IDENTIFICATION AND CHARACTERIZATION OF FACTORS INFLUENCING THE PERSISTENCE OF AVIAN INFLUENZA VIRUS IN SURFACE WATER OF AQUATIC HABITATS

by

SHAMUS PATRICK KEELER

(Under the Direction of David E. Stallknecht)

ABSTRACT

The natural reservoirs for avian influenza (AI) viruses are wild aquatic bird species of the orders Anseriformes and Charadriiformes. Infected birds readily excrete virus in their feces contaminating the surrounding aquatic environment with transmission occurring via the fecal-oral route. Previous studies have established that AI viruses can remain infectious in water for several weeks to months, suggesting that surface water may serve as both a transmission medium and potentially a long term reservoir. The majority of the studies focused on AI virus persistence in water have utilized sterile distilled water to simulate the surface water of aquatic ecosystems. The objective of this research was to identify and characterize the factors that affect the duration of AI virus persistence in natural surface water.

The duration of infectivity was determined for two common AI virus subtypes in 15 filtered surface water samples collected from waterfowl habitats in Georgia. Consistent with previous studies, viruses were less stable at warmer temperatures and in acidic water (pH<5). Variations in persistence times were observed between water samples with comparable pH and salinities indicating that other abiotic factors affect the stability of the virus in natural surface water. To identify the other important abiotic factors, viral persistence trials were performed in filtered surface water from 38 waterfowl habitats distributed across the United States and in-depth chemical analyses were performed. In addition to previously identified abiotic factors, the ammonia, chloride, and sulfate concentrations of surface water were determined to be significant predictors of virus persistence. To evaluate the effect of the biological component of surface water and examine virus strain-related variation in persistence, the loss of infectivity was estimated for nine wild duck isolated AI viruses in three types of water: distilled, filtered surface water, and intact surface water. All viruses persisted longest in distilled water followed by filtered surface water, suggesting that surface water may not readily facilitate the long term maintenance of AI viruses. These results contribute to our understanding of the role of surface water in the ecology of AI virus.

INDEX WORDS: influenza A virus, avian influenza virus, persistence, water, waterfowl, wild bird, environmental persistence

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DEDICATION

This work is dedicated to my parents for their sacrifices and unending love and support.

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I would not have pursued a PhD at SCWDS if it were not for Jane Huffman and for that I owe her a great debt. Her mentorship has been invaluable and she continuously provides me with support and opportunities. I look forward to our continued friendship and collaborations.

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CHAPTER ONE

INTRODUCTION

Wild birds are considered to be the natural reservoirs for avian influenza (AI) virus (Stallknecht and Shane, 1988; Webster et al., 1992). Naturally occurring infections have been reported in over 100 species representing 12 orders but species within the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds) are considered to be the most important hosts in the epidemiology of AI virus (Olsen et al., 2006; Stallknecht and Brown, 2008; Stallknecht and Shane, 1988; Webster et al., 1978). The species within these orders are diverse in both their geographic distribution and biological life history but most are associated with aquatic habitats (Webster et al., 1992). Viral replication occurs primarily in the epithelial cells of the intestinal tract of wild birds (Webster et al., 1978). High concentrations of virus are shed in the feces of infected birds, which contaminate surface water and the surrounding area of aquatic habitats (Hinshaw et al., 1979). Avian influenza viruses are stable in water and transmission between wild birds is thought to occur through an indirect route via contaminated surface water (Stallknecht et al., 1990b). Numerous studies have determined that the environmental persistence of AI virus plays an important role in the maintenance and transmission of the virus within wild bird populations (Breban et al., 2009; Roche et al., 2009; Rohani et al., 2009).

The current understanding of the persistence of AI virus in water is based almost entirely on studies using modified distilled water as a model system (Brown et al., 2009;

Brown et al., 2007; Lebarbenchon et al., 2011; Nazir et al., 2010; Stallknecht et al., 2010; Stallknecht et al., 1990b). The distilled water system allows for easy modification and control of various parameters, and adequately simulates the water column. Based on these studies, it's known that the virus can remain infectious in water for several weeks to months with the duration of persistence dependent on the temperature, pH, and salinity of the water (Brown et al., 2009; Stallknecht et al., 1990a). The optimal conditions for AI virus persistence are low temperature (<17C), neutral-basic pH (7.4-8.2), and fresh to brackish water (<25ppt) (Brown et al., 2009). Variation between the stability of viral strains in water has also been reported (Brown et al., 2009; Brown et al., 2007). Some studies have used surface water for AI virus persistence trials and these studies support the general trends defined using the distilled water model but the number and geographic distribution of the water samples included in these studies is limited (Nazir et al., 2010; Stallknecht et al., 1990a; Zarkov, 2006). Additionally, many of these studies used biologically intact surface water and made no attempt to differentiate or identify the wide range of abiotic and biotic factors that could be affecting viral persistence (Nazir et al., 2010; Zarkov, 2006).

The aquatic habitats of migratory waterfowl vary considerably, and the surface water at these sites is equally diverse with a wide range of physicochemical and biological factors (Stallknecht et al., 2010). The complexity of these aquatic systems limits the applicability of data collected using the modified distilled water model. To truly gain an understanding of AI virus persistence in natural surface water, a systematic and layered experimental approach should be applied. One such approach would be to perform laboratory based studies using large numbers of surface water samples under

semi-controlled conditions that limit the influence of confounding factors. The goal of this research was to identify and characterize the factors that affect AI virus persistence in surface water of aquatic habitats. Our hypothesis is that the persistence of AI virus in the surface water of aquatic habitats is affected by abiotic factors other than pH, salinity, and temperature, and by the microbial community. The research described herein provides a greater understanding of the persistence of AI virus in aquatic habitats, which enhances our understanding of the epidemiology of AI virus in wild birds and could be used to improve the current wild bird surveillance programs.

The specific aims of this study are the following:

- Evaluate the predictive ability of pH, salinity, and temperature on the persistence of AI virus in natural surface water without the confounding influence of biological organisms.
- 2. Identify the abiotic factors other than pH, salinity, and temperature that affect the persistence of AI virus in natural surface water.
- 3. Evaluate whether different strains of AI virus vary in their duration of persistence in distilled and natural surface water.

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CHAPTER TWO

LITERATURE REVIEW

INFLUENZA A VIRUS

Virus classification and nomenclature

Influenza A virus belongs to the genus *Influenzavirus A* in the family *Orthomyxoviridae*. In addition to the genus *Influenzavirus A*, the family *Orthomyxoviridae* contains three additional recognized genera; Influenzavirus B and *Influenzavirus C*, and *Thogotovirus*. An additional genera, *Quarjavirus*, has been proposed for several newly discovered tick-borne viruses including Wellfleet Bay virus, which has been associated with mortality events in common eiders (*Somateria mollissima*) (Presti et al., 2009; ProMed, 2012).

Influenza A virus strains are categorized into subtypes based on the viral antigens hemagglutinin (HA) and neuraminidase (NA), which can be differentiated using traditional serological methods or molecular techniques(Suarez, 2008). There are sixteen HA subtypes (H1-H16) and nine NA subtypes (N1-N9) formally recognized as subtypes but a seventeenth HA subtype (H17) was recently proposed based on the description of a novel virus isolated from bats in Guatemala (Alexander, 2007; Fouchier et al., 2005; Munster et al., 2007; Tong et al., 2012). An internationally standardized nomenclature, established by the World Health Organization, is used for naming individual influenza strains (WHO, 1980). Influenza strains are designated as follows: virus genus (A, B, or C)/animal host of origin/location of isolation/laboratory strain number/year of isolation followed by the HA and NA subtype of the virus. If the virus is isolated from humans, the animal host of origin is omitted. An example of this nomenclature is

A/Mallard/MN/199036/99 (H3N2). Additionally, influenza A viruses can be categorized based on pathogenicity into two pathotypes either low pathogenic (LP) or highly pathogenic (HP). Virus are designated HP on the following criteria established by the U.S. Animal Health Association: 1) An intravenous pathogenicity index greater than 1.2 in 6-week-old chickens or cause at least 75% mortality in 4-to-8-week old chickens after intravenous inoculation and 2) have multiple basic amino acids at the HA cleavage site characteristic of a HP virus (Suarez, 2008). The vast majority of the influenza A viruses are LP and only viruses of the subtypes H5 and H7 have been reported to be HP (Suarez, 2008). The terms avian influenza (AI) virus, human influenza virus, swine influenza virus, bat influenza virus are commonly used for viruses adapted to a specific host type but susceptibility to these viruses is not always restricted to the eponymous host type as evidenced by the ongoing HP H5N1 epizootic (Hinshaw et al., 1983; Sims and Brown, 2008).

Virus structure and replication

Influenza A viruses are pleomorphic with isolates ranging in shape from spherical to filamentous depending on the genetics of the virus and on whether the virus is a direct isolate from the host or an isolate that has been passaged in eggs or tissue culture (Burleigh et al., 2005; Choppin et al., 1960; Chu et al., 1949; Elleman and Barclay, 2004; Smirnov Yu et al., 1991; Webster and Bean, 1978). The size of the virions is equally as variable with lengths ranging from 80-120nm for spherical and rod-shaped virions to 300-400nm for filamentous virions (Webster et al., 1992). The virus has a single

stranded, negative sense RNA genome composed of eight independent segments. Each gene segment codes for one to two proteins for a total of 10-11 depending on the virus strain. The gene segments are designated by numbering 1-8 from longest to shortest. The gene segments one, three, four, five, and six code for single proteins, which are the polymerase basic protein 2 (PB2), polymerase acidic protein (PA), HA, nucleocapsid protein (NP), and NA, respectively. In all virus strains, gene segment two codes for the polymerase basic protein 1 (PB1) but in some strains the segment has an additional overlapping reading frame coding for the protein PB1-F2. Gene segment eight also codes for two proteins, nonstructural 1 (NS1) and nonstructural 2 (NS2 or sometimes NEP).

Virions have a host cell derived lipid envelope containing three viral encoded surface proteins, HA, NA, and M2. Underlying the lipid envelope is a protein shell made up of the major structural protein M1. The segments of the viral genome are coated by NP and each segment has a bound RNA dependent RNA polymerase (RDRP) formed by the PB1, PB2 and PA. This association between the RNA gene segments and viral proteins is referred to as the ribonuceloprotein (RNP) complex. The RNP complexes are not free floating inside the virion but associated with the M1. The NS2 protein is present in small amounts inside the virion. The NS1 and PB1-F2 proteins are not commonly found inside the virion. The HA is the major antigenic surface glycoprotein and is necessary for attachment to host cells and viral entry. Before the HA can initiate host cell binding, the protein must be cleaved by extracellular proteases forming two smaller polypeptides, HA1 and HA2. The HA1 is responsible for binding sialic acid-containing

receptors on the host cell surface, which initiates endocytosis. The HA of influenza virus strains can differ in their preference for binding different types of sialic acids and this feature influences the host range of viral strains (i.e. AI viruses preferentially bind alpha 2-3 and human influenza viruses preferentially bind alpha 2-6). The virus containing vesicle fuses with an endosome and hydrogen ions are pumped into the endosomal vesicle. The decrease in pH caused by the influx of hydrogen ions causes a conformational change in the HA2 allowing fusion of the viral envelope and endosomal membrane. Simultaneously, the M2 protein pumps hydrogen ions into the virion causing a decrease in the internal pH. The decreased pH allows the disassociation of the RNP complexes and M1. The fusion between the viral envelope and endosomal membrane releases the RNPs into the cytoplasm of the host cell. Unlike other RNA viruses, influenza replicates in the host nucleus not the cytoplasm. All four proteins in the RNP have nuclear localization sequences and are trafficked by the host cell into the nucleus as intact complexes. Inside the nucleus, the RDRP produces messenger RNA (mRNA) using the viral RNA (vRNA) segments as templates. The mRNAs are not exact copies of the vRNAs, they lack sequences at the 5' and 3' end, and require a primer for production. The primers are derived through a process called cap snatching, where the RDRP cleave the 5' caps off of host cell RNA polymerase II transcripts. The mRNAs are shipped out into the cytoplasm for translation. All viral proteins are produced in the cytoplasm using the host cell proteins. The viral surface proteins (HA, NA, and M2) are translated and trafficked to the cell membrane. The internal viral proteins (PA, PB1, PB2, NP, M1, and NS2) are translated by free ribosomes and trafficked back to the nucleus. The NS1 protein is found in the nucleus and cytoplasm, and disrupts normal host cell function and

defense allowing virus transcription to occur. PB1-F2 is not produced by all influenza strains and is thought to be involved in mediating apoptosis in host immune cells enhancing infection (Chen et al., 2001; Coleman, 2007). Inside the nucleus, the RDRP makes full length positive-stranded RNA copies of the vRNA, which acts as template for the production of vRNA gene segments. The RNP complexes form inside the nucleus, and it is thought that M1 and NS2 mediate the nuclear export of the RNPs since both proteins have nuclear export sequences. The M1 and RNP cell are trafficked to the cell membrane bind to the portion of the membrane where the HA, NA, and M2 have been inserted. Virions form through budding of the host cell membrane. The NA glycoprotein mediates release of viruses from the surface of the host cell by cleaving sialic acids.

Maintenance of Genetic Diversity

Particular structural features of influenza A virus allows for the maintenance of very broad antigenic and genetic diversity within this species of virus. One mechanism of generating genetic diversity called antigenic drift is a result of features of the viral polymerase (RDRP). The RDRP has a low fidelity and no proof reading mechanism resulting in 10⁻³ to 10⁻⁵ mutations per nucleotide copied (Buonagurio et al., 1986; Webster et al., 1980). The second mechanism of diversity generation is the segmented genome, which allows the virus to undergo reassortment or antigenic shift (Burnet and Lind, 1951; Burnet et al., 1949). If a host cell is infected simultaneously with two different viruses, the resulting progeny will not all be genetically identical, with some viruses similar to each parent virus and others being made up of a mix of gene segments from both viruses. A final mechanism of generating genetic diversity is through recombination between parental viruses. Recombination had long been considered a rare

event in influenza A viruses with little significance but recent studies have highlighted the potential importance of this mechanism (He et al., 2012; Wright and Webster, 2006).

