounts of virus are shed in the nasal discharges of infected swine (Landolt et al., 2003) and transmission occurs by a respiratory route. The incubation period is 1 – 3 days, and recovery typically occurs quickly from 4 days to one week after onset. Swine have an abundance of a-2,3 SA and galactose a-2,6 sialic acid (a-2,6 SA) linkages (the latter preferred by human influenza viruses) in their respiratory tracts (Ito and Kawaoka, 2000; Nelli et al., 2010). As such, they have been considered a prime "mixing vessel", or intermediate host, for IAV of avian, human, and swine origin (Webster et al., 1992; Scholtissek, 1995). As related to the emergence of new influenza viruses, if swine become coinfected with influenza viruses of different origins, the segmented viral genome can reassort, and potentially give rise to novel IAV. Occasionally, SIV have infected humans, and of note most recently, given rise to human pandemics like that caused in 2009 by pdm H1N1 (Garten et al., 2009; Smith et al., 2009a; Smith et al., 2009b).

HUMAN INFLUENZA AND TRANSMISSION

Influenza pandemics dot and underscore the pages of human history. Much like SIV in swine, infection with human influenza A often causes acute respiratory symptoms; historically, viruses of only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have caused sustained, person-to person transmission in humans. Annually in the United States, a typical endemic influenza season results in approximately 200,000 hospitalizations and claims 36,000 lives (Taubenberger and Kash, 2010). Novel influenza viruses can emerge through antigenic shift and drift and present new antigenic epitopes to a naïve population, causing significant increases in morbidity and mortality. The emergence of such influenza outbreaks and pandemics can lead to the infection of a large proportion of the world's population. The 1918 H1N1 "Spanish Flu" is considered one of the deadliest disease outbreaks in human history; in 16 months, the pandemic killed more than 50 million people worldwide, and infected more than three times that many (Patterson and Pyle, 1991). In April 2009, a new pandemic strain of human H1N1 IAV was identified in Mexico, and quickly spread across the globe. The pdm H1N1 virus represented a unique reassortment of gene segments from both North American and Eurasian swine origin; the NA and M genes of pdm H1N1 are derived from the European avian-like swine H1N1 lineage, and the other six genes (PB2, PB1, PA, HA, NP, and NS) from the North American swine triple reassortant H1N1 lineage (Garten et al., 2009; Taubenberger and Kash, 2010). This H1N1 virus now circulates in the endemic human influenza pool (World Health Organization, 2014).

Multiple IAV transmission models have been investigated in humans, none of which are necessarily mutually exclusive. Transmission events by aerosols, large droplets, and direct or indirect contact (the latter via fomites) (Brankston et al., 2007) are all mediated by the ability of

the virus to remain viable and infectious in the environment. Nearly 50 years ago, it was reported that aerosolized IAV strains of human and swine origin decayed more quickly than those derived from equine or avian sources (Mitchell et al., 1968; Mitchell and Guerin, 1972). Since then, much research has focused on elucidating the mechanisms by and conditions under which IAV is transmitted. Low relative humidity has been shown to be key in increased IAV aerosol stability (Schaffer et al., 1976), and aerosol transmission of human IAV is most efficient under cold and dry conditions in a guinea pig model (Lowen et al., 2007). Due to the close proximity of infected and susceptible individuals required for contact transmission, this mechanism is largely unaffected by an increase in ambient temperature from 20°C to 30°C (Lowen et al., 2008). It has also been shown that the 2009 pdm H1N1 virus is not transmitted more efficiently at elevated humidity or temperatures when compared to seasonal H3N2 human strains (Steel et al., 2011). IAV have been shown to persist on a variety of surfaces including paper currency, tissues, glass, and stainless steel (Bean et al., 1992; Noyce et al., 2007; Thomas et al., 2008; Wood et al., 2010; Dublineau et al., 2011) with an inverse relationship between increasing surface porosity and decreasing infectivity. If surfaces, especially non-porous ones, are heavily contaminated with virus, persistence times may be adequate to allow for transmission to uninfected individuals via fomites (Bean et al., 1992). It is important to note that the potential environmental role in transmission is not restricted solely to the stability of the virus particle itself, but can also have effects at the level of host (immune response, behavior) and delivery vehicle (particle sizes: desiccation versus accumulation) (Tellier, 2006; Lowen et al., 2007; Lowen and Steel, 2014). In a water medium, seasonal human H1N1 and H5N1 viruses derived from a mammalian cell line remained infectious for 15 and 12 days, respectively, at a temperature of 35°C, while the same viruses propagated on an avian cell line showed a reduced

infectivity of 19 and 5 days at the same temperature (Shigematsu et al., 2014). The role of the viral lipid bilayer in viral survival highlights that persistence in an exterior milieu is not dictated solely by the viral genome, but also by factors at the host and environmental levels.

AVIAN INFLUENZA, SHOREBIRDS, AND THE ENVIRONMENT

In wild birds, environmental persistence as related to IAV transmission has primarily been investigated in relation to ducks (Anseriformes). Considering the behavioral differences between ducks and other IAV reservoirs in the Charadriiformes (gulls and shorebirds), the importance of environmental persistence to transmission may vary. Incredibly high numbers (historically, on the order of hundreds of thousands) of *charadriiform* birds from the *Scolopacidae* (shorebirds) and *Laridae* (gulls) avian families descend on the Delaware Bay, USA, shoreline in May – June every year to feed on horseshoe crab (*Limulus polymephus)* eggs (Myers et al., 1987; Clark et al., 1993). Six shorebird species in particular depend on this critical stopover site: Dunlin (*Calidris alpina*), Red knot (*Calidris canutus rufa*), Ruddy Turnstone (*Arenaria interpres*), Sanderling (*Calidris alba*), Semipalmated sandpiper (*Calidris pusilla*), and Short-billed dowitcher (*Limnodromus griseus*) (Niles et al., 2009). Driven by lunar cycles and increasing water temperature (Smith et al., 2010), spawning horseshoe crabs converge on the shores of Delaware Bay at the same time annually to lay protein and energy-rich eggs on the beaches, at depths of up to 20 cm (Botton et al., 1992). Horseshoe crab egg densities as high as 1.2 million per meter of New Jersey shoreline have been reported (Smith et al., 2002). Disruption of sand layers due to wave action, and the actions of spawning female horseshoe crabs and feeding shorebirds serve to bring the eggs closer to the surface where they are available to foraging shorebirds (Botton et al., 1994; Smith, 2007). These small birds migrate from as far south as Tierra del Fuego, South

America towards their breeding grounds in the Arctic and utilize this critical energy-rich shoreline as a stopover point to refuel, gaining as much as twice their arrival weight in two weeks (Robinson et al., 2003). The spring migration of shorebirds is time-restricted; facing a short Arctic summer, they need to quickly gain weight to reach their breeding grounds, initiate nesting, and fledge their young. At Delaware Bay, avian IAVs have been consistently isolated from fecal and cloacal swabs from Ruddy Turnstones (*Arenaria interpres*) and to a lesser degree, some gull species, since the late 1980s (Krauss et al., 2010; Stallknecht et al., 2012). This unique convergence of susceptible migrating birds, horseshoe crab eggs, and IAV infection is unlike any other ecosystem yet discovered in the world (Munster et al., 2007; Winker et al., 2008; Gaidet et al., 2012). Delaware Bay has been coined an ecological "hotspot" for influenza; in over a decade of surveillance, from 1998 to 2008, the estimated average IAV prevalence in *Charadriiforme*s at Delaware Bay was more than 5% as compared to less than 0.5% at all other sites worldwide (Krauss et al., 2010). Dissecting the fundamental and interactive factors of host, ecology, and environment are key to beginning to explain this unique phenomenon and understanding what is required to be an ecological "hotspot" for IAV.

IAV infection at Delaware Bay appears to be an annual, localized epidemic, with most exposures (as measured by antibody levels and IAV prevalence) occurring after shorebirds have arrived at the site (Maxted et al., 2012a). Under conditions of high bird density, reduced flock immunity, and an increasing exposure to IAV, Ruddy Turnstones function largely as a short-term amplifying host in this system (Hanson et al., 2008; Maxted et al., 2012a). Though IAV has not been shown to be maintained in Delaware Bay over long periods of time, as shown by a narrow epidemic curve (Maxted et al., 2012a), there is likely an environmental role in the short-term persistence of virus in this unique system. The role of the environment, coupled with bird

behavior and physiology (species differences in receptivity to infection, role of humoral immunity, feeding/roosting practices), allows for a heterogeneous transmission schema (Gaidet et al., 2012) in which Ruddy Turnstones are the dominant player for several weeks. The fecal-oral route of IAV transmission may be occurring in the "traditional" sense, that is, Ruddy Turnstones infected with IAV may be defecating and roosting near shallow marshland pools, which could yield concentrated microcosms of water and virus, different from the situation in fresh-water lakes utilized by ducks, in which virus is readily diluted in larger lake volumes. Additionally, the foraging behavior of these birds, which involves probing into the sandy substrate, may present another opportunity for fecal-oral transmission.

Once virus-laden feces is deposited on the surface of the sand, processes that control the movement of viable, infectious virus in the subsurface include survival and migration/percolation though the sand column (Jin et al., 1997). Both of these processes are influenced by a multitude of factors, not limited to the nature of the soil/substrate, salt and pH conditions, temperature, moisture content, microbial antagonism, and aggregation of virus (Yates et al., 1987; Jin et al., 1997). Tidal washing may help push virus through the sand column, with the potential for it to be stabilized within a cooler gradient of temperatures than would be found at the surface of the sand/air interface. Influenza viruses also have distinct adsorption kinetics related to the isoelectric points conferred by their surface glycoproteins (Miller et al., 1944; Molodkina et al., 2006; Michen and Graule, 2010). Additionally, increased salt concentration as occurs in Delaware Bay can cause viral aggregation (Molodkina et al., 2006). Virus deposited in feces thus has the potential to become bound to other viral particles, to lectins within a matrix of horseshoe crab eggs, or potentially adsorbed to sand particles. These conditions all may protect the virus from UV degradation (Sagripanti and Lytle, 2007), high temperatures, and mechanical

perturbation. If these viruses percolate several centimeters through the sand substrate, they may be perfectly positioned for consumption by Ruddy Turnstones, adding a new dimension to the well-established idea of fecal-oral transmission.

Decades of surveillance data indicate that the predominant IAV subtypes differ between ducks and Delaware Bay shorebirds. However, these viruses share common sublineages, indicating a high degree of intermixing with less of a geographic component than had been previously thought (Widjaja et al., 2004; Obenauer et al., 2006). Analyses of matrix, NS, HA, and NP genes from IAVs isolated from 1969 through 2003 demonstrated no clear grouping by year of isolation, North American geographic location, or species of origin, with amino acid identities ranging from 85.7 – 99.1% for NS1 to as high as 95.7 – 99.6% for M1 (Spackman et al., 2005). Viruses collected from the same species at the same time and location often showed less identity to one another than to viruses with no inherent spatial or temporal relationship (Widjaja et al., 2004; Spackman et al., 2005). These viruses are ultimately derived from a common North American gene pool, with transmission likely occurring where migrating waterfowl and shorebird species share wintering or breeding grounds (Bahl et al., 2013). The interplay at Delaware Bay of ducks (both migratory and resident), shorebirds, gulls, and a potential environmental reservoir allows for the involvement of numerous host species; the Delmarva Peninsula on which Delaware Bay is positioned also serves as a major poultry producing area in the United States. Given that IAV prevalence in shorebirds (and ducks) falls to very low levels during different times of the year it has been proposed that 1) IAV are maintained in the environment during the winter in Northern breeding grounds (ducks) (Ito et al., 1995; Okazaki et al., 2000) and 2) there is low-level circulation of IAV in flocks when birds are widely dispersed along their wintering grounds, that then migrate with the birds upon return to

breeding areas (ducks and shorebirds) (Krauss et al., 2004). Shorebirds also may be implicated in maintaining the gene pool of IAV in nature as eight of 11 shorebird origin isolates were replication competent in a duck model (Kawaoka et al., 1988); however, the failure of three of these isolates (and four of seven gull isolates) to replicate in ducks implies that at least part of the respective IAV gene pools in Anseriformes and Charadriiformes is distinct (Kawaoka et al., 1988).

In the spring, Delaware Bay is considered an ecological hotspot for influenza; however, recovery of IAV from shorebirds in different parts of the world, and/or during different times of year, is rare (Gaidet et al., 2012; De Araujo et al., 2014; Hurtado et al., 2015). Recovery of IAV from shorebirds on wintering grounds is further complicated by their wide dispersal, propensity for rocky coastlines (Nettleship, 2000), and grouping in mixed-species assemblages. The prevalence of IAV in Ruddy Turnstones at Delaware Bay in May can approach 18% (Stallknecht et al., 2012), but the source of virus into this unique system remains largely unknown. The isolation of H5N9, H11N9, H13N2, and H13N9 LP IAV from shorebirds and gulls on their wintering grounds in South America (De Araujo et al., 2014; Ghersi et al., 2009; Mathieu et al., 2015) supports the idea of low-level circulation and maintenance of IAV outside of Delaware Bay. If this is the case, viruses would be available for transmission to this ecological hotspot during subsequent migration towards shorebird breeding grounds.

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CHAPTER 3

ENVIRONMENTAL STABILITY OF SWINE AND HUMAN PANDEMIC INFLUENZA VIRUSES IN WATER UNDER VARIABLE CONDITIONS OF TEMPERATURE, SALINITY, AND PH[1]

[1] Poulson, R. L., S. M. Tompkins, R. D. Berghaus, J. D. Brown, and D. E. Stallknecht. 2016. *Applied and Environmental Microbiology* 82: 3721-3726. Reprinted here with permission of the publisher.

ABSTRACT

The movement of influenza A viruses (IAVs) from wild bird reservoirs to domestic animals and humans is well established, but the transmission mechanisms that facilitate efficient movement across and within these host populations are not fully defined. Although predominant routes of transmission vary between host populations, the extent of environmental stability needed for efficient IAV transmission also may vary. Because of this, we hypothesized that virus stability would differ in response to varied host-related transmission mechanisms; if correct, such phenotypic variation might represent a potential marker for the emergence of novel animal or human influenza viruses. Here, the objective was to evaluate the ability of eight swine and six human IAV isolates to remain infective under various pH, temperature, and salinity conditions using a pre-established distilled water system. Swine and human viruses persisted longest at near-neutral pH, at cold temperatures, or under "freshwater" conditions. Additionally, no significant differences in persistence were observed between pandemic and nonpandemic IAVs. Our results indicate that there have been no apparent changes in the environmental stability of the viruses related to host adaptation.

IMPORTANCE

This study assessed the environmental stability of eight swine and six human influenza A viruses (IAVs), including viruses associated with the 2009 H1N1 pandemic, in a distilled water system. The important findings of this work are that IAV persistence can be affected by environmental variables and that no marked changes were noted between human and swine IAVs or between pandemic and nonpandemic IAVs.

INTRODUCTION

Influenza A viruses (IAVs) have been isolated from numerous avian and mammalian hosts, and cross-species transmission commonly occurs between wild bird reservoirs, domestic animals, and humans (Webster et al., 1977). Swine are susceptible to infection by IAVs of both avian and mammalian origin (Webster et al., 1992) and are recognized as an intermediate host for the evolution and adaptation of IAVs with pandemic potential (Webster et al., 1977; Webster et al., 1992). From 1930 to 1990, classic H1N1 swine influenza virus (SIV) underwent little genetic change, but by the late 1990s, the "triple-reassortant" SIV viruses H1N1, H3N2, and H1N2 had become predominant in swine in North America (Olsen, 2002; Vincent et al., 2008). The pandemic H1N1 influenza A virus (pH1N1) was first detected in April 2009 in two human cases in California (2009), and it quickly spread across the world. The emergence of this virus was subsequently traced to the reassortment of recent North American avian/human/swine triple-reassortant viruses with Eurasian swine viruses (Dawood et al., 2009). This virus has since infected swine and continues to reassort with other SIVs (Vijaykrishna et al., 2010; Ducatez et al., 2011); pH1N1 is now a part of the endemic human influenza pool (World Health Organization, 2014).

The potential for viruses such as pH1N1 to emerge as a result of reassortment between human, swine, and avian viruses involves unlikely transmission events. Transmission mechanisms for IAVs not only are poorly understood in all of these host systems but also vary between them. With birds, transmission of IAVs occurs primarily via a fecal-oral route (Webster et al., 1977; Ito and Kawaoka, 2000), in which water plays a large role. While the primary mode(s) of transmission of influenza between humans is believed to be an aerosol, droplet, or direct contact route (Olsen, 2002; Brankston et al., 2007), indirect transmission through

contaminated surfaces may also contribute to transmission. Likewise, swine influenza viruses are transmitted primarily via direct contact or through aerosols or droplets (Olsen et al., 2006), but indirect contact through fomites or shared environments cannot be discounted.

In this study, the infectivities of eight swine and six human (historical, seasonal, and pandemic) influenza viruses were evaluated under variable pH, temperature, and salinity conditions using a distilled water laboratory model system (Brown et al., 2009). Although modes of transmission for swine and human viruses may not directly involve contact with contaminated water, avian-origin IAVs have been well characterized in this medium (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009; Stallknecht and Brown, 2009; Lebarbenchon et al., 2011; Lebarbenchon et al., 2012) and, as such, provide a baseline for comparison. Furthermore, the potential implications of fomites in the transmission of IAVs in mammalian systems and swine husbandry practices that utilize common drinking troughs make this laboratory investigation applicable. The mechanisms that facilitate or allow for efficient movement across different host populations are varied, but they all require some degree of environmental stability. Because of this, we hypothesized that virus stability (i.e., environmental fitness) would differ in response to varied host-related transmission mechanisms. If correct, such phenotypic variation could represent a potential marker for the emergence of novel animal or human influenza viruses.

MATERIALS AND METHODS

Viruses

One avian-origin H1N1, eight swine, and six human influenza viruses were assessed using a previously described distilled water system (Stallknecht et al., 1990b; Brown et al.,

2009); the viruses used in this study are listed in Table 3.1. Viruses were propagated in Madin-Darby canine kidney (MDCK) cells (ATCC CRL-2936) following the method of Szretter et al. (2006) with modifications. Briefly, a 1:1,000 dilution of virus in minimal essential medium (MEM) supplemented with antibiotics and 1 mg/ml trypsin {treated with TPCK [L-(tosylamido-2-phenyl) ethyl chloromethyl ketone], Worthington Biochemical Corporation, Lakewood, NJ} was added to 75-cm^2 flasks of confluent and washed MDCK cells. Cells were incubated at 35°C or 37°C, and supernatants were harvested at 75% to 90% monolayer destruction. Stock viruses were stored at -70°C; stock virus titers ranged from $10^{6.5}$ to $10^{8.1}$ 50% tissue culture infectious dose ($TCID_{50}$)/ml, as determined by titration in MDCK cells.

Virus persistence trials

Prior to all treatment adjustments, distilled water was buffered with 10mM HEPES (Sigma, St. Louis, MO). Temperatures evaluated included 4°C, 10°C, 17°C, 23°C, 28°C, 32°C (human) or 35°C (swine) and 37°C. A temperature of -70°C was included as a control. Water used in temperature trials had a pH of 7.2 and a salinity of 0 ppt.

For pH trials, water pH was adjusted from 5.4 to 9.0 at 0.4-unit increments with the addition of 1N HCl or NaOH. A pH of 7.2 was also included in the analysis. All pH trials were completed at 17°C and a salinity of 0 ppt. Each pH treatment was measured at the start of the study and confirmed at the completion of each trial. In all cases, it did not vary more than 0.1 unit from the starting pH.

Salinity trials were completed in water at 17°C and pH 7.2. Salinity was adjusted with commercially available sea salt (Morton, Chicago, IL) to 0, 5, 10, 15, 20, 25, and 30 ppt.

For each trial, virus stock was diluted 1:25 to 1:100 in the respective water treatments to achieve a starting titer of approximately $10^{5.0}$ to $10^{6.0}$ $TCID_{50}$/ml. Virus-inoculated water was

aliquoted as single-use 1-ml volumes into 5-ml polystyrene tubes which were allowed to incubate in environmental chambers or water baths at defined temperatures; tubes were removed from the respective temperatures at the predetermined sampling time points and disposed of following titration. For each trial, virus-inoculated water was titrated at the time of inoculation (0 days postinoculation [dpi]) and at variable time points (from 1 to 14 days); these varied by treatment and were based on data from previous infectivity assays (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). Sampling frequencies ranged from daily (under conditions that have been shown to quickly inactivate viruses) to monthly (under conditions in which viruses have been shown to be long-lived). The numbers of time points recorded are indicated in Tables S3.1A to S3.3B in the supplemental material. Virus titrations with MDCK cells were performed as previously described (Brown et al., 2009). Endpoints were measured via hemagglutination assay using 0.5% chicken red blood cells as described previously (Killian, 2008).

Statistical analysis

Titers were calculated by using the method of Reed and Muench (1938) and reported as TCID50/ml. Linear regression was used to determine a 90% reduction time (Rt) for each virus-treatment combination; Rt values correspond to the time required for a decrease in viral titer by 1 $\log_{10}$ $TCID_{50}$/ml. Regression equations are shown in Tables S3.1A to S3.3B in the supplemental material. The minimum detectable limit for this procedure is $10^{1.8}$ $TCID_{50}$/ml.

Because Rt values were not normally distributed and their variances differed across environmental conditions, separate nonparametric comparisons of viruses originating from swine and humans and of pandemic and nonpandemic viruses were performed for each condition using the Wilcoxon rank sum test. All tests assumed a two-sided alternative hypothesis, and a P value

of < 0.05 was considered statistically significant. Analyses were performed using commercially available software (JMP, version Pro 12, 1989-2007; SAS Institute, Cary, NC).

RESULTS

Temperature

The MDCK-adapted swine and human viruses persisted longest at cold temperatures and were inactivated at temperatures greater than 17°C (Fig. 3.1); this pattern of responses is similar to that seen with the single avian control isolate (data shown in Tables S3.1A and S3.1B in the supplemental material). Treatments of 32°C and 35°C were excluded from the statistical comparisons, because none of the swine isolates were evaluated at 32°C, and only one of the human isolates was evaluated at 35°C. No reduction in persistence over the course of the trial (up to 300 days) was observed for viruses held at -70°C (data not shown). The variations in response within swine viruses and within human viruses were greatest at low temperatures. At 4°C, Rt values ranged from 55 to 250 days for swine viruses and from 30 to 160 days for human viruses; at 37°C, Rt values ranged from 0.5 to 3.4 days for swine viruses and 0.9 to 4 days for human viruses. Swine viruses persisted longer than human viruses at 10°C and 23°C (P = 0.045 and 0.010, respectively). There were no significant differences in environmental persistence between pandemic and nonpandemic viruses (Fig. 3.2).

pH

All swine viruses included in this study were most stable at near-neutral pH and were quickly inactivated at the extremes of the ranges tested; viruses of human origin showed a similar response (Fig. 3.3). There was rapid inactivation at pHs less than 6.2 and greater than 8.2 for all swine and human viruses, and the majority of viruses in all the groups were most stable at

pH 7.2. There were no significant differences in the responses of swine and human viruses at any pH. Seasonal and pandemic human viruses also did not differ significantly in their responses at any pH (Fig. 3.4). Rt values for all of the individual viruses and pH levels are provided in Table S3.2A and S3.2B in the supplemental material.

Salinity

Increased salinity had a detrimental effect on virus stability. The Rt values of all viruses assessed, regardless of host origin, were greatest in freshwater at a salinity of 0 ppt. Persistence of viruses from either host group (swine or human) showed similar declines with increasing salinity, with a marked decrease in stability as salinity was raised from 0 to 5 ppt (Fig. 3.5); the response was similar for the single avian control isolate (see Table S3.3A and S3.3B in the supplemental material). Swine viruses persisted longer than human viruses at 20 ppt ($P = 0.010$), but the two groups did not differ significantly at any other saline concentration. As was the case at colder temperatures, viruses within each host group were most variable in their persistence at a salinity of 0 ppt. Excluding A/Swine/Utah/02861/2009 (H1N2), with an Rt value of 6.3 days at a salinity of 0 ppt, the range of Rt values for all other swine viruses, regardless of subtype, was 33 to 72 days. While A/Mexico/INDRE/2009 (H1N1) was the least stable at 0 ppt, with an Rt value of 14 days, all other human viruses, seasonal and pandemic combined, had Rt values ranging from 21 to 46 days in freshwater. As was the case with temperature and pH, pandemic and nonpandemic viruses responded similarly to salinity, with no significant differences between groups at any saline concentration (Fig. 3.6). Regression equations for all viruses in the salinity trials are provided in Table S3.3A and S3.3B in the supplemental material.

DISCUSSION

Understanding the environmental stability of IAVs has relevance in defining transmission risks both within the avian reservoir and across domestic poultry and mammalian species. Persistence in water may represent a critical factor in virus maintenance and transmission in wild avian populations but also may play a minor role in the transmission of human and swine IAVs, which are transmitted primarily by contact and respiratory droplets. Although transmission mechanisms differ between these host groups, our results indicate no consistent or significant adaptations related to changes in environmental stability as determined by temperature, pH, and salinity. The general trends described for swine and human IAVs are similar to the well-documented responses of avian IAVs.

The interspecies and intraspecies transmission and maintenance of IAVs are dependent on factors at the host, viral, and environmental levels. To be transmitted and maintained, viruses must remain infectious. It has been well established that IAVs persist longest at cold temperatures (Stallknecht et al., 1990a; Brown et al., 2007; Brown et al., 2009; Negovetich and Webster, 2010; Dublineau et al., 2011); human and swine viruses analyzed in this study lasted longest at 4°C, and results were consistent with those previously reported for pandemic virus A/Paris/2590/2009 (H1N1), which had a reported Rt value of 178 days at 4°C (Dublineau et al., 2011). The swine and human viruses included in this study, however, demonstrated greater persistence at low temperatures than did a suite of viruses of avian origin that were previously analyzed (Brown et al., 2009). This result may be an artifact of how the human and swine viruses tested in our study were propagated; it has been shown that both human and avian viruses grown on MDCK cells are more stable at higher temperatures than are the same viruses when grown in chicken eggs (Shigematsu et al., 2014). At higher temperatures, all the viruses

assessed, regardless of their subtype and origin, were quickly inactivated, especially at temperatures higher than 28°C; this is similar to the reduction in persistence seen for a 2009 pandemic H1N1 and a 1999 seasonal H1N1 from >150 days at 4°C to just 2 days at 35°C (Dublineau et al., 2011). While swine viruses persisted significantly longer than human viruses at 10°C ($P = 0.045$) and 23°C ($P = 0.010$) in this study, such differences were not consistent across the broader range of temperatures evaluated, and given the small sample sizes, the statistical power is low. This study evaluates the thermal stability of swine influenza viruses at a range of temperatures likely encountered in both laboratory and natural settings. Thermal neutrality is important in swine production systems and is dependent largely on age strata and weight (Ames, 1980). Generally, preferred thermal conditions for swine range from 10°C to 32°C (Baker, 2004); under the laboratory conditions of this study, some currently circulating swine influenza viruses can remain infectious for several weeks (35°C) to more than 1 year (10°C) within this temperature range. The temperature stability of these viruses at temperatures consistent with host environments, such as swine production systems, may suggest that environmental adaptation is not necessary for movement of the virus across species barriers, despite the disparate body temperatures seen in avian (42°C), swine (39°C), and human (37°C) hosts.

The role of pH in IAV hemagglutinin (HA) membrane fusion has been well characterized (Maeda and Ohnishi, 1980; Matlin et al., 1981; Skehel et al., 1982; Doms et al., 1985), and pH might play a role in adaptation of IAV to a new host. From an environmental perspective, the effect of low pH on the integrity of external proteins on the surface of the virus may serve as a preemptive trigger, rendering HAs inactive (Junankar and Cherry, 1986). In the present study, human and swine viruses were most stable within a neutral pH range, as has been found with

avian viruses (Brown et al., 2009). We observed some subtle and nonsignificant differences in persistence across hosts and subtypes, but these differences did not provide clear evidence of pH-related environmental adaptation. Very small differences in the pH of fusion (between 5.0 and 5.7) have been associated with IAV host adaptation and cross-species transmission (Galloway et al., 2013; Cotter et al., 2014). Changes in environmental stability at pH 7.4 also have been reported with recombinant H5N1 viruses where a 0.5-unit change in the pH of fusion was shown to decrease environmental stability more than 45 days, while a decrease by the same amount increased persistence nearly 20 days compared to that of the wild type (Reed et al., 2010). While we did not detect any clear evidence of differences in pH tolerance between swine and human IAVs, additional evaluation may be necessary to detect fine-scale differences that may occur early in host adaption, especially those related to virus adaptation from avian to mammalian hosts.

Of the three variables investigated here, salinity is the least understood in its effect or mechanism of action relating to influenza stability in water. As was expected from results of previous studies, virus persistence decreased as the salinity of water increased for all viruses assessed. This loss in infectivity was most marked from 0 to 5 ppt for all viruses except A/Swine/Utah/02861/2009 (H1N2). Increasing osmotic pressure might serve to disrupt the integrity of the virus membrane and/or lead to premature inactivation. The response of individual avian virus stocks to increasing salinity has been shown to take on a number of forms, from negative log-linear to Gaussian (Brown et al., 2009). While many avian viruses tend to persist longest in moderately saline water, most viruses in our study showed greatest persistence in water with a salinity of 0 ppt. This difference might be due to the host from which the lipid bilayer of the virus was derived, the glycosylation moieties of the surface proteins (also a

function of the host cell), or potentially a marker of adaptation. Assessing these markers of adaptation, as well as determining the potential relevance of increased persistence of swine over human viruses at 20 ppt ($P = 0.045$) in this study, would require further investigation with a larger diversity of viruses. Given that IAV transmission in human and swine systems often involves respiratory secretions, salt concentrates in droplets likely play a role in virus viability. Yang et al. (2012) proposed three conditions related to relative humidity (RH)—physiological, concentrated, and dry—that play a role in the infectivity of influenza in droplets. Under this schema, virus stability was most jeopardized under conditions of RH between 50% and 99%, at which the evaporation of a given droplet of medium with salts led to an increase in the concentration of solutes; the interaction of mucus and RH yielded a similar effect on virus persistence.

