Filter-feeding bivalves can remove avian influenza viruses from water and reduce infectivity

Christina Faust^{1,*}, David Stallknecht², David Swayne³
and Justin Brown²

¹Odum School of Ecology, University of Georgia, Athens, GA 30602, USA

²Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

³Southeast Poultry Research Laboratory, Agriculture Research Service, U.S. Department of Agriculture, Athens, GA 30605, USA

Avian influenza (AI) viruses are believed to be transmitted within wild aquatic bird populations through an indirect faecal–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 titres in water with clams were significantly lower at 24 and 48 h 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 supernatants, 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 was inoculated with 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*

1. 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 used 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 faeces (Webster *et al.* 1978; Hinshaw & Webster 1982). Transmission of AI viruses among individuals within these aquatic bird populations is believed to occur through an indirect faecal–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 (Brebner *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 using 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 (Stallknecht *et al.* 1990a,b; Brown *et al.* 2007b). The ability to persist in water varied among different AI viruses, but generally viruses remained infective for months if not years under optimal conditions that include cold temperatures, fresh-to-brackish salinities and slightly basic pH (Stallknecht *et al.* 1990a,b; Brown *et al.* 2007b). AI viruses have been isolated from surface waters where waterfowl are found, demonstrating field support for these laboratory results (Hinshaw *et al.* 1980; Halvorson *et al.* 1985; Ito *et al.* 1995).

* Author for correspondence (christina.faust@gmail.com).

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2009.0572> or via <http://rspb.royalsocietypublishing.org>.

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 behaviour. 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 faeces and pseudofaeces (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 5–30 000 μm^2 (Wallace *et al.* 1977; Dame 1996).

The potential for filter-feeding bivalves to play a role in the transmission of viral diseases is not novel (Rippey 1994). Other viruses that use a faecal–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 (*Crassostrea glomerata*) within 24 h (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 40° latitude (Counts 1986). *C. fluminea* was selected as a 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 $\text{l h}^{-1} \text{g}^{-1}$ (McMahon 1983; Cohen *et al.* 1984).

Our objectives in this research are twofold: (i) to determine whether the filtering behaviour of *C. fluminea* reduces the concentration of AI virus in water and therefore influences viral persistence in an aquatic habitat and (ii) to determine whether H5N1 HPAI-infected water and clams after 48 h of exposure are infective to wood ducks (*Aix sponsa*), a highly susceptible waterfowl species. For objective 1, an LPAI virus (A/Mallard/MN/190/99 (H3N8)) was used to determine if *C. fluminea* could affect viral titre 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.

2. MATERIAL AND METHODS

(a) Experiment 1: LPAI viral persistence in water in the presence of *C. fluminea*

(i) Virus

A wild bird-origin LPAI virus A/Mallard/MN/190/99 (H3N8) was propagated in 9- to 11-day-old specific

pathogen-free (SPF) embryonating chicken eggs using previously described protocols (Swayne 1998). Infective amino-allantoic fluid (AAF) was stored at -70°C until trials were performed. All trials were performed with low-passage virus isolates (second or third passages).

(ii) Model invertebrate organism

C. fluminea were collected from sediments in a northeast Georgia piedmont stream (Sandy Creek, Upper Oconee Watershed, GA, USA). Clams of similar size, approximately 3 cm in 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.

(iii) 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: (i) if aeration or the presence of the airstone affected viral titre; (ii) if clams significantly affected pH over the duration of the trials; and (iii) to determine if a loss of viral titre resulted from adherence of virions to the shells. Flasks with and without aeration were set up and monitored over 48 h using established protocols (Stallknecht *et al.* 1990a,b; Brown *et al.* 2007b). 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 h post-inoculation (PI)) and stored at 4°C until viral titration in tissue culture as described below. Aliquots were also collected at 24 and 48 h 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 (Stallknecht *et al.* 1990a,b; Brown *et al.* 2007b). A sample of water from flasks with and without clams was measured with a SympHony Meter (VWR, Beverly, MA, USA) over a 48 h period. Water pH in flasks with clams increased on average over the 48 h period from 6.53 to 7.22 ($n = 6$), which was greater than the 6.62–6.91 change observed in the control without clams ($n = 1$) (ESM1).