Host Range

Influenza A virus has a diverse host range infecting many mammalian and avian species with distinct lineages of influenza A virus being endemic in multiple host species. Currently, two human-adapted subtypes (H1N1, H3N2) circulate within the world population but other subtypes (H2N2) have circulated in humans in the past and infection of humans with avian-adapted viruses have been reported (Alexander and Brown, 2009; Potter, 2001; Wright and Webster, 2006). Influenza infection in humans is a highly contagious, acute respiratory illness with transmission occurring through the air via sneezing or coughing. Seasonal epidemics occur in the winter with transmission enhanced by lower temperatures and low relative humidity (Lowen et al., 2007; Shaman and Kohn, 2009). The emergence of novel viral strains and limited population immunity to these strains can result in an influenza pandemic (Kilbourne, 2006; Osterholm, 2005).

Endemic lineages of influenza circulate within commercial swine (H1N1, H3N2) (Wright and Webster, 2006). Influenza infection in swine can have economic consequences for the swine industry and can be a source for the emergence of novel human-adapted influenza viruses (Neumann et al., 2009; Olsen et al., 2002). Additional endemic lineages circulate within horses (H3N8, H7N7) (Wright and Webster, 2006), dogs (H3N8) (Crawford et al., 2005; Peek et al., 2004) and poultry (various H1, H3, H5, H7, H9 subtypes) (Swayne, 2008). Infection of other species have also been reported including cats, mink, seals, and whales (Chambers et al., 1989; Geraci et al., 1982; Klingeborn et al., 1985; Kuiken et al., 2006; Kuiken et al., 2004; Lvov et al., 1978). Tong

et al. (2012) recently described a novel lineage of influenza virus isolated from yellowshouldered bats (*Sturnira lilium*) in Guatemala, which may represent the first known nonavian origin influenza virus.

Although the host range of influenza A virus is broad, the vast majority of known influenza viruses are adapted to wild aquatic birds (Olsen et al., 2006; Stallknecht and Shane, 1988). The majority of HA (1-16) and NA (1-9) subtypes circulate within wild bird populations and wild aquatic birds are considered to be the primordial reservoir for the virus (Stallknecht and Brown, 2008; Stallknecht and Shane, 1988; Suarez, 2008; Webster et al., 1992; Wright and Webster, 2006).

AVIAN INFLUENZA VIRUS IN WILD BIRDS

Avian Host Species

To date, influenza A virus has been isolated from over 100 different avian species, a majority of which inhabit or utilize aquatic environments with the exception of a few species within the avian orders Columbiformes (doves and pigeons), Galliformes (turkey, quail, and partridge), Passeriformes (songbirds), and Piciformes (woodpeckers) (Fouchier et al., 2005; Fuller et al., 2010; Hinshaw et al., 1982; Hinshaw et al., 1980; Olsen et al., 2006; Stallknecht and Brown, 2008; Stallknecht and Shane, 1988; Webster et al., 1992). The avian orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds) are considered to be the primary avian hosts for influenza A virus with 16 HA and 9 NA subtypes circulating within species of these two bird groups (Stallknecht and Brown, 2008; Stallknecht and Shane, 1988). Within the Anseriformes, most of the host species have been from the subfamily Anatinae (dabbling and diving ducks) with particularly high isolation rates in mallards (Anas platyrhynchos) (Krauss et al., 2004). The AI virus isolations from species within the order Charadriiformes have come from two avian families, the Scolopacidae (shorebirds) and Laridae (gulls). Viruses have been isolated from at least nine species within the family Scolopacidae but the ruddy turnstone (Arenaria interpres) accounts for a vast majority of the isolations with the highest prevalences reported at Delaware Bay in the northeastern United States during their annual spring migration (Hanson et al., 2008; Kawaoka et al., 1988; Krauss et al., 2010). For the family Laridae, most viral isolations are from species of gull (e.g. Blackheaded gull, Larus ridibundus and Herring gull, L. argentanus) although virus has been isolated from multiple species of tern including the first wild bird influenza A virus isolation from a common tern (Sterna hirundo) in 1961 (Becker, 1966; Hanson et al., 2008; Munster et al., 2007). The subtypes H13 and H16 are most commonly isolated from gulls and it is thought these viruses may be specifically adapted to charadriiform species representing an independent epidemiological cycle (Fouchier et al., 2005; Kawaoka et al., 1988).

Epidemiological Cycle

Replication of low pathogenic AI virus occurs in the intestinal tract of wild birds and large amounts of virus are shed in the feces (Webster et al., 1978). The duration of viral excretion can vary between individual birds depending on a variety of factors including immunological status and age of the host (Costa et al., 2010a, b; Webster et al., 1978). Based on most experimental infection studies, viral excretion lasts 3-12 days but excretion up to 28 days has been reported in experimentally inoculated Pekin ducks (*Anas platyrhynchos*) (Costa et al., 2010a, b; Hinshaw et al., 1980; Webster et al., 1978).

Transmission between individuals occurs via the fecal-oral route with the aquatic habitat serving as a transmission medium (Hinshaw et al., 1979).

Seasonal AI virus infections have been documented within anseriiform species in North America and Europe (Krauss et al., 2004; Munster et al., 2007; Wallensten et al., 2007). Peak prevalence occurs during summer and early fall coinciding with annual staging and migration (Krauss et al., 2004; Wallensten et al., 2007). During these periods, high concentrations of immunologically naïve juvenile birds congregate in migratory staging and stopover sites facilitating rapid spread of the virus (Krauss et al., 2004; Wallensten et al., 2007). The subtype diversity within anseriiform species varies based on the year, geographic location, and taxonomic diversity of the host population. The HA subtypes H3, H4, and H6, and the NA subtypes N2, N6, and N8 are most commonly isolated from North American waterfowl (Krauss et al., 2004; Stallknecht and Shane, 1988; Stallknecht et al., 1990c).

The epidemiological cycle of AI virus within charadriiform species (shorebirds and gulls) is not as well understood particularly among gulls. The only site in the world to experience seasonal AI virus infections in shorebirds is in Delaware Bay along the Atlantic coast of the United States. A variety of shorebird species migrating up the Atlantic flyway to breeding sites in North America, stopover at Delaware Bay in May-June to feed on horseshoe crab (*Limulus polyphemus*) eggs. The shorebird species congregate in high densities and feed along the water-beach interface, which is thought to facilitate transmission (Hanson et al., 2008; Krauss et al., 2010). Although AI viruses have been isolated from shorebird populations outside of Delaware Bay, the prevalence within these populations are very low (Fouchier et al., 2003; Krauss et al., 2010;

Stallknecht and Shane, 1988). The HA subtypes H13 and H16 have been primarily isolated from gulls and are thought to be a distinct lineage of viruses representing a unique epidemiological cycle within these hosts (Fouchier et al., 2005; Kawaoka et al., 1988; Olsen et al., 2006). Gulls can be infected with other AI viruses and often viruses circulating within shorebird populations will spillover into gulls since these species often utilize similar habitats (Hanson et al., 2008; Olsen et al., 2006).

ENVIRONMENTAL PERSISTENCE AND TRANSMISSION

While the duration and degree of viral shedding by AI virus infected wild birds varies, experimental infection studies have demonstrated that birds can shed virus for as long as 28 days and release up to 1×10^{10} EID₅₀ viruses per day (Hinshaw et al., 1980; Webster et al., 1978). Infected birds excrete virus via feces and contaminate the surrounding environment. The most commonly infected and ecologically important wild bird species are all highly gregarious and primarily utilize aquatic habitats (lakes, wetlands, and seacoasts). Contamination of the aquatic habitat is thought to facilitate indirect transmission (fecal-oral) between individuals and potentially these habitats can serve as a reservoir for short-term (within season) and long-term (between seasons) viral persistence and transmission. Avian influenza viruses have been isolated from surface water of waterfowl habitats when infected ducks were present (Halvorson et al., 1985; Hinshaw et al., 1980; Ito et al., 1995; Markwell and Shortridge, 1982; Sivanandan et al., 1991), and from surface water and sediment of aquatic habitats following bird migration (Ito et al., 1995; Lang et al., 2008; Lebarbenchon et al., 2011). Lebarbenchon et al. (2011) genetically characterized two AI viruses isolated from surface lake water on

consecutive years and determined that while the subtype of viruses isolated from water were not the dominant subtype recovered from birds during that year, they were prevalent in birds during the following season. Although these results are supportive of surface water acting as a long-term reservoir, water isolates and isolates recovered from birds during the following year were genetically distinct (Lebarbenchon et al., 2011). Experimental infection studies have shown that ducks will become infected with AI virus when utilizing contaminated water sources (VanDalen et al., 2010). In addition, Models of AI virus epidemics in wild birds have suggested that indirect fecal-oral transmission via contaminated water is an important determinant of transmission and should be considered when attempting to understand AI virus dynamics in wild birds (Breban et al., 2009; Roche et al., 2009; Rohani et al., 2009).

The stability of AI virus in surface water was first evaluated by Webster et al. (1978) using a single duck isolate and it was demonstrated that the virus could remain infectious in unchlorinated surface water for over a month. Laboratory trials using distilled water supported this environmental stability with viruses (10⁶ median tissue culture infectious doses [TCID₅₀]) remaining infectious for 207 days at 17°C (Stallknecht et al., 1990b) Using this same system, it was determined that persistence of AI virus was inversely related to temperature and salinity, and that stability was greatest in water with a neutral to basic pH (Stallknecht et al., 1990b). Filtered surface water samples from waterfowl habitats in Louisiana, USA also were tested, and the stability of AI virus in these samples was consistent with the results of the distilled water trials (Stallknecht et al., 1990a). However, these trials included only four surface water samples with limited variation in pH and salinity. Additional work with surface water collected in Bulgaria

supported earlier reports of the effects of pH, salinity, and temperature on AI virus persistence, and determined that AI virus persistence was reduced in biologically intact water compared to filtered water samples (Zarkov, 2006). Brown et al. (2009) evaluated the effect of pH, salinity, and temperature on twelve low pathogenic AI virus isolates representing HA subtypes 1-12 using the distilled water laboratory model. Avian influenza virus isolates showed variable response to the abiotic factors but stability was determined to be greatest at a slightly basic pH (7.4-8.2), low temperatures ($<17^{\circ}$ C), and fresh to brackish water (0-20,000 parts per million) (Brown et al., 2009). Direct comparisons of natural surface water and comparable distilled water support the inverse relationship between water temperature and AI virus stability (Nazir et al., 2010). In surface water samples with pHs outside the 7.4-8.2 range a reduced AI virus persistence was observed. (Zarkov, 2006). Freeze-thaw cycles have been shown to rapidly reduce viral infectivity in water but oscillations between different low temperatures (i.e. 10C and 17C) have no effect on AI virus stability (Lebarbenchon et al., 2011). Additionally, chlorine concentrations commonly found in drinking water have been shown to readily inactivate AI virus (Rice et al., 2007).

The role of aquatic invertebrates in the environmental maintenance and transmission of AI virus has also been evaluated but the studies have been few in number and have evaluated with a limited number of organisms. Faust et al. (2009) determined that the Asian clam (*Corbicula fluminea*), an invasive filter-feeding bivalve species, can remove and inactivate AI virus from water. Conversely, the zebra mussel (*Dreissena polymorpha*) has been shown to be able to concentrate AI virus from contaminated water and maintain infectious virus for several weeks even after being transferred to virus-free

water (Stumpf et al., 2010). Studies with water fleas (Daphnia magna) have demonstrated that these invertebrates may accumulate virus from contaminated water but the results are based on PCR and it's unknown whether these organisms would inactivate or maintain the virus (Abbas et al., 2012).

Viral persistence trials incorporating multiple components of aquatic ecosystems have demonstrated the difficulty in elucidating the confounding influence of various components on the duration of AI virus persistence. Horm et al. (2012) performed persistence trials in 20L aquariums and included intact surface water, sediment, plants, fish, bivalves and other organisms in various experimental setups. Although viral RNA was detected in water and several of the aquatic organisms, very little infectious virus was isolated during the study and the overall results of the study were inconsistent with previous viral persistence trials (Horm et al., 2012). Much of the difficulty in interpreting the results of the study by Horm et al. (2012) could be attributed to the volume and complexity of the experiment system.

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CHAPTER THREE

PERSISTENCE OF LOW PATHOGENIC AVIAN INFLUENZA VIRUSES IN FILTERED SURFACE WATER FROM WATERFOWL HABITATS IN GEORGIA,

 \mathbf{USA}^1

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ABSTRACT

The natural reservoirs for avian influenza virus (AIV) are wild bird species of the orders Anseriformes and Charadriiformes. The primary route of transmission for wild birds is through fecally contaminated surface water on shared aquatic habitats. Based on results from a distilled water model, AIV have been shown to remain infectious in water for many weeks to months with pH, salinity, and temperature affecting stability. To evaluate the effect of pH, salinity, and temperature on AIV persistence in natural surface water, the duration of infectivity was determined for two common low pathogenic AIV subtypes in 15 filtered surface water samples collected from major waterfowl habitats in Georgia, USA. Trials were performed at three incubation temperatures 10 C, 17 C, and 28 C. Consistent with previous studies, pH and temperature were found to have a significant effect on the stability of AIV in filtered surface water. Both viruses were less stable at warmer temperatures and in acidic water (pH<5). Due to the limited range of salinity of the field water samples, the role of salinity in AIV stability in surface water could not adequately be evaluated. Variations in persistence times were observed between water samples with comparable pH and salinities indicating that other factors affect AIV stability in natural surface water. These results contribute to our understanding of AIV persistence in aquatic habitats and may help in identifying areas with an increased likelihood of AIV persistence and potential transmission.