This study provides general response models for the individual effects of temperature, pH, and salinity on the ability of IAVs of different subtypes and from different hosts to remain infective in a distilled water milieu. Altogether, these results shed some light on the dynamics of these avian, swine, human, and pandemic H1N1 viruses under laboratory-simulated "environmental" conditions. Our data indicate that influenza persistence can be modulated by environmental variables and support previous findings for avian influenza viruses (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009). Furthermore, and perhaps of greater relevance to the swine and 2009 H1N1 outbreak strains, these data might inform decisions regarding preventive and management practices in both the laboratory and the field.

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We declare no competing interests.

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Table 3.1. Description of influenza viruses used in this study.

Origin	Strain	Status[a]	Passage[b]	Subtype	Titer ($TCID_{50}$/mL)
Avian	A/green-winged teal/Louisiana/213/1987	control	SPFE1/C1	H1N1	6.9
Swine	A/Swine/Minnesota/02719/2009	Non-PDM	C1/E1/C1	H3N2	7.6
Swine	A/Swine/North Carolina/02744/2009	Non-PDM	C1/E1/C1	H1N2	7.3
Swine	A/Swine/Minnesota/02746/2009	pH1N1	C1/E1/C1	H1N1	8.3
Swine	A/Swine/Minnesota/02749/2009	pH1N1	C1/E1/C1	H1N1	8.1
Swine	A/Swine/Minnesota/02751/2009	pH1N1	C1/E1/C1	H1N1	7.7
Swine	A/Swine/Illinois/02860/2009	pH1N1	C1/E1/C1	H1N1	7.4
Swine	A/Swine/Utah/02861/2009	Non-PDM	C1/E1/C1	H1N2	7.5
Swine	A/Swine/Iowa/15/1930	Non-PDM	?/E1/C1	H1N1	6.5
Human	A/New Jersey/08/1976	Non-PDM	SPFE7/E1/C1	H1N1	7.7
Human	A/Texas/15/2009	pH1N1	C2/E1/C1	H1N1	7.4
Human	A/Mexico/INDRE4487/2009	pH1N1	E2/E1/E1/C1	H1N1	7.1
Human	A/Brisbane/10/2007	Non-PDM	E2/E2/E1/C1	H3N2	7.9
Human	A/Brisbane/59/2007	Non-PDM	E2/E2/E1/C1	H1N1	6.5
Human	A/California/04/2009	pH1N1	C1/C1/C1	H1N1	6.7

[a] pH1N1 = 2009 pandemic H1N1 viruses; Non-PDM = non-pandemic viruses

[b] Sequential passage history: C = Madin Darby Canine Kidney cells, E = embryonated chicken eggs, SPFE = specific-pathogen-free embryonated chicken eggs. ? = unknown primary isolation source. Numbers indicate number of passages within each source / passage in a new source.

Supplementary Table S3.1A. Regression equations (R^2 value), Rt value (days) and the number of time points collected for swine viruses within each temperature treatment.

Temperature (C)		4	10	17	23	28	32	35	37
Iowa Swine	regr eq (R^2)	-0.007x+3.891 (0.802)	-0.010x+3.958 (0.842)	-0.024x+3.819 (0.864)	-0.0278x+3.865 (0.803)	-0.155x+3.846 (0.850)	*Not assessed at this temperature*	-0.184x+3.883 (0.931)	-0.315x+4.027 (0.919)
	Rt (days)	153.846	98.039	42.017	35.842	6.443		5.438	3.180
	# time pts	12	12	11	11	7		6	5
Illinois Swine	regr eq (R^2)	-0.006x+4.153 (0.719)	-0.006x+3.865 (0.735)	-0.018x+4.054 (0.897)	-0.033x+4.126 (0.887)	-0.092x+3.936 (0.873)		-0.220x+4.087 (0.958)	-0.468x+4.212 (0.959)
	Rt (days)	178.571	158.730	55.249	30.030	10.834		4.541	2.136
	# time pts	14	12	10	11	9		7	4
MN2719 Swine	regr eq (R^2)	-0.004x+4.402 (0.977)	-0.007x+4.224 (0.869)	-0.015x+4.149 (0.713)	-0.021x+4.168 (0.837)	-0.106x+4.451 (0.917)		-0.178x+4.297 (0.960)	-0.314x+4.291 (0.994)
	Rt (days)	250.00	147.06	66.23	48.31	9.48		5.63	3.19
	# time pts	12	11	8	9	7		7	6
MN2746 Swine	regr eq (R^2)	-0.005x+5.580 (0.655)	-0.016x+5.575 (0.849)	-0.028x+5.230 (0.711)	-0.034x+4.932 (0.783)	-0.158x+5.022 (0.844)		-0.378x+4.883 (0.819)	-0.727x+5.171 (0.841)
	Rt (days)	192.308	63.694	36.364	29.326	6.337		2.646	1.376
	# time pts	13	13	9	9	8		7	5
MN2749 Swine	regr eq (R^2)	-0.006x+5.268 (0.767)	-0.008x+5.112 (0.776)	-0.018x+5.152 (0.816)	-0.027x+5.147 (0.821)	-0.113x+5.369 (0.975)		-0.317x+5.358 (0.989)	-0.655x+5.038 (0.916)
	Rt (days)	172.414	125.000	54.348	36.765	8.873		3.158	1.526
	# time pts	14	10	9	9	9		5	4
MN2751 Swine	regr eq (R^2)	-0.005x+5.551 (0.927)	-0.011x+5.617 (0.757)	-0.021x+5.409 (0.820)	-0.035x+5.417 (0.915)	-0.193x+5.316 (0.898)		-0.208x+5.470 (0.918)	-0.386x+5.550 (0.936)
	Rt (days)	200.000	88.496	47.393	28.490	5.179		4.817	2.590
	# time pts	15	11	13	13	9		9	6
NC2744 Swine	regr eq (R^2)	-0.006x+4.510 (0.729)	-0.008x+4.328 (0.906)	-0.018x+4.414 (0.907)	-0.027x+4.283 (0.931)	-0.101x+4.365 (0.946)		-0.229x+4.522 (0.981)	-0.294x+4.036 (0.873)
	Rt (days)	178.571	123.457	55.556	37.594	9.911		4.359	3.407
	# time pts	12	14	14	13	10		7	6
Utah2861 Swine	regr eq (R^2)	-0.018x+3.604 (0.528)	-0.035x+3.496 (0.403)	-0.104x+4.173 (0.693)	-0.086x+3.817 (0.507)	-0.776x+4.408 (0.636)		-3.230x+5.000 (0.997)	-3.230x+5.000 (0.997)
	Rt (days)	56.180	28.986	9.634	11.628	1.289		0.310	0.310
	# time pts	17	12	10	13	4		3	3

Supplementary Table S3.1B. Regression equations (R^2 value), Rt value (days) and the number of time points collected for human (shaded gray) and one avian virus within each temperature treatment.

Temperature (C)		4	10	17	23	28	32	35	37
NJ/1976 Human	regr eq (R^2)	-0.005x+4.687 (0.605)	-0.013x+5.129 (0.722)	-0.021x+4.833 (0.909)	-0.053x+5.066 (0.805)	-0.114x+5.136 (0.861)	-0.139x+5.051 (0.937)	*Not assessed at this temperature*	*-0.310x+4.460 (0.916)*
	Rt (days)	222.222	80.000	47.847	19.011	8.780	7.215		3.223
	# time pts	17	19	20	12	11	11		10
Texas2009 Human	regr eq (R^2)	-0.007x+6.108 (0.829)	-0.018x+5.991 (0.739)	-0.026x+6.613 (0.878)	-0.056x+5.877 (0.669)	-0.102x+5.825 (0.892)	-0.144x+5.910 (0.927)		-0.299x+6.130 (0.948)
	Rt (days)	135.135	55.866	38.023	17.794	9.814	6.959		3.342
	# time pts	21	19	18	10	12	13		11
Brisbane10 Human	regr eq (R^2)	-0.008x+5.733 (0.940)	-0.010x+5.503 (0.692)	-0.013x+5.649 (0.572)	-0.049x+5.577 (0.798)	-0.091x+5.603 (0.863)	-0.116x+5.776 (0.977)		-0.315x+5.065 (0.849)
	Rt (days)	125.000	98.039	76.923	20.243	10.989	8.591		3.173
	# time pts	12	16	17	11	12	8		9
Brisbane59 Human	regr eq (R^2)	-0.033x+3.861 (0.518)	-0.042x+3.590 (0.642)	-0.049x+3.620 (0.669)	-0.094x+3.583 (0.703)	-0.136x+3.566 (0.653)	-0.158x+3.731 (0.847)		-0.248x+3.150 (0.694)
	Rt (days)	30.030	23.697	20.492	10.695	7.358	6.329		4.027
	# time pts	6	8	10	10	9	9		6
MX/INDRE Human	regr eq (R^2)	-0.029x+2.967 (0.482)	-0.034x+2.958 (0.693)	-0.0467x+2.797 (0.711)	-0.106x+3.625 (0.653)	-0.179x+3.955 (0.762)	-0.260x+4.143 (0.750)		-1.095x+3.960 (0.971)
	Rt (days)	34.602	29.412	21.413	9.416	5.593	3.849		0.913
	# time pts	7	7	8	9	7	6		3
CA04/2009 Human	regr eq (R^2)	-0.010x+5.265 (0.406)	-0.021x+5.322 (0.710)	-0.038x+5.662 (0.647)	-0.055x+5.559 (0.756)	-0.114x+5.272 (0.852)	-0.352x+5.166 (0.947)	-0.214x+5.566 (0.937)	-0.366x+5.646 (0.959)
	Rt (days)	101.010	47.619	26.110	18.248	8.787	2.838	4.682	2.734
	# time pts	18	20	12	12	10	5	7	7
LA/GWT Avian	regr eq (R^2)	-0.011x+4.364 (0.751)	-0.018x+4.179 (0.829)	-0.024x+4.456 (0.739)	-0.136x+4.297 (0.983)	-0.329x+4.125 (0.924)	-0.650x+4.190 (0.927)	*Not assessed at this temperature*	-1.840x+4.400 (0.880)
	Rt (days)	87.719	57.143	42.553	7.358	3.038	1.538		0.543
	# time pts	10	10	10	5	5	4		3

Supplementary Table S3.2A. Regression equations (R^2 value), Rt value (days) and the number of time points collected for swine viruses within each pH treatment.

	pH	5.4	5.8	6.2	6.6	7.0	7.2	7.4	7.8	8.2	8.6	9.0
Iowa Swine	regr eq (R^2)	-0.226x+3.728 (0.456)	-0.176x+3.480 (0.690)	-0.114x+3.606 (0.679)	-0.024x+4.178 (0.924)	-0.076x+3.477 (0.860)	-0.028x+4.292 (0.883)	-0.048x+3.537 (0.671)	-0.095x+4.088 (0.969)	-0.108x+4.309 (0.919)	-0.059x+3.644 (0.836)	-0.075x+3.548 (0.717)
	Rt	4.421	5.679	8.795	41.841	13.123	35.211	21.053	10.571	9.285	16.835	13.316
	# time	5	5	7	16	5	12	9	11	7	11	9
Illinois Swine	regr eq (R^2)	-0.392x+4.318 (0.913)	-0.317x+4.232 (0.955)	-0.037x+4.118 (0.921)	-0.022x+4.428 (0.915)	-0.019x+4.012 (0.827)	-0.014x+4.271 (0.846)	-0.018x+4.148 (0.813)	-0.021x+4.223 (0.813)	-0.029x+4.238 (0.835)	-0.060x+4.207 (0.948)	-0.102x+4.162 (0.900)
	Rt	2.554	3.158	27.322	45.455	52.632	71.942	54.348	48.309	34.364	16.611	9.843
	# time	6	6	14	16	19	17	19	18	17	9	9
MN02719 Swine	regr eq (R^2)	-0.183x+3.284 (0.556)	-0.129x+3.855 (0.621)	-0.024x+4.349 (0.853)	-0.020x+4.474 (0.893)	-0.035x+3.777 (0.789)	-0.021x+4.395 (0.735)	-0.025x+3.980 (0.852)	-0.025x+4.427 (0.723)	-0.039x+4.598 (0.950)	-0.027x+4.252 (0.787)	-0.037x+4.088 (0.715)
	Rt	5.456	7.764	42.373	50.251	28.490	47.170	39.683	40.323	25.707	36.496	27.027
	# time	6	7	15	19	15	16	17	16	13	16	14
MN02746 Swine	regr eq (R^2)	-0.446x+5.821 (0.780)	-0.415x+5.991 (0.774)	-0.120x+5.232 (0.837)	-0.031x+5.775 (0.919)	-0.062x+5.994 (0.930)	-0.019x+5.818 (0.840)	-0.049x+5.383 (0.793)	-0.059x+5.207 (0.921)	-0.083x+4.649 (0.706)	-0.065x+6.067 (0.936)	-0.067x+5.647 (0.973)
	Rt	2.244	2.410	8.333	32.468	16.207	53.191	20.284	16.920	11.947	15.408	14.837
	# time	5	5	8	20	15	19	15	12	9	15	14
MN02749 Swine	regr eq (R^2)	-0.472x+4.910 (0.821)	-1.264x+4.313 (0.930)	-0.482x+4.339 (0.623)	-0.028x+5.424 (0.943)	-0.036x+4.352 (0.794)	-0.016x+5.369 (0.885)	-0.043x+4.581 (0.864)	-0.045x+4.601 (0.732)	-0.037x+4.548 (0.904)	-0.032x+4.903 (0.745)	-0.039x+4.819 (0.773)
	Rt	2.117	0.791	2.075	36.101	27.624	61.728	23.256	22.124	26.954	31.746	25.575
	# time	12	3	5	18	16	18	13	14	17	17	16
MN02751 Swine	regr eq (R^2)	-0.627x+4.805 (0.951)	-0.645x+4.506 (0.841)	-0.097x+3.978 (0.739)	-0.074x+4.606 (0.640)	-0.047x+5.011 (0.819)	-0.019x+5.655 (0.702)	-0.035x+4.855 (0.773)	-0.054x+4.360 (0.714)	-0.585x+5.272 (0.937)	-0.030x+4.691 (0.809)	-0.041x+4.606 (0.853)
	Rt	1.596	1.551	10.288	13.550	21.322	53.763	28.986	18.416	1.708	33.784	24.631
	# time	4	7	11	8	15	15	17	15	4	14	16
NC02744 Swine	regr eq (R^2)	-0.527x+4.076 (0.872)	-0.958x+3.685 (0.964)	-0.417x+3.439 (0.803)	-0.021x+4.013 (0.868)	-0.113x+3.795 (0.992)	-0.022x+3.721 (0.516)	-0.118x+3.895 (0.931)	-0.114x+3.699 (0.885)	-0.185x+3.881 (0.821)	-0.051x+3.912 (0.848)	-0.076x+3.806 (0.828)
	Rt	1.897	1.044	2.399	48.780	8.889	45.455	8.467	8.787	5.414	19.646	13.089
	# time	4	3	7	18	7	16	7	7	6	12	12
Utah02861 Swine	regr eq (R^2)	-0.682x+3.666 (0.568)	-1.735x+3.505 (0.989)	-1.710x+3.480 (0.999)	-2.125x+3.895 (0.973)	-0.228x+4.510 (0.987)	-0.160x+3.471 (0.532)	-0.219x+4.400 (0.998)	-0.153x+4.220 (0.989)	-1.701x+3.480 (0.994)	-0.268x+4.047 (0.836)	-0.464x+4.579 (0.988)
	Rt	1.466	0.576	0.585	0.471	4.380	6.270	4.562	6.545	0.585	3.729	2.154
	# time	3	2	2	2	3	8	2	4	4	6	5

Supplementary Table S3.2B. Regression equations (R^2 value), Rt value (days) and the number of time points collected for human (shaded gray) and one avian virus within each pH treatment.

	pH	5.4	5.8	6.2	6.6	7.0	7.2	7.4	7.8	8.2	8.6	9.0
NJ/1976 Human	regr eq (R^2)	-0.685x+4.393 (0.956)	-0.660x+4.370 (0.911)	-0.218x+3.815 (0.624)	-0.042x+4.633 (0.943)	-0.055x+4.370 (0.708)	-0.021x+4.833 (0.909)	-0.041x+4.243 (0.750)	-0.035x+4.331 (0.727)	-0.182x+3.851 (0.771)	-0.087x+4.998 (0.937)	-0.084x+4.692 (0.871)
	Rt	1.460	1.515	4.583	24.038	18.282	47.847	24.570	28.736	5.501	11.521	11.976
	# time	7	7	9	13	12	20	15	16	4	10	11
Texas2009 Human	regr eq (R^2)	-0.814x+5.848 (0.897)	-0.766x+6.126 (0.890)	-0.060x+5.674 (0.879)	-0.031x+6.011 (0.925)	-0.043x+5.663 (0.853)	-0.026x+6.613 (0.878)	-0.039x+5.775 (0.912)	-0.038x+5.941 (0.920)	-0.120x+5.752 (0.922)	-0.063x+5.955 (0.912)	-0.087x+5.843 (0.906)
	Rt	1.229	1.306	16.584	31.949	23.419	38.023	25.510	26.178	8.313	15.823	11.547
	# time	5	6	17	18	17	18	18	19	8	16	17
Brisbane10 Human	regr eq (R^2)	-0.568x+4.653 (0.714)	-0.755x+5.107 (0.865)	-0.115x+4.515 (0.786)	-0.031x+4.597 (0.870)	-0.024x+5.753 (0.757)	-0.013x+5.649 (0.572)	-0.014x+5.617 (0.745)	-0.030x+5.572 (0.923)	-0.060x+4.862 (0.923)	-0.032x+4.422 (0.511)	-0.049x+4.262 (0.796)
	Rt	1.761	1.324	8.696	32.787	40.984	76.923	69.930	33.003	16.584	31.447	20.367
	# time	6	4	10	18	18	17	18	19	10	15	15
Brisbane59 Human	regr eq (R^2)	-0.375x+4.057 (0.583)	-0.411x+4.217 (0.810)	-0.127x+3.385 (0.665)	-0.042x+3.346 (0.605)	-0.085x+4.422 (0.941)	-0.048x+4.056 (0.918)	-0.086x+3.206 (0.826)	-0.078x+4.374 (0.922)	-0.237x+3.830 (0.855)	-0.108x+3.423 (0.803)	-0.108x+3.172 (0.540)
	Rt	2.667	2.434	7.893	23.753	11.737	21.008	11.574	12.821	4.228	9.259	9.268
	# time	4	5	9	11	5	9	4	6	5	8	8
MXINDRE Human	regr eq (R^2)	-0.405x+5.593 (0.861)	-0.341x+5.422 (0.825)	-0.191x+3.429 (0.495)	-0.092x+2.943 (0.572)	-0.054x+4.402 (0.910)	-0.069x+3.081 (0.581)	-0.059x+4.468 (0.860)	-0.088x+4.623 (0.844)	-0.474x+4.145 (0.746)	-0.143x+3.960 (0.795)	-0.239x+3.893 (0.743)
	Rt	2.470	2.934	5.236	10.893	18.587	14.430	16.978	11.429	2.108	6.978	4.184
	# time	5	5	7	7	11	8	11	9	5	9	6
CA04/2009 Human	regr eq (R^2)	-0.671x+3.802 (0.483)	-0.275x+4.379 (0.693)	-0.052x+4.462 (0.567)	-0.021x+4.258 (0.484)	-0.027x+5.445 (0.822)	-0.016x+4.745 (0.418)	-0.021x+5.214 (0.814)	-0.030x+4.628 (0.633)	-0.035x+5.055 (0.872)	-0.049x+4.663 (0.813)	-0.057x+4.983 (0.878)
	Rt	1.490	3.640	19.231	48.544	37.313	64.516	48.077	33.333	28.818	20.534	17.575
	# time	5	6	10	17	17	6	14	14	14	11	13
LA/GWT Avian	regr eq (R^2)	-2.345x+4.115 (0.975)	-0.065x+3.536 (0.550)	-0.033x+4.927 (0.829)	-0.023x+5.121 (0.689)	-0.023x+4.540 (0.862)	-0.024x+4.456 (0.739)	-0.021x+4.138 (0.730)	-0.026x+4.166 (0.716)	-0.035x+4.004 (0.704)	-0.076x+4.329 (0.935)	-0.060x+4.253 (0.745)
	Rt	0.426	15.456	30.769	42.194	43.860	42.553	46.729	38.760	28.490	13.210	16.750
	# time	4	14	17	17	18	10	18	18	11	6	8

Supplementary Table S3.3A. Regression equations (R^2 value), Rt value (days) and the number of time points collected for swine viruses within each salinity treatment.

Salinity (ppt)		0ppt	5ppt	10ppt	15ppt	20ppt	25ppt	30ppt
Iowa Swine	regr eq	-0.028x+4.292	-0.070x+4.236	-0.143x+4.036	-0.150x+3.938	-0.155x+3.411	-0.160x+3.716	-0.170x+3.385
	(R^2)	(0.883)	(0.929)	(0.933)	(0.968)	(0.816)	(0.882)	(0.667)
	Rt	35.211	14.388	6.978	6.676	6.472	6.227	5.869
	# time	12	10	7	7	6	9	11
Illinois Swine	regr eq	-0.0140x+4.271	-0.037x+4.395	-0.065x+4.206	-0.033x+4.164	-0.042x+3.896	-0.0420x+3.883	-0.055x+3.750
	(R^2)	(0.846)	(0.893)	(0.890)	(0.838)	(0.873)	(0.629)	(0.870)
	Rt	71.942	26.738	15.361	25.445	23.753	23.866	18.083
	# time	17	13	10	13	13	12	9
MN02719 Swine	regr eq	-0.021x+4.395	-0.037x+4.602	-0.035x+4.423	-0.031x+4.410	-0.040x+4.139	-0.097x+4.647	-0.100x+4.455
	(R^2)	(0.735)	(0.906)	(0.910)	(0.883)	(0.856)	(0.909)	(0.888)
	Rt	47.170	26.810	28.409	32.680	24.752	10.341	10.050
	# time	16	15	17	17	15	12	13
MN02746 Swine	regr eq	-0.019x+5.818	-0.046x+5.820	-0.096x+5.752	-0.062x+5.687	-0.070x+5.533	-0.077x+5.526	-0.112x+5.605
	(R^2)	(0.840)	(0.953)	(0.911)	(0.924)	(0.915)	(0.888)	(0.865)
	Rt	53.191	21.692	10.406	16.077	14.388	13.055	8.945
	# time	19	17	12	13	14	14	15
MN02749 Swine	regr eq	-0.016x+5.369	-0.061x+4.980	-0.067x+5.009	-0.069x+4.901	-0.099x+4.763	-0.099x+4.887	-0.131x+4.964
	(R^2)	(0.885)	(0.954)	(0.948)	(0.777)	(0.860)	(0.887)	(0.838)
	Rt	61.728	16.529	14.859	14.556	10.142	10.060	7.634
	# time	18	12	13	16	12	12	12
MN02751 Swine	regr eq	-0.019x+5.655	-0.040x+5.069	-0.061x+5.149	-0.065x+5.004	-0.039x+4.277	-0.103x+4.790	-0.118x+4.873
	(R^2)	(0.702)	(0.884)	(0.928)	(0.916)	(0.807)	(0.896)	(0.856)
	Rt	53.763	24.814	16.313	15.480	25.707	9.709	8.496
	# time	15	13	13	11	14	13	12
NC02744 Swine	regr eq	-0.022x+3.721	-0.059x+3.976	-0.078x+3.817	-0.034x+4.230	-0.050x+4.211	-0.088x+3.797	-0.078x+4.134
	(R^2)	(0.516)	(0.908)	(0.862)	(0.682)	(0.798)	(0.889)	(0.781)
	Rt	45.455	16.892	12.804	29.155	19.920	11.364	12.903
	# time	16	10	11	14	16	12	14
Utah02861 Swine	regr eq	-0.160x+3.471	-0.088x+4.913	-0.104x+4.812	-0.084x+3.539	-0.072x+3.738	-0.090x+3.881	-0.133x+4.060
	(R^2)	(0.532)	(0.930)	(0.948)	(0.795)	(0.886)	(0.700)	(0.898)
	Rt	6.270	11.325	9.653	11.976	13.947	11.173	7.547
	# time	8	9	10	11	11	10	10

Supplementary Table S3.3B. Regression equations (R^2 value), Rt value (days) and the number of time points collected for human (shaded gray) and one avian virus within each salinity treatment.

Salinity (ppt)		0ppt	5ppt	10ppt	15ppt	20ppt	25ppt	30ppt
Brisbane10 Human	regr eq (R^2)	-0.013x+5.649 (0.572)	-0.081x+5.994 (0.957)	-0.188x+5.287 (0.784)	-0.052x+4.804 (0.744)	-0.115x+4.696 (0.883)	-0.251x+5.419 (0.931)	-0.241x+5.312 (0.813)
	Rt	76.923	12.346	5.333	19.417	8.726	3.981	4.143
	# time pts	17	9	9	14	14	8	8
Brisbane59 Human	regr eq (R^2)	-0.048x+4.056 (0.918)	-0.048x+4.264 (0.915)	-0.175x+3.546 (0.762)	-0.127x+3.476 (0.657)	-0.105x+3.154 (0.558)	-0.138x+3.235 (0.812)	-0.135x+3.540 (0.694)
	Rt	21.008	20.877	5.701	7.868	9.569	7.231	7.386
	# time pts	9	11	6	10	10	9	10
MXINDRE Human	regr eq (R^2)	-0.069x+3.081 (0.581)	-0.086x+3.894 (0.809)	-0.108x+3.678 (0.641)	-0.098x+3.650 (0.752)	-0.226x+4.674 (0.853)	-0.112x+3.594 (0.710)	-0.124x+3.771 (0.741)
	Rt	14.430	11.614	9.302	10.256	4.433	8.953	8.039
	# time pts	8	7	11	12	7	10	9
CA04/2009 Human	regr eq (R^2)	-0.016x+4.745 (0.418)	-0.061x+4.315 (0.767)	-0.090x+4.218 (0.846)	-0.122x+4.560 (0.901)	-0.173x+4.550 (0.948)	-0.201x+4.941 (0.940)	-0.194x+4.214 (0.850)
	Rt	64.516	16.474	11.099	8.210	5.767	4.975	5.152
	# time pts	6	7	8	6	4	4	4
LA/GWT Avian	regr eq (R^2)	-0.016x+4.493 (0.536)	-0.073x+4.351 (0.859)	-0.084x+4.302 (0.795)	-0.074x+4.012 (0.755)	-0.096x+4.020 (0.733)	-0.148x+4.489 (0.926)	-0.188x+4.798 (0.828)
	Rt	64.103	13.624	11.976	13.532	10.471	6.775	5.330
	# time pts	10	6	7	7	6	4	4

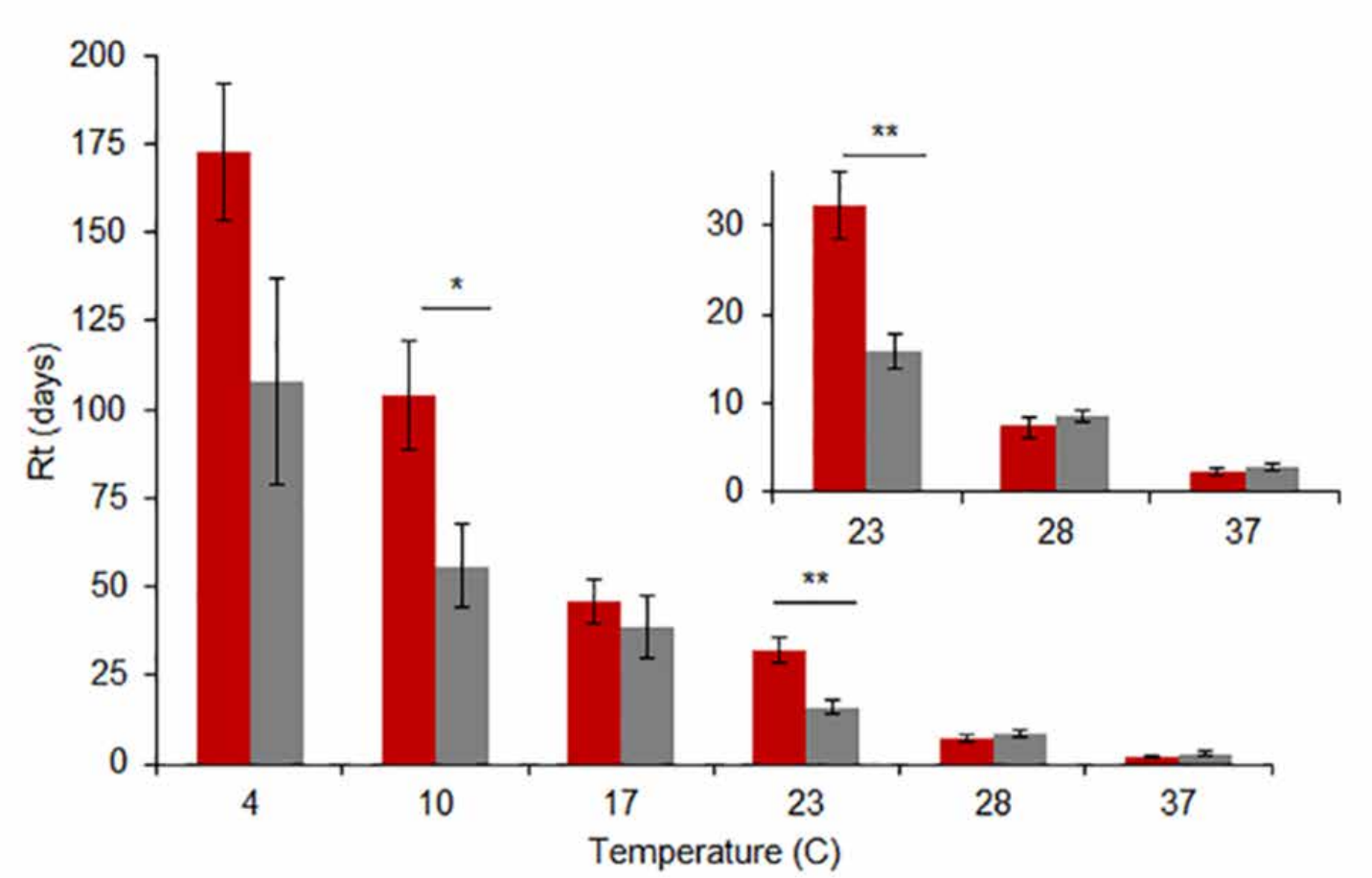

Figure 3.1. Mean Rt values (± standard error [SE]) for eight swine (red) and six human (gray) viruses in distilled water at temperatures ranging from 4°C to 37°C. The pH was held constant at 7.2, and salinity was 0 ppm. Significant differences in the responses for swine and human viruses exist at temperatures of 10°C (*, $P < 0.05$) and 23°C (**, $P < 0.01$). No other statistically significant differences were observed at an α value of 0.05.

