DISSERTATION

THE ROLE OF FRESHWATER SNAILS IN THE TRANSMISSION OF INFLUENZA A VIRUSES

Submitted by

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ABSTRACT

THE ROLE OF FRESHWATER SNAILS IN THE TRANSMISSION OF INFLUENZA A VIRUSES

Waterfowl are the natural reservoirs for avian influenza (AI) viruses. Avian influenza virus infections in these birds are generally subclinical, but they shed infectious virus through feces for several days, typically into water. Further, AI viruses can remain infectious in water for weeks. This characteristic enhances transmission of AI viruses among waterfowl because transmission is not constrained by direct contact. The prevalence of AI virus infection in waterfowl populations follows a cyclical pattern; prevalence is highest in the population after the breeding season. Shedding of AI viruses is nearly undetectable in these waterfowl populations by winter, yet the prevalence cycle repeats itself annually. Somehow, AI viruses are reintroduced to host populations. The mechanisms that drive the prevalence patterns observed in waterfowl are likely numerous and complex, but AI viral persistence in water is probably critical. Persistence of AI viruses in water also potentially exposes other organisms to the virus. Aquatic invertebrates, such as snails, are likely exposed to AI viruses while feeding on detritus in aquatic habitats, and gastropods are a common food source for many species of waterfowl. This trophic interaction may potentially serve as an additional route of AI virus transmission and maintenance. In this study, two species of freshwater snails (*Physa acuta* and *P. gyrina*) were experimentally

exposed to avian influenza virus (H3N8) to determine: 1) whether the snails have cellular receptors capable of binding to AI viruses, 2) whether snails can bioaccumulate AI viruses, 3) how long bioaccumulated AI viruses are maintained and remain infectious in snail tissues, and 4) whether *Physa* spp. can serve as mechanical vectors of AI viruses. My results indicated that, while *Physa* spp. snails sequestered infectious AI virus, the duration was short-lived and no transmission occurred. These data suggest that the snail species examined do not directly impact AI virus transmission among waterfowl; however, in the process of feeding on snails, waterfowl may be exposed to AI viruses both via water and ingestion such that other avenues of investigation are warranted.

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INTRODUCTION

Influenza A Viruses

Influenza A viruses (family *Orthomyxoviridae*, genus *Influenzavirus A)* are some of the most widespread and important pathogens in the world. These viruses are primarily pathogens of aquatic birds, but have adapted to infect a wide range of avian and mammalian hosts including humans. The clinical signs of the resulting disease range from subclinical to high mortality, depending on host species and viral strain (1992). Disease associated with influenza A viruses has caused regional epizootic events in poultry and wildlife leading to massive mortality events. In addition, influenza A viruses are the etiologic agents responsible for recurring annual epidemics in humans, and have caused three pandemics leading to nearly 60 million human deaths in the last century (Webster et al. 1992).

Although influenza A viruses have become established in a wide range of animal species, birds belonging to the Orders Anseriformes and Charadriiformes are considered to be the primordial reservoirs for all influenza A viruses (Stallknecht and Brown 2008). Viruses carried by these reservoirs afford sufficient genetic diversity for the emergence of an epizootic in other species. The influenza A viruses that typically cause disease in humans, swine, horses, and dogs demonstrate both antigenic drift and genetic shift, while avian influenza (AI) viruses currently appear to be in evolutionary stasis (Webster et al. 1992).

Most AI viruses target the intestinal epithelial cells of susceptible birds. Infected cells release viral particles into the intestines and are subsequently shed through feces into water (Stallknecht and Brown 2008). Avian influenza viruses are capable of remaining viable in water for several months (Stallknecht et al. 1990b, Brown et al. 2007). Viability in water is dependent on several factors, including water salinity and pH and viral subtype (Stallknecht et al. 1990a). Transmission between individuals and populations includes not only direct contact, but indirect contact via exposure to infectious virus in aquatic environments. This can occur several days or weeks after infected birds have left the area (Stallknecht et al. 1990b, Brown et al. 2007).

Biological Properties

Influenza A viruses are enveloped RNA viruses with an eight-segmented, single-stranded, negative-sense genome. The eight gene segments of influenza A viruses encode 10 proteins. These proteins include surface proteins hemagglutinin (HA) and neuraminidase (NA), and a membrane ion channel protein. Internal proteins include nucleoproteins, matrix protein, and three polymerase proteins: a polymerase basic protein 1, a polymerase basic protein 2, and a polymerase acidic protein (Webster et al. 1992). In addition, the genome codes for two nonstructural proteins, nonstructural protein 1, and nonstructural protein 2 (Palese and Shaw 2007).

Morphologically, influenza A viruses are extremely variable, ranging from spherical (80 – 120 nm diameter) to long filamentous forms (up to several microns in length). Influenza A virus isolates collected directly from animals are

usually characterized by the presence of a significant proportion of filamentous virions (Chu et al. 1949). However, multiple passages in eggs often results in mostly spherical virions (Choppin et al. 1960). These morphological changes appear to be controlled by the M1 and M2 proteins (Hughey et al. 1995, Bourmakina and Garcia-Sastre 2003).

Influenza A virus subtypes are identified by their surface proteins. Currently, 16 hemagglutinin (H1 – H16) subtypes and nine neuraminidase (N1 – N9) subtypes have been identified. Influenza A viruses are further categorized by their host species, location, and the year they were isolated. For example, A/Chicken/Alabama/75 (H4N6) is an influenza A virus sample collected from a chicken in Alabama in 1975 with H4 and N6 surface proteins. Subtypes isolated from birds are categorized as avian influenza viruses, and are classified further as either high-pathogenicity avian influenza (HPAI) or low-pathogenicity avian influenza (LPAI) viruses depending on the virulence of the virus in young chickens. An AI virus is considered HPAI if it causes 75% mortality in 4 – 8 week-old chicken chicks; conversely, LPAI viruses often cause only mild to severe respiratory disease in chickens with limited mortality (Perdue and Swayne 2005).

Epidemiology

The host's cellular membrane consists of biological molecules and carbohydrates including glycoproteins; the principal sugars of glycoproteins are sialic acids and galactose. Sialic acids (SA) are terminal sugars on the glycoprotein; the SAs are classified by the conformation of the linkage to

galactose by an α-2 carbon. Hemagglutinin of influenza A viruses attaches to the SA on the host cell during the initial phase of an infection. The HA proteins have a strong specificity to either the α2,3- or the α2,6-SA-galactose linkage. The expression of the SA receptors on the hosts' cells varies by both tissue type and animal species. The differential expression of receptors and the affinity of the HA protein for a specific SA-galactose linkage contribute to host specificity. The intestinal epithelial cells of birds often express α2,3 SA receptors and thus are susceptible to infection by influenza A viruses with an affinity for α2,3 SA-galactose linkages (i.e., avian influenza viruses). The respiratory epithelial cells of humans express α2,6 SA receptors and are susceptible to influenza A viruses capable of binding with a 2,6 SA-galactose linkages (Suarez 2008).

In addition to HA specificity and cellular membrane attributes, pathogenicity is also determined by available proteases in the host. During the primary phase of all influenza A virus infections, HA is cleaved into HA1 and HA2 subunits by proteases found in the cells in the host. Trypsin (or trypsin-like) proteases, precipitated by the presence of extra-cellular arginine, cleave all HA subtypes, but trypsin is typically only locally available in the respiratory tissues of mammals and the enteric tracts of birds (Klenk et al. 1975). However, the HA protein of HPAI is also cleaved by furin, a protease found in most mammalian and avian cells, in the presence of multiple basic extra-cellular amino acids (lysine and arginine). This allows HPAI viruses to replicate efficiently in a wide range of cell types, including brain, heart, muscle, and pancreas, thereby allowing for a systemic infection (Suarez 2008).

Genetic Plasticity

Influenza A viruses are genetically extremely variable due to a high mutation frequency. This genetic plasticity provides influenza A viruses the means to evade host immune responses through genetic drift and genetic shift. Genetic drift can occur in any of the eight genes, including the surface glycoproteins; mutations in the surface proteins can potentially alter the severity of disease and possibly the host range (Burnet 1951, Peiris et al. 2007, Suarez 2008). The segmented genome of influenza A viruses provides a mechanism for genetic shift – the recombination of two distinct influenza A virus subtypes. Genetic shift allows for sudden and significant changes in the genome, potentially creating a new influenza virus strain, and possibly leading to dramatic changes in the pathogenicity and the extent of the host range (Webster 1997).

Despite the genetic variability of influenza A viruses, AI viruses are thought to be in evolutionary stasis within waterfowl hosts, and have established tight host-pathogen relationships. Evolutionary changes are generally slow within the host populations as most of the coding for internal genes show little change at the amino acid level (Kida et al. 1987). However, the surface glycoproteins of the virus evolve more rapidly, showing a greater genetic diversity with multiple antigenic subtypes (Stallknecht and Brown 2008). Such changes may allow new influenza A virus strains to cross the species barrier. Once the species barrier is broken, the new influenza A virus strain may evolve rapidly, driven by high mutation rates and the new host's immune response (Webby et al. 2007). Genetic analysis of human influenza strains demonstrates that antigenic

drift has occurred extensively (Kida et al. 1987). Further, as the virus becomes adapted to the new host, it may lose the ability to replicate in the old host (Perdue 2008), potentially creating a unique viral lineage (Peiris et al. 2007).

Viral strains resulting from antigenic drift are largely responsible for annual influenza A outbreaks in humans, while viral changes due to antigenic shift have precipitated catastrophic pandemics (Webby et al. 2007). Pandemics are relatively rare, but can happen when new subtypes are efficiently transmitted from human-to-human. In 1957, the reassortment of an H1N1 influenza A virus subtype resulted in a pandemic that killed nearly 4 million people. The reassorted H1N1 subtype had HA, NA, and PB1 genes from an avian influenza virus (Kawaoka et al. 1989). The pandemic of 1968 (H3N2) occurred when the HA (H3) and the PB1 genes were replaced with avian-like influenza virus genes (Bean et al. 1992). Finally, the pandemic of 1918 (H1N1), which killed an estimated 50 million people, may have arisen through the direct adaptation of an avian influenza virus from an unknown source (Taubenberger and Morens 2006).

Ecology

Avian influenza viruses are nearly ubiquitous in aquatic birds throughout the world (Webster and Bean 1998). Waterfowl and shorebirds are considered to be the natural reservoirs for these viruses (Stallknecht and Brown 2008). Aquatic birds are generally considered to be ‘donors’ of AI viruses; that is, they introduce the virus into human or domestic animal populations but their role in the spread of epidemic agents is thought to be limited (Sims et al. 2005). This hypothesis may not be supported for highly pathogenic strains of AI viruses. The HPAI virus

(H5N1) responsible for the recent epizootic in Asia probably mutated from a LPAI virus strain that was initially introduced to domestic poultry by waterfowl. The new more virulent strain was introduced back into migratory wild waterfowl, and these birds may have subsequently served as traffickers of this new strain (Webby et al. 2007).

Transmission of influenza A viruses can occur through a variety of mechanisms and varies by host species and subtype. In birds, AI viruses are primarily transmitted through a fecal-oral route. Virus replicates in the intestinal epithelial cells in infected birds and is shed into the environment via feces. Exposure of immunologically naïve birds can occur through ingestion of the infectious AI virus present in the environment (Suarez 2008). This allows AI viruses to be transmitted among waterfowl with the aquatic environment acting as a viral source (Webster et al. 1978). Outbreaks on poultry farms have been traced to untreated environmental water (Alexander 1993), and, in an experimental setting, VanDalen et al. (2010) demonstrated transmission between mallards via contaminated water.

Mammalian strains, such as swine and human influenza viruses, cause respiratory diseases and are transmitted by the nasopharyngeal route (Kothalawala et al. 2006). Equine influenza viruses (H3N8) have been transmitted to racing dogs via horsemeat (Crawford et al. 2005), and HPAI virus (H5N1) was transmitted experimentally to herring gulls via infected chicken meat (Brown et al. 2008). Finally, outbreaks of HPAI virus (H5N1) in waterfowl have preceded influenza A virus detection of the same viral strain in both domestic

cats (*Felis catus*) and stone martens (*Martes foina*) indicating scavenging or predation may have facilitated transmission of AI viruses to mammals (Amonsin et al. 2007, Klopfleisch et al. 2007, Leschnik et al. 2007)

Water is considered to be the primary transmission facilitator of AI viruses among waterfowl (Webster et al. 1978). Influenza A viruses can remain viable in water for up to four months (Stallknecht et al. 1990b, Brown et al. 2007), and like most viruses, influenza A viruses have an affinity for suspended solids in the aquatic environment. Viruses that bind to these solids survive longer in natural water and may settle and accumulate in the sediment (Bitton 1980). The affinity of viruses for suspended solids is based on the electrostatic attraction of virions and the abiotic particulate matter; viruses are negatively charged, and many sediment particles are positively charged (Bitton 1980, Goyal et al. 1980, Chang et al. 1981). This creates an environment that exposes animals feeding in the sediment to infectious influenza A viruses.

Influenza A Viruses In Wildlife

As reservoirs of AI viruses, waterfowl infected with LPAI viruses typically exhibit no clinical signs, yet an infected bird may continue to shed viable virus for up to 4 weeks (Webster and Bean 1998). Additionally, as previously mentioned, AI viruses can remain viable in water for long periods. Ecologically, these characteristics of AI viruses are important for maximizing transmission potential, as viral shedding and the viral persistence in water eliminates the need for direct contact among individuals and populations (Stallknecht et al. 1990b, Brown et al. 2007).

In North America, waterfowl breed in Alaska and the northern territories of Canada. Prior to post-breeding migration in July and August, adult and juvenile birds congregate in large interspecific groups in Canada and the United States (Webster and Bean 1998, Elphick et al. 2001). The prevalence of AI virus infections in these waterfowl populations is highest during the marshalling period, primarily affecting hatch-year birds, with as many as 30% of juveniles shedding AI viruses (Webster and Bean 1998). North American waterfowl are typically infected with H3, H4, and H6 hemagglutinin subtypes (Stallknecht et al. 1990c). During fall (southward) migration prevalence of AI virus infection declines as the birds move south (Webster and Bean 1998). By the time the population reaches Mississippi in November, prevalence has declined to nearly undetectable levels. However, each fall the cycle repeats on the marshalling areas (Webster et al. 1976, Stallknecht et al. 1990c).The mechanisms that allow this cycle to continue are poorly understood.

Evidence exists for the presence of continuous low level infections and transmission among wild ducks. Blue-winged teal (*Anas discors*) may be an important species in this scenario, as they migrate southward early, prior to peak AI virus prevalence in juvenile ducks. This provides the wintering grounds with a potentially large population of immunologically naïve birds (Stallknecht et al. 1990c). Additionally, AI viruses have been detected in several species of duck (*A. crecca, A. cyanoptera, A. discors*, *A. fulvigula*, and *A. acuta*) in Texas during February, prior to northward migration (Hanson et al. 2005), and ducks (including

A. platyrhynchos, A. discors, and *A. acuta*) in Alberta, Canada were shedding AI viruses after spring migration (Sharp et al. 1993).

Charadriiformes, especially the families Scolopacidae (sandpipers and turnstones) and Laridae (gulls and terns), are also major avian reservoirs of AI viruses (Olsen et al. 2006). Similar to waterfowl, shorebirds are relatively resistant to AI virus-induced clinical signs. However, unlike waterfowl, shorebirds are most commonly infected with H13 and H16 subtypes (Fouchier et al. 2005), and annual AI virus epizootics occur during spring (northward), rather than fall, migration (Krauss et al. 2004).

Aquatic Invertebrates

As filter feeders, bivalves pump large amounts of water across their gills, filtering particulate matter including potential food, microscopic plants and animals, and other benthic debris (Elston 1997). This feeding mechanism can result in the bioaccumulation of viruses that are in the water column. Typically, these viruses do not infect or replicate in bivalves, but these animals act as transient reservoirs for infectious virus particles (Elston 1997). The trapped viruses can be mechanically transmitted to susceptible vertebrate hosts through predation or scavenging (Elston 1997). Infectious viruses detected in bivalve tissues include enteroviruses, hepatitis A viruses, noroviruses, and rotaviruses (Meyers 1984, Le Guyader et al. 1994, Le Guyader et al. 2000, Lees 2000).

There have been mixed results of experiments investigating the potential for the bioaccumulation of influenza A viruses by bivalves. Stumpf et al. (2010) demonstrated that zebra mussels (*Dreissena polymorpha*) bioaccumulated an

infectious LPAI virus (H5N1) and maintained detectable infectious virus for 14 days. Huyvaert et al. (*unpublished data*) detected LPAI viral RNA in Asiatic clam (*Corbicula fluminea*) tissues from clams that were exposed to infected water for up to24 hours.. However, a study by Faust et al. (2009) indicated that Asiatic clams remove infectious LPAI and HPAI viruses (H3N8 and H5N1 subtypes, respectively) from the water, rendering the viruses innocuous to wood ducks (*Aix sponsa*).

Freshwater gastropods, such as snails, are primarily herbivores and detritivores, scraping algae and other food particles off of sediment in aquatic habitats such as lakes and ponds (Vaughn 2009). This feeding mechanism exposes snails to any contaminants that are electrostatically attracted to the sediments (Bitton 1980). Although no information exists regarding the capability of snails to retain viruses that are non-infectious to snails, freshwater snails are capable of bioaccumulating electrostatically charged environmental contaminants such as copper (Frakes et al. 2008). In addition, *Physa* spp. are known to accumulate toxins such microcystins (hepatotoxins produced by cyanobacteria) and likely transmit these toxins to predators via ingestion (Zurawell et al. 1999).

