AVIAN DISEASES 63:230–234, 2019

Research Note—

The Pathogenesis of H7 Highly Pathogenic Avian Influenza Viruses in Lesser Scaup (*Aythya affinis*)

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Received 4 June 2018; Accepted 14 December 2018; Published ahead of print 18 December 2018

SUMMARY. Waterfowl are the natural hosts of avian influenza virus (AIV), and through migration spread the virus worldwide. Most AIVs carried by wild waterfowl are low pathogenic strains; however, Goose/Guangdong/1996 lineage clade 2.3.4.4 H5 highly pathogenic (HP) AIV now appears to be endemic in wild birds in much of the Eastern Hemisphere. Most research efforts studying AIV pathogenicity in waterfowl thus far have been directed toward dabbling ducks. In order to better understand the role of diving ducks in AIV ecology, we previously characterized the pathogenesis of clade 2.3.4.4 H5 HPAIV in lesser scaup (*Aythya affinis*). In an effort to further elucidate AIV infection in diving ducks, the relative susceptibility and pathogenesis of two North American lineage H7 HPAIV isolates from the most recent outbreaks in the United States was investigated. Lesser scaup were inoculated with either A/turkey/IN/1403-1/2016 H7N8 or A/chicken/TN/17-007147-2/2017 H7N9 HPAIV by the intranasal route. The approximate 50% bird infectious dose (BID_{50}) of the H7N8 isolate was determined to be 10^3 50% egg infectious doses (EID_{50}), and the BID_{50} of the H7N9 isolate was determined to be $<10^2$ EID_{50}, indicating some variation in adaptation between the two isolates. No mortality or clinical disease was observed in either group except for elevated body temperatures at 2 and 4 days postinoculation (DPI). Virus shedding was detected up to 14 DPI from both groups, and there was a trend for shedding to have a longer duration and at higher titer levels from the cloacal route. These results demonstrate that lesser scaup are susceptible to both H7 lineages of HPAIV, and similar to dabbling duck species, they shed virus for long periods relative to gallinaceous birds and don't present with clinical disease.

RESUMEN. *Nota de investigación*- Patogenia de los virus de influenza aviar altamente patógenos H7 en porrones bola (*Aythya affinis*).

Las aves acuáticas son los hospedadores naturales del virus de la influenza aviar y a través de la migración, propagan el virus en todo el mundo. La mayoría de los virus de influenza transportados por aves acuáticas silvestres son cepas de baja patogenicidad; sin embargo, actualmente el virus altamente patogénico Ganso/Guangdong/1996 linaje 2.3.4.4 H5 parece ser endémico en aves silvestres en gran parte del hemisferio oriental. La mayor parte de los esfuerzos de investigación que estudian la patogenicidad del virus de influenza aviar en aves acuáticas hasta ahora se han dirigido a los patos chapuceadores. Para comprender mejor el papel de los patos buceadores en la ecología del virus de influenza aviar, se caracterizó previamente la patogenia del virus de alta patogenicidad clado 2.3.4.4 H5 en porrones bola (*Aythya affinis*). En un esfuerzo por dilucidar aún más la infección por el virus de influenza aviar en patos buceadores, se investigó la susceptibilidad relativa y la patogénesis de dos aislamientos H7 de alta patogenicidad con linaje de América del Norte que circularon en brotes recientes en los Estados Unidos. Los porrones bola se inocularon con los virus de alta patogenicidad A/pavo/IN/1403-1/2016 H7N8 o con el virus A/pollo/N/17-007147-2/2017 H7N9 por vía intranasal. Se determinó que la dosis infecciosa aproximada de aves 50% (BID_{50}) del aislado H7N8 era de 10^3 dosis infecciosas embrión de pollo 50% (EID_{50}), y se determinó que el BID_{50} del aislado H7N9 era $<10^2$ EID_{50}, lo que indica alguna variación en la adaptación entre dos aislamientos. No se observó mortalidad ni enfermedad clínica en ninguno de los grupos, con excepción de elevadas temperaturas corporales a los dos y cuatro días posteriores a la inoculación. La eliminación del virus se detectó hasta 14 días después de la inoculación en ambos grupos y hubo una tendencia de que la eliminación por la ruta cloacal mostrara una mayor duración y niveles más altos de títulos. Estos resultados demuestran que el porrón bola es susceptible a ambos linajes H7 del virus de la influenza aviar de alta patogenicidad y similar a las especies de patos chapuceadores, eliminan el virus durante largos períodos en relación con las aves gallináceas y no presentan enfermedad clínica.