INTRODUCTION

Species of wild aquatic birds in the orders Anseriformes and Charadriiformes have been identified as the reservoirs for avian influenza virus (AIV) (Stallknecht and Shane, 1988) and transmission of AIV between wild birds occurs through the fecal-oral route involving surface water in aquatic habitats (Hinshaw et al., 1979, 1980). Replication of low pathogenic AIVs occurs primarily in the intestinal tract of infected ducks and large amounts of infectious viruses are shed in their feces (Webster et al., 1978). The duration of shedding can be as long as 28 days as reported in experimentally infected Peking ducks (Anas platyrhynchos) with large virus concentrations shed early in infection (Hinshaw et al., 1980). Experimentally infected Muscovy ducks (Cairina *moschata*) have been reported to shed up to $1 \times 10^{10} \text{ EID}_{50}$ in a 24 hr period (Hinshaw et al., 1980; Webster et al., 1978). Contaminated aquatic habitats may serve as both a transmission medium for short term transmission and potentially a reservoir for longer term transmission. Avian influenza viruses have been isolated from the surface water of waterfowl habitats when infected ducks were present (Halvorson et al., 1985; Hinshaw et al., 1980; Ito et al., 1995; Markwell and Shortridge, 1982; Sivanandan et al., 1991), and from surface water and sediment of aquatic habitats following bird migration (Ito et al., 1995; Lang et al., 2008). It has been suggested based on results from attempts to model AIV infection events in wild ducks, that indirect fecal-oral transmission via contaminated water is an important source of transmission and should be considered when attempting to understand AIV dynamics in wild birds (Breban et al., 2009; Roche et al., 2009; Rohani et al., 2009).

Most of the studies evaluating the persistence of AIV in water have utilized distilled water as a model system (Brown et al., 2009; Brown et al., 2007; Stallknecht et al., 1990a; Stallknecht et al., 1990b). The distilled water system allows for easy modification and control of pH, salinity, and temperature, and adequately simulates the water column of aquatic systems. Laboratory trials using distilled water demonstrated that the virus could remain infectious in water for weeks to months and the duration of persistence showed an inverse relationship to temperature and salinity (Stallknecht et al., 1990a; Stallknecht et al., 1990b). Filtered surface water samples from waterfowl habitats in Louisiana, USA also were tested, and the stability of AIV in these samples was consistent with the results of the distilled water trials (Stallknecht et al., 1990a). However, these trials included only four surface water samples with limited variation in pH and salinity. Additional work with surface water collected in Bulgaria supported earlier reports of the effects of pH, salinity, and temperature on AIV persistence, and determined that AIV persistence was reduced in biologically intact water compared to filtered water samples (Zarkov, 2006). Brown et al. (2009) evaluated the effect of pH, salinity, and temperature on twelve low pathogenic AIV isolates representing hemagglutinin subtypes 1-12 using the distilled water laboratory model. Viral stability was determined to be greatest at a slightly basic pH (7.4-8.2), low temperatures ($<17^{\circ}$ C), and fresh to brackish water (0-20,000 parts per million) (Brown et al., 2009). Direct comparisons of natural surface water and comparable distilled water support the inverse relationship between water temperature and AIV stability (Nazir et al., 2010).

Application of the laboratory results to natural systems is difficult due to the complexity of aquatic habitats (Stallknecht et al., 2010). To increase the understanding of

the environmental persistence of influenza viruses, it's necessary to perform laboratorybased trials utilizing surface water from waterfowl habitats but the complexity of natural water samples may hinder or distort our understanding. Multiple studies have utilized surface water for persistence trials but the number of surface water samples has been limited Furthermore, the experimental approaches have varied between studies, limiting their comparability. Nazir et al. (2010) showed that bacterial colony forming units increased significantly during the course of persistence trials with natural surface water, which could have a significant effect on viral stability and artificially alter the duration of persistence. To limit the number of confounding factors and allow comparisons between the studies that have utilized distilled water as a model system and the current study, natural surface water was filtered to remove the majority of biological material. The objectives of this study were to evaluate the effect of water temperature, pH, and salinity on the duration of low pathogenic (LP) AIV persistence in filtered surface water and to determine if these factors alone could account for the duration of AIV persistence in surface water samples in aquatic habitats that are naturally utilized by ducks.

MATERIALS AND METHODS

Water Collection

The major waterfowl habitats within Georgia, USA were identified and fifteen sites distributed across the state were selected for sampling. At each site, the body of water that was most likely to have at least a seasonal population of waterfowl was identified for sampling. In 2008, sites were sampled from March 13 to March 19. Water samples were taken within 1m of the shoreline and about 3 cm below the surface; 1 L was collected in a LDPE wide-mouth bottle (Thermo Fisher Scientific, Inc, Waltham,

Massachusetts, USA) and placed on ice for transport back to the lab. At each site, pH and specific conductance readings were taken using a YSI 556 MPS handheld instrument (YSI, Inc, Yellow Springs, Ohio, USA), which had been calibrated before each use. In the laboratory, water samples were filtered using a bottle-top vacuum filter system with a 0.22µm polyethersulfone membrane (Corning Inc, Corning, New York, USA) to remove most biological material. The pH and specific conductance was determined for the filtered samples using a VWR sympHony SB80PC bench top meter (VWR International, Radmor, Pennsylvanian, USA). Filtered water samples were stored at 4°C until experimental trials.

Pre-screening water for viruses

RNA was extracted from two 50µl aliquots of each filtered water sample using the MagMax 96 AI/ND Viral Isolation Kit (Ambion,Inc, Austin, Texas, USA) and a semiautomated nucleic acid purification system, KingFisher 24 (Thermo Scientific, Waltham, Massachusetts, USA) following previously published protocols (Das et al., 2009). Realtime reverse transcriptase PCR (rRT-PCR) was used to screen all samples for the matrix protein of influenza virus. Primers and cycling parameters were identical to those determined by Spackman et al. (Spackman et al., 2002) and were run on a SmartCycler (Cepheid, Inc, Sunnyvale, California, USA). Water samples were considered to be negative if the cycle threshold (Ct) value was 0 or greater than 40.

Two 500µl aliquots of each filtered water sample were diluted 1:1 in 2x serumfree Eagle's minimal essential medium (MEM). The diluted water samples were subjected to the same infectivity assay as the experimental water samples to screen for

environmentally deposited cytopathic agents and ensure that cytopathicity observed during the experiment was due to the AIV added during the study.

Viruses and virus titrations

Two LP AIV isolated from wild ducks were utilized in this study:

A/Mallard/MN/199036/99 (H3N2) and A/Mallard/MN/199057/99 (H4N6). The viruses were selected because these isolates were used for previous persistence trials performed in our laboratory and they represent common subtypes isolated from wild birds in North America (Brown et al., 2009; Wilcox et al., 2011). Stocks of both viruses were propagated in 9-11 day old specific pathogen free (SPF) embryonated chicken eggs with viral-infected amnioallantoic fluid (AAF) harvested at 4 days post inoculation (dpi). The median tissue culture infectious dose (TCID₅₀) for each stock was determined on Madin Darby canine kidney (MDCK) cells (described below). The titers of the H3N2 (third passage in eggs) and H4N6 (second passage in eggs) were 10^{8.3} TCID₅₀/ml and 10^{7.6} TCID₅₀/ml, respectively. Viral stocks were stored at -80°C until experimental trials. All titrations were performed using MDCK cells as described in Brown et al., 2009.

Experimental design

For both viruses, infective AAF was diluted 1:100 in each of the 15 filtered surface water samples. Inoculated water samples were placed into 5.0 ml polystyrene tubes at a volume of 4.0 ml. Each virus and water sample combination was maintained at 10 C and 17 C in low-temperature incubators and 28 C in a water bath. The three incubation temperatures were selected because they are temperatures that have been used extensively in the literature and represent water temperatures that would be encountered in aquatic environments throughout the year in Georgia (Brown et al., 2009; Brown et al.,

2007; Stallknecht et al., 1990a; Stallknecht et al., 1990b). In addition, 15mls of each water sample with no infective AAF was maintained at each temperature and the pH of the water samples was evaluated using a VWR sympHony SB80PC benchtop meter (VWR International, Radnor, Pennsylvania, USA) at all time points. Virus inoculated water was sampled at the time of inoculation (0 DPI) and at least five times thereafter. The frequency of the additional time points was determined based on estimates of the time required for a viral titer reduction of $2 \log_{10} \text{TCID}_{50}/\text{ml}$ (Brown et al., 2009; Stallknecht et al., 1990a; Stallknecht et al., 1990b). In cases where the viral titer became undetectable before five samples were collected, the trial was rerun with a shorter sampling frequency. If a $2 \log_{10} \text{TCID}_{50}/\text{ml}$ reduction of viral titer was not observed after five sampling times, additional samples were taken maintaining the same sampling frequency. The sampling frequency ranged from every two hrs for water samples with a low pH and high salinity at 28 C to every five days for water samples with a neutral pH and low salinity at 10 C. All titrations were performed in duplicate. A $TCID_{50}/ml$ was determined for each time as described (Brown et al., 2009).

Statistical analysis

The titration data for all trials were log_{10} transformed. Simple linear regression was used to calculate the virus log reduction time (Rt), which is the time in days required for a decrease of viral titer by 1 log_{10} TCID₅₀/ml (i.e. a 90% reduction in infectivity) (Minitab 15, Minitab Inc, State College, Pennsylvania, USA).

The variance of Rt values increased with the mean, so a log transformation was used for statistical analysis. Factorial repeated measures ANOVA was used to evaluate the effects of water temperature, pH category, and virus type on the log-transformed Rt values. Pairwise comparisons were performed using Tukey's procedure. A predictive model was obtained using multivariable linear regression with robust standard errors to account for repeated measurements on the same water samples. Multivariable model selection began with a maximum model that contained main effect terms for all variables, with subsequent step-wise elimination of any variables having P > 0.10. After reaching a preliminary main effects model, all possible two-way interactions were evaluated. All testing assumed a two-sided alternative hypothesis and P<0.05 was considered statistically significant. Statistical analysis was performed using commercially available software (Stata version 11.1, StataCorp LP, College Station, Texas, USA).

RESULTS

Study sites included most of the physiogeographic regions of Georgia including the ridge and valley region, piedmont region and the coastal plain (Figure 3.1). All collected water samples tested negative for AIV by rRT-PCR with all water samples having a Ct value of 0 and no cytopathic agents were detected in any of the filtered water samples. The pH and salinity of the collected water samples are summarized in Table 3.1 along with the calculated Rt values.

Water samples with a relatively low pH (4-5) had shorter reduction times than did those with a near neutral pH (7), and higher water temperatures also yielded shorter reduction times (Figure 3.2). Based on the repeated measures ANOVA of logtransformed Rt values, there was a significant effect of both pH (P<0.001) and temperature (P<0.001), but there was no difference between virus types (P=0.49). There were also no significant interactions between pH and virus (P=0.38); temperature and virus (P=0.17); temperature and pH (P=0.21); or pH, temperature, and virus (P=0.14).

The geometric mean Rt values for temperature and pH categories are summarized in Table 3.2. The mean log₁₀ Rt values for the pH 6.8-7.3 and 7.4-7.6 categories were not significantly different from each other, but both were significantly higher than for the pH 4.2-5.0 category. The mean log₁₀ Rt values differed significantly between all three temperatures (10 C, 17 C, and 28 C), with the longest reduction times at 10 C and the shortest at 28 C.

The final multivariable linear regression model is summarized in Table 3.3. Consistent with the results of the repeated measures ANOVA, only temperature and pH were identified as significant predictors (P<0.001). Compared to water samples at 10 C, the mean log₁₀ Rt value was 0.11 units lower at 17 C and 0.74 units lower at 28 C, corresponding to relative reductions in the untransformed Rt values of 22.4% and 81.7%, respectively. For every one unit increase in pH, the mean log₁₀ Rt increased by 0.39 units, corresponding to a relative increase in the untransformed Rt values of 144%. The final regression model explained 78.6% of the variability in log₁₀ Rt values (i.e., R^2 =0.786).

The majority of the water samples collected during this study had salinities <2.49 ppt making them well below the threshold for fresh water (<0.5ppt). Two water samples, Savannah NWR (Figure 3.1: Site 9) and Altamaha NWR (Figure 3.1: Site 10), could be considered brackish with salinities of 2.49 ppt and 2.43 ppt, respectively. Even these water samples are well below the salinity at which a significant reduction in the duration of viral persistence is observed in distilled water trials (Brown et al., 2009). Due to this limited range of salinity concentrations, the effect of salinity could not be adequately evaluated. Salinity was not statistically evaluated using the repeated measures ANOVA

but salinity was included in the multivariable linear regression and found to have no significant predictive value.

DISCUSSION

While it is known that AIV is stable in distilled water for months, our understanding of the factors which affect persistence of the virus in natural surface water are limited. The importance of the abiotic factors pH, salinity, and temperature have been evaluated in previous studies but these studies have mainly utilized distilled water as a laboratory model with a small number of surface water samples. Wild birds utilize a wide range of habitats and it is reasonable to presume that individual habitats will vary widely in their abiotic constituents. Evaluating the role of pH, salinity, and temperature in filtered surface water from waterfowl habitats enhances our understanding of the role of these abiotic factors in determining AIV stability and allows us to compare the laboratory determined persistence trends to natural water without the confounding effect of biological material.

The results of the trials with filtered surface water are consistent with some of the general trends of AIV persistence established by previous studies (Brown et al., 2009; Brown et al., 2007; Stallknecht et al., 1990a; Stallknecht et al., 1990b; Zarkov, 2006). The pH and temperature of surface water have a significant effect on the stability of AIV in filtered surface water. Both viruses persisted longer at the two lower temperatures (10 C and 17 C) compared to the highest temperature (28 C) evaluated in this study which is consistent with the inverse relationship between AIV persistence and temperature reported in previous studies (Brown et al., 2009; Nazir et al., 2010; Stallknecht et al., 1990a). The pH of the surface water samples used in this study ranged from 4.23 to 7.57

with most samples having a pH close to neutral (6.81-7.57). Studies using the distilled water model have established that AIV is most stable at a slightly basic pH (7.4-8.2) (Brown et al., 2009; Stallknecht et al., 1990a). Six of the water samples evaluated in this study had neutral pHs (6.8-7.3), which were slightly outside the laboratory established ideal range. There was no statistical difference between AIV stability in these six water samples compared to the five water samples with pHs in the ideal range. These results may indicate that the ideal pH range for the stability of AIV in surface water is wider (neutral to slightly basic) than previously established. Four of the field water samples used in this study had acidic pHs (4.23-4.83) and AIV persistence was significantly reduced in these samples The pHs observed in these samples were much lower than those used in previous experimental studies (Brown et al., 2009; Stallknecht et al., 1990a).