Freshwater snails, *Physa acuta* and *P. gyrina*, are distributed throughout North America. These species are habitat generalists and utilize a wide range of aquatic environments (Dillon et al. 2005, Turner and Montgomery 2009). *Physa* spp. are a prey item of several aquatic bird species including black-bellied tree ducks (*Dendrocygna autumnalis*), spectacled eiders (*Somateria fischeri*), upland sandpipers (*Bartramia longicauda*) and mottled ducks (*Anas fulvigula*) (Bolen and

Forsyth 1967, Brooks 1967, Robinson et al. 1997, Petersen et al. 2000, Houston and Bowen 2001, Bielefeld et al. 2010). In addition, gastropods are a common prey item of waterfowl such as mallards (*Anas platyrhynchos*) and mallards are generally considered important to the ecology of AI viruses (Swanson et al. 1985, Stallknecht and Shane 1988).

Research Questions

. The annual cyclical pattern of AI virus prevalence in waterfowl is driven by many ecological factors including environmental persistence and the migration patterns of key waterfowl species (Brown 2007). Avian influenza viruses persist in water for long periods and early migrating waterfowl species are factors in maintaining this cyclical pattern (Webster et al. 1978, Hinshaw et al. 1980, Stallknecht et al. 1990c). But there are likely other contributing factors driving this phenomenon.

All animals that share the aquatic environment with waterfowl are likely exposed to any AI viruses in the environment. However, no information exists on the influence of non-waterfowl species on AI virus ecology. Aquatic invertebrates, such as snails, are exposed to AI viruses whenever waterfowl are shedding virus into the shared habitat. What happens to AI viruses when snails are exposed? Do snails bioaccumulate AI viruses? How long do bioaccumulated AI viruses persist in snail tissues? Do bioaccumulated AI viruses remain infectious in snail tissues? Can snails act as mechanical vectors of AI virus via ingestion by waterfowl predators? And finally, do snails contribute to the annual cyclical pattern of AI virus prevalence observed in waterfowl?

These questions formed the motivation for a series of studies reported here evaluating: a) whether *Physa* spp. snails have SA receptors commonly found in aquatic bird species in the orders Anseriformes and Charadriiformes, b) whether and to what degree *Physa* spp. snails bioaccumulate a LPAI virus, and c) whether mallards allowed to ingest infected *Physa* spp. snails develop AI infection and transmit infectious virus to conspecifics.

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CHAPTER 1

EVALUATING A UBIQUITOUS FRESHWATER SNAIL (*PHYSA* SPP.) FOR INFLUENZA A VIRUS CELLULAR RECEPTORS

SYNOPSIS

Waterfowl are reservoirs for avian influenza (AI) viruses. Although there are multiple routes of transmission, these viruses are often transmitted via water. Persistence of infectious AI viruses in water allows transmission to occur days or possibly months later. This component of AI viral ecology enhances transmission of the virus between waterfowl because transmission among susceptible waterfowl is not constrained by direct contact. Aquatic invertebrates, such as snails, are also likely exposed to AI viruses while feeding on detritus in aquatic habitats and many waterfowl species feed extensively on gastropods. Any substances ingested by snails are likely ingested, secondarily, by waterfowl, thus potentially providing an additional transmission mechanism for AI viruses. In the following studies, I used lectin binding staining assays to determine whether *Physa* spp. snails have sialic acid receptors that are capable of binding to influenza A viruses. My results suggest that *Physa* spp. snails have sialic acid receptors that can bind waterfowl AI virus subtypes. The ability of snails to bind AI viruses suggests these snails are physiologically capable of bioaccumulating the viruses. Further, the bioaccumulation of AI viruses by snails may lead to

transmission of AI viruses through ingestion of the snails by susceptible species such as waterfowl.

INTRODUCTION

Aquatic birds are considered natural reservoirs for avian influenza (AI) viruses (Stallknecht and Shane 1988, Webster et al. 1992). Avian influenza (family *Orthomyxoviridae*, genus *Influenzavirus A*) viral infections in aquatic birds are subclinical, generally limited to the intestines, and are nearly ubiquitous in aquatic bird populations. Infected birds can shed infectious virus for several weeks (Webster et al. 1978, Hinshaw et al. 1980, Alexander 1993). Avian influenza virus is shed through feces into the environment, often into water and water is considered the primary transmission facilitator of AI viruses among waterfowl (Webster et al. 1978, Hinshaw et al. 1979, VanDalen et al. 2010).

Prevalence of AI virus infection among waterfowl populations is cyclical, with the highest shedding rate in North American birds during fall, prior to migration (Hinshaw et al. 1985). After breeding, North American waterfowl congregate in large interspecific groups in Canada and the United States prior to fall migration (Webster and Bean 1998, Elphick et al. 2001). Typically, the prevalence of AI viruses in these waterfowl populations is highest during this marshalling period, but once the fall migration begins, AI prevalence declines as birds move south (Webster and Bean 1998). Detection of AI viruses in waterfowl populations reaches very low levels by the time these birds reach their wintering grounds in late fall or early winter (Webster et al. 1976, Stallknecht et al. 1990c). The cyclical pattern of AI virus infection in waterfowl is likely due to an interaction

of factors including environmental viral persistence, and the migration patterns of multiple waterfowl species (Brown 2007).

Most research investigating environmental persistence of AI viruses has mainly centered on abiotic mechanisms. Avian influenza viruses can remain viable in water for up to four months allowing transmission to occur among individuals and populations without the need for direct contact (Hinshaw et al. 1979, Stallknecht et al. 1990b, Brown et al. 2007). Viral persistence in water is enhanced in turbid water because influenza A viruses have an affinity for suspended solids in the aquatic environment. Viruses that bind to these solids remain viable longer than unbound virus and bound particles may accumulate in the sediment (Bitton 1980). The affinity of viruses for suspended solids is due to the opposing electrostatic charges of virions and the particulate matter (Bitton 1980, Goyal et al. 1980, Chang et al. 1981), so animals feeding in the sediment are likely exposed to AI viruses when they are present.

Tadpole snails (*Physa acuta* and *P. gyrina*) have an extensive range in North America; they are found in virtually all freshwater habitats from the arctic regions south to Texas (Baker 1972, Dillon et al. 2005, Turner and Montgomery 2009). These snails feed exclusively on green algae and detritus on virtually all surfaces including pond sediment (Dillon 2000, Vaughn 2009). Tadpole snails are also known to bioaccumulate toxins including hepatotoxins; it is likely these toxins are transmitted to predators that consume affected snails (Zurawell et al. 1999). Predators of tadpole snails include fish, mammals, and other aquatic invertebrates (Dillon et al. 2005, Turner and Montgomery 2009). In addition,

tadpole snails are a prey item of several aquatic bird species including black-bellied tree ducks (*Dendrocygna autumnalis*), spectacled eider (*Somateria fischeri*), upland sandpiper (*Bartramia longicauda*), and mottled duck (*Anas fulvigula*) (Bolen and Forsyth 1967, Brooks 1967, Robinson et al. 1997, Petersen et al. 2000, Houston and Bowen 2001, Bielefeld et al. 2010). Further, gastropods in general are a prey item of several aquatic bird species including mallard (*Anas platyrhynchos*), northern shoveler (*A. clypeata*), ruddy duck (*Oxyura jamaicensis*), red knot (*Calidris canutus*), herring gull (*Larus smithsonianus*), great black-backed gull (*L. marinus*), and glaucous gull (*L. hyperboreus*) (Tinbergen 1961, Siegfried 1976, Swanson et al. 1985, Thompson et al. 1992, The Birds of North America 2004).

A requirement of AI virus infection is cellular binding; the primary event of infection is the binding of the viral hemagglutinin to sialic acid receptors on the surface of host epithelial cells (Suarez 2008). The conformation of the sialic acids is important in determining the susceptibility of the cells to AI viral binding. Most AI viruses bind to receptors with sialic acids having an α2,3 linkage to the penultimate galactose; swine influenza viruses bind preferentially to sialic acids with an α2,6 linkage (Wan and Perez 2006, Suarez 2008). Humans, swine, and several species of birds have been evaluated for sialic acid receptors capable of binding with influenza A viruses (Gambaryan et al. 2003, Thompson et al. 2006, Wan and Perez 2006), but very little information exists about the presence of receptors in aquatic invertebrates.

Bioaccumulation refers to the accumulation of substances (e.g., viruses, bacteria, toxicants) in the tissues of an organism through any exposure route, such as respiration, ingestion, or absorption (Farris and Van Hassel 2007). Aquatic invertebrates have been shown to bioaccumulate infectious viruses including enteroviruses, hepatitis A viruses, noroviruses, and rotaviruses (Meyers 1984, Le Guyader et al. 1994, Le Guyader et al. 2000, Lees 2000). Bioaccumulation occurs because these viruses bind to the intestinal epithelial cells of oysters, mussels, and clams (Tian et al. 2007). The ability of aquatic invertebrates to bioaccumulate viruses may play an important role in the transmission cycle of avian influenza viruses by providing an additional route of transmission. In order to examine snails as a viable candidate for AI viral bioaccumulation, it is important to demonstrate that there are sites within snail tissues that are capable of binding the viruses.

In this study, I stained snail tissues with lectins that bind to specific sialic acids (SA) to evaluate whether AI virus binding sites exist on snail epithelial cells. Using three lectins, I tested tissues from *Physa* spp. snails for the presence of three different sialic receptors: receptors found in two orders of aquatic birds (Anseriformes and Charadriiformes) and swine. The presence of these different lectins on the stained snail tissues would indicate that influenza A viruses that typically infect Anseriformes, Charadriiformes, and swine may also bind to snail tissues. The binding of AI viruses would represent the potential ability of the snail tissues to capture the viruses. In addition, depending on the location of the sialic acid receptors within the snails, potential binding sites may suggest that

bioaccumulation may occur. These potential binding sites may also lead to transmission of AI viruses to animals that ingest the snails.

METHODS

Sample collection and preparation. Snails were gathered during two collection events in fall 2010. Tadpole snails (*P. acuta* and *P. gyrina*, n = 300) were collected by hand from a private lake in Loveland, Colorado, USA. In both collection events, the snails were placed in 5-gallon buckets with lake water and transported to the National Wildlife Research Center in Fort Collins, Colorado, USA. The snails were transferred to four 12-gallon aquaria (Marineland, Cincinnati, OH) and fed algae wafers (Hikari, Himeji, Japan) and fresh greens. Water was partially (25%) changed weekly and completely changed once every 5 weeks.

In December 2010, 6 large snails (0.6 – 0.8g) were selected from the aquaria. The snails were placed in a 10% formalin solution, allowed to fix for 48 hours, and then transferred to 70% ethanol. After fixation, the snails were removed from the shell, sliced longitudinally, embedded in paraffin, cut with a microtome, and the cut sections were placed on positively charged microscope slides (Fisher Scientific, Pittsburgh, PA).

Tissue staining. I used two lectin binding assays to detect influenza A virus receptors in snails. One assay was designed to detect sialic acids that have an affinity for AI viruses (SAα2,3Gal). Further, this assay employs two lectins (MAA1 and MAA2) to differentiate between sialic acid receptors that are capable of binding with AI viruses that are typically found in Anseriformes

(SAα2,3Galβ1,4GlcNAc) and those typically found in Charadriiformes (SAα2,3Galβ1,3GalNAc). A second assay used *Sambucus nigra* agglutinin (SNA) to detect sialic acids that have an affinity for swine influenza viruses (SAα2,6) (Ito et al. 1998, Suzuki et al. 2000, Thompson et al. 2006).

These assays were performed several times on tissues from all 4 individual snails using multiple sections (n = 4) per individual. Each assay included three sections (slides) from two individuals; one received the lectin treatments, while the other served as a negative control and received only phosphate-buffered saline (PBS). Additionally, each assay included positive control tissues known to express the sialic acid receptors of interest. The positive control tissues included mallard intestines for the MAA1 and MAA2 assays, and swine trachea for the SNA assay (Ito et al. 1998).

I used protocols developed at the University of Georgia, Department of Pathology (see Appendix I). Briefly, slides were warmed at 62°C for 15 – 30 minutes, deparaffinized in xylene and ethanol baths and then rinsed in double distilled water. The slides were transferred immediately to warm citrate buffer and heated in a steamer (Black and Decker, New Briton, CT) for 35 minutes. After rinsing with double distilled water, the slides were incubated at room-temperature in 3% hydrogen peroxide for 15 minutes, and then rinsed again in double distilled water. These steps were conducted for both the MAA and SNA assays.

The assay to detect the AI virus receptors continued with multiple room-temperature incubation steps. First, slides were incubated in Carbo-Free

blocking solution (Vector Laboratories, Burlingame, CA) for 15 minutes, followed by a second incubation in fluorescein-conjugated *Maackia amurensis*-1 lectin (MAA1; Vector Laboratories, Burlingame, CA) for 1 hour. Subsequently, the slides received a second treatment with fluorescinated *Maackia amurensis*-2 lectin (MAA2; Vector Laboratories, Burlingame, CA), with an additional 1-hour incubation. The slides were incubated a fourth time for 2 hours with Streptavidin Alexa Fluor 546 (Invitrogen, Carlsbad, CA) to counterstain the tissues. The negative control slides were incubated with PBS in place of MAA1, MAA2, and Streptavidin. Finally, a drop of Prolong Gold (Invitrogen, Carlsbad, CA) was applied directly over each tissue section, and a cover slip was quickly placed on all slides. The slides were allowed to cure in the dark for 24 hours and then visualized under fluorescence microscopy.

The SNA assay to detect swine influenza virus receptors continues after the hydrogen peroxide treatment. Treated slides received fluorescein-conjugated *Sambucus nigra* and were incubated at room temperature for 1 hour, while the negative control slides received only PBS. After this step and a PBS rinse, Prolong Gold and a cover slip were placed on all slides. The slides were allowed to cure for 24 hours and visualized under fluorescence microscopy.

RESULTS

Results from the MAA 1 and MAA 2 assays demonstrated that only MAA 1 lectin bound to *Physa* spp. snail tissues, with all (n = 8 from 6 different snails) stained sections showing lectin binding; however, there was no evidence of MAA 2 binding in any snail tissue. The receptors were distributed in the digestive

system of the snails with Anseriformes-type sialic acid receptors found in the stomach and intestines (Figures 1.1 and 1.2). There was no evidence of fluorescence in any of the negative control tissues (n = 4; Figure 1.1). The positive-control mallard intestinal tissues showed lectin binding sites, and the untreated mallard tissues did not display any auto-fluorescence (negative control; Figures 1.3).

The SNA assays revealed no lectin-binding to snail tissues. The snail tissues did not show any evidence that swine or human influenza viruses would be able to bind to sialic acid receptors of the snail epithelial tissues. The positive control swine trachea sections showed lectin binding sites and the swine trachea did not display any auto-fluorescence (negative control; Figure 1.4).

DISCUSSION

The mechanisms that drive the cyclical pattern of AI virus infection prevalence in waterfowl are likely only partially understood. Water is generally considered an essential component in the transmission cycle of AI virus; however the source of AI virus that leads to inter-seasonal transmission is unknown. Viral persistence in water and early migrating waterfowl, such as blue-winged teal (*Anas discors*), may contribute to maintaining the cyclical prevalence patterns observed in waterfowl (Stallknecht et al. 1990c, Sharp et al. 1993, Hanson et al. 2005), but there may be other factors that preserve seasonal prevalence patterns.

As infected waterfowl shed AI virus into the water of lakes, ponds, and streams along the North American flyways, freshwater aquatic invertebrates are

potentially exposed to these viruses. The results from this study indicate that *Physa* spp. have sialic acid receptors on the gastrointestinal epithelial cells capable of binding to these AI viruses. The presence of these cellular receptors suggests that these snails may also be capable of bioaccumulating AI viruses.

The ability of tadpole snails to bioaccumulate AI viruses may impact AI ecology by extending the window for potential transmission of AI virus to susceptible avian hosts. Bioaccumulated AI virus in snails may be mechanically transmitted to snail predators such as mallards during periods when the viral titer of the water is too low to initiate direct transmission. Mallards are important in AI virus transmission cycles; these waterfowl are commonly infected with AI viruses, and shed high quantities of AI virus (Stallknecht and Shane 1988, Olsen et al. 2006). Mallards often share the aquatic habitat with *Physa* spp., and 25% of the typical mallard diet consists of gastropods (Swanson et al. 1985, Turner and Montgomery 2009). In addition, if *Physa* spp. snails are effective bioaccumulators of infectious AI viruses, snails have the potential to serve as mobile traffickers of AI virus. Tadpole snails commonly attach to floating debris such as leaves and logs (Vaughn 2009). As the flotsam moves with the currents, the snails, and any bioaccumulated AI virus, would move to new areas. This may provide AI viruses with a mechanism to move to locales that do not have infectious AI virus in the water and thus expose additional waterfowl to the virus through a non-traditional route. The presence of AI receptors in these snails suggests that the snails may contribute in some way to maintaining the cyclical transmission pattern seen in waterfowl.

Finally, I found no evidence that the snails used in this study possessed any of the cellular sialic acid receptors capable of binding to AI viruses that typically infect Charadriiformes, such as H13 and H16 subtypes (Fouchier et al. 2005). Several gull species consume freshwater snails (Tinbergen 1961, The Birds of North America 2004), but this study indicates that it is unlikely tadpole snails could act as a mechanical vector of these AI viruses. In addition, the snails lack influenza receptors capable of binding to swine influenza viruses, suggesting that swine foraging in aquatic habitats would be unlikely to be exposed to influenza A viruses via snails.

