DOI:10.1111/j.1750-2659.2011.00238.x
www.influenzajournal.com

Original Article

Experimental challenge and pathology of highly pathogenic avian influenza virus H5N1 in dunlin (*Calidris alpina*), an intercontinental migrant shorebird species

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Accepted 16 January 2011, Published Online 14 March 2011.

Background Shorebirds (Charadriiformes) are considered one of the primary reservoirs of avian influenza. Because these species are highly migratory, there is concern that infected shorebirds may be a mechanism by which highly pathogenic avian influenza virus (HPAIV) H5N1 could be introduced into North America from Asia. Large numbers of dunlin (*Calidris alpina*) migrate from wintering areas in central and eastern Asia, where HPAIV H5N1 is endemic, across the Bering Sea to breeding areas in Alaska. Low pathogenic avian influenza virus has been previously detected in dunlin, and thus, dunlin represent a potential risk to transport HPAIV to North America. To date no experimental challenge studies have been performed in shorebirds.

Methods Wild dunlin were inoculated intranasally and intrachoanally various doses of HPAIV H5N1. The birds were monitored daily for virus excretion, disease signs, morbidity, and mortality.

Results The infectious dose of HPAIV H5N1 in dunlin was determined to be $10^{1.7}$ EID_{50}/100 μl and that the lethal dose was $10^{1.83}$ EID_{50}/100 µl. Clinical signs were consistent with neurotropic disease, and histochemical analyses revealed that infection was systemic with viral antigen and RNA most consistently found in brain tissues. Infected birds excreted relatively large amounts of virus orally (10^4 EID_{50}) and smaller amounts cloacally.

Conclusions Dunlin are highly susceptible to infection with HPAIV H5N1. They become infected after exposure to relatively small doses of the virus and if they become infected, they are most likely to suffer mortality within 3–5 days. These results have important implications regarding the risks of transport and transmission of HPAIV H5N1 to North America by this species and raises questions for further investigation.

Keywords Dunlin, experimental infection, highly pathogenic avian influenza virus, pathology, shorebird.

Please cite this paper as: Hall *et al.* (2011). Experimental challenge and pathology of highly pathogenic avian influenza virus H5N1 in dunlin (*Calidris alpina*), an intercontinental migrant shorebird species. Influenza and Other Respiratory Viruses 5(5), 365–372.

Introduction

The primary reservoirs of avian influenza virus (AIV) are wild waterfowl (Anseriformes), and gulls and shorebirds (Charadriiformes).[1] Many of these species are highly migratory and could transport AIV, including highly pathogenic avian influenza virus (HPAIV), over long distances, possibly between continents.

Many surveillance efforts have examined the roles of wild waterfowl in the disease ecology of AI. Fewer studies have examined shorebirds, with the majority of reported AIV recoveries in shorebirds occurring in Delaware Bay on the eastern coast of North America. Interestingly, the highest seasonal occurrence of AIV in Delaware Bay shorebirds occurs during spring migration, when adult birds are moving northward to their breeding grounds in the Arctic from wintering areas in Central and South America.[2] This contrasts with the usual prevalence of AIV in waterfowl that peaks during the southward migration in autumn when large numbers of young, immunologically naïve birds congregate at stopover and staging areas. AIV in Delaware Bay shorebirds also occurs predominantly in only a few species, primarily ruddy turnstones (*Arenaria interpres*) and red knots (*Calidris canutus*).[3,4] Other common Delaware Bay shorebird species such as sanderlings (*Calidris alba*), willets (*Catoptrophorus semipalmatus*), plovers (*Charadrius* sp.), and the *hudsonia* race of dunlin (*Calidris alpina hudsonia*) have lower prevalences of

infection, similar to those found in shorebird species from other locations.[5–16]