Key words: diving duck, lesser scaup, highly pathogenic avian influenza, H7N8, H7N9

Abbreviations: AIV = avian influenza virus; BID_{50} = 50% bird infectious dose; CL = cloacal; DPI = days postinoculation; ECE = embryonated chicken eggs; EID_{50} = 50% egg infectious dose; HI = hemagglutination inhibition; HP = highly pathogenic; OP = oropharyngeal; PWRC = Patuxent Wildlife Research Center; RT-PCR = reverse transcription–polymerase chain reaction; USNPRC = U.S. National Poultry Research Center

Aquatic birds such as waterfowl, shorebirds, and gulls are the natural hosts for avian influenza virus (AIV) (22). Most efforts in surveillance and research on AIV in waterfowl have been in dabbling ducks (Anatidae, subfamily Anatinae), especially mallards (*Anas platyrhynchos*), due to their species abundance, easy sampling because of hunter harvesting, accessible habitats, and relatively high prevalence. Other types of ducks such as the lesser scaup (*Aythya affinis*), a diving duck, have also been shown to carry AIV (2). In the United States, although the lesser scaup's range is more restrictive than the mallard's, there is overlap which may facilitate interactions between the two types of ducks during certain times of year (3,18). Recent reports have also demonstrated that lesser scaup can be

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infected by, and subsequently shed clade 2.3.4.4 H5 highly pathogenic (HP) AIV (15). Other diving ducks including adult ruddy ducks (*Oxyura jamaicensis*) and common pochards (*Aythya ferina*) are also susceptible to clade 2.3.4.4 H5 HPAIV, and show no clinical signs after experimental infection (15,21). In contrast, juvenile ruddy ducks, and tufted ducks (*Aythya fulisula*), which are also susceptible to these influenza lineages, do present with clinical signs and mortality (12,15,23). Given these factors, it is important to continue to investigate the role of diving ducks in AIV ecology.

In this study we further investigate the pathobiology of AIV in lesser scaup by challenging them with two unrelated HPAIV isolates from recent outbreaks in domestic birds in the United States, both of which are North American lineage isolates (A/turkey/IN/1403-1/2016 H7N8 and A/chicken/TN/17-007147-2/2017 H7N9 HPAIV) (7,8). Each H7 isolate was evaluated by using three different doses of each isolate to evaluate the infectious dose, pathogenesis, and virus shed.

MATERIALS AND METHODS

Ducks. Lesser scaup viable eggs were obtained from the U.S. Geological Survey captive breeding colonies at Patuxent Wildlife Research Center (PWRC). The ducks were hatched at Southeast Poultry Research Laboratory, U.S. National Poultry Research Center (USNPRC), U.S. Department of Agriculture–Agricultural Research Service and were reared to 5–6 wk of age. All animal procedures were approved by the USNPRC and PWRC institutional animal care and use committees.

Viruses. Both A/turkey/IN/1403-1/2016 H7N8 and A/chicken/TN/17-007147-2/2017 H7N9 HPAIVs were obtained from the repository at USNPRC. The viruses were propagated in embryonating chicken eggs (ECEs) according to standard procedures (17). Dilutions were prepared in brain heart infusion broth and the titers were determined in ECEs using standard methods and the Reed-Muench method (13,14).

Pathogenesis study. Blood was collected from 20% of the ducks prior to challenge to confirm the absence of preexisting AIV antibodies by hemagglutination inhibition (HI) assay as described below. Each duck was banded for identification. The ducks were then divided into groups of four or five (except for the noninoculated control, which was one duck) and were inoculated with 0.l mL of either H7N8 or H7N9 via the intranasal route at a low dose (10^2 EID_{50}/bird), a medium dose (10^4 50% egg infectious dose [EID_{50}]/bird), or a high dose (10^6 EID_{50}/bird). The clinical condition of each duck was monitored a minimum of daily.

