

Migration strategy affects avian influenza dynamics in mallards (*Anas platyrhynchos*)

NICHOLA J. HILL,*¶** JOHN Y. TAKEKAWA,* JOSHUA T. ACKERMAN,† KEITH A. HOBSON,‡ GARTH HERRING,† CAROL J. CARDONA,§ JONATHAN A. RUNSTADLER¶ and WALTER M. BOYCE**¹

*U.S. Geological Survey, Western Ecological Research Center, San Francisco Bay Estuary Field Station, 505 Azuar Drive, Vallejo, CA, 94592, USA, ¶Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA, 02139, USA, **Wildlife Health Center, School of Veterinary Medicine, University of California, One Shields Ave, Davis, CA, 95616, USA, †U.S. Geological Survey, Western Ecological Research Center, University of California, Davis Field Station, One Shields Ave, Davis, CA, 95616, USA, ‡Environment Canada, 11 Innovation Rd, Saskatoon, SK, S7N 3H5, Canada, §College of Veterinary Medicine, University of Minnesota, 1971 Commonwealth Ave, St Paul, MN, 55108, USA

Abstract

Studies of pathogen transmission typically overlook that wildlife hosts can include both migrant and resident populations when attempting to model circulation. Through the application of stable isotopes in flight feathers, we estimated the migration strategy of mallards (*Anas platyrhynchos*) occurring on California wintering grounds. Our study demonstrates that mallards— a principal host of avian influenza virus (AIV) in nature, contribute differently to virus gene flow depending on migration strategy. No difference in AIV prevalence was detected between resident (9.6%), intermediate-distance (9.6%) and long-distance migrants (7.4%). Viral diversity among the three groups was also comparable, possibly owing to viral pool mixing when birds converge at wetlands during winter. However, migrants and residents contributed differently to the virus gene pool at wintering wetlands. Migrants introduced virus from northern breeding grounds (Alaska and the NW Pacific Rim) into the wintering population, facilitating gene flow at continental scales, but circulation of imported virus appeared to be limited. In contrast, resident mallards acted as AIV reservoirs facilitating year-round circulation of limited subtypes (i.e. H5N2) at lower latitudes. This study supports a model of virus exchange in temperate regions driven by the convergence of wild birds with separate geographic origins and exposure histories.

Keywords: animal migration, Pacific Flyway, pathogen transmission, stable isotopes, waterfowl

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Introduction

Animals using seasonal habitats migrate to avoid food scarcity, seek protected habitats for breeding and the physiological benefits of exploiting an optimal climate (Ashmole 1963). Avoidance of disease has also been proposed as a factor in the evolution of migration (Piersma 1997; Altizer *et al.* 2011). To minimize exposure to disease, animals may migrate from regions that

support high pathogen loads to Arctic or marine habitats that are less hospitable for pathogens (Piersma 1997; Mendes *et al.* 2005). Migrants may also avoid infection by moving away from densely populated habitats that are conducive to disease outbreaks (Alerstam *et al.* 2003). On the other hand, animal migration connects geographically isolated populations, potentially assisting the movement of disease. Birds are a prime example having been linked to the global expansion of West Nile virus (Rappole *et al.* 2000), Lyme disease (Ogden *et al.* 2008) and avian influenza virus or 'AIV' (Newman *et al.* 2012). Therefore, migration can be a mechanism for both limiting pathogen exposure and

¹Correspondence: Walter M. Boyce, Fax: 530 752 3318; E-mail: wboyce@ucdavis.edu

facilitating the movement of pathogens between populations.

While migration is a common strategy among species using seasonal habitats, there is high variation in the propensity to migrate. Many bird species show a combination of migration strategies including residency, intermediate-distance and long-distance migration (Alerstam *et al.* 2003). Residents are the most sedentary individuals on the spectrum, remaining in habitats characterized by mild temperatures with foraging opportunities throughout the year, or moving locally between wintering and breeding or moulting grounds. By providing a stable host population, residents may play an important role in the dynamics of pathogen transmission. Studies of elk (*Cervus elaphus*) indicate that high densities of non-migrating individuals can lead to increased local transmission of parasites (Hines *et al.* 2007). Permanent year-round populations that have prolonged exposure to pathogens may facilitate transmission by acting as reservoirs (Altizer *et al.* 2011). In some cases, residency may be triggered by human practices such as agriculture that provides an aseasonal food supply, with implications for spillover of pathogens to humans or domestic animals (Ewers *et al.* 2009; Gaukler *et al.* 2009).