Dunlin (*Calidris alpina*) is a common shorebird species, occurring on all continents except Antarctica. In Alaska, two races are present. *C. a. pacifica* is a common breeder in western Alaska which migrates primarily through the western flyway of North America and *C. a. arcticola* migrates from wintering areas in central and eastern Asia to breeding areas in northern Alaska. It is estimated that 200 000–750 000 individuals of this race cross the Bering Sea[17] and represents the largest population of transcontinental avian migrants between Asia and North America.[18] Small numbers of wild dunlin in that region have been found infected with AIV, including a bird sampled in Japan and a single bird in Alaska that was excreting a reassortant low pathogenic avian influenza virus (LPAIV) with Asian and North American genetic lineages.[5,14] Thus, migratory dunlin, especially the race *C. a. arcticola*, represents a potential mechanism for transporting HPAIV H5N1 to Alaska from their wintering areas in Asia where HPAIV H5N1 is known to occur. Once in Alaska, the virus could subsequently be transmitted to, and disseminated throughout North America by other species of migratory birds. Therefore, dunlin is considered an important species specifically targeted by surveillance efforts as high risk for transporting HPAIV H5N1 to North America,[19] yet these risks have not been adequately defined and most surveillance studies have not targeted and sampled spring migrants from Asia, the birds most likely to be involved.

Experimental studies on the effects of HPAIV H5N1 in North American wild birds have focused primarily on waterfowl, several species of gulls, and American kestrels (*Falco sparverius*).[20–26] To the best of our knowledge, no experimental challenge studies in shorebirds have been published. Herein we present the dose response of wild-caught dunlin to experimental infection of HPAIV H5N1 in terms of morbidity, survival, levels of virus shedding, histopathology, minimum infectious dose, and lethal dose. These findings have important implications regarding mode of transmission and potential risks of HPAIV H5N1 movement by shorebirds.

Materials and methods

Dunlin acquisition and husbandry

Wild dunlin were captured in mist nets along the coast of the Yukon-Kuskokwim Delta, Alaska. Birds were aged, measured (culmen and straight wing), weighed, leg banded, and assigned to races based on wing pattern.[27] Only juvenile birds were captured for this study as they were seasonally, the most available and the age class most likely to have no prior exposure to AIVs, and thus be seronegative. Birds were held up to 3 days in a rectangular enclosure (0·4 m high × 0·6 m wide × 2·0 m long) placed on short vegetation. Drinking water was provided *ad libitum*, and food was provided 2–4 times/day (commercially raised larval wax moths (*Gallenia mellonella*), fish food pellets, and locally harvested polychaete worms (*Arenicola* spp.). Birds were transported in commercially available pet carriers to anchorage, AK, held in a slightly larger communal holding facility (as described earlier) and provided wax moth larvae twice a day in quantities approximating *ad libitum* conditions. Birds were weighed and monitored daily between capture and transport via commercial aircraft to the USGS National Wildlife Health Center (NWHC), Madison, Wisconsin.

At the NWHC, the birds ($n = 24$) were placed in a plastic-coated wire-mesh enclosure (~2·0 m high × 2·0 m wide × 4·0 m long) within a Biosafety level 3 facility and allowed to acclimate. The dunlin were provided larval wax moths and mealworms (*Tenebrio molitor*) *ad libitum*. After 2 days, the birds were eating well and all were maintaining body weight. They were randomly assigned to study groups and transferred to individually HEPA-filtered isolator cages (2–3 birds/cage). All housing, transport, and animal care procedures were approved by USGS National Wildlife Health Center and Alaska Science Center institutional animal care and use committees.

Experimental design and highly pathogenic avian influenza virus inoculation

Six individuals in each of three groups were inoculated intranasally with one of three doses ($10^{0·7}$ EID_{50}/100 μl, $10^{2·7}$ EID_{50}/100 μl, $10^{4·7}$ EID_{50}/100 μl) of HPAIV H5N1 (A/whooper swan/Mongolia/244/05) diluted in brain/heart infusion broth. Three uninoculated dunlin served as controls, and one additional uninoculated bird at each dose was co-housed with two inoculated cagemates to test for contact transmission. Inocula were diluted in brain/heart infusion broth and viral titer of the highest dose confirmed in 10-day-old (37·5°C, 50% humidity), embryonating chicken eggs.[28] This method was also used to determine the infectious and lethal doses. After 14 days post-inoculation (DPI), 13 birds survived and appeared healthy, including negative controls and contact transmission subjects. We evaluated the immunological status and virus excretion of these birds (see Methods below) and determined that 12/13 remained influenza antibody negative and had not shed virus orally or cloacally after inoculation (see Results). These uninfected subjects were assumed to have remained naïve and were reassigned to new cohorts and inoculated with either $10^{0·7}$ EID_{50}/100 μl (two birds), $10^{1·7}$ EID_{50}/100 μl (six birds), or $10^{2·7}$ EID_{50}/100 μl (two birds), with two birds serving as negative controls.