Oropharyngeal (OP) and cloacal swabs (CL) were collected at 2, 4, 7, 10, and 14 days postinoculation (DPI). Body temperatures and body weights were recorded from the ducks challenged with the highest dose of each virus at 0, 2, 4, and 7 DPI (data were logged by individual duck). Body temperatures were taken cloacally with a digital pediatric thermometer. At 4 DPI, the negative control duck, and one duck from each of the H7N8 and H7N9 high-dose groups were euthanatized and evaluated for gross lesions. Samples of trachea, lung, brain, heart, spleen, thymus, bursa, liver, kidney, adrenal glands, intestine, pancreas, proventriculus, and skeletal muscle were also taken at this time for microscopic evaluation. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. Serial sections were processed for immunohistochemical staining with antibody for type A influenza to analyze the viral antigen distribution in the tissues (9). Serum was collected from all surviving ducks at 14 DPI and the ducks were then euthanatized. For the 50% bird infectious dose (BID_{50}) calculations, ducks that shed virus and/or were positive for AIV antibody by 14 DPI were considered infected.

Serology. The HI assay using homologous antigen was used to test for AIV antibodies pre- and postchallenge using standard procedures (11). A serum dilution of 1:8 and above that fully inhibited agglutination was considered positive, and dilutions of 1:4 or less were considered nonspecific.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR targeting the matrix gene was used to evaluate virus shed using methodology described previously (4,16). The standard curves were run in triplicate and used RNA from the same viruses that were used to prepare each inoculum. Virus quantity was reported as equivalents to infectious titer.

RESULTS AND DISCUSSION

None of the ducks inoculated with the H7N8 or H7N9 isolates showed clinical signs. However, ducks inoculated with the high dose (body temperatures were only recorded from the high-dose groups) of either the H7N8 or H7N9 isolates demonstrated a trend (the groups were too small for reliable statistical analysis) of increased body temperatures at 2 and 4 DPI (Fig. 2), which returned to preinfection levels by 7 DPI. Previous studies have reported elevated temperatures in Pekin ducks following HPAIV infection (7), but mallards infected with this same isolate did not show an increase in body temperature (10). Body weights continued to increase throughout the experiment, indicating no apparent decrease in appetite in infected ducks (data not shown). Thus, while no clinical disease is apparent, it is unclear whether the short febrile response, as observed in the high-dose group, would affect behavior in the wild.

No gross lesions were observed in the two inoculated ducks and one noninoculated control duck that were euthanatized and necropsied. Both inoculated ducks were shedding low levels of virus from both the OP and CL routes. The only microscopic lesions were mild lymphoplasmacytic rhinitis and tracheitis, and no viral antigen staining was present in any of the tissues examined. Similar microscopic lesions were observed in tissues from mallards infected with this same isolate. Additionally, viral antigen was found in epithelial cells of the air sac, intestine, and cloacal bursa, and in isolated single cells in lung, heart, and spleen of mallards (10).

Lesser scaup inoculated with the low dose of the H7N8 isolate did not shed detectable levels of virus by either the OP or CL routes and did not seroconvert, indicating they did not become infected. In contrast, all ducks in the medium- and high-dose groups shed detectable levels of virus and were antibody positive at the end of the study (Table 1; Fig. 1). OP shedding was detected from both groups at 2 DPI, but not at 4 DPI, with the exception of one bird in each group. CL shedding continued through 14 DPI for the medium-dose group, and was not detected after 7 DPI from the high-dose group. Shed patterns and titers in the medium-dose group were similar to what was seen in mallards with the same isolate (10).

Unexpectedly, the mean viral titers were higher in the medium-dose group. This was confirmed by repeating the RNA extraction and rRT-PCR. Typically the peak titers are consistent for an AIV isolate in a specific host regardless of dose (although lower doses may have later peak shed). One possible explanation for the differences in shed titers observed between the medium and high doses could be that the high challenge dose resulted in a stronger innate immune response in the ducks. This stronger response may have more effectively mediated the infection, resulting in the lower level of virus shed that was observed. However, the high dose did not produce this effect in mallards, chickens, or turkeys (10).