To determine the effects of AI virus adherence to clam shells on viral titre, 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 titre was determined by real-time RT-PCR (RRT-PCR) and compared to previous results from flasks with clams ($n = 13$) and without clams ($n = 3$) that were also analysed by RRT-PCR at 0, 24 and 48 h PI. The presence of clam shells resulted in a decrease in titre that was not significantly different than control flasks but was a significantly smaller decrease from flasks with live clams at 48 h PI ($p < 0.01$; electronic supplementary material 2).

(iv) 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: (i) 48 h trial ($n = 33$): 1.0 ml aliquots of water were collected at 0, 24 and 48 h PI; (ii) short time interval 48 h trial ($n = 8$): 1.0 ml aliquots of water were collected approximately every 6 h from 0 to 48 h PI; (iii) 96 h trial ($n = 7$): 1.0 ml aliquots of water were collected at 0, 24, 48, 72 and 96 h PI. Regardless of the study group, water samples were collected at 0, 24 and 48 h 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 h PI) and stored at 4°C until viral titration assays and RRT-PCR were performed at the end of the 48 h trial or during the 96 h trial. Water samples were tested no more than 50 h after collection (samples from 96 h trials were tested in two groups). Previous studies by our laboratory have shown that viral titres of AI are stable in distilled water for months to years at 4°C (Stallknecht *et al.* 1990a).

(v) Infectivity assays

The infectivity of AI virus in water samples was quantified using a microtitre 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 titres were calculated as previously described (Reed & Muench 1938) and recorded as 50 per cent tissue culture infective doses, TCID₅₀ ml⁻¹. The minimal detectable limit of the assay is 10^{2.17} TCID₅₀ ml⁻¹.

(vi) RNA extraction, primer sets and RRT-PCR

Viral RNA was extracted from 50 µl of water samples with clams ($n = 13$) and without clams ($n = 2$) and from 50 µl 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 RRT-PCR.

RRT-PCR was carried out with a Qiagen one-step kit using a 25 µL mixture containing 8 µl of RNA, 6.95 µl of H₂O, 5 µl of 5X buffer, 1.25 µl of 25 mM MgCl₂, 1 µl of enzyme mixture, 0.5 µM forward primer (M+25, 5'-AGATGAGTCTTCTAACCGAGGTCTG -3'), 0.5 µM reverse primer (M-124, 5'-TGCAAAAACATCTTCAAGTCTC TG-3'), 0.8 µl of dNTPs, 0.5 µl of RNase inhibitor and 0.5 µl of photosensitive probe (M+64 5'-FAM-TCAGG CCCCCTCAAAGCCGA-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 s and 60.0°C for 20 s. A threshold cycle (C_T) of 40 cycles was used as a diagnostic cutoff for determining positive samples during analysis.

(vii) Analysis of exposed clam tissue

At the end of 48 h 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 of DPBS and rinsed three times in 20 ml of fresh DPBS; each clam was handled separately and fresh solution was used for each wash. Tissue was transferred to a 5 ml tube containing 1 ml of antibiotic (Ab) solution (10 000 U of Penicillin G, 10 mg of streptomycin, 25 µg of amphotericin B). To homogenize the tissue, a tissue tearer (Model 985-370, Biospec Products, Inc., Racine, WI, USA) was used for 15 s. Between individual clam tissues, the blade was cleaned by running the homogenizer for 30 s in each of the three following washes: 100 per cent EtOH, distilled water and serum-free Eagle's minimum essential medium. 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 min at 50 Hz. Seven hundred microlitres of resulting supernatant was removed and centrifuged for an additional 10 min at 166.67 Hz. 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 (VI) in embryonating chicken eggs attempted on supernatant from six exposed clams and one unexposed clam was analysed using VI following standard procedures (Swayne *et al.* 1998). The remaining supernatant from the remaining eight exposed clams and one unexposed clam was analysed by RRT-PCR as described above.