Defining the migration strategy of wild birds may shed light on the maintenance and movement of AIV along migratory flyways. Wild birds, particularly species from aquatic habitats, are thought to be the reservoir for AIV (Webster *et al.* 1992). Decades of surveillance has aimed at clarifying the role of wild birds in transmission of the virus (Olsen *et al.* 2006), yet few studies consider that migrant and resident populations may co-exist. Migrant and resident birds differ in their geographic origin, with consequences on body condition, immune defences and pathogen exposure (Moller & Erritzoe 1998; Yohannes *et al.* 2008), and are predicted to play different roles in AIV dynamics. Resident birds may act as a year-round reservoir, but support for this is incongruous (Stallknecht *et al.* 1990; Hanson *et al.* 2005; Ferro *et al.* 2010). Mottled ducks (*Anas fulvigula*) are among the few resident duck species that have been investigated; however, small sample sizes ($n < 100$) have hindered attempts to accurately assess prevalence through the annual cycle. Migrants, on the other hand, have been implicated in the spread of AIV between breeding and wintering areas, facilitated by relay transmission at stopover or staging sites (Olsen *et al.* 2006).

Logistical challenges impede following migratory animals throughout the annual cycle and hinder the study of AIV and other infectious agents (Hoye *et al.* 2010). This has resulted in a lack of studies comparing migrant and resident populations of the same species

to investigate infection patterns. Measurements of naturally occurring stable isotopes in tissues of migrant animals have become powerful tools for determining the origins of a variety of birds, mammals and insects (reviewed in: Hobson 1999; Hobson & Wassenaar 2008; Rubenstein & Hobson 2004). Stable hydrogen isotopes ($\delta^2\text{H}$) are particularly useful in migratory bird studies in North America, because precipitation patterns result in predictable changes in isotopic ratios along latitudinal gradients. Mean growing season $\delta^2\text{H}$ values in precipitation are reflected in the flight feathers of birds grown at natal or moulting sites, providing an intrinsic marker of the origin of the bird (Hobson & Wassenaar 1997; Clark *et al.* 2006). This technique holds promise for improving the design of studies seeking to identify migrant and resident birds and their roles in pathogen dynamics (Gunnarsson *et al.* 2012).

The mallard (*Anas platyrhynchos*) has been reported as a principal host of AIV in North America (Hinshaw *et al.* 1980, 1986; Alfonso *et al.* 1995; Ip *et al.* 2008) and Europe (Munster *et al.* 2007). Mallards have migrant and resident populations across their Holarctic distribution and are therefore a model species to compare patterns of AIV infection according to migration strategy. Within the Pacific Flyway of North America, mallards breed from Mexico to Alaska. The Central Valley of California is a major over-wintering destination for Pacific Flyway mallards (Gilmer *et al.* 1982). However, there is also a large local-breeding population of mallards within the Central Valley, with 60–80% of mallards harvested in California estimated to be locally born (Zuwerink 2001). Benefits of residency may include higher rates of nest success and potentially earlier recruitment of young than counterparts that breed in Alaska (McLandress *et al.* 1996). Thus, the Central Valley provides a unique system to examine how AIV transmission may be facilitated by migrant and resident hosts.

The specific aims of our study were to determine whether (i) migration status is linked with prevalence and diversity of AIV, (ii) migrants introduce virus to wintering grounds and (iii) residents act as an AIV reservoir on the wintering grounds. Our study took a cross-disciplinary approach by applying stable isotopes and phylogenetic tools to clarify how migration ecology influences AIV transmission.

Materials and Methods

Sample populations

Mallards were collected during the winter at Sacramento National Wildlife Refuge (39.4079°N, 122.1591°

W) and Conaway Ranch Duck Club (38.6485°N, 121.6673°W), approximately 120 km apart in the Central Valley. The Central Valley supports 60% of the migratory waterfowl in the Pacific Flyway during the winter and nearly 20% of waterfowl in North America (Gilmer *et al.* 1982). Hunter-killed mallards were collected for sampling when hunters brought harvested birds to check stations at Sacramento Refuge and Conaway. Sampling consisted of cloacal swabbing and collection of a primary feather and occurred up to three times per week between 18 October 2008 and 25 January 2009.

Mallards sampled during the summer breeding period provided a 'known-source' population and validated the stable hydrogen isotope ($\delta^2\text{H}$) signature at major breeding grounds. Feathers were collected from individuals at four breeding locations along the Pacific Flyway 1) Minto Flats State Game Refuge, Alaska (64.9105°N, 151.1984°W); 2) Lower Klamath National Wildlife Refuge, California (41.9724°N, 121.7242°W); 3) Upper Butte Basin, California (39.383498°N, 121.88043°W); and 4) Suisun Marsh, California (38.1635°N, 121.9703°W). Mallards were live-captured with baited swim-in traps constructed of 1" by 2" wire mesh during the post-breeding moult, between 21 July and 26 August 2009. Sampling for AIV during summer was limited to the three California breeding grounds.