While these results suggest that the potential exists for bioaccumulation of Anseriformes-specific viruses to occur in freshwater snails, a great deal of research will be required before definitive conclusions about the role of aquatic snails in AI virus transmission can be made. In order to implicate *Physa* spp. snails in AI virus transmission, experiments to show actual viral bioaccumulation, the persistence of detectable infectious virus, and the likelihood of transmission via snail tissues will need to be conducted. Nevertheless, my results showing the presence of SAα2,3Galβ1,4GlcNAc receptors – those most commonly found in the enteric epithelial tissues of Anseriformes – suggests that the capacity for snail tissues to bind AI viruses exists and this highlights the notion that *Physa* spp. snails may play a role in the transmission of AI viruses among waterfowl.

FIGURES

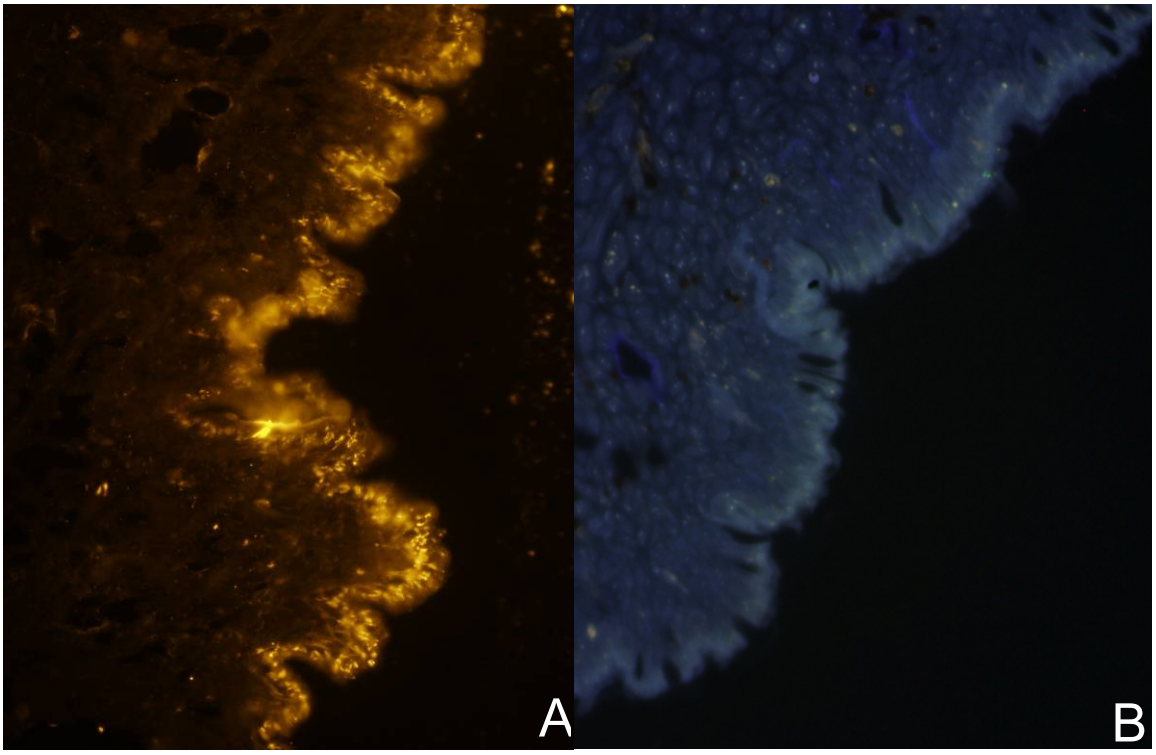

Figure 1.1. Intestinal tissues of tadpole snails (*Physa* spp.) stained for the presence of sialic acid receptors capable of binding with avian influenza viruses. A) is stained with the lectins Maackia amurensis (MAA) 1 and 2. The MAA1 (gold) has bound to potential sialic acids most commonly found in waterfowl (SAα2,3Galβ14GlcNAc), while there is no binding of MAA2 (green), a sialic acid receptor more commonly found in Charadriiformes species. B) is the corresponding negative control tissue; these tissues received only PBS in place of MAA1 and 2 lectins.

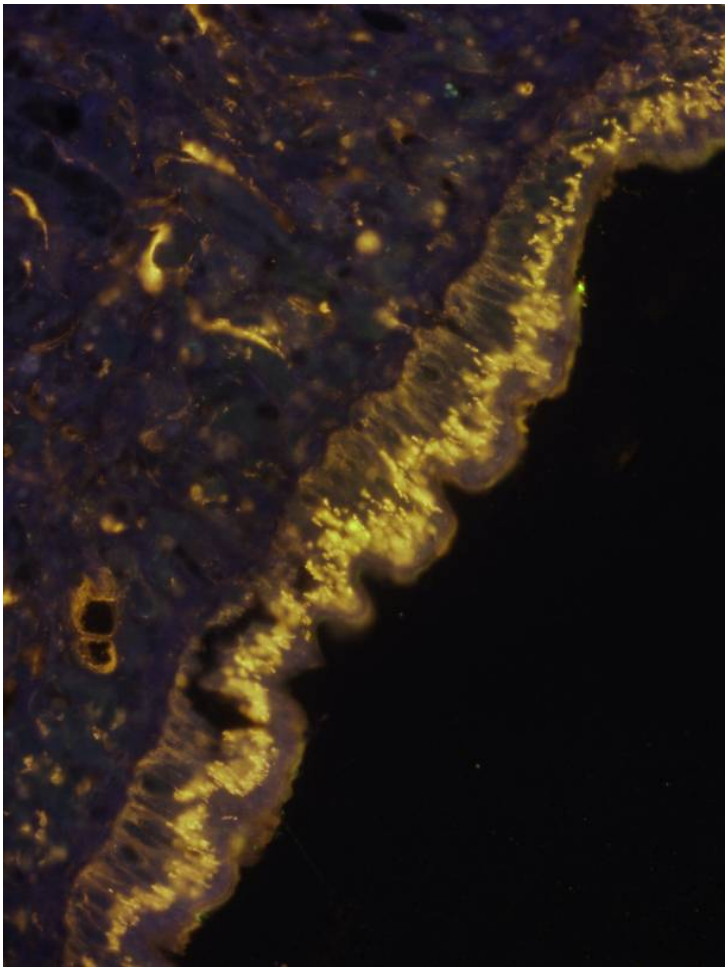

Figure 1.2. Stomach tissues of tadpole snails (*Physa* spp.) stained for the presence of sialic acid receptors capable of binding with avian influenza viruses. The tissues were stained with *Maackia amurensis* (MAA) 1 and 2 lectins. The MAA1 (gold) has bound to potential sialic acids most commonly found in Anseriformes species (SAα2,3Galβ14GlcNAc), while there is no binding of MAA2 (green) which would indicate the sialic acid more commonly found in Charadriiformes species.

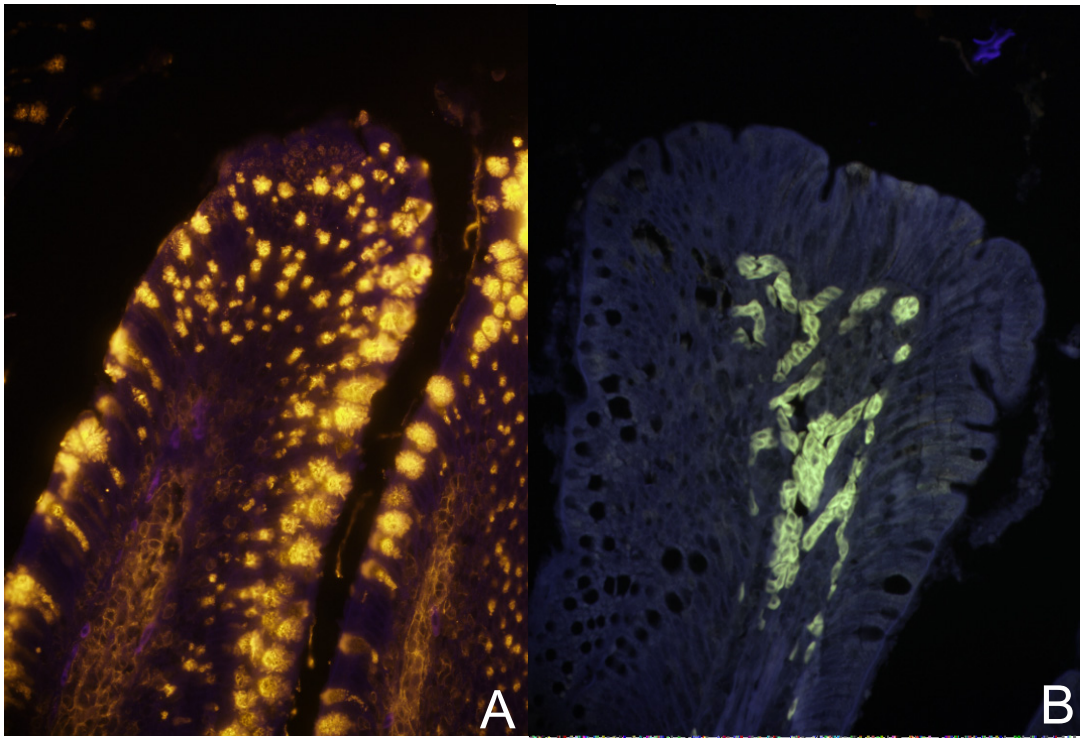

Figure 1.3. Mallard (*Anas platyrhynchos*) intestines stained for the presence of sialic acid receptors capable of binding with avian influenza viruses. The tissues, serving as positive control tissues, were stained with the lectins Maackia amurensis (MAA) 1 and 2. A) MAA1 (gold) has bound to sialic acid receptors most commonly found in Anseriformes (SAα2,3Galβ14GlcNAc), and MAA2 (green, not visible) preferentially binds with sialic acid receptors more commonly found in Charadriiformes (SAα2,3Galβ1,3GalNAc). B) shows the corresponding negative control tissue; this tissue received only PBS in place of MAA1 and 2 lectins.

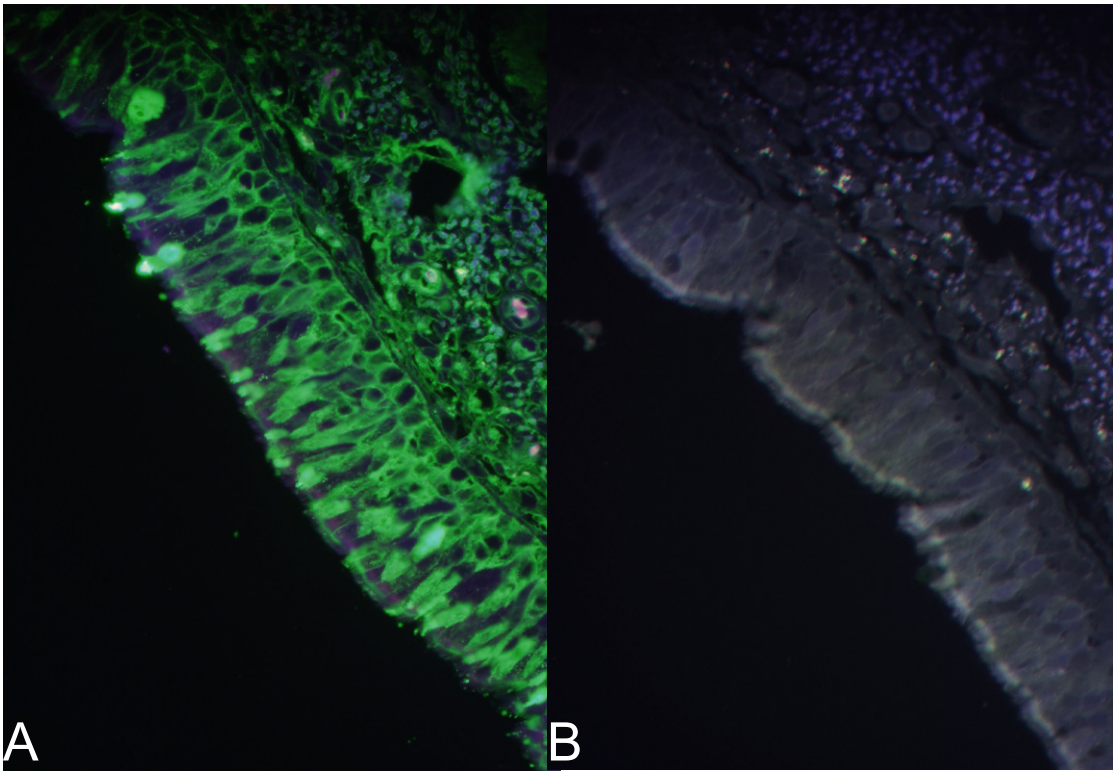

Figure 1.4. Swine (*Sus scrofa*) trachea stained with Sambucus nigra (SNA) for the presence of sialic acid receptors (SAα2,6Gal) capable of binding with swine influenza viruses. These tissues served as positive control tissues for the SNA assay. A) is stained with SNA represented by green demonstrating the presence of influenza A virus receptors. B) is the corresponding negative control tissue; this tissue received only PBS in place of SNA lectins.

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CHAPTER 2

BIOACCUMULATION AND PERSISTENCE OF AVIAN INFLUENZA VIRUS IN TADPOLE SNAILS (*PHYSA* SPP.)

SYNOPSIS

Environmental persistence of avian influenza (AI) viruses provides a mechanism for viral transmission among waterfowl without direct contact of individuals. Infected waterfowl shed AI viruses into the environment and AI viruses remain infectious in water for long periods, likely exposing multiple animal host species to the virus. Freshwater snails are likely exposed to AI viruses when infected waterfowl shed virus into lakes, ponds, and streams, and these snails are a common food source of many species of waterfowl. This trophic interaction may serve as a novel route of AI virus transmission. In the experiments described here, I exposed *Physa* spp. snails to an AI virus (H3N8) to determine whether snails can bioaccumulate the virus and, if so, how long the virus persists in the snail tissues. The snail tissues were destructively sampled and tested by quantitative real-time RT-PCR. The experiments demonstrated that *Physa* spp. snails do bioaccumulate AI viral RNA in their tissues at low titers for at least 96 hours. These results indicate that it may be possible for transmission to occur between waterfowl via ingestion of a natural invertebrate prey item.

INTRODUCTION

Aquatic birds, especially species in the orders Anseriformes (waterfowl such as ducks and geese) and Charadriiformes (gulls and terns) are considered the natural reservoirs for avian influenza (AI) viruses (Stallknecht and Brown 2008). Avian influenza (family *Orthomyxoviridae*, genus *Influenzavirus A*) virus infections in these birds are generally subclinical, but the birds can shed infectious virus for several weeks (Webster et al. 1978, Hinshaw et al. 1980, Alexander 1993). These viruses replicate well in the intestines and are shed in high concentrations in the feces; this results in infectious virus being deposited into the environment, especially water (Webster et al. 1978). Once in water, AI viruses can remain viable for up to four months allowing transmission to occur among individuals and populations without the need for direct contact (Stallknecht et al. 1990b, Brown et al. 2007).

Prevalence of AI virus infection among waterfowl populations is cyclical, with the highest shedding rate in North American birds occurring during fall, prior to migration (Hinshaw et al. 1985). After breeding but before migration, North American waterfowl congregate in large interspecific groups in Canada and the United States (Webster and Bean 1998, Elphick et al. 2001). Typically, the prevalence of AI viruses in these waterfowl populations is highest during this marshalling period, but once the fall migration begins, AI prevalence declines as the birds move south (Webster and Bean 1998). The frequency of AI virus infections in waterfowl populations is low by the time these birds reach their wintering grounds in late fall and early winter (Webster et al. 1976, Stallknecht et

al. 1990c). However, this AI virus transmission cycle begins again the next fall on the marshalling areas. The mechanisms that drive this AI virus transmission cycle are likely related to an interaction of several factors including environmental viral persistence, and the ecology of multiple waterfowl species (Brown 2007).

Most research investigating environmental persistence of AI viruses has centered on abiotic mechanisms. Avian influenza virus persistence in water is influenced by temperature, pH, salinity, and AI virus subtype (Stallknecht et al. 1990b, Brown et al. 2007). In addition, AI viruses have an affinity for suspended solids in the aquatic environment (Bitton 1980). Viruses that bind to these solids remain viable longer, allowing viruses to settle and accumulate in the sediment (Bitton 1980). Further work has demonstrated that AI viruses persist in ice, mud, and soil (Zhang et al. 2006, Vong et al. 2008). Few studies have examined potential biotic factors associated with AI viral persistence in aquatic environments.

Bioaccumulation refers to the accumulation of any substance (e.g., viruses, bacteria, toxins) in the tissues of an organism through any exposure route, such as respiration, ingestion, or absorption (Farris and Van Hassel 2007). Molluscs are known to bioaccumulate infectious viruses such as enteroviruses, hepatitis A viruses, noroviruses, and rotaviruses (Meyers 1984, Le Guyader et al. 1994, Le Guyader et al. 2000, Lees 2000). The viruses bind to the intestinal epithelial cells of the shellfish (Tian et al. 2007). Similarly, freshwater aquatic invertebrates are potentially exposed to AI viruses during the seasonal shedding of virus by waterfowl and may bioaccumulate virus in their epithelial cells. Zebra

mussels (*Dreissena polymorpha*) bioaccumulated and maintained infectious AI virus (low pathogenic H5N1) in their tissues for 14 days (Stumpf et al. 2010). In contrast, Asiatic clams (*Corbicula fluminea*) bioaccumulated LPAI, but the tissues failed to transmit AI virus to wood ducks (*Aix sponsa*) via ingestion (Faust et al. 2009). Nonetheless, no studies have examined the potential role of aquatic snails in AI virus transmission.

