BIOLOGICAL FACTORS AFFECTING PERSISTENCE OF AVIAN INFLUENZA VIRUS IN AQUATIC ENVIRONMENTS

by

CHRISTINA LYNN FAUST

(Under the Direction of Sonia Altizer)

Traditional research on avian influenza (AI) viruses in wild birds has focused on interactions between the viral agent and host; however, understanding the ecology and natural history of AI requires addressing interactions among host, pathogen, and environment. Avian influenza viruses are transmitted within wild aquatic bird populations through an indirect fecal-oral route involving contaminated water. The persistence of AI viruses in water and subsequent transmission to susceptible individuals is thought to play an essential role in the transmission dynamics of avian influenza. Previous studies have shown that the persistence of AI viruses in water is dependent on abiotic environmental factors (pH, temperature, salinity), but no research has been conducted to determine the role of biological factors in the environment on AI persistence.

The influence of filter feeding bivalves on the of AI in water was evaluated utilizing the freshwater, filter-feeding Asian clam, *Corbicula fluminea*. *C. fluminea* reduced the persistence and infectivity of low pathogenic (LP) and high pathogenic (HP) AI viruses, respectively, over a 48-hr period. The results of this study highlights

the role that invertebrate species inhabiting aquatic environments- specifically filter-feeding bivalves- can play in the persistence and transmission of AI viruses.

INDEX WORDS: AVIAN INFLUENZA, PERSISTENCE, ENVIRONMENTAL FACTORS, WATER, BIVALVES

BIOLOGICAL FACTORS AFFECTING PERSISTENCE OF AVIAN INFLUENZA VIRUS IN AQUATIC ENVIRONMENTS

by

CHRISTINA LYNN FAUST

B.S., The University of Georgia, 2009

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2009

© 2009

CHRISTINA LYNN FAUST

All Rights Reserved

BIOLOGICAL FACTORS AFFECTING PERSISTENCE OF AVIAN INFLUENZA VIRUS IN AQUATIC ENVIRONMENTS

by

CHRISTINA LYNN FAUST

Major Professor: Sonia Altizer

Committee: David Stallknecht
Carl Ronald Carroll

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2009

DEDICATION

I would like to dedicate my thesis to my wonderful mother, Lynn Diane Olyha Faust, who instilled a love of the environment into her children at a young age. She has always encouraged and supported me, and for that, I am forever grateful.

ACKNOWLEDGEMENTS

There are many I would like to thank for their help over the years: for reading through a draft, for discussing ideas with, or simply sharing a laugh over an experiment gone wrong. I will not be able to name everyone that has meant so much over the past two years, but I will try to do some of them justice. Foremost I would like to thank my committee: Sonia Altizer, David Stallknecht, and Ron Carroll for their inspiration and guidance. I would also like to thank my family: my mother, my brother and sister-in-law, my grandparents, and the rest of my extended family for their love and patience during my academic career at UGA. I also must acknowledge the many friendships that have taught me humility, kindness, and compassion through their actions, including my fellow ecologists, the water polo team, my roommates, and many more.

Many people not mentioned on the title pages have been instrumental in the formation of this project and the work conducted throughout. Everyone at the Southeastern Cooperative Wildlife Disease Study has been very helpful throughout the time I have worked there, especially Justin Brown, Ginger Goekjian, Shamus Keeler, and Deb Carter. I would also like to thank everyone in the Altizer lab including Julie Rushmore, Jean Chi, Byron Ledbetter, Becky Bartel, Barbara Han, Jamie Winternitz, and Daniel Streiker for sharing wisdom of disease ecology and their persistent pursuit of knowledge. Additionally, people in the greater Odum School of Ecology community, including Misha Boyd and Jim Richardson, have always been a source of inspiration. Also, I greatly appreciate the patience of Andrew Durso, Joel Mittleman, and Kurt Hertzer while helping me with

statistics. Lastly, I would like to thank everyone in the Honors Program for making a joint Bachelor’s/Master’s degree possible to obtain and for all their kindness, guidance, and support over the years.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

1 INTRODUCTION 1

WORKS CITED 3

2 LITERATURE REVIEW ON THE TRANSMISSION AND PERSISTENCE OF AVIAN INFLUENZA VIRUSES IN THE ENVIRONMENT 4

SUMMARY 4

CLASSIFICATION OF AVIAN INFLUENZA VIRUSES 4

HOST RANGE OF AVIAN INFLUENZA 6

INFECTION DYNAMICS OF AVIAN INFLUENZA WITHIN WILD BIRD POPULATIONS 10

AQUATIC TRANSMISSION OF AVIAN INFLUENZA 11

PERSISTENCE OF AVIAN INFLUENZA IN THE ENVIRONMENT 12

BIOLOGICAL FACTORS IN THE AQUATIC ENVIRONMENT THAT COULD INFLUENCE AVIAN INFLUENZA PERSISTENCE 16

CONCLUSIONS AND OBJECTIVES 22

WORKS CITED 24

3 FILTER-FEEDING BIVALVES CAN REMOVE AVIAN INFLUENZA VIRUSES FROM WATER AND REDUCE INFECTIVITY 40

SUMMARY 41

KEYWORDS 41

INTRODUCTION 42

METHODS 45

RESULTS 54

DISCUSSION 59

ACKNOWLEDGEMENTS 62

WORKS CITED 64

4 CONCLUSION 78

WORKS CITED 81

LIST OF TABLES

Page

Table 3.1: Comparison of rate of decrease in viral titers based on RT values. 74

Table 3.2: Summary of wood duck trials...75

Table 3.3: Supplementary Material- Persistence of virus over 48 hrs compared to pH levels

in water with (n=7) and without (n=1) *C. fluminea* 76

LIST OF FIGURES

Page

Figure 3.1: Summary of the persistence of LPAI virus in the presence and absence of *C. fluminea* over 48 hrs. 70

Figure 3.2: Comparison of virus quantification by infectivity assay and real-time RT-PCR (RRT-PCR) 71

Figure 3.3: Viral persistence in water in the presence of clams over short time intervals....73

Figure 3.4: Supplementary Material- Effect of clam shell surface on virus concentration over 48 hrs 77

CHAPTER 1

INTRODUCTION

Avian species in the orders Anseriformes and Charadriiformes are widely accepted as the natural reservoirs for avian influenza (AI) viruses (Webster et al. 1992). AI infects birds globally (Olsen et al. 2006), yet little is known of the ecology and dynamics of AI virus within its natural hosts. Wild birds are of concern to public health and agricultural groups for the their risk of introduction of H5 and H7 strains into domestic poultry, so much of the research on AI in wild populations has concentrated on seroprevalence and virus isolation surveys.

Transmission of AI between individuals is believed to occur via a fecal- oral route, utilizing contaminated water in aquatic habitats as a mechanism for disseminating infective virus (Hinshaw et al. 1979, Webster et al. 1992). Despite this environmental link in the transmission cycle of AI, little is known about the factors that affect the persistence and infectivity of the virus within this potential environmental reservoir. Previous research that has been conducted has focused on abiotic factors, specifically pH, temperature, and salinity and shown significant impacts of these factors on the persistence of AI viruses in water (Stallknecht et al. 1990a, Stallknecht et al. 1990b, Brown et al. 2007, Brown et al. 2008).

Under optimal conditions AI viruses can persist in water for over 100 days, and because of this, viruses will inevitably be exposed to biotic components of the

aquatic environment. The purpose of this study is to examine biological factors that may influence the persistence and infectivity of AI in water.

Studies conducted on other viruses that utilize a fecal-oral transmission routes have shown that they accumulate and remain infective within filter-feeding bivalves (Lees 2000). In this study, the Asian clam (*Corbicula fluminea*) will be used as a model species to examine whether filter feeders affect the persistence of AI in water. If this is the case, such data can be used to further detail the link between biotic factors and AI in the environment.

Understanding the transmission routes and factors that affect them will lead to a better understanding of the ecology of AI and its circulation among avian hosts. Doing so may ultimately aid mitigation of the emerging economic and public health challenges that AI poses.

WORKS CITED

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. 2008. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. Veterinary Microbiology.

Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht. 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Diseases **51**:285-289.

Hinshaw, V. S., R. G. Webster, and B. Turner. 1979. Water-borne Transmission of Influenza A Viruses. Intervirology **11**:66-68.

Lees, D. 2000. Viruses and bivalve shellfish. International Journal of Food Microbiology **59**:81-116.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. Osterhaus, and R. A. M. Fouchier. 2006. Global patterns of influenza A virus in wild birds. Science **312**:384-388.

Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990a. Effects of pH, Temperature, and Salinity on Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:412-418.

Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990b. Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:406-411.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and Ecology of Influenza-a Viruses. Microbiological Reviews **56**:152-179.

CHAPTER 2

LITERATURE REVIEW ON THE TRANSMISSION AND PERSISTENCE OF AVIAN INFLUENZA VIRUSES IN THE ENVIRONMENT

SUMMARY

This chapter reviews published literature on the transmission of avian influenza viruses (AIV) in the environment, beginning with the natural history and general understanding of the ecology of AIV. Persistence in the environment is understood to be an important part of the transmission cycle and has been shown to be dependent on abiotic factors (temperature, pH, salinity). However there is limited research conducted on the persistence of AIV within the aquatic environment, so the review also draws from research on related viruses to bring attention to biological factors that may play a role in the transmission and epidemiology of AIV.

CLASSIFICATION OF AVIAN INFLUENZA VIRUS

Avian influenza viruses (AIV) are classified in the family *Orthomyxoviridae* of single-stranded RNA viruses. There are five genera within the family: *Influenza A, B*, and *C*, as well as *Isavirus* and *Thogotovirus*. All AIV are classified as *Influenza A* based on their antigen types. Influenza A viruses infect many species of avian and

mammalian hosts, including humans, swine, and horses (Webster et al. 1992). Understanding the basic structure of AIV will aid in investigations of AIV ecology, including identifying factors that most strongly affect their persistence in the environment.

RNA viruses are the most mutable of any biological entity (Hanada et al. 2004). They are highly variable (Fenner et al. 1987) because they lack proofreading and are subject to point mutations in 1 out of 10^4 bases (Holland et al. 1982, Steinhauer and Holland 1987). AIV have eight gene segments that encode for ten viral proteins: three surface proteins, five internal proteins, and two non-structural proteins (O'Neill et al. 1998, Palese and Shaw 2006).

Avian influenza viruses are serotyped based on two of their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). HA proteins appear as spikes on the membrane surface and are the most abundant surface protein (Compans et al. 1970). The HA protein is responsible for binding the virus to host cells (Webster et al. 1992) and is the major antigen associated with virus neutralization. NA proteins free virus from the host cells in which they were formed, facilitating virus spread by cleaving the terminal sialic acid from glycoproteins or gylcolipids (Webster et al. 1992). Overall, there are 16 subtypes of HA (H1-H16) and 9 subtypes of NA (N1-N9), and most combinations of these two proteins have been isolated from wild birds (Alexander 2000, Fouchier et al. 2005).

AIV are also classified based upon pathogencity in chicken. Most serotypes are low pathogenic (LP) AIV, but some H5 and H7 viruses can also be classified as high pathogenicity based on guidelines from the World Organization for Animal

Health, formally known as the OIE. High pathogenic notifiable avian influenza viruses (HPNAIV) "have an IVPI [intravenous pathogenicity index] in 6-week-old chickens greater than 1.2 [or], as an alternative, cause at least 75% mortality in 4- to 8-week-old chickens infected intravenously" (World Organization for Animal Health 2008). H5 and H7 viruses that do not meet the aforementioned qualifications but have an amino acid motif similar to known HPNAIV isolates are also considered HPNAIV. Subtypes of H5 and H7 that are not considered HPNAIV are considered low pathogenic notifiable avian influenza (LPNAI) viruses for purposes of international trade because of their threat for infection of poultry and must be reported to WOAH.

For the purposes of this paper, HPNAIV will be referred to as HPAIV and all other AIV will be considered LPAIV. Research suggests that LPAIV become HPAIV through mutation within domestic bird populations (Li et al 1990). Only one HPAIV outbreak in wild birds not associated with a known outbreak in domestic birds has been recorded (Becker 1966).

HOST RANGE OF AVIAN INFLUENZA

AIVV infect domestic and wild birds, as well as several mammalian species, but this review focuses on wild birds and aquatic transmission within these species. In 1961, the first AIV from a free-living bird was isolated from *Sterna hirundo*, common tern, in South Africa during an outbreak in which 1300 birds died from a H5N1 HPAIV (Becker 1966). To date, AIV have been isolated from more than 100 species in 12 avian orders from Africa, Asia, Australia, Europe, North America, and

South America (Hinshaw et al. 1980a, Hinshaw et al. 1982, Stallknecht and Shane 1988, Süss et al. 1994, Olsen et al. 2006, Spackman et al. 2006). There is also evidence from seroprevalence surveys that AIV exist in bird species in Antarctica (Austin and Webster 1993, Wallensten et al. 2006).

Most isolations of AIV are from species in the orders Anseriformes (ducks, geese, and swan) and Charadriiformes (gulls, terns, and shorebirds) (Stallknecht and Shane 1988, Hanson 2002, Olsen et al. 2006). However, AIV are not evenly detected within species of these orders and virus isolations are often concentrated within smaller taxonomic groupings. The majority of isolations from Anseriformes have been reported from the subfamily Anatinae (dabbling ducks and diving ducks) and within this, mallards are the source of the greatest number of isolations (Hinshaw et al. 1980c, Kocan et al. 1980, Deibel et al. 1985, Hinshaw et al. 1986). Some studies suggest that mallards may be as susceptible to AIV infection as other waterfowl species (Alfonso et al. 1995), but their high abundance in the U.S. drives their importance as a reservoir species (Hinshaw et al. 1986, Slemons et al. 1991).

An unequal distribution of AIV isolations also occurs within species in the order Charadriiformes. Infection is most common in two families: Scolopacidae, which include sandpipers and turnstones (shorebirds), and Laridae, which includes gull and tern species (Hanson 2002, Olsen et al. 2006). Particularly interesting is the largest concentration of infections of Scolopacidae occur within one species, ruddy turnstones (*Arenaria interpres*), at one location, Delaware Bay, USA (Hanson 2002, Stallknecht 2003, Krauss et al. 2004, Stallknecht and Brown 2007). Ruddy turnstones comprised 11.2% of the birds that were sampled but accounted for over

40% of the total AIV isolations (Kawaoka et al. 1988). It is unknown why this species has such high prevalences compared to other species, but it is hypothesized that interactions with anseriforms during migration, susceptibility to AIV, behavior, or habitat usage may play a role in the dynamics of AIV within ruddy turnstones (Hanson 2002).

AIV have also been isolated from bird species that are associated with aquatic habitats, but are not within the orders Anseriformes and Charadriiformes. Isolates have been detected from species in Ciconiiformes, Gaviiformes, Gruiformes, Pelecaniformes, Podicipediformes, and Procellariiformes (Stallknecht and Brown 2008). Some isolates are from species in the orders Columbiformes, Piciformes, and Passeriformes that do not associate with aquatic habitats, but the prevalence of AIV is very low within these species (Hinshaw et al. 1982, Deibel et al. 1985, Nettles et al. 1985).