Ethics statement

This study was carried out in strict accordance with the recommendations of the Ornithological Council 'Guidelines to the Use of Wild Birds in Research'. Procedures for capture and handling were approved by the University of California, Davis Animal Care and Use Committee (Protocol 16120).

Collection of samples

The sex of all mallards was determined, and morphometric measurements (bill length, flat wing and short tarsus) were collected. The right wing was removed and stored until aged on the basis of plumage by trained waterfowl biologists (Pacific Flyway Wing Bee) as either hatch year (HY) or after hatch year (AHY). To collect AIV samples, a polyester-tipped swab (MicroPur™, PurFybr Inc., Munster, IN, USA) was inserted into the cloaca of the bird. The swab tip was removed and preserved in cryovial tubes (Remel Inc., Lenexa, KS, USA) containing viral transport media (VTM). Samples were kept on ice for <8 h or transferred to a liquid nitrogen vapour shipper before storage in a -80 °C freezer prior to laboratory analyses.

Testing for AIV

Samples were screened for AIV by virus isolation in embryonating chicken eggs followed by testing for haemagglutinating activity with red blood cells from chickens. In brief, 150 µL of VTM was inoculated into the allantoic cavity of 9- to 11-day-old embryonating SPF chicken eggs (SPAFAS, Charles River) and incubated at 37.5 °C for 6 days or until embryo death, as detected by daily candling. Three passages were attempted to determine the presence of virus. The virus allantoic fluid (VAF) from live embryos was tested for haemagglutinating activity with chicken red blood cells following standard methods (Swayne *et al.* 1998). We extracted RNA from VAF harvested from all dead embryos and the haemagglutinating VAF from live embryos using the MagMAX-96 Viral Isolation Kit (Ambion Inc., Austin, TX, USA). We generated cDNA using the M-MLV as per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and then tested for AIV using real-time PCR targeting the matrix gene (Spackman *et al.* 2003). Whole-genome sequencing was performed at the J. Craig Venter Institute (JCVI) in Rockville, MD (Nelson *et al.* 2009), and all sequences generated have been assigned Genbank accession numbers (Table S1).

Stable isotope analysis

Flight feathers of HY mallards are grown on the breeding grounds before fledging, and flight feathers of AHY are completely moulted near breeding regions immediately following the nesting season (Young & Boag 1981; Klint 1982). Once grown, feathers remain isotopically inert, reflecting the signature of the geographic region in which they were grown. Therefore, we used feather isotopic signatures in breeders to discriminate among breeding locations and then assign wintering mallards to a migrant or resident status.

We conducted stable isotope analysis on a total of 525 feathers from wintering mallards. This included all birds that tested positive for AIV ($n = 47$) and the remaining feathers were randomly sub-sampled from the 1420 collected. Months were similarly represented; October ($n = 129$), November ($n = 117$), December ($n = 126$) and January ($n = 153$). A total of 121 feathers from known-source breeding mallards in California and Alaska were also collected during the summer 2009 and analysed to describe the range of isotopic values broadly associated with breeding locations.

A primary feather from each wing was cleaned of surface oils using a hexane-acetone solvent wash and air-dried. Vanes were cut from the same position on each feather and weighed using a microbalance transferred into isotope grade silver capsules (Costech

Analytical Technologies, Valencia, CA, USA) and analysed at the Stable Isotope Facility, University of California, Davis, CA, USA.

For $\delta^2\text{H}$ analyses, samples were allowed to equilibrate with laboratory ambient water vapour along with keratin standards for 96 h as described by Wassenaar & Hobson (2003). Stable hydrogen isotope measurements were performed using a HEKAtech HT-O AnalyzerTM (HEKAtech, Wegberg, Germany) interfaced with a PDZ Europa 20–20 isotope-ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Final $\delta^2\text{H}$ values were expressed relative to the Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation scale.

For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses, measurements were performed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope-ratio mass spectrometer. Samples were combusted at 1000 °C in a reactor packed with chromium oxide and silvered copper oxide. Final $\delta^{13}\text{C}$ values were expressed relative to the Vienna PeeDee Belemnite, and $\delta^{15}\text{N}$ values were expressed relative to air.

Samples were interspersed with duplicates (per 10 samples) to ensure repeatability of isotope results, separately for wintering and summer mallards. For further details of isotope methods and laboratory standards used for calibration, refer to <http://stableisotopefacility.ucdavis.edu/>.