Tadpole snails (*Physa acuta* and *P. gyrina*) are distributed throughout North America; these species are habitat generalists allowing them to utilize a wide range of aquatic environments (Dillon et al. 2005, Turner and Montgomery 2009). These species feed exclusively on green algae and detritus (Dillon 2000, Vaughn 2009), and are known to accumulate toxins such as microcystins (hepatotoxins produced by cyanobacteria) through ingestion. These toxins are likely to be transmitted to predators as they consume these invertebrates (Zurawell et al. 1999). Tadpole snails are a prey item of several aquatic bird species including black-bellied tree ducks (*Dendrocygna autumnalis*), spectacled eider (*Somateria fischeri*), upland sandpiper (*Bartramia longicauda*) and mottled duck (*Anas fulvigula*) (Bolen and Forsyth 1967, Brooks 1967, Robinson et al. 1997, Petersen et al. 2000, Houston and Bowen 2001, Bielefeld et al. 2010). In addition, at least 51 species of aquatic bird species, including mallard (*Anas platyrhynchos*), northern shoveler (*A. clypeata*), ruddy duck (*Oxyura jamaicensis*), red knot (*Calidris canutus*), herring gull (*Larus smithsonianus*), great black-backed gull (*L. marinus*), and glaucous gull (*L. hyperboreus*)

consume gastropods (Tinbergen 1961, Siegfried 1976, Swanson et al. 1985, Thompson et al. 1992, The Birds of North America 2004).

Tadpole snails share aquatic environments with waterfowl, so it is likely they are exposed to AI viruses that have been shed into the water by infected birds. However, no information exists regarding the potential effects to predators and scavengers consuming these exposed snails. In chapter 1, I demonstrated that *Physa* spp. snails have sialic acid receptors capable of binding to AI viruses. To further study the potential relationship between freshwater snails and AI viruses, here I report on an experimental exposure study to evaluate the potential of *Physa* spp. snails to bioaccumulate AI viruses. In the following experiments, I exposed tadpole snails (*Physa acuta* and *P. gyrina*) to an AI virus to determine whether: 1) snails bioaccumulate AI viruses, 2) snails bioaccumulate AI viruses differently depending on exposure method, 3) the presence of a snail affects the viral concentration of the water in aquaria, and 4) how long AI virus persists in snail tissues.

METHODS

Snail collection. Freshwater snails (*P. acuta* and *P. gyrina*, n = 300) were collected by hand from a private lake in Loveland, CO on two occasions in August 2010. In both collection events, the snails were placed in 5-gallon buckets with lake water and transported to the National Wildlife Research Center in Fort Collins, Colorado. The snails were transferred to four 12-gallon aquaria (Marineland, Cincinnati, OH) and fed algae wafers (Hikari, Himeji, Japan) and fresh greens. Water was partially (≈25%) changed weekly and completely

changed once every 5 weeks. Prior to each experiment, snails were transferred to small containers (BacT Bottle, Fisher Scientific, Pittsburgh, PA), one snail per aquarium, these served as mini-aquaria. The mini-aquaria were partially filled with well water (collected from Colorado State University Foothills Fisheries Laboratory, Fort Collins, CO) leaving space (3 – 5 cm) above the waterline. The mini-aquaria were loosely covered to allow air exchange but to prevent snail escape. In addition to water, the snails were provided with algae and greens as food items, and each time the aquaria water was replaced, new food items were added to the aquaria. Only snails greater than 80 mm were used in the following experiments and the temperature in the room that the aquaria were housed was maintained at 17 – 20°C.

Exposure

Virus preparation. An AI virus isolate (A/H3N8/mallard/CO/2008) from a mallard used in an unrelated experimental infection study at the National Wildlife Research Center (originally collected from wild bird feces A/H3N8/mallard/C0/187718/2008) was propagated in embryonated chicken eggs, resulting in a viral stock of 10^7 EID_{50}/mL. The inoculum was made by diluting the virus stock in BA-1 (M199, 0.05 M Tris, pH 7.6,1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin, and1 g/ml amphotericin B) resulting in an inoculum of approximately 10^5 EID_{50}/mL.

Experiment 1. To test for viral bioaccumulation in snail tissues, the aquaria with snails were randomly assigned to one of two treatment groups: inoculation (n =

40), and negative control (n = 10) and snails were transferred to new mini-aquaria with fresh well water (50 mL). The inoculation group received 0.5mL of the AI virus inoculum making the concentration of virus in the aquaria water approximately 10^3 EID_{50}, while the negative control received 0.5 mL of BA-1.

Immediately after inoculation, a 1 mL water sample was collected from each aquarium (beginning-exposure water), and the snails were allowed to remain in the inoculated water for 36 hrs. After this exposure period, the ending-exposure water sample (1 mL) was collected, and the snails were rinsed with 10 mL of fresh well-water and transferred to new aquaria with fresh well water (50 mL); this is the depuration period. After 2 hours of depuration, half of the aquaria were sampled (inoculated n = 20, and negative-control n = 5); a sample of the depuration water (1 mL), and the snail were collected. After 24 hours of depuration, the remaining aquaria were sampled (depuration water and snail; inoculated, n = 20; negative control, n = 5).

Snails collected at the end of the depuration periods were removed from the shell by cutting along the inner spiral of the shell until the body became free. The shell-less snail was placed in a microcentrifuge tube (Fisher Scientific, Pittsburg PA) with 1mL BA-1, and a single 4 mm stainless steel ball bearing (Grainger, Fort Collins, CO). The samples were stored on wet ice until processing. Once in the lab, the tissues were placed in chilled racks (TissueLyser Adapter Set, Qiagen, Valencia, California) and agitated for 10 min at 25 Hertz using a Mixer Mill homogenizer (Retsch MM301, Newton,

Pennsylvania), and then centrifuged (10,000×*g*) for 3 minutes. The supernatant was transferred to cryovials and frozen at -80°C.

Experiment 2. To test for the best exposure method, 144 aquaria with 100 mL well-water were randomly assigned to 2 treatments, single- or multiple-inoculation (n = 72/treatment). These groups were further divided into 3 groups each: the inoculation group (TX) received a snail and an inoculation (n = 40/treatment), no-snail negative control (SC) received an inoculation but no snail (n = 16/treatment), and no-virus negative control (VC) received a snail but no virus (n = 16/treatment). Each group was further divided equally into 4 subgroups corresponding to sampling and inoculation time stamps, detailed below and in Figure 2.1.

On the first day of exposure, all TX and SC aquaria were inoculated with the viral stock (10^5 EID_{50}), with the single-inoculation group receiving a full dose (1 mL) of inoculum creating an estimated 10^3 PCR EID_{50}, and the multiple-group received a one-quarter dose (0.25 mL) creating an estimated $10^{2.4}$ EID. The single- and multiple-inoculation VC subgroups received 1 mL or 0.25 mL of BA-1 respectively.

After 24 hours, the aquaria water was replaced with fresh well-water in all TX and VC subgroups (both single- and multiple-inoculation). Snails from all single-inoculation TX and SC aquaria subgroups, one multiple-inoculation TX subgroup, and one multiple-inoculation VC subgroup were allowed to depurate. The remaining multiple-inoculation TX and SC aquaria were re-inoculated with another one-quarter dose (0.25 mL) of viral stock, and multiple-inoculation VC

subgroups received another 0.25 mL aliquot of BA-1. The following day, the depurating snails were removed from aquaria and sampled and processed as outlined above (Figure 2.1).

Each subsequent day, from 2 – 4 days post-inoculation (DPI), the water in the aquaria was replaced with fresh well-water in all remaining TX and VC subgroups (multiple inoculation). One subgroup from the multiple-inoculation treatment group was allowed to begin depurating, while the remaining multiple-inoculation TX and SC subgroups were re-inoculated with another one-quarter dose (0.25 mL) of viral stock, and remaining multiple-inoculation TX and SC subgroups received 0.25mL BA-1 on each of these days. Lastly, the multiple-inoculation SC subgroup corresponding to the newly depurating subgroup was removed after sampling.

On the final day of the experiment (5 DPI), the remaining subgroup of depurating snails (single-inoculation TX and VC, and multiple-inoculation TX and VC) were removed from the depurating water, sampled and processed as above.

A water sample (1 mL) was collected from each aquarium after each inoculation (beginning-exposure water) and again 24 hours later (ending-exposure water). After the depuration period, a sample of the depuration water (1 mL) and the entire snail were collected. The snail tissue was processed as previously described for experiment 1. All samples were initially stored on wet ice and then frozen at -80°C after each sampling session.

Experiment 3. To test for the effects of snails on viral persistence, 72 new aquaria were prepared with fresh well water (100 mL). Snails (n = 60) were

transferred to the new aquaria (1 snail/aquarium), and the aquaria with snails were randomly assigned to one of two treatment groups: inoculated (INOC, n = 48), or no virus negative control (NV, n = 12). The remaining 12 aquaria served as the no snail negative control (NS). The INOC and NS groups received 1 mL of the AI virus inoculum pipetted directly into the water of each aquarium resulting in a viral titer of approximately 10^3 EID_{50}.mL, while the negative virus control group received 1 mL of BA-1.

The snails were allowed to remain in the inoculated water for 96 hours. At the end of this exposure period, each snail was removed from the inoculated water, rinsed with 10 mL fresh well water using a serological pipette, and transferred to a new aquarium with fresh well water (100 mL). The NS group aquaria were sampled and removed (detailed below). The INOC and NV groups were allowed to depurate for 24 – 192 hours.

After 24 hours of depuration, one-quarter of the snails from INOC (n = 12) and NV (n = 3) aquaria were removed. The remaining snails were allowed to continue to depurate. This step was repeated at 48 and 96 hours for an additional one-quarter of the snails from both the INOC and NV groups. The remaining snails from the INOC and NV aquaria (n = 12 and n = 3 respectively) were sampled after 192 hours of depuration. In addition, depuration water was replaced at 48, 96, and 144 hours for the aquaria that continued to depurate past 48 hours.

Immediately after inoculation, a 1 mL water sample was collected from each aquarium (beginning-exposure water), and a second water sample (1 mL;

ending-exposure water) was collected at the end of the exposure period (96 hours). Samples of depuration water (1 mL) and snail tissues were collected at the end of the depuration period for each snail and its aquarium. The snail tissue was processed as previously described for experiment 1. All samples were initially stored on wet ice and then frozen at -80°C after each sampling session.

Sample testing. All samples were tested in duplicate by quantitative real-time RT-PCR (qrtRT-PCR) for viral RNA detection and quantification using a protocol developed at the National Veterinary Services Laboratories (United States Department of Agriculture, Ames, IA). Ribonucleic acid (RNA) was extracted using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Primers and probe specific for the influenza A virus matrix gene developed by Spackman et al (2003) were used in conjunction with the ABI One-step RT-PCR master mix and the ABI 7900 Real Time PCR System (Life Technologies Corporation, Carlsbad, CA) with thermocycler conditions developed by Aguero et al (2007). All samples related to the same aquarium (beginning-exposure water, ending-exposure water, depuration water, and snail tissue) were tested in duplicate on the same extraction plate to reduce the possibility of inducing an extraction effect on the analysis. Calibrated controls with known viral titers (10^2 EID_{50}/mL–10^5 EID_{50}/mL) were included on each plate to construct a 4-point standard curve. Sample viral RNA quantities were extrapolated from the standard curves and presented as PCR EID_{50} equivalents/mL. Tissue samples were considered positive if both replicate qrtRT-PCR results were greater than 10^0 PCR EID_{50} equivalents. Experiments conducted at the National Wildlife

Research Center demonstrate that the qrtRT-PCR assay used to analyze the samples can detect known influenza A viral titers of 10^0 EID_{50} 90% of the time (unpublished data); both of the replicate samples tested were required to be above this threshold to be considered positive.

Data Analyses

The qrtRT-PCR data from both experiments were analyzed using SAS 9.2 software (SAS Institute, Cary, NC). In addition, Akaike's Information Criterion, with small sample size correction (AICc) was used for model selection and multimodel inference (Burnham and Anderson 2002). Models with a ΔAICc < 2 retained enough weight to be considered viable.

Experiment 1. In the first snail exposure experiment, I used Proc MIXED implemented in SAS to determine whether AI virus RNA was retained in the snail tissues. I constructed 5 models to determine the effects of a viral inoculation and depuration hours had on the detection of AI viral RNA in the snail tissues. The model set included a model with no effects, two models with a continuous inoculation effect, or depuration hours (2 and 24 hours; categorical), and two models with additive and interactive effects. The two main effects (viral inoculation and depuration hours) were considered fixed effects and the detection of viral RNA in tissues was the dependent variable (continuous).

Experiment 2. For the second experiment, I analyzed the PCR data using Proc MIXED to examine the effects of exposure method (single vs. multiple inoculations) on the detection of AI viral RNA in snail tissues and depuration water. To accomplish this, I constructed 5 models to determine the effects of the

exposure method, and viral titer of the beginning-exposure water. The model set included a model with no effects, two models with exposure method (single or multiple inoculation) or a continuous inoculation effect (EID_{50} equivalents/mL), and two models with additive and interactive effects. The exposure method and inoculation effect were considered fixed and the detection of viral RNA in tissues was the dependent variable.

Experiment 3. In the third experiment I performed two analyses of the PCR data. In the first analysis, I used Proc GLIMMIX to examine the effects of the viral titer of the beginning-exposure water and depuration duration on the detection of virus in the snail tissues. I constructed 5 models to evaluate the effects of depuration time and viral titer of beginning-exposure water on the likelihood of a PCR-positive snail. The model set included a model with no effects, two models with viral titer of beginning-exposure water (continuous) and depuration duration effect (categorical; 1, 2, 4, or 8 days), and two models incorporating both additive and interactive effects of these variables. The two main effects (beginning-exposure water and depuration duration) were considered fixed effects and the detection of viral RNA in tissues (binomial) was the dependent variable.

In the second analysis, I used Proc MIXED implemented in SAS to evaluate the presence of snails on viral RNA decline in water. Viral decline was determined by change in viral titers between the beginning-exposure water and the ending-exposure water (96 hour exposure). All samples without virus were excluded from the analysis. I developed 5 models including a model with no

effects, two single effect models (i.e., presence of a snail and viral titer of the beginning-exposure water), a model incorporating additive effects of snail presence and viral titer of the beginning-exposure water, and a fully interactive model. The variables snail presence (categorical) and viral titer of the beginning-exposure water (continuous) were treated as fixed effects while the dependent variable, viral decline, was continuous.

RESULTS

Experiment 1. The data from the first experiment indicate that AI viruses appear to be retained at low titers in the tissues of snails for up to 24 hours, with 22% (9 of 40) of snail tissues testing positive for AI viral RNA, with 30% (n = 20) of snail tissues testing positive after 2 hours depuration, and 15% (n = 20) of snail tissues testing positive after 24 hours of depuration (Table 2.1). The top model included the single effect of beginning-exposure water viral titer and held an AICc weight of 0.65 (Table 2.2). The top three models carried a total AICc weight of more than 0.95. The cumulative AICc weights for the variables within the model set were 0.32 for depuration hours and 0.96 for viral titer of the inoculating dose (Table 2.3).

Experiment 2. The data from the second experiment regarding inoculation method showed that snail tissues also retained AI viruses with 14% (11 of 80) testing positive for AI virus RNA (Table 2.4)**.** In addition, the top model in the analysis evaluating the effects of exposure method on viral titer carried an AICc weight of 0.88 and included the single effect of exposure method (Table 2.5). Using estimates of the total quantity of virus detected in the snail tissue as a

function of exposure method from the top model, higher viral titers resulted from the single inoculation group compared to the multiple inoculation group (single inoculation 3.86 PCR EID_{50} equivalents, SE 1.61; multiple inoculation 1.93 PCR EID_{50} equivalents, SE 0.81). Nevertheless, the two exposure methods had overlapping confidence intervals (Figure 2.2). The evidence ratio of the top model to the only other model carrying any weight (no effects; AICc weight = 0.12) was 7.3:1, indicating that the top model was seven times more likely than the model of no effect.

Experiment 3. Results from the third experiment showed that the proportion of qrtRT-PCR-positive tissue samples declined from 33% (4/12) at 24 hours to 0% by 96 hours post-depuration (Figure 2.3). In addition, viral RNA was undetectable in the depuration water by 48 hours post-depuration. Both the viral titer of the beginning-exposure water and depuration duration influenced the detection of AI virus by qrtRT-PCR. The top two models were the interactive model, which held an AICc weight of 0.66, and the additive model which carried an AICc weight of 0.22 (Table 2.6). Thus, the likelihood of detecting AI virus in snail tissues is dependent on both viral titer of the beginning-exposure water and depuration duration. The remaining models, the two single effect models (viral titer of the beginning-exposure water or depuration duration) and the model of no effect carried a cumulative AICc weight of 0.12, demonstrating that these variables alone had little influence on the detection of AI virus RNA in snail tissues. The results indicate that exposing snails to AI viruses may lead to bioaccumulation of AI viruses, but viral persistence in the tissues may be short-

lived as demonstrated by the lack of positive samples past 48 hours of depuration (Figure 2.3).

Analysis of the effect of snails on viral decline in the aquarium water suggested that the presence of snails had an effect on viral decline. The top model was the additive model which included both the viral titer of the beginning-exposure water and the presence of a snail; this model carried an AICc weight > 0.99 (Table 2.7).