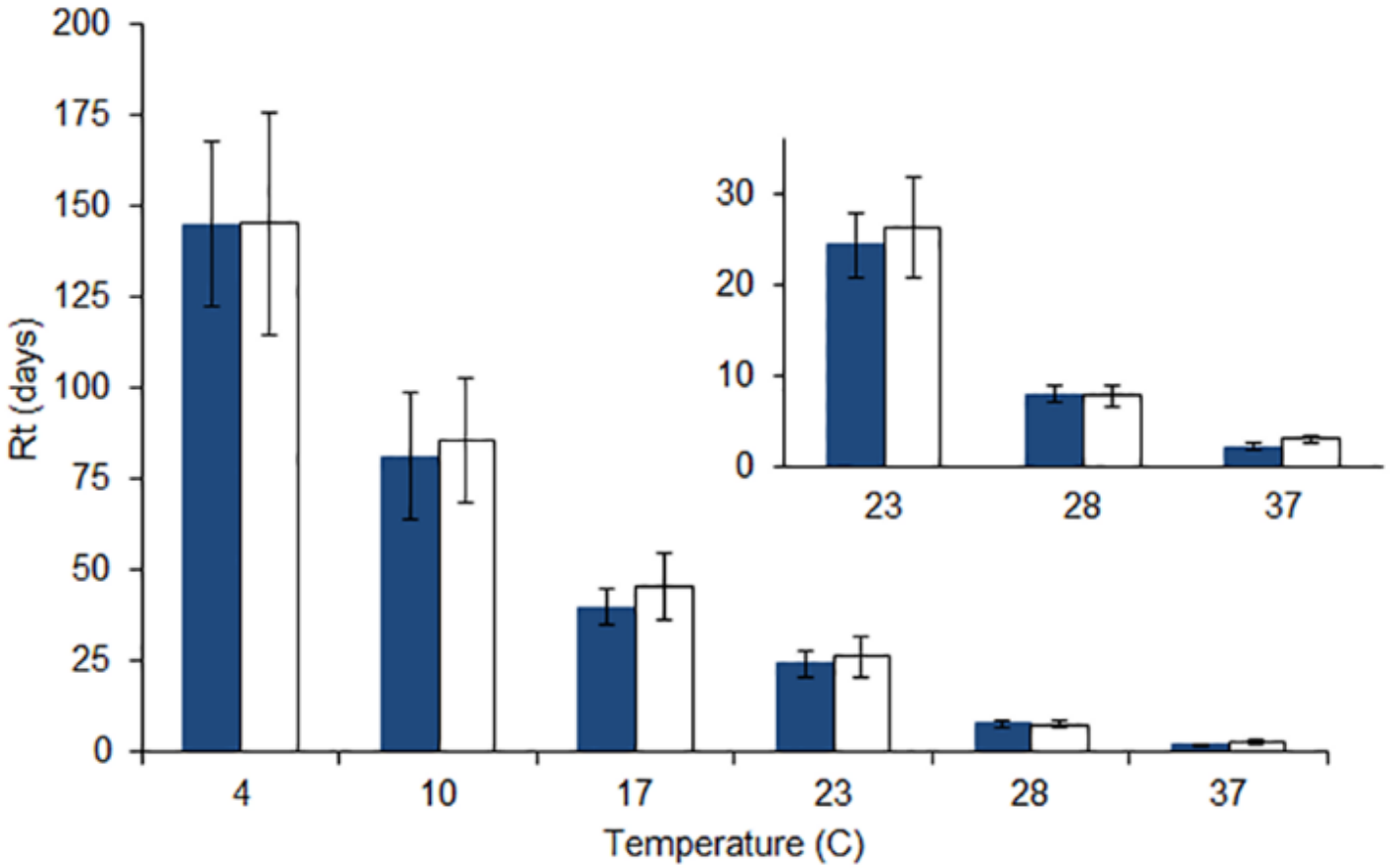

Figure 3.2. Mean Rt values (±SE) for seven pandemic (blue) and seven non-pandemic (white) viruses in distilled water at temperatures ranging from 4°C to 37°C. The pH was held constant at 7.2 and salinity was 0ppm. No statistically significant differences were observed in the response for pandemic and non-pandemic viruses at any temperature at an a value of 0.05.

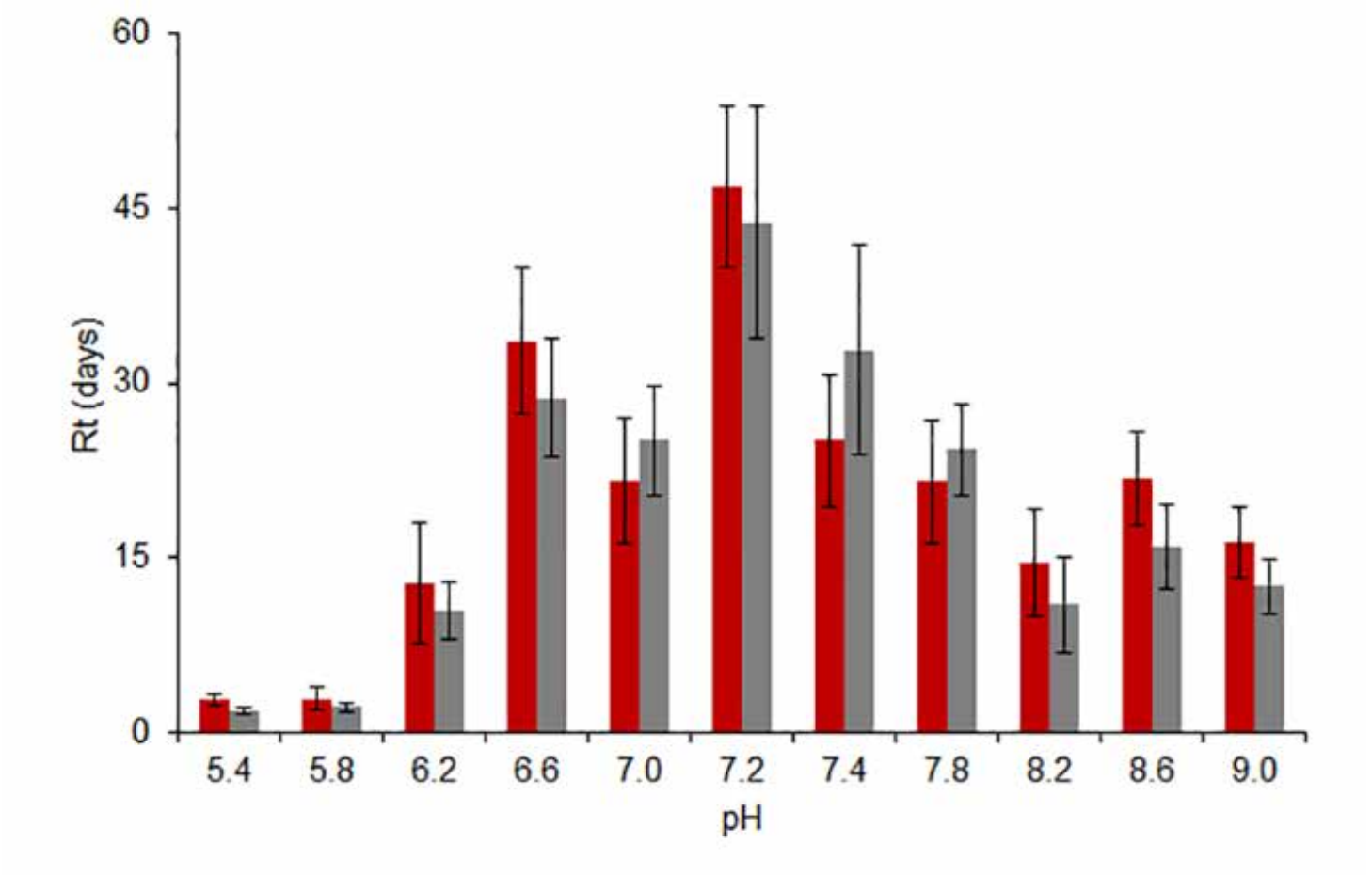

Figure 3.3. Mean Rt values (±SE) for eight swine (red) and six human (gray) viruses in distilled water at pHs ranging from 5.4 to 9.0. The temperature was held constant at 17°C and salinity was 0ppm. No statistically significant differences were observed in the response for swine and human viruses at any pH at an a value of 0.05.

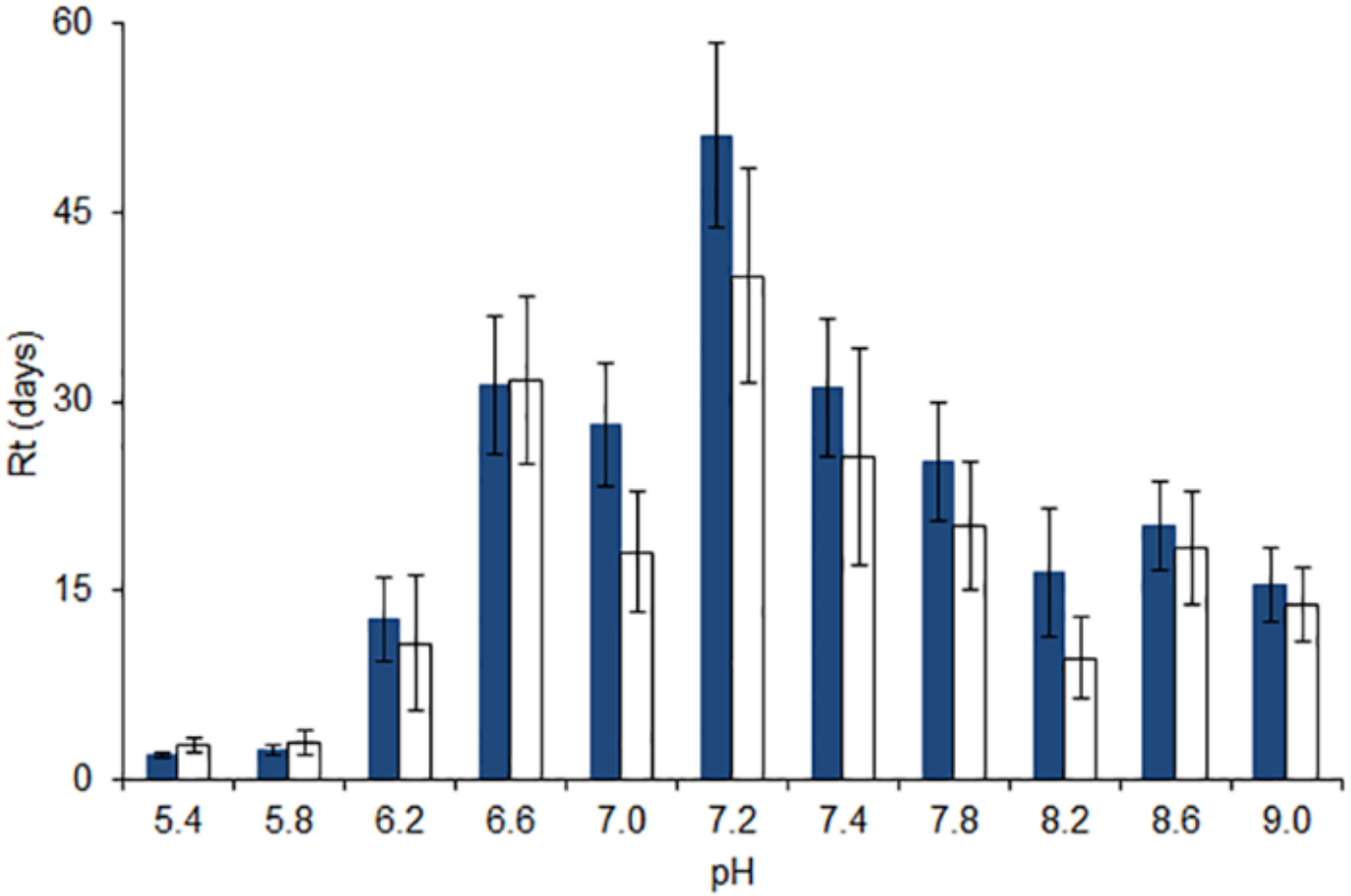

Figure 3.4. Mean Rt values (±SE) for seven pandemic (blue) and seven non-pandemic (white) viruses in distilled water at pHs ranging from 5.4 to 9.0. The temperature was held constant at 17°C and salinity was 0ppm. No statistically significant differences were observed in the response for pandemic and non-pandemic viruses at any pH at a value of 0.05.

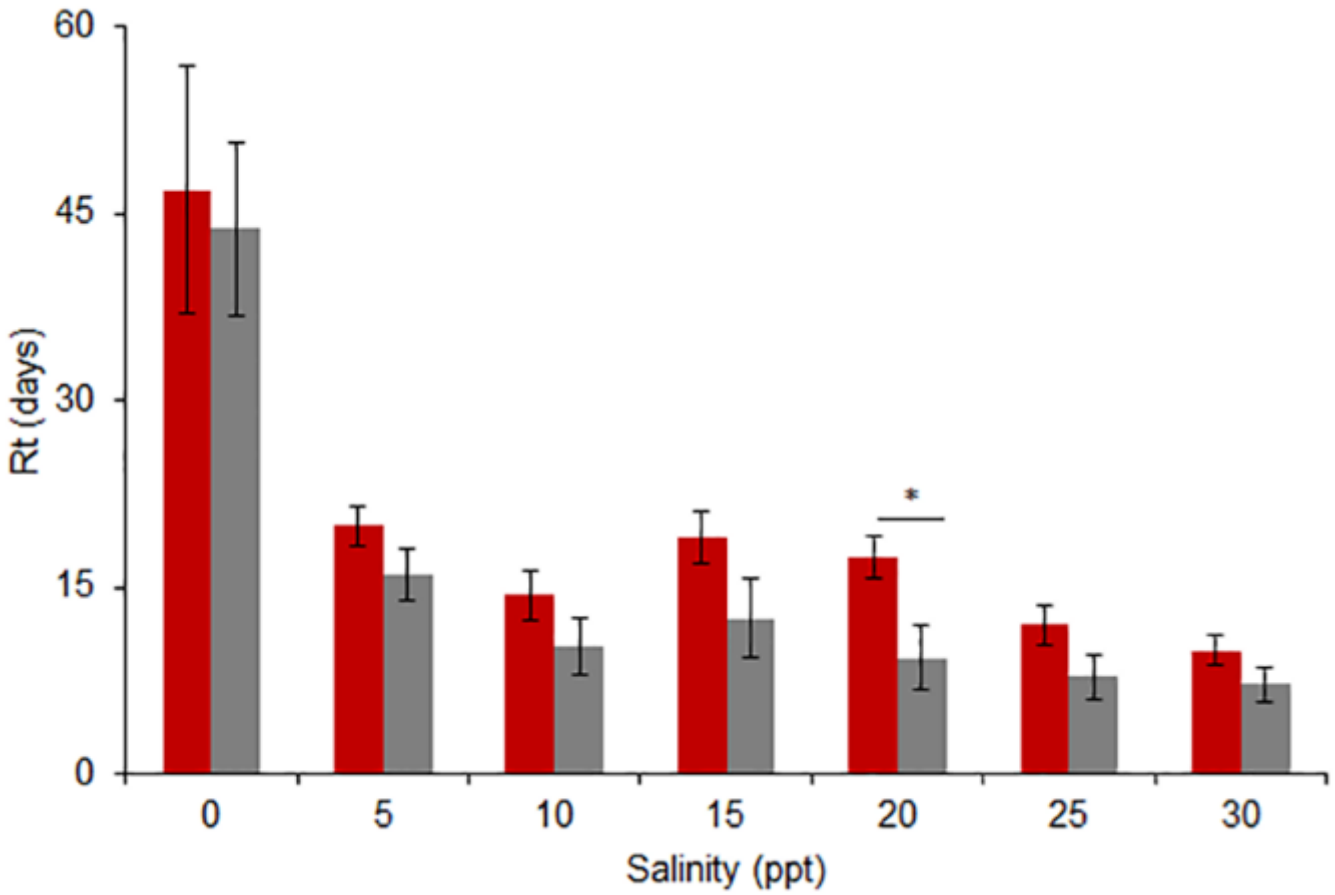

Figure 3.5. Mean Rt values (±SE) for eight swine (red) and six human (gray) viruses in distilled water at salinities ranging from 0ppt to 30ppt. The temperature was held constant at 17°C and the pH was 7.2. Significant differences in the response for swine and human viruses exist at 20ppt (*; P-value < 0.05). No statistically significant differences were observed in the response for swine and human viruses at any other saline concentration at an a value of 0.05.

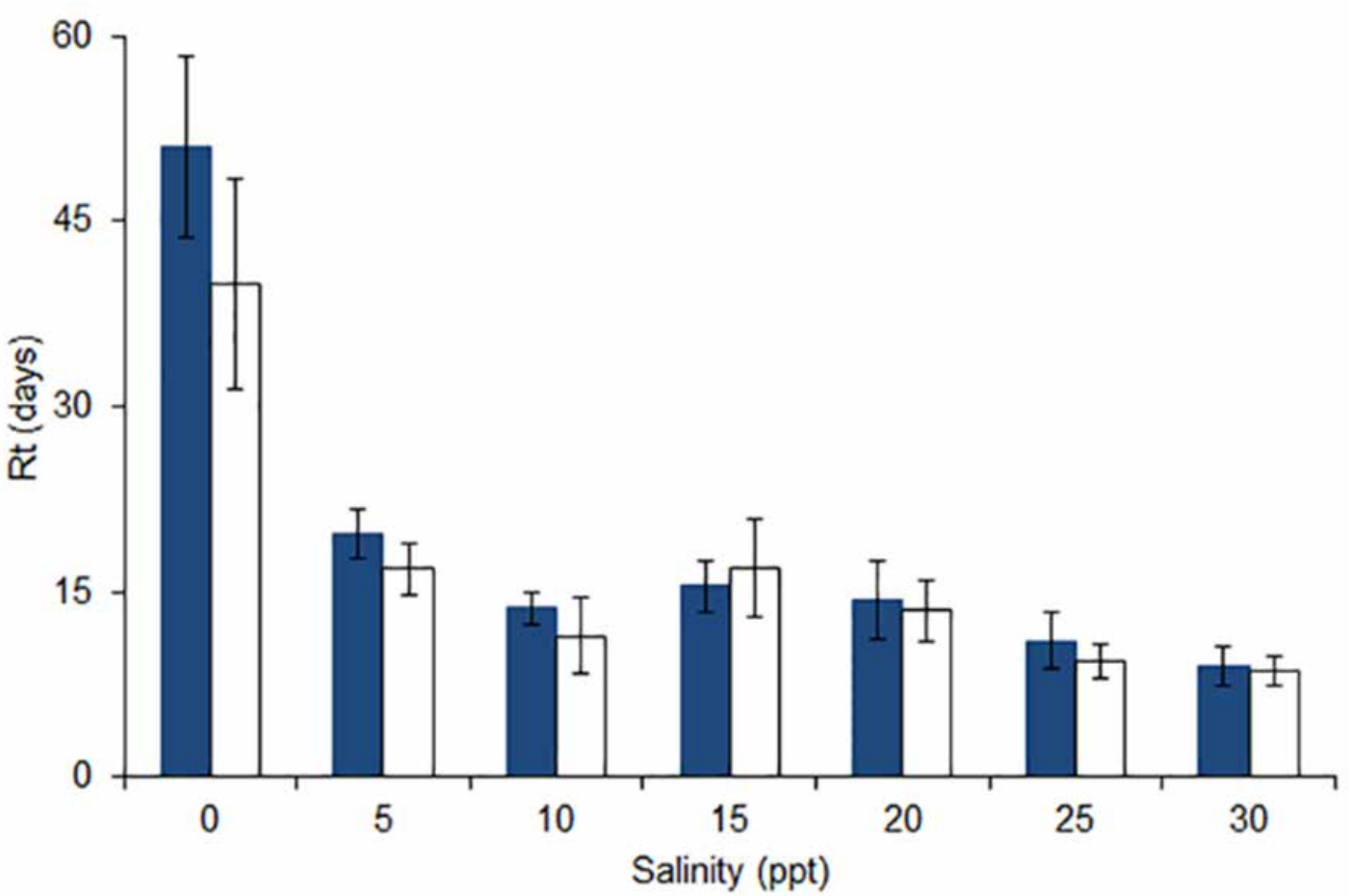

Figure 3.6. Mean Rt values (±SE) for seven pandemic (blue) and seven non-pandemic (white) viruses in distilled water at salinities ranging from 0ppt to 30ppt. The temperature was held constant at 17°C and pH was 7.2. No statistically significant differences were observed in the response for pandemic and non-pandemic viruses at any salinity at a value of 0.05.

CHAPTER 4

INFLUENZA A VIRUS: SAMPLING OF THE UNIQUE SHOREBIRD HABITAT AT DELAWARE BAY, USA[2]

[2] Poulson, R. L., P. M. Luttrell, M. J. Slusher, B. R. Wilcox, R. D. Berghaus, L. J. Niles, A. D. Dey, S. Krauss, R. G. Webster, and D. E. Stallknecht. To be submitted to *Proceedings of the Royal Society of London B: Biological Sciences*

ABSTRACT

Delaware Bay, in the northeastern United States, has long been recognized as a hotspot for avian influenza A viruses (IAVs); every spring, this coastal region serves as a brief stopover site for thousands of long-distance migrating shorebirds, en route to breeding grounds in the Arctic. During these stopovers, IAV have been consistently recovered from Ruddy Turnstones (*Arenaria interpres*) that are likely infected as they feed by probing sand and cobble in search of food. In May 2010 – 2012, we successfully isolated 19 IAV from environmental samples (sand, $n = 18$; horseshoe crab eggs, $n = 1$) obtained from Delaware Bay sites. Two of these viruses were subjected to laboratory conditions similar to those in the Delaware Bay spring-time environment, and remained infectious for seven days. Here, through the recovery of IAV from environmental samples, temperature monitoring at and below the sand surface, and simulated laboratory trials, we provide evidence that the beach environment may enhance localized transmission and short-term maintenance of IAV in this unique ecosystem.

Key words: influenza A virus, environment, Delaware Bay, avian influenza

INTRODUCTION

In avian populations, namely birds in the orders Anseriformes (ducks) and Charadriiformes (shorebirds and gulls), influenza A viruses (IAVs) are primarily transmitted through an indirect fecal-oral route involving fecal-contaminated water (Webster et al., 1992). Maintenance of IAVs in these populations is not only dependent on the ability of the virus to remain viable in the environment utilized by these avian hosts, but also on viral availability to susceptible hosts. This availability can be enhanced through specific feeding behaviors that increase viral contact with the host. Dabbling ducks, which perpetuate most hemagglutinin (HA)

subtypes (Webster et al., 1992; Krauss et al., 2004; Munster et al., 2007), feed on surface waters; when in close contact/high densities such as might be found on migratory staging areas, such feeding behaviors could lead to enhanced transmission of IAV via the fecal-oral route (Munster and Fouchier, 2009).

Tens of thousands of shorebirds (Scolopacidae) and gulls (Laridae) annually utilize beach habitats at Delaware Bay in May – June to feed on horseshoe crab (*Limulus polymephus*) eggs. Many of these birds, especially the shorebirds, rely on this food source for refueling during long-distance spring migration flights to breeding grounds in the Arctic (Robinson et al., 2003). At Delaware Bay during these stopovers, IAVs have been consistently isolated from fecal and cloacal swabs collected from shorebirds and gulls; most isolations have originated from Ruddy Turnstones (*Arenaria interpres*) (Krauss et al., 2010; Stallknecht et al., 2012). This unique convergence of IAV infection, susceptible migrating birds, and horseshoe crab eggs is unlike any other ecosystem yet discovered (Munster et al., 2007; Winker et al., 2008; Gaidet et al., 2012). Driven by lunar cycles and increasing water temperatures (Smith et al., 2010), spawning horseshoe crabs annually lay energy-rich eggs on the beaches, at depths of up to 20cm (Botton, 1992). Though dependent on shoreline characteristics, egg densities as high as 1.2 million per meter of New Jersey shoreline have been reported (Smith et al., 2002). This substantial food source is critical to the survival of migrating Ruddy Turnstones and other shorebirds stopping at this site (Tsipoura and Burger, 1999); as such, feeding on horseshoe crab eggs plays an important role in the complex annual ecology of IAV at this location.

Ruddy Turnstones utilize a foraging behavior that involves probing several centimeters into sand and cobble (Colwell and Landrum, 1993) in an effort to locate invertebrates, and when available, buried horseshoe crab eggs (Tsipoura and Burger, 1999). Although this feeding

behavior is not directly consistent with the water-borne transmission route suggested as important in IAV transmission in waterfowl populations (Hinshaw et al., 1979; Lebarbenchon et al., 2007; Rohani et al. 2009), it is conceivable that IAV deposited in feces could be protected from inactivation in a wet sand substrate and be available to feeding Ruddy Turnstones through their probing efforts. In infected Ruddy Turnstones, IAV is routinely shed in feces; feces could easily be covered by sand on the beach by either wave or wind action and be available to birds either probing or excavating to feed on horseshoe crab eggs.

To determine if such a scenario is plausible, we attempted to: 1) recover IAVs directly from the beach environment at substrate depths to which Ruddy Turnstones are capable of probing; 2) measure the temperature profiles present in this environment; and 3) experimentally assess the persistence of these viruses at the observed temperatures within a relevant media (sand/cobble).

MATERIALS AND METHODS

Beach samples

Environmental samples (sand) were collected from four Delaware Bay beaches during 2010, 2011, and 2012 (Figure 4.1): May 2010 at Reeds Beach, New Jersey (NJ) (39°07’09.47” N, 74°53’28.07”W); May 2011 at Cooks Beach, NJ (39°06'34.92" N, 74°53'34.08" W) and Pierces Point, NJ (39°05'02.00"N, 74°54'20.99"W); and in 2012, at Pierces Point and Kimbles Beach, NJ (39°06'20.41" N, 74°53'44.62" W). For sand sampling, 3mL syringes were modified to serve as coring devices via the removal of the bottom-most tip of the syringe. Sand was sampled in the afternoon, on a receding tide, and at locations at which there had been a shorebird catch the same morning. In 2010, 2011 and at Pierces Point in 2012, 15m long transects were

divided into three zones defined as (1) backshore, characterized by sporadic shorebird feeding; (2) high tide line; and (3) foreshore/swash zone, at the edge of the receding tide. Approximately 20 samples were collected in each of three transects within each zone. At the Kimbles Beach collection site in 2012, five transects were spaced approximately 1.5m apart from backshore to the water's edge, with 10 samples per transect. Samples were not taken in obvious horseshoe crab depressions. Modified syringe cores were plunged into the sand/substrate to a depth of approximately 5cm. Care was taken to avoid introducing surface sand into the sample; as such, the bottom-most 0.5mL of sand was then dispensed into 4mL cryovials containing 2mL of chilled viral transport media (VTM) consisting of brain heart infusion media (Becton Dickinson and Co., Sparks, Maryland, USA) supplemented with antibiotics [penicillin G (1,000 units/mL), streptomycin (1 mg/mL), kanamycin (0.5 mg/mL), gentamicin (0.25 mg/mL), and amphotericin B (0.025 mg/mL)] (Sigma Chemical Company, St. Louis, Missouri, USA) and the remaining volume of sand in the syringe was discarded. In 2011, opportunistic sampling of eggs in horseshoe crab excavations was carried out at Pierces Point. Small batches of freshly deposited eggs were wrapped in moistened paper towels. All samples were stored at 4°C for 24 – 48 hr and shipped to the laboratory for storage at -80°C or immediate processing.