Establishing mallard origins

We considered a 3-isotope ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^2\text{H}$) likelihood of assignment model following the approach of Royle & Rubenstein (2004). However, we concluded that feather $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ had poor ability to differentiate among terrestrial-freshwater sources of mallards throughout their range along the western seaboard of North America. Instead, feather $\delta^2\text{H}$ showed good segregation among the potential sources considered (Fig. 1). We examined the distributions of feather $\delta^2\text{H}$ values of 121 known-source mallards collected at four major breeding grounds and categorized these sites by thresholds associated with extreme feather $\delta^2\text{H}$ values (i.e. we considered the total range of our samples). Known-source mallards were not sampled during the same precipitation year as wintering mallards (2008–2009), and therefore, feathers collected in the summer only provided approximate feather isotope values expected from the breeding grounds. Mallards were assigned to one of three migratory origins: residents (RES), intermediate-distance migrants (IDM) or long-distance migrants (LDM). In addition, mallards sampled during the breeding season in California (Jul–Aug 2009) were classified BRE. This population included both locally born HY and AHY birds ($n = 371$).

Statistical analyses

To test whether AIV prevalence of wintering mallards varied according to age (HY, AHY) and migration status (RES, IDM, LDM), a binomial logistic model (link function = logit) for main effects was constructed, where the dependent variable was AIV infection status. Biologically relevant two-way interactions were included in the model: migration status and age, and migration status and collection date. Collection date was applied as a covariate for models. When significant differences were detected, a pairwise comparison of estimated marginal means was run to identify which variables were different from one another, applying a Bonferroni correction.

To relate changes in body condition to AIV infection, we conducted a principal component analysis (PCA) to determine which morphometric measure (flat wing length, bill length and short tarsus) best described variation in structural body size. Principal component one (Eigenvalue = 1.936, proportion = 0.646) indicated correlation between the three morphometrics, flat wing ($\alpha = 0.606$), bill length ($\alpha = 0.553$) and short tarsus ($\alpha = 0.572$). Body mass was added as a factor to the global logistic model (described above), and flat wing was applied as a covariate to control for structural size differences in subsequent analyses. Two-way interactions between migration status and body mass were also investigated.

To assess viral diversity, the Shannon Wiener Index (Shannon 1948) was used to calculate the distribution of HA and NA subtypes infecting mallards. The formula, $H' = -\sum p_i \ln(p_i)$ was used, whereby p_i is the proportion of an individual subtype relative to the sum of all subtype proportions infecting either RES, IDM, LDM or BRE. A high H' indicates high viral diversity. Statistical analyses were conducted using SPSS VERSION 16 software for Macintosh (logistic regressions) or SAS VERSION 9.1 for PCA (principal components analysis).

Phylogenetic analyses

Phylogenetic analyses focused on the HA and NA genes because of the utility of these segments to assess rapid genomic changes likely to occur between stages of the annual cycle of mallards. We analysed 34 AIV isolates collected from RES ($n = 23$), IDM ($n = 7$) and LDM ($n = 4$) mallards in California during the winter of 2008–09. This was supplemented with 21 isolates collected from locally breeding mallards (BRE) during the summer of 2009. The 55 isolates from this study were compared with reference sequences originating from virus sampled between 2005 and 2010 from regions of migratory connectivity with California (based on band returns: Zuwerink 2001) including Alaska, British