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

(i) 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 (SEPR), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Athens, GA, USA.

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 titre of 10⁵ TCID₅₀ per 0.1 ml (single bird inoculum) for the intra-nasal (IN) inoculum. The back-titre on this inoculum was determined in Madin-Darby Canine Kidney (MDCK) cells using standard techniques (Brown *et al.* 2008) and was 10^{5.3} TCID₅₀ 0.1 ml⁻¹.

(ii) 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 three, 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*.

(iii) Experimental setup

The H5N1 HPAI water trials were performed with a slightly modified study design from the LPPI clam trials described

above. The *C. fluminea* ($n = 9$) were collected as described in the LPAI trials. Nine 75 cm² 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 TCID₅₀ ml⁻¹. 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 h PI. The 0 h PI titres on the flasks ranged from $10^{5.5}$ to $10^{5.7}$ TCID₅₀ ml⁻¹. The viral-infected water in each flask was sampled 0, 24 and 48 h PI as described for the LPAI-clam trials for viral titration in MDCK cells. At 48 h PI, 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 that 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 that 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: (i) IN-Mongolia/05 (positive control)—three wood ducks were inoculated IN with 10^5 EID₅₀ 0.1 ml⁻¹ of the Mongolia/05 stock diluted in BHI; (ii) IN-virus/water/clam—three wood ducks were inoculated IN with 0.1 ml of water from flasks with clams 48 h after inoculation with the Mongolia/05 virus; (iii) IN-virus/water/no clam—three wood ducks were inoculated IN with 0.1 ml of water from flasks without clams 48 h after inoculation with the Mongolia/05 virus; (iv) Ingestion-virus/clam—three wood ducks were each fed a single shucked clam 48 h PI with the Mongolia/05 virus; (v) IN-No virus/clam supernatant (negative control)—three wood ducks were inoculated IN with 0.1 ml of clam supernatant from flasks that were not inoculated with the Mongolia/05 virus; and (iv) IN-Virus/clam supernatant—three wood ducks were inoculated IN with 0.1 ml of clam supernatant 48 h 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 used in the inoculation trials detailed below.

(iv) Experimental trials of HPAI infectivity

All the experiments with H5N1 HPAI virus were performed in the USDA-certified BSL 3-Ag facility at SEPRL (Barbeito *et al.* 1995).

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⁻¹ of gentamicin, 4000 units ml⁻¹ of penicillin and 5 µg ml⁻¹ of amphotericin B) from all birds on 0, 1, 2, 4, 7 and 9 days 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 (P37861, Sigma-Aldrich, St Louis, MO, USA) (5 grains ml⁻¹) so that each bird received 100 mg of sodium pentobarbital per kg body weight.

(v) 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 titres were expressed as TCID₅₀ ml⁻¹ and the minimal detectable limit of this assay was $10^{1.96}$ TCID₅₀ ml⁻¹.

Oropharyngeal and cloacal swabs collected during the experimental infection trials were stored at -70°C until VIs were performed. VI 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-inoculation and PI serum with the commercially available AI virus antibody test kit ELISA (MultiS-Screen) (IDEXX, Westbrook, ME, USA). The serologic testing was performed following the manufacturer's instructions and using 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 haemagglutinin-inhibition test.

(c) Statistical analysis

Data from microtitre endpoint titration in CEF and MDCK was log₁₀-transformed. Data for flasks with clams (independent variable, $n = 48$) and flasks without clams (controls, $n = 10$) were analysed with a one-way repeated-measures analysis of variances (rm-ANOVA), with clam presence/absence as the between-subject variable and viral titre 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 titres between flasks with clams and without clams at each time point (0, 24 and 48 h 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).

3. RESULTS

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

At 24 h PI, 72.9 per cent ($n = 35$) of the water samples from flasks with clams had undetectable levels of virus, and at 48 h PI, that percentage increased to 83.3 per cent ($n = 40$). Flasks with *C. fluminea* ($n = 48$) had a significant effect on viral titres compared to flasks without clams ($n = 10$) over time according to a one-way rm-ANOVA (treatment $\times$ 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 $\times$ time; $F_{16,90} = 4.16$, $p < 0.0001$ adjusted using Greenhouse-Geisser Epsilon). A profile analysis revealed that change in viral titre between 0 and 24 h 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 to 48 h 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 titre was significantly lower in flasks with *C. fluminea* at 24 and 48 h PI ($p < 0.0001$ at both time points, figure 1).