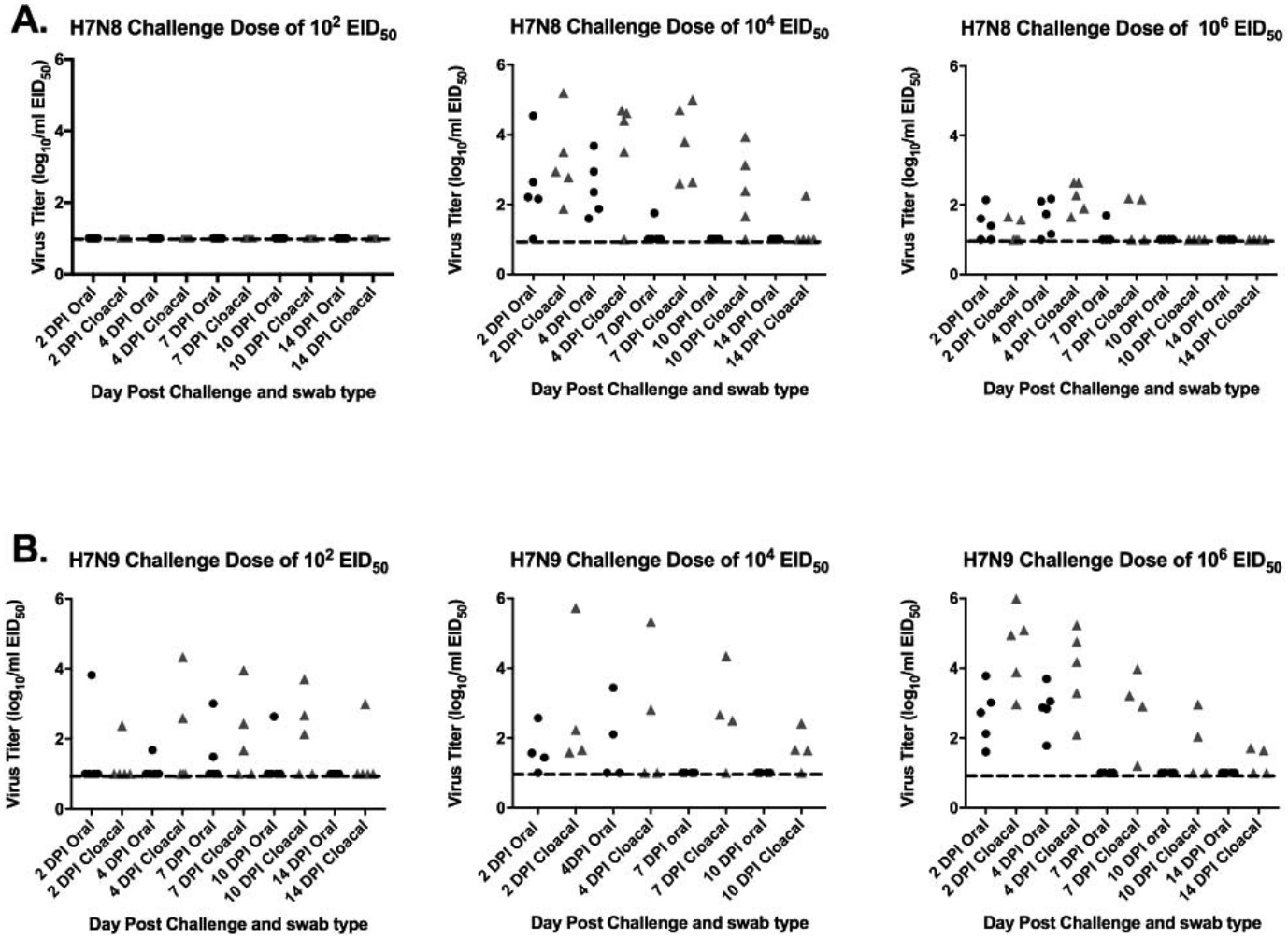

Fig. 1. Virus shed from ducks inoculated with either (A) A/turkey/IN/1403-1/2016 H7N8 HPAIV or (B) A/chicken/TN/17-007147-2/2017 H7N9 HPAIV (titers expressed as log $_{10}$ EID_{50} equivalents) at 2, 4,7, 10, and 14 DPI as detected by real-time RT-PCR in OP and CL swabs. Sample sizes are too small for accurate mean and error bars. The approximate limit of detection for the real-time RT-PCR assay (1.1 log $_{10}$ EID_{50}/ml) is indicated by a dashed line. Black circles represent OP swabs, and CL swabs are represented by grey triangles.

