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Interspecific exchange of avian influenza virus genes in Alaska: the influence of trans-hemispheric migratory tendency and breeding ground sympatry

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Abstract

The movement and transmission of avian influenza viral strains via wild migratory birds may vary by host species as a result of migratory tendency and sympatry with other infected individuals. To examine the roles of host migratory tendency and species sympatry on the movement of Eurasian low pathogenic avian influenza (LPAI) genes into North America, we characterized migratory patterns and LPAI viral genomic variation in mallards (*Anas platyrhynchos*) of Alaska in comparison to LPAI diversity of northern pintails (*Anas acuta*). A 50-year band recovery data set suggests that unlike northern pintails, mallards rarely make trans-hemispheric migrations between Alaska and Eurasia. Concordantly, fewer (14.5%) of 62 LPAI isolates from mallards contained Eurasian gene segments compared to those from 97 northern pintails (35%), a species with greater intercontinental migratory tendency. Aerial survey and banding data suggest that mallards and northern pintails are largely sympatric throughout Alaska during the breeding season, promoting opportunities for interspecific transmission. Comparisons of full genome isolates confirmed near-complete genetic homology (>99.5%) of seven viruses between mallards and northern pintails. This study found viral segments of Eurasian lineage at a higher frequency in mallards than previous studies, suggesting transmission from other avian species migrating inter-hemispherically or the common occurrence of endemic Alaskan viruses containing segments of Eurasian origin. We conclude that mallards are unlikely to transfer Asian origin viruses directly to North America

via Alaska, but that they are likely infected with Asian origin viruses via interspecific transfer from species with regular migrations to the Eastern Hemisphere.

Keywords

Anas platyrhynchos; avian influenza; migratory; reassortment; transmission

Introduction

Wild migratory birds, primarily in the avian orders of Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds), are the natural reservoirs of a large diversity of low pathogenic avian influenza (LPAI) virus subtypes (Webster *et al.* 1992; Kim *et al.* 2009). Nomenclature of influenza viral subtypes (e.g., H5N1) is based on phylogenetic characteristics of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA types have been identified in LPAI viruses found in wild water birds (Clark & Hall 2006) and these subtypes have the potential to evolve into highly pathogenic avian influenza (HPAI) strains, such as H5N1 (Guan *et al.* 1999; Hoffmann *et al.* 2000). Continued outbreaks of the HPAI H5N1 strain in wild and domestic birds and its transfer to humans (World Health Organization 2010) has led to a marked increase in monitoring and research of wild and domestic birds as hosts of HPAI and LPAI viral strains.

Phylogenetic analyses of low pathogenic avian influenza (LPAI) viral RNA segments from Eurasia and North America have revealed two distinct groupings of lineages that correspond to hemispheric origins (Gorman *et al.* 1990a, b; Ito *et al.* 1991). However, several recent studies have documented genetic exchange between these hemispheric lineages (Widjaja *et al.* 2004; Wallensten *et al.* 2005; Jackwood & Stallknecht 2007; Krauss *et al.* 2007) resulting from periodic gene flow via migratory birds and persistence following introduction (Bahl *et al.* 2009). In North America, a greater number of avian influenza genes of Eurasian origin have been documented in peripheral areas where divergent viral gene pools overlap, such as in Alaska and eastern North America (Pearce *et al.* 2010; Ramey *et al.* 2010a, b). In contrast, birds sampled in areas further from continental margins appear to have viruses with fewer lineages of Eurasian origin (e.g., Krauss *et al.* 2007; Pearce *et al.* 2009).

In Alaska, the bulk of our knowledge about the frequency of reassortment events between Eurasian and North American LPAI viruses and thus, connectivity of hemispheric viral populations comes from the northern pintail (*Anas acuta*; Koehler *et al.* 2008; Ramey *et al.* 2010a). Multiple data sources suggest the higher frequency of Eurasian LPAI lineages in northern pintails in Alaska results from sympatry between birds that winter in Eurasia and North American and breed in the North Pacific (Miller *et al.* 2005; Flint *et al.* 2009; Yamaguchi *et al.* 2010). However, it remains unclear if the level of gene reassortment observed in LPAI viruses of northern pintails is common to all avian hosts that breed in Alaska or only those with strong migratory connections to Eurasia. While there are numerous birds that migrate from Australasian and Eurasian wintering areas to Alaska each year (Winker & Gibson 2010), there are also many species and populations that remain in Alaska for breeding and within North America for wintering. Determining the degree of migratory and population connectivity of avian taxa between Asia and Alaska is important for the prioritization of species to target for highly pathogenic avian influenza (HPAI) surveillance and for understanding what factors influence the introduction of novel pathogens to the North American landscape. However, it has not been determined if migratory birds that do not regularly migrate between hemispheres can be effective sentinels for detecting foreign origin viruses in North America.