Differences between sampling times, from 0 to 24 h PI and from 24 to 48 h PI, were used to calculate RT values—the average time in days for the virus to reduce infectivity by 90 per cent, equivalent to a decrease in viral titre by 1 $\log_{10}$ TCID₅₀ ml⁻¹. The RT values were 2.52 days in control flasks, compared to just over 14 h in flasks with clams (table 1). And to further evaluate the reduction in viral titre over time, RRT-PCR was carried out simultaneously on 13 flasks with clams and two without clams. A reduction in AI virus RNA, as indicated by increasing threshold cycles (C_T), was typically observed 24 h after a drop in infectious viral titre; the average time lag was 33 h but ranged from 0 to 48 h after a decrease in viral titre was observed in infectivity assays (figure 2).

A subset of the flasks containing AI-infected water and clams was also sampled at 72 and 96 h PI ($n = 8$) to evaluate whether clams released virions back into the water in faeces or pseudofaeces. As reported above, viral titres decreased to below detectable limits within 48 h PI, and the titres remained below limits of detection at 72 and 96 h PI. Additionally, another subset of eight flasks containing AI-infected water and clams was sampled approximately every 6 h over the 48 h trial to obtain 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 titre reduction within the 48 h trial period, but once the titre began to decrease, it declined at a rapid rate (figure 3).

The supernatant from homogenized clams that were exposed to AI virus was analysed with RRT-PCR and 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. VI attempts from a separate subset of AI-exposed clam tissue ($n = 6$) and control clam tissue ($n = 1$) were also negative.

(b) 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 titre, whereas viral titre in flasks with clams ($n = 6$) dropped below the minimal detectable limit ($10^{2.17}$ TCID₅₀ ml⁻¹) within 48 h.

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 2. All three wood ducks intranasally (IN) inoculated with the viral stock solution containing 10^6 EID₅₀ of the H5N1 HPAI virus (positive control group), and all of the wood ducks IN inoculated with virus-inoculated

Figure 1. Summary of the persistence of LPAI virus in the presence and absence of *C. fluminea* over 48 h. The average decrease of viral titre, quantified as $\log_{10}$ -transformed tissue culture infective dose ($\log_{10}$ TCID₅₀ ml⁻¹), within flasks with clams ($n = 48$) was significantly different than flasks without clams (control flasks, $n = 10$) at 24 and 48 h PI. Data points represent means and an asterisk denotes a significant difference ($p < 0.0001$) according to a Tukey–Kramer *post hoc* test. Bars indicate 95 per cent confidence intervals.

Table 1. Comparison of rate of decrease in viral titres based on RT values. Calculated by dividing the days post-inoculation (PI) by the change in $\log_{10}$ -transformed TCID₅₀ ml⁻¹. Indicates a rough estimate of the amount of time in days that it takes to reduce infectivity by 90 per cent, equivalent to a decrease in viral titre by 1 $\log_{10}$ TCID₅₀ ml⁻¹.

	RT ₁ (0–24 h PI)	RT ₂ (24–48 h PI)
clams absent ($n = 10$)	2.9305	2.1055
clams present ($n = 48$)	0.4529	0.7294

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: (i) IN inoculated with virus-infected water with clams; (ii) IN inoculated with supernatant from clams that filtered virus-infected water; (iii) IN inoculated with supernatant from clams that were in non-infected water (negative controls); and (iv) that ingested a single shucked clam that filtered virus-infected water.