Waterfowl within the orders Anseriformes and Charadriiformes are widely recognized as the reservoirs for AIV (Hinshaw et al. 1979, Webster et al. 1992). No wild birds have exhibited clinical signs of infection in prevalence surveys, but influenza A viruses can cause significant morbidity and mortality in other species, including domestic birds and mammals (Webster et al. 1992). Most laboratory studies that infected wild birds with LPAIV did not report lesions or other pathological signs in birds, even when they were shedding virus (Homme and Easterday 1970, Alexander et al. 1978, Hinshaw et al. 1980b, Kida et al. 1980, Kawaoka et al. 1988), however one study reported lesions in the lungs of ducks infected with LPAIV (Cooley et al. 1989). Recently, analyses of long term data sets

suggest that AIV affects the body condition of migrating mallards but more research is needed to confirm these conclusions (Latorre-Margalef et al. 2009).

All 9 neuraminidase and all but 2 hemagglutinin types have been detected in Anseriformes (Hinshaw et al. 1985). The remaining subtypes, H13 and H16, have primarily been isolated in gulls, and genetic evidence suggests they have been distinct from subtypes circulating amongst ducks and geese for some time (Fouchier et al. 2005). The most common AIV isolated from wild birds belong to H3, H4 and H6 subtypes (Stallknecht and Shane 1988, Sharp et al. 1993, Krauss et al. 2004), with some studies also detecting a high prevalence of H11 (Ottis and Bachmann 1983, Stallknecht and Shane 1988, Slemons et al. 1991). There are higher prevalences of H5, H7, and H9 subtypes in Charadriiformes, but these levels are still low (Krauss et al. 2004).

Some believe that because not all subtypes are recovered from wild aquatic birds frequently at high prevalences, there may be alternative reservoirs for these subtypes (Sharp et al. 1993). Low detection of some subtypes may be due in part to biased sampling of common and easily studied species (Spackman 2009). Targeting new species for surveillance studies may help elucidate reservoir species of more subtypes of AIV. Using data from studies over the past 30 years, ecological and biological traits associated with AIV isolations determined that a long migration and surface-feeding are the two most predictive characteristics that a species will have high prevalences of LPAIV (Garamszegi and Møller 2007). These findings can be used to direct studies on new bird species.

INFECTION DYNAMICS OF AVIAN INFLUENZA WITHIN WILD BIRD POPULATIONS

Virus detection peaks in the fall in anseriform species, when AIV preferentially infects naive young waterfowl (Hinshaw et al. 1980c, Hinshaw et al. 1985, Slemons et al. 1991). Longitudinal studies at marshalling sites prior to migration show a mean prevalence of 30% in juvenile birds of influenza virus infection, with prevalence in some years as high as 61% positive isolates (Hinshaw et al. 1985). Many surveys have confirmed that young birds are infected at significantly higher rates compared to adults (Hinshaw et al. 1980a, Hinshaw et al. 1985, Alfonso et al. 1995, Fouchier et al. 2003). The prevalence of AIV decreases during migration (Rosenberger et al. 1974, Webster et al. 1976), and in wintering grounds the prevalence is lower than 1-2% (Stallknecht and Shane 1988). Ducks returning to their breeding grounds in April show very low prevalence, only 1 of 348 samples (0.3%) from lakes in Alberta were positive for AIV (Sharp et al. 1993).

The peak prevalence of AIV occurs in high-density premigration marshalling sites in ducks and geese, whereas the highest prevalence in shorebirds is observed upon their northward migration in May in Delaware Bay (Hanson 2002, Stallknecht 2003, Stallknecht and Brown 2008).

AIV show not only seasonality (Sharp et al. 1993), but studies show strong evidence for cyclic patterns in which AIV subtypes show high prevalence within populations in some years followed by reduced prevalence for several years (Hinshaw et al. 1985, Sharp et al. 1993, Krauss et al. 2004). Because mallards switch breeding sites between years (Del Hoyo et al. 1996), some authors have argued that

subtypes are introduced to populations when individuals carry viruses from one distinct population to another.

Although understanding the ecology and disease dynamics of AIV is important for domestic birds, agriculture, and human health, there have been a limited number of research projects focused on understanding the mechanism by which multiple subtypes circulate through the multi-host system. More research is needed to understand transmission within wild birds before outbreaks in domestic birds can be fully understood. Gathering more information about the circulation of AIV within its reservoir will aid in the prevention of transmission events to domestic birds.

AQUATIC TRANSMISSION OF AVIAN INFLUENZA

AIV are transmitted within wild bird populations via a fecal-oral route utilizing contaminated water as a transport media (Webster et al. 1992). This initial hypothesis was presented by Webster et al. (1978) after laboratory studies demonstrated replication of AIV within the small intestine and high viral titers shed in feces for extended period (6 days). The same study also demonstrated the persistence of AIV in duck feces for 30 days and 7 days at 4°C and 20°C, respectively. The following year, a field study supported the hypothesis by isolating AIV from unconcentrated water samples from six lakes and from fecal samples from the shores of three lakes in Canada (Hinshaw et al. 1979). Two other studies have isolated AIV from unconcentrated water in Minnesota (Halvorson et al. 1985) and

Alaska (Ito et al. 1995). Additionally, no AIV have been isolated from water where duck populations are not actively feeding (Hinshaw et al. 1986).

Once a bird is infected with AIV, it replicates within cell lining in the lower intestinal tract (Slemons and Easterday 1977) and is excreted in high concentrations within the feces (Webster et al. 1978, Hinshaw and Webster 1982). In laboratory infection experiments, Muscovy ducks (*Carina moschata*) shed feces with a high viral titer of $10^{7.8}$ median embryo infectious dose (EID_{50})/ml (Webster et al. 1978). Host excrete virus for a long period; one species, Peking white duck (*Anas platyrhynchos*), sheds virus for over 30 days (Hinshaw et al. 1980a).

PERSISTENCE OF AVIAN INFLUENZA IN THE ENVIRONMENT

Most research on AIV has concentrated on the interactions between the viruses and hosts, despite the important linkage to the environment through fecal-oral transmission (Hinshaw et al. 1979, Sinnecker et al. 1983). The initial study used an H3N2 virus (A/duck/Memphis/546/74) to examine the persistence of AIV in non chlorinated Mississippi water and fecal matter (Webster et al. 1978). The stability of virus in the water was tested at two temperatures: 1) at 4°C the virus titer did not decrease significantly during the first two weeks but decreased noticeably in the following two weeks and 2) at 22°C the virus was detected 8 days after the trial began, but not 13 days afterward. Virus in diluted fecal samples showed a similar pattern at the two temperatures: 1) at 4°C viral titer remained stable for the first 7 days and then decreased but was still infective at 32 days and 2) at 22°C the virus titer decreased rapidly and was undetectable after 8 days.

Field surveillance studies supported these findings by isolating virus from unconcentrated water in habitats used by waterfowl in Canada, Minnesota, and Alaska (Hinshaw et al. 1980c, Halvorson et al. 1985, Ito et al. 1995) and from feces sampled from the shore of a lake in Great Britain (Alexander et al. 1980).

A series of laboratory distilled water models were used to evaluate the effects of physical and chemical properties of water on the persistence of LP and HP AIV (Stallknecht et al. 1990a, Stallknecht et al. 1990b, Brown et al. 2007, Brown et al. 2009). Five different subtypes isolated from four waterfowl species in Louisiana were diluted 1:50 in distilled water and infectivity was evaluated over time at two temperatures (Stallknecht et al. 1990b). Linear regression models were created to explain the data and estimate persistence of virus with a starting concentration of 10^6 $TCID_{50}$/ml. Virus persistence at 17°C ranged from 126 to 194 days and persistence at 28°C ranged from 30 to 104 days. In addition to confirming AIV sensitivity to temperature, the study also showed variable stability between virus subtypes. A follow up study examined the effects of temperature (17°C and 28°C), pH (6.2, 7.2, 8.2) and salinity (0ppt and 20 ppt) on the persistence of three isolates from waterfowl in Louisiana and found that viruses persisted longest at 17°C, pH 8.2, and 0ppt (Stallknecht et al. 1990a). Because water conditions were similar to those found in natural habitats of waterfowl, these data support theory the viruses persist in the environment and can infect hosts over an extended period of time.

An extensive evaluation of temperature, pH, and salinity was conducted on 12 wild-bird origin AIV isolated from anseriform and chardriiform species (Brown et al. 2009). Distilled water buffered with 10mM HEPES was manipulated to include

the following variables: two temperatures (17°C and 28 °C), eight pH values (5.8, 6.2, 6.6, 7.0, 7.4, 7.8, 8.2, and 8.6), and seven salinities (0, 5,000, 10,000, 15,000, 20,000, 25,000 and 30,000 parts per million (ppm)). The persistence of all AIV tested decreased with high temperature, low pH, and high salinity. The degree of response to each variable varied between subtypes, but overall reconfirmed the importance of physical and chemical properties on the persistence of AIV in the environment. HPAIV viruses react similarly to temperature, pH, and salinity, but do not persist as long as wild-type (LP) AIV (Brown et al. 2007).

In response to an outbreak of H5N1 in poultry and humans in Cambodia, samples from the environment were tested by real time reverse transcriptase- PCR (rRT-PCR) (Vong et al. 2008). A total of 167 samples from areas around 43 households that had infected domestic birds was collected, and 77 samples from 14 households were positive for subtype H5N1. Viral RNA was found in poultry feces (50%), soil swabs (50%), water plants in household ponds (50%), swabs from feathers of recently dead birds (50%), and mud (29%). These environmental samples were taken within 3 months of the outbreaks at each home, indicating that viruses were shed into the environment but because no viruses were isolated through passage in Maxine-Darby Canine Kidney (MDCK) cells it is unknown whether viruses can remain infective in these media.

Another study was performed in Asia was conducted in response to the reemergence of HPAIV H5N1 in China. Since 2004, the World Health Organization (WHO) has confirmed 38 human cases and 25 deaths over a large geographic range in mainland China (WHO 2009). Fang (2008) examined multiple environmental

factors in 128 outbreaks of HPAIV H5N1 in poultry and wild birds in 26 provinces and townships. Environmental factors included mean annual precipitation, poultry density, and minimal distances to lakes, wetlands, national highways, and main cities. This analysis of environmental factors showed that risk of infection of HPAIV H5N1 was positively correlated with increasing annual precipitation, decreasing minimal distances to the nearest lake and/or wetland, and proximity to the nearest national highway (Fang et al. 2008). This study emphasizes the importance of understanding the persistence of AIV in water, as it has implications for poultry and human health.

Lastly, and perhaps most convincing of the importance of environmental persistence, a recent study emphasized the importance of understanding persistence for elucidating the epidemiology of LPAIV in wild birds. Utilizing North American prevalence data (from Krauss et al. 2004), an SIR model was created that follows seasonal and multi year cycles of AIV (Breban et al. 2009). Stochastic and deterministic models that incorporated fecal-oral transmission, migration, seasonality, and pulsed reproduction were not able to show AIV dynamics. However, when fecal-oral and environmental transmission were incorporated into the model, the epidemiology of AIV was captured most successfully. Environmental transmission did not account for a large number of infections, but only 2-3 cases per year were necessary to sustain the dynamics of AIV that are observed in wild birds (with 2-4 year intervals between peaks of high prevalence). Environmental persistence was also shown to be more important in smaller populations of birds than in larger ones.

As demonstrated through several field, laboratory, and modeling studies, the persistence of AIV in the environment is important to understand because 1) AIV can remain infective in water reservoirs for extended periods (Stallknecht et al. 1990a, Stallknecht et al. 1990b, Brown et al. 2007, Brown et al. 2009), 2) aquatic sources of virus serve as a route of transmission within and between species (Hinshaw et al. 1979), 3) environmental transmission is a necessary part of the epidemiology of AIV within wild birds (Breban et al. 2009), and 4) understanding AIV transmission in wild bird species can help predict the risk of HPAIV emergence and infection in poultry and humans (Fang et al. 2008).

BIOLOGICAL FACTORS IN THE AQUATIC ENVIRONMENT THAT COULD INFLUENCE AVIAN INFLUENZA PERSISTENCE

Because AIV is spread via a fecal-oral route in contaminated water, the virus is exposed to properties of the water (as discussed above) but also organisms that live in the habitats of waterfowl. The habitats where AIV have been detected from water sources and isolated within wild birds cover a range of latitudes, but the greatest incidence of infection in freshwater waterfowl occurs in young birds at marshalling sites prior to migration (Hinshaw et al. 1980c, Hinshaw et al. 1985, Slemons et al. 1991, Sharp et al. 1993). At this time, mallards congregate in freshwater in high densities (Del Hoyo et al. 1996), thus providing conditions for AIV virus transmission. After breeding and prior to migration, ducks select habitats that have abundant food resources dense vegetative cover, resting areas, and isolation from disturbances (Gilmer et al. 1977, Hohman et al. 1992). To evaluate

the potential biological factors within aquatic habitats of pre migratory (post breeding) waterfowl that may affect AIV transmission, a more thorough understanding of the habitats and ecosystems where AIV are isolated from is necessary.

The majority of isolates from ducks are from dabbling species (feed from the surface of shallow water) compared to diving species (Olsen et al. 2006, Munster et al. 2007), and surface feeding is a predictor of the prevalence of LPAIV in a species (Garamszegi and Møller 2007). Mallards, one species of dabbling duck, primarily feed in shallow waters, sieving water for water plants, insects and mollusks (Arzel et al. 2006). In addition to understanding the host range of AIV, habitat requirements and feeding strategies are important when considering an environmentally transmitted virus.

There was one study conducted on biological components of the aquatic environment that may influence the persistence of AIV, however the results are inconclusive. Zarkov (2006) evaluated viral persistence in filtered and unfiltered water from five different sources of surface water. Chemical properties (i.e. nitrates, calcium, pH) and physical (temperature) were measured before trials and recorded for each sample. Experiments were not replicated and the difference of viral persistence between filtered and unfiltered waters was not determined to be statistically significant. Because the authors used a 0.45 μm filter, there may have been a substantial amount of microorganisms left in the filtered samples. In order to draw conclusions on the affect of microorganisms, more standardized trials starting with a higher initial viral titer need to be run and control samples should be filtered

with a 0.22 μm filter to ensure that the quantity of microorganisms is at a minimum. In a review of chemical and physical methods to inactivate AIV, the authors suggest organic material will inhibit decontamination processes in the environment but no data supports this claim (De Benedictis et al. 2007). It is unknown at this point whether factors in the aquatic environmental will inhibit AIV, increase its persistence or lead to an alternative transmission route.

Over 100 human and animal viruses are excreted in waste (Melnick 1984, Craun 1991), and are transmitted by water, food, formites, and direct contact (Fong and Lipp 2005). The viruses are collectively referred to as enteric viruses and cause illnesses such as gastroenteritis, conjunctivitis, and hepatitis (Bosch 1998). Like AIV in birds, the viruses typically replicate within the gastrointestinal tract of the host (Fong and Lipp 2005) and infected hosts will shed high concentrations of the virus, 10^5 to 10^{11} viral particles per gram of stool (Farthing 1989).

Enteric viruses are transmitted via contaminated water by drinking the water, using recreational water sources and urban rivers, and consuming shellfish exposed to contaminated waters (Rose et al. 1987, Gerba and Rose 1990, Cecuk et al. 1993, Lipp and Rose 1997, Jiang et al. 2001). Similar to AIV, enteric viruses can survive and remain infective in seawater for up to 130 days, in freshwater up to 120 days, and in soil (20°C to 30°C) up to 100 days (Environmental Protection U.S. Environmental Protection Agency 1992, Jiang et al. 2001). An early study also demonstrated a correlation between the persistence of enteric viruses and the activity of the native flora in natural water (Metcalf and Stiles 1967). While in the environment, enteric viruses accumulate in sediments, and have been shown to

persist longer than if suspended in the water column (Smith et al. 1978). These sediments can serve as reservoirs for the virus when active viruses are resuspended into the water column, and therefore can extend the infectivity period of viruses (Schaiberger et al. 1982, Rao et al. 1984, Bosch et al. 1988).