Sampling

Serum samples were collected from all birds on DPI 0 and DPI 14, and when possible, immediately prior to euthana-

sia by CO_2 asphyxiation. Sera were stored at −20°C. All birds were weighed daily and monitored as needed to ascertain health status. Daily cloacal and oropharyngeal swabs were obtained using Dacron tipped applicators, placed in cryovials containing viral transport media (Hanks Balanced Salt Solution, 0·05% gelatin, 5% glycerin, 1500 U/ml penicillin, 1500 μg/ml streptomycin, 0·1 mg/ml gentamicin, 1 μg/ml fungizone) and stored at −80°C until analyses.

Serology

The pre-inoculation (day 0) and final immunological status were determined using a commercially available competitive enzyme-linked immunosorbant assay (cELISA) kit according to the manufacturer's directions (IDEXX Laboratories, Westbrook, ME, USA). This assay detects antibodies to the nucleoprotein of influenza viruses in a wide variety of wild avian species.[29]

RNA extraction and real time reverse transcription–polymerase chain reaction (RT-PCR)

Viral RNA was extracted from cloacal and oropharyngeal swabs using the MagMAX™-96 AI/ND Viral RNA Isolation kit (Ambion, Austin, TX, USA) following the manufacturer's procedures. Tissue samples were macerated in viral transport media and 300 μl of suspension was added to 300 μl viral lysis buffer and extracted as described earlier. Real time RT-PCR was performed using the published procedures for the detection of H5 influenza isolates.[30] RT-PCR assays used reagents provided in the Qiagen OneStep® RT-PCR kit (Valencia, CA, USA) and performed on a Stratagene Mx3005P thermal cycler (Agilent Technologies, Santa Clara, CA, USA). Quantification of viral shedding of each subtype was performed by comparing C_t values of swab samples to standard curves generated from viral RNA extracted from the initial inocula. The presence or absence of viable virus was confirmed in embryonating egg culture from all swab samples and virus titrations were determined in embryonating egg culture on swabs from a subset of the infected birds.

Necropsy, immunohistochemical, and histological analyses

All birds were examined at necropsy when portions of brain, trachea, lung, heart, liver, kidney, gonad, adrenal, spleen, duodenum, pancreas, jejunum, cecal tonsil, and cloaca with bursa of Fabricius were collected for histopathology. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. Additional portions of brain, intestine, liver, lung, spleen, and kidney were collected for RNA extraction and RT-PCR analyses as described earlier.

Immunohistochemistry processing was performed at the Department of Pathology Histology Laboratory, College of Veterinary Medicine, University of Georgia. Following deparaffinization, proteinase K treatment was used for antigen retrieval and endogenous peroxidase was blocked using 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ, USA). Mouse monoclonal antibody to Influenza A virus nucleoprotein (1 mg/ml; Biodesign International, Saco, ME, USA), 1:200 in Dako® Antibody Diluent (Dako, Carpinteria, CA, USA), was applied to slides for 60 minutes. This was followed by biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA, USA) and streptavidin conjugated with horseradish peroxidase (Dako's LSAB® 2, Dako). The substrate-chromogen system used was DAB (Dako), and slides were counterstained with Gills II hematoxylin. Positive tissue controls consisted of formalin fixed, paraffin-embedded heart from AIV-infected chicken. As a negative control, primary antibody was substituted with Universal Negative Control (Dako).