The mallard (*Anas platyrhynchos*) is the most abundant and ubiquitous waterfowl species of both Old and New World (Kear 2005). While phylogeographic analysis of Eurasian and North American mallards suggests substantial differentiation, gene flow may occur asymmetrically from Eurasia to North America through Alaska (Kulikova *et al.* 2005). Within North America, breeding birds from Alaska move south to areas in the Pacific, Central, and Mississippi flyways for winter (Bellrose 1980). Additionally, some populations in Alaska are thought to be non-migratory, such as in the Aleutian Islands (Kulikova *et al.* 2005). Satellite telemetry tracking of mallards from wintering sites in Japan (Yamaguchi *et al.* 2008) observed birds moving up to 2,000 km to likely breeding areas in Russia, although not in the direction of Alaska as noted in northern pintails (Yamaguchi *et al.* 2010). Band-recovery data of mallards that winter in Japan mirrors the pattern observed with satellite telemetry with birds moving primarily to the north east (Yamashina Institute for Ornithology 2002). Band-recovery data from mallards banded in North America have not previously been examined for trans-hemispheric movements.

Mallards have some of the highest levels of LPAI and HPAI prevalence among waterfowl species (Munster *et al.* 2007; Ip *et al.* 2008; Kou *et al.* 2009). Studies on the effectiveness of mallards as long distances virus carriers, however, are incongruent. One HPAI H5N1 challenge study suggests they are more likely to act as long distance vectors for this strain than several other common duck species (Keawcharoen *et al.* 2008), whereas recaptures of wild mallards at staging areas suggest shedding times for LPAI are too low to make mallards an effective vector across continental and intercontinental scales (Latorre-Margalef *et al.* 2009). HPAI infections in mallards appear to be largely influenced by previous exposure to LPAI. Sero-positive individuals mostly showed no clinical disease and reduced cloacal excretion and shedding time post H5N1 infection (Fereidouni *et al.* 2009), providing arguments both for and against a potentially healthy carrier of HPAI in mallards. While these studies investigate important aspects of LPAI and HPAI ecology within a single host, they do not resolve complex issues such as immigration of foreign viruses to a mixed host community, and the relationship of viruses shared by sympatric host species.

Our overall objectives in this study were to assess the migratory connectivity of mallards between Eurasia and North America using band-recovery data, examine the degree of breeding and post-breeding sympatry of mallards and northern pintails in Alaska with aerial survey and banding data, and compare genomic diversity of LPAI viruses in mallards of Alaska to those from recent investigations of the northern pintail (Koehler *et al.* 2008; Ramey *et al.* 2010a) using both phylogenetic and genotypic methods. These data comparisons allow us to evaluate whether mallards in Alaska, that may have little or no migratory connection to Eurasia, show lower levels of Asian origin LPAI viral genes than the northern pintail, a species that moves between East Asia and North America. Furthermore, we address the frequency of apparent interspecific transmission by investigating viral similarity between waterfowl hosts using comparisons of subtype distributions and genomic homology.

Materials and Methods

Migratory tendency

To compare incidence of trans-hemispheric band recoveries of mallards to those observed in northern pintails by Flint *et al.* (2009), we examined data from the U.S. Geological Survey's Bird Banding Laboratory. We summarized the number of mallards of known sex banded in winter (November to March) between 1951 and 2005 within North America and recovered from Asia and Russia between 1951 and 2010. Captive-reared or sick individuals were excluded from the banding data set.