Although the effect of bacteria and other microorganisms on the persistence of AIV or enteric viruses has not been evaluated, there are several studies that examine chemical and biological characteristics of seawater that influence the infectivity of bacteriophages over time. Although the structure and transmission cycles of bacteriophages are very different than AIV and enteric viruses, researchers can use the parameters examined in the following studies to evaluate biological factors in the aquatic environment that influence viral persistence. The first study evaluated the persistence of the ΦX174 strain of bacteriophage and concluded that there were three mechanisms influencing the virus: microorganisms inactivated viruses, chemical components of the water affected the virus, and organic particulate matter protected the virus and decreased the rate of degradation (Mitchell and Jannasch 1969). A later study contradicted one conclusion, and showed that dissolved organic matter increased rates of inactivation (Noble and Fuhrman 1997).

Multiple studies showed a strong correlation between UV radiation and high rates of inactivation (Suttle and Chen 1992, Noble and Fuhrman 1997, 1999). Although bacteria decreased the persistence of viruses, proteases, nucleases and other enzymes had a greater influences on decay rates (Suttle and Chen 1992, Noble and Fuhrman 1997). However, as Noble and Fuhrman (1999) point out, the

presence of bacteria and protists increase the production of free and membrane bound nucleases and proteases, so bacteria and protists may not have as great direct influence on the persistence of viruses, but their combined direct and indirect affects may be crucial for understanding persistence of viruses in water. Lastly, these experiments also showed that there were differences of tolerances between the types of viruses and the origins of marine water samples, with viruses from certain environments persisting longer in the water they were extracted from (Noble and Fuhrman 1997, 1999).

Macroorganisms in the environment can also impact transmission cycles of viruses. The majority of research concerning macroorganisms focuses on filter-feeders within the ecosystem. Filter-feeding bivalves perform important functions in fresh- and salt water (Jorgensen 1990, Vaughn and Hakenkamp 2001, Newell 2004, Spooner and Vaughn 2006, Coen et al. 2007) and are ideal organisms to study for their influence on virus transmission. In many ecosystems, bivalves are the dominant filter-feeders and high densities can filter the entire flow of water in an aquatic system (Strayer et al. 1999). The maximum filtration rates recorded for mussels range from 0.5 to 1.0 L/ hour (Vaughn et al. 2008). In addition to processing a large amount of water, bivalves are very efficient and can take up particles as small as 3 μm (Jorgensen 1990, Bayne and Hawkins 1992). Filtration rates depend on the species of bivalve, the size of the individual, the population density, the temperature of the water, particle size and concentration, and flow regimes (Vaughn et al. 2008).

By filtering water, bivalves perform many vital ecosystem services. They reduce turbidity of the water and areas that have higher densities of bivalves are less cloudy. They play an important role in aquatic foodwebs through top down control of phytoplankton populations (Dame 1996). Filtration feeding by bivalves produces feces and pseudofeces (undigested material) that settle 40 times faster than unaggregated material (Kautsky and Evans 1987). By processing the water, the suspension feeding bivalves increase the rate of biodeposits (Jorgensen 1990), alter nutrient dynamics and link the benthic environment to the water column (Strayer et al. 1999, Spooner and Vaughn 2006). All of these ecosystem impacts have potential to alter the persistence and transmission of viruses in water.

Mollusks, particularly bivalves, can become infected by viruses by filter-feeding from contaminated water (Renault and Novoa 2004), and they can also bioaccumulate viruses from vertebrates (Meyers 1984). Sometimes they serve as transient hosts, reinfecting vertebrate hosts when ingested (Lees 2000). Human viruses that are transmitted via a fecal-oral in the families Calciviridae, Astroviridae, Rotoviridae, Adenoviridae, and Picornaviridae have been detected in bivalve mollusks (review in Lees 2000). Although there are over 100 viruses that are shed in human feces, only a few of these have been associated with shellfish-borne illnesses. Outbreaks associated with the consumption of shellfish include hepatitis A virus (Ohara et al. 1983, Sanchez et al. 2002), Norwalk virus (Grohmann et al. 1980, Grohmann et al. 1981, Gunn et al. 1982, Schwab et al. 1998), Snow Mountain agent (Truman et al. 1987), nonspecified SRSV (small round structured viruses) (Appleton 1987, Sekine et al. 1989), cockle agent (Appleton and Pereira 1977,

Appleton 1987), nonspecified parvovirus (Appleton 1987), astrovirus (Appleton 1987, Kurtz and Lee 1987), and calcivirus (Appleton 1987, Cubitt 1991). The effect of filter feeders on viral transmission also depends on the ecology of the virus host, the species of filter feeders, the density of the filter feeders, and the rate of filtration.

Laboratory studies conducted on commercially important mollusks and enteric diseases can be used as examples for studying the impact of filter feeders on AIV persistence and infectivity. Hepatitis A accumulated within tissues in concentrations up to 100x greater than virus in the water and persisted for 7 days in mussels (Enriquez et al. 1992). Accumulation of the viruses occurs within 24 hours and in as little as 3 hours. A study evaluating reovirus type III and Semliki Forest virus uptake by the rock oyster, *Crassotrea glomerata*, suggested that the uptake of virus has an upper limit, and once the sites for virus absorption are occupied filter-feeders can no longer remove virus from the water (Bedford et al. 1978). Bivalves are present in habitats where birds congregate and feed, and there is a possibility that filter-feeders in the habitat play a role in the persistence and infectivity of virus in the environment.

CONCLUSIONS AND OBJECTIVES

Avian influenza is an important global disease and has implications for wild and domestic bird health, human health, and agriculture (Clark and Hall 2006). Understanding the dynamics of AIV within wild bird populations will help elucidate risks factors in transmission to domestic birds and humans. Although environmental persistence is thought to play a crucial role in the maintenance of

AIV in wild birds (Breban et al. 2009), little is known about biological factors that may affect the persistence and transmission of AIV in the aquatic environment.

Because there is much potential for bivalves to accumulate virus and possible infect hosts, it should be a priority for investigating biological factors that influence the persistence and infectivity of AIV in the aquatic environment. The following chapter examines how filter-feeding bivalves affect the persistence and transmission of avian influenza. The Asian clam, *Corbicula fluminea* (Müller, 1774) (Mollusca: Corbiculidae), is used as a model organism. Previous studies have shown that clam populations affect both plankton abundance and water clarity, providing ecosystem services to their environs (Sousa et al. 2008). *C. fluminea* is also one of the most invasive bivalve species and has great potential to exert an influence on AIV persistence because of its high growth, filtration, and assimilation rates (McMahon 2002).

The objectives of the study were two-fold: 1) to determine if biological factors in the aquatic environment, in this case filter-feeding bivalves, influenced the persistence of AIV in water and 2) to ask whether filter-feeding bivalves increased viral persistence by harboring AIV (and infecting new hosts), decreased persistence by inactivating the virus, or did not influence the infectivity of AIV in water.

<u>WORKS CITED</u>

Alexander, D. J. 2000. A review of avian influenza in different bird species. Veterinary Microbiology **74**:3-13.

Alexander, D. J., W. H. Allan, D. G. Parsons, and G. Parsons. 1978. The pathogenicity of four avian influenza viruses for fowls, turkeys, and ducks. Research in Veterinary Science **24**:242-247.

Alexander, D. J., M. S. Collins, and R. E. Gough. 1980. Isolation of an influenza A virus from avian faeces samples collected at a London reservoir. The Veterinary Record **107**:41-42.

Alfonso, C. P., B. S. Cowen, and H. Van Campen. 1995. Influenza A viruses isolated from waterfowl in two wildlife management area of Pennsylvania. Journal of Wildlife Diseases **31**:179-185.

Appleton, H. 1987. Small round viruses: classification and role in food-borne infections. Pages 108-125 *in* Ciba Foundation Symposium. John Wiley and Sons, Chichester, UK.

Appleton, H. and M. S. Pereira. 1977. A possible virus aetiology in outbreaks of food-poisoning from cockles. Lancet **1**:780.

Arzel, C., J. Elmberg, and M. Guillemain. 2006. Ecology of spring-migrating Anatidae: a review. Journal of Ornithology **147**:167-184.

Austin, F. J. and R. G. Webster. 1993. Evidence of ortho-and paramyxoviruses in fauna from Antarctica. Journal of Wildlife Diseases **29**:568.

Bayne, B. L. and A. J. S. Hawkins. 1992. Ecological and physiological aspects of hebivory in benthic suspension feeding molluscs. Pages 265-288 *in* D. M.

John, S. J. Hawkins, and J. H. Price, editors. Plant-animal interactions in the marine benthos. Clarendon Press, Oxford.

Becker, W. B. 1966. The isolation and classification of tern virus: influenza virus A/tern/South Africa/1961. The Journal of Hygiene:309-320.

Bedford, A. J., G. Williams, and A. R. Bellamy. 1978. Virus accumulation by the rock oyster Crassostrea glomerata. Applied and Environmental Microbiology **35**:1012-1018.

Bosch, A. 1998. Human enteric viruses in the water environment: a minireview. Int. Microbiol **1**:191.

Bosch, A., F. Lucena, R. Girones, and J. Jofre. 1988. Occurrence of enteroviruses in marine sediment along the coast of Barcelona, Spain. Canadian Journal of Microbiology **34**:921-924.

Breban, R., J. M. Drake, D. E. Stallknecht, and P. Rohani. 2009. The Role of Environmental Transmission in Recurrent Avian Influenza Epidemics. PLoS Computational Biology **5**.

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. 2009. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. Veterinary Microbiology **136**:20-26.

Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht. 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Diseases **51**:285-289.

Cecuk, D., V. Kruzic, B. Turkovic, and M. Grce. 1993. Human viruses in the coastal environment of a Croatian harbor. Revue d'épidémiologie et de santé publique **41**:487.

Clark, L. and J. Hall. 2006. Avian influenza in wild birds: status as reservoirs, and risks to humans and agriculture. Ornithological monographs **60**:3-29.

Coen, L. D., R. D. Brumbaugh, D. Bushek, R. Grizzle, M. W. Luckenbach, M. H. Posey, S. P. Powers, and S. G. Tolley. 2007. Ecosystem services related to oyster restoration. Marine Ecology-Progress Series **341**:303-307.

Compans, R. W., H.-D. Klenk, L. A. Caliguiri, and P. W. Choppin. 1970. Influenza virus proteins : I. Analysis of polypeptides of the virion and identification of spike glycoproteins. Virology **42**:880-889.

Cooley, A. J., H. Vancampen, M. S. Philpott, B. C. Easterday, and V. S. Hinshaw. 1989. Pathological lesions in the lungs of ducks infected with influenza A viruses. Veterinary Pathology **26**:1-5.

Craun, G. F. 1991. Causes of waterborne outbreaks in the United States. Water Science & Technology **24**:17-20.

Cubitt, W. D. 1991. Review of the epidemiology and diagnosis of waterborne viral infections. Water Science & Technology **24**:197-203.

Dame, R. F. 1996. Ecology of Marine Bivalves: An Ecosystem Approach. CRC Press.

De Benedictis, P., M. S. Beato, and I. Capua. 2007. Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. Zoonoses And Public Health **54**:51-68.

Deibel, R., D. E. Emord, W. Dukelow, V. S. Hinshaw, and J. M. Wood. 1985. Influenza Viruses and Paramyxoviruses in Ducks in the Atlantic Flyway, 1977-1983, Including an H5N2 Isolate Related to the Virulent Chicken Virus. Avian Diseases **29**:970-985.

Del Hoyo, J., A. Elliott, and J. Sargatal. 1996. Handbook of the Birds of the World Lynx Edicions.

Enriquez, R., G. G. Froesner, V. Hochstein-Mintzel, S. Riedemann, and G. Reinhardt. 1992. Accumulation and persistence of hepatitis A virus in mussels. Journal of Medical Virology **37**:174-179.

Fang, L. Q., S. J. de Vlas, S. Liang, C. W. Looman, P. Gong, B. Xu, L. Yan, H. Yang, J. H. Richardus, and W. C. Cao. 2008. Environmental factors contributing to the spread of H5N1 avian influenza in mainland China. Plos ONE **3**:e2268-e2268.

Farthing, M. J. G. 1989. Viruses and the gut. Smith Kline & French, Walwyn Garden City, Hertfordshire, United Kingdom.

Fenner, F., P. A. Bachmann, E. P. Gibbs, F. Murphy, M. J. Studdert, and D. O. White. 1987. Orthomyxoviridae. Pages 112-114; 473-484 *in* D. O. White, editor. Veterinary Virology. Academic Press, Inc., San Diego, CA.

Fong, T. T. and E. K. Lipp. 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiology and Molecular Biology Reviews **69**:357-371.

Fouchier, R. A. M., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. Osterhaus. 2005. Characterization of a

novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal Of Virology **79**:2814.

Fouchier, R. A. M., B. Olsen, T. M. Bestebroer, S. Herfst, L. Van der Kemp, G. F. Rimmelzwaan, and A. Osterhaus. 2003. Influenza A virus surveillance in wild birds in Northern Europe in 1999 and 2000. Avian Diseases **47**:857-860.

Garamszegi, L. Z. and A. P. Møller. 2007. Prevalence of avian influenza and host ecology. Proceedings of the Royal Society B: Biological Sciences **274**:2003.

Gerba, C. P. and J. B. Rose. 1990. Viruses in source and drinking water. IN: Drinking Water Microbiology: Progress and Recent Developments. Springer-Verlag New York, Inc., New York. 1990. p 380-396, 5 tab, 64 ref.

Gilmer, D. S., R. E. Kirby, I. J. Ball, and J. H. Riechmann. 1977. Post-Breeding Activities of Mallards and Wood Ducks in North-Central Minnesota. The Journal of Wildlife Management **41**:345-359.

Grohmann, G. S., H. B. Greenberg, B. M. Welch, and A. M. Murphy. 1980. Oyster-associated gastroenteritis in Australia: the detection of Norwalk virus and its antibody by immune electron microscopy and radioimmunoassay. Journal of Medical Virology **6**.

Grohmann, G. S., A. M. Murphy, P. J. Christopher, E. Auty, and H. B. Greenberg. 1981. Norwalk virus gastroenteritis in volunteers consuming depurated oysters. Aust. J. Exp. Biol. Med. Sci **59**:219-228.

Gunn, R. A., H. T. Janowski, S. Lieb, E. C. Prather, and H. B. Greenberg. 1982. Norwalk virus gastroenteritis following raw oyster consumption. American Journal of Epidemiology **115**:348-351.

Halvorson, D. A., C. J. Kelleher, and D. A. Senne. 1985. Epizootiology of avian influenza: effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota. Applied and Environmental Microbiology **49**:914-919.

Hanada, K., Y. Suzuki, and T. Gojobori. 2004. A large variation in the rates of synonymous substitution for RNA viruses and its relationship to a diversity of viral infection and transmission modes. Pages 1074-1080. SMBE.

Hanson, B. 2002. Temporal, spatial and species patterns of avian influenza viruses among wild birds. MS Dissertation. The University of Georgia, Athens, GA.