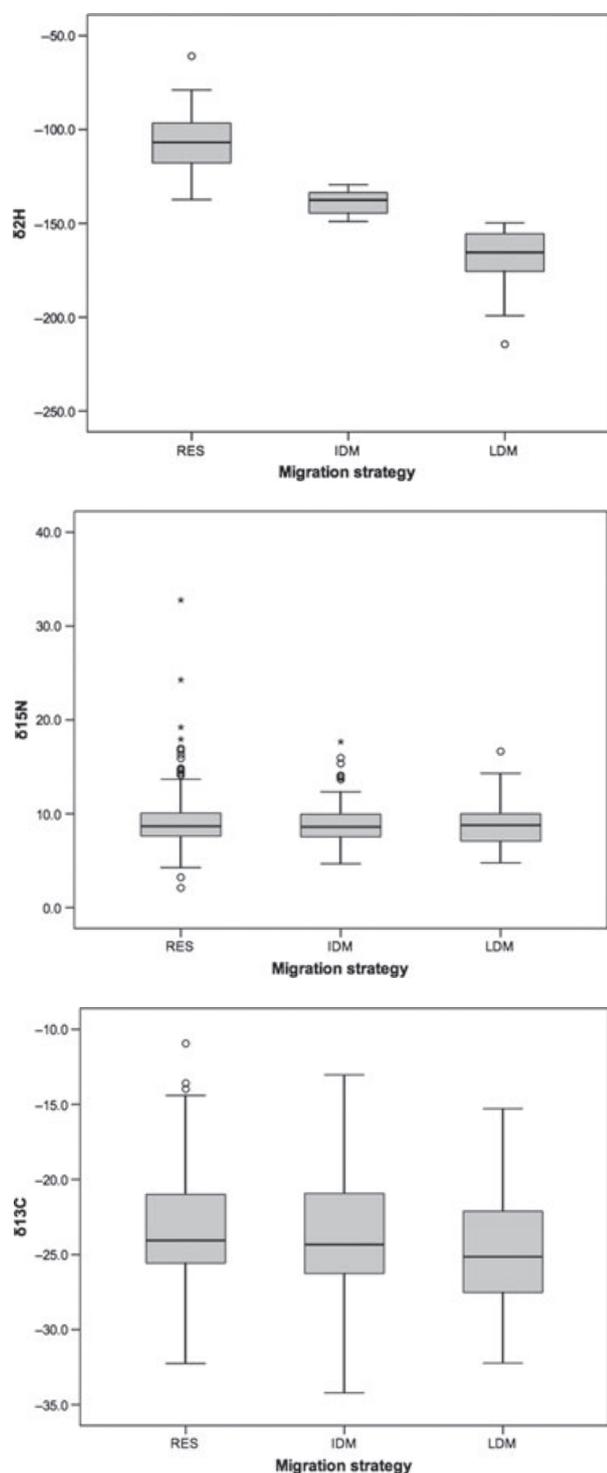


Fig. 1 Feather values of $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from mallards sampled in California, 2008–2009. Values of $\delta^2\text{H}$ were best able to differentiate among the geographic origin of resident (RES), intermediate-distance (IDM) and long-distance migrant (LDM) mallards. Horizontal lines represent median values, whiskers indicate sample minimum and maximum, and dots indicate outliers.

Columbia, Alberta, Washington and Oregon. This corresponded to the Pacific Flyway and regions of overlap with the Central Flyway. Waterfowl from seven species sympatric with mallards were included in the analysis: northern pintail (*Anas acuta*), American green-winged teal (*Anas carolinensis*), blue-winged teal (*Anas discors*), cinnamon teal (*Anas cyanoptera*), American wigeon (*Anas americana*), gadwall (*Anas strepera*) and northern shoveller (*Anas clypeata*). Searches were conducted using the Influenza Resource Database (IRD, [http://www/fludb.org](http://www.fludb.org)) based on sequences available as of 31 Jan 2012. Sequences from IRD that occurred in duplicate (100% nucleotide identity) were removed if there were multiples from a host species sampled within the same location and year. Total data set sizes for the HA segment were as follows: H1: $n = 34$ (1668 nt), H3: $n = 177$ (1632 nt), H4: $n = 90$ (1652 nt), H5: $n = 42$ (1651 nt), H6: $n = 75$ (1662 nt), H10: $n = 41$ (1619 nt), H11: $n = 29$ (1670 nt) and H12: $n = 27$ (1677 nt) and for the NA segment, N1: $n = 70$ (1416 nt), N2: $n = 60$ (1391 nts), N5: $n = 30$ (1426 nt), N6: $n = 73$ (1416 nt), N7: $n = 37$ (1453 nt), N8: $n = 119$ (1414 nt) and N9: $n = 21$ (1267 nt).

Alignments of individual segments were made using ClustalW with default parameters (Larkin *et al.* 2007) implemented in GENEIOUS 5.5.6. software (Biomatters, Ltd., New Zealand). A best-fit nucleotide substitution model was selected using MRMODELTEST 2.3 (Nylander, J. A.A. 2004. MRMODELTEST v2. Program distributed by author. Evolutionary Biology Centre, Uppsala University). For all genetic sublineages, the model with the lowest Akaike Information Criterion corrected score was the general time reversible (GTR) model accounting for estimates of invariable sites (I) and the gamma distribution parameter (G). Phylogenetic trees were generated with a Bayesian Markov chain Monte Carlo (MCMC) coalescent approach implemented in BEAST 1.6.2. (Drummond & Rambaut 2007). Sequences were time-calibrated according to sampling year. A Bayesian skyline coalescent tree prior was used in all simulations, and the starting tree was randomly generated. An optimal chain length was selected for each subtype (10–600 million generations, sampling frequency of 1000–60 000) to achieve an effective sample size >200 in TRACER 1.5 (Rambaut & Drummond 2007). Consensus trees with a 10% burnin value were generated using TREEANNOTATOR 1.6.2. and visualized in FIGTREE 1.3.1.

The branch placement of RES, IDM, LDM and BRE was assessed to determine the nearest relative (host species) and geographic origin of viruses. Sequences from RES, IDM and LDM that nested within clades from Alaska, British Columbia, Alberta, Washington and Oregon were considered long-distance imports. Conversely, sequences nested within California clades

were considered to be locally circulating or endemic to the wintering grounds.