DISCUSSION

The mechanisms that drive the cyclical pattern of AI virus infection prevalence in waterfowl are only partially understood. Environmental persistence and early migrating waterfowl species such as blue-winged teal (*Anas discors*) likely contribute significantly to maintaining AI viral infection in waterfowl species (Brown 2007). However, there are probably additional dynamics that influence the seasonal prevalence patterns of AI virus infection. The ability of aquatic invertebrates to bioaccumulate AI viruses may be a factor in the cyclical AI virus prevalence patterns seen in waterfowl, particularly if aquatic invertebrates harboring bioaccumulated virus in their tissues are consumed as prey items.

A previous study (Chapter 1), showed that *Physa* spp. snails have sialic acid receptors like those of Anseriformes (ducks, geese, swans) suggesting that, if *Physa* spp. snails bioaccumulate AI virus in their tissues, they may be an important biotic component of AI virus transmission in the wild. The results from these experiments indicate that tadpole snails do bioaccumulate AI viruses in their tissues. This is evidenced by snail tissues testing positive for viral RNA in

all three experiments, and data showing that the presence of a snail affects viral decline in aquaria water. While viral titers in snail tissues were relatively low (< $10^{1.8}$ PCR EID_{50} equivalents) and lasted less than four days, these results suggest that snails could act as mechanical vectors.

Both the first and third experiments reported here showed that the concentration of virus at the beginning of the exposure period was important in terms of whether or not virus was detected in snail tissues and that the duration of persistence of virus in snail tissues declined with lengthening depuration duration. These results indicate that snail exposure events of low concentration, as might occur when only small numbers of infected waterfowl are depositing virus into the aquatic environment, are likely less important for virus transmission and persistence compared to high concentration events as might happen at the beginning of the marshaling period.

In addition, freshwater snails are likely exposed to low titers of AI virus nearly continuously during marshaling and fall migration, so the multiple inoculation method is probably a more ecologically relevant exposure method. However, the results from the second experiment demonstrated that any difference between exposure methods for detecting AI viruses by RT-PCR is likely not biologically significant. Rather, a single exposure of a snail to virus, if delivered during a window of opportunity for transmission (e.g., pre-breeding or during the marshaling period) and is of high concentration, may contribute to viral persistence in a wild system.

Direct exposure to infectious virus in water is likely a more efficient transmission mechanism for AI viruses to susceptible hosts like waterfowl (Webster et al. 1978, Hinshaw et al. 1980, Alexander 1993, VanDalen et al. 2010). Avian influenza viruses remain infectious for long periods, as long as 54 days at 17°C (Stallknecht et al. 1990b), whereas, the AI virus in the snail tissues in these experiments was undetectable by four days post-depuration. This implies that water is an ideal transmission mechanism, and in a lake-type setting, where the water currents are with relatively limited, recently shed AI virus would remain in the water column, available to other waterfowl. However, in a stream-type setting, where waters are constantly flowing, any AI virus in the water column moves downstream. Avian influenza viruses that are bound to the sediment have an extended persistence and remain more stationary (Bitton 1980), making the infectious virus more readily available to animals that feed in the sediment, including aquatic invertebrates such as snails. This could mean that the viral titer of the snail tissues may be higher than the water, making the snails a source of infectious virus in stream settings.

Several aspects of AI virus ecology were not addressed in these experiments. The experiments described in this chapter were conducted using one AI virus subtype, and two snail species from the same genus, in well-water with no sediment, at 17 – 20°C yet all of these factors are known to contribute to AI virus persistence in water (Bitton 1980, Stallknecht et al. 1990b, Brown et al. 2007). It is likely these factors also affect the uptake of virus by snails and the persistence of AI in snail tissues. Decreasing the water temperature would have

increased viral persistence in the water (Brown et al. 2007), and presumably viral persistence in snail tissues. However, because the feeding rate of snails is influenced by temperature, food ingestion and viral uptake should be slower at lower temperatures (Navarro et al. 2002, Selck et al. 2006). This indicates that there may be an optimal temperature range for both AI virus persistence in water and nutrient uptake by snails; this optimal temperature likely varies depending on snail species (Navarro et al. 2002, Selck et al. 2006).

In addition, sediment was excluded from these experiments to reduce confounding variables, but the presence of sediment may change the viral uptake. Tadpole snails gleaning detritus and algae feed from the sediment, ingesting particulate matter (Vaughn 2009); and AI virus is electrostatically attracted to the sediments (Bitton 1980, Goyal et al. 1980, Chang et al. 1981). These aspects of snail ecology and viral properties suggest the presence of sediments would likely increase viral exposure of the snails

Additional studies are needed to show whether snails harboring bioaccumulated AI viruses in their tissues contribute to transmission. Studies to determine whether bioaccumulated virus remains infectious and experiments demonstrating whether snails can act as mechanical vectors via ingestion by susceptible waterfowl are essential. Mallards (*Anas platyrhynchos*) are important AI virus reservoirs and likely have a significant impact on the seasonal variation of AI virus prevalence in aquatic environments (Stallknecht and Shane 1988). Further, mallards consume gastropods, including snails, throughout the year and a typical female mallard's diet includes as much as 25% snails in the

pre-breeding season (Swanson et al. 1985). While water appears to be a better medium for the persistence of AI viruses based on the experiments presented here, no studies have yet examined whether transmission of AI viruses occurs via ingestion of snail prey by susceptible waterfowl and how this mechanism may fit into the larger question about the maintenance of the cyclical pattern of AI virus infection prevalence observed in waterfowl.

TABLES

Table 2.1. Summary of results from an experimental demonstration of the possible short-term bioaccumulation of AI viral RNA in snail (*Physa* spp.) tissues. Aquarium water titers are means of 20 aquaria per group. Snail tissues were tested after depuration and were considered positive if both PCR replicates were positive and had an average viral concentration of at least 10^0 PCR EID_{50} equivalents.

Depuration hours	Aquarium water titers (log_{10}+1)		Snail tissues			
			Number		Titers (log_{10}+1)	
	0 hrs	36 hrs	Tested	Positive	Average	Range
2	3.42	3.00	20	6	0.85	0.00 – 1.86
24	3.41	2.93	20	3	0.43	0.00 – 1.31

Table 2.2. Candidate model set and model rankings for an experiment (Experiment 1) analyzing viral RNA detection in snail tissues as a function of the viral titer of the inoculating water (IW, continuous) and depuration duration in hours (Dh; 2, 24). K is the number of parameters in each model, the -2logL is the -2log likelihood, AICc is Akaike's Information Criterion with a small sample size correction factor, ΔAICc is the AIC difference of each model relative to the best model in the model set, and the AICc weights quantify the probability a model is the best model given the data and the model set.

Model	K	-2logL	AICc	ΔAICc	AICc Weight
Beginning-exposure water (BEW)	**3**	**381.70**	**387.95**	**0.00**	**0.651**
Dh+BEW	4	380.50	388.92	0.97	0.247
Dh+BEW+(Dh*BEW)	5	379.70	390.34	2.39	0.060
Intercept-only	2	386.90	391.02	3.07	0.030
Depuration hours (Dh)	3	385.70	391.95	4.00	0.012

Table 2.3. Cumulative AICc weights for variables within the candidate model set assessing the effects of viral titer of the water and depuration duration on AI viral persistence in snail tissues (see also Table 2.2). The AICc weights represent the cumulative weight of evidence for the effect of a particular variable on viral RNA detection in snail tissues by qrtRT-PCR.

Effect	Cumulative AICc weights
Main effects	
Depuration hours	0.318
Beginning-exposure water (BEW)	0.958
Two-way interactive	
Depuration hours * Beginning-exposure water (BEW)	0.060

Table 2.4. Summary of results from an experimental demonstration of the possible short-term bioaccumulation of AI viral RNA in snail (*Physa* spp.) tissues. Aquarium water titers are means of 12 aquaria per group. The aquaria water samples from the multiple inoculation subsets were tested 24 hours after each inoculation. Snail tissues were tested after depuration and are considered positive if both PCR replicates were positive and had a viral concentration of $>10^0$ PCR EID_{50} equivalents.

		Aquarium water					Snail tissues			Depuration water		
	Sub-	Titers (log_{10}+1)		Inoc-	Depuration		RNA	Titers (log_{10}+1)		RNA	Titers (log_{10}+1)	
Group	set	0 hrs	24 hrs	ulation	hours	Aquaria	+	Avg	Max	+	Avg	Max
Multiple inoculation	1	1.90	1.49	1	24	10	1	0.08	0.34	1	0.29	1.02
	2	2.03	1.55	1	24	10	0	0.03	0.12	0	0.03	0.06
		1.88	1.67	2								
	3	2.01	1.53	1	24	10	1	0.11	0.51	0	0.05	0.21
		1.79	0.81	2								
		1.86	1.62	3								
	4	2.18	1.66	1	24	10	2	0.31	0.92	0	0.02	0.09
		2.16	1.79	2								
		2.12	2.02	3								
		2.18	1.77	4								
Single inoculation	1	2.74	2.49	1	24	10	4	0.92	1.76	1	0.15	0.47
	2	2.74	2.44	1	48	10	1	0.20	0.70	0	0.12	0.23
	3	2.76	2.38	1	72	10	1	0.08	0.43	0	0.06	0.17
	4	2.81	2.53	1	96	10	1	0.19	0.57	0	0.16	0.45

Table 2.5. Candidate model set and model rankings for an experiment (Experiment 2) evaluating viral RNA detection in snail tissues as a function of exposure method (EM, 1, 2) and viral titer of the inoculating water (IW, continuous). K is the number of parameters in each model, the -2logL is the -2log likelihood, AICc is Akaike's Information Criterion with a small sample size correction factor, ΔAICc is the AIC difference of each model relative to the best model in the model set, and the AICc weights quantify the probability a model is the best model given the data and the model set.

Model	K	-2logL	AICc	ΔAICc	AIC Weight
Exposure method (EM)	**3**	**538.60**	**544.92**	**0.00**	**0.885**
Intercept-only	2	542.80	546.96	2.04	0.115
Beginning-exposure water (BEW)	3	552.00	558.32	13.40	0.000
IW+ BEW	4	547.90	556.43	11.52	0.000
IW+ BEW +(IW* BEW)	5	555.90	566.71	21.80	0.000

Table 2.6. Candidate model set and model rankings for an experiment (Experiment 3) evaluating the effects of the viral titer of the beginning-exposure water and depuration duration on detection of AI virus RNA by qrtRT-PCR in *Physa* spp. snail tissues. Models in this set included viral RNA detection in snail tissues as a function of the viral titer of the inoculating water (IW, continuous) and depuration duration (DD; 1, 2, 4, 8 days). K is the number of parameters in each model, the -2logL is the -2log likelihood, AICc is Akaike's Information Criterion corrected for small sample sizes, ΔAICc is the AICc difefrence of each model relative to the best model (most supported model = 0) in the model set, and AICc weights quantify the probability a model is the best model given the data and the model set.

Model	K	-2logL	AICc	ΔAICc	AICc Weight
BEW+DD+(BEW*DD)	**4**	**609.67**	**617.00**	**0.00**	**0.663**
BEW+DD	3	612.78	618.78	1.78	0.218
Intercept-only	2	615.90	619.90	2.90	0.071
Beginning-exposure water (BEW)	3	614.76	620.76	3.76	0.030
Depuration duration (DD)	3	615.33	621.33	4.33	0.017

Table 2.7. Candidate model set and model rankings for an experiment (Experiment 3) evaluating viral decline in aquarium water over 24 hours as a function of the presence of a snail (Snail; 0, 1) and viral titer of the beginning-exposure water (IW). K is the number of parameters in each model, the -2logL is the -2log likelihood, AICc is Akaike's Information Criterion with a small sample size correction factor, ΔAICc is the AIC difference of each model relative to the best model, and the AICc weights quantify the probability a model is the best model given the data and the model set.

Model	K	-2logL	AICc	ΔAICc	AIC Weight
Snail+BEW	**4**	**596.30**	**604.67**	**0.00**	**0.996**
Beginning-exposure water (BEW)	3	604.80	611.02	6.35	0.002
Snail+BEW+(Snail* BEW)	5	600.10	610.67	5.99	0.002
Intercept-only	2	805.40	809.51	204.84	0.000
Presence of snail (Snail)	3	795.10	801.32	196.65	0.000

FIGURES

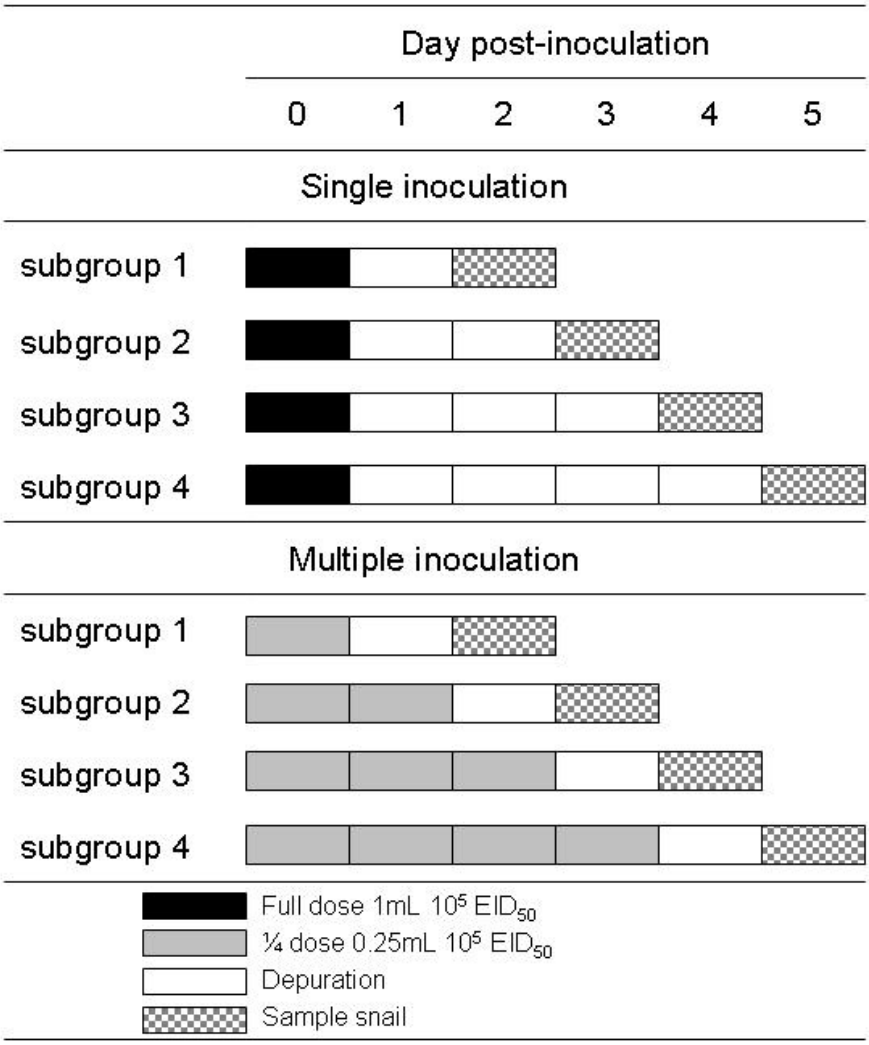

Figure 2.1. Experimental design and sampling scheme of exposure methods for an experiment evaluating the degree of virus accumulation by snails depending on inoculatuon mode (Experiment 2). *Physa* spp. snails were exposed to avian influenza virus (H3N8) in two different ways. One set of treatment groups was exposed in a single inoculation while the others received multiple inoculations. Both treatments had 3 groups, and each group was equally divided into four subgroups. All aquaria waters were sampled at the time of inoculation and again 24 hours later; water from the multiple-inoculation treatment groups were sampled multiple times, after each inoculation and again 24 hours later. The aquaria water and snail tissues were sampled at the end of depuration.

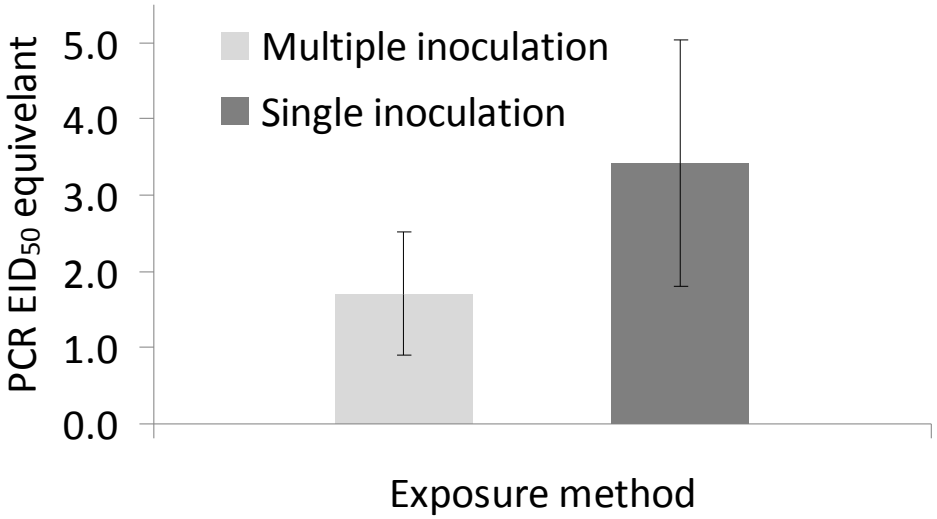

Figure 2.2. Comparison of two methods of exposure of a freshwater snail (*Physa* spp.) to avian influenza virus (AI; H3N8). Aquaria with 1 snail each (n = 40/group) were inoculated with either a single full dose (1 mL), or 1 – 4 ¼ doses (0.25 mL) of 10^5 EID_{50} (egg infectious dose 50). The snails were exposed, allowed to depurate and their tissues were tested by quantitative real-time RT-PCR. The PCR results were compared to positive control samples with known titers (EID_{50}) measured by virus isolation in embryonated chicken eggs.