At termination, 0%, 100%, and 50% of ducks in the low-, medium-, and high-dose groups, respectively, were positive for AIV antibodies by HI assay (Table 1). Based on serology and virus shedding, the BID_{50} for the H7N8 isolate was determined to be approximately 10^3 EID_{50}. The reported BID_{50} for this isolate is similar in chickens ($10^{3.2}$ EID_{50}), but is slightly higher than for turkeys and mallards ($<10^2$ and $10^{2.5}$ EID_{50}, respectively), indicating some variability in virus infectivity among species which is common for AIV (10). Also, chickens and turkeys did die from infection. Chickens infected with this same isolate had higher levels of virus shed from the OP route at 2 DPI, but had similar levels shed from the CL route as the lesser scaup. Infected turkeys shed higher levels from both the OP and CL routes compared to the lesser scaup, as did mallards who also shed for a longer amount of time (10). The increased duration of shed from the waterfowl is partly because they survived infection.

Table 1. Seroconversion (14 DPI), proportion of ducks shedding, and approximate BID_{50} for lesser scaup (*Aythya affinis*) inoculated with H7 HPAIV.

Challenge isolate	Dose: EID_{50}/bird	No. antibody positive/ no. tested	No. shedding/ no. tested[A]	Approximate BID_{50}[B]
A/turkey/IN/1403-1/2016 H7N8	10^2	0/5	0/5	10^3 EID_{50}
	10^4	5/5	5/5	
	10^6	2/4	5/5	
A/chicken/TN/17-007147-2/2017 H7N9	10^2	5/5	5/5	$<10^2$ EID_{50}
	10^4	4/4	5/5	
	10^6	4/4	5/5	

[A]Ducks were counted as positive for shed if virus was detected in oral or cloacal swabs at any time.
[B]Ducks were considered infected if they shed detectable levels of virus at any time and/or if they seroconverted.

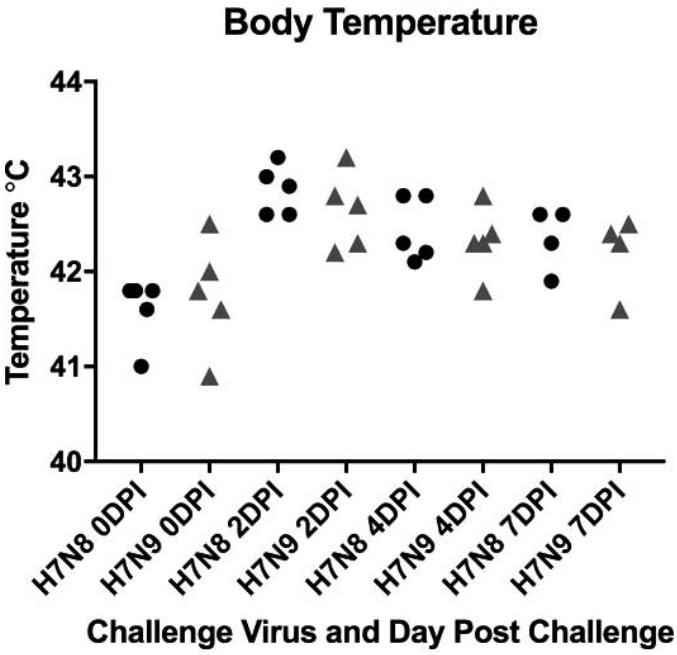

Fig. 2. Body temperatures were taken from the highest challenge groups taken by the cloacal route at 0, 2, 4, and 7 DPI. Sample sizes are too small for accurate mean and error bars. The H7N8 inoculated ducks are shown as black circles, and the H7N9 inoculated ducks are shown as grey triangles.

Ducks inoculated with all three doses of the H7N9 isolate shed virus from both OP and CL routes at 2 DPI (Fig. 1, Panel B). Shedding by the OP route was not detected after 10 DPI for the low-dose group, or after 4 DPI from the medium- and high-dose groups. Among the ducks in the lowest dose group, individual ducks did not shed detectable levels of virus more than two sample days in a row. This pattern suggests that the ducks shedding at later sample times were infected later by lateral spread from their cage-mates rather than by inoculation. Cloacal shedding was detected through 14 DPI from at least one duck in both the low- and high-dose groups, while in the medium-dose group, CL shedding was not detected after 10 DPI. All ducks in all three dose groups were positive for AIV antibody by HI assay (Table 1). Based on serology and virus shedding, the BID_{50} for this isolate was determined to be $<10^2$ EID_{50}. Mallards also had the same mean infectious dose with this virus, showed no clinical signs and shed similar titers of the virus (Pantin-Jackwood, unpubl. data). Chickens also demonstrated a similar mean infectious dose of $<10^2$ EID_{50} indicating similar adaptation to the two species. Infected chickens also shed higher levels of virus by the OP route before dying compared to the lesser scaup, but the lesser scaup shed higher levels of virus by the CL route (Pantin-Jackwood, unpubl. data).