Virus isolation from environmental samples and birds

Horseshoe crab eggs were carefully removed from paper towels with sterile forceps, and 2 – 3 were placed into each of five tubes containing 2mL of VTM. For virus isolation preparation, sand samples were vigorously vortexed for 15 sec and horseshoe crab eggs were mechanically homogenized (Tissue-Tearor, Biospec Products, Inc., Bartlesville, Oklahoma, USA). Tubes containing sand or homogenized eggs were centrifuged at 1500 x g for 15 mins and supernatant was inoculated (0.33mL/egg) into three 9 – 11 day old specific-pathogen-free

(SPF) embryonating chicken eggs (ECE) via the allantoic route. SPF ECE were incubated at 37°C for 120 hr; amnio allanotic fluid (AAF) was then harvested and tested by hemagglutination assay using 0.5% chicken red blood cells (Killian 2008). RNA from all hemagglutination assay positive AAF was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA) as per the manufacturer's instructions, and tested by matrix gene real-time reverse transcriptase (rrt)-PCR (Fouchier et al., 2000). Identified IAV were further subtyped using hemagglutinin (HA) and neuraminidase (NA) rt-PCR specific primers as previously described (Lee et al., 2001; Fereidouni et al., 2009; Tsukamoto et al., 2009). Shorebirds were also sampled and tested for IAV by virus isolation in SPF ECE during the years of environmental sampling (2010 – 2012) as previously described by the University of Georgia (UGA) and collaborators at St. Jude Children's Research Hospital (SJCRH) (Stallknecht et al., 2012).

Temperature profiles

Temperature loggers (DS1922L iButton, Maxim Integrated, San Jose, California, USA) were programmed to collect temperature data every 1800 sec at an accuracy of ± 0.5°C for approximately 48 hr. Loggers were deployed on May 23, 2012 between 17:40 and 18:40, and recovered on May 25, 2012 at 17:10. In total, 16 paired temperature loggers were placed at approximately 15cm depth in sand/substrate and at the surface of the sand; they were attached to 30cm long pieces of rebar with zip-ties for easy retrieval. Loggers were situated in eight locations that corresponded to high tide (HT), low tide (LT), and far low tide (far-LT) zones at Cooks Beach and Pierces Point, and at HT and LT zones at Reeds Beach (Figure 4.1).

Virus persistence

The three viruses used in laboratory trials were isolated in May 2012 from environmental sources (A/Sand/New Jersey/Sand2012 4-4/2012(H12N3) [Sand 4-4] and A/Sand/New

Jersey/Sand2012 5-8/2012(H12N1) [Sand 5-8]) and from a Ruddy Turnstone fecal sample: A/Ruddy Turnstone/New Jersey/AI12-2976/2012(H12N3) [RUTU-2976]. First passage isolates of stock viruses were propagated in SPF ECE and were stored at -80°C. Viruses were titrated on Madin Darby canine kidney cells (MDCK, American Type Culture Collection, Manassas, Virginia, USA) (Brown et al., 2009); viral titers ranged from $10^{6.90}$ to $10^{7.70}$ median tissue culture infectious dose ($TCID_{50}$)/mL. Table 4.1 provides a description of viruses used in this study.

Water used in the persistence trials consisted of distilled water that was buffered with 10mM HEPES, and pH was adjusted with 1N solutions of NaOH or HCl to provide a pH of 6.0 or 7.2. Salinity was adjusted to 20 ppt with the addition of commercially available sea salt (Morton, Chicago, Illinois, USA). These conditions were chosen to fall within the range of Delaware Bay water pH values (pH 6.0 – 8.5) measured in the same year in Zone 6 where sampling sites were located in our study (Delaware Bay River Commission, 2012) and to most closely match the average salinity of Delaware Bay waters in 2012 (Pyle, 2013). Aliquots of water were allowed to condition in a 22°C incubator. This temperature was chosen based upon thermal data collected from environmental data loggers placed in the sand/substrate in Delaware Bay in 2012 (described above).

A large container of sand was collected from DE Bay in 2012, and was covered and stored at indoor ambient temperature until use in the experiment. To prepare treatments, sand was weighed and aliquoted at 25g into 50mL conical tubes (approximately 14.5mL of sand per tube). Tubes of sand were placed in an environmental incubator at 22°C while viral dilutions were prepared; stock viruses were diluted in the 22°C temperature acclimated water at between 1:10 to 1:100 to achieve a starting titer of approximately $10^{5.30}$ to $10^{6.30}$ $TCID_{50}$/mL. Tubes of sand were then hydrated with 8mL of the viral-inoculated water; viral-inoculated water was also

added to 50mL conical tubes with no sand to serve as controls. Given the granularity of the sand used, 8mL of water was found to fully hydrate approximately the bottom 14.5mL of sand. Experimental conditions are summarized in Table 4.1.

Water samples from the water (W) and sand + water (SW) treatments were collected at 0 days post-inoculation (dpi) and 1, 2, 3, 4, 5, and 7 dpi. For the W treatments, tubes were vortexed, and 0.70mL water was removed. For SW treatments, a sterile 1cc syringe with 20G needle was inserted into each sand sample to 2.5cm depth and approximately 0.70mL of water was removed. At 7 dpi, a 1mL sand core (SC) also was taken from all SW treatments and processed for virus titration and matrix rrt-PCR as previously described for beach samples. Virus titers in sand and water samples were determined by microtiter endpoint titration in MDCK cells as described (Stallknecht et al., 1990b). Additionally, RNA from most samples was extracted as described for AAF, and screened and quantified for the matrix gene of IAV on a SmartCycler (Cepheid, Inc., Sunnyvale, California, USA) using primers and probe as described (Spackman et al., 2002); cycle threshold (Ct) values were recorded for all RNAs extracted.

Statistical Analyses

Viral titers were calculated according to the method of Reed and Muench (Reed and Muench, 1938). Linear regression was used to determine a 90% reduction time (Rt) for each virus/treatment combination that demonstrated more than a 1 $\log_{10}$ $TCID_{50}$/mL reduction in viral titer over the course of the trial; Rt values correspond to the time required for a decrease in viral titer by 1 $\log_{10}$ $TCID_{50}$/mL. The minimum detectable limit for this procedure is $10^{1.77}$ $TCID_{50}$/mL.

The effects of experimental factors on viral titers and Ct values were evaluated using linear mixed models with virus as a random effect. Models included main effects for sample

type (W or SW), pH (6.0 or 7.2), and day post-inoculation, as well as all possible two-way interactions between these three fixed effect variables. All tests assumed a two-sided alternative hypothesis, and P-values less than 0.05 were considered statistically significant. Analyses were performed using commercially available statistical software (JMP version PRO 12, 1989-2007; SAS Institute Inc., Cary, NC).

RESULTS

Natural environment

Viruses were successfully isolated from Delaware Bay sand core samples in 2010 and 2012. In 2010, a total of 17 H13N6 IAV viruses were isolated from 192 sand samples, with at least four recovered from every zone sampled at Reeds Beach. In 2012, no IAV were isolated from 229 sand samples collected at Pierces Point; one H12N1 and one H12N3 from 230 sand samples were isolated on different transects at Kimbles Beach. No viruses were isolated from 360 sand cores collected at either Cooks Beach or Pierces Point in 2011; however one low pathogenicity H7N3 IAV was obtained from one of five homogenized horseshoe crab egg samples collected at approximately 2cm depth at Pierces Point. Matching HA/NA subtype combinations were also recovered from swabs or environmental samples collected from shorebirds at the same sites and in the same years (Table 4.2) by UGA and/or SJCRH.

Temperature profiles obtained from data loggers on the surface of the sand were highly variable, ranging as much as 22°C over a 48 hr period as compared to only 9°C when buried at 15cm at the same site in the same time period (Pierces Point, HT; Supplementary Table S4.1). Temperature loggers in areas that were more frequently covered by water (those in far-LT and LT zones) were also less susceptible to temperature fluctuations than those placed in HT areas

which were covered with water less often over the two day period (Supplementary Table S4.1). Temperature loggers buried at 15cm in the far-LT zone at Cooks and LT zone at Reeds Beaches were recovered at 2.5cm and 10cm depth, respectively. Shallow retrieval of loggers initially buried in the LT zone was likely a function of extreme tidal washing/erosion in areas that sustained wave action. Interestingly, the logger placed at 15cm depth in the HT zone at Reeds Beach was recovered on the sand surface. The Reeds Beach site differs from the others surveyed here in that it is more residential with permanent inhabited structures, a steep, narrow beach, and a bulkhead. The interaction of these parameters may have contributed to the displacement of the logger to the surface after just two days. The potential role of tidal cycles on buried and surface substrate temperature at the Cooks Beach HT zone is shown in Figure 4.2; probes placed in the LT zone at Reeds Beach responded similarly, with the buried probe having a more constant temperature, regardless of tidal cycle (Supplementary Figure S4.1). The temperature profiles of probes buried at 15cm in far-LT and LT zones (and therefore under water more often) at Pierces Point did not undergo substantial temperature variation, regardless of tide height, while the buried probe in the HT zone was more responsive to changing tide (Figure 4.3).

Environmental persistence-experimental studies

Infectious virus was detected for 7 days in all water only (W) treatments (Table 4.3). Infectious viruses were isolated from the water of sand + water (SW) treatments for a shorter duration than from W alone (to at least 5 days for all SW treatments except the shorebird isolate, RUTU-2976, at pH 6.0 [only detected to 3 days]); infectious virus was retrieved from all sand cores (SC) at 7 dpi. RNA was detected from all treatments for all days. As expected, Ct values increased in value over the 7 days of the trial for all viruses and treatments. Environmental isolate Sand 4-4 showed an appreciable decrease in titer at 0 dpi in the SW treatment at a pH of

6.0 (3.80 $TCID_{50}$/mL), as compared to the W control at a pH of 6.0 (5.90 $TCID_{50}$/mL). Interestingly, despite a starting titer more than 1 $\log_{10}$ $TCID_{50}$/mL lower than the same virus in SW at pH 7.2, the SC sample from Sand 4-4 at 7 dpi had an Rt value approximately 2 days longer than that from the SC from the more neutral pH treatment (3.53 and 1.55 days, respectively).

With respect to viral titers, there was a significant main effect of sample type (W or SW) ($P < 0.001$) and a significant interaction between sample type and day ($P < 0.001$), indicating that titers were significantly lower in the SW samples compared to the matched W controls on day 0, and the titers in the SW samples decreased significantly more quickly over time. There was no significant main effect of pH ($P = 0.461$) and no significant interaction between pH and day ($P = 0.353$), suggesting that there was no difference in titers for samples with pH 6.0 compared to those with pH 7.2. Likewise, there was no significant interaction between sample type and pH ($P = 0.905$). Results for the Ct values were consistent with those of the viral titers, with Ct values being significantly higher in the SW samples compared to the W samples on day 0 ($P < 0.001$) and increasing significantly more quickly for the SW samples over time ($P < 0.001$). There was no significant main effect of pH on the Ct values ($P = 0.116$) and no significant interaction between pH and day ($P = 0.140$). Finally, there was no significant interaction between sample type and pH in their effects on Ct values ($P = 0.280$).

DISCUSSION

Within the Delaware Bay ecosystem, the unique foraging strategies of Ruddy Turnstones, coupled with their population density and propensity to amplify IAVs, provides an opportunity to explore the interplay where the potential environmental persistence of IAV, host behavior, and

viral transmission intersect. To be transmitted, viruses must remain infectious in the environmental milieu that exists between hosts and susceptible individuals, for an adequate duration, and in a form that will allow for transfer to new hosts. Given that Ruddy Turnstones probe substrate and debris in search of food, transmission of infectious virus buried by foraging efforts of other birds, by spawning of horseshoe crabs, or by virus/feces percolated into sand could be an expansion of the indirect fecal-oral route long recognized as the primary mode of transmission of avian IAV.

At least one IAV was successfully isolated from environmental (sand and/or horseshoe crab egg) samples every year from 2010 – 2012. In relation to viruses recovered from sand cores, care was taken to collect samples from a depth at which foraging shorebirds could naturally probe. Surface sand was not included, in order to avoid (day of sampling) contamination from recently deposited shorebird feces. Although the IAV subtype(s) isolated from sand cores/horseshoe crab eggs matched the IAV subtypes isolated directly or indirectly (feces) from shorebirds, it is unlikely that recently deposited virus would percolate to this depth without subsequent tidal action or through the action of Ruddy Turnstones excavating sites while feeding. Therefore it is likely that some of the infectious viruses recovered were excreted at least several hours, and possibly a number of tidal cycles, earlier.

Temperature profiles from buried and surface probes reveal a number of factors relevant to the environmental maintenance of IAV. First, buried temperature loggers, regardless of tidal positioning, showed a narrower range of temperature extremes whereas those on the sand surface were influenced more by ambient air temperature and solar irradiation when not underwater, and by water temperature as the tide covered them. Also, a more constant layer of water on loggers (such as was the case at LT and Far-LT zones) tended to further restrict temperature fluctuation

(Figure 4.3). The ability of IAV to remain infectious is inversely related to increasing temperature; most IAV tend to be fairly quickly inactivated at high temperatures in both distilled (Stallknecht et al., 1990a; Stallknecht et al., 1990b; Brown et al., 2007; Brown et al., 2009; Stallknecht et al., 2010) and natural (Stallknecht et al., 1990a; Nazir et al., 2010; Lebarbenchon et al., 2011; Keeler et al., 2012) water studies. Modulation of the temperature in a given natural microenvironment, such as could be found below the surface of the sand, could play an important role in the ability of IAV to remain infectious. In addition to temperature, buried virus would also be protected from UV inactivation and depending on the elevation of the beach, from desiccation. The movement of several loggers from buried to surface positions could provide evidence of a natural mechanism that may serve to increase the availability of these viruses to feeding birds; the observed sand movement may have resulted from wave, tide, current, or wind action. Additionally, given their large body size and burrowing tendencies, the movements of horseshoe crabs have been shown to reactivate sediment and alter the release of eggs back onto the surface, often from depths not penetrated from wave action alone (Botton et al., 1994; Jackson et al., 2005). These results not only provide evidence of a microenvironment that is compatible with IAV persistence but also of a means for increasing the availability of these viruses to birds after deposition.

Under laboratory conditions at average water salinity (20ppt) and sand temperature (22°C) observed at Delaware Bay in 2012, all three IAV (Sand 4-4, Sand 5-8, and RUTU-2976) remained viable in sand and were detectable by IAV matrix rrt-PCR for the duration of the experiment (7 dpi). When these relevant IAV were utilized in persistence trials, variation was seen across all treatments and viruses; this was expected based on previous results from experiments using distilled and natural water models (Stallknecht et al., 1990a; Brown et al.,

2009; Negovetich and Webster, 2010; Keeler et al., 2012; Keeler et al., 2013). Viral titers for all viruses at both pH levels decreased more quickly in SW samples than in W samples alone, indicating an effect related to the presence of sand. The sand used in these trials was not sterile nor chemically characterized and could have contained both biological and chemical components that could have had a detrimental effect on virus infectivity. Factors that may have been associated with the sand that potentially inactivate viruses include increased concentrations of salt(s), proteolytic enzymes (Yates et al., 1990), and microbial antagonism (Sobsey and Meschke, 2003).

Infectious virus was isolated from the water portion of SW for 5 – 7 days with the two environmental isolates (Sand 4-4 and Sand 5-8) and between 3 – 5 days for the bird-origin RUTU-2976 isolate. All viruses were re-isolated from the sand portion of all SC samples at the termination of the experiment (at 7 dpi), and in most cases, at a higher titer than those recovered from the water portion. The water portions of the SW samples were removed via syringe at all sampling points, and as such, very little particulate matter was included in the aliquots. The sand portions of these samples (day 7) were vigorously vortexed in media of a higher pH before supernatants were removed for titration and RNA extraction. Based on these results (virus not detected in water (SW) but detected in sand (SC) from the same sample), it appears that infective virus was bound or sequestered within the sand matrix. Sand 4-4 showed more than a 2 $\log_{10}$ $TCID_{50}$/mL reduction in viral titer when inoculated into the SW treatment at pH 6.0 (Table 4.3), but the SC taken from this same treatment at 7 dpi had an Rt value of 3.53 days, the highest of any of the SC samples. Given that sand treatments were not homogenous in particle size, abiotic or biotic components, or exact chemical composition, this may represent the case where a particular viral microcosm was quickly sequestered into a heterogenous surrounding sand matrix

and protected from the potentially detrimental effects of a low environmental pH. Factors such as aggregation (Young and Sharp, 1977) and adsorption onto surfaces or particles (Gerba and Schaiberger, 1975) can stabilize or help protect viruses from degradation; however, there is currently little information available related to the ability of IAV to adsorb to sandy substrates and other sediment types. Such binding may have facilitated the isolation of an IAV from horseshoe crab eggs. These eggs were naturally contaminated in the beach environment which further supports the potential role of environmental components (sand, cobble, detritus, food sources, feces) as contributing to viral availability in the Delaware Bay system. The role of horseshoe crab eggs in sequestering infectious IAV as related to transmission to foraging hosts warrants further investigation.

Electrostatic charges can serve to bind or sequester viruses within columns of sand/sediment in laboratory and natural settings and the morphology, dimension, and isoelectric point(s) (IEP) of viral particles and surface proteins play major roles in the fate of a given viral population in an environment. The IEP of IAV, which corresponds to the pH value at which the virus has neutral charge, has been shown to range between 4.0 to 7.0 (Michen and Graule, 2010), dependent upon purification technique and viral origin. For a given virus, the more positively charged the surrounding sediment is, the more likely the virus is to be adsorbed, whereas increasingly more alkaline pH would favor desorption of virus from sediment through the generation of strong repulsive forces (Taylor et al., 1981; Rao et al., 1986). Finally, the potential implications of the properties of AAF (in which the viruses used in our persistence trial were propagated) bear mentioning. Factors such as the pH of AAF, recorded to be between 6.6 – 7.1 at 14 days of chicken embryo development (Boutilier et al., 1977; Kanmaz et al., 2001), as well as the varied biochemical and physical properties of its primary components albumen (Willems

et al., 2014) and other proteinaceous matter, may be contributing to the tendency of these IAV to adsorb to or be repelled from the sand particles.

In the Delaware Bay IAV/shorebird relationship, viruses that can persist at relevant ambient temperatures and in/on natural substrates might be more readily available to susceptible shorebirds. Those viruses that can remain infectious longer under such conditions could serve to facilitate both the maintenance and transmission of IAV. Additionally, the combining of virus with sand may protect it from being dislodged by tidal washing. Our study demonstrates that (1) viable virus can be isolated from the beach environment at Delaware Bay during periods when shorebirds are infected with IAV; (2) mechanisms exist related to Ruddy Turnstone feeding and the natural movement of sand to make these viruses available to feeding birds; and (3) unknown mechanisms exist that appear to hold and stabilize these viruses in the sand matrix. Based on these observations, it is possible that the local beach environment may contribute to IAV transmission and short-term maintenance during these annual outbreaks. The isolation of IAV subtypes only present in shorebirds at the time of sampling, however, suggests that the beach environment does not contribute to long-term IAV maintenance at Delaware Bay, but the infection of shorebirds in this unique ecosystem, and the subsequent spread of IAV as a result of migration may play a role in the global maintenance of IAV.

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Table 4.1. Description of influenza viruses and experimental conditions used in laboratory persistence trials.

Strain Name	ID in trial	Passage[a]	Titer ($TCID_{50}$/mL)	pH	Substrate[b]
A/Sand/New Jersey/Sand2012 4-4/2012(H12N3)	SAND 4-4	SPFE2	7.78	6.0	SW, SC
					W
				7.2	SW, SC
					W
A/Sand/New Jersey/Sand2012 5-8/2012(H12N1)	SAND 5-8	SPFE2	7.42	6.0	SW, SC
					W
				7.2	SW, SC
					W
A/Ruddy Turnstone/New Jersey/AI12-2976/2012(H12N3)	RUTU-2976	SPFE2	6.98	6.0	SW, SC
					W
				7.2	SW, SC
					W

[a] SPFE2 = specific-pathogen-free embryonated chicken egg passage 2
[b] SW = sand + water; SC = sand core; W = water only

Table 4.2. Influenza A viruses recovered from environmental samples (sand from transects in 2010 and 2012; horseshoe crab eggs in 2012) collected from three beach locations at Delaware (DE) Bay, USA, and matching HA/NA subtypes from shorebirds collected at DE Bay in the same year.

Collection Year	Location	Transect Location	Strain Name	Subtype	Recovery of matched HA/NA subtype from shorebirds
2010	Reeds Beach	Backshore Zone#1 (behind AM bird net)	A/sand/NJ/Sand2010 1-17/2010	H13N6	SJCRH[#] (1): Reeds Beach
			A/sand/NJ/Sand2010 1-19/2010		
			A/sand/NJ/Sand2010 1-24/2010		
			A/sand/NJ/Sand2010 1-27/2010		
		Swash/high tide Zone#2 (AM net area)	A/sand/NJ/Sand2010 2-11/2010		
			A/sand/NJ/Sand2010 2-35/2010		
			A/sand/NJ/Sand2010 2-37/2010		
			A/sand/NJ/Sand2010 2-38/2010		
			A/sand/NJ/Sand2010 2-42/2010		
			A/sand/NJ/Sand2010 2-43/2010		
			A/sand/NJ/Sand2010 2-47/2010		
			A/sand/NJ/Sand2010 2-49/2010		
			A/sand/NJ/Sand2010 2-56/2010		
		Foreshore Zone#3 (7.5-9m from end of AM net)	A/sand/NJ/Sand2010 3-1/2010		
			A/sand/NJ/Sand2010 3-7/2010		
			A/sand/NJ/Sand2010 3-26/2010		
			A/sand/NJ/Sand2010 3-72/2010		
2011	Pierces Point	N/A	A/horseshoe crab egg/NJ/AI11-673/2011	H7N3	UGA[¥] (7): Cooks Beach (2); Pierces Point (1); Reeds Beach (3); Villas Beach (1)
					SJCRH[#] (8): Cooks Beach (4); Kimbles Beach (2); Pierces Point (2)
2012	Kimbles Beach	Transect#4 (1.5m behind receding water's edge)	A/sand/NJ/Sand2012 4-4/2012	H12N3	UGA (20): Baycove (2); Cooks Beach (1); Kimbles Beach (12); Pierces Point (4); Reeds Beach (1)
					SJCRH[#] (7): Norburys Landing (1); Pierces Point (6)
		Transect#5 (at receding tide edge)	A/sand/NJ/Sand2012 5-8/2012	H12N1	UGA (14): Baycove (4); Cooks Beach (2); Kimbles Beach (5); Pierces Point (3)

[#] St. Jude (SJCRH) IAV isolates were obtained from fecal swabs;

[¥] UGA IAV isolates were obtained from fecal swabs or oropharyngeal/cloacal swabs taken directly from shorebirds or gulls.

Table 4.3. Viral titers reported as $TCID_{50}$/mL and IAV matrix gene Ct values, in brackets, for all viruses and treatments analyzed for seven days as part of the experimental laboratory persistence trial. Regression equations and Rt values were calculated for all treatments yielding at least a one log reduction in viral titer over the length of the trial.

			Titer ($TCID_{50}$/mL) [Ct Values]								**SC[+]**	
			Days post-inoculation (dpi) water samples								**dpi sand**	**Regression Equation, Rt value (days)**
Virus	**Treatment**	**pH**	0	1	2	3	4	5	*6*	7	7	
Sand 4-4	W[#]	6.0	5.90 [21.66]	5.57 [21.86]	5.55 [21.38]	5.52 [21.36]	5.44 [22.24]	5.41 [21.97]	**NT[a]** [22.42]	5.49 [22.63]	***N/A*[b]**	***N/A***
		7.2	5.94 [21.60]	5.59 [21.6]	5.76 [21.65]	5.63 [21.89]	5.47 [21.99]	5.56 [23.02]	**NT** [22.48]	5.69 [22.43]	***N/A***	***N/A***
	SW[*]	6.0	3.80 **[NT]**	2.94 **[NT]**	1.91 [34.30]	2.76 **[NT]**	1.77 **[NT]**	1.77 [32.50]	**NT** [NT]	1.84 [38.93]	1.83 [36.85]	y = -0.28x + 3.24, 3.53
		7.2	5.17 [29.39]	3.16 [31.36]	1.96 [30.07]	2.30 [33.05]	1.77 [32.66]	1.80 [32.43]	**NT** [32.02]	**ND[c]** [33.03]	1.80 [36.37]	y = -0.64x + 4.18, 1.55
Sand 5-8	W	6.0	6.32 [18.91]	6.10 [18.74]	6.01 [18.80]	6.10 [18.75]	5.63 [19.14]	6.10 [19.58]	**NT** [19.60]	6.10 [19.31]	***N/A***	***N/A***
		7.2	6.32 [19.13]	6.04 [18.85]	6.10 [18.75]	6.10 [19.23]	5.17 [19.33]	5.01 [19.54]	**NT** [19.90]	4.90 [19.54]	***N/A***	y = -0.23x + 6.36, 4.44
	SW	6.0	4.81 [22.61]	3.44 [27.99]	4.01 [25.65]	2.90 [28.02]	2.47 [27.55]	1.93 [28.39]	**NT** [30.42]	**ND** [26.83]	3.88 [29.36]	y = -0.52x + 4.56, 1.91
		7.2	5.17 [21.71]	4.12 [26.68]	3.56 [25.59]	3.36 [26.78]	1.79 [27.95]	1.77 [28.34]	**NT** [27.68]	1.79 [26.73]	2.93 [30.27]	y = -0.50x + 4.65, 2.01
RUTU-2976	W	6.0	5.57 [20.41]	5.10 [20.76]	5.17 [20.82]	5.01 [20.39]	5.22 [20.81]	4.17 [21.70]	**NT** [23.55]	3.24 [24.60]	***N/A***	y = -0.31x + 5.72, 3.18
		7.2	5.52 [20.36]	5.49 [20.54]	5.44 [20.58]	5.69 [20.25]	4.60 [20.50]	4.90 [21.33]	**NT** [22.14]	5.01 [21.39]	***N/A***	***N/A***
	SW	6.0	4.59 [24.00]	3.32 [28.58]	2.31 [28.01]	1.84 [28.82]	**ND** [29.15]	**ND** [31.13]	**NT** [31.85]	**ND** [30.61]	2.22 [31.53]	y = -0.98x + 4.43, 1.02
		7.2	5.00 [23.49]	3.28 [28.55]	2.47 [27.65]	1.93 [29.21]	1.79 **[NT]**	1.77 [30.33]	**NT** [29.16]	**ND** [29.35]	3.03 [31.35]	y = -0.64x + 4.21, 1.57

[a] NT = not tested, [b] *N/A* = not applicable, [c] ND = tested, but below limit of detection

[#] W = water only treatment; * SW = sand + water treatment; [+] SC = sand core taken at 7 dpi

Supplemental Table 4.1. Maximum, minimum, mean, and the range in temperatures (°C) for individual temperature loggers in the beach environment, by site, tidal zone, and initial positioning on (surface) sand or buried at 15 cm.