Hinshaw, V. and D. Webster. 1982. The natural history of influenza A viruses. Pages 79-104 *in* A. S. Beare, editor. Basic and Applied Influenza Research CRC Press, Inc., Boca Raton, FL.

Hinshaw, V. S., G. M. Air, A. J. Gibbs, L. Graves, B. Prescott, and D. Karunakaran. 1982. Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. Journal Of Virology **42**:865-872.

Hinshaw, V. S., W. J. Bean, R. G. Webster, and G. Sriram. 1980a. Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102**:412-419.

Hinshaw, V. S., V. F. Nettles, L. F. Schorr, J. M. Wood, and R. G. Webster. 1986. Influenza Virus Surveillance in Waterfowl in Pennsylvania after the H5N2 Avian Outbreak. Avian Diseases **30**:207-212.

Hinshaw, V. S., R. G. Webster, W. J. Bean, and G. Sriram. 1980b. The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies Comparative Immunology Microbiology and Infectious Diseases **3**:155-164.

Hinshaw, V. S., R. G. Webster, and B. Turner. 1979. Water-borne Transmission of Influenza A Viruses. Intervirology **11**:66-68.

Hinshaw, V. S., R. G. Webster, and B. Turner. 1980c. The Perpetuation of Orthomyxoviruses and Paramyxoviruses in Canadian Waterfowl. Canadian Journal of Microbiology **26**:622-629.

Hinshaw, V. S., J. M. Wood, R. G. Webster, R. Deibel, and B. Turner. 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bulletin of the World Health Organization **63**:711.

Hohman, W. L., C. D. Ankney, and D. H. Gordon. 1992. Ecology and Management of Postbreeding Waterfowl. Pages 128-189 *in* B. D. J. Batt, A. D. Afton, M. G. Anderson, C. D. Ankney, D. H. Johnson, J. A. Kadlec, and G. L. Krapu, editors. Ecology and Management of Breeding Waterfowl. University of Minnesota Press, Minneapolis, MN.

Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol. 1982. Rapid evolution of RNA genomes. Science **215**:1577-1585.

Homme, P. J. and B. C. Easterday. 1970. Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A/turkey/Wisconsin/1966 virus. Avian Diseases **14**:285-290

Ito, T., K. Okazaki, Y. Kawaoka, A. Takada, R. G. Webster, and H. Kida. 1995. Perpetuation of Influenza A Viruses in Alaskan Waterfowl Reservoirs. Archives of Virology **140**:1163-1172.

Jiang, S., R. Noble, and W. Chu. 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. Applied and Environmental Microbiology **67**:179-184.

Jorgensen, C. B. 1990. Bivalve Filter Feeding: Hydrodynamics, Bioenergetics, Physiology and Ecology. Olsen & Olsen, Fredensborg, Denmark.

Kautsky, N. and S. Evans. 1987. Role of biodeposition by Mytilus edulis in the circulation of matter and nutrients in a Baltic coastal ecosystem. Marine Ecology Progress Series **38**:201-212.

Kawaoka, Y., T. M. Chambers, W. L. Sladen, and R. Gwebster. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology **163**:247-250.

Kida, H., R. Yanagawa, and Y. Matsuoka. 1980. Duck influenza lacking evidence of disease signs and immune response. Infection and Immunity **30**:547-553.

Kocan, A. A., V. S. Hinshaw, and G. A. Daubney. 1980. Influenza A viruses isolated from migrating ducks in Oklahoma. Journal of Wildlife Diseases **16**:281.

Krauss, S., D. Walker, S. P. Pryor, L. Niles, L. Chenghong, V. S. Hinshaw, and R. G. Webster. 2004. Influenza A Viruses of Migrating Wild Aquatic Birds in North America. Vector-Borne & Zoonotic Diseases **4**:177-189.

Kurtz, J. B. and T. W. Lee. 1987. Astroviruses: human and animal. Pages 92-107 *in* Ciba Foundation Symposium. John Wiley and Sons, Chichester, UK.

Latorre-Margalef, N., G. Gunnarsson, V. J. Munster, R. A. M. Fouchier, A. D. M. E. Osterhaus, J. Elmberg, B. Olsen, A. Wallensten, P. D. Haemig, T. Fransson, L. Brudin, and J. Waldenström. 2009. Effects of influenza A virus infection on

migrating mallard ducks. Proceedings of the Royal Society B-Biological Sciences **276**:1029-1036.

Lees, D. 2000. Viruses and bivalve shellfish. International Journal of Food Microbiology **59**:81-116.

Lipp, E. K. and J. B. Rose. 1997. The role of seafood in foodborne diseases in the United States of America. Revue scientifique et technique (International Office of Epizootics) **16**:620.

McMahon, R. F. 2002. Evolutionary and physiological adaptations of aquatic invasive animals: r selection versus resistance. Canadian Journal of Fisheries and Aquatic Sciences **59**:1235-1244.

Melnick, J. L. 1984. Etiologic agents and their potential for causing waterborne virus diseases. Enteric viruses in water **15**:1–16.

Metcalf, T. G. and W. C. Stiles. 1967. Survival of enteric viruses in estuary waters and shellfish. Pages 439-444 *in* G. Berg, editor. Transmission of Viruses by the Water Route. Interscience, New York.

Meyers, T. R. 1984. Marine bivalve mollusks as reservoirs of viral finfish pathogens: significance to marine and anadromous finfish aquaculture. Marine Fisheries Review **46**:14-17.

Mitchell, R. and H. W. Jannasch. 1969. Processes controlling virus inactivation in seawater. Environmental Science & Technology **3**:941-943.

Munster, V. J., C. Baas, P. Lexmond, J. Waldenstrom, A. Wallensten, T. Fransson, G. F. Rimmelzwaan, W. E. P. Beyer, M. Schutten, B. Olsen, A. Osterhaus, and R. A. M.

Fouchier. 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. Plos Pathogens **3**:630-638.

Nettles, V. F., J. M. Wood, and R. G. Webster. 1985. Wildlife Surveillance Associated with an Outbreak of Lethal H5N2 Avian Influenza in Domestic Poultry. Avian Diseases **29**:733-741.

Newell, R. I. E. 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. Journal of Shellfish Research **23**:51-62.

Noble, R. T. and J. A. Fuhrman. 1997. Virus Decay and Its Causes in Coastal Waters. Appl. Environ. Microbiol. **63**:77-83.

Noble, R. T. and J. A. Fuhrman. 1999. Breakdown and microbial uptake of marine viruses and other lysis products. Aquatic microbial ecology **20**:1-11.

O'Neill, R. E., J. Talon, and P. Palese. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. Embo Journal **17**:288-296.

Ohara, H., H. Naruto, W. Watanabe, and I. Ebisawa. 1983. An outbreak of hepatitis A caused by consumption of raw oysters. The Journal of Hygiene:163-165.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. Osterhaus, and R. A. M. Fouchier. 2006. Global patterns of influenza A virus in wild birds. Science **312**:384-388.

Ottis, K. and P. A. Bachmann. 1983. Isolation and characterization of ortho-and paramyxoviruses from feral birds in Europe. Zentralblatt für Veterinärmedizin. Reihe B. Journal of veterinary medicine. Series B **30**:22.

Palese, P. and M. L. Shaw. 2006. Orthomyxoviridae: The viruses and their replication. Pages 1648-1689 *in* D. M. Knipe and P. M. Howley, editors. Fields virology. Lippincott Williams & Wilkins, Philadelphia, PA.

Rao, V. C., K. M. Seidel, S. M. Goyal, T. G. Metcalf, and J. L. Melnick. 1984. Isolation of enteroviruses from water, suspended solids, and sediments from Galveston Bay: survival of poliovirus and rotavirus adsorbed to sediments. Applied and Environmental Microbiology **48**:404-409.

Renault, T. and B. Novoa. 2004. Viruses infecting bivalve molluscs. Aquating Living Resources **17**:397-409.

Rose, J. B., R. L. Mullinax, S. N. Singh, M. V. Yates, and C. P. Gerba. 1987. Occurrence of rotaviruses and enteroviruses in recreational waters of Oak Creek, Arizona. Water Research **21**:1375-1381.

Rosenberger, J. K., W. C. Krauss, and R. D. Slemons. 1974. Isolation of Newcastle Disease and Type-A Influenza Viruses from Migratory Waterfowl in the Atlantic Flyway. Avian Diseases **18**:610-613.

Sanchez, G., R. M. Pinto, H. Vanaclocha, and A. Bosch. 2002. Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak. Journal of clinical microbiology **40**:4148-4155.

Schaiberger, G. E., T. D. Edmond, and C. P. Gerba. 1982. Distribution of enteroviruses in sediments contiguous with a deep marine sewage outfall. Water Research **16**:1425-1428.

Schwab, K. J., F. H. Neill, M. K. Estes, T. G. Metcalf, and R. L. Atmar. 1998. Distribution of Norwalk Virus within Shellfish Following Bioaccumulation and

Subsequent Depuration by Detection Using RT-PCR. Journal of Food Protection **61**:1674-1680.

Sekine, S., S. Okada, Y. Hayashi, T. Ando, T. Terayama, K. Yabuuchi, T. Miki, and M. Ohashi. 1989. Prevalence of small round structured virus infections in acute gastroenteritis outbreaks in Tokyo. Microbiology and Immunology **33**:207.

Sharp, G. B., Y. Kawaoka, S. M. Wright, B. Turner, V. Hinshaw, and R. G. Webster. 1993. Wild Ducks Are the Reservoir for Only a Limited Number of Influenza A Subtypes. Epidemiology and Infection **110**:161-176.

Sinnecker, R., H. Sinnecker, E. Zilske, and D. Köhler. 1983. Surveillance of pelagic birds for influenza A viruses. Acta virologica **27**:75.

Slemons, R. D. and B. C. Easterday. 1977. Type-A influenza viruses in the feces of migratory waterfowl. Journal of the American Veterinary Medical Association **171**:947-948.

Slemons, R. D., M. C. Shieldcastle, L. D. Heyman, K. E. Bednarik, and D. A. Senne. 1991. Type A Influenza Viruses in Waterfowl in Ohio and Implications for Domestic Turkeys. Avian Diseases **35**:165-173.

Smith, E. M., C. P. Gerba, and J. L. Melnick. 1978. Role of sediment in the persistence of enteroviruses in the estuarine environment. Applied and Environmental Microbiology **35**:685-689.

Sousa, R., C. Antunes, and L. Guilhermino. 2008. Ecology of the invasive Asian clam Corbicula fluminea (Müller, 1774) in aquatic ecosystems: an overview. Pages 85-94. edpsciences. org.

Spackman, E. 2009. The ecology of avian influenza virus in wild birds: What does this mean for poultry? Poultry Science **88**:847-850.

Spackman, E., K. G. McCracken, K. Winker, and D. E. Swayne. 2006. H7N3 avian influenza virus found in a South American wild duck is related to the Chilean 2002 poultry outbreak, contains genes from equine and North American wild bird lineages, and is adapted to domestic turkeys. Journal Of Virology **80**:7760-7764.

Spooner, D. E. and C. C. Vaughn. 2006. Context-dependent effects of freshwater mussels on stream benthic communities. Freshwater Biology **51**:1016-1024.

Stallknecht, D. E. 2003. Ecology and Epidemiology of Avian Influenza Viruses in Wild Bird Populations: Waterfowl, Shorebirds, Pelicans, Cormorants, Etc. Avian Diseases:61-69.

Stallknecht, D. E. and J. D. Brown. 2007. Wild birds and the epidemiology of avian influenza. Journal of Wildlife Diseases **43**:S15.

Stallknecht, D. E. and J. D. Brown. 2008. Ecology of Avian Influenza in Wild Birds. Pages 43-58 *in* D. E. Swayne, editor. Avian Influenza. Blackwell Publishing, Ames, Iowa.

Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990a. Effects of pH, Temperature, and Salinity on Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:412-418.

Stallknecht, D. E. and S. M. Shane. 1988. Host Range of Avian Influenza Virus in Free Living Birds. Veterinary Research Communications **12**:125-141.

Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990b. Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:406-411.

Steinhauer, D. A. and J. J. Holland. 1987. Rapid evolution of RNA viruses. Annual Reviews in Microbiology **41**:409-431.

Strayer, D. L., N. F. Caraco, J. J. Cole, S. Findlay, and M. L. Pace. 1999. Transformation of Freshwater Ecosystems by Bivalves. Bioscience **49**:19.

Süss, J., J. Schäfer, H. Sinnecker, and R. G. Webster. 1994. Influenza virus subtypes in aquatic birds of eastern Germany. Archives of Virology **135**:101-114.

Suttle, C. A. and F. Chen. 1992. Mechanisms and rates of decay of marine viruses in seawater. Applied and Environmental Microbiology **58**:3721-3729.

Truman, B. I., H. P. Madore, M. A. Menegus, J. L. Nitzkin, and R. Dolin. 1987. Snow Mountain agent gastroenteritis from clams. American Journal of Epidemiology **126**:516-525.

U.S. Environmental Protection Agency. 1992. Manual on guidelines for water reuse. Center for Environmental Reservation Information, Cincinnati, Ohio.

Vaughn, C. C. and C. C. Hakenkamp. 2001. The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology **46**:1431-1446.

Vaughn, C. C., S. J. Nichols, and D. E. Spooner. 2008. Community and foodweb ecology of freshwater mussels. Journal of the North American Benthological Society **27**:409-423.

Vong, S., S. Ly, S. Mardy, D. Holl, and P. Buchy. 2008. Environmental contamination during influenza A virus (H5N1) outbreaks, Cambodia, 2006. Emerging Infectious Diseases **14**:1303-1305.

Wallensten, A., V. J. Munster, A. Osterhaus, J. Waldenström, J. Bonnedahl, T. Broman, R. A. M. Fouchier, and B. Olsen. 2006. Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region. Antarctic Science **18**:353-356.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and Ecology of Influenza A Viruses. Microbiological Reviews **56**:152-179.

Webster, R. G., M. Morita, C. Pridgen, and B. Tumova. 1976. Ortho- and Paramyxoviruses from Migrating Feral Ducks: Characterization of a New Group of Influenza A Viruses. J Gen Virol **32**:217-225.

Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. Copal Murti. 1978. Intestinal influenza: Replication and characterization of influenza viruses in ducks. Virology **84**:268-278.

World Health Organization. 2009. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO.

World Organization for Animal Health. 2008. Avian Influenza 10.4.1. Terrestrial Animal Health Code-2008. World Organization for Animal Health, Paris, France.

Zarkov, I. S. 2006. Survival of avian influenza viruses in filtered and natural surface waters of different physical and chemical parameters. Revue De Medecine Veterinaire **157**:471.

Zhang, G., D. Shoham, D. Gilichinsky, S. Davydov, J. D. Castello, and S. O. Rogers. 2006. Evidence of Influenza A Virus RNA in Siberian Lake Ice. Journal Of Virology **80**:12229-12235.