Overall, a multivariable linear regression model including only temperature and pH explained 78.6% of the variability in log₁₀ Rt values. Despite the satisfactory predictive ability of the model, other factors may also be important for determining the duration of AIV persistence. Under experimental conditions, salinity could not be evaluated in the present study, but it also can affect AIV stability (Brown et al., 2009; Brown et al., 2007; Stallknecht et al., 1990a). In Georgia, saline habitats are not available to dabbling ducks outside of coastal regions and for this reason most of the water samples were from fresh water with salinities less than 0.5ppt. Such habitats may be important in other areas of North America. A large range of variability of persistence was observed between the field water samples, particularly between water samples with neutral pHs and at lower temperatures, which are the more favorable conditions for AIV persistence.

While other studies have reported variability of persistence between viral subtypes and isolates (Brown et al., 2009; Brown et al., 2007), virus subtype was not found to be a significant determinant of AIV stability in this study. The surface water samples used in this study were filtered with a 0.2µm filter to remove the majority of biological material but filtering does not entirely eliminate the influence of biological material. The filter would not have removed small molecules secreted or released by biological organisms and the filtering process itself may have lysed organisms releasing cellular contents into the water. Previous studies have shown that biological constituents of surface water reduce AIV persistence but it is unclear whether microorganisms or their byproducts cause the reduction in infectivity (Nazir et al., 2010; Zarkov, 2006). While the observed variability could be related to these or other biological factors, these results may also indicate that other abiotic factors beyond pH, salinity, and temperature affect AIV persistence in surface water. Additionally, in natural aquatic systems, other factors not represented in the laboratory trials such as UV light and water current could reduce the duration of AIV infectivity by either inactivating the virus or dispersing the virus and reducing the localized viral titer.

As we gain a better understanding of AIV stability in surface water, we may be better able to predict how long AIV will persist in a particular aquatic habitat. With this type of information we could target the areas with the greatest capacity for AIV persistence for environmental sampling, which could be used to enhance current surveillance programs or for scientific studies focused on AIV ecology and transmission. Based on the laboratory persistence trials, several of the sites sampled in this study may be habitats where AIV could persist longer term (i.e. weeks to months) and be sites with

an increased likelihood of environmental transmission risk between migratory waterfowl populations but further trials and environmental sampling would be required before particular sites could be identified as habitats that facilitate long term persistence. In addition to identifying areas of high environmental persistence, we could also identify areas with limited capacity for AIV persistence. In this study, the four sample sites with the lowest pHs were found to have a much lower capacity for surface water persistence and could be considered of low concern for long term stability and transmission of AIV. Waterfowl habitats are made up of many different types of aquatic systems, each with their own abiotic and biotic constituents, and all undergo changes over time. Unfortunately, our current understanding of the factors affecting AIV persistence is limited and any conclusions about the persistence capacity of an area based on laboratory persistence trials are limited to the water sample collected for the study. For example, the water sample from Altamaha WMA evaluated in this study indicated that this area has a lower capacity for surface water persistence of AIV. Altamaha WMA is considered to be one of the premier waterfowl migrating and wintering areas in Georgia, and encompasses an area of about 109km² with about 20 impoundments ranging from tidal freshwater to brackish water. The surface water persistence capacity of Altamaha WMA cannot be judged on a single water sample taken from one impoundment but the persistence trials do give an indication of suitability of the immediate area surrounding the sampling site. In time, it may be possible to estimate the persistence capacity of an aquatic system without performing laboratory persistence trials

This study evaluated the role of pH and temperature in determining AIV stability in natural surface water by limiting the influence of biotic components through filtration.

The study was designed to be complementary to previous studies that had utilized sterile distilled water as a model system. The results indicated that many of the previously established persistence trends are applicable to natural surface water; in particular the duration of viral persistence increases as water temperature decreases and as the pH approaches neutral to slightly basic. We also stressed the necessity to develop research on brackish to salt water to evaluate precisely the effects of salinity in AIV persistence in such habitats. Further studies utilizing surface water are necessary to differentiate the effect of biotic and abiotic constituents, and to identify the other factors affecting AIV persistence in water, and predict sites with high potential for virus transmission in wild waterfowl populations

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			A/Mallard/MN/199036/99 (H3N2) Rt Values		A/Mallard/MN/199057/99 (H4N6) Rt Values			
Site (Reference #)	рН	Salinity (ppt)	10 C	17 C	28 C	10 C	17 C	28 C
Carter's Lake in Coosawatte WMA (1) Morris Creek in	7.2	0.04	21.2	22.8	2.1	16.8	16.5	1.6
Fishing Creek WMA (2)	7.3	0.04	24.9	20.6	6.7	32.4	25.7	6.9
B.F. Grant WMA (3)	7.4	0.09	15.8	11.9	7.9	23.2	9.6	8.1
Joe Kurz WMA (4)	6.8	0.05	58.1	44.8	6.5	61.4	37.2	9.7
Riverband WMA (5)	7.3	0.06	57.5	43.9	7.8	61.7	44.8	10.0
Eufala NWR (6)	7.4	0.06	88.5	47.4	9.5	68.0	73.0	12.7
Mayhaw WMA (7)	4.7	0.03	5.7	3.8	1.9	2.6	4.1	1.2
Evans County PFA (8)	6.9	0.05	51.0	49.8	7.0	79.4	66.7	11.6
Savannah NWR (9)	7.6	2.49	30.6	18.3	5.1	24.7	14.7	6.9
Altamaha WMA (10)	4.8	2.43	12.9	18.0	2.1	11.8	10.6	2.2
Laura Walker SP – Dixon Memorial (11)	4.3	0.04	0.7	0.8	0.3	3.8	0.7	0.2
Grand Bay WMA (12)	4.2	0.05	0.9	1.0	0.7	3.1	1.8	0.2
Phinizy Swamp WMA (13)	7.2	0.10	73.0	41.3	9.2	62.1	48.8	10.0
Lake Seminole	7.4	0.06	70.9	51.3	18.3	63.3	48.1	11.1
Thomas Co. (14) Plantation Ponds in Rivercreek WMA (15)	7.6	0.10	26.0	28.1	7.0	50.3	52.9	8.2

Table 3.1. Summary of water sample characteristics and virus log reduction times (Rt) by virus type and temperature. The Rt values are the time (days) required for a decrease of viral titer by $1 \log_{10} \text{TCID}_{50}/\text{ml}$.

WMA: Wildlife Management Area; NWR: National Wildlife Refuge; PFA: public fishing area; SP: state park

Table 3.2. Geometric mean (min-max) virus log reduction times (days) in filtered water samples by temperature and pH category for both virus types (H3N2 and H4N6) combined.

17 C	28 C	[†] Total
1 / C		
	20 C	
2.8 (0.8, 18)	0.7 (0.3, 2.1)	$1.9^{a}(0.3, 18)$
36 (21, 50)	6.6 (2.1, 9.2)	$22^{b}(2.1,73)$
• •••••••••••••••••••••••••••••••••••		ach (r . 1, ac)
29 (12, 51)	8.9 (5.1, 18)	22° (5.1, 89)
17b (0.0 51)	4.05 (0.2, 10)	11 (0 2 00)
17 (0.8, 51)	4.0 (0.3, 18)	11 (0.3, 89)
	2.8 (0.8, 18) 36 (21, 50) 29 (12, 51) 17 ^b (0.8, 51)	17.0° 20.0° $2.8 (0.8, 18)$ $0.7 (0.3, 2.1)$ $36 (21, 50)$ $6.6 (2.1, 9.2)$ $29 (12, 51)$ $8.9 (5.1, 18)$ $17^{\circ} (0.8, 51)$ $4.0^{\circ} (0.3, 18)$

^{*}Number of water samples in each pH category

[†]Within rows and columns, marginal means with a superscript in common do not differ with a level of significance of 5% over all comparisons

Variable	Coefficient (Robust Standard Error)	95% Confidence Interval	[†] P value
Temperature			
10 C	0.74 (0.05)	0.64, 0.83	< 0.001
17 C	0.63 (0.06)	0.51, 0.75	
28 C	Referent	Referent	
рН	0.39 (0.07)	0.24, 0.53	< 0.001
Constant	-1.93 (0.46)	-2.92, -0.94	0.001

Table 3.3. Multivariable linear regression model for the prediction of log_{10} virus reduction time (Rt) in water samples collected from 15 locations (R²=78.6%).

[†]Based on Wald statistics



Figure 3.1. A map of Georgia, USA with flags marking the location of the fifteen sampling sites. The collection sites were: 1. Carter's Lake in Coosawatte Wildlife Management Area (WMA) (34°35'51.11"N, 84°41'40.67"W), 2. Morris Creek in Fishing Creek WMA (33°52'23.66"N, 82°34'54.80"W), 3. B.F. Grant WMA (33°25'30.54"N,

83°29'06.61"W), 4. Joe Kurz WMA (33°07'31.62"N, 84°31'31.22"W), 5. Riverband WMA (32°27'33.12"N, 82°50'15.40"W), 6. Eufala National Wildlife Refuge (NWR) (31°59'31.74"N, 85°03'10.80"W), 7. Mayhaw WMA (31°12'13.07"N, 84°47'34.76"W), 8. Evans County public fishing area (32°07'33.49"N, 81°48'08.06"W), 9. Savannah NWR (32°09'48.47"N, 81°08'10.38"W), 10. Altamaha WMA (31°21'17.78"N, 81°27'06.44"W), 11. Laura Walker State Park – Dixon Memorial (31°08'26.45"N, 82°12'52.16"W), 12. Grand Bay WMA (30°56'03.59"N, 82°12'52.16"W), 13. Phinizy Swamp WMA (33°25'01.88"N, 81°59'05.68"W), 14. Lake Seminole (30°47'07.51"N, 84°44'35.66"W), and 15. Thomas Co. Plantation Ponds in Rivercreek WMA (30°54'39.42"N, 84°00'27.79"W). The charts next to each sampling site summarize the experimentally determined average viral log reduction time for that water sample at all three incubation temperatures.



Figure 3.2. The virus log reduction times (Rt) for two influenza virus isolates in fifteen filtered surface water samples with varying pH from 4.23 to 7.57 at three temperatures. (A) The Rt values for A/Mall/MN/199036/99 (H3N2) with general trend line for all Rt values at each temperature; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C. (B) The Rt values for A/Mall/MN/199057/99 (H4N6) with general trend line for all Rt values at each temperature; black diamonds and solid line: 10 Rt values at each temperature; black diamonds and solid line for all Rt values for A/Mall/MN/199057/99 (H4N6) with general trend line for all Rt values at each temperature; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C. (B) The Rt values at each temperature; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C.

CHAPTER FOUR

ABIOTIC FACTORS AFFECTING PERSISTENCE OF AVIAN INFLUENZA VIRUS IN FILTERED SURFACE WATER FROM WATERFOWL HABITATS¹

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Abstract

Avian influenza (AI) virus can remain infectious in water for months and virus contaminated surface water is considered to be a source of infection within wild waterfowl populations. Previous work has characterized the effect of pH, salinity, and temperature on viral persistence in water but a majority of that work has been done in modified distilled water. The objective of this study was to identify the abiotic factors that influence the duration of AI virus persistence in natural surface water. Water samples were collected from 38 waterfowl habitats distributed across the United Sates. Samples were submitted to the USGS Georgia Water Science Center for chemical analysis and the University of Georgia (UGA) for viral persistence trials. Samples were filtered using $0.22\mu m$ filters and the duration of persistence within each water sample was determined for three wild-bird origin influenza A viruses at 10C, 17C, and 28C. The effects of the physicochemical factors on the duration of viral persistence were evaluated using multivariable linear regression with robust standard errors. The duration of AI virus persistence in filtered water was determined to be longest in surface water with low temperature (<17 C), neutral-basic pH (7.0-8.5), low salinity (<0.5ppt), and low ammonia concentrations (<0.5mg/L). Our results also highlighted potential strain-related variation in the stability of AI virus in surface water. These results bring us closer to being able to predict the duration of AI virus persistence in surface water of waterfowl habitats.

Introduction

Wild birds are considered to be the primordial reservoir for influenza A virus with species within the orders Anseriformes and Charadriiformes having the largest and most diverse genetic pool of viruses (Stallknecht and Brown, 2008; Stallknecht and Shane, 1988). Within these wild bird hosts, replication of avian influenza (AI) virus occurs primarily in the epithelial cells of the intestinal tract and large amounts of virus are shed in feces (Hinshaw et al., 1980; Webster et al., 1978). The virus contaminates the surrounding aquatic environment, where it remains infectious, facilitating indirect transmission between birds (Hinshaw et al., 1979; Nazir et al., 2010; Nazir et al., 2011; Stallknecht et al., 1990b). Environmental persistence of AI virus has been determined to be important for the epidemiology of the virus within wild bird populations and within aquatic habitats, surface water is considered to be the major site of environmental contamination (Breban et al., 2009; Franklin et al., 2011; Roche et al., 2009; Rohani et al., 2009).

The persistence of AI virus in water has been confirmed through environmental surveillance and laboratory-based investigations (Brown et al., 2009; Lang et al., 2008; Stallknecht et al., 2010; Stallknecht et al., 1990a; Stallknecht et al., 1990b). The temperature, pH, and salinity of the water have been identified as important determinants of the duration of persistence (Brown et al., 2009; Brown et al., 2007; Lebarbenchon et al., 2011; Nazir et al., 2010; Stallknecht et al., 1990a; Zarkov, 2006). Using modified distilled water as a laboratory model, Brown et al. (2009) determined that AI viruses are most stable in water with neutral-basic pH (7.4-8.2), low salinity (<20 ppt), and at low temperatures (<17 C). These general trends are supported by further laboratory

investigations utilizing natural surface water samples (Keeler et al., In Press; Nazir et al., 2010; Stallknecht et al., 1990a; Zarkov, 2006). In a recent publication, Keeler et al. (Keeler et al., In Press) demonstrated that variability in AI virus stability in filtered surface water from waterfowl habitats could not be entirely accounted for by only considering pH, salinity and temperature, particularly under conditions historically considered to be ideal for persistence.