		Zone											
		High Tide (HT)				Low Tide (LT)				Far Low Tide (far-LT)			
Probe Location	**Site**	**Max**	**Min**	**Mean**	**Range**	**Max**	**Min**	**Mean**	**Range**	**Max**	**Min**	**Mean**	**Range**
Surface	Cooks	32.1	17.6	23.1	14.5	32.1	17.6	23.4	14.5	27.2	18.2	22.0	9.0
	Pierces	39.1	17.2	25.7	21.9	28.7	18.1	22.7	10.6	26.6	18.1	21.9	8.5
	Reeds	38.7	17.7	25.4	21.0	27.6	18.6	26.0	9.0	--	--	--	--
Buried (15 cm)	Cooks	24.1	20.1	22.1	4.0	21.1	19.1	20.2	2.0	22.6[#]	20.1	21.4	2.5
	Pierces	28.6	20.1	23.7	8.5	23.6	19.6	21.7	4.0	22.7	20.2	21.4	2.5
	Reeds	35.6[*]	19.1	25.4	17.0	21.7[¥]	19.7	20.7	2.0	--	--	--	--

[*] Buried probe at Reeds HT was retrieved at the surface of the sand at 48 hours

[¥] Buried probe at Reeds LT was retrieved at 10cm depth at 48 hours

[#] Buried probe at Cook's far-LT was retrieved at 2.5cm depth at 48 hours

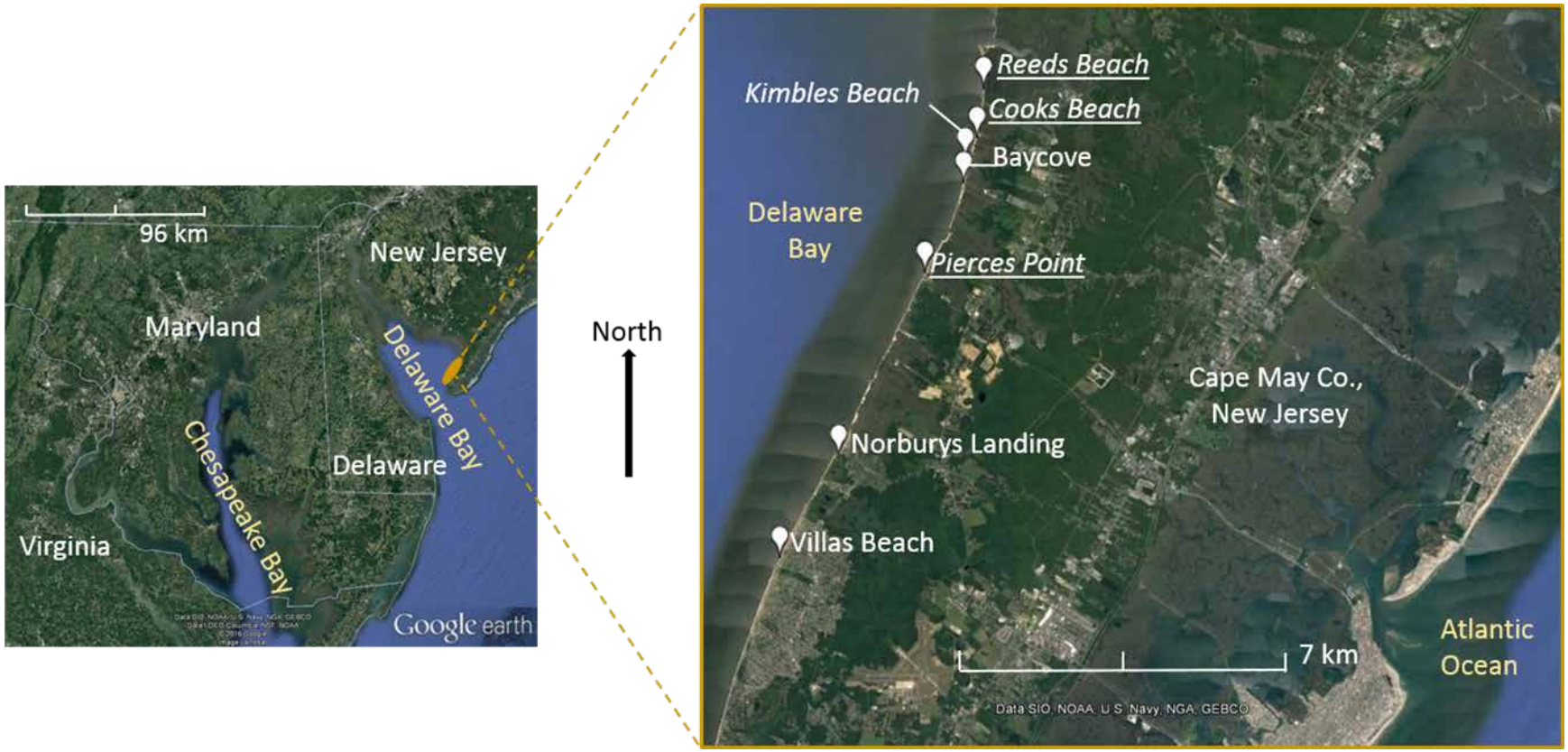


Figure 4.1. Delaware Bay sampling locations from which IAVs were isolated from shorebird samples (2010 – 2012; white markers), at which temperature loggers were placed (2012; underlined location names) and the locations of environmental sampling (2010 – 2012; *italicized* location names).

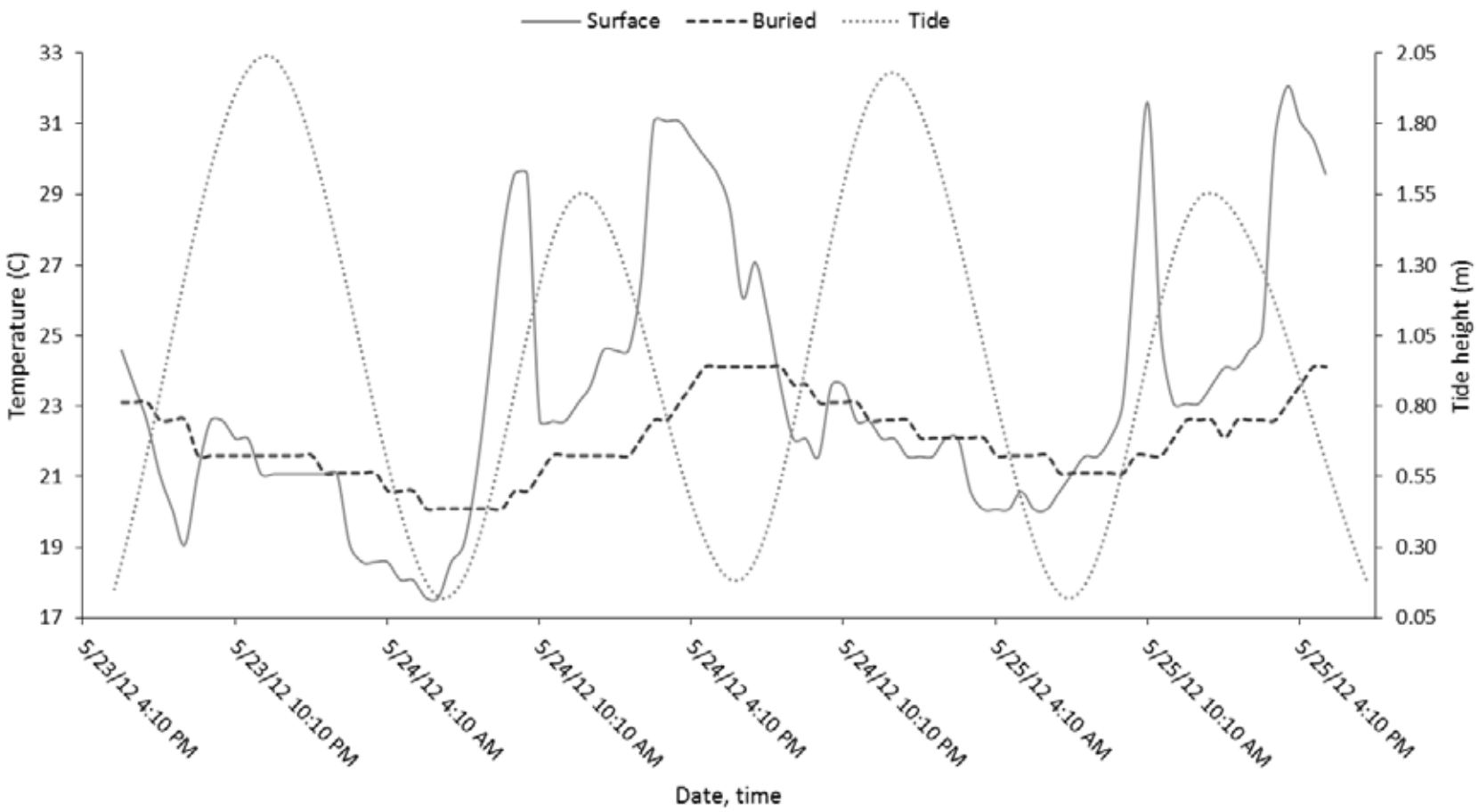


Figure 4.2. Cooks Beach HT temperature (°C) profiles for surface (solid, gray) and buried (dashed, black) temperature loggers over a 2 day time period. Tide height in meters is reflected on the secondary axis (dotted, light gray).

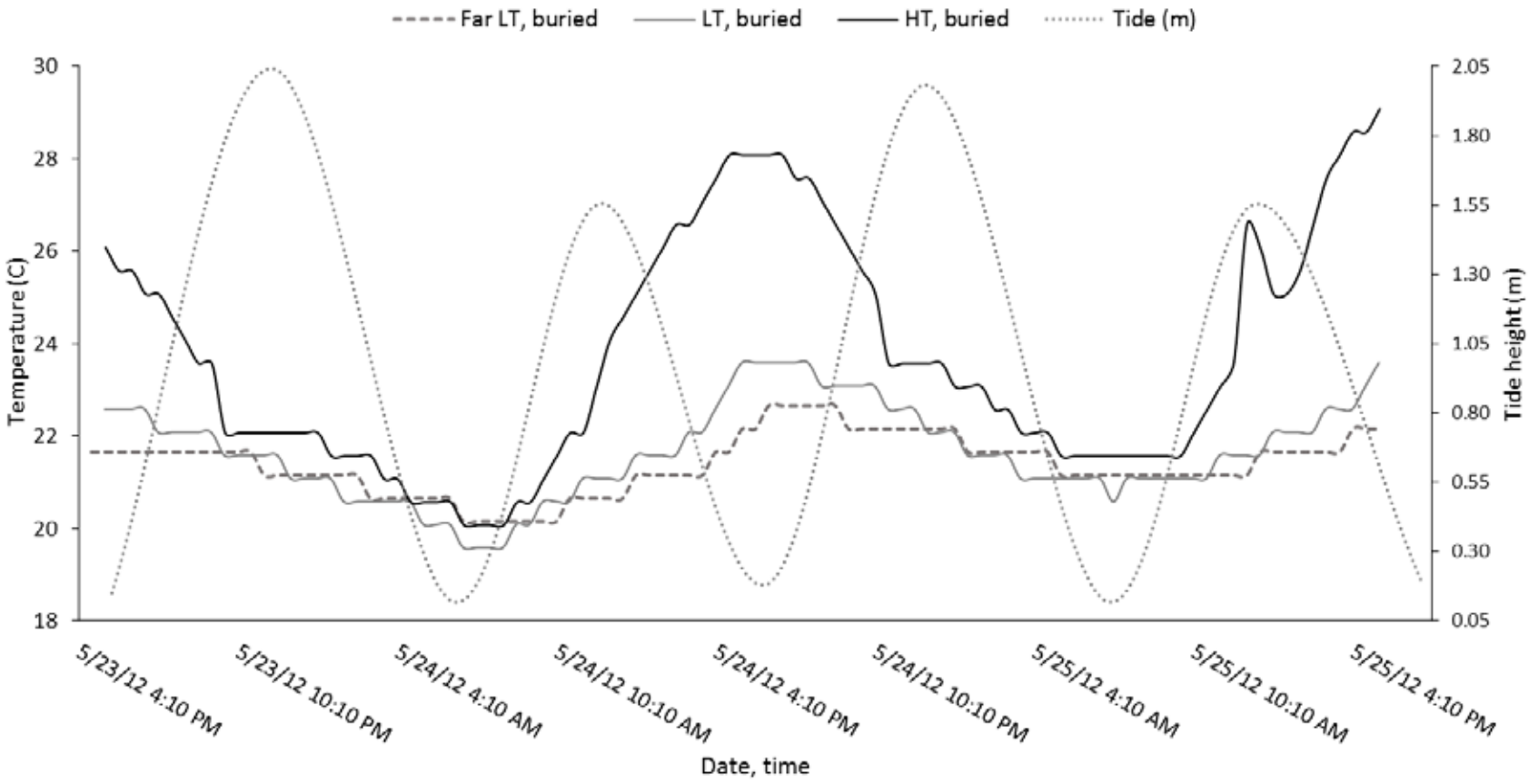
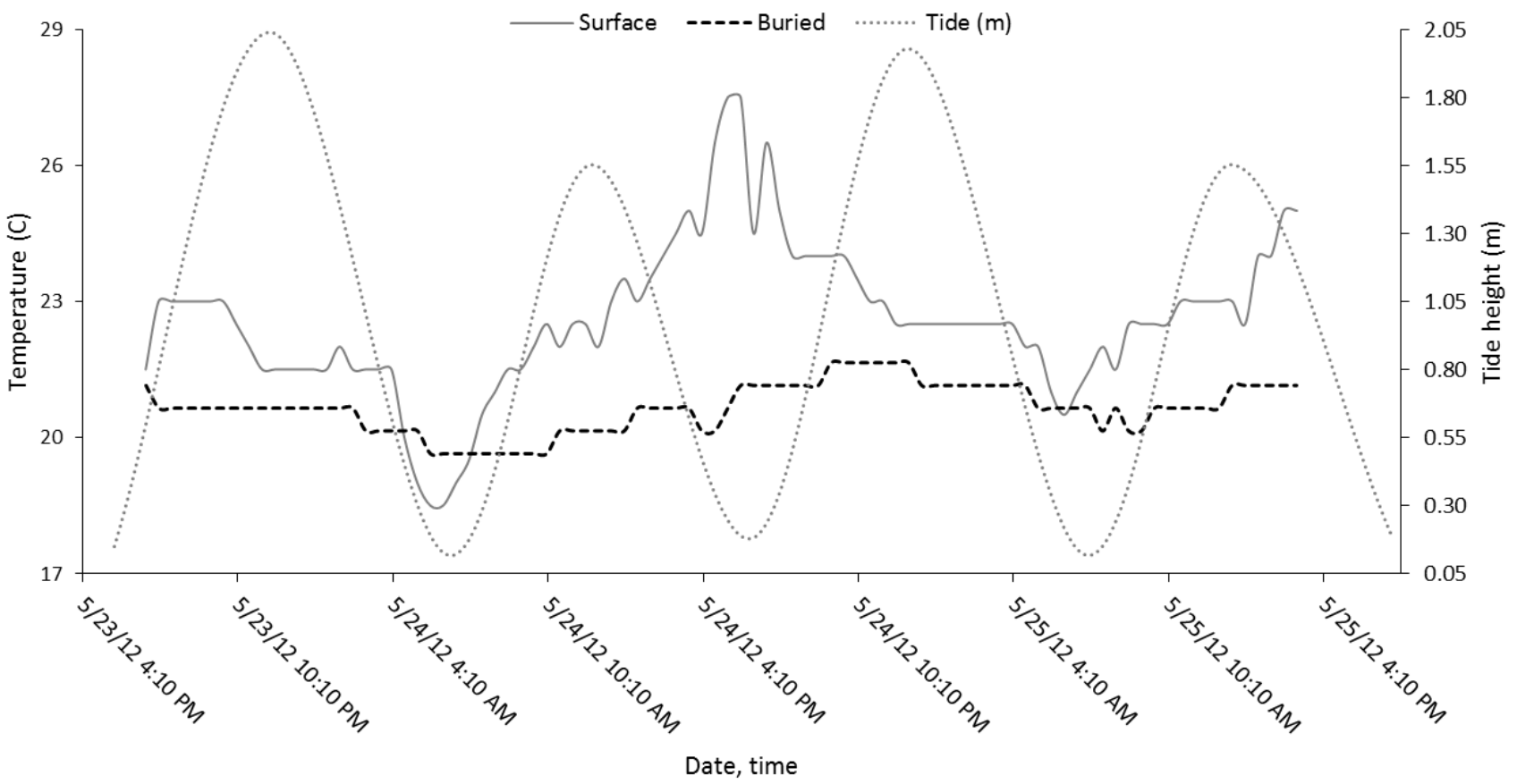


Figure 4.3. Pierces Point temperature (°C) profiles for buried temperature loggers over a 2 day time period in far-LT (dashed, gray), LT (solid, gray) and HT (solid, black) zones. Tide height in meters is reflected on the secondary axis (dotted, light gray).



Supplementary Figure 4.1. Reeds Beach HT temperature (°C) profiles for surface (solid, gray) and buried (dashed, black) temperature loggers over a 2 day time period. Tide height in meters is reflected on the secondary axis (dotted, light gray).

CHAPTER 5

INFLUENZA A VIRUSES IN WINTERING AND MIGRATING RUDDY TURNSTONES (*ARENARIA INTERPRES*) AND THEIR CONNECTION WITH AN ECOLOGICAL HOTSPOT AT DELAWARE BAY[3]

[3] Poulson, R.L., D. L. Carter, A. M. Ramey, S. Belville, L. J. Niles, A. D. Dey, C. Minton, S. Krauss, R. G. Webster, and D.E. Stallknecht. To be submitted to *Scientific Reports*

ABSTRACT

For more than three decades, avian influenza A viruses (IAV) have been annually isolated from shorebirds and gull hosts in the order Charadriiformes at Delaware Bay, USA, during May. This site serves as a critical migratory stopover site for shorebirds on their northward migration to arctic breeding grounds. Although the majority of IAV isolates at this site have been recovered from Ruddy Turnstones (*Arenaria interpres*), it is unknown if this species is involved in either the maintenance or movement of these viruses outside of the Delaware Bay hotspot. To address this, we sampled and tested Ruddy Turnstones on wintering areas in Florida and Georgia from 2010-2015 and recovered 25 low pathogenicity IAV. In most cases, the HA and/or NA subtype of IAVs recovered from Ruddy Turnstones on wintering areas matched viruses recovered at Delaware Bay during the previous year or that year's migratory cycle. Given the recovery of these "matched" antigenic surface proteins, we hypothesized that viral gene segments were transported into Delaware Bay with migrating Ruddy Turnstones. Genetic analyses of all eight IAV gene segments from four H5N9 and 15 H6N1 viruses recovered from Ruddy Turnstones during the winter of 2012 and the following May at Delaware Bay revealed a high level of genetic relatedness at the nucleotide level, suggesting that migrating Ruddy Turnstones are serving to move IAVs from wintering grounds to the Delaware Bay ecosystem.

Key words: influenza A virus, Ruddy Turnstone, shorebird migration, Delaware Bay

INTRODUCTION

The movement of migrating birds, at both small scales and across broad latitudinal and longitudinal gradients, can serve to disseminate avian influenza A viruses (IAV) and other

pathogens over potentially long distances (Rappole et al., 2000; Pearce et al., 2011; Ramey et al., 2015; Reeves et al., 2015; Reeves et al., 2016). Wild birds in the orders Anseriformes (ducks and geese) and Charadriiformes (shorebirds, gulls, and terns) are known to have a role in the movement and maintenance of IAV. While ducks and geese have been shown to be critical to the long-term maintenance of IAV, shorebirds are thought to be important in the spread of viruses over long distances but on a shorter time scale (Bahl et al., 2013). Transmission of IAV among ducks primarily occurs via a fecal-oral route (Hinshaw et al., 1979), but less is known about transmission of IAV among shorebirds and gulls. Within both Anseriformes and Charadriiformes, the maintenance of IAV is presumed to be continuous, though often intermittent and at low levels (Krauss et al., 2004), such as is the case when these birds are on their wintering grounds.

Delaware Bay, USA represents a critical stopover site where shorebirds replenish fat stores and energy reserves during long distant migratory flights from their wintering to Arctic breeding grounds (Robinson et al., 2003). During May, large numbers of shorebirds (Family Scolopacidae) and gulls (Family Laridae) annually congregate at high densities (up to 210 birds per square meter (Clark et al., 1993; Gillings et al., 2007)). For more than three decades at this location, IAVs have been consistently recovered from shorebirds and gulls with most isolates originating from Ruddy Turnstones (*Arenaria interpres*) (Krauss et al., 2010; Stallknecht et al., 2012). While at Delaware Bay, the increase in both IAV and antibody prevalence over time and the association of these increases with Ruddy Turnstones body mass indicates that the majority of birds are infected after arrival (Maxted et al., 2012). This is likely a function of the high density and co-mingling of susceptible birds. Migrating Ruddy Turnstones amplify and maintain a diversity of IAV subtypes while present at Delaware Bay (Krauss et al., 2010; Stallknecht et

al., 2012). At this site, the relationship between shorebirds and IAV is localized and short-term, with Ruddy Turnstone-specific onsite amplification (Maxted et al., 2012). In shorebirds, recovery of IAV outside of Delaware Bay and at other times of the year has been infrequent (Gaidet et al., 2012; De Araujo et al., 2014; Hurtado et al., 2015), and as such, this stopover site has been coined an ecological "hotspot" for IAV (Krauss et al., 2010).

Ruddy Turnstones in the Americas winter along both coasts of North and South America, and as far south as Tierra gel Fuego (Nettleship, 2000). Upon their southern migration during late summer/early fall, these birds become widely dispersed, in small flocks that frequent rocky shorelines and mudflats (Nettleship, 2000); winter densities are in stark contrast to those seen at DE Bay, which are estimated to be greater than 60 Ruddy Turnstones/m^2 (Gillings et al., 2007). These birds remain on wintering grounds for their first complete summer (Nettleship, 2000), and the majority of adult birds return to the same breeding and overwintering locations annually, via the same migratory routes (Metcalfe and Furness, 1985). In the past decade, surveillance efforts in Brazil targeting the fall return of shorebirds from their breeding grounds have resulted in the isolation of three H11N9 viruses (De Araujo et al., 2014), and several IAV have been isolated from Ruddy Turnstones in Peru during winter months (Ghersi et al., 2009; Nelson et al., 2016). However, there is a scarcity of data on IAV epidemiology and ecology in shorebirds on their wintering grounds.

Despite extensive surveillance for IAV in Delaware Bay, it remains unknown how these viruses become so rampant in the system in such a short window of time, annually diversify into dozens of different hemagglutinin (HA) and neuraminidase (NA) combinations, and what their original source is. In addition to the annual migration of shorebirds through this area every spring from many areas, this region supports breeding colonies of gulls and is host to resident

and migratory waterfowl. Also of potential importance is the nearby Delmarva Peninsula, which is a major poultry producing area in the United States. To determine if spring migrating Ruddy Turnstones serve as a source of IAV (gene segments or intact viruses) into Delaware Bay, we: 1) attempted to recover IAVs from this species in the winter; 2) continued ongoing IAV surveillance efforts of shorebirds at Delaware Bay; and 3) assessed the relatedness of isolates from these two sources.

METHODS

Surveillance of Arenaria interpres – overwintering and Delaware Bay sites

Fecal samples were collected from overwintering Ruddy Turnstones at numerous Florida and Georgia sites along the Southern USA coastline during 2010-2013, and 2015-2016 (Figure 5.1). At Delaware Bay beaches in May 2010 – 2014, paired oropharyngeal and cloacal swabs or fecal deposits were collected from Ruddy Turnstones, as part of long-term and ongoing surveillance efforts in this region carried out by researchers at St. Jude Children's Research Hospital (SJCRH) and the University of Georgia (UGA), as previously described (Krauss et al., 2010, Stallknecht et al., 2012).

Virus isolation

UGA samples were subjected to virus isolation in 9 – 11 day old specific pathogen free (SPF) embryonated chicken eggs (ECE) as previously described (Hanson et al., 2008) and returned to -80°C storage conditions. After incubation of SPF ECE at 37°C for 120 hours, amnioallanotic fluids were tested by hemagglutination assay using 0.5% chicken red blood cells. Viral RNA was extracted from all hemagglutinating samples using the QIAgen Viral RNA kit (Qiagen, Inc., Valencia, California, USA) as per manufacturer's recommendations. Influenza

viruses were identified by reverse-transcriptase PCR (RT-PCR) targeting the matrix gene (Fouchier et al., 2000), and IAV were further characterized into HA and NA subtypes by subtype-specific RT-PCR (Lebarbenchon et al., 2013) or HI/NI assay at the United States Department of Agriculture Animal and Plant Health Inspection Service National Veterinary Services Laboratory. Samples collected by SJCRH were subjected to molecular and virological testing as previously described (World Health Organization, 2002).

Molecular analyses

Influenza viral RNAs were sequenced using step-wise, overlapping RT-PCR for all eight IAV gene segments, as previously reported (Ramey et al., 2010). Briefly, cDNA fragments were amplified using the one-step RT-PCR kit (Qiagen, Inc.) and previously published primers (Zou, 1997; Hoffmann et al., 2001; Phipps et al., 2004; Bragstad et al., 2005; Obenauer et al., 2006; Li et al., 2007; Pearce et al., 2011). PCR products were treated with ExoSap-IT (USB Inc., Cleveland, Ohio, USA) or gel purified and extracted using the QIAquick gel extraction kit (Qiagen, Inc.) without additional purification prior to sequencing. Cycle sequencing was performed with identical primers used for RT-PCR and BigDye Terminator version 3.1 (Applied Biosystems, Foster City, California, USA); samples were analyzed on an Applied Biosystems 3730xl automated DNA sequencer (Applied Biosystems). Sequencher version 5.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) was used to assemble, edit, and trim contigs. MEGA version 6.0 (Tamura, 2013) was used to compute pair-wise distance (PWD) and phylogenetic comparisons of genes sequenced in this study with North and South American internal IAV gene sequences from 2000-2015 retrieved from the NCBI Genbank Influenza Virus Resource database (Bao et al., 2008) on July 21, 2016; given the paucity of sequence information available, reference sequences for surface genes (HA and NA) were not restricted by date. Trimmed

alignment lengths for gene segments used to generate phylogenies in nucleotides are as follows: PB2 (2,275), PB1 (2,215), PA (2,183), HA5 (1,691), HA6 (1,622), NP (1,438), NA1 (1,395), NA9 (1,409), M (928), and NS (816). For these analyses, gene segments with >99.0% nucleotide (nt) identity were considered highly similar (Reeves et al., 2011).

Barcoding fecal samples

Confirmation of the avian species of origin of viruses isolated from fecal samples was carried out by targeting cytochrome oxidase I (COI) mitochondrial DNA from host feces. When available, fecal swabs from which IAV were isolated were thawed a second time. Host DNA was extracted using the QIAamp DNA Stool mini kit (Qiagen, Inc.) as per the manufacturer's recommendations, with the following modification: 2 uL of 1ug/ul carrier RNA was added to the lysis buffer-proteinase K-sample mixture to increase DNA yield, as previously reported (Cheung et al., 2009). PCR amplification of the barcode regions of the COI gene were performed on DNA extracts using universal bird primers LTyr and COI907aH2 as previously described (Tavares and Baker, 2008). PCR products were treated with ExoSap-IT (USB Inc.) without further purification prior to sequencing. Cycle sequencing with identical primers used for PCR and sequence alignment were performed as described for influenza viral RNA. Reference Aves COI sequences (n = 2,473) were retrieved from BOLDSYSTEMS.ORG (accessed on July 18, 2016; Ratnasingham and Herbert, 2007), and MEGA version 6.0 (Tamura, 2013) was used to generate a maximum-likelihood phylogenetic tree.

RESULTS

Virus Isolation

From 2,968 fecal samples collected on overwintering beaches prior to the northern shorebird migration in 2010 – 2013, and 2014 – 2016, 25 IAV were isolated (overall prevalence = 0.84%). Subtypes (number) of viruses recovered on the Atlantic Coast of Northern Florida include H3N8 (1) in January 2011, low pathogenicity (LP) H5N9 (3) in March 2012, H6N1 (9) in May 2012, H12N2 (1) in January 2013, and H3N4 (11) in December 2014. Locations of 2012 overwintering IAV positive samples are shown with blue stars in Figure 5.1 and summaries of annual overwintering recovery of IAV by date of collection and location are shown in Table 5.1. In 2012, a shift was seen in the HA/NA subtypes recovered from northeast Florida samples from LP H5N9 in March (early winter), to H6N1 in May (late winter).

In six of eight instances, HA and/or NA subtypes isolated in the winter were also recovered from Delaware Bay collection sites by SJCRH or UGA in the spring preceding or following winter collections (Table 5.2); however, viruses recovered from birds on wintering grounds did not represent the predominant HA/NA combinations identified at Delaware Bay (Table 5.2). Subtype diversity and IAV prevalence at Delaware Bay were variable depending on the timing of collection as shown in Figure 5.2; over a 15-day period in 2012, there was a shift in the subtype combinations recovered by UGA over time, and a peak in IAV prevalence during May 20 – 24. Of the 14 different subtypes recovered from Delaware Bay in May 2012, three (H1N1, H1N8, and H12N1) were recovered in every time period while nine subtypes (H1N3, LP H5N3, LP H5N9, LP H7N3, LP H7N7, H9N1, H10N8, H12N8, and H13N6) were only isolated during one of the 5-day periods.

Molecular Analyses

Given that two different subtype combinations (LP H5N9 and H6N1) retrieved from 2012 winter samples were also seen in Delaware Bay three weeks to two months later, these 19 IAV (12 winter, 7 Delaware Bay) were selected for full genome sequencing. Because it shared an HA subtype with Florida viruses, one 2012 LP H5N3 from Delaware Bay was also included in these analyses. IAVs from Florida and Delaware Bay shared from greater than 99.0% to 100.0% nucleotide (nt) identity for individual gene segments (PWD matrices for all genes are shown in Supplementary Tables S5.1 – S5.8), but no two isolates from different locations shared more than 93.0% nt identity across eight gene segments compared (data not shown). Early winter isolates were nearly identical to each other at all eight gene segments, sharing greater than 99.6% nt identity. Late-winter isolates were very similar (greater than 99.0%) to those isolated in March for the PB2, PB1, PA, and NS genes when compared in PWD matrices (Supplementary Tables S5.1 – S5.4); NP and matrix genes from late-winter isolates were very similar to one another, but differed from those earlier in the winter, sharing 98.4% and 96.6% nt identity, respectively (Supplementary Tables S5.5 – S5.6). The sequences of the respective surface genes (HA5, HA6, NA1, and NA9) were well conserved across time and space, with Florida and Delaware Bay viruses sharing greater than 99.2% nt identity with one another within each subtype (Supplementary Tables S5.7 – S5.8). Table 5.3 depicts the relative phenotypes, based on the 99.0% nt identify threshold for the viruses analyzed here, as well as two reference viruses that were the best matches based on a BLAST search for multiple segment sequences generated in this study: (a) reference "contributing" virus – A/Ruddy turnstone/NJ/AI11-1678/2011(H7N7), isolated at Delaware Bay in the spring the year before this study, shares high identity at the nt level to numerous winter and Delaware Bay viruses for multiple gene segments,

and b) reference "receiving" virus – A/gull/MA/13JR00943/2013(H9N1), isolated in Massachusetts the year after the present study with four gene segments highly similar to ones identified here.