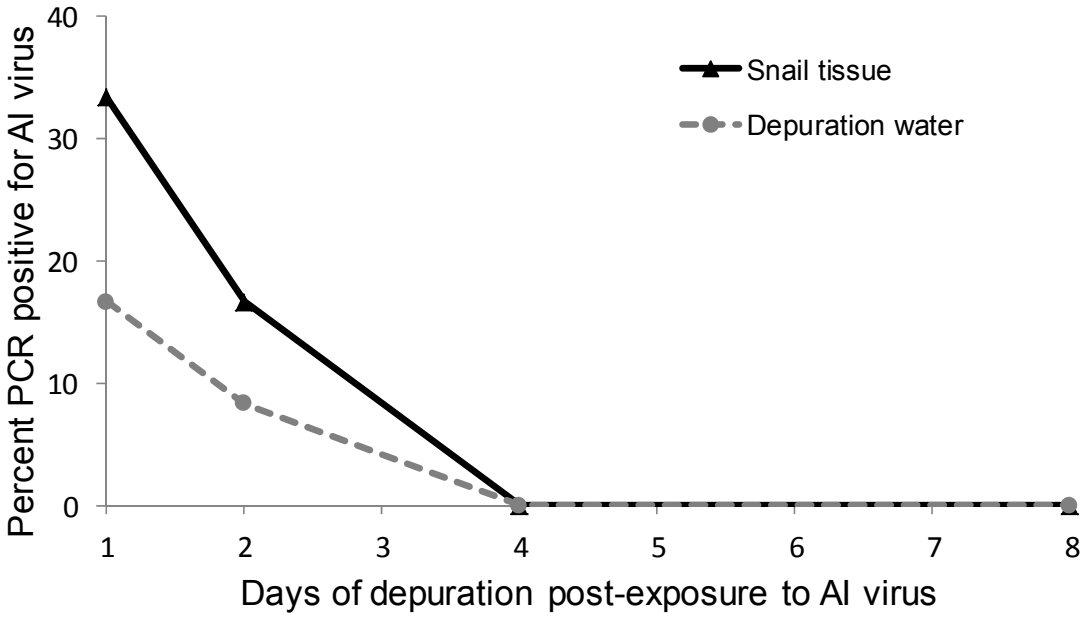

Figure 2.3. *Physa* spp. snails exposed to avian influenza virus for 24 hours and then sampled after depuration durations of 1, 2, 4, or 8 days (n = 12/time period). The samples were tested in duplicate by quantitative real time RT-RCR and tissues were considered positive if both wells were above 10^0 PCR EID_{50} equivalents.

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CHAPTER 3

EXPERIMENTAL TRANSMISSION OF A LOW PATHOGENIC AVIAN INFLUENZA VIRUS BETWEEN MALLARDS VIA FRESHWATER SNAILS (*PHYSA* SPP.)

SYNOPSIS

The ability of avian influenza (AI) viruses to remain infectious in water for extended periods provides the virus a mechanism that allows transmission to occur long after shedding birds have left the area. However, this also exposes other aquatic animals to AI viruses, including freshwater invertebrates. Previous experiments demonstrated that AI viral RNA can be sequestered in snail tissues. In this study, I determined whether freshwater snails (*Physa acuta* and *P. gyrina*) can serve as a mechanical vector of AI viruses via ingestion. In the first experiment, I exposed 20 *Physa* spp. snails to an avian influenza virus (H3N8) and inoculated embryonated chicken eggs with the homogenized snail tissues. The results indicate that sequestered AI viruses remain infectious in snail tissues, with 10% of the snails infecting eggs. In a second experiment, I exposed snails to water contaminated with the feces of AI virus-inoculated mallards. The exposed snails were fed to an immunologically naïve group of mallards to evaluate ingestion of freshwater snails as an alternate route of AI virus transmission. None of these naïve mallards developed an infection indicating

that transmission via ingestion likely did not occur. Results of this study suggest that this particular trophic interaction may not play an important role in the transmission of AI viruses in aquatic habitats.

INTRODUCTION

Wild birds, especially species in the orders Anseriformes (waterfowl such as ducks and geese) and Charadriiformes (gulls and terns) are considered the natural reservoirs for avian influenza viruses (Stallknecht and Shane 1988, Webster et al. 1992). Avian influenza (AI; family *Orthomyxoviridae*, genus *Influenzavirus A*) viruses are transmitted among aquatic birds primarily through an indirect fecal-oral route involving fecal-contaminated water in aquatic habitats (Webster et al. 1978). Avian influenza virus infections in these birds are generally subclinical, but the birds can shed infectious virus for several weeks (Webster et al. 1978, Hinshaw et al. 1980, Alexander 1993). Environmental persistence in water allows transmission to occur among individuals and populations without the need for direct contact (Stallknecht et al. 1990b, Brown et al. 2007).

Prevalence of AI virus infection among waterfowl populations is cyclical, with the highest shedding rate in North American birds occurring during fall, prior to migration (Hinshaw et al. 1985). After breeding but before migration, North American waterfowl congregate in large interspecific groups in Canada and the United States (Webster and Bean 1998, Elphick et al. 2001). Typically, the prevalence of AI viruses in these waterfowl populations is highest during this marshalling period, but once the fall migration begins, AI prevalence declines as

the birds move south (Webster and Bean 1998). The frequency of AI virus infections in waterfowl populations is virtually undetectable by the time these birds reach their wintering grounds in late fall and early winter (Webster et al. 1976, Stallknecht et al. 1990c). Prevalence reaches high levels again in the fall on the marshaling areas and this cycle of AI viral prevalence in waterfowl is repeated annually. Nevertheless, the mechanisms that drive this pattern are only partially understood. Environmental persistence and migration habits of multiple waterfowl species are likely critical to maintaining this cyclical pattern (Brown 2007), but there may be other factors that contribute to this facet of AI virus ecology.

Most research investigating environmental persistence of AI viruses has mainly centered on abiotic mechanisms. Avian influenza virus persistence in water is influenced by temperature, pH, salinity, and AI virus subtype. (Stallknecht et al. 1990b, Brown et al. 2007). In addition, AI viruses have an affinity for suspended solids in the aquatic environment (Bitton 1980). Viruses that bind to these solids remain viable longer, allowing viruses to settle and accumulate in the sediment (Bitton 1980). Other researchers have demonstrated that AI viruses persist well in ice, mud, and soil (Zhang et al. 2006, Vong et al. 2008).

Few studies have examined potential biotic factors associated with viral persistence in the aquatic environment. Bioaccumulation refers to the accumulation of substances (e.g., viruses, bacteria, toxicants) in the tissues of an organism through any exposure route, such as respiration, ingestion, or

absorption (Farris and Van Hassel 2007). Freshwater aquatic invertebrates are potentially continuously exposed to AI viruses during the seasonal shedding period of waterfowl. Bioaccumulation of virus by aquatic invertebrates may impact the ecology of AI viruses by creating a "reservoir" for the virus and extending the infectious period. Alternatively, aquatic invertebrates, such as Asiatic clams, appear to remove infectious viruses from the environment (Faust et al. 2009). Zebra mussels (*Dreissena polymorpha*) bioaccumulated and maintained infectious AI virus (low pathogenic H5N1) in their tissues for 14 days (Stumpf et al. 2010). Asiatic clams (*Corbicula fluminea*) bioaccumulated LPAI, but the tissues failed to transmit AI virus to wood ducks (*Aix sponsa*) (Faust et al. 2009). In any case, no studies have examined the potential role of aquatic snails in AI virus persistence and transmission.

Tadpole snails (*Physa acuta* and *P. gyrina*) are found throughout North America in virtually all freshwater habitats (Dillon et al. 2005, Turner and Montgomery 2009). These snails feed exclusively on green algae and detritus on the sediment (Dillon 2000, Vaughn 2009), and are known to accumulate toxins such as microcystins (hepatotoxins produced by cyanobacteria) through ingestion which they likely transmit to predators (Zurawell et al. 1999). *Physa* spp. are a prey item of several aquatic bird species including black-bellied tree ducks (*Dendrocygna autumnalis*), spectacled eiders (*Somateria fischeri*), upland sandpipers (*Bartramia longicauda*) and mottled ducks (*Anas fulvigula*) (Bolen and Forsyth 1967, Brooks 1967, Robinson et al. 1997, Petersen et al. 2000, Houston and Bowen 2001, Bielefeld et al. 2010). In addition, at least 51 species of

aquatic bird species, including mallards (*A. platyrhynchos*), northern shovelers (*A. clypeata*), ruddy ducks (*Oxyura jamaicensis*), red knots (*Calidris canutus*), herring gulls (*Larus smithsonianus*), great black-backed gulls (*L. marinus*), and glaucous gulls (*L. hyperboreus*) consume gastropods (Tinbergen 1961, Ingolfsson 1976, Siegfried 1976, Swanson et al. 1985, Thompson et al. 1992, The Birds of North America 2004).

Physa spp. share the aquatic environment with waterfowl and it is likely that these snails are exposed to AI viruses that have been shed into the water by infected birds. In Chapter 1, I demonstrated that *Physa* spp. snails have sialic acid receptors capable of binding avian influenza viruses, similar to those found in Anseriformes, and in Chapter 2, I showed that these snails can bioaccumulate AI viral RNA. In the following experiments, I exposed tadpole snails (*Physa acuta* and *P. gyrina*) to an AI virus to determine whether: 1) AI viruses remain infectious after bioaccumulation in snail tissues, and 2) AI viruses can be mechanically transmitted from infected mallards to naïve mallards via ingestion of snails harboring infectious virus.

METHODS

Snail collection. Tadpole snails (*P. acuta* and *P. gyrina*, n = 300) were collected by hand from a private lake in Loveland, CO on two occasions during August 2010. Snails were placed in 5-gallon buckets with lake water, transported to the National Wildlife Research Center (NWRC) in Fort Collins, CO, and transferred to four 12-gallon aquaria (Marineland, Cincinnati, OH). The snails were maintained for eight months and fed algae wafers (Hikari, Himeji, Japan), and spinach

supplied ad libitum. Adult snails in the colony repeatedly laid eggs and numerous eggs hatched. Subsequently, the offspring of captive snails were retained and used in these experiments.

Virus preparation. An AI virus isolate (A/H3N8/mallard/C0/2008) from a cloacal swab of an experimentally infected mallard (originally collected from wild bird feces A/H3N8/mallard/C0/187718/2008) was propagated in embryonated chicken eggs, resulting in a viral stock at 10^7 EID_{50}. The inoculum used here was made by diluting the viral stock in BA-1 (M199, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/ml penicillin, 100 g/ml streptomycin, and1 g/ml amphotericin B) to obtain an inoculum concentration of approximately 10^6 EID_{50}.

Snail Experiment

Inoculation. One day before inoculation, 36 large (shells 9 – 11 mm in length) adult snails (*Physa* spp.) were transferred to three 4L glass beakers (12 snails/beaker) and these were partially filled with 2L of well-water (collected from Colorado State University Foothills Fisheries Laboratory, Fort Collins, CO). A spinach leaf and algae wafers were provided as food items. The beakers were moved into a biosafety cabinet, and the snails were allowed to acclimate to this new environment for 24 hours. On inoculation day, two aquaria were inoculated with 2mL of prepared AI virus stock creating aquaria water with a virus concentration of approximately 10^3 EID_{50} and the third aquarium was inoculated with 2mL of BA-1 to serve as a negative control. Water in all three aquaria was agitated to create a homogeneous mixture.

Exposure and depuration. Snails were maintained in these beakers with virus-spiked water for 72 hours. After this exposure period, individual snails were removed from the aquaria, rinsed with 10 mL fresh well-water, and transferred to three clean glass beakers with 2 L of fresh well-water and new food items. The transferred snails were allowed to depurate for 24 hours.

Sampling. Immediately after inoculation, a 1 mL water sample (beginning-exposure water) was collected from each aquarium. At the end of the exposure period (72 hours), a second water sample (ending-exposure water; 1 mL) was collected. A depuration water sample (1 mL) was collected at the end of the depuration period. Finally, snail tissues were harvested, and any egg sacs laid during depuration were opportunistically collected. The beginning-exposure and ending-exposure water samples were stored at -80°C until testing and the depuration water, snail tissues, and egg sacs were stored at -20°C for <24 hours before testing.

Snails collected at the end of the depuration period were removed from the shell by cutting along the inner spiral of shell until the body was freed from the shell. The shell-less snail tissue was placed in a microcentrifuge tube (Fisher Scientific, Pittsburg, PA) with 1 mL BA-1, and a single 4 mm stainless steel ball bearing (Grainger, Fort Collins, CO). The samples were stored on wet ice until processing. Once in the lab, tissues were placed in chilled racks (TissueLyser Adapter Set, Qiagen, Valencia, California) and agitated for 10 min at 25 Hertz using a Mixer Mill homogenizer (Retsch MM301, Newton, Pennsylvania),

followed by centrifugation at 10,000×*g* for 3 min. The supernatant was then transferred to cryovials.

Viral Assays. To conserve resources, only subsets of samples were tested by virus isolation (VI; assay details are given below) and quantitative real-time reverse transcriptase polymerase chain reaction (qrtRT-PCR; assay details are given below). Thirty-seven samples were tested by VI in chicken embryos. To confirm viral inoculation of the water, the subset included three initial water samples (n = 1/treatment beaker), and three ending-exposure water samples (n = 1/treatment beaker). To detect viral bioaccumulation by snails the subset also included 25 snail tissues (n = 10/exposed beaker, and n = 5 negative control). To detect any release of virus by snails, five depuration water samples (n = 2/exposed beaker, n = 1 negative control), and one egg sac sample (exposed) were tested.

Thirty samples were tested by qrtRT-PCR for viral RNA detection and quantification. To confirm and quantify the viral titer of the beginning-exposure and ending-exposure water samples, this subset included three initial water samples (n = 1/ treatment beaker), three second water samples (n = 1/ treatment beaker), and inocula. In order to identify potential bioaccumulated virus and virus defecated into the depuration water as AI virus, 20 snail tissues (n = 8/exposed group, and n=4 negative control), five depuration water samples (n = 2/exposed group, n = 1 negative control), and one egg sac sample (exposed) were tested qrtRT-PCR. Table 3.1 summarizes these assay subsets.

Mallard Experiment

Mallards. Thirty one-day-old mallard ducklings were purchased in April 2011 (Stomberg's Chicks and Game Birds, Pine River, MN). The birds were raised indoors for 4 – 8 weeks (room size 3.8×3.7×2.6 m), fed commercially available chick starter feed, and provided with water enhanced with an electrolyte/vitamin powder (Durvet, Blue Springs, MO) ad libitum in poultry waterers; pools for bathing were added to the pens when mallards were 10 days old for enrichment. A pre-experiment blood sample (0.6 mL) and cloacal swabs (placed in BA-1) were collected from each individual when the ducklings were 20 days old. The blood was stored at -20°C for 24 hours until testing; the swab samples were stored at -80°C until testing.

Inoculated Group

Housing. A group of 12 mallards was randomly selected from the set of 30 animals at four weeks old. This cohort served as the inoculation group. These birds were separated into four sets of three animals each (3 inoculated sets and one negative control set) and housed apart from the remaining 18 in four pens (2.6×2.2×2.1m). A temporary wall (Zipwall® Arlington, MA) was used to partition a portion of the room to house the negative control birds. Each pen included a 60-liter stock tank to serve as a pond, a poultry waterer, and food bowls.

Snails were moved into the building on the same day as the mallards. Four 12-gallon aquaria (200 – 300 snails/aquarium) were placed adjacent to the treatment pens, and the entire volume of water in each snail aquarium was replaced with fresh well-water and food items. In addition, each pen/aquarium

combination had a 200-liter storage tank filled with fresh well-water to be used to replenish ponds (Figure 3.1). To facilitate water transfer from the ponds to the snail aquaria and from the storage tanks to the ponds, 12-volt pumps (LVM, Hoddesdon. Herts, UK) were placed in each mallard pond and water storage tank.

Inoculation. Two days after the first cohort of mallards was moved into the new building the mallards (n = 9 in 3 pens) were inoculated with 1 mL of approximately 10^6 EID_{50} of AI virus. The inoculum for each bird was divided between routes of exposure: each bird received a portion of the inoculum orally (0.6 mL), intranasally (0.2 mL), and intraocularly (0.2 mL). The intranasal and intraocular inoculations were equally divided between the nares or orbits of each bird. Three mallards serving as negative controls were sham-inoculated, as described above, with 1mL of BA-1 (Figure 3.2).

Transferring water. On 2 days post-exposure (DPE), approximately 40 L of water was siphoned out of each aquarium. The aquaria were refilled with water pumped directly from the mallard pond, and the mallard ponds were refilled with clean well-water from the storage tank. A similar process was repeated daily on 3 – 7 DPE; however, the water that was transferred into the aquaria was a 5:1 mixture of fresh well-water and mallard pond water. This change was made because the snails had moved out of the aquaria water likely due to the high concentration of nitrogen compounds in the mallard pond effluent.

Sampling. Oropharyngeal and cloacal swabs were collected from all 12 mallards on days 2 – 7 DPE; swabs were placed in BA-1 and stored at -80°C.