Breeding ground sympatry

To assess the degree of breeding ground sympatry of mallards and northern pintails in Alaska, we used data from the North American Waterfowl Breeding Population and Habitat Survey (U.S. Fish and Wildlife Service 2010). During the survey biologists count waterfowl while flying fixed-wing aircraft along established line transects in nesting areas. In Alaska approximately 2269 km² of potential nesting habitat across 11 geographic strata (Figure 1) was sampled each spring (Hodges et al. 1996). Each transect was divided into 13 to 26-km segments. Using survey data collected from 2003–2009 we assessed the proportion of segments on which both mallards and northern pintails were observed. We also averaged numbers of mallards and northern pintails observed on each segment across years and used a Pearson correlation analysis to assess strength of the relationship between mean abundance for each species. To temporally extend the assessment of breeding ground sympatry, we used late summer banding data from five study sites in Alaska where mallards and northern pintails are routinely captured in baited swim-in traps. We used long-term banding data from multiple areas, but only from the month of August and only if the banding sites were consistently within a single 10 degree latitude/longitude block. Banding sites were located in the following areas of Alaska: Yukon Flats National Wildlife Refuge (NWR), Koyukuk NWR, Yukon Delta NWR, and the Minto Flats and Susitna Flats State Game Refuges (Figure 1).

Virus sampling, isolation, and sequencing

Totals of 274, 273, and 161 cloacal swab samples were collected in 2006, 2007, and 2008, respectively, from live and hunter-shot mallards throughout Alaska. Samples were collected during spring and fall months on the Minto Flats and Susitna Flats State Game Refuges, the Yukon Delta National Wildlife Refuge (NWR), Izembek NWR (Alaska Peninsula), and the Mendenhall Wetlands State Game Refuge (southeast Alaska; Figure 1). Swab samples were subjected to viral isolation in embryonated eggs as previously described (Senne 1989). A total of 39 LPAI virus isolates were subjected to genomic sequencing. Viral RNA was extracted from allantoic fluid and amplified via RT PCR as described previously (Ramey *et al.* 2010a). PCR was carried out using primers of Zou (1997), Hoffmann *et al.* (2001), Phipps *et al.* (2004), Bragstad *et al.* (2005), Obenauer *et al.* (2006), Li *et al.* (2007), Koehler *et al.* (2008) or primers specifically designed for this study (Table S1). PCR products were purified and sequenced as described in Ramey *et al.* (2010a).

We obtained complete sequence data for 299 (out of a possible 312) gene segments from 39 viral isolates. However, we also included contemporary (2000 or after) LPAI virus sequences from mallards sampled in the interior of Alaska that were available on the GenBank database at the National Center for Biotechnology Information (Bao *et al.* 2008) for the following gene segments: M ($n = 20$), NP ($n = 21$), NS ($n = 22$), PA ($n = 20$), PB1 ($n = 20$), PB2 ($n = 21$), HA ($n = 17$), and NA ($n = 21$). The inclusion of these sequences increased the total number of sequences analyzed to 461 from a total of 62 isolates. Three of these sequences, all from a single mallard isolate (A/Mallard/AK/3211/2002), were previously published by Spackman *et al.* (2005) for the M, NS and NP gene segments. The total number of nucleotides analyzed for each RNA gene segment was: M (927), NP (1376), NS (642), PA (2137), PB1 (2221), PB2 (2217), HA (range = 1633–1689), and NA (range = 1331–1407). All sequences produced in this study were assembled and edited with Sequencher version 4.7 (Gene Codes Corp., Ann Arbor, MI). Chromatograms that contained multiple peaks for many or all nucleotides were considered co-infections and excluded from analysis (17 gene segments from 3 isolates). We compared subtype distributions of mallards (excluding those from GenBank) to those observed among northern pintails by Ramey *et al.* (2010a) using a chi-square (X^2) test of homogeneity. Prior to this test, we excluded all but one viral isolate of the same subtype that occurred within ducks sampled in the same

location and on the same day. The exact P value for the X^2 statistic was calculated using a randomized sampling distribution (1000 replicates) of our subtype data. GenBank accession numbers for gene segments obtained in this study are as follows: HM193551–HM193849.