Results

Migration status

Three migratory origins were identified among the 525 wintering mallards (i) RES birds hatched in California ($\delta^2\text{H}$: -137.3 to -60.9 , $n = 335$), (ii) IDM birds hatched in Oregon, Washington or British Columbia ($\delta^2\text{H}$: -149.0 to -129.4 , $n = 94$), and (iii) LDM birds hatched in Alaska ($\delta^2\text{H} < -149.8$, $n = 68$). For the remaining birds ($n = 28$), migratory origin was unassignable because feather $\delta^2\text{H}$ values fell outside the range described by known-source mallards. Feather $\delta^2\text{H}$ values of wintering mallards and their descriptive statistics are presented in Table 1. The median harvest date for RES mallards was 30 November, followed by a median harvest date of 7 December for IDM mallards and 17 December for LDM mallards.

AIV prevalence

A total of 47 of the 525 wintering mallards were positive for AIV, corresponding with a prevalence of 8.95%. The prevalence of AIV did not differ significantly between RES (9.55%, 95% CI: 6.40–12.70), IDM (9.57%, 95% CI: 3.63–15.52) or LDM mallards (7.35%, 95% CI: 1.15–13.56), and no significant interactions were detected between migratory status and date, age or body mass (Table 2). Prevalence differed significantly according to age ($\chi^2 = 28.003$, $P < 0.001$; Table 2). HY birds had a significantly higher prevalence of AIV (15.33%, 95% CI: 11.16–19.50) than AHY birds (1.26%, 95% CI: 0.05–2.68). Prevalence declined each month from October (13.95%, 95% CI: 7.97–19.93) to January (0.26%, 95% CI: 0.09–5.14).

A total of 46 of the 371 locally breeding mallards sampled during summer (July–August) shed AIV, a prevalence of 12.40%. This population consisted of a high percentage of HY birds born locally in California

($n = 262$, 70.4%). Similar to wintering birds, prevalence differed significantly according to age ($\chi^2 = 24.253$, $P < 0.001$). HY mallards had a significantly higher prevalence of AIV (16.79%, 95% CI: 10.74–22.84) than sympatric AHY from California (1.83%, 95% CI: 0.44–3.23). Prevalence was significantly higher in July (15.79%, 95% CI: 9.94–21.64) compared to August (3.81%, 95% CI 2.19–5.43).

Viral subtypes

Viruses were isolated, and complete HA-NA subtypes were identified for 91.50% (43/47) and 95.45% (21/22) of PCR positive allantoic fluid samples from wintering and breeding mallards, respectively. The subtype H5N1 was not detected, and all H5 viruses were characterized as low pathogenic because they lacked polybasic residues at the HA cleavage site.

Wintering mallards shed a total of 13 different viral subtypes during October 2008–January 2009 (Fig. 2). These subtypes represented 8 HA genes (H1, H3-6 and H10-12) and 7 NA genes (N1-2 and N5-9). RES mallards had the highest viral diversity for both the HA ($H' = 1.60$) and NA subtypes ($H' = 1.67$) compared to all groups on the wintering grounds. LDM birds had the next highest viral diversity (HA: 1.33, NA: 1.10), followed by IDM (HA: 1.14; NA: 0.90).

The most common subtype identified from RES and IDM was H6N1, occurring at frequencies of 35.71% and 62.50%, respectively (Fig. 2). This subtype was not detected in LDM, in which all subtypes (H1N6, H4N6, H10N2, H10N7 and H11N2) were as common as each other. Subtypes shared between mallards of different migratory status included H4N6, H5N2, H6N1, H10N7, H11N2 and H11N9 or 46.15% of subtypes identified in the winter (Fig. 3).

Breeding mallards shed a total of six viral subtypes during July to August 2009, and there were two cases of mixed infections (Fig. 2). A total of 4 HA (H1, H3-5) and 4 NA subtypes (N1-2, N6, N8) were detected. The viral diversity of BRE birds was the lowest of all mallard groups (HA: 0.93; NA: 0.64). The most common

Table 1 Migratory status of mallards sampled during winter in California and descriptive statistics of associated hydrogen ($\delta^2\text{H}$ in ‰). The percentage of hatch-year birds (HY) occurring in each migratory group is also presented

Migratory status	N	Mean	Min.	Max.	SD	HY (%)
Resident	355	-106.86	-137.30	-60.93	13.49	52.5 ($n = 176$)
Intermediate-distance migrant	94	-138.64	-148.96	-129.41	6.04	51.1 ($n = 48$)
Long-distance migrant	68	-166.63	-214.40	-149.78	13.25	58.8 ($n = 40$)