CHAPTER 3

FILTER-FEEDING BIVALVES CAN REMOVE AVIAN INFLUENZA VIRUSES FROM WATER AND REDUCE INFECTIVITY[1]

[1] Christina Faust, David Stallknecht, David Swayne, & Justin Brown. Accepted by *Proc. R. Soc. B*. Reprinted here with permission of publisher, 07/09/2009

SUMMARY

Avian influenza (AI) viruses are believed to be transmitted within wild aquatic bird populations through an indirect fecal-oral route involving contaminated water. This study examined the influence of filter-feeding bivalves, *Corbicula fluminea*, on the infectivity of AI virus in water. Clams were placed into individual flasks with distilled water inoculated 1:100 with a low pathogenic (LP)AI virus (A/Mallard/MN/190/99 (H3N8)). Viral titers in water with clams were significantly lower at 24- and 48-hr post-inoculation compared to LPAI-infected water without clams. To determine whether clams affected the infectivity of AI viruses, 18 wood ducks (*Aix sponsa*) were divided into test groups and inoculated with a variety of treatments of clam supernatant, whole clams, and water exposed to a high pathogenic (HP)AI (A/whooper swan/Mongolia/244/05 (H5N1)). None of the wood ducks inoculated with HPAI-infected water that was filtered by clams or that were inoculated or fed tissue from these clams exhibited morbidity or mortality. All wood ducks exposed to either HPAI-infected water without clams or the original viral inoculum died. These results indicate that filter-feeding bivalves can remove and reduce the infectivity of AI viruses in water, and demonstrate the need to examine biotic environmental factors that can influence AI virus transmission.

KEYWORDS

Avian influenza, transmission, environment, persistence, water, *Corbicula fluminea*

INTRODUCTION

Avian influenza (AI) viruses have been isolated from more than 105 wild bird species representing 13 orders (Olsen et al. 2006), but species in the Orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, and terns) are considered the natural reservoirs (Stallknecht & Shane 1988). Characteristics of this aquatic bird reservoir system include subclinical viral infections within these avian hosts (Slemons et al. 1974; Webster et al. 1992), an efficient transmission mechanism involving the aquatic habitats utilized by these avian species (Webster et al. 1992), and viral stability in water (Hinshaw et al. 1979; Stallknecht et al. 1990b). Viral replication within aquatic birds occurs within the epithelial cells lining the intestinal tract, and virus is excreted into the environment in high concentrations in feces (Hinshaw & Webster 1982; Webster et al. 1978). Transmission of AI viruses among individuals within these aquatic bird populations is believed to occur through an indirect fecal-oral route in which virus-contaminated water facilitates dissemination (Hinshaw et al. 1979). In addition to playing an important role in the transmission of AI virus within wild bird populations, the aquatic environment has also been suggested to contribute to the long-term maintenance of AI viruses within avian populations (Webster et al. 1992). Models of endemic AI in anseriform species in North America suggest that transmission of viruses that survive in the environment is essential for maintaining AI in populations, especially in small communities (Breban et al. 2009).

Although water potentially plays an important role in the transmission and maintenance of AI in wild avian hosts, very little is currently known about what environmental factors influence the ability of AI viruses to remain infectious in this medium. Most of what is understood about this topic has focused on the influence of abiotic conditions of water. Based on experiments utilizing a laboratory-based distilled water model system, persistence of low pathogenic (LP) and high pathogenic (HP) AI viruses in water has been shown to depend on water temperature, pH, and salinity (Brown et al. 2007b; Stallknecht et al. 1990a; Stallknecht et al. 1990b). The ability to persist in water varied among different AI viruses, but generally viruses remained infective for months if not years under optimal conditions which include cold temperatures, fresh to brackish salinities, and slightly basic pH (Brown et al. 2007b; Stallknecht et al. 1990a; Stallknecht et al. 1990b). AI viruses have been isolated from surface waters where waterfowl are found, demonstrating field support for these laboratory results (Halvorson et al. 1985; Hinshaw et al. 1980; Ito et al. 1995).

Despite the above effects of abiotic characteristics of the aquatic environment, little is known about the influence of biological factors on AI virus persistence in water. Filter-feeding bivalves have the potential to exert a significant influence on AI viruses in aquatic habitats due to their widespread distribution, overlapping habitat utilization, and feeding behavior. Filter-feeding bivalves are considered an important link between the water column and benthic communities, removing seston and excreting deposits in the substrate in the form of feces and pseudofeces (undigested material) (Strayer et al. 1999). Rates of filtration of bivalve populations

equal or exceed rates of other filter-feeders such as pelagic grazers; bivalves filter as much as 10-100% of the water column daily and can uptake particles sized 5 to 30,000 μm^2 (Dame 1996; Wallace et al. 1977).

The potential for filter feeding bivalves to play a role in the transmission of viral diseases is not novel (Rippey 1994). Other viruses that utilize a fecal-oral route of transmission have been shown to accumulate and remain infective in various bivalve species including hepatitis A virus in mussels and Norwalk virus in oysters and clams (Enriquez et al. 1992; Schwab et al. 1998). The removal of virus from water by filtering bivalves can occur rapidly; half of a reovirus type III concentration was removed from the water column by New Zealand rock oysters (*Crassotrea glomerata*) within 24 hrs (Bedford et al. 1978).

To examine the potential for filter feeding bivalves to remove AI viruses from aquatic habitats and to provide a source of virus to aquatic birds, the Asiatic clam *Corbicula fluminea* was selected as a model bivalve species. *C. fluminea* is native to eastern Asia but is currently established in lakes and rivers in the United States south of 40º latitude (Counts III 1986). *C. fluminea* was selected as the model organism because it was locally available, is tolerant to a variety of habitats and laboratory conditions, and has a great potential to remove particles from the water column due to the high densities in which it is found in the field (up to 10,000 individuals/m^2) as well as high filtration rates of 1-2 L/hr/g (Cohen et al. 1984; McMahon 1983).

Our objectives in this research are two-fold: (1) to determine whether the filtering behavior of *C. fluminea* reduces the concentration of AI virus in water and

therefore influences viral persistence in an aquatic habitat, and (2) to determine whether H5N1 HPAI-infected water and clams after 48-hr of exposure are infective to wood ducks (*Aix sponsa*)- a highly susceptible waterfowl species. For objective 1, a LPAI virus (A/Mallard/MN/190/99 [H3N8]) was used to determine if *C. fluminea* could affect viral titer in water over time using a laboratory-based system to simulate aquatic habitats. For objective 2, a Eurasian lineage H5N1 HPAI virus (A/whooper swan/Mongolia/244/05 [H5N1]) was used to evaluate the influence that clams have on AI virus infectivity *in vivo* within this model system.

METHODS

Experiment 1: LPAI Viral Persistence in Water in the Presence of *C. fluminea*

Virus. A wild bird-origin LPAI virus A/Mallard/MN/190/99 (H3N8) was propagated in 9- to11-day old specific-pathogen-free (SPF) embryonating chicken eggs using previously described protocols (Swayne 1998). Infective amnio-allantoic fluid (AAF) was stored at -70º C until trials were performed. All trials were performed with low passage virus isolates (second or third passages).

Model invertebrate organism. *Corbicula fluminea* were collected from sediments in a Northeast Georgia piedmont stream (Sandy Creek, Upper Oconee Watershed, Georgia, USA). Clams of similar size, approximately 3 cm width, were collected from the field for each experiment. Shells were rinsed thoroughly with distilled water to remove any surface debris, and clams were immediately used in laboratory trials.

Experimental setup. Individual clams were placed in a 75-cm^2 cell culture flasks (Corning Life Sciences, Lowell, MA, USA) fitted with an airstone and containing 200

ml of distilled water. All flasks were kept in a biosafety cabinet. Because the water was aerated and live, wild clams were used during trials; preliminary experiments were conducted to determine: 1) if aeration or the presence of the airstone affected viral titer; 2) if clams significantly affected pH over the duration of the trials; and 3) to determine if a loss of viral titer resulted from adherence of virions to the shells. Flasks with and without aeration were set up and monitored over 48-hr using established protocols (Brown et al. 2007b; Stallknecht et al. 1990a; Stallknecht et al. 1990b). Each flask was inoculated 1:100 with the AI-infected AAF. After inoculation, the water was mixed with a 1 ml pipette, and a 1.0 ml aliquot was removed (0-hr PI) and stored at 4° C until viral titration in tissue culture as described below. Aliquots were also collected at 24- and 48-hr PI for titration.

The duration of AI virus infectivity was not significantly influenced by the presence of tubing and airstones for aeration. The potential effect of clams on the pH of the water was also evaluated because previous studies have shown that AI persistence in water varies with pH (Brown et al. 2007b; Stallknecht et al. 1990a; Stallknecht et al. 1990b). A sample of water from flasks with and without clams was measured with a SympHony Meter (VWR, Beverly, MA, USA) over a 48-hr period. Water pH in flasks with clams increased on average over the 48-hr period from 6.53 to 7.22 (n=6), which was greater than the 6.62 to 6.91 change observed in the control without clams (n=1) (table 3.3).

To determine the effects of AI virus adherence to clam shells on viral titer, clam tissue was removed from shells (n=2). Valves were glued together to control surface area because previous observation showed that live clams remained closed

for a much larger portion of time in captivity. Viral titer was determined by RRT-PCR and compared to previous results from flasks with clams (n=13) and without clams (n=3) that were also analyzed by RRT-PCR at 0-, 24-, and 48-hr PI. The presence of clam shells resulted in a decrease in titer that was not significantly different than control flasks but was a significantly smaller decrease than flasks with live clams at 48-hr PI ($p<0.01$) (figure 3.4you).

Experimental trials of LPAI virus persistence. To fully characterize the temporal dynamics of viral persistence in the presence of *C. fluminea,* we conducted 48 trials that were separated into three study groups which varied in the timing and frequency of water sampling: 1) 48 hr trial (n=33): 1.0 ml aliquots of water were collected at 0-, 24-, and 48-hr PI, 2) short time interval 48 hr trial (n=8): 1.0 ml aliquots of water were collected approximately every 6 hrs from 0- to 48-hr PI; 3) 96 hr trial (n=7): 1.0 ml aliquots of water were collected at 0-, 24-, 48-, 72-, and 96-hr PI. Regardless of study group, water samples were collected at 0-, 24-, and 48-hr PI in all 48 trials.

In each trial, a single clam (n=48) was placed into a flask immediately after viral inoculation. After inoculation, the water was mixed with a 1 ml pipette and a 1.0 ml aliquot was removed (0-hr PI) and stored at 4ºC until viral titration assays and RRT-PCR were performed at the end of the 48- or during the 96-hr trials. Water samples were tested no more than 50 hrs after collection (samples from 96-hr trials were tested in 2 groups). Previous studies by our lab have shown that viral titers of AI are stable in distilled water for months to years at 4 C (Stallknecht et al. 1990a).

Infectivity assays. The infectivity of AI virus in water samples was quantified using a microtiter endpoint titration in primary cultures of chicken embryo fibroblasts (CEF) prepared from 9- to 11- day old SPF embryonating chicken eggs as previously described (Stallknecht et al. 1990b). Viral titers were calculated as previously described (Reed 1938) and recorded as 50% tissue culture infective doses, $TCID_{50}$/ml. The minimal detectable limit of the assay is $10^{2.17}$ $TCID_{50}$/ml.

RNA Extraction, Primer Sets, and RRT-PCR. Viral RNA was extracted from 50µl of water samples with clams (n=13) and without clams (n=2) and from 50µl of clam tissue supernatant from clams exposed (n=8) and unexposed (n=1) to virus-inoculated water using a RNeasy Qiagen kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Viral stock of the A/Mallard/MN/190/99 subtype was used as a positive control for RNA extractions, and distilled water was used as a negative control. RNA extracts were immediately tested using real-time RT-PCR (RRT-PCR).

RRT-PCR was carried out with a Qiagen one-step kit using a 25µL mixture containing 8 µL RNA, 6.95 µL H_2O, 5µL 5X buffer, 1.25µL 25mm $MgCl_2$, 1 µL of Enzyme Mixture, 0.5 µM forward primer (M +25, 5'- AGA TGA GTC TTC TAA CCG AGG TCG -3'), 0.5 µM reverse primer (M -124, 5'- TGC AAA AAC ATC TTC AAG TCT CTG -3'), 0.8 µL dNTPs, 0.5 µL RNAse inhibitor, and 0.5 µL of photosensitive probe (M + 64 5'- FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1 -3'). Primers were for the conserved region of the Matrix gene in Influenza A viruses (Spackman et al. 2002). The reaction was carried out in a SmartCycler thermocycler (Cepheid Sunnyvale, CA, USA) for 30 min at 50.0°C, followed by 15 min at 95.0°C, and amplifying for 45

subsequent cycles alternating between 95.0°C for 1 sec and 60.0°C for 20 sec. A threshold cycle (C_T) of <40 cycles was used as a diagnostic cutoff for determining positive samples during analysis.

Analysis of exposed clam tissue. At the end of 48-hr trials, clams exposed to AI virus-infected water (n=14) and unexposed control clams (n=2) were removed from shells with a sterile scalpel. Tissue was washed in 10 ml DPBS and rinsed 3 times in 20 ml fresh DPBS; each clam was handled separately and fresh solution was used for each wash. Tissue was transferred to a 5mL tube containing 1 ml antibiotic(Ab) solution (10,000 u Penicillin G, 10 mg Streptomycin, 25 ug Amphotericin B). To homogenize the tissue, a Tissue Tearer (Model 985-370, Biospec Products, Inc., Racine, WI, USA) was used for 15 seconds. Between individual clam tissues, the blade was cleaned by running the homogenizer for 30 seconds in each of three following washes: 100% EtOH, distilled water, and serum-free Eagle's minimum essential medium (MEM). The resulting homogenate solution was transferred to a 1.5 ml centrifuge tube (Fisherbrand G-Tubes, Fisher Scientific, Pittsburgh, PA, USA) and centrifuged for 15 minutes at 3,000 rpm. 700µl of resulting supernatum was removed and centrifuged for an additional 10 minutes at 10,000 rpm. The supernatant was removed, filtered through a 0.8/0.2 µm membrane low protein binding acrodisc syringe filter (Pall Corporation, Ann Arbor, MI, USA), and serially diluted from 10^{-1} to 10^{-6} in the antibiotic solution described above. Virus isolation in embryonating chicken eggs was attempted on supernatant from 6 exposed clams and 1 unexposed clam were analyzed using virus isolation following standard procedures (Swayne et al. 1998). The remaining supernatant from the remaining 8

exposed clams and 1 unexposed clam were analyzed by RRT-PCR as described above.

Experiment 2: Effect of *C. fluminea* on infectivity of H5N1 HPAI in water

Virus. The H5N1 HPAI virus strain A/whooper swan/Mongolia/244/05 (H5N1) (Mongolia/05) used in this study was obtained from the virus repository at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, Georgia.

Stock of the H5N1 HPAI virus was prepared by second passage in 9- to 11-day-old SPF embryonating chicken eggs using standard procedures (Swayne 1998). The viral stock was diluted in brain-heart infusion (BHI) medium to yield a final titer of 10^5 $TCID_{50}$ per 0.1 ml (single bird inoculum) for the intra-nasal (IN) inoculum. The back-titer on this inoculum was determined in Madin-Darby Canine Kidney (MDCK) cells using standard techniques (Brown et al. 2008) and was $10^{5.3}$ $TCID_{50}$/0.1 ml.

Model indicator organism. Eighteen captive-bred wood ducks, *Aix sponsa,* were acquired from a private breeder at approximately 16-weeks of age (Chenoa Farms, Martin, TN, USA). This age was selected to correspond with the age at which previous infectious and lethal dose determination trials were performed (Brown et al. 2007a). Positive and negative control wood ducks were housed in groups of 3, and all other ducks were housed individually. All birds were housed in self-contained isolation units, which were ventilated under negative pressure with high efficiency particulate air (HEPA)-filtered air. The birds were maintained under continuous lighting, and food and water were provided *ad libitum*.