Quantifying intercontinental reassortment

We used phylogenetic methods similar to that of Koehler *et al.* (2008) and Ramey *et al.* (2010a) to assign gene segments from each Alaska isolate into either Eurasian or North American clades. Sets of reference LPAI viral sequences (Table S2) that originated from either Eurasian or North American sampling locations were obtained from GenBank. At least nine reference sequences from duck species from across the United States and Canada, isolated between 2000 and 2009, were selected for each gene segment to represent North American lineages. Sequence information for all LPAI gene segments isolated from duck species from East Asia (China, Japan, and South Korea) between 2000 and 2009 was selected to represent Asian lineages: M ($n = 165$), NP ($n = 71$), NS ($n = 105$), PA ($n = 41$), PB1 ($n = 44$), PB2 ($n = 40$), HA ($n = 47$), and NA ($n = 185$). Additional reference samples from previous LPAI genomic analyses (Krauss *et al.* 2007; Dugan *et al.* 2008; Bahl *et al.* 2009; Ramey *et al.* 2010a) were also included to represent major phylogenetic clades. Sequences were aligned using Sequencher version 4.7 (Gene Codes Corp., Ann Arbor, MI).

To determine levels of support for hemispheric clades of LPAI viral gene segments and characterize geographic affinity of mallard LPAI sequences, we used MEGA version 4.0.2 (Tamura *et al.* 2007) and MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). In MEGA, we used the Maximum Composite Likelihood model for nucleotide sequences with 10,000 bootstrap replicates to generate neighbor-joining (NJ) trees. In MrBayes, each analysis was run for at least 1×10^6 generations (or until the standard deviation of split frequencies was < 0.01) using four heated chains following a burn-in of 5000 generations. Average posterior probabilities of the 50% majority rule consensus tree topologies were estimated using a sampling of likelihood parameters every 100 generations. Trees were visualized using FigTree (Rambaut & Drummond 2007).

Upon creation of phylogenetic trees, branch placements of sequences for each gene segment were compared relative to each other and reference samples. Sequences from isolates collected in North America that were nested within Asian clades were considered intercontinental reassortment events. As closely related reassortment events could be representative of subsequent spread of foreign origin lineages after initial introduction (Krauss *et al.* 2007), sequences that formed monophyletic or near monophyletic clades within Asian origin lineages were assumed to be the result of a single outsider event.

Identifying homologous viruses between host species

Full genomic sequence data was acquired from 74 northern pintail isolates (Koehler *et al.* 2008; Ramey *et al.* 2010a), 17 mallard isolates from Alaska available on GenBank, and 39 mallard isolates from this study. To determine levels of genomic homology between viruses from mallards and northern pintails, all eight gene segments from each isolate were assigned alleles using FluGenome (Lu *et al.* 2007). Alleles for gene segments were determined using the BLAST method with default settings for coverage ($>95\%$) and identity ($>95\%$). Genotypes of each virus were then created by concatenating alleles for each gene segment. Viruses from mallard and northern pintail isolates with identical genotypes that were collected within the same year were selected for an analysis of genetic homology between host species using pairwise distances (PWD) in PAUP 4.0b (Swofford 1998). Strains with PWD values <0.01 at all eight gene segments were considered homologous.

Results

Despite the banding of nearly 1.1 million male and 650,000 female mallards in North America during winter months between 1951 and 2005, only two recoveries are known from Eurasia. One bird was recovered on the Lena River Delta, Russia (N 71.750°, E 129.083°) and the other near Krest Bay, Russia (Dzubin 1962). Additionally, there are no recoveries in Eurasia of the > 15,000 mallards banded in Alaska during summer months since 1951. In contrast, there are eight recoveries in Eurasia from the > 57,000 northern pintails banded in Alaska during summer months since 1951.

Approximately 222 aerial survey segments were flown in Alaska each year from 2003 to 2009. Both northern pintails and mallards were observed in all sample strata in all years. Across years, mallards were observed on 86% of transect segments, and on most (76%) of those segments northern pintails were also observed. Mean abundance of mallards was positively correlated ($r = 0.21$, $P < 0.001$) with average numbers of northern pintails observed on a transect segment. August banding data revealed that northern pintails are more often encountered in northern and western portions of Alaska (Figure 1). Near equal numbers of the two species were captured in southcentral Alaska (Susitna Flats).