Results

Susceptibility, clinical signs, and mortality

In the initial round of experimental infections, we inoculated dunlin with one of three doses of HPAIV H5N1 ($10^{0.7}$, $10^{2.7}$, $10^{4.7}$ EID_{50}/100 μl). At the highest dose, $10^{4.7}$ EID_{50}/100 μl, all birds excreted virus and all died within 5 days of inoculation. At the middle dose, $10^{2.7}$ EID_{50}/100 μl, all birds excreted virus but only 5/6 died, whereas at the lowest dose none of the birds became infected based on the absence of detectable viral RNA in oral and cloacal swabs. None of the contact transmission subjects (one per dose) became infected even though two of these were co-housed with two infected birds. A second round of inoculations using surviving seronegative and virus-negative birds was performed to more accurately define the HPAIV infectious and lethal doses. Two birds were inoculated with $10^{2.7}$ EID_{50}/100 μl, both of which died on DPI 4, similar to the results of the first round of inoculation at that dose. Of the six birds inoculated with $10^{1.7}$ EID_{50}/100 μl, three became infected and died while the others excreted no viral RNA and survived to 14 DPI. One of the two birds inoculated with $10^{0.7}$ EID_{50}/100 μl died for undetermined reasons on DPI 3 but did not shed virus or contain viral RNA in any tissue and was therefore assumed to be not infected with HPAIV. The other bird at that dose was uninfected and survived to 14 DPI. None of the control birds from either round of inoculations became infected. The infection and survival of HPAIV H5N1 inoculated dunlin are summarized in Figure 1. Based on these data, the infectious dose (ID_{50}) of HPAIV H5N1 in dunlin was calculated as $10^{1.7}$ EID_{50}/100 μl and the lethal dose (LD_{50}) was $10^{1.83}$ EID_{50}/100 μl.

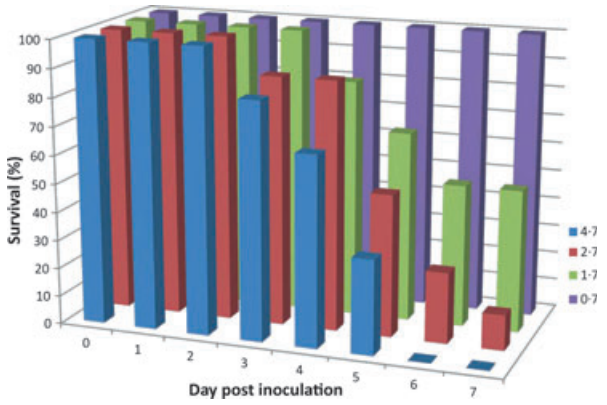

Figure 1. Survival of dunlin after intranasal inoculation with various doses of highly pathogenic avian influenza virus H5N1 (A/whooperswan/Mongolia/244/05). Virus doses are those administered (100 μl) to each dunlin, expressed as Log_{10} Egg Infectious Dose $(EID)_{50}$.

Disease signs were similar in all but one infected subjects and consisted of feather fluffing, ataxia, loss of appetite, tremors, and loss of motor control and balance. We could reliably identify the race of all but four of the birds and found no difference between the two different races in terms of disease signs, shedding, and infection. Infected birds lost more than 10% of their body mass in the 24-hour period prior to death or euthanasia, and this was the most consistent indication of pending morbidity/mortality. There were no overt signs in any bird of respiratory or intestinal disease. Birds were euthanized after severe clinical signs were observed. Mortality or euthanasia of infected dunlin occurred typically between DPI 4 and 5 (range 3–6). As previously mentioned, one bird (#254) was infected with $10^{2·7}$ EID_{50}/100 μl and excreted virus yet exhibited no overt signs of disease and survived for the full term of the study. Thus, other than this one bird, it appears that if a dunlin does become infected with HPAIV, they are most likely to die from the infection within a week following exposure to the virus.

Virus shedding

All birds that became infected excreted virus orally, beginning on DPI 1 and continued shedding virus until they were euthanized or died (Table 1). Based on RT-PCR analysis, peak amounts of virus found in oral swabs typically ranged from 10^3 to 10^4 EID_{50} equivalents/ml; however, the DPI of peak shedding was variable. The exception was the bird that survived infection (#254) orally excreted only $10^{1·7}EID_{50}$/100 μl of virus for 2 days (DPI 3, 4).