Genetic relationships between internal IAV gene segments sequenced as part of this study and contemporary (year 2000 – 2015) North and South American segments were inferred in maximum-likelihood (ML) phylogenies created with 1,000 bootstrap iterations. Compressed ML phylogenetic sub-trees for the surface HA5, HA6, NA1, and NA9 genes are depicted in Figures 5.3 – 5.6 with North and South American reference sequences; no time restriction was imposed on reference surface genes. ML phylogenies for HA5, HA6, NA1, and NA9 that include all sequences downloaded from the NCBI Genbank Influenza Virus Resource database are shown as topologies in Supplementary Figures S5.1 – S5.4, respectively. Sequences within a respective HA or NA subtype grouped closely together in all cases with the exception of the HA5 of AI12-2871 and the NA1 of AI12-2375. The NA3 segment of AI12-2871 was most closely related to KJ413442.1 A/blue-winged teal/TX/AI12-614/2012(H10N3).

Fecal Barcoding

Host DNA was successfully extracted from three fecal swabs (overwintering, $n = 2$ and DE Bay, $n = 1$) from which IAV were isolated, and identified to be most closely related to *Arenaria interpres* through analysis of a 431 base pair section of the coding region of the COI mitochondrial gene (Figure 5.7). Clades were compressed based upon the taxonomic order of bird species; the majority of bird COI sequences included here were in order Passeriformes. Birds within order Charadriiformes were further sub-classified by family to retain the resolution necessary to visualize the relationship(s) between fecal COI DNA from samples AI12-1244, AI12-1340, AI12-2010 (collected in this study) and Aves reference sequences.

DISCUSSION

Delaware Bay is the only known "hotspot" for IAV in shorebirds worldwide, but the dynamics of this system – namely the expansion, amplification and reassortment of IAV subtypes – are fairly short-lived and not fully understood. This important migratory stopover site represents a location where IAV can be reliably isolated from shorebirds during a brief four- to five-week window of time every year. While IAV prevalence in Ruddy Turnstones in May can be as high as 18% (Stallknecht et al., 2012), the source of virus into this unique system remains largely unknown. Recovery of viruses from shorebirds on their wintering grounds along the Atlantic flyway is difficult due to the wide dispersal of Ruddy Turnstones across the southern United States and into Central and South America. Reports of the isolation of IAV from this species prior to spring migration are rare but genetic analyses of several H11N9 IAV isolated from Ruddy Turnstones in Brazil in November indicate that Ruddy Turnstones migrating south in the late summer may be transporting IAV with them (De Araujo et al., 2014; Hurtado et al., 2015). Genetic analyses of IAV recovered in Peru from Ruddy Turnstones and other Charadriiformes across several years in both early and late winter have shown that they are of North American lineage; segments from some of these viruses group closely with those from Delaware Bay collected the previous year (Ghersi et al., 2009; Nelson et al., 2016). Gene segments from Chilean IAV isolates collected in early winter months from Franklin's gulls (*Leucophaeus pipixcan*; H13N2 and H13N9) and a kelp gull (*Larus dominicanus*; LP H5N9) also were very similar to segments from Delaware Bay viruses isolated a year or two prior; all but the NA and matrix gene of the H13N9 virus were most closely matched to Delaware Bay IAV gene segments (Mathieu et al., 2015). Though historically rare, such isolations support the

idea of 1) the movement of viruses or viral gene segments with migrating shorebirds upon their fall southward migration and/or 2) the low-level circulation of IAV on wintering grounds.

The work presented here reports the successful isolation of 25 IAV from fecal deposits collected at several sites along the Atlantic coast of Florida prior to the spring migration of shorebirds, between February 2010 and December 2015. In most cases, the same HA and/or NA subtypes were recovered from shorebirds sampled at Delaware Bay the spring prior to or immediately after these winter collections. Full genome sequencing of the matched HA and NA subtype combinations in 2012 indicates a high degree of genetic relatedness for all eight gene segments between both early and late winter and spring viruses, including for the highly mutable surface antigens (HA and NA). Reassortment plays an important role in the expansion and maintenance of IAV diversity at Delaware Bay (Bahl et al., 2013; Barton et al., 2014) and the dynamics of this diversity "machine" are evident (Figure 5.2, Table 5.3), even with the small number of viruses compared here. Though we did not detect a Delaware Bay full IAV genome homologous to those of overwintering viruses, overwintering viral gene segments are being transported to and becoming assimilated into the larger IAV gene pool at Delaware Bay (Table 5.3). Successful COI barcoding of several of the fecal samples from which IAV were derived to the level of avian species indicates that these viruses were isolated from Ruddy Turnstones.

Samples from Ruddy Turnstones on wintering grounds differ from samples collected at Delaware Bay as the former potentially include hatch-year birds. Because we were sampling feces on wintering grounds, we cannot discount the inclusion of samples from this age class. Ruddy Turnstones do not migrate north to Arctic breeding grounds in their first year of life (Nettleship, 2000) and the circulation of viral subtypes in this age class might not be expected to be reflected in viruses recovered in Delaware Bay. However, the strong genetic relationships for

all eight IAV gene segments between one or more wintering and Delaware Bay viruses suggests that the infection of post-breeding birds is occurring on the wintering grounds, with subsequent movement of viral gene segments upon northern migration. The viruses recovered on wintering grounds also do not represent the predominant subtype combination recovered at Delaware Bay prior to or after winter sampling (Table 5.2). Winter isolations of IAV from Ruddy turnstones and gulls of H11N9 in Brazil (De Araujo et al., 2014), LP H5N9, H13N2, and H13N9 in Chile (Mathieu et al., 2015), and H10N9 in Peru (Ghersi et al., 2009) also are not reflective of the predominant subtypes recovered at Delaware Bay in those years (Stallknecht et al., 2012). It is possible, especially related to those viral subtypes that were detected prior to migration, that the lower prevalence of infection at Delaware Bay was limited by pre-exposure on the wintering grounds.

Although beyond the scope of this study, the potential involvement of non-breeding age classes on wintering grounds in the annual maintenance of these viruses deserves additional study. Such maintenance could be enhanced through favorable environmental conditions during winter. The daily air temperature when the LP H5N9 viruses were recovered in Florida in March 2012 was 19°C (range 13°C – 25°C) (National Oceanic and Atmospheric Association, 2012). In laboratory models, avian IAV can persist, on average, more than 20 days in distilled water at 23°C; at 17°C, average IAV persistence increases to more than 30 days (Brown et al., 2009). Given that shorebirds tend to be widely dispersed on wintering grounds, the longevity of infectious IAV in this cool winter temperature range may serve to increase viral availability to susceptible hosts. The environmental/substrate persistence of IAV reported in this dissertation in Chapter 4 and elsewhere (Lang et al., 2008; Nazir et al., 2010; Nazir et al., 2011), especially as

related to the unique foraging/scavenging behavior of Ruddy Turnstones, also may play a role in the maintenance and transmission of virus during the winter.

Genetic analysis of other overwintering IAV viruses and continued surveillance of winter populations of Ruddy Turnstones will allow us to better elucidate the relatedness of viruses recovered at different points along a migratory pathway, both from breeding to wintering sites, and vice versa. Given that we did not detect the movement of an intact winter virus into Delaware Bay, connecting other species- or location-related sources of viruses and their gene segments to this ecological hotspot also warrants further investigation.

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Table 5.1. Estimated influenza A virus prevalence and subtype recovery from shorebird fecal samples collected at overwintering sites on the Atlantic or Gulf of Mexico Coasts, prior to shorebird migration through Delaware Bay, from 2010 – 2016, organized by collection date and location.

Overwintering Year	Month	Location (Coast)	IAV Prevalence (%)	Subtypes (number)
2010	February	Duval Co. FL; Chatham Co. GA (Atlantic)	0/34	
	March	Chatham Co. GA (Atlantic)	0/50	
		Pinellas Co. FL (Gulf)	0/106	
	April	Duval Co. FL; Chatham Co. GA (Atlantic)	0/184	
		Longboat Keys (Gulf)	0/14	
2011	January	Duval Co. FL; Chatham Co. GA (Atlantic)	1/46 (2.2)	H3N8 (1)
	February	Duval Co. FL; Chatham Co. GA (Atlantic)	0/180	
	March	Duval Co. FL; Chatham Co. GA (Atlantic)	0/203	
	April	Duval Co. FL; Chatham Co. GA (Atlantic)	0/98	
2012	January	Duval Co. FL; St. Johns Co. FL (Atlantic)	0/99	
	March	Duval Co. FL (Atlantic)	3/64 (4.7)	LPAI H5N9 (3)
		Pinellas Co. FL (Gulf)	0/19	
	May	Duval Co. FL (Atlantic)	9/378 (2.4)	H6N1 (9)
2013	January	Nassau Co. FL (Atlantic)	1/36 (2.8)	H12N2 (1)
	March	Duval Co. FL (Atlantic)	0/164	
	April	Duval Co. FL; Nassau Co. FL (Atlantic)	0/200	
2015	December	Duval Co. FL; Nassau Co. FL; Palm Beach Co. FL (Atlantic)	11/238 (4.6)	H3N4 (11)
	January	Chatham Co. GA; Glynn Co. GA; Nassau Co. FL (Atlantic)	0/241	
	February	Duval Co. FL; Nassau Co. FL (Atlantic)	0/100	
		Hillsborough Co. FL; Pinellas Co. FL (Gulf)	0/87	
	March	Duval Co. FL; Nassau Co. FL (Atlantic)	0/174	
2016	December	Duval Co. FL; Nassau Co. FL (Atlantic)	0/211	
	February	Duval Co. FL; Nassau Co. FL (Atlantic)	0/42	
		All Sites	25/2,968 (0.8)	
		Atlantic Coast	25/2,742 (0.9)	
		Gulf Coast	0/226	

Table 5.2. The number of influenza A viruses, by subtype (number), isolated from shorebird samples collected at Delaware (DE) Bay by St. Jude Children's Research Hospital (SJCRH, fecal samples) or at DE Bay and overwintering sites by the University of Georgia (UGA, fecal or CL/OP swabs) from Ruddy Turnstones. HA or NA subtypes colored red were seen in a preceding or subsequent sampling effort, also designated by a red arrow along the top of the table. Cells shaded gray indicate the recovery of a matched HA and NA subtype in a preceding and/or subsequent collection, also designated by a black arrow along the top of the table.

DE Bay 2010	Winter[c] 2011	DE Bay 2011	Winter 2012	DE Bay 2012	Winter 2013	DE Bay 2013	Winter 2014	DE Bay 2014	Winter 2015	DE Bay 2015
H6N1 (13)[b]		H7N3 (16)[b]		H1**N1** (38)		H10N7 (90)[b]		H12**N4** (69)[b]		H7N3 (99)[b]
H8N4 (4)		H9N7 (10)[b]		**H12**N3 (27)[b]	**H12N2** (1)	H10N8 (33)[b]		H13N6 (16)[b]		H1N1 (71)[b]
H5N2 (3)		**H5**N2 (9)[b]		**H12N1** (15)		H10N1 (22)[b]		H6N2 (9)[b]		H1N3 (21)[b]
H6**N8** (2)[b]		H9N2 (3)[b]		H1N8 (10)[b]		H10**N2** (20)[b]		H11N2 (8)[b]		H7N1 (12)
H2N3 (1)		H7N7 (2)	**H6N1(9)**	**H6N1 (8)**[b]		H10N9 (18)[b]		H6**N4** (6)[a]		H1N2 (2)[a]
H2N9 (1)		H10N6 (2)[a]		**H6**N4 (4)[a]		H11**N2** (17)[b]	***No Collection***	**H3**N6 (5)[b]		H1N8 (2)[b]
H3N2 (1)		H10**N9** (2)		H7N7 (4)		H11N8 (7)		H6N1 (5)		H11N2 (2)[b]
H3N8 (1)	**H3N8 (1)**	**H5**N3 (1)[a]		**H12**N8 (2)		H11N7 (5)[b]		**H3N4 (2)**	**H3N4 (11)**	H16N3 (2)[a]
H6N4 (1)[a]		**H5**N6 (1)		H13N6(2)[b]		H6N8 (3)[b]		H6N8 (2)[b]		H6N8 (1)[a]
H13N6 (1)[a]		H7N2 (1)[a]		H16N6 (2)[a]		H1N8 (1)[a]		H1N8 (1)		
		H9N6 (1)[a]		H1N3 (1)		H16N3 (1)		H4**N4** (1)		
		H10N3 (1)		**H5**N3(1)				H7N3 (1)		
		H5N9 (1)[a]	**H5N9 (3)**	**H5N9(1)**				H12N1 (1)		
		H11N2 (1)[a]		H7N3 (1)				H16N6 (1)[a]		
		H11N3 (1)		H9**N1** (1)						
		H12N2 (1)		H10N8 (1)						

[a] Number of isolates recovered from SJCRH fecal samples; [b] Number of combined isolates recovered at DE Bay from SJCRH fecal samples and UGA fecal and/or CL/OP samples; [c] All winter viruses are from UGA fecal swabs.

Table 5.3. Phenotype of viruses recovered during this study that share differing degrees of nucleotide (nt) identity within each gene segment. Viruses with the same color code within a given gene segment share >99.0% nt identity. The HA and NA of the first H5N9 (AI12-1161) and H6N1 (AI12-1541) viruses isolated are the reference sequences within those respective gene segments.

	Percent nucleotide similarity to reference sequence[1]								
Virus	**PB2**	**PB1**	**PA**	**NP**	**MA**	**NS**	**HA**	**NA**	
A/Ruddy turnstone/NJ/AI11-1678/2011(H7N7)	***ref***	***ref***	***ref***	***ref***	***ref***	***ref***	*N/A (H7)*	*N/A (N7)*	reference[1]
A/Ruddy turnstone/FL/AI12-1161/2012/H5N9	92.0	99.6	99.6	91.9	96.9	95.1	***ref***	***ref***	Overwintering viruses - this study
A/Ruddy turnstone/FL/AI12-1244/2012/H5N9	92.0	99.6	99.6	91.9	96.9	95.1	99.9	100.0	
A/Ruddy turnstone/FL/AI12-1476/2012/H5N9	91.9	99.6	99.6	91.9	96.9	95.2	99.8	100.0	
A/Ruddy turnstone/FL/AI12-1541/2012/H6N1	91.9	99.3	99.4	91.7	99.1	95.2	***ref***	***ref***	
A/Ruddy turnstone/FL/AI12-1590/2012/H6N1	91.7	99.3	99.4	91.7	99.1	95.2	100.0	100.0	
A/Ruddy turnstone/FL/AI12-1591/2012/H6N1	91.9	99.2	99.4	91.7	99.1	95.2	99.9	100.0	
A/Ruddy turnstone/FL/AI12-1592/2012/H6N1	91.9	99.2	99.4	91.7	99.1	95.2	99.9	100.0	
A/Ruddy turnstone/FL/AI12-1593/2012/H6N1	91.9	99.3	99.4	91.7	99.1	95.2	100.0	100.0	
A/Ruddy turnstone/FL/AI12-1683/2012/H6N1	91.9	99.3	99.4	91.7	99.1	95.2	99.9	100.0	
A/Ruddy turnstone/FL/AI12-1638/2012/H6N1	91.9	99.3	99.4	91.7	99.1	95.2	99.9	99.9	
A/Ruddy turnstone/FL/AI12-1340/2012/H6N1	91.9	99.3	99.4	91.7	99.1	95.2	100.0	100.0	
A/Ruddy turnstone/FL/AI12-1368/2012/H6N1	92.0	99.3	99.5	93.7	99.1	95.1	99.9	99.9	
A/Ruddy turnstone/NJ/AI12-2120/2012/H6N1	99.6	99.5	87.4	91.7	99.7	98.0	99.6	99.6	DE Bay viruses - this study
A/Ruddy turnstone/NJ/AI12-2279/2012/H5N9	99.6	99.5	87.4	91.7	99.5	95.3	99.4	99.3	
A/Ruddy turnstone/NJ/AI12-2166/2012/H6N1	99.6	96.1	87.4	91.7	96.7	98.2	99.5	99.5	
A/Ruddy turnstone/NJ/AI12-2356/2012/H6N1	99.6	99.4	87.5	91.7	99.7	98.2	99.6	99.6	
A/Ruddy turnstone/NJ/AI12-2375/2012/H6N1	91.8	93.5	99.4	93.8	96.8	95.1	99.2	93.0	
A/Ruddy turnstone/NJ/AI12-2871/2012/H5N3	96.8	94.1	87.4	91.6	96.9	97.8	97.7	*N/A*	
A/Ruddy turnstone/NJ/AI12-2010/2012/H6N1	99.6	99.4	87.4	91.7	99.6	98.2	99.6	99.6	
A/Ruddy turnstone/NJ/AI12-2210/2012/H6N1	99.6	96.2	87.4	91.7	96.9	98.3	99.5	99.6	
A/gull/MA/13JR00943/2013(H9N1)	91.9	96.0	87.7	93.7	97.5	97.0	*N/A (H9)*	93.0	reference[2]

[1] Virus A/Ruddy turnstone/NJ/AI11-1678/2011/H7N7 was used as a "contributing" reference virus for internal genes; [2] Virus A/gull/MA/13JR00943/2013/H9N1 was used as a "receiving" reference sequence for internal genes and NA1.

Supplemental Table S5.1. Pairwise distance matrix for 2,275 nucleotides of the PB2 gene (segment 1) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												**DE Bay viruses**								**Ref**
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY186001**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	99.3	99.3																			
H6N1	LW	1368	99.4	99.4	99.8																		
H5N9	EW	1476	100.0	100.0	99.3	99.4																	
H6N1	LW	1541	99.3	99.3	100.0	99.8	99.3																
H6N1	LW	1590	99.3	99.3	100.0	99.7	99.3	100.0															
H6N1	LW	1591	99.3	99.3	100.0	99.8	99.3	100.0	100.0														
H6N1	LW	1592	99.3	99.3	100.0	99.8	99.3	100.0	100.0	100.0													
H6N1	LW	1593	99.3	99.3	100.0	99.8	99.3	100.0	100.0	100.0	100.0												
H6N1	LW	1638	99.3	99.3	100.0	99.8	99.3	100.0	100.0	100.0	100.0	100.0											
H6N1	LW	1683	99.3	99.3	100.0	99.8	99.3	100.0	100.0	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	91.8	91.8	91.7	91.8	91.8	91.7	91.7	91.7	91.7	91.7	91.7	91.7									
H6N1	DB	2120	91.8	91.8	91.7	91.9	91.8	91.7	91.8	91.7	91.7	91.7	91.7	91.7	100.0								
H6N1	DB	2166	91.8	91.8	91.7	91.8	91.8	91.7	91.7	91.7	91.7	91.7	91.7	91.7	100.0	100.0							
H6N1	DB	2210	91.8	91.8	91.7	91.8	91.8	91.7	91.7	91.7	91.7	91.7	91.7	91.7	100.0	100.0	100.0						
H5N9	DB	2279	91.8	91.8	91.7	91.8	91.8	91.7	91.7	91.7	91.7	91.7	91.7	91.7	99.7	99.7	99.7	99.7					
H6N1	DB	2356	91.8	91.8	91.7	91.9	91.8	91.7	91.8	91.7	91.7	91.7	91.7	91.7	100.0	99.9	100.0	100.0	99.7				
H6N1	DB	2375	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	99.0	91.6	91.6	91.6	91.6	91.6	91.6			
H5N3	DB	2871	91.5	91.5	91.4	91.6	91.5	91.4	91.5	91.4	91.4	91.4	91.4	91.4	96.5	96.4	96.5	96.5	96.5	96.4	91.3		
H7N7	Ref[e]	CY186001	92.0	92.0	91.9	92.0	92.0	91.9	92.0	91.9	91.9	91.9	91.9	91.9	99.6	99.6	99.6	99.6	99.5	99.6	91.8	96.8	
H9N1	Ref[f]	CY195824	97.1	97.1	97.0	97.1	97.1	97.0	97.0	97.0	97.0	97.0	97.0	97.0	91.7	91.7	91.7	91.7	91.7	91.7	96.7	91.7	91.9

[a] Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY186001 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195824 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.2. Pairwise distance matrix for 2,215 nucleotides of the PB1 gene (segment 2) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												DE Bay viruses								Ref
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY186000**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	99.5	99.5																			
H6N1	LW	1368	99.5	99.5	99.7																		
H5N9	EW	1476	100.0	100.0	99.5	99.5																	
H6N1	LW	1541	99.5	99.5	100.0	99.7	99.5																
H6N1	LW	1590	99.5	99.5	100.0	99.7	99.5	100.0															
H6N1	LW	1591	99.5	99.5	100.0	99.6	99.5	100.0	100.0														
H6N1	LW	1592	99.5	99.5	100.0	99.6	99.5	100.0	100.0	99.9													
H6N1	LW	1593	99.5	99.5	100.0	99.7	99.5	100.0	100.0	100.0	100.0												
H6N1	LW	1638	99.5	99.5	100.0	99.7	99.5	100.0	100.0	100.0	100.0	100.0											
H6N1	LW	1683	99.5	99.5	100.0	99.7	99.5	100.0	100.0	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	99.6	99.6	99.3	99.3	99.6	99.3	99.3	99.2	99.2	99.3	99.3	99.3									
H6N1	DB	2120	99.7	99.7	99.4	99.4	99.7	99.4	99.4	99.3	99.3	99.4	99.4	99.4	99.7								
H6N1	DB	2166	96.0	96.0	95.7	95.9	96.0	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.8	95.8							
H6N1	DB	2210	96.0	96.0	95.7	95.9	96.0	95.7	95.7	95.7	95.7	95.7	95.7	95.7	95.8	95.8	100.0						
H5N9	DB	2279	99.7	99.7	99.4	99.4	99.7	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.5	99.6	95.8	95.8					
H6N1	DB	2356	99.6	99.6	99.3	99.3	99.6	99.3	99.3	99.3	99.3	99.3	99.3	99.3	100.0	99.8	95.8	95.8	99.5				
H6N1	DB	2375	93.3	93.3	93.1	93.1	93.3	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93.2	93.2			
H5N3	DB	2871	93.8	93.8	93.7	93.8	93.8	93.7	93.7	93.6	93.6	93.7	93.7	93.7	93.6	93.7	93.9	93.9	93.7	93.6	96.4		
H7N7	Ref[e]	CY186000	99.6	99.6	99.3	99.3	99.6	99.3	99.3	99.2	99.2	99.3	99.3	99.3	99.4	99.5	96.2	96.2	99.5	99.4	93.5	94.1	
H9N1	Ref[f]	CY195823	95.8	95.8	95.5	95.7	95.8	95.5	95.5	95.5	95.5	95.5	95.5	95.5	95.6	95.6	99.4	99.4	95.7	95.7	92.8	93.6	92.8

[a] Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY186000 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195823 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.3. Pairwise distance matrix for 2,183 nucleotides of the PA gene (segment 3) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												DE Bay viruses								Ref
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY185999**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	99.3	99.3																			
H6N1	LW	1368	99.4	99.4	99.9																		
H5N9	EW	1476	99.9	99.9	99.2	99.3																	
H6N1	LW	1541	99.3	99.3	100.0	99.9	99.2																
H6N1	LW	1590	99.3	99.3	100.0	99.9	99.2	100.0															
H6N1	LW	1591	99.3	99.3	100.0	99.9	99.2	100.0	100.0														
H6N1	LW	1592	99.3	99.3	100.0	99.9	99.2	100.0	100.0	100.0													
H6N1	LW	1593	99.3	99.3	100.0	99.9	99.2	100.0	100.0	100.0	100.0												
H6N1	LW	1638	99.3	99.3	100.0	99.9	99.2	100.0	100.0	100.0	100.0	100.0											
H6N1	LW	1683	99.3	99.3	100.0	99.9	99.2	100.0	100.0	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	87.2	87.2	87.3	87.4	87.2	87.3	87.3	87.3	87.3	87.3	87.3	87.3									
H6N1	DB	2120	87.4	87.4	87.6	87.7	87.4	87.6	87.6	87.6	87.6	87.6	87.6	87.6	99.6								
H6N1	DB	2166	87.2	87.2	87.3	87.4	87.2	87.3	87.3	87.3	87.3	87.3	87.3	87.3	99.7	99.7							
H6N1	DB	2210	87.2	87.2	87.3	87.4	87.2	87.3	87.3	87.3	87.3	87.3	87.3	87.3	99.7	99.7	100.0						
H5N9	DB	2279	87.2	87.2	87.3	87.4	87.2	87.3	87.3	87.3	87.3	87.3	87.3	87.3	99.7	99.4	99.5	99.5					
H6N1	DB	2356	87.3	87.3	87.4	87.5	87.3	87.4	87.4	87.4	87.4	87.4	87.4	87.4	99.9	99.5	99.6	99.6	99.7				
H6N1	DB	2375	99.2	99.2	99.4	99.5	99.1	99.4	99.4	99.4	99.4	99.4	99.4	99.4	87.3	87.5	87.3	87.3	87.3	87.4			
H5N3	DB	2871	98.3	98.3	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	87.4	87.7	87.4	87.4	87.4	87.5	98.1		
H7N7	Ref[e]	CY185999	99.6	99.6	99.4	99.4	99.5	99.4	99.4	99.4	99.4	99.4	99.4	99.4	87.4	87.6	87.4	87.4	87.4	87.5	99.4	98.6	
H9N1	Ref[f]	CY195822	87.5	87.5	87.6	87.7	87.4	87.6	87.6	87.6	87.6	87.6	87.6	87.6	90.2	90.3	90.3	90.3	90.1	90.3	87.6	87.4	87.7

[a] Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY185999 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195822 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.4. Pairwise distance matrix for 816 nucleotides of the NS gene (segment 8) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												**DE Bay viruses**								**Ref**
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY185995**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	99.6	99.6																			
H6N1	LW	1368	99.5	99.5	99.9																		
H5N9	EW	1476	99.9	99.9	99.8	99.6																	
H6N1	LW	1541	99.6	99.6	100.0	99.9	99.8																
H6N1	LW	1590	99.6	99.6	100.0	99.9	99.8	100.0															
H6N1	LW	1591	99.6	99.6	100.0	99.9	99.8	100.0	100.0														
H6N1	LW	1592	99.6	99.6	100.0	99.9	99.8	100.0	100.0	100.0													
H6N1	LW	1593	99.6	99.6	100.0	99.9	99.8	100.0	100.0	100.0	100.0												
H6N1	LW	1638	99.6	99.6	100.0	99.9	99.8	100.0	100.0	100.0	100.0	100.0											
H6N1	LW	1683	99.6	99.6	100.0	99.9	99.8	100.0	100.0	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	95.1	95.1	95.2	95.1	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2									
H6N1	DB	2120	94.7	94.7	94.9	94.7	94.9	94.9	94.9	94.9	94.9	94.9	94.9	94.9	99.1								
H6N1	DB	2166	94.9	94.9	95.0	94.9	95.0	95.0	95.0	95.0	95.0	95.0	95.0	95.0	99.3	99.6							
H6N1	DB	2210	95.0	95.0	95.1	95.0	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	99.4	99.8	99.9						
H5N9	DB	2279	99.8	99.8	99.9	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	95.3	95.0	95.1	95.2					
H6N1	DB	2356	95.1	95.1	95.2	95.1	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2	100.0	99.1	99.3	99.4	95.3				
H6N1	DB	2375	99.3	99.3	99.4	99.3	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	95.1	94.7	94.9	95.0	99.5	95.1			
H5N3	DB	2871	95.8	95.8	96.0	95.8	96.0	96.0	96.0	96.0	96.0	96.0	96.0	96.0	97.4	97.3	97.4	97.5	96.1	97.4	95.8		
H7N7	Ref[e]	CY185998	95.1	95.1	95.2	95.1	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2	98.2	98.0	98.2	98.3	95.3	98.2	95.1	97.8	
H9N1	Ref[f]	CY195821	95.5	95.5	95.3	95.2	95.6	95.3	95.3	95.3	95.3	95.3	95.3	95.3	96.8	96.4	96.6	96.7	95.5	96.8	95.2	98.2	96.9

[a] Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY185998 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195821 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.5. Pairwise distance matrix for 1,438 nucleotides of the NP gene (segment 5) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												DE Bay viruses								Ref
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY185997**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	98.4	98.4																			
H6N1	LW	1368	92.1	92.1	91.9																		
H5N9	EW	1476	100.0	100.0	98.4	92.1																	
H6N1	LW	1541	98.4	98.4	100.0	91.9	98.4																
H6N1	LW	1590	98.3	98.3	99.9	91.9	98.3	99.9															
H6N1	LW	1591	98.4	98.4	100.0	91.9	98.4	100.0	99.9														
H6N1	LW	1592	98.4	98.4	100.0	91.9	98.4	100.0	99.9	100.0													
H6N1	LW	1593	98.4	98.4	100.0	91.9	98.4	100.0	99.9	100.0	100.0												
H6N1	LW	1638	98.4	98.4	100.0	91.9	98.4	100.0	99.9	100.0	100.0	100.0											
H6N1	LW	1683	98.4	98.4	100.0	91.9	98.4	100.0	99.9	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	99.9	99.9	98.4	91.9	99.9	98.4	98.3	98.4	98.4	98.4	98.4	98.4									
H6N1	DB	2120	99.9	99.9	98.4	91.9	99.9	98.4	98.3	98.4	98.4	98.4	98.4	98.4	100.0								
H6N1	DB	2166	99.9	99.9	98.4	91.9	99.9	98.4	98.3	98.4	98.4	98.4	98.4	98.4	100.0	100.0							
H6N1	DB	2210	99.8	99.8	98.5	91.9	99.8	98.5	98.4	98.5	98.5	98.5	98.5	98.5	99.9	99.9	99.9						
H5N9	DB	2279	99.5	99.5	98.3	91.7	99.5	98.3	98.3	98.3	98.3	98.3	98.3	98.3	99.5	99.5	99.5	99.4					
H6N1	DB	2356	99.8	99.8	98.3	91.9	99.8	98.3	98.3	98.3	98.3	98.3	98.3	98.3	99.9	99.9	99.9	99.9	99.4				
H6N1	DB	2375	92.1	92.1	92.0	99.9	92.1	92.0	91.9	92.0	92.0	92.0	92.0	92.0	92.0	92.0	92.0	91.9	91.8	91.9			
H5N3	DB	2871	93.3	93.3	93.4	91.5	93.3	93.4	93.3	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93.4	93.3	93.3	93.3	91.6		
H7N7	Ref[e]	CY185997	91.9	91.9	91.7	93.7	91.9	91.7	91.7	91.7	91.7	91.7	91.7	91.7	91.8	91.8	91.8	91.7	91.7	91.7	93.8	91.6	
H9N1	Ref[f]	CY195820	92.2	92.2	92.1	99.7	92.2	92.1	92.0	92.1	92.1	92.1	92.1	92.1	92.1	92.1	92.1	92.0	91.9	92.0	99.8	91.7	93.7