We observed eight HA and eight NA LPAI virus subtypes among the mallard isolates obtained in this study ($n = 39$; Figure 2). The most common subtype combination was H4N6 (35.9% of isolates) followed by H3N2 (17.9%) H3N8 (12.8%), and H7N3 (7.7%). All other subtype combinations (H1N1, H2N3, H3N6, H7N2, H10N7, H11N9, and H12N5) accounted for less than 25.6% of the total (2.6–5.1% each). There was no significant difference between the subtype combinations of mallards and northern pintails sampled in Alaska from 2006–2008 ($X^2 = 20.7$, $P = 0.35$).

Phylogenetic analysis of all 461 mallard sequences to determine hemispheric affinities revealed 10 genes from nine isolates (14.5% of all 62 isolates) in three gene segments (HA, H3, NP, and PA) that were more closely related to Eurasian than North American clades (Figure 3). NP reassortment events were observed in isolates from samples collected in each year of the study (2006–2008) and in all sampling locations except the Yukon-Kuskokwim Delta. Only one isolate (A/mallard/interior Alaska/7MP0709/2007/H3N8) contained >1 gene segment of Eurasian origin (one each from the H3 and NP). After excluding all but one closely related strain that formed a clade within strains from the opposing hemisphere (Krauss *et al.* 2007), the total number of independent reassortment events was reduced to three (0.65% of 461 gene segments), which occurred in the PA, H3 and NP gene segments. Of these three, the only reassortment event independent of those previously identified in Alaska waterfowl by Ramey *et al.* (2010a) was the PA sequence of isolate A/mallard/Alaska/44430-056/2008/H11N9 (Figure 4). The remaining reassortment events grouped within the Eurasian Group 1 clades of the NP and H3 as described in Ramey *et al.* (2010a).

Genotypic analysis of 126 northern pintail and mallard LPAI viruses from Alaska revealed 50 unique complete genotypes. A total of 36 genotypes occurred in northern pintails and 24 in mallards. Ten genotypes were shared between species. Eight genotypes were observed in both northern pintail and mallard isolates within the same year, which were subsequently investigated for evidence of interspecies viral transmission. Of the 50 resulting pairwise comparisons between mallard and northern pintail viruses of identical genotype, seven were homologous (PWD <0.01) across all eight gene segments (Table S3). The range of geographic distances among collection locations for these “identical” viruses was 2.1 to 960.3 km and came from isolates collected 4 to 32 days apart.

Discussion

Inter-hemispheric connectivity

Band-recovery data suggest no regular migratory connectivity between North American and Eurasian populations of mallards. Nearly ten times more mallards (~1.7 million) were banded in North America (outside of Alaska) during winter than northern pintails (~173,000) over the same time period (Flint *et al.* 2009), yet there are 65 times more recoveries of northern pintails in Russia ($n = 130$) than mallards ($n = 2$). Although inferences of migratory patterns based solely on banding data can be biased by both recovery and reporting rates of metal leg bands, the pattern of low inter-continental movements was also observed with mitochondrial DNA across Eurasia and North America (Kulikova *et al.* 2005) and logically follows from satellite telemetry data from Japan (Yamaguchi *et al.* 2008). In concordance with these data, we observed a smaller proportion of trans-hemispheric reassortment events in mallards (0.65%) in comparison to northern pintails (3.5%) over the same time period. Similarly, reassortment events were detected in 17.9% of the 39 Alaska mallard isolates obtained in this study and 8.7% of the 23 Alaska mallard isolates from GenBank. These proportions are well below the average of 35% of isolates from northern pintails in Alaska (Ramey *et al.* 2010a). Thus, we conclude that the rate of intercontinental migratory movements and occurrence of Asian reassortment events within North American waterfowl hosts are correlated.