Table 2 Generalized linear model results of the main effects of age, body mass and migration status and two-way interactions on avian influenza infection of mallards wintering in California

Factor	df	Wald – χ^2	<i>P</i> -value
Collection date	1	13.639	<0.001
Flat wing	1	2.921	0.087
Age	1	28.003	<0.001
Body mass	1	0.128	0.721
Migration status	2	1.611	0.447
Migration status*date	2	3.469	0.177
Migration status*age	2	4.589	0.101
Migration status*body mass	2	0.225	0.894

Significant *P*-values (≤ 0.05) are highlighted in bold.

subtype identified from BRE mallards was H5N2 (71.43%). Subtypes common to both wintering (Oct 2008–Jan 2009) and breeding mallards included H1N1, H3N8, H4N6 and H5N2 or 66.67% of subtypes identified during the summer (Jul–Aug 2009) (Fig. 3).

Phylogenetic assignment

Support for the introduction of virus into California by LDM mallards was evident for both HA and NA segments. LDM isolates of H1, H4 and N6 originated from parent strains shed by Alaskan mallards and northern pintail in 2007–2008, indicating a possible introduction by LDM mallards that migrated from Alaska (Fig. 4a,b, Fig. S1). LDM isolates of H1 and H4 viruses formed a separate clade from RES and BRE mallards, evidence for multiple introduction events involving mallards of different migratory origin in California. There were no cases of LDM isolates subsequently occurring in dabbling ducks during the winter 2008–2009 and following breeding season in California, 2009. Clustering of LDM within the California clades of the H10 and H11 viruses suggested local infection by strains shed by the northern shoveller on the wintering ground during 2008 (Fig. S1). LDM mallards appeared to acquire N2 and N7 viruses in California (Fig. S2), with evidence of direct gene flow of N6 viruses from RES to LDM mallards (Fig. 4b).

Isolates from IDM mallards clustered with the California clade for both HA (H6, H11) and NA (N1, N9) genes, suggesting these migratory mallards were primarily infected on the wintering grounds. However, relatedness between IDM and mallard 2007 viruses from Washington and Oregon was also evident for N1 and N2 viruses (Fig. 4b, Fig. S2). This pattern indicated IDM may facilitate periodic introduction of these two viruses from Pacific NW breeding grounds. The N2 was the most evolutionary divergent subtype (29.81×10^{-2} :

Table 3) and was shed by mallards of all migratory status in winter and BRE mallards in summer 2009. Isolates from IDM and LDM mallards consistently formed separate clades, indicating limited gene exchange between these migratory host populations (Fig. S2).

Isolates from RES mallards were most closely related to dabbling duck viruses collected during the winter in California, 2008. Isolates from the American green-wing teal were identified as the parent strain for H4, H5, H10, H11 and N2, N7, N9 viruses shed by RES mallards. Occurrence of this pattern across a wide range of subtypes suggests frequent inter-species transmission between teals and RES mallards in California, 2008. Additionally, RES isolates showed high relatedness to viruses shed by mallards or northern pintail from Alaska (H4, H10: Fig. S1, N1: Fig. 4b), British Columbia (N2: Fig. S2), Alberta (N8: Fig. S2) and Washington (H12: Fig. S1, N5: Fig. S2, N6: Fig. 4b). Hence, long- and intermediate-distance movement of virus by ducks from northern breeding grounds into the RES mallard population in California may have periodically occurred.

Relatedness between BRE and RES isolates was evident for both HA (H3: Fig. S1, H5: Fig. 4a) and NA (N1: Fig. 4b, N8: Fig. S2) genes. The subtype H5 was limited in its host range to RES and BRE mallards and was not detected in migratory mallards (IDM, LDM), indicating transmission was limited to California. The H5 sequences also showed the least evolutionary divergence compared to other subtypes (0.69×10^{-2} ; Table 3). For both H5 and N2, RES sequences from 2008 were ancestral to isolates from BRE mallards (Fig. 4a, Fig. S2), indicating gene flow from wintering populations to subsequent breeding mallards in the summer, 2009. Isolates from BRE mallards were the closest relatives of H4 and N6 viruses shed by California mallards sampled in 2010 (Fig. S1, Fig. 4b).