All but three birds that became infected excreted RT-PCR detectable viral RNA cloacally (Table 2). However, the duration and amounts of virus shedding were much reduced and less consistent in comparison with the oral shedding. The three exceptions, birds 254, 969, 987, did not excrete detectable virus cloacally yet did orally.

Seroconversion

Based on cELISA analysis, all dunlin had no detectable influenza antibodies prior to inoculation. When possible, serum was collected from subjects immediately prior to euthanasia. Sera from birds euthanized before DPI 4 were all influenza antibody negative, while sera from DPI 5 or afterward were seropositive (data not shown). Of sera from birds that died on DPI 4, 3/5 had detectable antibodies to AIV, independent of dose, indicating that an immune response to HPAIV H5N1 occurred relatively quickly (by DPI 4–5) in infected birds.

Necropsy and histopathology

Twenty-one inoculated dunlin and two controls were examined at necropsy. Gross lesions were not observed in any tissues. The two controls had greater amounts of subcutaneous and mesenteric fat than most of the birds that succumbed to infection, but fat reserves in other inoculated birds were similar to controls.

Examination of hematoxylin and eosin (HE)-stained sections of brain revealed mild lymphoplasmacytic meningitis and perivascular cuffing without other evidence of encephalitis in 11 of the 23 dunlin. Only one dunlin had segmental loss of Purkinje cells with no associated inflammation. All of the dunlin observed to have neurologic pathology were infected with AIV. No inflammation or necrosis was observed in HE-stained sections of the heart, aorta, syrinx, pancreas, small intestine and ceca, esophagus, kidney, adrenal gland, bursa, fat, ovary, fallopian tube, or testes. Livers from nine of 23 birds, including the two controls, had mild pigment accumulation in hepatocytes and Kupffer cells and three birds had periportal lymphocytes, plasma cells with mild bile duct hyperplasia. Spleens from eight of 21 birds, including both controls, had hemosiderin-like pigment and droplets of amorphous red material, suggesting red blood cell fragments, in the cytoplasm of cells in the arteriolar sheaths. One bird had a solitary focus of mild subacute inflammation in the lung, and another bird had mild focal subacute airsacculitis. All other sections of lung and air sac were unremarkable.

Tissues from seven infected dunlin were immunohistochemically (IHC) stained to detect AIV antigen. Staining was inconsistent except that all brainstems showed intense staining in the cytoplasm and nuclei of neurons and glial cells (Figure 2). Other regions of brain had less consistent AIV antigen staining. Four dunlin had mild AIV antigen staining in the mucosal and scattered interstitial cells in the small intestine. Two of these had mild staining in the mucosa of ceca or large intestine and one had heavy staining in the large intestine. The three remaining dunlin each

Table 1. Oral excretion of highly pathogenic avian influenza virus H5N1 by experimentally infected dunlin.