Reassortment events in mallards were observed in three gene segments: NP, H3, and PA. Interestingly, Ramey *et al.* (2010a) observed most Eurasian sequences in these same three genes among LPAI viruses isolated from northern pintails. All of the Eurasian NP and H3 genes in mallards occurred within the Group 1 lineages for both of these segments as identified in figures 4 and 5 of Ramey *et al.* (2010a). Additionally, for both mallards and northern pintails, reassortment events in the NP and H3 genes were observed in >1 year of sampling, suggesting possible establishment of these lineages among multiple waterfowl hosts in Alaska or in the environment. In *post hoc* analyses of all contemporary North American waterfowl NP and H3 sequences available on GenBank, we observed additional isolates with similar genetic characteristics for these genes. Two NP sequences (A/mallard/Maryland/802/2007/H5N1 (LPAI) and A/northern shoveler/California/HKWF1026/2007/H7N3) and four H3 sequences (A/green-winged teal/Alaska/4/2007, A/American wigeon/Alaska/1/2007, A/northern shoveler/Alaska/7MP1708/2007, and A/northern shoveler/Alaska/7MP1606/2007) clustered within the Eurasian Group 1 clades for these genes as noted by Ramey *et al.* (2010a). Whether the presence of Eurasian NP and H3 lineages in these additional avian hosts represents transmission from intercontinental migrants such as northern pintails or direct connection with Eurasia remains a matter of speculation. The prevalence of Eurasian origin genes for the NP, H3, and PA segments in Alaska, as compared to elsewhere in North America, suggests that the geographic position of Alaska in relation to Eurasia influences the genomic diversity of LPAI viruses of avian hosts that migrate to Alaska from Eurasia and, to a lesser degree, those hosts that remain within the North American continent for breeding and wintering.

Sympatry and interspecific viral exchange

Because viral transmission occurs between hosts via fecal and oral routes in water and sediments (Stallknecht & Brown 2007) and potentially through environmental reservoirs where viruses can persist (Brebner *et al.* 2009), shared breeding and staging areas of multiple waterbird species likely contribute to common LPAI genomic diversity. Aerial survey and banding data shows that mallards and northern pintails were sympatric across breeding grounds of Alaska, but densities vary by location. Both species were observed on all 11 survey strata in Alaska, but mallards were seen in higher densities on interior survey strata

(Hodges *et al.* 1996). In contrast, northern pintails were more abundant in strata located in the western and northern portions of the state. August banding data suggest a similar pattern, with more northern pintails captured in northern and western areas.

The highest frequencies of Eurasian gene segments in northern pintail isolates came from the Alaska Peninsula (70%) and Yukon-Kuskokwim Delta (50%) both in western Alaska (Ramey *et al.* 2010a, table 2). We suggest that these higher frequencies may be, at least partially, dependent on the closer proximity of these areas to Asia. Few mallard isolates were available from these western Alaska localities (Alaska Peninsula, $n = 1$; Yukon-Kuskokwim Delta, $n = 2$) likely due to their lower densities in these regions. If frequency of Eurasian genes is spatially dependent, the difference in frequencies observed between mallards and northern pintails could be explained by this disparate species distribution. Some evidence for this is suggested by the fact that of the three western Alaska mallard samples, the single Alaska Peninsula isolate contained a Eurasian-lineage NP gene.

We observed all HA and NA subtypes previously reported in northern pintails (Ramey *et al.* 2010a) except for H5 and H6. Additionally, Ramey *et al.* (2010a) found eight subtype combinations (H1N3, H1N8, H3N1, H5N9, H6N1, H6N2, H6N4, and H6N8) that were not detected in our study. Two subtype combinations (H3N2 and H7N2) found in mallard isolates were not previously identified in Alaska pintails. The finding of unique subtypes and combinations may reflect differences in size and distribution of samples and suggests that subtypes or combinations are not dependent on host species (Chen & Holmes 2009). Furthermore, the distribution of HA and NA subtype combinations did not vary significantly between mallards and northern pintails in Alaska. However, the predominant subtypes observed among mallards and northern pintails in Alaska (H3, H4, N6 and N8) were also the most common in long-term studies of North American waterfowl species (Sharp *et al.* 1993).

The high level of genetic similarity between mallard and northern pintail isolates using a genotypic approach indicates that these species can share identical (>99.5%) viruses in the wild. Of the seven genotype comparisons determined to be homologous across all eight gene segments, six were made from mallard and northern pintail isolates collected less than two weeks apart (4–12 days) and one 32 days apart. None of the fifty comparisons involved isolates collected on the same day or from the same location. Our definition of an identical virus (>99% homologous across all eight segments) could be viewed as too stringent and certainly other highly similar viruses would be observed within and between species if this level of homology was reduced. As additional full LPAI genomes are sequenced from other migratory species in Alaska, future investigations could remove the homology cut-off of 99% we imposed to examine how levels of genetic similarity change across landscapes, species, and years.