Experimental setup. The H5N1 HPAI water trials were performed with a slightly modified study design from the LPAI clam trials described above. The *C. fluminea* (n=9) were collected as described in the LPAI trials. Nine 75-cm^2 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA) were inoculated with the Mongolia/05 virus to achieve a starting viral concentration in the water of 10^5 $TCID_{50}$/ml. This concentration was selected based on filtering rate data from the LPAI-clam experiments with the intent of achieving the highest starting concentration of virus in water that the clams would completely filter out by 48-hr PI. The 0-hr PI titers on the flasks ranged from $10^{5.5}$ $TCID_{50}$/ml to $10^{5.7}$ $TCID_{50}$/ml. The viral infected water in each flask was sampled 0-, 24-, and 48-hr PI as described for the LPAI-clam trials for viral titration in Madin-Darby Canine Kidney (MDCK) cells. At 48-hr PI, the water was collected for use in the different inoculation treatment groups described below.

Prior to inoculation, oropharyngeal and cloacal swabs were collected from each wood duck to ensure they were not actively infected and shedding AI virus at the start of the study. In addition, pre-inoculation serum was collected from each bird to confirm they were serologically negative to influenza A type specific antigens by using a commercially available blocking enzyme-linked immunosorbent assay (bELISA) test (IDEXX, Westbrook, ME, USA). The wood ducks were then evenly separated into the following six experimental groups: 1) IN-Mongolia/05 (positive control)- three wood ducks were inoculated IN with 10^5 EID_{50}/0.1ml of the Mongolia/05 stock diluted in BHI; 2) IN-virus/water/clam - three wood ducks were inoculated IN with 0.1ml of water from flasks with clams 48-hr after inoculation

with the Mongolia/05 virus; 3) IN-virus/water/no clam – three wood ducks were inoculated IN with 0.1ml of water from flasks without clams 48-hr after inoculation with the Mongolia/05 virus; 4) Ingestion-virus/clam – three wood ducks were each fed a single shucked clam 48-hr PI with the Mongolia/05 virus; 5) IN-No virus/clam supernatant (negative control)- three wood ducks were inoculated IN with 0.1ml of clam supernatant from flasks that were not inoculated with the Mongolia/05 virus; and 6) IN-Virus/clam supernatant - three wood ducks were inoculated IN with 0.1ml of clam supernatant 48-hr PI with the Mongolia/05 virus. For the feeding trials the clam was shucked and rinsed thoroughly with distilled water, as described above. The clam meat was digitally placed in the caudal oral cavity of each wood duck and the beak gently held shut until the birds swallowed. The ducks were then monitored to ensure they did not regurgitate the clam meat. Clams processed for IN inoculation with tissue supernatant were shucked and rinsed as described above. Each individual clam was then placed in 1.0 ml of BHI, homogenized by hand using sterilized Ten Broeck tissue grinders. The tissue supernatant was transferred to a 4.0 ml cryogenic vial (Corning Incorporated, Corning, NY, USA). The supernatant was clarified by centrifugation and immediately utilized in the inoculation trials detailed below.

Experimental trials of HPAI infectivity. All of the experiments with H5N1 HPAI virus were performed in the USDA-certified BSL 3-Ag facility at SEPRL (Barbeito et al. 1995). The ducks in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Craig et

al. 1999) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at SEPRL.

After inoculation, all birds were monitored daily for clinical signs of disease or death. Oropharyngeal and cloacal swabs were collected and then placed in BHI with antibiotics (400 μg/ml gentamicin, 4000 units/ml penicillin, and 5 μg/ml amphotericin B) from all birds on 0-, 1-, 2-, 4-, 7-, and 9- days post-inoculation (PI) and from any birds that died during the study. The experiment was terminated on 14 days PI, at which time blood was collected from the surviving birds for serologic testing via the bELISA. At 14 days PI, the surviving birds were killed via intravenous injection of a solution of pentobarbital sodium salt (P 37861, Sigma-Alrich, St. Louis, MO, USA) (5 grains/ml) so that each bird received 100 mg of sodium pentobarbital per kg body weight.

Infectivity and Serologic Assays. H5N1 HPAI virus-infected water in the clam filtering trials was titrated in MDCK cells using published protocols (Brown et al. 2008). Viral titers were expressed as $TCID_{50}$/ml and the minimal detectable limit of this assay was $10^{1.96}$ $TCID_{50}$/ml.

Oropharyngeal and cloacal swabs collected during the experimental infection trials were stored at -70º C until virus isolations were performed. Virus isolation was performed in 9- to 11-day old SPF embryonating chicken eggs using previously described procedures (Swayne 1998). Serologic testing was performed on the pre- and post-inoculation serum with the commercially-available AI virus antibody test kit, ELISA (MultiS-Screen) (IDEXX, Westbrook, ME, USA). The serologic testing was performed following manufacturer's instructions and utilizing the test kit reagents

and controls. Previous studies performed in our laboratory indicate that this serologic test is as sensitive and accurate in wood ducks infected with H5N1 HPAI virus as other traditional serologic tests, including the agar-gel immunodiffusion and H5 specific hemagglutinin-inhibition test.

Statistical analysis

Data from microtiter endpoint titration in CEF and MDCK was log_{10}-transformed. Data for flasks with clams (independent variable, n=48) and flasks without clams (controls, n=10) were analyzed with a one-way repeated measures analysis of variances (rm-ANOVA), with clam presence/absence as the between-subject variable and viral titer at each time points as the repeated measure. To control variation between trials that were conducted in different test groups on separate days, the analysis was blocked for the effect of testing group. A Tukey-Kramer *post hoc* test was used to determine significant differences between viral titers between flasks with clams and without clams at each time point (0-, 24-, and 48-hr PI). Analyses were conducted using Statistical Analysis System 9.1 (SAS Inc., Cary, NC, USA) and graphics were created in SigmaPlot (Systat Software, Inc., San Jose, CA, USA).

RESULTS

LPAI viral persistence in water in the presence of *C. fluminea*

At 24-hr PI, 72.9% (n=35) of the water samples from flasks with clams had undetectable levels of virus, and at 48-hr PI, that percentage increased to 83.3% (n=40). Flasks with *C. fluminea* (n=48) had a significant effect on viral titers compared to flasks without clams (n=10) over time according to a one-way rm-

ANOVA (treatment x time; $F_{2,90}$=394.40, p<0.0001 adjusted using Greenhouse-Geisser Epsilon). A block effect for each group of trials was also significant over time (block x time; $F_{16,90}$=4.16, p<0.0001 adjusted using Greenhouse-Geisser Epsilon). A profile analysis revealed that change in viral titer between 0- and 24-hr PI was significant for both the treatment ($F_{1,45}$=704.96, p<0.0001) and block ($F_{8,45}$=6.16, p<0.0001) effect but that change from 24-hrs PI to 48 hrs-PI was not for either effect ($F_{1,45}$=3.10, p=0.0850 and $F_{8,45}$=1.97, p=0.0720). According to a Tukey-Kramer *post hoc* analysis, viral titer was significantly lower in flasks with *C. fluminea* at 24- and 48- hr post-inoculation (PI) (p< 0.0001 at both time points, figure 3.1).

Differences between sampling times, from 0-hr to 24-hr PI and from 24-hr to 48-hr PI, were used to calculate RT values- the average time in days for the virus to reduce infectivity by 90%, equivalent to a decrease in viral titer by 1 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$)/ml. The RT values were 2.52 days in control flasks, compared to just over 14 hrs in flasks with clams (table 3.1). And to further evaluate the reduction in viral titer over time, real-time RT-PCR (RRT-PCR) was carried out simultaneously on 13 flasks with clams and 2 without clams. A reduction in AI virus RNA, as indicated by increasing threshold cycles (C_T), was typically observed 24 hrs after a drop in infectious viral titer; the average time lag was 33 hrs but ranged from 0 to 48 hrs after a decrease in viral titer was observed in infectivity assays (figure 3.2).

A subset of the flasks containing AI-infected water and clams was also sampled at 72- and 96-hr PI (n=8) to evaluate whether clams released virions back into the water in feces or pseudofeces. As reported above, viral titers decreased to

below detectable limits within 48-hr PI, and the titers remained below limits of detection at 72- and 96-hr PI. Additionally, another subset of 8 flasks containing AI-infected water and clams was sampled approximately every 6 hr over the 48-hr trial in order to get a more sensitive measure of the temporal effect of bivalve filtration on AI persistence. The results of these trials sampled at shorter intervals indicated that individual clams varied in the onset of viral titer reduction within the 48-hr trial period, but once titer began to decrease, it declined at a rapid rate (figure 3.3).

Supernatant from homogenized clams that were exposed to AI virus was analyzed with RRT-PCR and virus isolation (VI) in specific pathogen free 9- to 11-day old embryonating chicken eggs. All attempts to detect virus through RRT-PCR in AI-exposed clam tissue (n=8) and unexposed clam tissue (n=1) were negative. Virus isolation attempts from a separate subset of AI-exposed clam tissue (n=6) and control clam tissue (n=1) were also negative.

Effect of *C. fluminea* on infectivity of H5N1 HPAI in water

Following experimental procedures from the water persistence trials with the LPAI virus described above, clams were exposed to water inoculated with H5N1 HPAI virus strain A/whooper swan/Mongolia/244/05. Control flasks without clams (n=3) had minimal change in titer, whereas viral titer in flasks with clams (n=6) dropped below the minimal detectable limit ($10^{2.17}$ $TCID_{50}$/ml) within 48 hrs.

Eighteen wood ducks were used in the experiment, and all of the birds were negative for antibodies to type A influenza prior to the experiment. Mortality, viral shedding, and seroconversion data are summarized in table 3.2. All three of the wood ducks intranasally (IN) inoculated with the viral stock solution containing 10^6

EID_{50} of the H5N1 HPAI virus (positive control group), and all of the wood ducks IN inoculated with virus-inoculated water without clams died. All but one of the birds in these two treatment groups died at 5 days PI; a single bird inoculated with virus-inoculated water died at 6 days PI. Oropharyngeal and cloacal swabs from all of the ducks in these groups were positive for AI virus on 1-, 2-, and 4-days PI and the day the individual died. None of the wood ducks in the remaining exposure groups exhibited morbidity or mortality, excreted virus, or developed post-exposure antibodies to type A influenza viruses. These remaining groups included wood ducks that were: 1) IN inoculated with virus-infected water with clams; 2) IN inoculated with supernatant from clams that filtered virus-infected water; 3) IN inoculated with supernatant from clams that were in non-infected water (negative controls); and 4) that ingested a single shucked clam that filtered virus-infected water.

DISCUSSION

This study is the first to evaluate the influence of biological components of aquatic habitats on AI persistence in water and the potential implications on viral transmission within the aquatic bird reservoir system. Traditional research on AI in wild birds has focused on interactions between the viral agent and host; however, fully understanding the ecology and natural history of AI requires addressing interactions among host, pathogen, and environment. The transmission of AI within the wild bird reservoir is environmentally dependent, and the results of this research underscore the value of considering biotic environmental factors when

studying AI. Our research indicates that invertebrate species inhabiting aquatic environments- in particular bivalves- can influence the persistence and viral loads of AI in water. Considering that AI infection in birds is a dependent on the concentration of AI viruses (Swayne & Slemons 2008) and that our findings show that filtering clams do not serve as an alternative transmission route, filter-feeding bivalves could negatively affect AI virus transmission within aquatic bird populations.

Within 48 hrs, the presence of *C. fluminea* had a significant effect on viral titers in water compared to viral-infected water without clams. The viral titers in flasks with clams were significantly lower than control flasks at both 24- and 48-hr PI. Although according to analysis with rm-ANOVA the treatment (clams) was only significant from 0- to 24-hrs PI, during the 24- to 48-hr PI interval, most viral titers were undetectable, and the insignificance of the second time interval was most likely influenced by the high minimal detectable limit of the infectivity assay used ($10^{2.17}$ $TCID_{50}$/ml). Difference between test groups was most likely driven by the difference in initial viral titer.

In addition to titration in CEF, a subset of the trials was measured using RRT-PCR. In these trials, AI virus was detected with RRT-PCR an average of 33-hr after it was no longer detectable in cell culture assays. This difference in results between the assays most likely reflects the higher sensitivity of RRT-PCR for viral detection; however, it is important to note that the viral RNA detected with PCR does not reflect infective virions whereas titration in CEF reflects infectivity.

Several factors or mechanisms associated with the presence of the clams in water could contribute to the reduction in viral titer, but we believe that these factors are not as significant as the filtering behavior of *C. fluminea*. Previous studies utilizing a distilled water model system indicate that AI persistence is highest in the neutral or slightly basic pH ranges (Brown et al. 2007b; Stallknecht et al. 1990a). The levels of pH were more basic in flasks with clams compared to unaltered distilled water (table 3.3); therefore, this pH difference, if there was an effect, would have increased the ersistence of AI in the water and reduced the magnitude of the observed effect of *C. fluminea* on viral titer. Additionally, a decrease in titer in flasks containing only shells did not have as high a rate of reduction as live clams of equivalent shell surface area (figure 3.4). Attachment to the shell surface may have contributed to the observed titer reduction but does not account for the variability in onset of decrease or rapid rates of decline of AI virus titers in trials with live clams. Presumably, if shell attachment were a significant factor in the reduction of titer, virus would have decreased from the beginning of the trial, but data did not show this trend for all trials.

Testing the water at shorter time intervals showed variation in the onset and rate of viral titer reduction in trials with clams compared to the constant and slower rates in flasks without clams. Filtration by clams is often sporadic and varies in rates and timing of uptake between individuals (Lauritsen 1986). Evaluation of pH and clam shell effects and the characterization of viral titers within short time intervals suggests that the filtrating behavior of the *C. fluminea* was likely the dominant process producing the titer reduction. Although *C. fluminea* and AI virus

persistence has not been investigated under field conditions, previous studies with other viruses support our results. *C. fluminea* have been shown to accumulate Norovius, average virion diameter 38.0 nm, from infected environments, and influenza A viruses are much larger, 80nm to 120nm in diameter (Prasad et al. 1994; Saitoh et al. 2007; Webster et al. 1992).

By using H5N1 HPAI virus and a highly susceptible species of waterfowl, the effects of *C. fluminea* on infectivity of different components of the laboratory model system were evaluated. Wood ducks are a sensitive model species for the detection of H5N1 HPAI virus with a very low infectious and lethal dose; an estimated 50% bird infectious dose of less than 1 $\log_{10}$ EID_{50} (Brown et al. 2007a). In spite of this low infectious dose, inoculation with clam tissue or clam supernatant that containing filtered virus or inoculation with water that contained clams for 48 hrs failed to produce infection. Wood ducks exposed to virus inoculated water without clams, however, were infected with AI determined by mortality, viral excretion, and seroconversion.

The results of our study suggest that AI viruses are inactivated or sequestered in the clam tissue after filtration, rendering the virus non-infective. While some viruses remain infective in bivalves and transmit to hosts through consumption of infected bivalves (Lees 2000), the presence of infective AI virus or intact AI virus RNA was not detected in clam tissue by virus isolation in eggs or *in vivo* studies in a highly susceptible avian species or through RRT-PCR. Although these trials were carried out in a simplified distilled water model, we believe that it has applications for natural ecosystems where transmission events between wild birds occur.