[a] Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY185997 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195820 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.6. Pairwise distance matrix for 928 nucleotides of the matrix gene (segment 7) for viruses analyzed in this study, and two reference sequences. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

			Overwintering Viruses												DE Bay viruses								Ref
Subtype	**Season**	**Virus ID**	**1161[d]**	**1244**	**1340**	**1368**	**1476**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2279**	**2356**	**2375**	**2871**	**CY185995**
H5N9	EW[a]	1244	100.0																				
H6N1	LW[b]	1340	96.0	96.0																			
H6N1	LW	1368	96.0	96.0	100.0																		
H5N9	EW	1476	100.0	100.0	96.0	96.0																	
H6N1	LW	1541	96.0	96.0	100.0	100.0	96.0																
H6N1	LW	1590	96.0	96.0	100.0	100.0	96.0	100.0															
H6N1	LW	1591	96.0	96.0	100.0	100.0	96.0	100.0	100.0														
H6N1	LW	1592	96.0	96.0	100.0	100.0	96.0	100.0	100.0	100.0													
H6N1	LW	1593	96.0	96.0	100.0	100.0	96.0	100.0	100.0	100.0	100.0												
H6N1	LW	1638	96.0	96.0	100.0	100.0	96.0	100.0	100.0	100.0	100.0	100.0											
H6N1	LW	1683	96.0	96.0	100.0	100.0	96.0	100.0	100.0	100.0	100.0	100.0	100.0										
H6N1	DB[c]	2010	96.4	96.4	99.4	99.4	96.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4									
H6N1	DB	2120	96.6	96.6	99.5	99.5	96.6	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.9								
H6N1	DB	2166	100.0	100.0	96.0	96.0	100.0	96.0	96.0	96.0	96.0	96.0	96.0	96.0	96.4	96.6							
H6N1	DB	2210	100.0	100.0	96.0	96.0	100.0	96.0	96.0	96.0	96.0	96.0	96.0	96.0	96.4	96.6	100.0						
H5N9	DB	2279	96.6	96.6	99.2	99.2	96.6	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.7	99.8	96.6	96.6					
H6N1	DB	2356	96.6	96.6	99.5	99.5	96.6	99.5	99.5	99.5	99.5	99.5	99.5	99.5	99.9	100.0	96.6	96.6	99.8				
H6N1	DB	2375	99.9	99.9	95.9	95.9	99.9	95.9	95.9	95.9	95.9	95.9	95.9	95.9	96.3	96.4	99.9	99.9	96.4	96.4			
H5N3	DB	2871	97.4	97.4	96.2	96.2	97.4	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.4	96.6	97.4	97.4	96.6	96.6	97.3		
H7N7	Ref[e]	CY185995	96.9	96.9	99.1	99.1	96.9	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.6	99.7	96.9	96.9	99.5	99.7	96.8	96.9	
H9N1	Ref[f]	CY195818	96.4	96.4	96.7	96.7	96.4	96.7	96.7	96.7	96.7	96.7	96.7	96.7	97.1	97.2	96.4	96.4	97.0	97.2	96.3	96.6	97.5

[a]Early winter recovered virues (EW); [b] Late winter recovered viruses (LW); [c] Delaware Bay recovered viruses (DB); [d] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9; [e] Reference sequence accession CY185995 strain name is A/ruddy turnstone/NJ/AI11-1678/2011/H7N7; [f] Reference sequence accession CY195818 strain name is A/gull/MA/13JR00943/2013/H9N1.

Supplemental Table S5.7. Pairwise distance matrix for (a) 1,622 nucleotides of the HA6 gene (segment 4) and (b) 1,691 nucleotides of the HA5 gene for viruses analyzed in this study. Values are in percentages and shaded according to nucleotide identity: dark gray ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

7A			Overwintering Viruses									DE Bay viruses				
Subtype	**Season**	**Virus ID**	**1340[c]**	**1368**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2356**
H6N1	LW[a]	1368	99.9													
H6N1	LW	1541	100.0	99.9												
H6N1	LW	1590	100.0	99.9	100.0											
H6N1	LW	1591	99.9	99.8	99.9	99.9										
H6N1	LW	1592	99.9	99.8	99.9	99.9	100.0									
H6N1	LW	1593	100.0	99.9	100.0	100.0	99.9	99.9								
H6N1	LW	1638	99.9	99.8	99.9	99.9	99.9	99.9	99.9							
H6N1	LW	1683	99.9	99.8	99.9	99.9	99.9	99.9	99.9	99.9						
H6N1	DB[b]	2010	99.6	99.4	99.6	99.6	99.5	99.5	99.6	99.5	99.5					
H6N1	DB	2120	99.6	99.4	99.6	99.6	99.5	99.5	99.6	99.6	99.5	99.8				
H6N1	DB	2166	99.5	99.4	99.5	99.5	99.4	99.4	99.5	99.4	99.4	99.7	99.7			
H6N1	DB	2210	99.5	99.4	99.5	99.5	99.4	99.4	99.5	99.4	99.4	99.7	99.7	100.0		
H6N1	DB	2356	99.6	99.4	99.6	99.6	99.5	99.5	99.6	99.5	99.5	99.9	99.8	99.7	99.7	
H6N1	DB	2375	99.2	99.1	99.2	99.2	99.1	99.1	99.1	99.1	99.1	99.4	99.4	99.3	99.3	99.4

[a] Late winter recovered viruses (LW); [b] Delaware Bay recovered viruses (DB); [c] Virus AI12-1340 is a late winter (LW) virus, subtype H6N1.

7B			Overwintering Viruses			DE Bay virus
Subtype	**Season**	**Virus ID**	**1161[c]**	**1244**	**1476**	**2279**
H5N9	EW[a]	1244	99.9			
H5N9	EW	1476	99.8	99.9		
H5N9	DB[b]	2279	99.4	99.4	99.3	
H5N3	DB	2871	97.7	97.7	97.7	97.5

[a] Early winter recovered viruses (EW); [b] Delaware Bay recovered viruses (DB); [c] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9.

Supplemental Table S5.8. Pairwise distance matrix for (a) 1,395 nucleotides of the NA1 gene (segment 6) and (b) 1,409 nucleotides of the NA9 gene for viruses analyzed in this study and one reference sequence. Values are in percentages and shaded according to nucleotide identity: dark gray, ≥ 99.0%; medium gray ≥ 98.0%; light gray ≥ 97.0%.

8A			Overwintering Viruses									DE Bay viruses					
Subtype	**Season**	**Virus ID**	**1340[c]**	**1368**	**1541**	**1590**	**1591**	**1592**	**1593**	**1638**	**1683**	**2010**	**2120**	**2166**	**2210**	**2356**	**2375**
H6N1	LW[a]	1368	99.9														
H6N1	LW	1541	100.0	99.9													
H6N1	LW	1590	100.0	99.9	100.0												
H6N1	LW	1591	100.0	99.9	100.0	100.0											
H6N1	LW	1592	100.0	99.9	100.0	100.0	100.0										
H6N1	LW	1593	100.0	99.9	100.0	100.0	99.9	99.9									
H6N1	LW	1638	99.9	99.9	99.9	99.9	99.9	99.9	99.9								
H6N1	LW	1683	100.0	99.9	100.0	100.0	100.0	100.0	100.0	99.9							
H6N1	DB[b]	2010	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6						
H6N1	DB	2120	99.6	99.5	99.6	99.6	99.6	99.6	99.6	99.5	99.6	99.9					
H6N1	DB	2166	99.5	99.4	99.5	99.5	99.5	99.5	99.5	99.4	99.5	99.9	99.8				
H6N1	DB	2210	99.6	99.5	99.6	99.6	99.6	99.6	99.6	99.5	99.6	99.9	99.9	99.9			
H6N1	DB	2356	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	99.6	100.0	99.9	99.9	99.9		
H6N1	DB	2375	93.0	92.9	93.0	93.0	93.0	93.0	93.0	92.9	93.0	92.8	92.7	92.6	92.7	92.8	
H9N1	Ref[d]	CY195824	92.8	92.7	92.8	92.8	92.8	92.8	92.8	92.7	92.8	92.5	92.5	92.4	92.5	92.5	99.4

[a] Late winter recovered viruses (LW); [b] Delaware Bay recovered viruses (DB); [c] Virus AI12-1340 is a late winter (LW) virus, subtype H6N1;
[d] Reference sequence accession CY195824 strain name is A/gull/MA/13JR00943/2013/H9N1.

8B			Overwintering Viruses		
Subtype	**Season**	**Virus ID**	**1161[c]**	**1244**	**1476**
H5N9	EW[a]	1244	100.0		
H5N9	EW	1476	100.0	100.0	
H5N9	DB[b]	2279	99.3	99.3	99.3

[a] Early winter recovered viruses (EW); [b] Delaware Bay recovered virus (DB); [c] Virus AI12-1161 is an early winter (EW) virus, subtype LP H5N9.

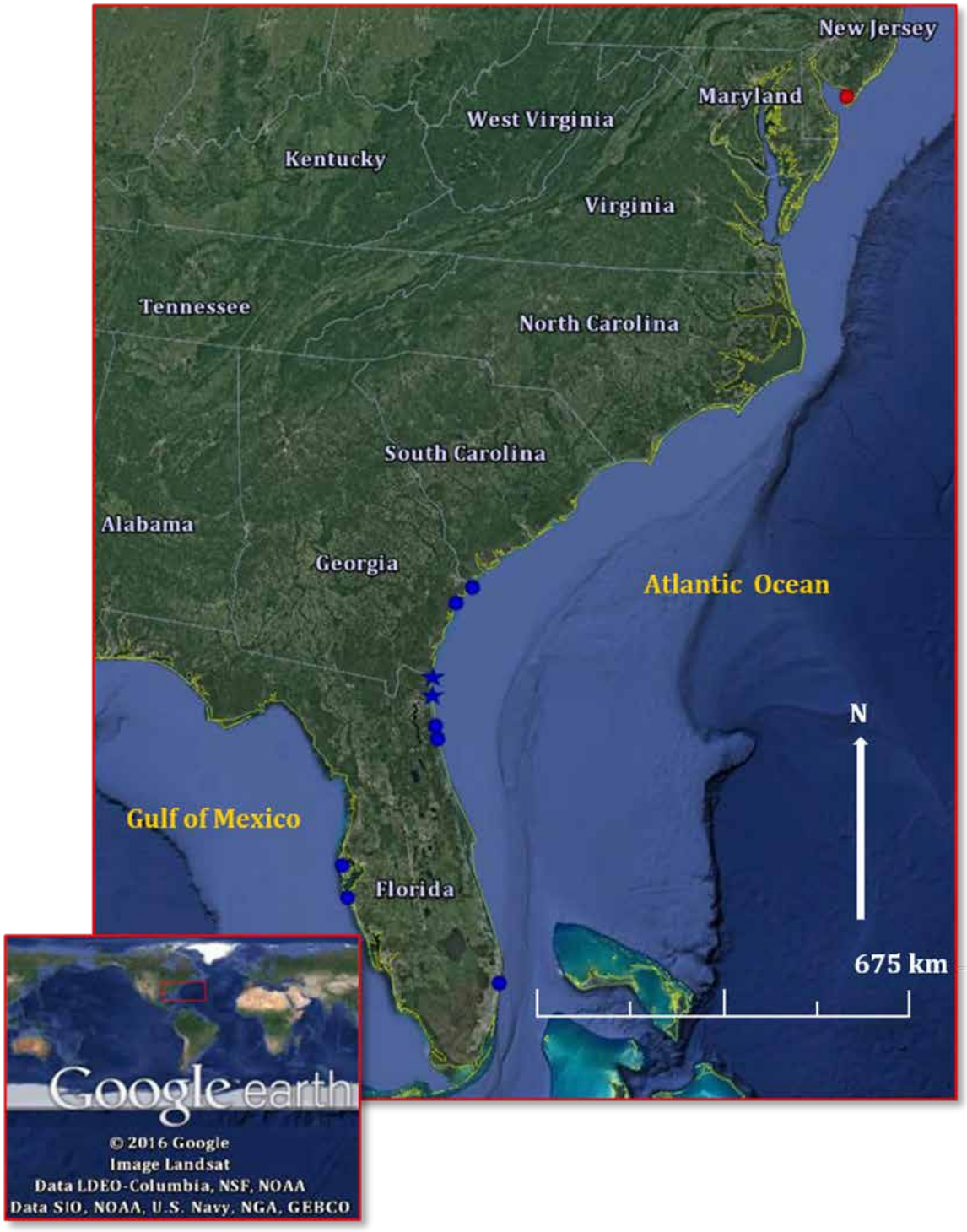


Figure 5.1. Relative location of overwintering collection sites in Florida, Georgia, and South Carolina, USA, depicted by blue markers. Blue stars represent overwintering locations from which IAV were isolated in 2012. The Delaware Bay spring migratory stopover site is designated with a red marker.

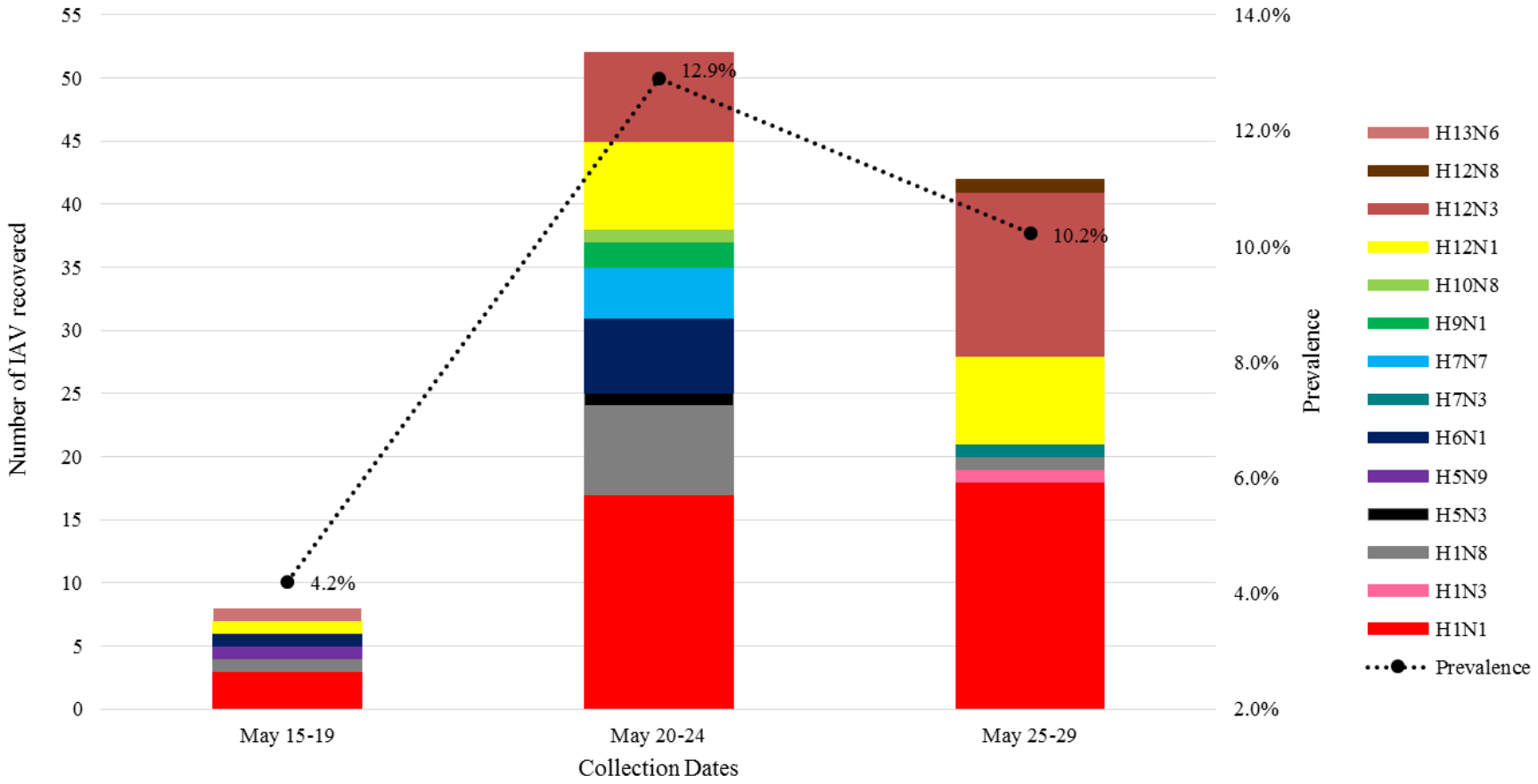


Figure 5.2. The number of influenza A viruses recovered by UGA at Delaware Bay in three 5-day time periods during May 2012. Estimated overall IAV prevalence in Ruddy Turnstones within each time period is designated by the black dotted line.

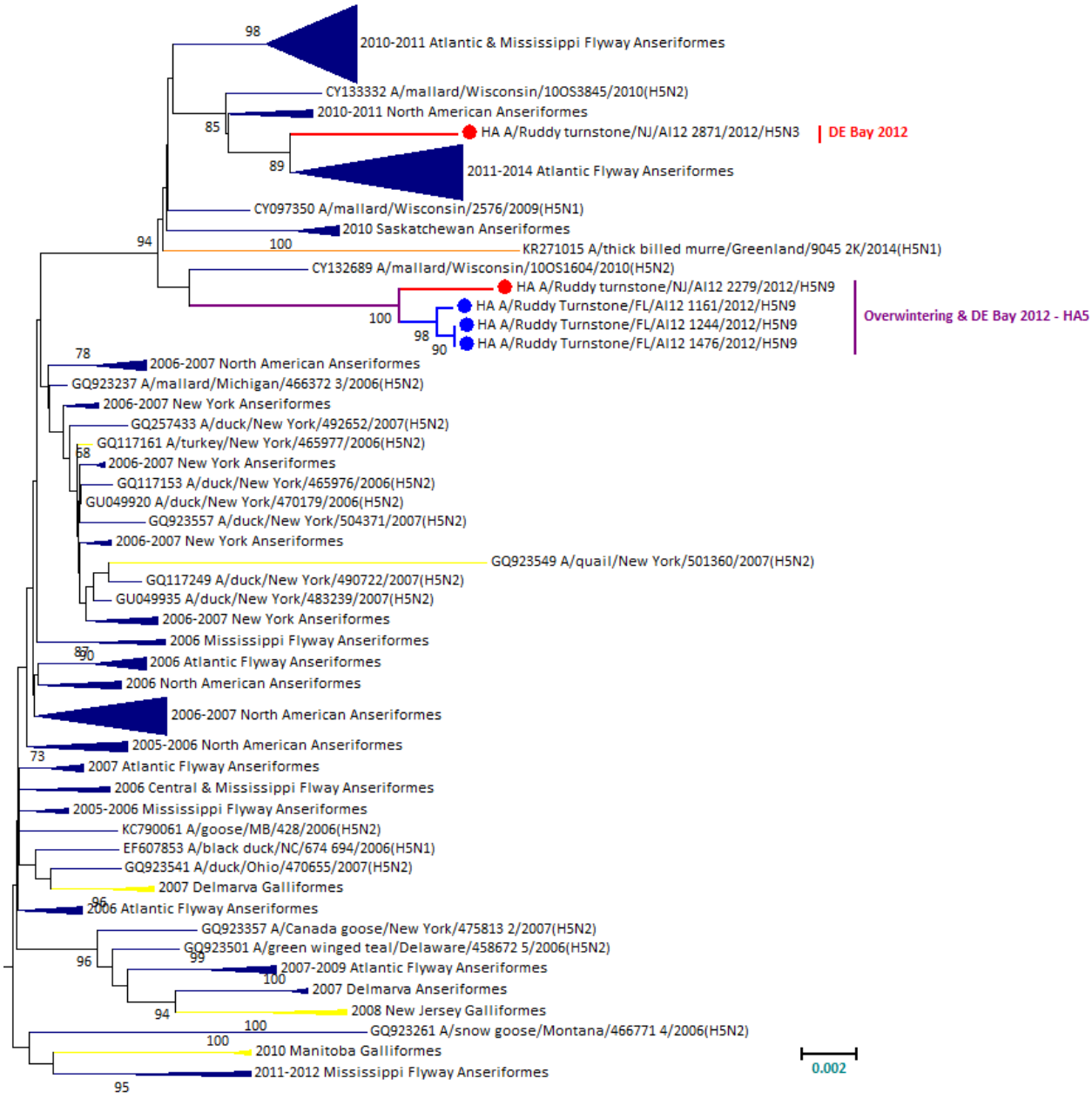


Figure 5.3 Compressed maximum-likelihood (ML) phylogenetic sub-tree for hemagglutinin HA5 gene segments derived from influenza A viruses (IAV) isolated from wild and domestic birds in North and South America (excluding Alaska), without date restriction. Nodes for HA5 segments identified in this study are colored in red (Delaware Bay) or blue (overwintering) circles. Bootstrap values lower than 65 are omitted. The ML topology that includes all 549 sequences is available as supplemental material (Supplemental Figure S5.1).

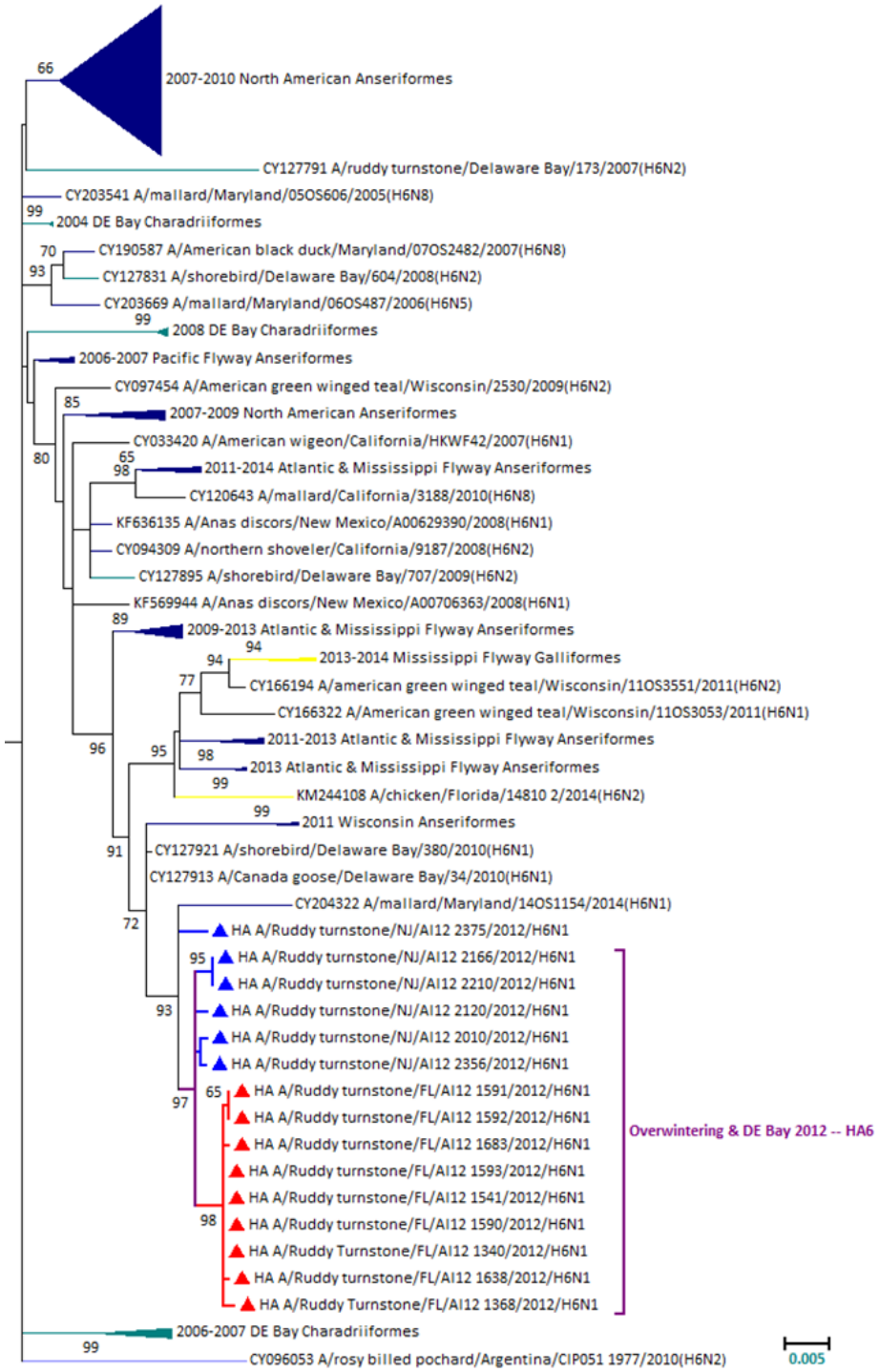
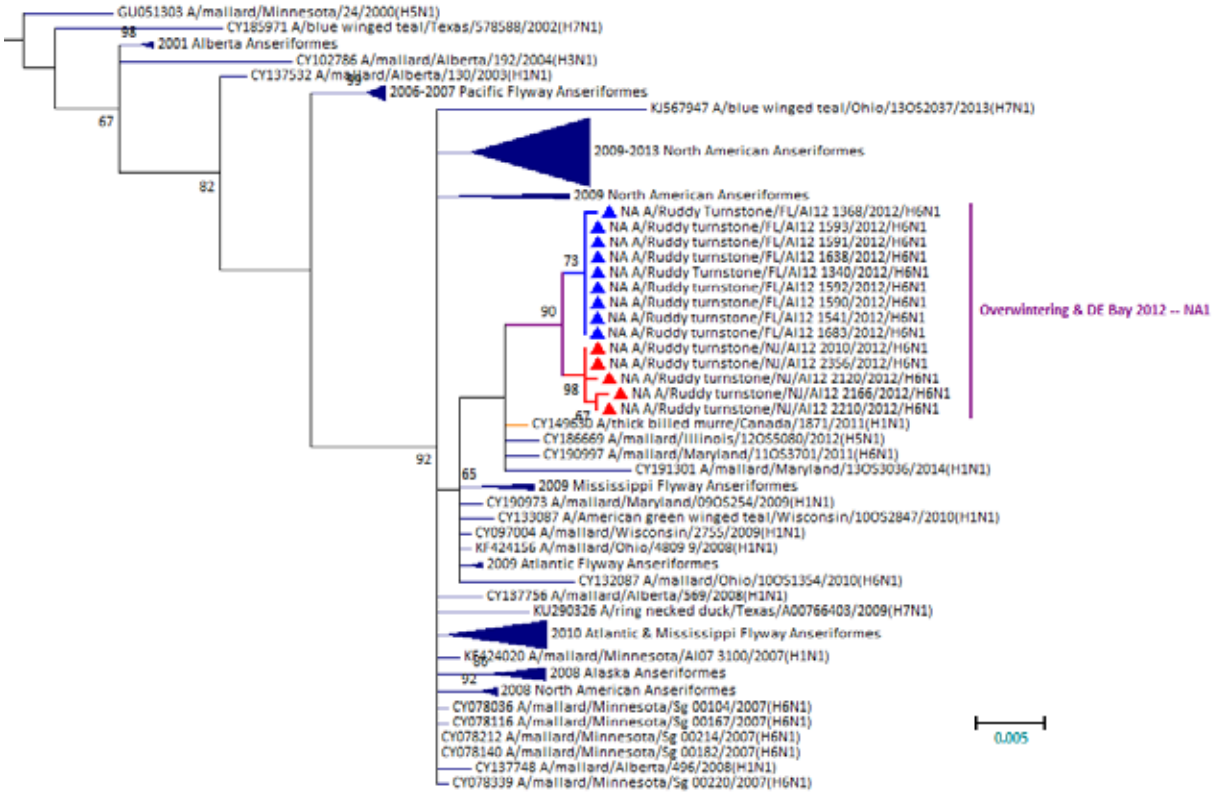


Figure 5.4. Compressed ML phylogenetic subtree for hemagglutinin HA6 gene segments derived from IAV isolated from wild and domestic birds in North and South America (excluding Alaska), without date restriction. Nodes for HA6 segments identified in this study are colored in red (Delaware Bay) or blue (overwintering) triangles. Bootstrap values lower than 65 are omitted. The ML topology that includes all 506 sequences is available as supplemental material (Supplemental Figure S5.2).