4. DISCUSSION

This study is the first to evaluate the influence of biological components of aquatic habitats on AI persistence in

Figure 2. Comparison of virus quantification by infectivity assay and real-time RT-PCR (RRT-PCR). Water at 0, 24, 48, and 72 h PI was analysed 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 microtitre endpoint titration in chicken embryo fibroblasts and was reported as $\log_{10}$ -transformed tissue culture infective dose ($\log_{10}$ TCID₅₀ ml⁻¹) 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 per cent confidence intervals are shown. There was an average 33 h ‘delay’ in the decrease in RRT-PCR values, but the delay ranged from 0 to 48 h 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. 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 h over a 48 h period. Water from three of eight trials had undetectable levels of virus at 7 h PI. Data points represent $\log_{10}$ -transformed tissue culture infective doses, with a minimal detectable limit of $10^{2.17}$ TCID₅₀ ml⁻¹. One sample increased in titre, but based on longer term evaluations of persistence, the observed increase is most probably due to human error in infectivity assays.

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

Table 2. Summary of wood duck trials. 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 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.

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

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 depends 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 h, the presence of *C. fluminea* had a significant effect on viral titres in water compared to viral-infected water without clams. The viral titres in flasks with clams were significantly lower than control flasks at both 24 and 48 h PI. According to analysis with rm-ANOVA, the treatment (clams) was only significant from 0 to 24 h PI, during the 24 to 48 h PI interval most viral titres 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 ($10^{2.17}$ TCID₅₀ ml⁻¹). Difference between the test groups was most likely driven by the difference in initial viral titre.

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 h 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 titre, but we believe that these factors

are not as significant as the filtering behaviour of *C. fluminea*. Previous studies using a distilled water model system indicate that AI persistence is highest in the neutral or slightly basic pH ranges (Stallknecht *et al.* 1990a; Brown *et al.* 2007b). The levels of pH were more basic in flasks with clams compared to unaltered distilled water (electronic supplementary material 1); therefore, this pH difference, if there was an effect, would have increased the persistence of AI in water and reduced the magnitude of the observed effect of *C. fluminea* on viral titre. Additionally, a decrease in titre in flasks containing only shells did not have as high a rate of reduction as live clams of equivalent shell surface area (ESM2). Attachment to the shell surface may have contributed to the observed titre reduction but does not account for the variability in onset of decrease or rapid rates of decline of AI virus titres in trials with live clams. Presumably, if shell attachment were a significant factor in the reduction of titre, 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 titre 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 titres within short time intervals suggest that the filtering behaviour of the *C. fluminea* was likely the dominant process producing the titre reduction. Although *C. fluminea* and AI virus persistence have not been investigated under field conditions, previous studies with other viruses support our results. *C. fluminea* have been shown to accumulate Norovirus, average virion diameter 38.0 nm, from infected environments, and influenza A viruses are much larger, 80–120 nm in diameter (Webster *et al.* 1992; Prasad *et al.* 1994; Saitoh *et al.* 2007).

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 per cent bird infectious dose of less than $1 \log_{10} \text{EID}_{50}$ (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 h 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 VI 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 (Phelps 1994; McMahon 2002), 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-feeding 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 titre 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.

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.

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 commenting on drafts of this manuscript. Funding for this work was provided through Cooperative Agreement 1U19CI0004501 by the Centers for Disease Control.