The effect of filter-feeding bivalves on AI virus transmission depends on many factors. In ecosystems that support a high concentration of filter-feeding bivalves that inactivate the virus, bivalves could reduce the risk of infection by lowering the viral load in aquatic environments. Filtration feeding is dependent on the species of bivalve, size of the individuals, population density, temperature of the water, particle size and concentration, and flow regimes (Vaughn et al. 2008). In particular, *C. fluminea* reduce eutrophication and increase the clarity of water because of their high filtration rates and dense populations (McMahon 2002; Phelps 1994), and would presumably remove viruses, either attached to food particles or free floating, from the water column. Clearance times, the length of time it takes bivalves to filter an entire volume of a water body, in estuaries and coastal communities vary between a few days to several years in different ecosystems dependent on populations of bivalves and flow regimes (Dame & Prins 1997). Reduction of virus would also depend on the time between shedding of the virus and infection of a susceptible individual, with longer periods resulting in reduced risks.

Removal and inactivation of AI virus from aquatic environments has implications for theories that viruses overwinter in northern latitudes to infect returning waterfowl in the spring. Viruses may persist from fall until spring but only in environments in which filter feeing bivalve populations are not near the carrying capacity (Dame & Prins 1997). Ephemeral, intermittent, and seasonal wetlands have uniquely adapted communities and these habitats and the organisms they support may help or hinder transmission of AI viruses. Studies should examine

the importance of the aquatic transmission route and investigate ecosystems that help facilitate the spread of AI viruses between individuals by limiting removal of AI virus from the water.

The results of this study provide evidence that biotic environmental factors can influence the persistence and potential transmission of AI viruses in an aquatic habitat. Clams reduced the viral titer in water, presumably through filtration, but the virus did not remain infective within the clam tissue. Taken together, these results suggest that filter feeding bivalves exert a negative impact on the transmission of AI virus in an aquatic environment and suggest an additional ecosystem service (i.e. disease control) provided by filter-feeding bivalves. This research represents a preliminary examination into a novel topic relating to AI ecology, the role of biotic environmental factors. Additional studies are warranted to better understand the influence and significance of filter-feeding bivalves and other biological variables on AI transmission within the wild bird reservoir system under field conditions.

ACKNOWLEDGEMENTS

We thank Virginia Goekjian and everyone at the Southeastern Cooperative Wildlife Disease Study for their guidance and expertise throughout the project. We also thank Carlos Estevez and Joan Beck of the Southeast Poultry Research Laboratory for technical assistance during the wood duck experimental infection trial. Additionally, we acknowledge Britta Hansen for the initial work on this project,

Andrew Durso and Rebecca Bartel for assistance with statistical analysis, and Sonia Altizer, Elijah Carter, Ronald Carroll, and two anonymous reviewers for commentating on drafts of this manuscript. Funding for this work was provided through Cooperative Agreement 1U19Cl0004501 with the Centers for Disease Control.

WORKS CITED

Barbeito, M. S., Abraham, G., Best, M., Cairns, P., Langevin, P., Sterritt, W. G., Barr, D., Meulepas, W., Sanchez-Vizcaino, J. M. & Saraza, M. 1995 Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. First International Veterinary Biosafety Workshop. *Rev Sci Tech* **14**, 873-87.

Bedford, A. J., Williams, G. & Bellamy, A. R. 1978 Virus Accumulation by Rock Oyster Crassostrea Glomerata. *Applied and Environmental Microbiology* **35**, 1012-1018.

Breban, R., Drake, J. M., Stallknecht, D. E. & Rohani, P. 2009 The Role of Environmental Transmission in Recurrent Avian Influenza Epidemics. *PLoS Computational Biology* **5**.

Brown, J. D., Goekjian, G., Poulson, R., Valeika, S. & Stallknecht, D. E. 2008 Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. *Veterinary Microbiology*.

Brown, J. D., Stallknecht, D. E., Valeika, S. & Swayne, D. E. 2007a Susceptibility of Wood Ducks to H5N1 Highly Pathogenic Avian Influenza Virus *Journal of Wildlife Diseases* **43**, 660-667.

Brown, J. D., Swayne, D. E., Cooper, R. J., Burns, R. E. & Stallknecht, D. E. 2007b Persistence of H5 and H7 avian influenza viruses in water. *Avian Diseases* **51**, 285-289.

Cohen, R. R. H., Dresler, P. V., Phillips, E. J. P. & Cory, R. L. 1984 The Effect of the Asiatic Clam, Corbicula fluminea, on Phytoplankton of the Potomac River, Maryland. *Limnology and Oceanography* **29**, 170-180.

Counts III, C. L. 1986 The zoogeography and history of the invasion of the United States by Corbicula fluminea(Bivalvia: Corbiculidae). *American Malacological Bulletin.* **Special Ed. No. 2**, 7-39.

Craig, J. V., Dean, W. F., Havenstein, G. B., Kruger, K. K., Nestor, K. E., Purchase, G. H., Siegel, P. B. & van Wicklen, G. L. 1999 Guidelines for poultry husbandry. In *Guide for the care and use of agricultural animals in agricultural research and teaching*, pp. 55-66. Illinois: Federation of American Societies of Food Animal Science.

Dame, R. F. 1996 *Ecology of Marine Bivalves: An Ecosystem Approach*: CRC Press.

Dame, R. F. & Prins, T. C. 1997 Bivalve carrying capacity in coastal ecosystems. *Aquatic Ecology* **31**, 409-421.

Enriquez, R., Froesner, G. G., Hochstein-Mintzel, V., Riedemann, S. & Reinhardt, G. 1992 Accumulation and persistence of hepatitis A virus in mussels. *Journal of Medical Virology* **37**, 174-179.

Halvorson, D. A., Kelleher, C. J. & Senne, D. A. 1985 Epizootiology of Avian Influenza-Effect of Season on Incidence in Sentinel Ducks and Domestic Turkeys in Minnesota. *Applied and Environmental Microbiology* **49**, 914-919.

Hinshaw, V. & Webster, D. 1982 The natural history of influenza A viruses. In *Basic and Applied Influenza Research* (ed. A. S. Beare), pp. 79-104. Boca Raton, FL: CRC Press, Inc.

Hinshaw, V. S., Webster, R. G. & Turner, B. 1979 Water-borne Transmission of Influenza A Viruses. *Intervirology* **11**, 66-68.

Hinshaw, V. S., Webster, R. G. & Turner, B. 1980 The Perpetuation of Orthomyxoviruses and Paramyxoviruses in Canadian Waterfowl. *Canadian Journal of Microbiology* **26**, 622-629.

Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R. G. & Kida, H. 1995 Perpetuation of Influenza A Viruses in Alaskan Waterfowl Reservoirs. *Archives of Virology* **140**, 1163-1172.

Lauritsen, D. D. 1986 Filter-feeding in Corbicula fluminea and its effect on seston removal. *Journal of the North American Benthological Society* **5**, 165-172.

Lees, D. 2000 Viruses and bivalve shellfish. *International Journal of Food Microbiology* **59**, 81-116.

McMahon, R. F. 1983 Ecology of an invasive pest bivalve, Corbicula. *The Mollusca* **6**, 505-561.

McMahon, R. F. 2002 Evolutionary and physiological adaptations of aquatic invasive animals: r selection versus resistance. *Canadian Journal of Fisheries and Aquatic Sciences* **59**, 1235-1244.

Olsen, B., Munster, V. J., Wallensten, A., Waldenstrom, J., Osterhaus, A. & Fouchier, R. A. M. 2006 Global patterns of influenza A virus in wild birds. *Science* **312**, 384-388.

Phelps, H. L. 1994 The Asiatic clam (Corbicula fluminea) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. *Estuaries and Coasts* **17**, 614-621.

Prasad, B. V., Rothnagel, R., Jiang, X. & Estes, M. K. 1994 Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. *J. Virol.* **68**, 5117-5125.

Reed, L. J., and H. Muench. 1938 A simple method for estimating fifty per cent endpoints. *American Journal of Hygiene* **27**, 493-497.

Rippey, S. R. 1994 Infectious diseases associated with molluscan shellfish consumption. *Clinical Microbiology Reviews* **7**, 419.

Saitoh, M., Kimura, H., Kozawa, K., Nishio, O. & Shoji, A. 2007 Detection and phylogenetic analysis of norovirus in Corbicula fluminea in a freshwater river in Japan. *Microbiology and Immunology* **51**, 815-822.

Schwab, K. J., Neill, F. H., Estes, M. K., Metcalf, T. G. & Atmar, R. L. 1998 Distribution of Norwalk Virus within Shellfish Following Bioaccumulation and Subsequent Depuration by Detection Using RT-PCR. *Journal of Food Protection* **61**, 1674-1680.

Slemons, R. D., Johnson, D. C., Osborn, J. S. & Hayes, F. 1974 Type-A influenza viruses isolated from wild free-flying ducks in California. *Avian Dis* **18**, 119-24.

Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T. & Suarez, D. L. 2002 Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of clinical microbiology* **40**, 3256-3260.

Stallknecht, D. E., Kearney, M. T., Shane, S. M. & Zwank, P. J. 1990a Effects of Ph, Temperature, and Salinity on Persistence of Avian Influenza-Viruses in Water. *Avian Diseases* **34**, 412-418.

Stallknecht, D. E. & Shane, S. M. 1988 Host Range of Avian Influenza Virus in Free Living Birds. *Veterinary Research Communications* **12**, 125-141.

Stallknecht, D. E., Shane, S. M., Kearney, M. T. & Zwank, P. J. 1990b Persistence of Avian Influenza Viruses in Water. *Avian Diseases* **34**, 406-411.

Strayer, D. L., Caraco, N. F., Cole, J. J., Findlay, S. & Pace, M. L. 1999 Transformation of freshwater ecosystems by bivalves - A case study of zebra mussels in the Hudson River. *Bioscience* **49**, 19-27.

Swayne, D. E. 1998 *A laboratory manual for the isolation and identification of avian pathogens*: American Association of Avian Pathologists, University of Pennsylvania Kennett Square, PA.

Swayne, D. E., Glisson, J. R., Jackwood, M. W., Pearson, J. E. & Reed, W. M. 1998 *A laboratory manual for the isolation and identification of avian pathogens*: American Association of Avian Pathologists, University of Pennsylvania Kennett Square, PA.

Swayne, D. E. & Slemons, R. D. 2008 Using Mean Infectious Dose of High-and Low-Pathogenicity Avian Influenza Viruses Originating from Wild Duck and Poultry as One Measure of Infectivity and Adaptation to Poultry. *Avian Diseases* **52**.

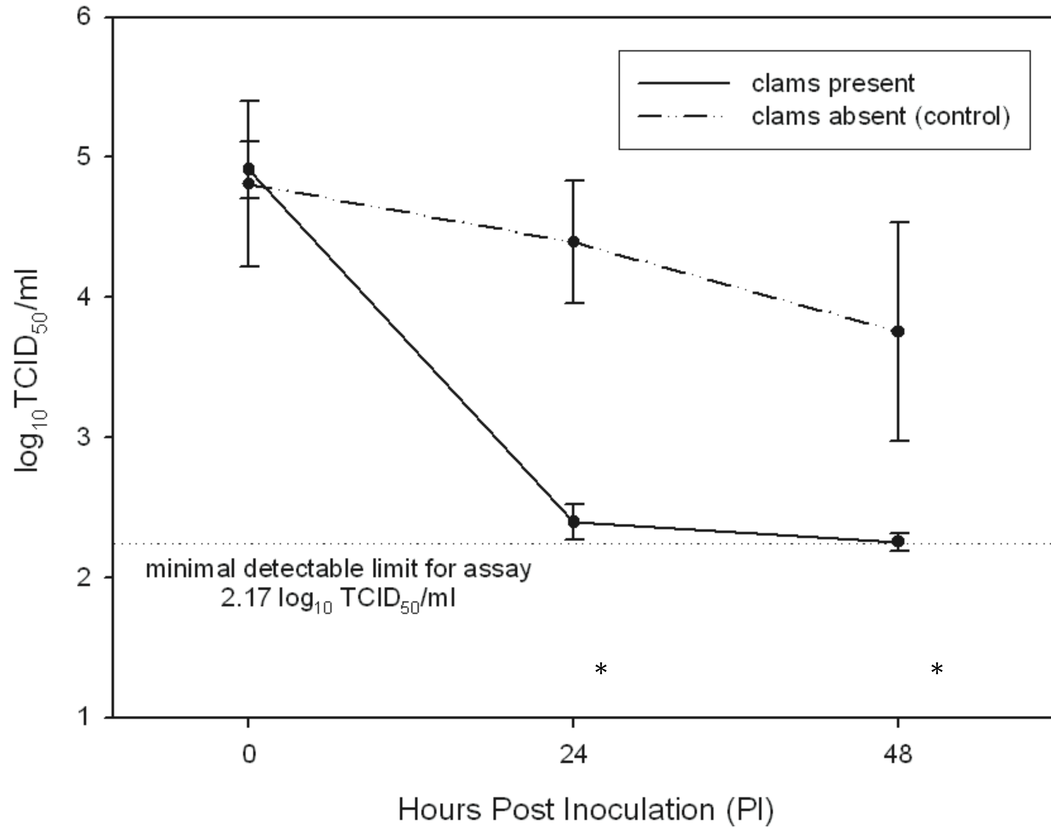
Vaughn, C. C., Nichols, S. J. & Spooner, D. E. 2008 Community and foodweb ecology of freshwater mussels. *Journal of the North American Benthological Society* **27**, 409-423.

Wallace, J. B., Webster, J. R. & Woodall, W. R. 1977 Role of Filter Feeders in Flowing Waters. *Archiv Fur Hydrobiologie* **79**, 506-532.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. 1992 Evolution and Ecology of Influenza-a Viruses. *Microbiological Reviews* **56**, 152-179.

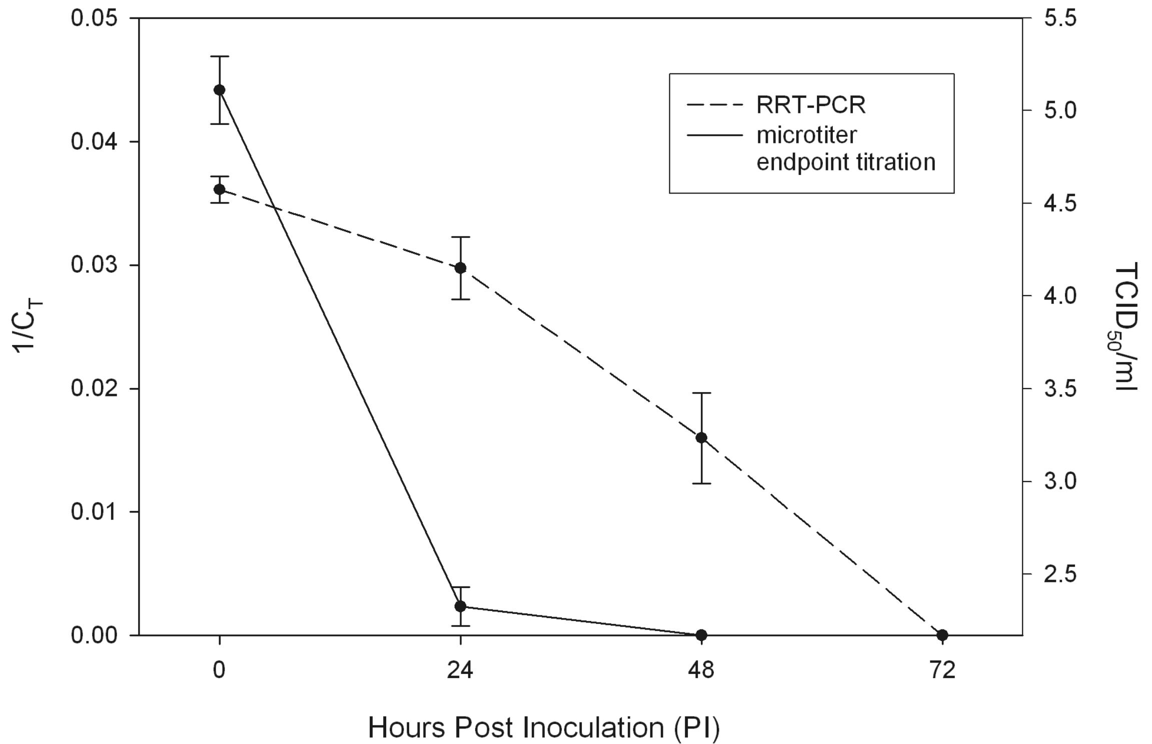
Webster, R. G., Yakhno, M., Hinshaw, V. S., Bean, W. J. & Murti, K. G. 1978 Intestinal Influenza- Replication and Characterization of Influenza Viruses in Ducks. *Virology* **84**, 268-278.