Bird ID	Dose*	Day post-inoculation (DPI)							
		DPI-0	DPI-1	DPI-2	DPI-3	DPI-4	DPI-5	DPI-6	DPI-7**
971	Control	nc***,†	nc	nc	nc	nc	nc	nc	nc
981	Control	nc	nc	nc	nc	nc	nc	nc	nc
962	Control	nc	nc	nc	nc	nc	nc	nc	nc
966††	Control	nc	nc	nc	nc	nc	nc	nc	nc
983††	Control	nc	nc	nc	nc	nc	nc	nc	nc
251	0·7	nc	nc	nc	nc	nc	nc	nc	nc
964	0·7	nc	nc	nc	nc	nc	nc	nc	nc
987	0·7	nc	nc	nc	nc	nc	nc	nc	nc
980	0·7	nc	nc	nc	nc	nc	nc	nc	nc
966	0·7	nc	nc	nc	nc	nc	nc	nc	nc
983	0·7	nc	nc	nc	nc	nc	nc	nc	nc
971††	0·7	nc	nc	nc	nc				
962††	0·7	nc	nc	nc	nc	nc	nc	nc	nc
980††	1·7	nc	2·55‡	1·86‡	1·72‡	1·61‡			
251††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
965††	1·7	nc	2·92 (>2·0†††)	4·51 (>4·0)	2·89 (>2·0)	2·55 (>2·0)	4·11 (3·64)		
964††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
981††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
987††	1·7	nc	nc	2·30‡	2·07‡	2·23‡	1·39‡	2·15‡	
258	2·7	nc	3·01‡	3·03‡	1·59	1·75‡	3·60‡		
963	2·7	nc	1·21‡	1·87‡	1·05‡	0·93‡	2·10‡	2·28	
254	2·7	nc	nc	nc	1·30‡	1·76	nc	nc	nc
259	2·7	nc	2·76‡	2·84‡	3·24‡	2·60‡	2·65‡		
986	2·7	nc	3·57‡	3·19‡	3·07‡	4·03‡			
968	2·7	nc	3·90‡	3·31‡	2·41‡				
970††	2·7	nc	3·02‡	2·83‡	1·65‡	1·92‡			
257††	2·7	nc	3·56‡	2·76‡	2·37‡	3·29‡			
974	4·7	nc	2·99‡	2·11‡	2·42‡				
973	4·7	nc	4·11 (3·75)	3·16 (>3·0)	2·74 (2·0)	2·86 (1·75)	2·81 (1·4)		
969	4·7	nc	3·05‡	2·84‡	2·50‡	1·98‡			
967	4·7	nc	3·98‡	3·5‡	3·95‡				
256	4·7	nc	3·68‡	2·97‡	2·09‡	2·15‡	3·12‡		
252	4·7	nc	3·44‡	2·64‡	2·17‡	2·79‡			

*Inoculation dose (log_{10} EID_{50}/100 μl).
**Infected birds died/euthanized on last day sampled. Those surviving to DPI 7 lived to DPI 14 (not shown).
***nc = no C_t value obtained by H5 specific RT-PCR analysis and confirmed in embryonating egg culture.
†Virus amounts represented as Log_{10} EID_{50} equivalents based on RT-PCR standard curves generated from original inocula.
††Second round of inoculation using seronegative, non-infected birds from the first round of inoculation, including contact transmission subjects 965, 970, and 257.
†††Numbers in parentheses represent viral titers as determined in embryonating egg culture.
‡Presence of infectious virus confirmed by virus isolation in embryonating eggs.

had a single small cluster of AIV antigen-positive myocytes (5–12 cells) and a subset of these cells had very condensed nuclei suggesting necrosis. One of five aorta examined was positive for antigen associated with a small focus of endarteritis. Mild AIV antigen-positive mucosal cells were seen in isolated mesobronchi of four of seven dunlin and these same four birds had mild mucosal staining of the syrinx. Two of these and one additional bird had mild multifocal staining of the air sac. Antigen staining in adrenal glands was mild and focal in one bird and multifocally moderate in two other birds. There was mild random staining for AI antigen in four of six spleens examined. Four bursae were stained for avian influenza antigen and none of the lymphocytes were positive; however, the overlying mucosal epithelium was positive in three birds.

Discussion

To assess the risks of dunlin transporting and transmitting HPAIV H5N1 into North America and/or dispersing the

Table 2. Cloacal excretion of highly pathogenic avian influenza virus H5N1 by experimentally infected dunlin.