A) Clade of overwintering and Delaware Bay NA1 sequences



B) One outlier Delaware Bay NA1 sequence

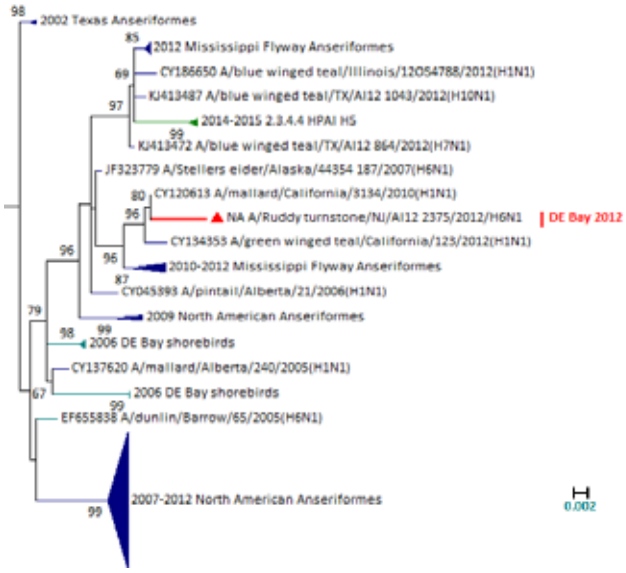


Figure 5.5. Compressed ML phylogenetic subtrees for neuraminidase NA1 gene segments derived from IAV isolated from wild and domestic birds in North and South America without date restriction. Nodes for NA1 segments identified in this study are colored in red (Delaware Bay) or blue (overwintering) triangles. Bootstrap values lower than 65 are omitted. The ML topology that includes all 403 sequences is available as supplemental material (Supplemental Figure S5.3). A) Position of 14 of 15 viruses of NA1 subtype isolated in this study; B) position of one DE Bay NA1 that shares 93% nt identity with other viruses isolated in this study.

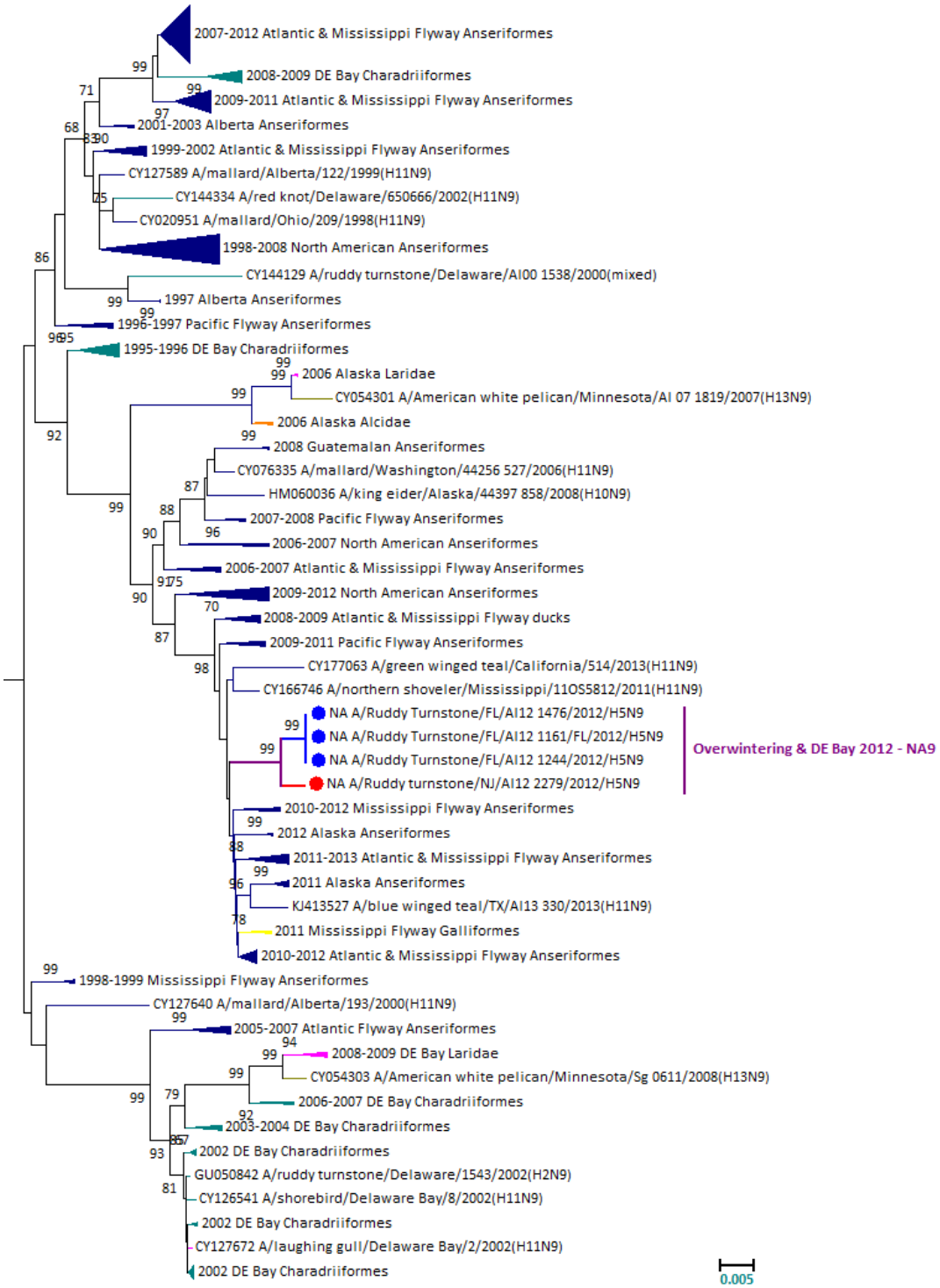


Figure 5.6. Compressed ML phylogenetic subtree for neuraminidase NA9 gene segments derived from IAV isolated from wild and domestic birds in North and South America, without date restriction. Nodes for NA9 segments identified in this study are shown in red (Delaware Bay) or blue (overwintering) circles. Bootstrap values lower than 65 are omitted. The ML topology that includes all 335 sequences without compression is available as supplemental material (Supplemental Figure S5.4).

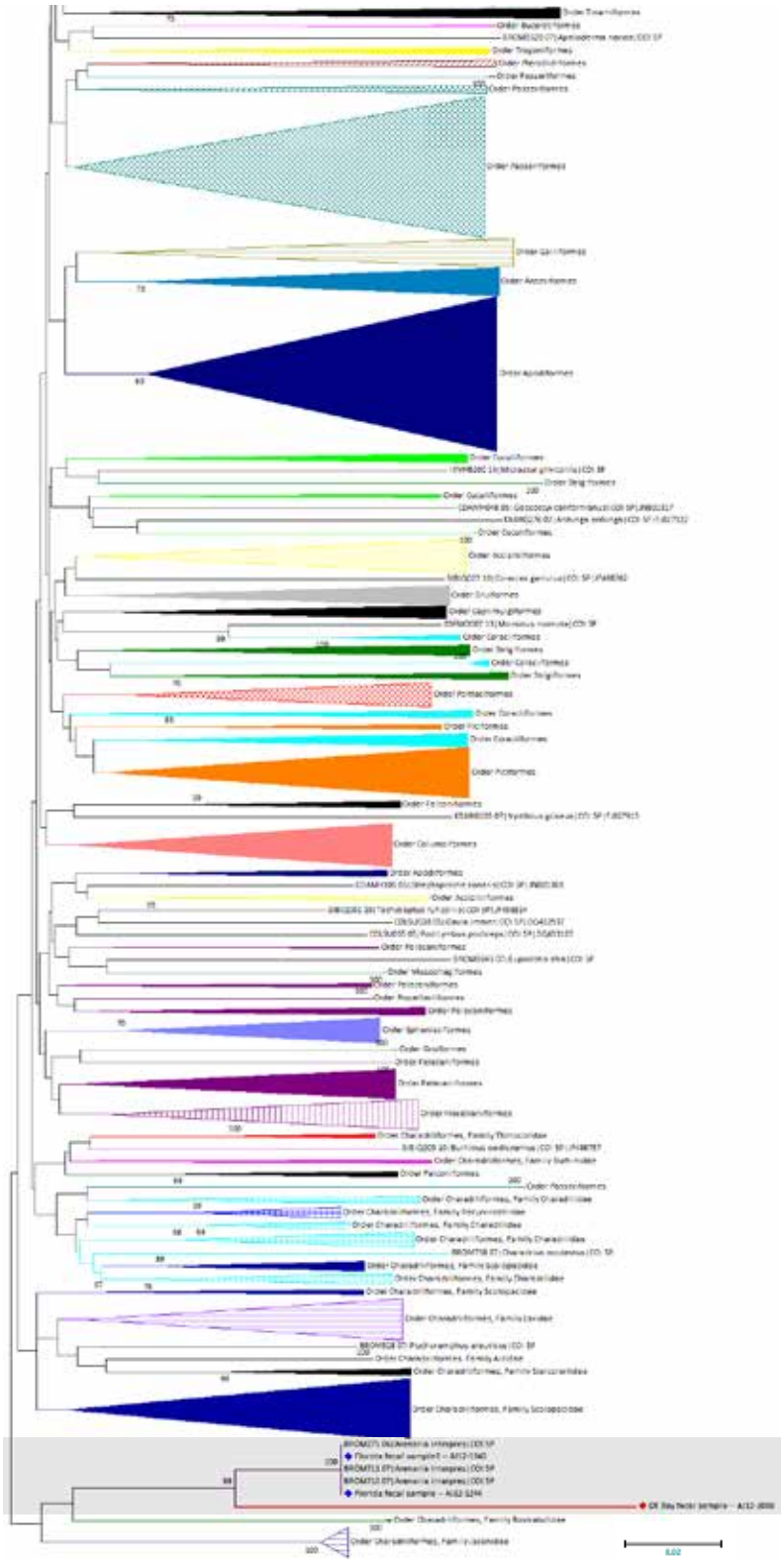
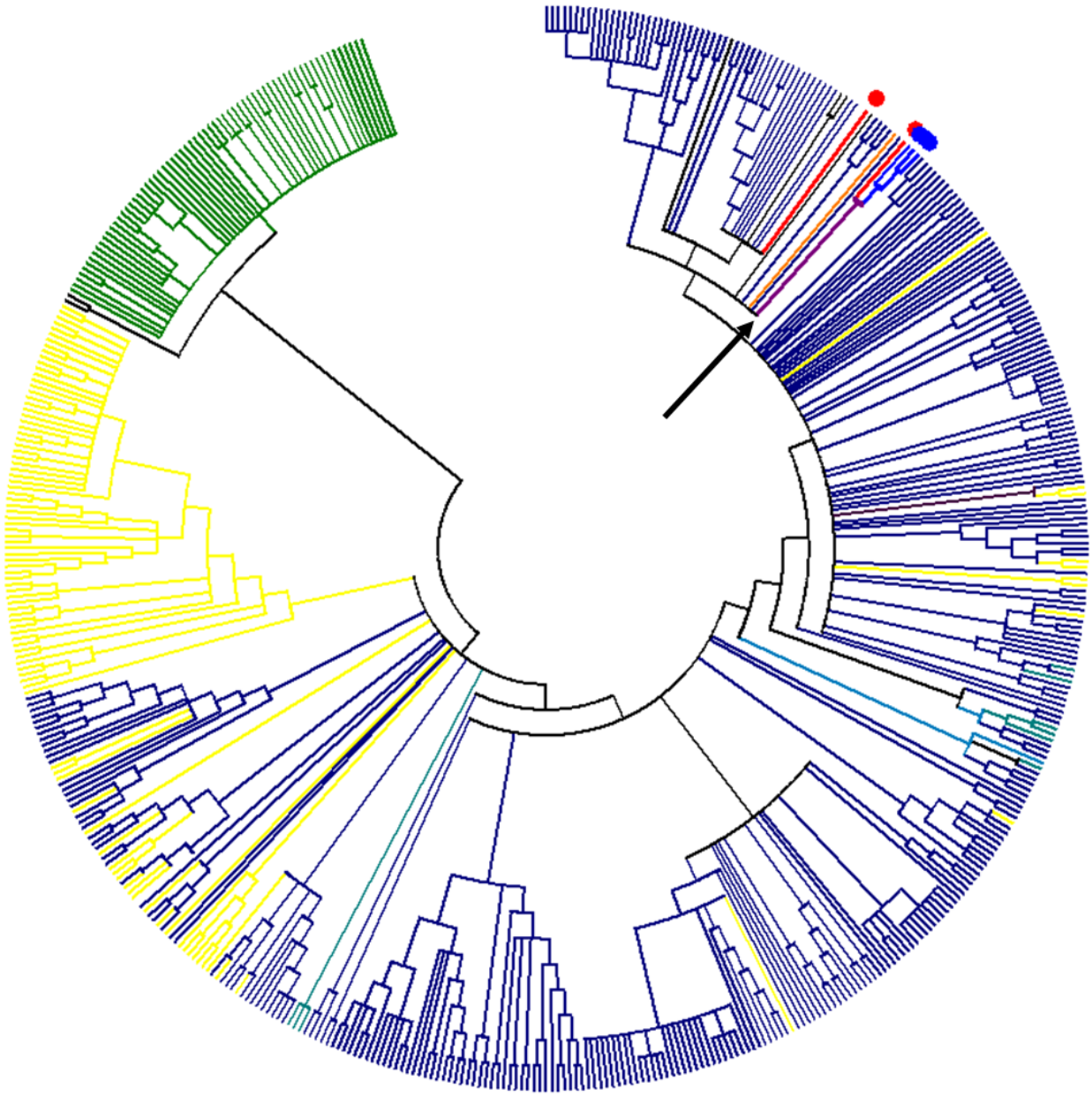
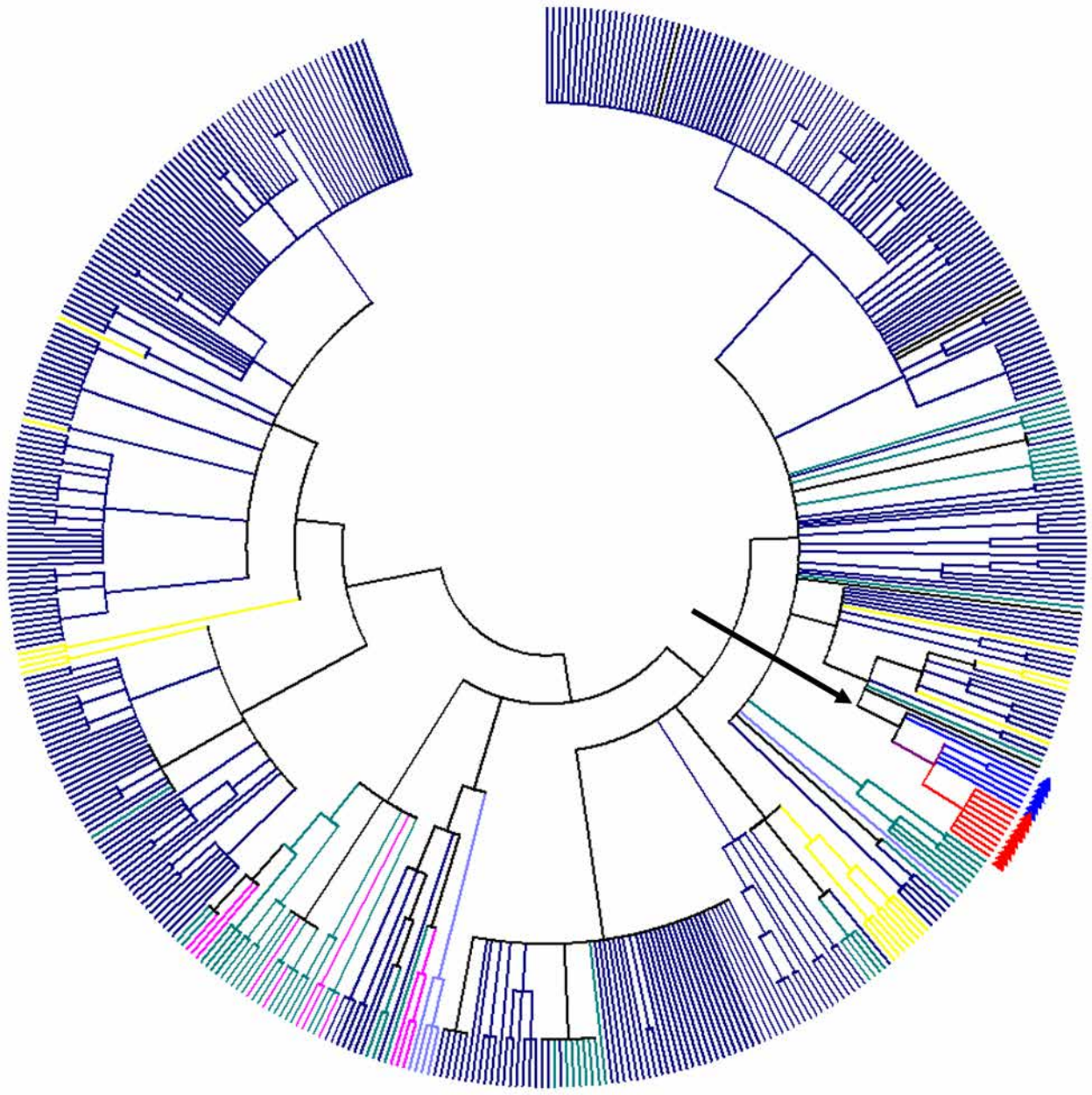


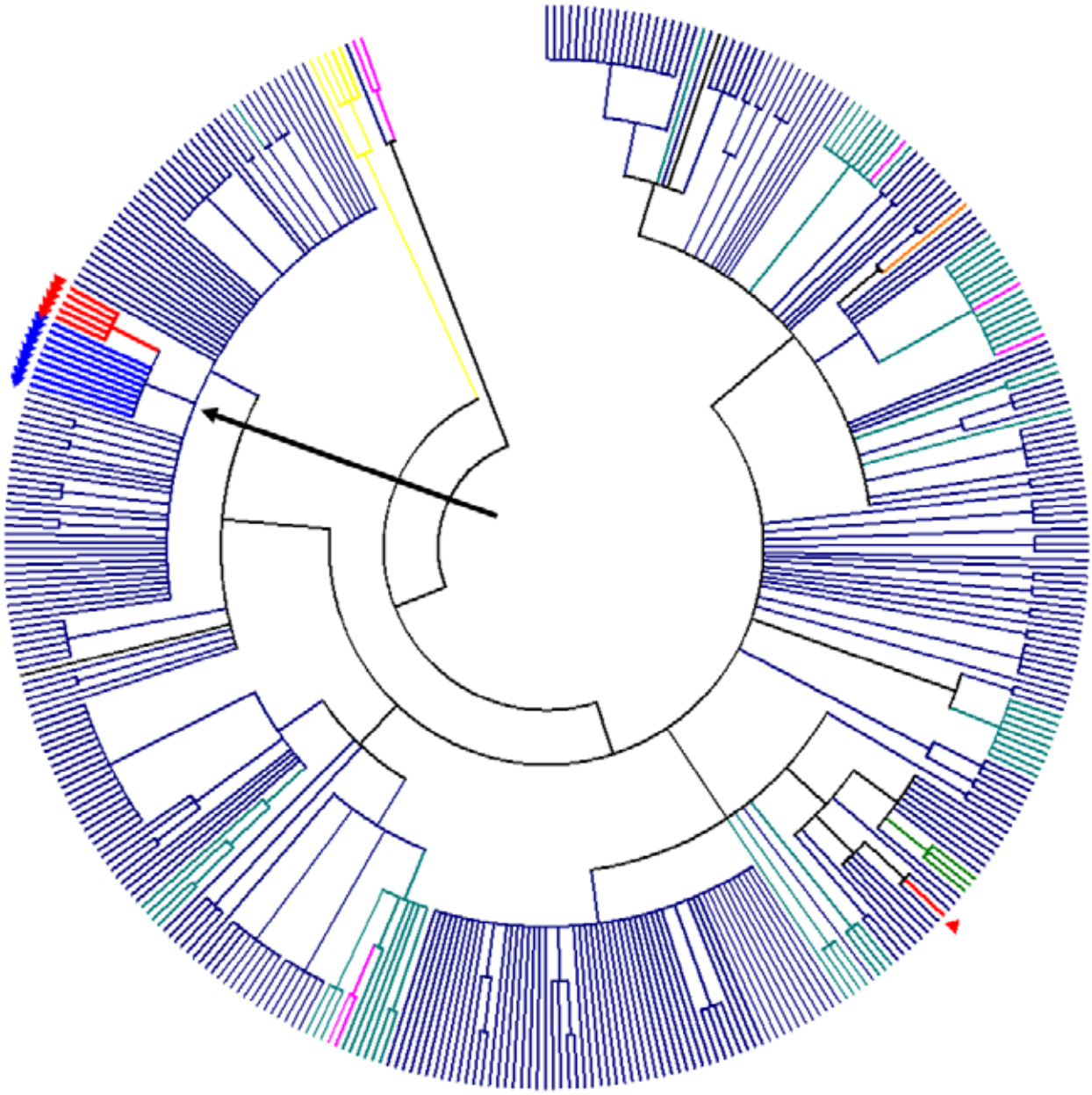
Figure 5.7. Compressed ML phylogenetic tree for 2,473 avian COI DNA sequences retrieved from BOLD.ORG and avian fecal samples (n = 3) from this study. Nodes for species identified in this study are shown in blue (overwintering) or red (DE Bay) diamonds, and shaded in gray with reference *A. interpres* sequences. Bootstrap values lower than 60 are omitted.



Supplementary Figure S5.1. ML phylogenetic tree depicting the inferred topology for 549 sequences of HA5 hemagglutinin gene segments from wild and domestic birds in North and South America. Position of the three overwintering H5N9 and Delaware (DE) Bay H5N3 (1) or H5N9 (1) viruses isolated in this study are shown with blue or red dots at branch tips, respectively. The bootstrap support for the overwintering/DE Bay node indicated by a black arrow is 100%. Color coding for reference branches is as follows: green = 2014-2015 HPAI 2.3.4.4 H5, navy = Anseriformes, orange = *Alcidae*, teal = *Scolopacidae*, yellow = Galliformes.



Supplementary Figure S5.2. ML phylogenetic tree depicting the inferred topology for 506 sequences of HA6 hemagglutinin gene segments from wild and domestic birds in North and South America. Position of the nine overwintering and six Delaware (DE) Bay H6N1 viruses isolated in this study are shown with blue or red triangles at branch tips, respectively. The bootstrap support for the overwintering/DE Bay node indicated by a black arrow is 97%. Color coding for reference branches is as follows: lavender = Rosy-billed pochards, navy = Anseriformes, orange = *Alcidae*, pink = *Laridae*, teal = *Scolopacidae*, yellow = Galliformes.



Supplementary Figure S5.3. ML phylogenetic tree depicting the inferred topology for 403 sequences of NA1 neuraminidase gene segments from wild and domestic birds in North and South America. Position of the nine overwintering and six Delaware (DE) Bay H6N1 viruses isolated in this study are shown with blue or red triangles at branch tips, respectively. The bootstrap support for the overwintering/DE Bay node indicated by a black arrow is 90%. Color coding for reference branches is as follows: green = 2014-2015 HPAI 2.3.4.4 H5N1, navy = Anseriformes, orange = *Alcidae*, pink = *Laridae*, teal = *Scolopacidae*, yellow = Galliformes.

Supplementary Figure S5.4. ML phylogenetic tree depicting the inferred topology for 335 sequences of NA9 neuraminidase gene segments from wild and domestic birds in North and South America. Position of the three overwintering H5N9 and one Delaware (DE) Bay H5N9 viruses isolated in this study are shown with blue or red dots at branch tips, respectively. The bootstrap support for the overwintering/DE Bay node indicated by a black arrow is 93%. Color coding for reference branches is as follows: navy = Anseriformes, orange = *Alcidae*, pink = *Laridae*, teal = *Scolopacidae*, yellow = Galliformes.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

The transmission of pathogens to susceptible hosts depends upon appropriate conditions at the levels of host, pathogen, and environment; with influenza viruses, this is complicated by the diversity of viruses and hosts, as well as the environments they occupy. Although the environmental tenacity of influenza A viruses (IAVs) is well described, it is unknown if environmental adaptation plays a part in host adaptation such as occurred in 2009 with the swine-origin H1N1 human pandemic. In the case of IAV in the wild bird reservoir, both environmental transmission through water and the movement of viruses during migration are important components of the IAV transmission cycle in ducks. These host and environmental relationships, however, may not be as important in shorebirds that occupy very different habitats and exhibit very different host behaviors related to both wintering and migration. The overall objectives of this research related to understanding host and environmental factors affecting IAV transmission and maintenance. In Chapter 3, we investigated if IAV environmental adaptation and fitness varied between viruses associated with different mammalian hosts (swine and human). In Chapters 4 and 5, we described discrete environmental and host drivers that are potentially involved in the ecology of IAV (through experimental and field studies) in migratory shorebirds at a unique ecological and seasonal system: Delaware Bay.

Persistence: In Chapter 3, the environmental stabilities of eight swine and six human IAV isolates were assessed under different temperature, pH, and salinity conditions in a

laboratory distilled water system. Viruses represented historical and current, as well as seasonal and pandemic, H1N1, H1N2, and H3N2 IAV strains. A single variable was manipulated in each set of trials, and viral tenacity was measured at either 4°C – 37°C (n = 6 treatments), at a pH of 5.4 – 9.0 (n = 11 treatments), or at a salinity of 0 – 30ppt (n = 7 treatments), conditions that have been well-established in distilled water laboratory models utilizing avian IAV. All viruses, regardless of subtype or species of origin, persisted longest at cold temperatures, at near neutral pH, and in low saline environments. While there were significant differences in persistence profiles for swine and human viruses at 10°C, 23°C, and 20ppt salinity (swine viruses persisted longer than human), no consistent or significant adaptations related to changes in environmental stability were observed between swine and human IAV, nor between pandemic and seasonal (non-pandemic) IAV. The general trends described were similar to the well-documented responses of avian IAV under similar conditions. This work further supports the effect of environmental variables on modulating influenza persistence; however, we did not detect any evidence of environmental adaptation associated with host.

Persistence and Transmission: The persistence of avian IAVs isolated from environmental samples taken during the spring migration of shorebirds through Delaware Bay, USA was assessed in Chapter 4. Viruses were retrieved from sand cores (H13N6, n = 17; H12N1, n = 1; and H12N3, n = 1) and horseshoe crab eggs (LP H7N3, n = 1) recovered from approximately 5cm depth in sand/surrounding substrate; these viruses matched the IAV subtypes isolated from shorebirds at Delaware Bay in the same years. In addition, temperature probes were deployed in May 2012 on the surface and at subsurface depths of sand at several Delaware Bay beaches. The substrate temperatures measured by these probes, as well as publically

available climatologic and hydrologic data, were utilized to create laboratory microenvironments similar to those at Delaware Bay. The persistence capabilities of the environmental H12N1 and H12N3 isolates, as well as an H12N3 recovered from a Ruddy Turnstone (*Arenaria interpres*) fecal sample were then evaluated in a laboratory system. Viral stability was monitored under treatments that included sand and distilled water at 22°C, 20ppt salinity and at a pH of 6.0 or 7.2. All viruses remained infectious within sand columns for 7 days, a time-frame suitable for localized transmission to shorebirds upon subsequent foraging efforts. The local substrate at Delaware Bay may serve to stabilize IAV, protecting it from the harsh effects of dessication and UV degradation. Microcosms of detritus, sand, horseshoe crab eggs, and shorebird fecal matter may in turn increase the availability of environmentally persistent viruses to susceptible shorebird hosts.

Maintenance and Transmission: The final objective of this work was to determine what role overwintering shorebirds may play in the maintenance and transmission of IAV at Delaware Bay every May. Surveillance of Ruddy Turnstones on their overwintering grounds in the southeastern United States resulted in the recovery of 25 IAV from 2010 – 2015. In most cases, the hemagglutinin and neuraminidase subtypes recovered from Ruddy Turnstones on wintering grounds matched those identified at Delaware Bay during the previous year or that year's migratory cycle. Given the recovery of these "matched" antigenic surface proteins, we hypothesized that viral gene segments were transported into Delaware Bay with migrating Ruddy Turnstones. Genetic analyses of all eight IAV gene segments from four H5N9 and 15 H6N1 viruses recovered from Ruddy Turnstones during the winter of 2012 and the following May at Delaware Bay revealed the high level of genetic relatedness at the nucleotide level, suggesting

that migrating Ruddy Turnstones are serving to redistributre IAVs from wintering grounds to the Delaware Bay ecosystem. However, the transfer of a homologous genome from winter sites to Delaware Bay was not identified, indicating that 1) winter gene segments are transferred to and assimilated into the larger IAV gene pool at Delaware Bay, and 2) other resident or migratory birds utilizing the Delaware Bay region may be an additional source of IAV gene segments at this ecological hotspot for IAV.

Assessing viral *persistence*, *maintenance*, and *transmission* is important to our understanding of IAV ecology, especially in natural systems. Further, dissecting the complex interaction(s) of these components is critical to defining IAV-host dynamics. The studies presented here were an attempt to assess persistence as related to host adaptation (Chapter 3), and environmental transmission (Chapter 4) and finally, to better understand the maintenance and subsequent transmission of IAV in and to susceptible shorebird hosts (Chapter 5). Further work, especially as related to the exact role/scale of environmental IAV transmission and the identification of alternate sources of influenza viruses into the Delaware Bay system, will be important to our continued understanding of the global ecology and epidemiology of IAV in natural hosts.