Additionally, water samples (1 mL) were collected from the mallard pond and aquaria daily during this period. After sample collection on 7 DPE, all 12 mallards were euthanized with an intravenous injection of Beuthanasia®-D Special (1 mL/kg; Schering-Plough, Summit, NJ).

Snails. Snails were initially exposed to mallard pond water two days after the mallards were inoculated, and were continuously exposed to the mallard pond water for more than 8 days. Following this exposure period, the snails were removed from the aquaria and rinsed with fresh well-water. The food items were removed, the aquaria were disinfected (10% bleach solution), rinsed, and refilled with fresh well-water. The snails were placed back into the aquaria, and new food items were added to the aquaria. The snails were allowed to depurate for 24 – 36 hours.

Snail-exposed Group

Housing. Twelve more mallards were randomly selected from the remaining set of 18 at six weeks of age. This cohort served as the snail-exposed group. Like the first cohort, these birds were separated into four sets of three animals each (3 inoculated sets and one negative control set) and housed in the same pens as the inoculated ducks.

Exposure. Twelve hours after these mallards were placed in the new pens, each was caught, restrained, hand-fed 1g of virus-exposed snails, and released back into the pen. The following day, 6 – 7 g of virus-exposed snails was offered in a bowl in each pen and the mallards were allowed to free-feed until the snails were consumed. At the time of feeding, ten snails from each aquarium were

retained to assess tissue concentrations of virus in the snails offered to mallards. These snails were removed from the shell, placed in 1.0mL BA-1 (1 snail/vial), and homogenized as described earlier.

Sampling. Oropharyngeal and cloacal swabs were collected from all 12 mallards on days 2 – 7 DPE; swabs were placed in BA-1 and stored at -80°C. Additionally, water samples (1 mL) were collected from the aquaria daily during this period. At 14 DPE, blood was collected from all 12 mallards (0.6 mL) and the mallards were euthanized after sampling with an intravenous injection of Beuthanasia®-D Special (1 mL/kg).

Unexposed Group. The final six mallards remained in the pre-exposure housing room. These birds were maintained as described earlier and were isolated from the exposed mallards and snails; these mallards served as additional negative controls for the ELISA assays. At eight weeks old, after the preceding mallard experiment, these mallards were bled for a second time (0.6 mL). The blood samples were centrifuged (10,000×*g* for 5 min) and the serum was tested for influenza A virus antibodies by ELISA prior to storage.

Sample Testing

Swabs, Water and Snails. Oral and cloacal swabs, water samples, and snail tissues were temporarily stored on wet ice and then frozen at -80°C prior to testing. These samples were tested by qrtRT-PCR for the presence of the influenza A virus matrix gene. To conserve resources, inoculated mallard swab samples (n = 9) were only tested on alternating days; swab samples collected from four mallards on days 2, 4, and 6 DPE were tested, and swab samples

collected from the remaining five mallards on days 3, 5, 7 DPE were tested (Table 3.1). All swabs collected from the snail-inoculated mallards, all water samples from both mallard cohorts and snail tissue samples were tested by qrtRT-PCR as detailed below.

Blood. All blood samples were centrifuged (10,000×*g* for 5 min) and the serum was evaluated for influenza A virus antibodies using a commercially available blocking enzyme-linked immunosorbent assay (bELISA; FlockCheck AI MultiS-Screen antibody test kit, IDEXX Laboratories, Westbrook, ME).

Assays

Virus Isolation. I used published protocols (Szretter et al. 2006) for this assay. In brief, 200 embryonated specific pathogen-free (SPF) chicken eggs were incubated for 10 days. Eggs were inoculated (0.5 mL) with water or snail sample supernatant (0.5 mL/snail) in replicate (five eggs/sample); positive and negative control eggs were included in the assay. The eggs were incubated for five additional days. Each day, eggs were inspected for signs of egg death such as unresponsive embryo or degraded blood vessels. Allantoic fluid was collected from dead eggs (eggs that were dead by 1 day post-inoculation were discarded), and tested by hemagglutination assay (HA) for virus following Spackman (2008). Allantoic fluid was also cultured on blood agar to detect any pathogenic bacteria. Samples that caused egg death after 1 day post-inoculation and were HA positive were tested by qrtRT-PCR to confirm the presence of influenza A virus RNA.

Real time RT-PCR. I used a protocol developed at the National Veterinary Services Laboratories (United States Department of Agriculture, Ames, IA) for the qrtRT-PCR. All samples were tested in duplicate. RNA was extracted using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Primers and probe specific for the influenza type A matrix gene developed by Spackman et al. (2003) were used in conjunction with the ABI one-step RT-PCR master mix and run on an ABI 7900 Real Time PCR System (Life Technologies Corp, Carlsbad, CA) with thermocycler conditions developed by Aguero et al (2007). Calibrated controls with known viral titers (10^2 EID_{50}/mL–10^5 EID_{50}/mL) were included on each plate to construct 4-point standard curves. Sample viral RNA quantities were interpolated from the standard curves and presented here as PCR EID_{50} equivalents/mL. Samples were considered positive if both replicate results were greater than 10^1 PCR EID_{50} equivalents.

RESULTS

Snail Experiment

Two of twenty (10%) of the virus-exposed snail tissue samples caused egg death and were positive by HA and by qrtRT-PCR. All of the initial water (2/2) and second water (2/2) samples of both exposed beakers were positive by both VI and qrtRT-PCR. The mean titer of the initial water was $10^{3.0}$ PCR EID_{50} equivalents, and declined to $10^{2.0}$ PCR EID_{50} equivalents in the second water samples. Only 50% (2/4) of the depuration water was VI and HA positive for infectious influenza A viruses and none were PCR positive. All of the negative control samples were negative by HA, VI, and qrtRT-PCR.

Mallard Experiment

Prior to the experiment, all 30 mallards were negative for AI virus antibodies by bELISA. All 12 inoculated mallards became infected with AI virus as indicated by oral and cloacal shedding. Oral and cloacal swabs from the inoculated mallards (2 and 7 DPE) tested positive for high titers of influenza A virus by qrtRT-PCR (Figure 3.2). Pond water samples taken from pens of inoculated mallards all tested positive for the presence of AI virus by qrtRT-PCR. Aquarium water samples tested positive for AI virus RNA for all days between 2 and 11 DPE.

The swabs collected from snail-exposed mallards were all negative by qrtRT-PCR. In addition, the blood samples from these birds tested negative by bELISA. However, there was serologic activity in the snail-exposed mallards with two individual mallards with a absorbance change between the pre-exposure bleed and the post-exposure bleed >0.25 absorbance units. The negative control mallards as well as the unexposed group all had an absorbance change < 0.05 units. Finally, 13% (4/30) of the snail tissues exposed to the duck pond water were positive for AI viral RNA by RT-PCR.

DISCUSSION

The prevalence of AI virus infection in waterfowl follows an annual cycle, but our knowledge of the factors that influence this cycle is incomplete. Early migrating waterfowl species may contribute to maintaining AI viral infection in waterfowl species, because this migration pattern leaves large segments of the waterfowl population naïve to the currently circulating AI virus strain. The

persistence of AI viruses in water most likely plays a vital role in maintaining the annual cycle because it allows the exposure of immunologically naïve waterfowl to AI viruses weeks after infected birds leave the area (Brown 2007). Additional factors, such as the persistence of virus in aquatic invertebrate prey items, may also contribute to the seasonal prevalence patterns of AI virus infection.

Avian influenza virus persistence in water enhances viral transmission among waterfowl, but it also exposes other animals to the virus including aquatic invertebrates. Bioaccumulation of AI viruses by these invertebrates could provide an additional route of exposure particularly through ingestion of them as prey items. Previous experiments (Chapters 1 and 2) showed that *Physa* spp. snails are capable of bioaccumulating AI viruses at low titers for at least 48 hours. This experiment provides additional data, revealing that bioaccumulated AI viruses remain infectious in snail tissues and that mallards will readily eat virus-exposed snails, but the quantity of snails fed to the mallards was likely too low to produce viral shedding, or a significant antibody response.

The quantity of snails fed to the mallards in the second experiment was likely smaller than what some waterfowl consume regularly. Mallards are important AI virus reservoirs and likely have a significant impact on the seasonal variation of AI virus prevalence in aquatic habitats like marshaling and breeding areas (Stallknecht and Shane 1988). Mallards consume snails throughout the year, but snail consumption increases in spring, prior to the breeding season; 25% of the female mallard diet consists of snails during this period (Swanson et al. 1985). While no published data exist describing the total volume of food

consumed by wild mallards, the recommended volume of feed for captive mallards is 120g of feed/day (Ash 1969). Assuming that wild mallards eat as much food as captive mallards, they may consume as much as 30g of snails/day. Consumption of a larger quantity of virus-exposed snails, even at the low titers in tissues demonstrated in chapter 2, suggests that infection of mallards via ingestion of snails could occur.

Although transmission did not occur in this study, two mallards that had fed on exposed snails demonstrated a change in antibody levels, while the mallards fed on unexposed snails showed virtually no change in antibody levels. All of the sera samples were considered negative for antibodies to influenza A virus, but the difference in the change of absorbance levels for two of the fed mallards suggests that the concept of AI virus transmission by consumption of freshwater snails may be worth exploring further.

These experiments demonstrated that infectious AI virus is maintained briefly in snail tissues, long enough for tissue samples to be infectious to embryonated eggs. However, transmission of an AI virus (H3N8) to mallards through ingestion failed to occur. This study, along with previous experiments described in this dissertation, demonstrates that, while snails may be capable of serving as mechanical vectors for the transmission of AI viruses, the window of opportunity would likely be short. This brief period of infectiousness would not likely have a substantial impact on the transmission or maintenance of AI viruses. Nevertheless, snails may play a minor role in the maintenance of AI virus infections in wild waterfowl either directly through ingestion of larger quantities of

infected snails, through ingestion of more snails with a higher viral titer or indirectly through the act of waterfowl feeding on snails which may expose the birds to any AI viruses in the environment, thus facilitating transmission. These questions and others regarding the optimal environmental conditions for virus persistence and transmission in aquatic environments warrant additional careful study under both laboratory and wild conditions.

TABLES

Table 3.1. A synopsis of the samples collected and tested from both the snail exposure experiment and the mallard/snail transmission experiment. Due to financial limitations, not all samples collected were tested. The sample testing scheme was weighted towards the exposed snails in the snail experiment and the snail-exposed mallards in the mallard experiment.

Samples			Assays		
Type	Collected	Tested	PCR	VI	ELISA
Snail Experiment					
Aquaria (n = 3)					
Initial water	6	6	3	3	
Second water	6	6	3	3	
Depuration water	6	6	5	5	
Snails (n = 36)					
Tissues	36	25	20	25	
Mallard Experiment					
Inoculated mallards (n = 12)					
Oral swabs	72	36	36		
Cloaca swabs	72	36	36		
Pens (n=4)					
Pond water	24	24	24		
Aquaria	24	24	24		
Snails (n=1000)					
Tissues	40	40	40		
Snail-exposed mallards (n=12)	72	72	72		
Oral swabs	72	72	72		
Cloaca swabs	12	12			12
Blood					
Unexposed mallards (n = 6)	6	6			6
Blood					

FIGURES

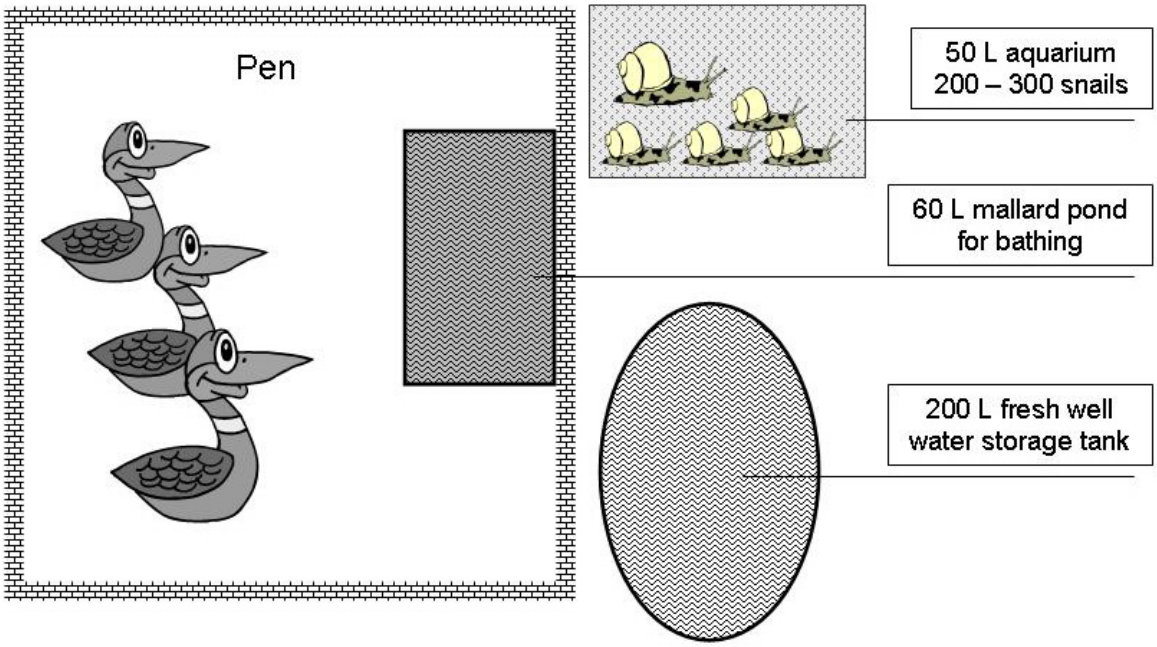

Figure 3.1. Mallard pen configuration with aquarium, duck pond, and water storage. Each pen had a mallard pond inside for the mallards to bathe, and an aquarium and extra water storage tank placed just outside of the pen. After the mallards were inoculated (2 – 7 days post-exposure), a portion of aquarium water was drained, pond water was pumped into the aquarium to replace the drained water, and then water was pumped from the storage tank into the mallard pond.

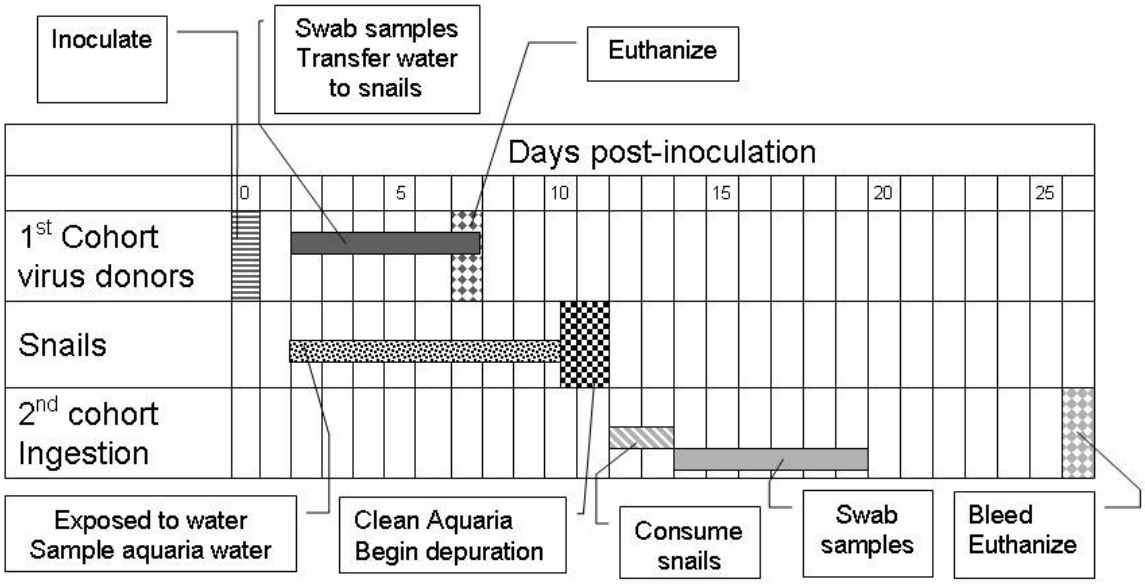

Figure 3.2. Experimental design of the mallard snail-exposure experiment detailing the timelines of the three groups of animals. This experiment was designed to test for mechanical transmission of avian influenza virus between mallards (*Anas platyrhynchos*) via ingestion of *Physa* spp. snails.