REFERENCES

- Barbeito, M. S. *et al.* 1995 Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. First International Veterinary Biosafety Workshop. *Rev. Sci. Tech.* **14**, 873–887.
- Bedford, A. J., Williams, G. & Bellamy, A. R. 1978 Virus accumulation by rock oyster *Crassostrea glomerata*. *Appl. Environ. Microbiol.* **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 Comput. Biol.* **5**, e1000346. (doi:10.1371/journal.pcbi.1000346)
- Brown, J. D., Stallknecht, D. E., Valeika, S. & Swayne, D. E. 2007a Susceptibility of wood ducks to H5N1 highly pathogenic avian influenza virus. *J. Wildl. 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 Dis.* **51**, 285–289. (doi:10.1637/7636-042806R.1)
- 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. *Vet. Microbiol.* **136**, 20–26.
- 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. *Limnol. Oceanogr.* **29**, 170–180.
- Counts III, C. L. 1986 The zoogeography and history of the invasion of the United States by *Corbicula fluminea* (Bivalvia: Corbiculidae). *Am. Malacol. Bull. 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. Savoy, IL: Federation of American Societies of Food Animal Science.
- Dame, R. F. 1996 *Ecology of marine bivalves: an ecosystem approach*. Boca Raton, FL: CRC Press.
- Dame, R. F. & Prins, T. C. 1997 Bivalve carrying capacity in coastal ecosystems. *Aquatic Ecol.* **31**, 409–421. (doi:10.1023/A:1009997011583)
- Enriquez, R., Froesner, G. G., Hochstein-Mintzel, V., Riedemann, S. & Reinhardt, G. 1992 Accumulation and persistence of hepatitis A virus in mussels. *J. Med. Virol.* **37**, 174–179. (doi:10.1002/jmv.1890370305)
- 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. *Appl. Environ. Microbiol.* **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. (doi:10.1159/000149014)
- Hinshaw, V. S., Webster, R. G. & Turner, B. 1980 The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. *Can. J. Microbiol.* **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. *Arch. Virol.* **140**, 1163–1172. (doi:10.1007/BF01322743)
- Lauritsen, D. D. 1986 Filter-feeding in *Corbicula fluminea* and its effect on seston removal. *J. North Am. Benthol. Soc.* **5**, 165–172. (doi:10.2307/1467704)
- Lees, D. 2000 Viruses and bivalve shellfish. *Int. J. Food Microbiol.* **59**, 81–116. (doi:10.1016/S0168-1605(00)00248-8)
- McMahon, R. F. 1983 Ecology of an invasive pest bivalve, *Corbicula*. *Mollusca* **6**, 505–561.
- McMahon, R. F. 2002 Evolutionary and physiological adaptations of aquatic invasive animals: *r*-selection versus resistance. *Can. J. Fish. Aquat. Sci.* **59**, 1235–1244. (doi:10.1139/f02-105)
- 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. (doi:10.1126/science.1122438)
- Phelps, H. L. 1994 The Asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. *Estuar. Coasts* **17**, 614–621. (doi:10.2307/1352409)
- 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. & Muench, H. 1938 A simple method for estimating fifty per cent endpoints. *Am. J. Hygiene* **27**, 493–497.
- Rippey, S. R. 1994 Infectious diseases associated with molluscan shellfish consumption. *Clin. Microbiol. Rev.* **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. *Microbiol. Immunol.* **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. *J. Food Prot.* **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**, 119–124. (doi:10.2307/1589250)
- 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. *J. Clin. Microbiol.* **40**, 3256–3260. (doi:10.1128/JCM.40.9.3256-3260.2002)
- Stallknecht, D. E. & Shane, S. M. 1988 Host range of avian influenza virus in free living birds. *Vet. Res. Commun.* **12**, 125–141. (doi:10.1007/BF00362792)
- 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 Dis.* **34**, 412–418. (doi:10.2307/1591429)
- Stallknecht, D. E., Shane, S. M., Kearney, M. T. & Zwank, P. J. 1990b Persistence of avian influenza viruses in water. *Avian Dis.* **34**, 406–411. (doi:10.2307/1591428)

- 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. (doi:10.2307/1313490)
- Swayne, D. E. 1998 *A laboratory manual for the isolation and identification of avian pathogens*. Kennett Square, PA: American Association of Avian Pathologists, University of Pennsylvania.
- 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 Dis.* **52**, 455–460. (doi:10.1637/8229-012508-Reg.1)
- 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*. Kennett Square, PA: American Association of Avian Pathologists, University of Pennsylvania.
- Vaughn, C. C., Nichols, S. J. & Spooner, D. E. 2008 Community and foodweb ecology of freshwater mussels. *J. North Am. Benthol. Soc.* **27**, 409–423. (doi:10.1899/07-058.1)
- Wallace, J. B., Webster, J. R. & Woodall, W. R. 1977 Role of filter feeders in flowing waters. *Arch. Hydrobiol.* **79**, 506–532.
- 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. (doi:10.1016/0042-6822(78)90247-7)
- Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. 1992 Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179.