Figure 3.1. Summary of the persistence of LPAI virus in the presence and absence of *C. fluminea* over 48 hrs



The average decrease of viral titer, quantified as $\log_{10}$- transformed tissue culture infective dose ($\log_{10}$ $TCID_{50}$/ml), within flasks with clams (n=48) was significantly different that flasks without clams (control flasks, n=10) at 24- and 48-hr PI. Data points represent means and a * denotes a significant difference ($p<0.0001$) according to a Tukey-Kramer *post hoc* test. Bars indicate 95% confidence intervals.

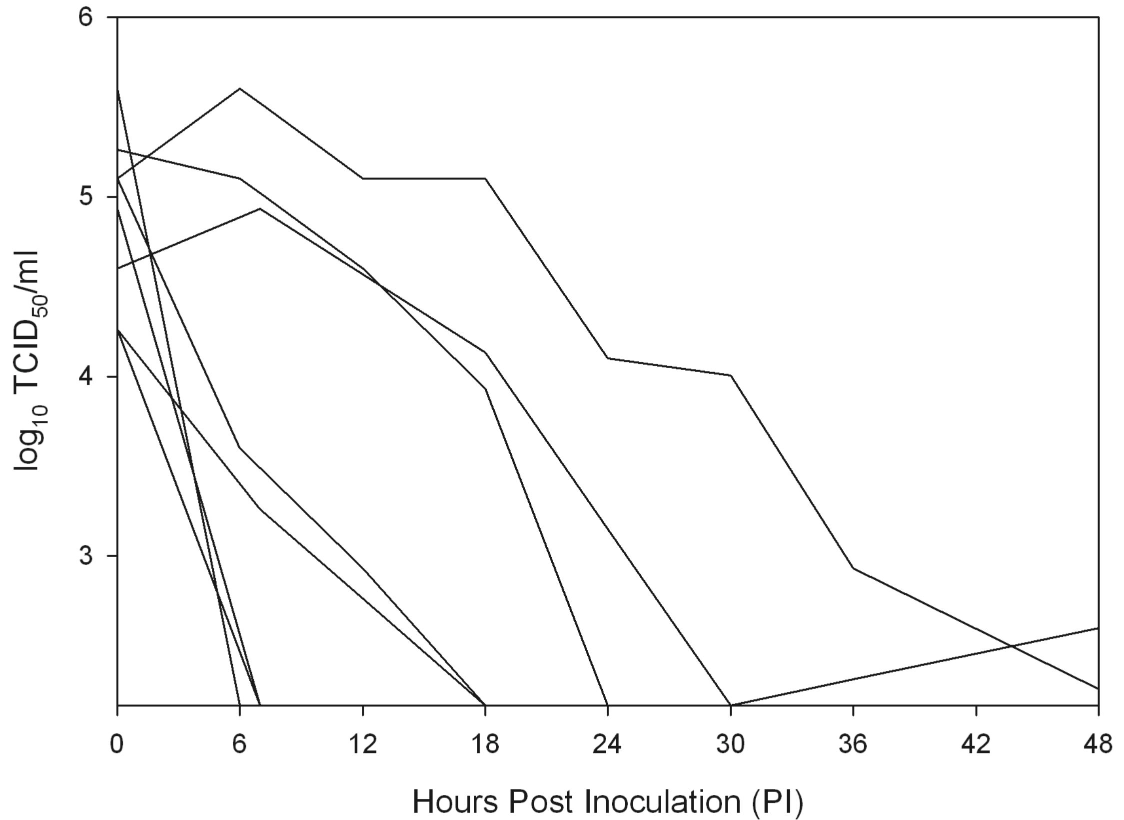
Figure 3.2. Comparison of virus quantification by infectivity assay and real-time RT-PCR (RRT-PCR)



Water at 0-, 24-, 48-, and 72-hr PI was analyzed with both tests on the same day. Persistence was evaluated from 13 flasks with clams. Control flasks (n=2), those without a clam, were also evaluated but are not shown in the graph. The infectivity assay was conducted using microtiter endpoint titration in chicken embryo fibroblasts and was reported as $\log_{10}$- transformed tissue culture infective dose ($\log_{10}$ TCID$_{50}$/ml) on the right y-axis. Values for RRT-PCR were recorded as inverse threshold cycle values ($1/C_T$) on the left y-axis, and the cutoff for C_T was considered 40 cycles. The data points represent means and 95% confidence intervals are

shown. There was an average 33-hr "delay" in the decrease in RRT-PCR values, but the delay ranged from 0- to 48-hr after the virus was undetectable in the infectivity assay. Note that scales are not equal and values are not relative to one another.

Figure 3.3. Viral persistence in water in the presence of clams over short time intervals.



Water samples from flasks with clams (n=8) were taken approximately every 6 hr over a 48-hr period. Water from 3 of 8 trials had undetectable levels of virus at 7-hr PI. Data points represent $\log_{10}$-transformed tissue culture infective doses, with a minimal detectable limit of $10^{2.17}$ $TCID_{50}$/ml. One sample increased in titer, but based on longer term evaluations of persistence, the observed increase is most likely die to human error in infectivity assays.

Table 3.1. Comparison of rate of decrease in viral titers based on RT values.

	RT_1 (0-hr PI to 24-hr PI)	RT_2 (24-hr PI to 48-hr PI)
clams absent (n=10)	2.9305	2.1055
clams present (n=48)	0.4529	0.7294

Calculated by dividing the days post-inoculation by the change in log_{10}-transformed $TCID_{50}$/ml. Indicates a rough estimate of the amount of time in days that it takes to lose one log of virus.

Table 3.2. Summary of wood duck trials.

treatment applied to wood ducks	*mortality*	*viral shedding*	*serology*
intranasal (IN)-virus/water/no clam	3/3	3/3	n/a
IN-virus/water/clam	0/3	0/3	0/3
IN-clam supernatant/virus	0/3	0/3	0/3
Ingest-virus/clam	0/3	0/3	0/3
IN-clam supernatant/no virus (negative control)	0/3	0/3	0/3
IN-virus stock (positive control)	3/3	3/3	n/a

Prior to inoculation, oropharyngeal and cloacal swabs were collected from each bird to ensure they were not actively infected and shedding AI virus at the start of the study. After inoculation, all birds were monitored daily for clinical signs of disease or death. Oropharyngeal and cloacal swabs were collected from all birds on 0-, 1-, 2-, 4-, 7-, and 9-days post-inoculation (PI) and from any birds that died during the study. The experiment was terminated on 14-days PI, at which time blood was collected from the surviving birds for serologic testing via the bELISA, birds that died during the study were not tested for serology.

Table 3.3. Supplementary Material- Persistence of virus over 48 hrs compared to pH levels in water with (n=7) and without (n=1) *C. fluminea.*

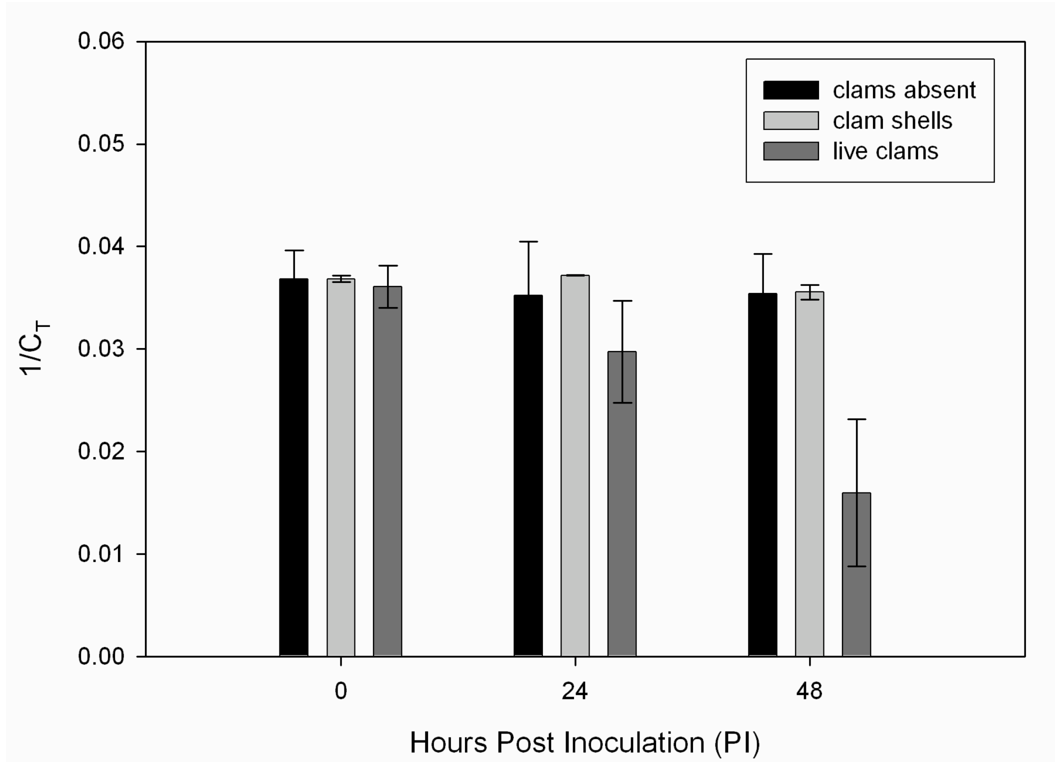
titer	clams present	clams absent
0-hr PI	4.5278	5.1
24-hr PI	2.1963	4.6
48-hr PI	2.1832	--

pH	clams present	clams absent
0-hr PI	6.56	6.62
24-hr PI	7.17	6.73
48-hr PI	7.41	6.91

The pH was measured alongside viral titers to determine if pH caused significant bias in data collection. Although the pH did change over time, more basic pH levels were consistently observed in flasks with clams. Because viral persistence has been shown to increase with basicity (Stallknecht et al. 1990), the decrease in titer in water with clams cannot be explained by changing pH levels. The titer for 48-hr PI in the flask without a clam was not reported because of an error with cells used for microtiter endpoint titration.

Stallknecht, D. E., Kearney, M. T., Shane, S. M. & Zwank, P. J. 1990 Effects of Ph, Temperature, and Salinity on Persistence of Avian Influenza-Viruses in Water. *Avian Diseases* **34**, 412-418.

Figure 3.4. Supplementary Material- Effect of clam shell surface on virus concentration over 48 hrs.



Clam tissue was removed from clam shells (n=2). The two valves (sides) of the shell were glued to control surface area with live clams. Viral concentration was quantified by RRT-PCR and compared to flasks without clams (n=3) and flasks with live clams (n=13). Values are reported as inverse threshold cycles (C_T) and 95% confidence intervals are shown.

CHAPTER 4

CONCLUSION

Epidemiology studies focus on the host, the pathogen, the environment, and interactions between the three. The majority of research conducted on avian influenza has focused on the viruses (different subtypes, HPAI vs. LPAI) and hosts (wild and domestic birds and humans). AI is transmitted via a fecal-oral route in aquatic habitats and the environmental transmission route plays a vital role in the maintenance of AI viruses in wild bird populations (Hinshaw et al. 1979, Breban et al. 2009). Despite this, there has been limited focus on environmental factors that impact the transmission and infectivity of AI viruses in water.

Abiotic factors, including temperature, pH, and salinity of water, have been shown to affect the infectivity of viruses in water (Stallknecht et al. 1990a, Stallknecht et al. 1990b, Brown et al. 2007, Brown et al. 2008), but no studies have examined affects of biological components of ecosystems on AI persistence. This study is the first to conclude that biological factors in the aquatic environment affect the persistence of AI viruses.

Results of the experiments demonstrated that *Corbicula fluminea*, a filter-feeding freshwater clam, can remove LPAI virus from distilled water within 48-hours. The rate of uptake was dependent on individual clams, and most likely reflected time actively filtering water. *C. fluminea* also removed HPAI viruses from

water within 48-hours, and virus remaining in the water was not sufficient enough to cause infection in *Aix sponsa* (wood duck), a highly susceptible avian species. Therefore, *C. fluminea* is effective in reducing the persistence and infectivity of AI viruses in water.

Whole clams and supernatant from *C. fluminea* that had been exposed to water inoculated with HPAI were fed to wood ducks to determine the infectivity of AI virus within clams' tissues. No viral shedding or infection was observed in any of the wood ducks in the trials; therefore *C. fluminea* was a dead-end host for AI viruses. *C. fluminea* reduced the persistence of AI in water and did not serve as an alternative transmission route through consumption of bivalves by susceptible birds.

Although using bivalves as control mechanisms for phytoplankton and nutrients has been suggested (review in Newell 2004), the use of bivalves to control water transmitted viruses is a novel concept. Managed correctly, *C. fluminea* could be used to control outbreaks of AI, but perhaps it and other filter feeders already perform such a service in healthy environments with resident populations of bivalves.

The results of this study emphasize the importance of ecosystem health. Intact aquatic ecosystems that are healthy enough to support populations of bivalves might benefit from services that the filter feeders provide by clearing water of viruses and removing harmful toxins and metals from circulation in the system.

Avian influenza poses risks to agriculture, wild and domestic birds, and humans. Incidence of HPAI outbreaks have increased in recent years, and it is more

vital than ever to understand the ecology of AI and the role that AI hosts, viruses, and the environment play in the emergence of new HP strains. This study demonstrates one biological factor, filter-feeding bivalves, that reduces transmission and infectivity of AI in water. There are many more environmental factors that may affect the dynamics of AI within reservoir populations.

The result of the study is a promising step towards understanding the ecology of AI, but we must focus more efforts on the environmental factors that come into play when dealing with AI as a multi-host, multi-pathogen, environmentally transmitted zoonotic disease.

<u>WORKS CITED</u>

Breban, R., J. M. Drake, D. E. Stallknecht, and P. Rohani. 2009. The Role of Environmental Transmission in Recurrent Avian Influenza Epidemics. PLoS Computational Biology **5**.

Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht. 2008. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. Veterinary Microbiology.

Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht. 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Diseases **51**:285-289.

Hinshaw, V. S., R. G. Webster, and B. Turner. 1979. Water-borne Transmission of Influenza A Viruses. Intervirology **11**:66-68.

Lees, D. 2000. Viruses and bivalve shellfish. International Journal of Food Microbiology **59**:81-116.

Newell, R. I. E. 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. Journal of Shellfish Research **23**:51-62.

Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990a. Effects of pH, Temperature, and Salinity on Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:412-418.

Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990b. Persistence of Avian Influenza Viruses in Water. Avian Diseases **34**:406-411.