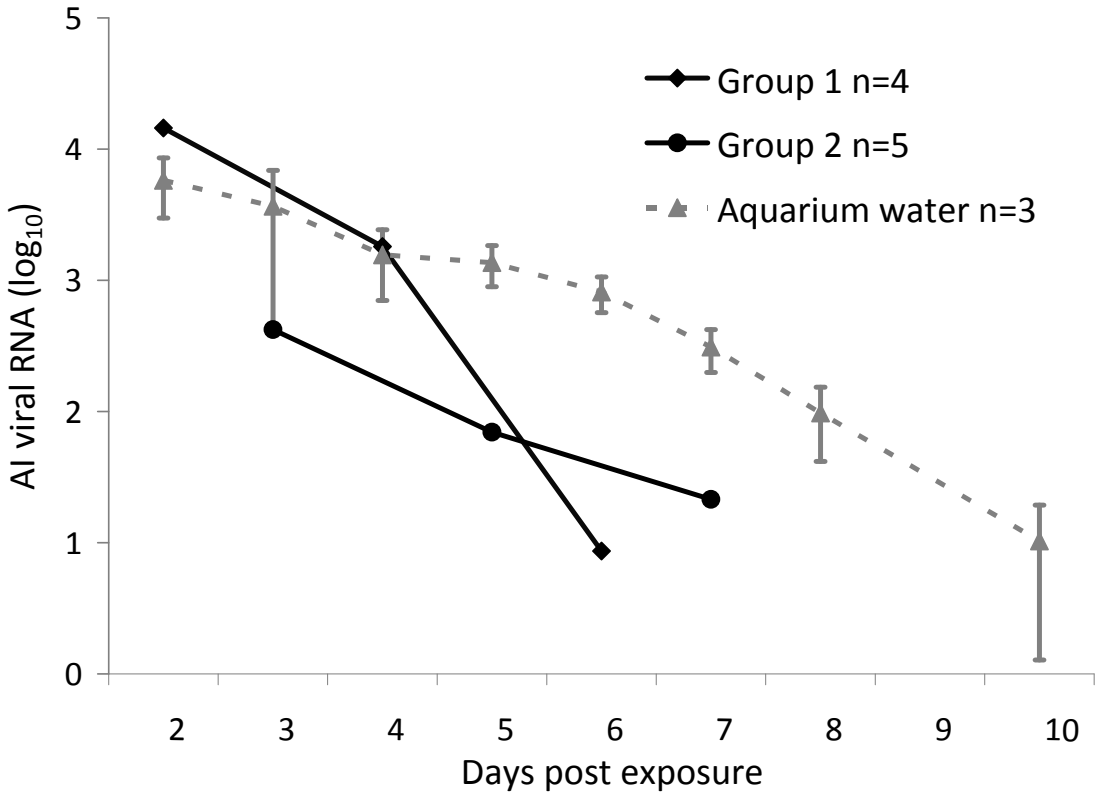

Figure 3.3. Avian influenza viral RNA detected in directly exposed mallards (cloacal swabs), and aquarium water (transferred from the mallard ponds). Samples from the two groups of mallards were tested on alternating days (group 1 on odd days and group 2 on even days, 95% confidence interval shown for aquarium water).

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APPENDIX I

Tissue Staining Procedure

Maackia amurensis I and II lectins

These assays were developed at by Elizabeth Howerth and Monique Silva Franca at the University of Georgia, College of Veterinary Medicine, Department of Pathology.

1.0 PURPOSE

1.1 For detection of sialic acid (SAα2,3Gal), the cellular binding site for avian influenza viruses and differentiating between gull influenza virus subtype receptors (SAα2,3Galβ14GlcNAc) and duck influenza virus subtype (SAα2,3Galβ13GalNAc).

2.0 PROCEDURES

2.1 Tissue preparation

2.1.1 Collect appropriate tissues and place in formalin for 2 – 3 days. Tissues should include positive controls such as duck intestines.

2.1.2 Remove tissues from formalin and place in 70% ethanol for at least 1 day.

2.1.3 Cut formalin-fixed tissue sections nickel-sized and place in tissue cassettes. Label cassettes appropriately, and place in fresh 70% ethanol.

2.1.4 Take tissue cassettes to Diagnostic Laboratory and request unstained positively charged ("+" on slide) slides. Get at least 2 slides cut from each tissue, 1 will be stained with the florescent marker, the other will serve as negative control to detect auto-florescence.

2.2 Supplies and reagents

Equipment

- Steamer (Black and Decker Flavor Scenter Steamer Plus™)
- Tissue staining system with at least 8 wells
- Upright slide rack
- 1-2 vertical staining jar with lid

- Parafilm, cut into slide sized pieces, enough for each slide

Reagents

- Xylene – in 3 wells
- 100% ETOH – in 2 wells
- 95% ETOH – in 1 well
- 75% ETOH – in 1 well
- DDH_2O – in 1 well (fresh each time)
- Sodium citrate solution:
  - Combine 1.457g powder with 200mL DDH20
  - Using a pH meter, add 1M HCl slowly to solution until pH is 6.0
  - Add 300mL DDH2O
- 3% H_2O_2 – if using 30% H_2O_2, make 1:10 dilution with methanol
- 1X Carbo-Free blocking solution – 1mL 10X Carbo-Free blocking solution (Vector labs SP-5040)
- PBS
- *Maackia amurensis* I lectin (MAAI; Vector Laboratories, F1311)
- *Maackia amurensis* II lectin (MAAII; Vector Laboratories, B1265)
- Streptavidin Alexa Fluor 546 (Invitrogen, S-11225)
- Prolong Gold (Invitrogen, P36931)

2.3 Staining procedure

2.3.1 Wear gloves whenever handling slides to protect slides from any oil and lotions on hands.

2.3.2 Prepare steamer by adding water.

2.3.3 Put the slides in the oven at 62-63C for at least 30 minutes (not above 63 C) – softens paraffin.

2.3.4 Deparaffinized the slides in xylene solutions and hydrate in ethanol and DDH_2O.

- Xylene well 1 (10 min)
- Xylene well 2 (5 min)
- Xylene well 3 (5 min)
  Note: These steps should be conducted in a fume hood as xylene fumes are hazardous. Additionally, care should be used when handling slide racks. The slide rack handles will not be immersed in the xylene, but racks should be gently immersed (while wearing gloves) to prevent splashing.

2.3.5 Hydrate the slides in ethanol solutions and DDH_2O (fresh water every time).

- 100% ethanol well 1 (5 min)
- 100% ethanol well 2 (5 min) – turn on steamer, fill clean

vertical staining jar with sodium citrate buffer

- 95% ethanol 5 min – start warming citrate buffer by placing vertical staining jar into steamer
- 75% ethanol 5 min
- Rinse in fresh DDH_2O

2.3.6 Transfer slides to warm citrate buffer, allow slides to steam for 35 minutes.

2.3.7 Remove slide bath with slides from steamer, carefully drain buffer, and rinse 3 times with DDH_2O by refilling and draining vertical staining jar.

2.3.8 Refill vertical staining jar with 3% H_2O_2, incubate slides at room temperature for 15 minutes.

2.3.9 Prepare 1X Carbo-Free blocking solution (1mL 10X Carbo-Free+10mL PBS) during incubation.

2.3.10 Drain H_2O_2 and rinse slides 3 times with DDH_2O as described in 2.2.7.

2.3.11 Place slides flat on rack in square bioassay dish ($245mm^2$). Pipette enough 1X Carbo-Free blocking solution to cover tissue, replace cover on dish, and allow slide to incubate at room temperature for 15 min.

2.3.12 MAAI lectin procedure

2.3.12.1 Prepare the MAAI lectin solution (F1311) at a 1:100 dilution during incubation step, 3µLMAA1 + 297µL PBS)/slide. Remember, only ½ of the slides will receive the lectin solution. Wrap vial in aluminum foil to keep dark and cool.

2.3.12.2 Fill conical vial with enough PBS for the negative control slides – 300µLPBS/slide.

2.3.12.3 Separate the negative control slides from treated slides.

2.3.12.4 Tip each slide individually to pour-off Carbo-Free blocking solution, but keep slide in dish.

2.3.12.5 Pipette 300µL MAAI lectin solution on to each treated slide making sure the tissue is covered.

2.3.12.6 Pipette 300µL PBS over each negative control slide making sure the tissue is covered.

2.3.12.7 Gently place un-stretched Parafilm strip over each slide, the capillary action of the liquid will form a seal between Parafilm and liquid.

2.3.12.8 Place cover over tray, cover with foil to block light, and place in dark place (e.g. drawer).

2.3.12.9 Allow to incubate at room temp for 1 hour.

2.3.13 MAA2 lectin procedure

2.3.13.1 Prepare the MAA2 lectin solution (F1265) at a 1:50 dilution during incubation step, 6µLMAA1 + 294µL PBS)/slide. Remember, only ½ of the slides will receive the lectin solution. Wrap vial in aluminum foil to keep dark and cool

2.3.13.2 Fill conical vial with enough PBS for the negative control slides – 300µLPBS/slide, and the PBS wash step – several mL PBS/slide.

2.3.13.3 Dump MAA1 solution and pat bottom of slides dry on paper towel.

2.3.13.4 Using your fingers, tilt slide to 45° angle, gently wash each slide 3x with several mL of PBS.

2.3.13.5 Pat bottom of slides dry on paper towel and replace on rack.

2.3.13.6 Pipette 300µL MAA2 lectin solution on to each treated slide making sure the tissue is covered.

2.3.13.7 Pipette 300µL PBS over each negative control slide making sure the tissue is covered.

2.3.13.8 Gently place un-stretched Parafilm strip over each slide, the capillary action of the liquid will form a seal between Parafilm and liquid.

2.3.13.9 Place cover over tray, cover with foil to block light, and place in dark place (e.g. drawer).

2.3.13.10 Allow in incubate at room temp for 1 hour.

2.3.14 Streptavidin Alexa Fluor 546 solution procedure

2.3.14.1 Prepare the streptavidin Alexa Fluor 546 solution at a 1:100 dilution during incubation step, 3µLMAA1 + 297µL PBS)/slide. Remember, only ½ of the slides will receive the lectin solution. Wrap vial in aluminum foil to keep dark and cool.

2.3.14.2 Pour-off MAA2 solution.

2.3.14.3 Using your fingers, tilt slide to 45° angle, gently wash each slide 3x with several mL of PBS.

2.3.14.4 Pat bottom of slides dry on paper towel and replace on rack.

2.3.14.5 Pipette 300µL Streptavidin solution on to each treated slide making sure the tissue is covered.

2.3.14.6 Pipette 300µL PBS over each negative control slide making sure the tissue is covered.

2.3.14.7 Gently place un-stretched Parafilm strip over each slide, the capillary action of the liquid will form a seal between Parafilm and liquid.

2.3.14.8 Place cover over tray, cover with foil to block light, and place in dark place (e.g. drawer).

2.3.14.9 Allow in incubate at room temp for 2 hours.

2.3.15 Prolong Gold procedure

2.3.15.1 Pull Prolong Gold (ProLong® Gold antifade reagent with DAPI, cat# P36931) from freezer about 15 minutes before incubation step is complete.

2.3.15.2 Pour-off Streptavidin solution.

2.3.15.3 Using your fingers, tilt slide to 45° angle, gently wash each slide 1x with several mL of PBS.

2.3.15.4 Pat bottom of slides dry on paper towel and move slides to clean dry rack on rack.

2.3.15.5 Using disposable pipettes, place 1 drop of Prolong Gold on each slide, and gently place cover slip over tissue. Hold slip cover edge along slide edge and allow cover slip to drop over Prolong Gold.

2.3.15.6 Check for bubbles, if bubbles near tissues, use fine clean, dry pipette tip to push bubble towards edge.

2.3.15.7 Replace slides on rack.

2.3.15.8 Replace tray cover and cover with foil and place in dark place (e.g. drawer) for 24 hours.

2.3.16 Visualization under the fluorescent/confocal microscope

2.3.16.1 Filters should include

- DAPI to visualize the nucleated cells
- FITC to visualize gull influenza virus subtype receptors (SAα2,3Galβ14GlcNAc) receptors
- TRITC to visualize duck influenza virus subtype (SAα2,3Galβ13GalNAc) receptors

2.3.16.2 Initially observe positive control tissues in the microscope to make sure assay worked.

2.3.16.3 View negative control positive tissue to check for auto-fluorescence.

2.3.16.4 View remaining tissues while checking negative controls for auto-fluorescence.

2.3.16.5 Protect slides from the light at all times, leaving out only long enough to microscopically view.

2.3.17 Store slides in refrigerator in the dark.

Tissue staining procedure

For detection of mammalian influenza virus receptors

Lectin Histochemistry

Fluorescein *Sambucus Nigra*

1.0 PURPOSE

1.1 For detection of sialic acid (SAα2,6Gal), the cellular binding site for human and pig influenza viruses.

2.0 PROCEDURES

2.1 Tissue preparation

2.1.1 Collect appropriate tissues and place in formalin for 2 – 3 days. Tissues should include positive controls such as pig trachea.

2.1.2 Remove tissues from formalin and place in 70% ethanol for at least 1 day.

2.1.3 Cut formalin-fixed tissue sections nickel-sized and place in tissue cassettes. Label cassettes appropriately, and place in fresh 70% ethanol.

2.1.4 Take tissue cassettes to Diagnostic Laboratory and request unstained positively charged ("+" on slide) slides. Get at least 2 slides cut from each tissue, 1 will be stained with the florescent marker, the other will serve as negative control to detect auto-florescence.

2.2 Supplies and reagents

Equipment

- Steamer (Black and Decker Flavor Scenter Steamer Plus™)
- Tissue staining system with at least 8 wells
- Upright slide rack
- 1-2 vertical staining jar with lid
- Parafilm, cut into slide sized pieces, enough for each slide

Reagents

- Xylene – in 3 wells
- 100% ETOH – in 2 wells
- 95% ETOH – in 1 well
- 75% ETOH – in 1 well
- DDH_2O – in 1 well (fresh each time)
- Sodium citrate solution:
  - Combine 1.457g powder with 200mL DDH20
  - Using a pH meter, add 1M HCl slowly to solution until pH is 6.0

- Add 300mL DDH2O
- 3% H_2O_2 – if using 30% H_2O_2, make 1:10 dilution with methanol 10mL PBS
- PBS
- Sambucus nigra (SNA; Invitrogen, F1301)
- Prolong Gold (Invitrogen, P36931)

2.3 Staining procedure.

2.3.1 Wear gloves whenever handling slides to protect tissues/slides from any oil and lotions on hands.

2.3.2 Prepare steamer (Black and Decker Flavor Scenter Steamer Plus™) by adding water.

2.3.3 Put the slides in the oven at 62-63C for at least 30 minutes (not above 63 C) – softens paraffin.

2.3.4 Deparaffinize the slides in xylene solutions and hydrate in ethanol and DDH_2O.

- Xylene well 1 (10 min)
- Xylene well 2 (5 min)
- Xylene well 3 (5 min)

Note: These steps should be conducted in a fume hood as xylene fumes are hazardous. Additionally, care should be used when handling slide racks. The slide rack handles will not be immersed in the xylene, but racks should be gently immersed (while wearing gloves) to prevent splashing.

2.3.5 Hydrate the slides in ethanol solutions and DDH_2O (fresh water every time).

- 100% ethanol well 1 (5 min)
- 100% ethanol well 2 (5 min) – turn on steamer, fill clean vertical staining jar with sodium citrate buffer
- 95% ethanol 5 min – start warming citrate buffer by placing vertical staining jar into steamer
- 75% ethanol 5 min
- Rinse in fresh DDH_2O

2.3.6 Transfer slides to <u>warm</u> citrate buffer, allow slides to steam for 35 minutes.

2.3.7 Remove slide bath with slides from steamer, carefully drain buffer, and rinse 3 times with DDH_2O by refilling and draining vertical staining jar.

2.3.8 Refill vertical staining jar with 3% H_2O_2, incubate slides at room temperature for 15 minutes.

2.3.9 Prepare the SNA lectin solution (F1301) at a 1:100 dilution during incubation step, 3µLSNA + 297µL PBS)/slide. Remember, only ½ of the slides will receive the lectin solution. Wrap vial in aluminum foil to keep dark and cool.

2.3.10 Fill conical vial with enough PBS for the negative control slides – 300µLPBS/slide.

2.3.11 Drain H_2O_2 and rinse slides 3 times with DDH_2O as described in 2.2.7.

2.3.12 SNA lectin procedure.

- 2.3.12.1 Place slides flat in slide staining system (or on rack in square bioassay dish), keeping negative control slides separate from treated slides.
- 2.3.12.2 Pipette 300µL SNA lectin solution on to each treated slide making sure the tissue is covered.
- 2.3.12.3 Pipette 300µL PBS over each negative control slide making sure the tissue is covered.
- 2.3.12.4 Gently place un-stretched Parafilm strip over each slide, the capillary action of the liquid will form a seal between Parafilm and liquid.
- 2.3.12.5 Place cover over tray, cover with foil to block light, and place in dark place (e.g. drawer).
- 2.3.12.6 Allow in incubate at room temp for 1 hour.

2.3.13 Prolong Gold procedure.

- 2.3.13.1 Pour-off SNA solution.
- 2.3.13.2 Using your fingers, tilt slide to 45° angle, gently wash each slide 3x with several mL of PBS.
- 2.3.13.3 Pat bottom of slides dry on paper towel and move slides to clean dry rack on rack.
- 2.3.13.4 Using disposable pipettes, place 1 drop of Prolong Gold on each slide, and gently place cover slip over tissue. Hold slip cover edge along slide edge and allow cover slip to drop over Prolong Gold. Several pipettes may be needed for this procedure.
- 2.3.13.5 Check for bubbles, if bubbles near tissues, use fine clean, dry pipette tip to push bubble towards edge.
- 2.3.13.6 Replace slides on rack.
- 2.3.13.7 Replace tray cover and cover with foil and place in dark place (e.g. drawer) for 24 hours.

2.3.14 Visualization under the fluorescent/confocal microscope

- 2.3.14.1 Filters should include:
  - DAPI to visualize the nucleated cells
  - FITC to visualize gull influenza virus subtype receptors (SAα2,3Galβ14GlcNAc) receptors
  - TRITC to visualize duck influenza virus subtype (SAα2,3Galβ13GalNAc) receptors

2.3.14.2 Initially observe positive control tissues in the microscope to make sure assay worked.
2.3.14.3 View negative control positive tissue to check for auto-fluorescence.
2.3.14.4 View remaining tissues while checking negative controls for auto-fluorescence.
2.3.14.5 Protect slides from the light at all times, leaving out only long enough to microscopically view.
2.3.14.6 Store slides in refrigerator in the dark.

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