Wild waterfowl utilize a wide range of aquatic habitats and these habitats have equally diverse surface water with highly variable physiochemical characteristics. While pH, salinity and temperature have been shown to be significant predictors of AI virus stability, the influence of other physicochemical characteristics has not been fully evaluated and other factors may be affecting the stability of the virus. Characterizing the relationship between these factors and viral stability would provide further insight into the role of surface water persistence in the epidemiology of AI virus and would bring us closer to being able to predict the duration of viral persistence within various aquatic habitats. The objective of this study was to determine the duration of persistence of several AI viruses in filtered surface water from geographically discrete waterfowl habitats and identify the abiotic factors that are significant predictors of the duration of viral stability.

Materials and Methods

Water collection

From June 2008 to April 2009, surface water samples were collected from 38 waterfowl habitats across the continental United States. Within each habitat, the body of water that was most likely to have at least a seasonal population of waterfowl was

identified for sampling. Water samples were collected as multi-vertical grabs (Survey, 2012). Field properties were measured at each sampling event and include water temperature, dissolved oxygen, specific conductance, and pH using a multi-parameter sonde, which was calibrated daily prior to use. Turbidity was analyzed using portable turbidity meters. Duplicate water samples were collected within 1m of the shoreline and about 3 cm below the surface in 1L LDPE wide-mouth bottles (Thermo Fisher Scientific, Inc, Waltham, Massachusetts, USA) and placed on ice for transport back to the lab. Half of the water sample was sent to the U.S. Geological Survey National Water Quality Lab in Denver, Colorado where it was analyzed for major ions and nutrients using methods described in Fishman and Friedman (Fishman and Friedman, 1989), Patton and Kryskalla (Patton and Kryskalla, 2011), Patton and Truitt (Patton and Truitt, 2000), Brenton and Arnett (Brenton and Arnett, 1993), and Fishman (Fishman, 1993).

The remaining sample water was sent to the Southeastern Cooperative Wildlife Disease Study (SCWDS) at the University of Georgia. Upon arrival at SCWDS, the water samples were filtered using a bottle-top vacuum filter system with a 0.22µm polyethersulfone membrane (Corning Inc, Corning, New York, USA) to remove the majority of biological material. The pH and specific conductance was determined for the filtered samples using a VWR sympHony SB80PC bench top meter (VWR International, Radmor, Pennsylvania, USA) and the samples were stored at 4°C until water persistence trials were performed.

Viruses

Three low pathogenic AI viruses isolated from wild ducks were utilized in this study: A/Mallard/MN/199036/99 (H3N2), A/Mallard/MN/199057/99 (H4N6), and

A/Northern Pintail/TX/421716/01 (H8N4). The viruses were selected because they were used for previous persistence trials performed in our laboratory and they are subtypes isolated from wild birds in North America (Brown et al., 2009; Wilcox et al., 2011). Stocks of the viruses were propagated in 9-11 day old specific pathogen free (SPF) embryonated chicken eggs with viral-infected amnioallantoic fluid (AAF) harvested at 4 days post inoculation (dpi). The median tissue culture infectious dose (TCID₅₀) for each stock was determined on Madin Darby canine kidney (MDCK) cells (Brown et al., 2009). Viral stocks were stored at -80°C until experimental trials.

Water persistence trials

Before water persistence trials were performed, all filtered water samples were screened for AI virus RNA and environmentally deposited cytopathic agents. RNA was extracted from two 50µl aliquots of each filtered water sample using the MagMax 96 AI/ND Viral Isolation Kit (Ambion,Inc, Austin, Texas, USA) and a semi-automated nucleic acid purification system, KingFisher 24 (Thermo Scientific, Waltham, Massachusetts, USA) following previously published protocols (Das et al., 2009). Realtime reverse transcriptase PCR (rRT-PCR) was used to screen all samples for the matrix protein of influenza A virus. Primers and cycling parameters were identical to those determined by Spackman et al. (Spackman et al., 2002) and were run on a SmartCycler (Cepheid, Inc, Sunnyvale, California, USA). Water samples were considered to be negative if the cycle threshold (Ct) value was greater than 40. Two 500µl aliquots of each filtered water sample were diluted 1:1 in 2x serum-free Eagle's minimal essential medium (MEM). The diluted water samples were subjected to the same infectivity assay

as the experimental water to ensure that the cytopathicity observed during the experiment was not due to environmental contamination.

For all viruses, infective AAF was diluted 1:100 in each of the 38 filtered surface water samples. Inoculated water samples were divided into 4 mL aliquots in 5 mL polystyrene round-bottom tubes. Each virus and water sample combination was maintained at 10 C and 17 C in low-temperature incubators and 28 C in a water bath. The three incubation temperatures were selected because they are temperatures that have been used extensively in the literature and they represent water temperatures that would be encountered in aquatic habitats of waterfowl (Brown et al., 2009; Brown et al., 2007; Stallknecht et al., 1990a; Stallknecht et al., 1990b). In addition, 15mls of each water sample with no infective AAF was maintained at each temperature and the pH of the water samples was evaluated using a VWR sympHony SB80PC benchtop meter (VWR International, Radnor, Pennsylvania, USA) at the start and completion of each trial. Virus inoculated water was sampled at the time of inoculation (0 DPI) and at least six times thereafter. The frequency of the additional time points was determined based on estimates of the time required for a viral titer reduction of $2 \log_{10} \text{TCID}_{50}/\text{ml}$ (Brown et al., 2009; Stallknecht et al., 1990a; Stallknecht et al., 1990b). In cases where the viral titer became undetectable before five samples were collected, the trial was rerun with a shorter sampling frequency. If a $2 \log_{10} \text{TCID}_{50}/\text{ml}$ reduction of viral titer was not observed after five sampling times, additional samples were taken maintaining the same sampling frequency. The sampling frequency ranged from every two hrs for water samples with a low pH and high salinity at 28 C to every five days for water samples with a neutral pH

and low salinity at 10 C. All titrations were performed in duplicate. A $TCID_{50}$ /ml was determined for each time point as previously described (Brown et al., 2009).

Statistical analysis

The titration data for all trials were log_{10} transformed. Simple linear regression was used to calculate the virus log reduction time (Rt), which is the time in days required for a decrease of viral titer by 1 log_{10} TCID₅₀/ml (i.e. a 90% reduction in infectivity) (Minitab 15, Minitab Inc, State College, Pennsylvania, USA).

The variance of Rt values increased with the mean, so a log transformation was used for statistical analysis. A predictive model was obtained using multivariable linear regression with robust standard errors to account for repeated measurements on the same water samples. Multivariable model selection began with a maximum model that contained main effect terms for all variables, with subsequent step-wise elimination of any variables having P > 0.10. After reaching a preliminary main effects model, all possible two-way interactions were evaluated. Residuals were plotted against the fitted values to screen for outliers and graphically evaluate the distributional assumptions. All testing assumed a two-sided alternative hypothesis and P<0.05 was considered statistically significant. Statistical analysis was performed using commercially available software (Stata version 11.1, StataCorp LP, College Station, Texas, USA).

Results

The water samples collected for the study came from a wide range of waterfowl habitats distributed across the continental United States (Table 4.1) and the samples were determined to have a wide range of physicochemical characteristics (Table 4.2). All water samples collected for the study were determined to be AI virus negative and no

environmentally deposited cytopathic agents were detected. Viral persistence trials were performed in all thirty-eight surface water samples and Rt values were calculated for each water/virus/temperature combination (Table 4.3) but due to incomplete physicochemical data for six sites only the data from 32 samples were used for the statistical analysis (Tables 4.1 and 4.2).

Previous studies have determined that the ideal pH for AI virus persistence in water is at or near neutral pH with reduced persistence observed in both acidic and basic water (Brown et al., 2009; Stallknecht et al., 1990a). Consequently, in the regression analysis pH was categorized into ideal (pH 7.0-8.5) and non-ideal (pH < 7.0 and > 8.5) ranges. The final multivariable linear regression model is summarized in Table 4.4. Virus, temperature, pH, salinity, ammonia concentrations, and sulfate concentrations, were determined to be significant predictors of the \log_{10} Rt values and a significant interaction was observed between virus and salinity. The magnesium, sodium, potassium, and chloride concentrations were highly correlated with salinity (r > 0.9), and were excluded from the model selection process. All other physicochemical factors evaluated in this study were determined to be non-significant predictors. The final regression model explained 64.0% of the variability in \log_{10} Rt values (i.e., R²=0.640). There was a significant interaction between virus and salinity, for every one unit increase in salinity (PPT), the mean log10 Rt decreased by 0.014 units for the H8N4 virus (95% CI: -0.027, -0.001), 0.016 units for the H3N2 virus (95% CI: -0.028, -0.004), and 0.019 units for the H4N6 virus (95% CI: -0.031, -0.006), which corresponded to relative decreases in the untransformed Rt values of 3.1%, 3.7%, and 4.2%, respectively. The duration of persistence for the H8N4 virus was significantly shorter than both the H3N2 and H4N6

viruses in fresh water (P < 0.001), but the differences between viruses were reduced in brackish and salt water (Table 4.5). Consistent with previous studies of AI virus persistence in water (Brown et al., 2009; Stallknecht et al., 1990a), viruses persisted longer at lower temperatures (geometric mean, Table 4.3), near neutral pH (Figure 4.1A), and low salinity (Figure 4.1B). Compared to water samples at 10 C, the mean $\log_{10} Rt$ value was 0.226 units lower at 17 C and 0.935 units lower at 28 C, corresponding to relative reductions in the untransformed Rt values of 40.6% and 88.4%, respectively. Compared to water samples with a pH less than 7.0 or greater than 8.5, the mean log10 Rt value was 0.40 units higher in water samples with a neutral to basic pH (7.0-8.5), which corresponds to a relative increase in the untransformed Rt values of 151%. An inverse relationship was observed between the Rt values and the ammonia concentrations (Figure 4.1C). For every one unit increase in ammonia (mg/L), the mean log10 Rt decreased by 0.796 units, corresponding to a relative decrease in the untransformed Rt values of 84.0%. Although there was an inverse univariate relationship between the Rt values and the sulfate concentration (Figure 4.1D), the relationship was positive after adjusting for the other variables in the multivariable analysis. For every one unit increase in sulfate (g/L), the mean log10 Rt increased by 0.082 units, corresponding to a relative increase in the untransformed Rt values of 20.7% with all other variables held constant. All water samples with relatively high (>1000mg/L) sulfate concentrations had pH ranges between 7.95 and 8.40 (Figure 4.2).

Discussion

The stability of AI virus in water has long been considered to be an important characteristic for the maintenance of the virus within wild bird populations (Hinshaw et

al., 1979; Webster et al., 1978). The results of this study provide supporting evidence for the role of surface water as a source of AI virus infection for wild waterfowl species and increases our understanding of the factors that influence the duration of persistence. All three AI viruses remained infectious in the filtered surface water samples for appreciable lengths of time with the duration of persistence being influenced by measurable abiotic factors including temperature, pH, salinity, ammonia concentrations, and sulfate concentrations. A significant difference in the duration of persistence was observed between the H8N4 virus and the other two subtypes indicating potential strain-related differences in aquatic stability similar to those reported in previous studies (Brown et al., 2009; Brown et al., 2007). Strain-related differences in stability could be an indication of the local environment putting selective pressure on the virus, resulting in more environmentally stable AI viruses. More environmentally stable viruses would be better suited for transmission within low density waterfowl populations and between different migratory populations or seasons. Further studies should be performed using natural surface water and a larger number of AI virus strains to determine whether some viruses are more environmentally stable than others.

Consistent with previous studies, temperature and pH were determined to be significant predictors of AI virus stability (Brown et al., 2009; Brown et al., 2007; Keeler et al., In Press; Nazir et al., 2010; Stallknecht et al., 1990a; Zarkov, 2006). The duration of viral persistence was highest at lower incubation temperatures and in water samples with neutral-basic pH. Greater variability in the duration of viral infectivity was observed between water samples with ideal pH conditions incubated at lower temperatures consistent with previous observations (Keeler et al., In Press). These results, coupled with
the results of previous studies, clearly highlight the importance of temperature and pH in determining the duration of persistence of AI virus in water. These factors provide useful criteria for ruling out particular aquatic habitats as longer term sources of virus. Any site with very acidic (pH<6) or basic (pH>8.5) water and/or high (\geq 28C) surface water temperatures would not be suitable for long-term persistence of AI virus.

The influence of salinity on AI virus persistence has been described previously (Brown et al., 2009; Stallknecht et al., 1990a). In general terms, AI viruses persist longer in freshwater (<0.5ppt) but some viruses have been shown to persist equally as well in fresh and brackish (0.5-30ppt) water (Brown et al., 2009; Brown et al., 2007). The H8N4 virus used in this study was also used by Brown et al (Brown et al., 2009) and in both studies the virus persisted equally as well in fresh and brackish water but showed a reduction in persistence in salt water. The H8N4 virus was isolated from a northern pintail (Anus acuta) along the coast of Texas. The area where the virus was isolated has a mix of fresh and brackish surface water and the higher salinity tolerance of the virus could be an indication of adaptation to the local aquatic environment but further studies are necessary to evaluate this hypothesis. Common ions contributing to the salinity of water are sodium, calcium, magnesium, and potassium. While these values were not included in the final model, the relative abundance of these ions determines the salinity of the water and so cumulatively they are determinative of the duration of persistence of the virus. Although other ions were excluded from the model, sulfate was determined to be predictive of AI virus persistence. Sulfate is one of the most abundant ions present in all natural water, with the concentrations varying considerably based on the source of the water (i.e. inland vs. coastal) and the mineral content of the area (Stumm and Morgan,

1996). In our multivariable model, sulfate concentrations of surface water enhance the duration of viral persistence but sulfate concentration is most likely a confounding variable. All the water samples with high sulfate concentrations (mg/L) had slightly basic pHs. As demonstrated in this study and others, the virus persists longest in water samples with a neutral to basic pH.