Previous work has shown that lesser scaup are susceptible to infection with a BID_{50} of $<10^4$ EID_{50}/bird with the U.S. 2014 clade 2.3.4.4 H5 HPAIVs but without clinical disease (15). The lesser scaup infected with the H7N8 isolate had a similar BID_{50}, while the lesser scaup infected with the H7N9 had a lower BID_{50} than the clade 2.3.4.4. H5 HPAIVs. Both the H7N8 and H7N9 groups tended to shed slightly higher levels of virus from both the OP and CL routes compared to lesser scaup challenged with the U.S. 2014 clade 2.3.4.4 H5 HPAIVs.

Lesser scaup infected with both H7 isolates displayed a trend to shed for a longer amount of time, and higher titer levels, from the CL route compared to the OP route. High levels of cloacal shedding are typical for ducks infected with AIV, and indicate an expected enteric tissue tropism for these viruses (19). Since ducks shed virus from the CL route for a longer period of time compared to gallinaceous species, and do not show disease signs, they can introduce the virus into the environment for a longer duration, which again highlights the importance of keeping wild birds out of contact with domestic poultry.

Based on these data, lesser scaup are susceptible to infection with both of the North American H7 HPAIV isolates used in this study. Similar to the clade 2.3.4.4 H5 HPAIV isolates, the H7 isolates did not cause apparent clinical disease (15). The lack of clinical disease confirms that the lesser scaup could serve as reservoirs for these HPAIVs. Further evidence that lesser scaup may serve as reservoirs is that a low pathogenicity H7N8 isolate was collected from a lesser scaup in Kentucky in 2015 that contained six gene segments with >99% sequence identity with A/turkey/IN/1403-1/2016 H7N8 (23). As AIV reservoirs with an expansive range and migratory behavior, lesser scaup could contribute to the spread of AI viruses.

The range of lesser scaup includes Central America, Mexico, and the continental United States extending north into Canada (1). High-density lesser scaup wintering areas include Florida, portions of the Gulf Coast, and areas along both the Atlantic and Pacific coasts, including the mid-Atlantic Coast and the Central Valley of California. Wintering lesser scaup are associated with large coastal wetlands including brackish bays and estuaries, but also utilize nearby freshwater resources, such as wetlands, lakes, ponds, reservoirs, and impoundments (1). Satellite telemetry has shown that lesser scaup captured and tagged in the Midwest migrate across the continental United States from Florida to Alaska (6).

With the belief that AI viruses are transmitted within the wild bird populations through the fecal-oral pathway of contaminated water, the diet and foraging behavior of the lesser scaup could facilitate contamination across individuals and species. Lesser scaup consume mainly aquatic invertebrates such as insects, crustaceans, and mollusks throughout their life cycle (1). With their primary prey being mollusks, filter-feeding bivalves, lesser scaup could contract the virus from their food sources. Faust *et al.* (5) showed that bivalves do indeed filter the viruses out of the water column, but they also indicated that the viruses were not bioaccumulated by wood ducks (*Aix sponsa*) when fed the tissue of infected clams. However, when foraging on benthic prey, lesser scaup insert their bill into the substrate at a 35°–45° angle and rapidly open and close their mandibles while swimming forward and moving their head in short, lateral arcs (20). This prey-searching behavior could expose the scaup to the viruses in the substrate and water column, thereby providing a migratory host for the viruses.

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ACKNOWLEDGMENTS

We gratefully acknowledge Scott Lee, Jesse Gallagher, Diane Smith, Suzanne DeBlois, Keith Crawford, Gerald Damron, and Roger Brock (U.S. Department of Agriculture) and Jeffery Sullivan (Natural Systems Analysts, Inc.) for technical assistance with this work. This research was supported by U.S. Department of Agriculture Agricultural Research Service CRIS Project 6612-32000-066-00D, with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) under IAA No. AAI12004-001-00001, and by the U.S. Geological Survey Ecosystems Mission Area. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. Mention of trade names or commercial products in this manuscript is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Government. The U.S. Government is an equal opportunity provider and employer. Data are available from the authors upon request.