Conclusions

Our comparison of genomic diversity in LPAI viruses isolated within the same time frame from mallard and northern pintail ducks in Alaska suggests that migratory connectivity to Eurasian viral gene pools, as well as LPAI prevalence, should be considered when determining suitable host species to sample for introduction of Asian H5N1 HPAI. Mallards were not initially included on the list of high priority species for surveillance sampling in Alaska because of their lack of migratory connectivity to Eurasia, but were subsequently added as a “species of concern” in Alaska (USFWS & USGS 2009) because of their high prevalence of avian influenza, including the HPAI H5N1 virus (Kou *et al.* 2009). There is strong evidence that wild sympatric mallards and northern pintails host homologous influenza gene segments and often near identical (>99.5%) influenza genomes. Thus, the high prevalence of avian influenza in mallards coupled with their tendency to share habitats

with a highly trans-hemispheric species suggests they could serve as an important wild sentinel species in Alaska and elsewhere for HPAI viruses. Indeed, some have argued for use of captive sentinel mallards in areas where migratory birds are present as part of a surveillance strategy for HPAI viruses (Globig *et al.* 2009).

The key risk is that viruses such as HPAI H5N1 may be introduced to North America by a single species then spread within and among species. Our data suggest that mallards are not transferring Asian viruses to North America, but they are being infected with Asian origin viruses through interspecific transfer. As such, the proposed pattern of HPAI H5N1 dispersal among continents appears to be occurring among LPAI viruses. This supports the general strategy for HPAI surveillance sampling of targeting areas with known migratory connections to areas where HPAI H5N1 currently exists, including both migrant and sentinel species, and considering interspecific transfer as mechanism for virus dispersal to other areas.

The use of phylogenetic analysis in genomic assessments of LPAI viruses from migratory birds is an integral part of a methodology for targeting species and regions for sampling to detect not only HPAI, but other avian-borne pathogens as well (Pearce *et al.* 2009). In Alaska, a greater number of reassortment events – combining Eurasian and North American influenza segments – are being discovered among LPAI virus isolates obtained from wild birds than was expected (Ramey *et al.* 2010a, b). This realization suggests a pattern of ‘dilution by distance’ as similar species sampled at greater distances from Alaska exhibit LPAI viruses with little or no evidence of trans-hemispheric reassortment. The commonality of this pattern, seen in this study for influenza, may be tested in future genomic surveys of other wildlife pathogens in Alaska in areas where different disease populations intermix. Additionally, disease genetics can help illustrate the complex network of environmental and host factors that contribute to pathogen emergence, persistence and spread (Megali *et al.* 2010; Njabo *et al.* 2010).

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Figure 1.

Map of Alaska showing U.S. Fish and Wildlife Service aerial breeding pair survey strata (shaded regions), bar charts of the total number of mallards and pintails banded in August across multiple years, and distribution of sampling locations for mallard swab samples that were either negative (solid circles) or positive (open circles) for low pathogenic avian influenza (LPAI). Numbers within open circles represent the total LPAI isolates characterized via phylogenetic and genotypic methods (see text).

Figure 2. Distribution of hemagglutinin (HA) and neuraminidase (NA) subtypes observed among 39 low pathogenic avian influenza viruses isolated from mallards in Alaska from 2006–2008.

Figure 3. Neighbor-joining phylograms for internal gene segments of avian influenza comparing isolated viruses from Alaskan mallards to Eurasian (shaded) and North American (unshaded) reference samples. Inferred inter-hemispheric gene movements within the PA ($n = 1$) and NP ($n = 8$) gene segments are shown (circled). Bootstrap and posterior probability values are given at major clade divisions that define North American lineages (filled circle).

Figure 4. Phylogram showing the position of the PA gene segment within the Eurasian lineage for HM193755 A/mallard/Alaska/44430-056/2008/H11N9 (labeled 3) as distinct from two reassortment events (labeled 1 and 2) in Alaska northern pintails identified by Ramey *et al.* (2010).