An additional ion that can contribute to salinity but was determined to be a nonsignificant predictor of AI virus persistence was fluoride. Fluoride is most widely known from the water fluoridation programs instituted in the USA and elsewhere as a way to reduce tooth decay (Jeffreys and Harmeson, 1951; Organization, 1994; Yeung, 2008). The recommended concentrations of fluoride in drinking water are 0.5-1.0 mg/L and most surface water has significantly lower concentrations but there are areas that have high fluoride concentrations (>1.0mg/L) including portions of the southwestern USA (Yeung, 2008). Although fluoride was determined to be statistically nonsignificant as a predictor, an inverse univariate relationship between fluoride concentrations and viral persistence was observed. Water with fluoride concentrations >0.5mg/L showed reduced durations of persistence. In this study, only two surface water samples were found to have high fluoride concentrations (>1.0 mg/L) and both came from the southwestern USA: New Camp Beach, Salton Sea State Recreational Area (Site 15, fluoride concentrations: 3.19mg/L) and Impound B, Bitter Lake NWR (Site 37, fluoride concentrations: 2.93mg/L). The Salton Sea State Recreational Area water sample had other abiotic conditions that would be unfavorable for AI virus persistence including the highest salinity (42.48 ppt) and ammonia concentration (1.04mg/L) observed in this study but the water sample from Bitter Lake NWR had more ideal conditions for longer term

persistence (pH=8.03, Salinity=7.58ppt, Ammonia=0.038mg/L). The lower persistence of all three viruses in the Bitter Lake NWR water sample may indicate that high fluoride concentrations can reduce the duration of viral persistence but further studies are required to confirm these observations. If fluoride was determined to have a detrimental effect on AI virus, many aquatic habitats in the southwestern USA would not be suitable for longer term persistence of AI virus.

The inverse relationship between ammonia concentrations and AI virus persistence was not surprising as ammonia has long been known to be toxic to viruses (Emmoth et al., 2011; Ward and Ashley, 1977; Warren, 1962). Single stranded RNA viruses have been shown to be particularly susceptible to ammonia as the mechanism of inactivation involves the cleavage of viral RNA in intact particles and single stranded genomes are more susceptible to this cleavage (Burge et al., 1983; Ward, 1978). The unionized form of ammonia is the most virucidal and the state of ammonia is affected by pH with more un-ionized ammonia present at higher pHs but no interaction was observed between ammonia and pH in this study (Ward, 1978; Warren, 1962). Emmoth et al. (Emmoth et al., 2011) recently demonstrated that ammonia can be used to inactivate AI virus in chicken hatchery waste supporting the results of this study. Ammonia is a natural waste product of aquatic organisms but is introduced into surface water in large quantities via agricultural and sewage runoff (Cheeke and Dierenfeld, 2010; Smith et al., 1999). The amount of ammonia present within an aquatic system is associated with the level of anthropogenic disturbance with highest ammonia concentrations usually found downstream of urban areas (Mueller and Helsel, 1995). Most pristine aquatic habitats would have lower ammonia concentrations and based on the other abiotic conditions of

the area, these habitats could be sources for longer term persistence of AI virus. Ammonia is not very stable in water and is readily oxidized by microorganisms into nitrite and nitrate but ammonia concentrations can remain high in some systems that experience continuous environmental deposition (Mueller and Helsel, 1995). High concentrations of ammonia can cause eutrophication of aquatic systems, where algae bloom in large numbers crowding out other organisms and depleting oxygen concentrations and other resources within the system (Smith et al., 1999). Recent studies have demonstrated that the biological components of surface water can reduce the duration of viral persistence but it's unknown what portion of the microbial community of water is affecting the virus (Nazir et al., 2010; Zarkov, 2006). The algae blooms caused by high ammonia concentrations may cause a reduction in viral persistence in addition to the reduction caused by the direct inactivation of virus. High phosphorous and nitrate concentrations can also cause eutrophication of aquatic habitats (Smith et al., 1999). While these two physicochemical factors were not determined to be predictive of AI virus stability in this study, under natural conditions these factors could also influence the microbial community, altering viral persistence. This potential link between eutrophication and AI virus persistence warrants further investigation.

Based on our results, the duration of AI virus persistence will be highest in surface water with low temperature (<17 C), neutral-basic pH (7.0-8.5), low salinity (<0.5ppt), and low ammonia concentrations (<0.5mg/L). Our results also highlighted potential strain-related variation in the stability of AI virus in surface water. These results bring us closer to being able to predict the duration of AI virus persistence in surface water of waterfowl habitats. This type of information could be used to develop

environmental surveillance programs or to identify areas that should be targeted by traditional surveillance. Coupling the multivariable linear regression model developed in this study with data on migratory and land use patterns of waterfowl could allow for the ranking of waterfowl sites based on the likelihood of surface water facilitating persistence and transmission. Although we are confident that the general trends described in this study are representative of natural phenomenon, any field application of our model should be done cautiously as our current understanding of the environmental persistence of AI virus remains incomplete. We focused on persistence in water and on identifying the influential abiotic constituents but the biological components of water have been shown to influence viral stability and other parts of the aquatic habitat, such as soil, have been proposed as alternative sources of virus (Franklin et al., 2011; Nazir et al., 2010; Nazir et al., 2011; Zarkov, 2006). Future studies should focus on identifying and characterizing the important biological components of water and on determining whether other potential environmental reservoirs exist within aquatic habitats.

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Table 4.1. The location of the surface water collection sites used in this study. Thirtyeight sites were sampled and used for avian influenza virus persistence trials but due to incomplete physicochemical information for six sites only the data from 32 samples were used for the statistical analysis.

Site	Site Name	Location	GPS Coordinates
ID		(City, State)	51.5 00014114445
1*	Flint Creek, Wheeler NWR	Priceville, AL	34°32'42.30"N
	,	,	86°55'53.52"W
2	Wildlife Sanctuary, Bald Knob	Bald Knob, AR	35°16'27.66''N
	NWR		91° 33'32.16"W
3	Aguirre Lake, Buenos Aires NWR	Sasabe, AZ	31°35'08.4"N
	-		111°30'21.4"W
4	New Camp Beach, Salton Sea	North Shore, CA	33°30'00.7"N
	State Recreational Area		115°54`50.2''W
5*	Windmill Pond, Sutter NWR	Yuba City, CA	39°03'38.1"N
			121°44'04.6"W
6	Big Pine Marsh, National Key	Big Pine, FL	24°43'44.9"N
	Deer NWR		81°23'30.9"W
7	C-1 Pond, Loxahatchee NWR	Delray Gardens,	26°29'50.4''N
		FL	80°13'03.7"W
8	C-9 Ditch, Loxahatchee NWR	Delray Gardens,	26°29'15.4''N
		FL	80°12'46.8"W
9	Oilpad Marsh, Ten Thousand	Goodland, FL	25°58'01.2"N
	Island NWR		81°33'36.3"W
10	Hardworking Bayou, Ding Darling	Sanibel, FL	26°27'46.3"N
	NWR		82°07'59.0"W
11	Lighthouse Pond, St Marks NWR	St Marks, FL	30°04'35.4"N
			84°10'48.1"W
12	Mounds Pool 1, St Marks NWR	St Marks, FL	32°06'57.2"N
		~	84°08'54.4"W
13	Otter Lake, St Mark NWR	St Marks, FL	30°01'17.1''N
		~	84°25'13.6"W
14	Tower Pond, St Mark NWR	St Marks, FL	30°05'16.3"N
		a 11 1 FF	84°09'32.3"W
15	Lucky Lake, Florida Panther NWR	Sunniland, FL	26°10′04.6″N
1.6			81°29′32.1″W
16	DT-2, Savannah NWR	Port Wentworth,	32°11′28.9″N
17		GA	81°04′4/.5″W
17	Impound 14, Savannah NWR	Port Wentworth,	32°09′01.30″N
10		GA Deut West 1	81°06'31.00" W
18	WD-1, Savannah NWR	Port Wentworth,	52°09′17.5″N
		GA	81°06′28.8″W

19	Patoka Oxbow, Patoka NWR	Glezen, IN	38°22'53.0"N
	·	,	87°22'18.0"W
20	Mudd Lake, LA	Holly Beach, LA	29°48'2.75''N
		5	93°28'26.60"W
21	Salt Pool, Rachel Carson NWR	Wells, ME	43°19'14.9''N
		,	70°34'27.4"W
22	Bullhead Creek, Shiawassee NWR	Saginaw, MI	43°23'21.7"N
		C ,	84°00'31.8"W
23*	Agassiz Pool, Agassiz NWR	Middle River, MN	48°19'41.39''N
		,	95°57'40.51''W
24	Pelican Pool, Squaw Creek NWR	Napier, MO	40°03'10.79"N
	· •	-	95°14'59.23"W
25	Main Pool, Benton Lake NWR	Swall School, MT	47°41'36.66''N
			111°20'47.52"W
26	Lake 1, Chase Lake NWR	Sykeston, ND	47°20'29.10"N
		-	99°21'39.90"W
27*	Pond 1, Ballards Marsh WMA	Simeon, NE	42°36'01.62"N
			100°33'21.54"W
28	Pond 1, Corson Wildlife	Corson, NJ	39°12'14.72''N
	Management Area		74°57'2.56"W
29	Impound B, Bitter Lake NWR	Roswell, NM	33°27'09.9"N
			104°23'57.5"W
30	Seneca Pond, Montezomma NWR	Seneca Falls, NY	42°58'22.5"N
			76°44'17.5"W
31	Bobcat Lake, Tishomingo NWR	Tishomingo, OK	34°10'27.5"N
			96°40'46.2"W
32*	McKay Creek Reservoir, McKay	Pendleton, OR	45°34'38.9"N
	Creek NWR		118°46'28.7"W
33	Pool J, Carolina Sandhills NWR	Angelus, SC	34°36'03.83''N
			80°15'27.09"W
34	Bear River Bay, Bear River	Corinne, UT	41°27'57.4"N
	Migratory Bird Refuge		112°16'39.6"W
35	Bear Lake, Bear Lake NWR	Laketown, UT	41°52'31.3"N
			111°17'47.3"W
36	Canal 1, Great Dismal Swamp	Suffolk, VA	36°37'9.35''N
	National Wildlife Refuge (NWR)		76°33'43.07''W
37	Public Boat Ramp, Lake	Burlington, VT	44°28'38.10"N
	Champlain		73°13'16.98''W
38*	Bayley Lake, Little Pend Oreille	Addy, WA	48°28'31.12"N
	NWR		117°38'4.22''W

*Sites not included in the statistical analysis

Variable	Mean	Range
Ammonia (mg/L)	0.14	0.02 - 1.04
Bromide (mg/L)	8.85	0.0 - 72.8
Calcium (g/L)	0.14	$8.20 \text{ x} 10^{-4} - 0.93$
Carbon Dioxide (mg/L)	11.5	0.10 - 110
Chloride (g/L)	3.82	$2.43 \text{ x}10^{-3} - 21.9$
Fluoride (mg/L)	0.51	0.04 - 3.19
Hardness (g/L)	1.41	$5.00 \text{ x}10^{-3} - 8.9$
Iron (g/L)	0.24	$8.00 \text{ x}10^{-3} - 2.05$
Magnesium (g/L)	0.25	7.00 x10 ⁻⁴ - 159
Manganese (mg/L)	81.2	0.9 - 1370
Nitrate and Nitrite (mg/L)	0.03	8.00x10 ⁻³ - 0.12
Organic Nitrogen (mg/L)	1.25	0.27 - 3.60
Orthophosphate (mg/L)	0.15	0.01 - 1.14
рН	8.00	5.84 - 9.40
Phosphorus (mg/L)	0.06	3.00x10 ⁻³ - 0.44
Potassium (g/L)	0.73	$5.00 \text{ x}10^{-5} - 0.43$
Salinity (PPT)	7.14	0.02 - 42.5
Silica (mg/L)	6.38	0.14 - 34.6
Sodium (g/L)	2.14	0.27 - 14.0
Sulfate (g/L)	1.05	1.00x10 ⁻⁴ -12.1
Total Nitrogen (mg/L)	1.35	0.37 - 4.70

Table 4.2. Summary of the physicochemical properties of the 32 surface water samplesused for the development of the multivariable linear regression model.

Table 4.3. Summary of virus log reduction times (Rt) by virus and temperature for all 38 water samples. The Rt value is the time (days) required for a decrease of viral titer by 1 $\log_{10} \text{TCID}_{50}/\text{ml}$, which is a 90% reduction in infectivity. Due to incomplete physicochemical information only the data from 32 of the 38 samples were used for statistical analysis.