Bird ID	Dose*	Day post-inoculation (DPI)							
		DPI-0	DPI-1	DPI-2	DPI-3	DPI-4	DPI-5	DPI-6	DPI-7**
971	Control	nc***,†	nc	nc	nc	nc	nc	nc	nc
981	Control	nc	nc	nc	nc	nc	nc	nc	nc
962	Control	nc	nc	nc	nc	nc	nc	nc	nc
966††	Control	nc	nc	nc	nc	nc	nc	nc	nc
983††	Control	nc	nc	nc	nc	nc	nc	nc	nc
251	0·7	nc	nc	nc	nc	nc	nc	nc	nc
964	0·7	nc	nc	nc	nc	nc	nc	nc	nc
987	0·7	nc	nc	nc	nc	nc	nc	nc	nc
980	0·7	nc	nc	nc	nc	nc	nc	nc	nc
966	0·7	nc	nc	nc	nc	nc	nc	nc	nc
983	0·7	nc	nc	nc	nc	nc	nc	nc	nc
971††	0·7	nc	nc	nc	nc				
962††	0·7	nc	nc	nc	nc	nc	nc	nc	nc
980††	1·7	nc	nc	nc	0·79	2·58†††			
251††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
965††	1·7	nc	nc	0·96	2·77†††	1·78	1·76†††		
964††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
981††	1·7	nc	nc	nc	nc	nc	nc	nc	nc
987††	1·7	nc	nc	nc	nc	nc	nc	nc	
258	2·7	nc	nc	nc	nc	1·96†††	nc		
963	2·7	nc	nc	nc	nc	0·05	nc	0·87	
254	2·7	nc	nc	nc	nc	nc	nc	nc	nc
259	2·7	nc	nc	nc	nc	nc	0·97		
986	2·7	nc	3·46†††	nc	0·27	nc			
968	2·7	nc	nc	nc	1·05				
970††	2·7	nc	nc	0·14	0·84	nc			
257††	2·7	nc	nc	1·50†††	0·48†††	0·97†††			
974	4·7	nc	nc	nc	2·48†††				
973	4·7	nc	nc	nc	nc	nc	0·28		
969	4·7	nc	nc	nc	nc	nc			
967	4·7	nc	2·01†††	nc	2·82†††				
256	4·7	nc	0·07	nc	nc	nc	0·10		
252	4·7	nc	0·05	nc	0·71	1·71			

*Inoculation dose ($\log_{10}$ EID_{50}/100 μl).
**Infected birds died/euthanized on last day sampled. Those surviving to DPI 7 lived to DPI 14 (not shown).
***nc = no C_t value obtained by H5 specific RT-PCR analysis and confirmed in embryonating egg culture.
†Virus amounts represented as Log_{10} EID_{50} equivalents based on standard curves from the original inocula.
††Second round of inoculation using seronegative, non-infected birds from the first round of inoculations, including contact transmission subjects 965, 970, and 257.
†††Presence of infectious virus confirmed by virus isolation in embryonating eggs.

virus once it gets to the continent, we must consider a variety of factors. Our results suggest that dunlin is most likely to suffer mortality as a result of infection with this HPAI virus. The infectious dose and lethal dose were nearly identical ($10^{1.7}$ EID_{50}/100 μl versus $10^{1.83}$ EID_{50}/100 μl respectively) with death occurring 4–5 days after exposure. Infection was systemic, occurring in all tissues examined with the highest amounts of oral shedding of virus occurring quickly at DPI 1–2. However, the one infected bird that did survive infection and excrete virus means that in a large population of migrating dunlin, significant numbers of infected birds could still potentially transport the virus into North America. Therefore, the pertinent questions are whether infected dunlin could migrate after infection with HPAIV H5N1 and, if so, how far? Reports that Bewick's swans (*Cygnus columbianus bewickii*) have reduced foraging and migratory behavior and that mallards (*Anas platyrhyncos*) show reduced body mass after infection with LPAIV dispute the assumption that waterfowl are generally asymptomatic carriers of those viruses.[31,32] The actual physiologi-

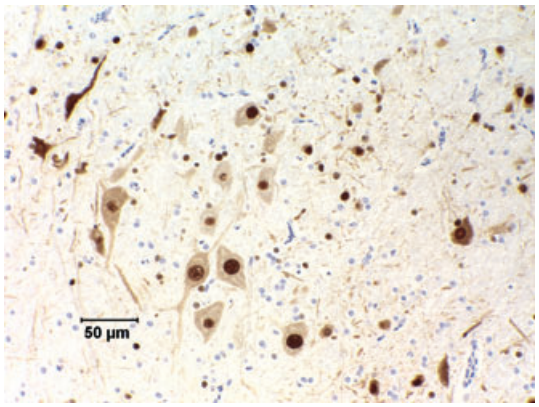

Figure 2. Immunohistochemistry for influenza A virus antigen using brown chromagen label on tissue from dunlin (*Calidris alpina)* experimentally inoculated with highly pathogenic avian influenza virus H5N1. Brainstem section with intense brown staining of nuclei and cytoplasm of neurons and glial cells representing immunohistochemical positive label for avian influenza virus antigen. Inflammation to the infected cells was not seen on hematoxylin and eosin-stained tissue.

cal costs of infection and subsequent immune response remain unknown and must be accurately defined to answer these critical questions for both low and highly pathogenic influenza viruses.