	H3N2				H4N6			H8N4		
Site	10 C	17 C	28 C	10 C	17 C	28 C	10 C	17 C	28 C	
1*	20.1	15.3	3.3	19.6	21.1	1.8	4.3	1.7	2.5	
2	12.9	14.0	3.5	21.0	15.4	2.1	18.2	15.2	1.8	
3	62.9	25.6	4.8	54.8	31.0	3.4	71.0	38.8	3.9	
4	10.1	6.4	1.4	8.6	4.2	0.8	8.7	5.8	1.0	
5*	33.3	19.0	5.2	11.2	15.0	4.8	11.2	6.6	2.8	
6	7.4	3.4	0.8	6.5	1.9	0.5	3.8	1.6	0.7	
7	26.8	23.5	3.1	25.2	26.7	3.6	20.9	24.9	3.3	
8	29.2	15.9	3.9	25.3	17.5	3.2	7.2	2.4	1.0	
9	37.0	17.4	6.0	44.0	17.1	6.3	37.3	15.2	2.7	
10	20.7	13.6	1.4	18.7	9.7	0.8	17.1	6.3	0.9	
11	19.9	12.4	2.7	19.3	8.0	1.9	15.5	5.3	1.3	
12	44.0	22.5	3.8	45.0	29.1	7.2	26.5	16.1	4.2	
13	4.6	1.5	1.2	7.4	2.4	2.2	5.1	2.0	1.2	
14	13.2	10.2	1.4	15.3	8.3	1.3	5.0	3.4	1.5	
15	81.6	30.1	4.1	69.1	32.7	3.1	48.4	20.1	2.2	
16	25.1	16.3	1.8	27.6	25.6	4.1	8.5	12.5	2.2	
17	41.9	21.8	1.8	28.8	25.2	0.7	9.2	9.0	0.5	
18	38.0	26.6	3.9	38.8	27.6	4.0	35.7	27.0	3.7	
19	5.6	3.1	1.9	5.9	1.7	1.4	4.2	1.4	1.6	
20	25.9	15.6	2.9	22.5	18.1	2.8	36.4	25.9	2.1	
21	3.6	2.4	1.0	4.6	4.1	0.4	4.8	3.6	0.4	
22	5.0	4.0	3.1	4.2	5.4	2.1	6.4	4.0	1.8	
23*	6.5	2.5	1.8	5.1	2.1	1.7	7.5	1.5	1.4	
24	23.2	24.2	2.7	22.0	18.8	2.5	26.4	22.5	1.9	
25	8.2	4.7	0.3	5.4	4.2	0.3	5.2	4.1	0.4	
26	16.1	11.8	0.7	22.3	14.4	0.5	17.9	9.7	0.4	
27*	40.1	19.2	4.3	29.7	17.8	3.0	24.7	18.9	1.6	
28	14.9	5.8	1.0	13.8	4.8	0.9	14.3	6.1	1.5	
29	23.6	17.9	1.3	15.4	15.2	1.6	12.0	14.7	1.1	
30	11.2	6.3	4.5	4.4	2.1	2.8	3.9	1.3	2.4	
31	82.0	44.5	4.9	86.2	49.2	2.6	46.6	12.4	2.5	

32*	51.5	29.0	5.5	46.3	37.8	3.5	3.9	4.1	1.7
33	13.7	8.9	6.7	16.9	7.2	6.5	8.9	5.1	4.3
34	18.4	11.2	4.6	22.9	11.4	2.7	22.1	8.8	3.2
35	92.6	43.1	3.0	86.2	49.3	3.6	20.6	11.8	2.9
36	3.2	2.7	0.3	1.0	0.9	0.3	0.5	0.4	0.2
37	11.2	6.9	2.6	9.7	6.7	3.2	9.7	7.4	2.1
38*	51.9	38.0	5.4	50.0	46.4	3.3	14.3	4.0	5.6
Geometric Mean	19.2	11.5	2.3	17.3	10.8	1.9	11.5	6.4	1.6

*Sites not included in the statistical analysis

Variable	Coefficient	95% CI	$^{\dagger}P$
	(Robust SE)		
Virus			< 0.001
H3N2	0.190 (0.044)	0.010, 0.280	< 0.001
H4N6	0.163 (0.036)	0.091, 0.236	< 0.001
H8N4	Referent	Referent	
Temperature			< 0.001
10 C	Referent	Referent	< 0.001
17 C	-0.226 (0.025)	-0.277, -0.176	< 0.001
28 C	-0.935 (0.057)	-1.05, -0.820	
pН			
<7.0 and >8.5	Referent	Referent	
7.0-8.5	0.400 (0.189)	0.015, 0.784	0.042
Salinity (PPT)	-0.014 (0.006)	-0.026, -0.001	0.040
Ammonia (mg/L)	-0.796 (0.227)	-1.26, -0.333	0.001
Sulfate (g/L)	0.082 (0.033)	0.015, 0.148	0.018
Virus x Salinity			0.004
H3N2	-0.003 (0.002)	-0.007, 0.002	0.241
H4N6	-0.005 (0.002)	-0.009, -0.001	0.008
H8N4	Referent	Referent	
Constant	0.878 (0.189)	0.493, 1.26	< 0.001

Table 4.4. Multivariable linear regression model for the prediction of log_{10} virus reduction time (Rt) in filtered water samples collected from 32 waterfowl habitats (R²=64.0%).

[†]Based on Wald statistics

Water Type	'n	H3N2	H4N6	H8N4
Fresh	17	9.5	8.3	5.5
(<0.5ppt)	17	(0.3, 92.6)	(0.3, 86.2)	(0.2, 80.0)
Brackish	12	6.0	6.0	5.0
(0.5-30ppt)	12	(0.3, 44.0)	(0.3, 45.0)	(0.4, 37.2)
Salt	2	4.4	3.1	3.0
(>30ppt)	3	(0.8, 20.7)	(0.5, 18.7)	(0.6, 17.1)
Total	22	7.4	6.7	5.0
Total	52	(0.3, 92.6)	(0.3, 86.2)	(0.2, 71.0)

Table 4.5. Geometric mean (min-max) virus log reduction times (days) in the 32 filtered

 water samples used for the statistical analysis by water type and virus.

*Number of water samples in of each water type



Figure 4.1. Comparison of the virus log reduction times (Rt) of all three viruses and the statistically significant abiotic characteristics of the 32 filtered surface water samples included in the statistical analysis; black diamonds and solid line: 10 C, grey squares and dashed line: 17 C, white triangles and dotted line: 28 C.



Figure 4.2. A univariate comparison of the pH and sulfate concentration for the 32 filtered surface water samples included in the statistical analysis.

CHAPTER FIVE

STRAIN-RELATED VARIATION IN THE PERSISTENCE OF INFLUENZA A VIRUS IN THREE TYPES OF WATER: DISTILLED WATER, FILTERED SURFACE WATER, AND INTACT SURFACE WATER¹

¹ Keeler, S.P., C. Lebarbenchon, and D.E. Stallknecht. In Review, Virology Journal.

ABSTRACT

Background

The persistence of influenza A (IA) virus in aquatic habitats has been demonstrated to be a determinant for virus transmission dynamics in wild duck populations. In this study, we investigated virus strain-related variation in persistence in water for nine wild duck isolated IA viruses of three subtypes (H3N8, H4N6 and H8N4).

Results

We experimentally estimated the loss of infectivity over time in three different types of water: distilled, filtered surface water, and intact surface water. All viruses persisted longest in distilled water followed by filtered surface water with markedly reduced durations of persistence observed in the intact surface water. Strain-related variations were observed in distilled and filtered surface water but limited variation was observed in the intact surface water.

Conclusions

The finding suggest that the role of surface water for long-time (between years) maintenance of AI viruses in the environment may be limited, and suggest that the physico-chemical characteristics of water as well as microorganisms may be of strong importance. Results also indicate that the extent of strain-related variation observed in distilled water may overestimate persistence abilities for IA viruses in the wild and supports the need to develop experiments that account for these effects to assess subtype, genotype as well as spatial and temporal variation in the persistence in aquatic habitats.

BACKGROUND

Influenza A (IA) virus persistence in aquatic habitats has been demonstrated to be determinant for virus transmission dynamics in wild duck populations (Breban et al., 2009; Roche et al., 2009; Rohani et al., 2009). In these hosts, viral replication mainly occurs in the epithelial cells of the intestinal tract, resulting in high virus concentration in feces (Hinshaw et al., 1979; Webster et al., 1978). Infected birds contaminate aquatic environments in which IA viruses can persist for extended periods of time, depending on water temperature and physico-chemical characteristics (Brown et al., 2009; Domanska-Blicharz et al., 2010; Lebarbenchon et al., 2011; Nazir et al., 2010; Stallknecht et al., 1990b). Biotic components including aquatic invertebrates and microorganisms also have recently been proposed as potential factors affecting virus removal or accumulation in the environment (Faust et al., 2009; Nazir et al., 2010; Stumpf et al., 2010; Zarkov, 2006).

Strain-related variations in the persistence of IA viruses have been investigated under experimental conditions using distilled water maintained at different temperatures, pH and salinity (Brown et al., 2009; Brown et al., 2007). In a recent study, Lebarbenchon et al. (Lebarbenchon et al., Submitted) suggested that differences in the persistence of IA viruses in water may be limited when considering co-circulating viruses in a single duck population. Variation in the duration of persistence has however been documented for viruses circulating in different locations or seasons (Brown et al., 2009), suggesting potential adaptive responses of IA viruses to local water characteristics and the ability to rapidly evolve toward optimal level of persistence in changing environments (Lebarbenchon et al., Submitted). While studies have compared the aquatic stability of multiple IA virus strains, to date, these studies have been limited to comparisons of persistence in distilled water (Brown et al., 2009; Brown et al., 2007; Lebarbenchon et al., Submitted). Direct comparison of the duration of persistence of viruses under more realistic conditions are limited, in particular the effects of the physico-chemical characteristics and microorganisms in surface water have remained poorly understood. In this study, we investigated virus strain-related variation for three IA virus subtypes in wild duck populations: H3N8, H4N6, and H8N4 (Krauss et al., 2004; Munster et al., 2007; Wilcox et al., 2011). We experimentally estimated the loss of infectivity over time of nine virus strains, in three different types of water: distilled water, filtered surface water, and intact surface water. We discuss results in the light of current knowledge on IA virus ecology in wild duck populations and the role for water-borne transmission in avian influenza epidemiology.

METHODS

Virus selection

All viruses were isolated from dabbling duck species. Six viruses were obtained from a population surveillance study in Minnesota conducted in 2007 (Wilcox et al., 2011): A/Mallard/Minnesota/Sg-00051/2007 (H3N8), A/Mallard/Minnesota/Sg-00048/2007 (H3N8), A/Mallard/Minnesota/Sg-00169/2007 (H3N8) (referred to hereafter as H3N8-07a, H3N8-07b and H3N8-07c); and A/Mallard/Minnesota/Sg-00050/2007 (H4N6), A/Mallard/Minnesota/Sg-00053/2007 (H4N6), A/Mallard/Minnesota/Sg-00063/2007 (H4N6) (referred to hereafter as H4N6-07a, H4N6-07b and H4N6-07c). Two additional viruses isolated in Mallards in Minnesota in 1999 were included: A/Mallard/Minnesota/199106/1999 (H3N8) and A/Mallard/Minnesota/199044/1999 (H4N6) (referred to hereafter as H3N8-99 and H4N6-99). Finally, a H8N4 virus isolated in Texas in 2001 was also included: A/Northern Pintail/TX/421716/01 (referred to hereafter as H8N4-TX-01).

Stock viruses were propagated in 9 to 11 day old specific pathogen free (SPF) embryonating chicken eggs with all viruses being second passage (Swayne, 2008). Serial titrations were performed in SPF embryonating chicken eggs and Madin Darby canine kidney (MDCK) cells to determine the median embryo infectious dose (EID₅₀) and the median tissue culture infectious dose (TCID₅₀) (Reed and Muench, 1938; Swayne, 2008).

Water-persistence trials

For each of the nine viruses, we tested the effect of the water type (distilled, filtered surface, and intact surface), on the decrease in infectivity over time. A surface water sample was collected from Memorial Pond (33°55'37.31"N, 83°23'2.71"W), a 12,141 m³ man-made lake inside a recreational park, on 06-May 2011. The site was selected for convenience as the site is close to the lab, and the pond was known to have a neutral pH and low salinity. The pond also has resident waterfowl species including peridomestic muscovy ducks (*Cairina moschata*) and mallards (*Anas platyrhynchos*). Three 1L water samples were collected in LDPE wide-mouth bottles (Thermo Fisher Scientific, Inc, Waltham, Massachusetts, USA) within 1 m of the shoreline and about 3 cm below the surface. Samples were placed on ice for transport back to the lab. Specific conductance and pH readings were taken at the site of collection using a YSI 556 MPS handheld instrument (YSI, Inc, Yellow Springs, Ohio, USA). In the lab, half of each 1L water sample was filtered using a bottle-top vacuum filter system with a 0.22µm

polyethersulfone membrane (Corning Inc, Corning, New York, USA) to remove most biological material. The pH was confirmed in the laboratory for both the filtered and intact surface water using a VWR sympHony SB80PC bench top meter (VWR International, Radmor, Pennsylvanian, USA). The average pH for all surface water samples was determined to be 7.2 and the salinity was 12 parts per million (ppm).

Distilled water buffered with 10 mM HEPES was adjusted with 1N solutions of NaOH or HCl to provide a pH = 7.2. For each virus, infective amnio-allantoic fluid was diluted 1:100 in the distilled water, filtered surface water, and intact surface water. Inoculated water samples were divided into 2 mL aliquots in 5 mL polystyrene roundbottom tubes and placed in incubators set to 17C. All experiments were run in triplicate. Virus-negative filtered and intact surface water controls were setup using the same methods as the experimental trials and run concurrently to ensure no environmentally deposited cytopathic agents including IA virus were present in the surface water samples.

For each water type, the TCID₅₀/mL was determined at the time of inoculation (day 0) and 11-15 times post-inoculation. The tubes containing the virus inoculated water samples were removed from the incubator and vortexed to resuspend any particulate matter and thoroughly mix suspension. Duplicate 0.5 mL aliquots were removed from each tube and diluted 1:1 by addition of 0.5 mL of 2X minimal essential medium (MEM). Ten-fold dilutions (10^{-1} to 10^{-6}) were made in 1X MEM supplemented with antibiotics (10000 U/mL Penicillin G, 10 mg/mL Streptomycin, $25\mu g/mL$ Amphotericin). Infectivity assays were performed on MDCK cells (Brown et al., 2009).

Statistical analyses

Statistical analyses were carried out in R 2.12.1 (www.R-project.org). An analysis of covariance (ANCOVA) was used to test the effects of time, virus strain and type of water on virus infectivity. Results from duplicate titrations were averaged and log₁₀ transformed prior to analyses. Linear regressions were used to calculate the time required for a 90% reduction of infectivity in water (i.e. time required for a decrease of the viral titer by 1 log₁₀ TCID₅₀/mL (Reed and Muench, 1938; Swayne, 2008). Fligner–Killeen tests were used prior to the ANCOVA to check for homogeneity of variance (Crawley, 2007).

<u>RESULTS</u>

Viral persistence in water significantly decreased over time ($F_{1,856} = 75$, P < 0.001), with evidence that this decrease strongly varied with the type of water (time by water type interaction: $F_{2,856} = 937$, P < 0.001). The main effect of water type was also significant ($F_{2,856} = 2392$, P < 0.001): all virus strains persisted longest in distilled water followed by filtered surface water with markedly reduced duration of persistence observed in intact surface water (Table 5.1, Figure 51). The effect of virus on the persistence was significant ($F_{8,856} = 88$, P < 0.001); however, this effect may be induced by slight differences in the initial doses used for each virus. A strong interaction was found between the time and the viral strain ($F_{8,856} = 21$, P < 0.001), indicating that the decrease in virus titer over time varied among viruses. Also, a significant interaction between the water type and the viral strain was found ($F_{16,856} = 21$, P < 0.001) suggesting that the pattern of infectivity loss induced by the water type differed between viruses.

($F_{16,856} = 2.3$, P < 0.01), indicating that the effect of the water type on viral persistence over time was different among virus strains.

To further investigate differences in the pattern of loss of infectivity over time we performed an independent ANCOVA for each water type. The main effects of the time and virus were significant for all water types. A significant interaction between time and virus strain was found for the distilled ($F_{8,414} = 4.96$, P < 0.001) and filtered ($F_{8,333} = 2.47$, P < 0.05) water. For the intact surface water however, this interaction was no longer significant ($F_{8,109} = 0.88$, P = 0.53). The tables 5.2-5.4 present differences in the loss of infectivity over time between viruses. H4N6-07c exhibited a significantly higher loss of infectivity as compared to other virus strains, in distilled water, however a note of caution is warranted since a lower dose of virus was used for this virus.

DISCUSSION

All viral strains showed a markedly reduced duration of persistence in intact surface water compared to both filtered surface water and distilled water. The surface water sample we used had pH and salinity levels considered to be ideal for long-term persistence (i.e. neutral to basic pH and low salinity (Brown et al., 2009)) and the trials were conducted at a constant incubation temperature (17°C), limiting previously documented temperature-variation effects (Lebarbenchon et al., Submitted; Lebarbenchon et al., 2011). These results are consistent with previous studies involving intact surface water (Nazir et al., 2010; Zarkov, 2006); the duration of IA virus persistence in the surface water of aquatic habitats can significantly be reduced by adverse physico-chemical conditions (Keeler et al., In Press) or due to the presence of a wide variety of biological organisms including bacteria, fungi, algae, and protozoa (Zarkov, 2006). The duration of persistence in the filtered surface water used in our experiments was significantly increased (ten-fold) as compared to intact surface water, suggesting that microorganisms or other nonorganic particulate matter present in the water sample could limit the ability of IA virus to remain infectious in aquatic habitats. The surface water sample used in this study was not biologically characterized. The pond that was sampled is located in an urban area inside a public park and the site could have higher microorganism counts compared to more pristine (less human impacted) ecosystems (Mueller and Helsel, 1995; Smith et al., 1999). If microorganisms are reducing the environmental stability of the virus, surface water with higher microbial counts maybe less hospitable to viral persistence so the degree of reduction of viral persistence observed in this study may not be universally applicable to all surface water. Overall, the reduction in the duration of persistence observed in intact surface water suggests that it may not represent a suitable environment for long-term maintenance (between years) of IA viruses as was suggested based on persistence trials performed entirely in modified distilled water. The importance in virus persistence of other components of aquatic ecosystems (e.g. soil (Lang et al., 2008; Nazir et al., 2011), aquatic invertebrates (Stumpf et al., 2010), in the transmission dynamics of IA viruses in wild duck population, remain poorly understood. These factors may however favor longterm (between-years) maintenance of viruses in the environment, in particular between epidemics, and require further investigation.

Consistent with previous studies involving multiple IA viruses, variation in the duration of viral persistence was observed between strains in distilled water and even in filtered surface water (Brown et al., 2009; Brown et al., 2007). The strain related differences were considerably reduced in the intact surface water indicating that viral strain or subtype may only have a limited effect on the persistence of viruses in surface water. These findings suggest that usually reported strain-related variations in the duration of infectivity in distilled water may not reflect realistic persistence abilities for IA viruses at least at the temperature evaluated in this study. Future experimental designs need to consider this aspect to evaluate potential subtype, genotype as well as spatial and temporal variation in the persistence of these viruses in aquatic habitats.

The viruses used in this study were isolated from waterfowl congregating in large numbers as part of a seasonal migratory pattern (Hanson et al., 2005; Wilcox et al., 2011). During these times of mass gatherings of birds, viral adaptation to local water conditions would not be necessary as the majority of the transmission would be driven by the density of birds occupying the site (Rohani et al., 2009). The viruses we selected for this project would have only required a minimal amount of environmental persistence to ensure indirect transmission during these periods of high bird density and this could explain why limited strain related differences were observed in intact surface water but further studies are required to evaluate the validity of this hypothesis.

The results of this study provide further insight into the role of surface water as a medium for virus transmission, but limited evidence that surface water could act as longtime environmental reservoir for IA viruses. Strain related differences in virus persistence were not observed in intact-surface water indicating that water physicochemical characteristics as well as microorganisms may significantly reduce maintenance abilities of IA viruses in water. This finding supports the need to develop experimental

designs reflecting natural conditions in order to assess strain-related variation in the maintenance in aquatic environments.

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Table 5.1. Summary of the average virus log reduction times. Average virus log reduction times (Rt) with standard deviation in parentheses for each virus strain and water type combination. The Rt values are the time (days) required for a decrease of viral titer by $1 \log_{10} \text{TCID}_{50}/\text{ml}$.

	Water Type								
Virus Strain	Distilled	Filtered Surface	Intact Surface						
H3N8-07a	65.9 (3.7)	29.2 (3.1)	3.7 (0.4)						
H3N8-07b	73.7 (10.8)	27.1 (1.5)	3.0 (0.8)						
H3N8-99	78.8 (2.9)	36.9 (5.9)	3.2 (0.1)						
H3N8-07c	59.1 (14.2)	41.4 (10.7)	3.2 (0.1)						
H8N4-TX-01	68.6 (18.8)	26.2 (6.2)	2.3 (0.1)						
H4N6-07a	75.6 (2.3)	24.4 (3.1)	3.5 (0.8)						
H4N6-07b	69.2 (14.4)	29.3 (2.3)	2.8 (0.1)						
H4N6-99	66.6 (17.2)	29.9 (5.3)	3.2 (0.7)						
H4N6-07c	46.2 (2.1)	25.6 (3.2)	3.1 (0.2)						
Average	67.1 (9.8)	30.0 (5.6)	3.1 (0.4)						
	H3N8- 07a	H3N8- 07b	H3N8-99	H3N8-07c	H8N4- TX-01	H4N6- 07a	H4N6- 07b	H4N6-99	H4N6-07c
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H3N8-07a		0.53	0.19	0.07	0.66	0.42	0.98	0.5	<0.001
H3N8-07b			0.5	<0.05	0.28	0.86	0.54	0.19	<0.001
H3N8-99				<0.01	0.08	0.62	0.2	<0.05	<0.001
H3N8-07c					0.18	<0.01	0.07	0.27	<0.05
H8N4-01						0.21	0.64	0.82	<0.001
H4N6-07a							0.43	0.14	<0.001
H4N6-07b								0.49	<0.001
H4N6-99									<0.01
H4N6-07c									

Table 5.2. Effect of the virus strain x time interaction on the virus infectivity, in distilled water (P values).

	H3N8- 07a	H3N8- 07b	H3N8-99	H3N8-07c	H8N4- TX-01	H4N6- 07a	H4N6- 07b	H4N6-99	H4N6-07c
H3N8-07a		0.6	0.16	0.06	0.31	0.15	0.96	0.97	0.3
H3N8-07b			0.06	<0.05	0.62	0.37	0.57	0.57	0.61
H3N8-99				0.63	<0.05	<0.01	0.18	0.18	<0.05
H3N8-07c					<0.01	<0.01	0.07	0.07	<0.01
H8N4- 01						0.69	0.28	0.29	0.99
H4N6-07a							0.14	0.14	0.69
H4N6-07b								0.99	0.28
H4N6-99									0.28
H4N6-07c									

Table 5.3. Effect of the virus strain x time interaction on the virus infectivity, in filtered surface water (P values).

	H3N8- 07a	H3N8- 07b	H3N8-99	H3N8-07c	H8N4- TX- 01	H4N6- 07a	H4N6- 07b	H4N6-99	H4N6-07c
H3N8-07a		0.26	0.28	0.2	<0.05	0.71	0.11	0.28	0.19
H3N8-07b			0.99	0.97	0.14	0.54	0.46	0.82	0.82
H3N8-99				0.98	0.14	0.54	0.48	0.83	0.83
H3N8-07c					0.13	0.49	0.46	0.83	0.83
H8N4-01						0.07	0.37	0.23	0.18
H4N6-07a							0.24	0.48	0.42
H4N6-07b								0.67	0.59
H4N6-99									0.96
H4N6-07c									

Table 5.4. Effect of the virus strain x time interaction on the virus infectivity, in intact surface water (P values).



Figure 5.1. Virus infectivity in water over time as a function of water type. Lines represent the least-squares regression lines for each

water type (Solid black diamonds: distilled water; Grey squares: filtered surface water; White triangles: intact surface water).

CHAPTER SIX

SUMMARY AND CONCLUSIONS

Since the recognition that wild aquatic birds are the primordial reservoirs of influenza A (AI) viruses, environmental stability has been recognized as an important determinant in both transmission and maintenance of these viruses in avian reservoirs. Avian influenza viruses infect a wide range of avian species but the most commonly infected species are highly gregarious and utilize a wide range of aquatic habitats including lakes, wetlands, and seacoasts. The surface water of these aquatic habitats becomes contaminated by the feces of infected birds and serves as a transmission medium and potentially a long-term reservoir for these viruses. Although, environmental sampling and laboratory-based studies support a role for surface water in the transmission and maintenance of the virus, these studies are few in number and are often limited in their applicability to natural surface water due to the use of sterile distilled water as a laboratory model. The physical and chemical characteristics of the surface water of aquatic habitats are complex and vary considerably between locations. The overall objective of this research was to identify and characterize the factors that influence AI virus persistence in natural surface water from aquatic habitats of wild waterfowl.

The duration of infectivity was determined for two common, low pathogenic AI virus subtypes (H3N2 and H4N6) in filtered surface water samples collected from 15 waterfowl habitats within Georgia (Chapter 3). Natural surface water was filtered to limit the influence of biological organisms and other material within the water column. The pH

and salinity was determined for each water sample and the persistence trials were performed at three incubation temperatures (10C, 17C and 28C). The study was designed to be complementary to previous studies that utilized sterile distilled water as a model system. The results of the trials were consistent with the general trends of AI virus persistence established by previous studies. The pH and incubation temperature of the filtered surface water had a significant effect on the stability of the virus. Both viruses persisted longer at the two lower incubation temperatures (10 C and 17 C) compared to the highest temperature (28 C) evaluated in this study. The pH of the surface water samples ranged from 4.2 to 7.6; the duration of viral persistence was longest for both viruses in the water sample with neutral to basic pH (6.8-7.6). The salinity of the surface water samples was limited so the effect of this factor could not be adequately evaluated. Variation in the duration of persistence was observed between water samples with comparable pH and salinities, which may indicate that other abiotic factors affect the stability of AI virus in natural surface water. The general trends established in this study could be used to broadly categorize aquatic habitats based on their capacity for surface water persistence, which could be used for studies focused on the ecology and transmission of AI virus.

Additional viral persistence trials were performed (Chapter 4) to identify and characterize the significant abiotic predictors of AI virus stability in natural surface water. Thirty-eight surface water samples were collected from waterfowl habitats distributed across the United Sates and trials were performed using three low pathogenic AI virus subtypes (H3N2, H4N6, and H8N4). Water samples were filtered to remove the majority of the biological material and in-depth chemical analysis of the water samples

was performed by the U.S. Geological Survey National Water Quality Lab (Denver, CO). The data was used to construct a multivariable linear regression model. In addition to pH, salinity, and temperature, ammonia and sulfate concentrations were determined to be significant predictors of the duration of viral persistence. Based on the results of the study, surface water with low temperature (<17 C), neutral-basic pH (7.0-8.5), low salinity (<0.5ppt), and low ammonia levels (<0.5mg/L) is the most conducive for AI virus persistence. The sulfate concentration of surface water was determined to enhance the duration of viral persistence but the mechanism of action is unknown and warrants further investigation. A significant difference in the influence of salinity on the duration of persistence was observed between the H8N4 virus and the other two subtypes indicating potential strain-related differences in aquatic stability. Coupling the multivariable linear regression model developed in this study with data on migratory and land use patterns of waterfowl could allow for the ranking of waterfowl sites based on the likelihood of surface water facilitating persistence and transmission. This type of information could be used to develop environmental surveillance programs or to identify areas that should be targeted by traditional surveillance.

The third study (Chapter 5) evaluated the duration of persistence of nine low pathogenic AI viruses of three subtypes (H3N8, H4N6 and H8N4) in three types of water: sterile distilled, filtered surface water, and intact surface water. The pH and salinity concentration of all three water types was identical (pH 7.2 and salinity 12PPM) and all trials were performed at 17C. These conditions are considered to be ideal for longterm persistence of AI virus (i.e. low temperature, neutral to basic pH and low salinity). All nine viruses persisted longest in distilled water followed by filtered surface water

with significantly reduced duration of persistence observed in intact surface water. Variation in the duration of viral persistence was observed between strains in distilled water and filtered surface water but the variation was considerably reduced in the intact surface water. The findings of this study indicate that viral strain or subtype may only have a limited effect on the persistence of viruses in natural surface water. The strainrelated variations in the duration of persistence reported in distilled water may not reflect realistic persistence abilities for the virus at least at the temperature evaluated in this study. The duration of viral persistence was significantly reduced in intact surface water compared to persistence estimates in modified distilled water, suggesting that surface water may not serve as a long-term environmental reservoir.

The information gained from these studies enhances our understanding of the role of surface water in the maintenance and transmission of AI virus. The results support a role for surface water as a transmission medium and a short-term (within season) reservoir but the reduced persistence observed in intact surface water (Chapter 5) suggests that surface water may not serve as a long-term (between seasons) reservoir. The duration of viral persistence in surface water will vary based on the measureable physicochemical characteristics identified in these studies. These finding could be used for ranking habitats based on their likelihood for supporting the environmental transmission of AI virus through surface water. Such a ranking system could be used to augment or enhance current surveillance programs or research projects focused on AI virus in wild bird populations. Any field application of the results of these studies should be done cautiously as the current understanding of the persistence of AI virus in surface water still remains incomplete. The biological components of water were shown to

reduce the duration of viral persistence and lessen strain-related variation but the microbial diversity of aquatic ecosystems is highly diverse. The important biological determinants of reduced AI virus persistence remain unknown. In addition to the biological components, other particulate matter within the water column, such as soil, could also be affecting the infectivity of the virus. Future studies should focus on identifying and characterizing the important filterable components of water (microbial and non-biological). An additional area for future studies would be the identification of other potential environmental reservoirs within aquatic habitats other than surface water.