Infected dunlin excreted considerable amounts of virus orally but much smaller amounts cloacally. Based on a dunlin ID_{50} of $10^{1.7}$ EID_{50}/100 μl, the amount of virus excreted orally by infected birds was more than sufficient to infect naïve dunlin; however, none of the uninoculated birds co-housed with infected birds became infected with HPAIV despite shared water/food containers, flooring, etc. While the sample size of these co-housed birds was small, the relatively low amounts of cloacal shedding of virus and the requisite infectious dosage suggest that fecal/oral transmission of HPAIV H5N1 in dunlin would be unlikely.

The birds' behavior, where direct physical contact was rare, apparently precluded contact transmission. This is also reflected in their natural settings where, even though dunlin can occur in high densities, direct contact, such as mutual preening does not happen to any great extent. Thus, with fecal/oral and contact transmission unlikely, that leaves aerosol transmission as the only other possible mechanism for transmission between dunlin. This would seem equally unlikely given the infectious dose and open air locations where the birds reside. While extreme caution must always be taken when extrapolating captive, controlled, experimental study results to the natural ecology in the field, our results raise questions that certainly warrant further investigation, including increased sampling of migrating populations in the spring, to ascertain the actual risks of these birds transporting HPAIV to North America.

While infected dunlin may not represent a significant risk for transmission of HPAIV H5N1 to other dunlin, they do pose risks to other, more susceptible species. Many other shorebirds, gulls, and waterfowl occupy the same habitats as dunlin and could become exposed to contaminated shorelines. Predators and scavengers are, perhaps, even more in danger of becoming infected by consuming dead and dying birds.[26,33] The tissues of infected dunlin contained large amounts of virus. A variety of gull species are common in habitats used by dunlin and several species of falcons, most notably, peregrine falcons (*Falco peregrinus*), are known to feed on dunlin.[34] Several falcon species have been shown to be extremely susceptible to HPAIV H5N1 with 100% mortality at all doses tested.[24,35,36]

In this study, we examined only immature birds obtained in late summer/autumn, a period when surveillance studies typically have revealed low virus prevalence in shorebirds. However, studies showing high prevalence of LPAIV in shorebirds mainly involve northward migrating adults in spring (i.e. ruddy turnstones and red knots in Delaware Bay). Moreover, because the potential route of HPAIV H5N1 being introduced into North America by dunlin would be through northbound spring migrants from Asia into Alaska, experimental studies in adult birds would be informative. As a primary reservoir of influenza, it is important to experimentally examine shorebirds' roles in the disease ecology of influenza. To date no experimental infection studies in shorebird species have been published with either LPAIV or HPAIV isolates. Using the techniques and experience gained from this study, we will be able to experimentally examine other shorebird species' roles. These are critical studies to determine the levels and duration of viral shedding, transmission mechanisms, survival, and ultimately the potential risks of influenza in these important species.

Acknowledgements

The authors express their sincere gratitude to Dan Ruthrauff, Matthew Sexson, Maksim Dementyev, Ty Donnelly, Dejan Stojanovic, and T. Lee Tibbitts for their dedicated efforts to capture, house, and transport these birds under field conditions. Jennifer Brunner helped with animal care at the USGS NWHC, and we thank Allison Klein and Douglas Berndt for their efforts trimming the histological samples and the staff of the Department of Pathology Histology Laboratory, College of Veterinary Medicine, University of Georgia, for their assistance with the IHC analyses. Dr. David Swayne at the USDA Southeast Poultry Research Laboratory generously provided the HPAI virus isolate. Funds for this study were provided by the USGS. Use of trade or product names does not imply endorsement by the United States government.

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