Discussion

Animal migration can enhance the spread of pathogens, but this has rarely been demonstrated, probably owing to logistical challenges in following migratory species (Altizer *et al.* 2011). Our study used measurements of feather stable isotopes to tease apart the roles of mallards exhibiting a range of migration strategies and found no detectable difference in AIV prevalence. Long-distance migration is often punctuated by multiple stopovers between wintering and breeding grounds, arguing for contact with a greater number and type of pathogens than resident counterparts (Moller & Erritzoe 1998; Waldenstrom *et al.* 2002). However, the prevalence among RES, IDM and LDM ranged from 7.35% to 9.55%, indicating little variation in rates of infection despite differences in geographic origin of the host and

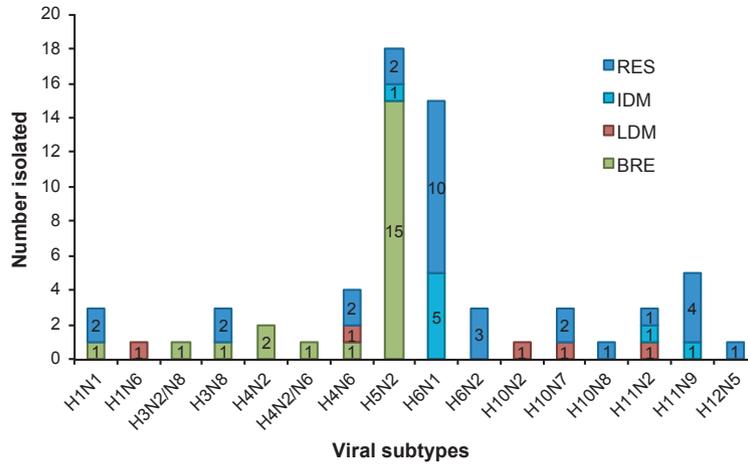


Fig. 2 Distribution of avian influenza subtypes isolated from mallards in California, 2008–2009. Subtypes are coloured according to migratory status of mallards: residents (RES: dark blue), intermediate-distance migrants (IDM: teal blue), long-distance migrants (LDM: red) or breeding season mallards (BRE: green). Values within bars indicate the number of isolates.

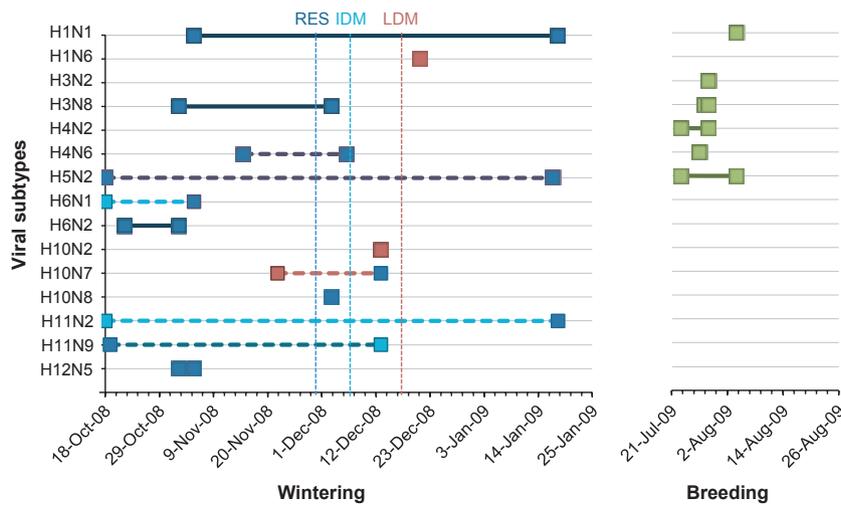


Fig. 3 Circulation and longevity of avian influenza subtypes in mallards from California, 2008–2009. Colours indicate migration status of the first and last hosts: residents (RES: dark blue), intermediate-distance migrants (IDM: teal blue), long-distance migrants (LDM: red) or breeding season mallards (BRE: green). Solid line indicates circulation among mallards of a single migratory status, and a dashed line indicates circulation among mallards of different migration status. Vertical lines indicate median date of harvest for RES (30 November), IDM (7 December) and LDM (17 December) mallards.

distance travelled. Changes in body condition that accompany long-distance migration can influence susceptibility to infection (Buehler *et al.* 2010), but no link between condition and AIV prevalence was detected. Instead, age of birds accounted for observed differences in prevalence, with HY mallards showing a prevalence 12 times higher than AHY birds. This is consistent with the large body of AIV literature that proposes HY birds act as immuno-naïve hosts driving the transmission of the virus (Hinshaw *et al.* 1980; Guberti *et al.* 2007; Hoye *et al.* 2010).

Viral diversity was similar between mallards of different geographic origin. RES birds had marginally higher viral subtype diversity for the HA and NA subtypes, compared to IDM and LDM populations. This is in contrast to waterfowl studies that show migrants are infected by a higher diversity of haemoprotozoan parasites than sedentary species, evidence that migration exposes birds to a richer parasite fauna (Figuerola & Green 2000). The equivalent viral diversity observed in this study may be explained by the mixing of virus pools between RES, IDM and LDM mallards on the

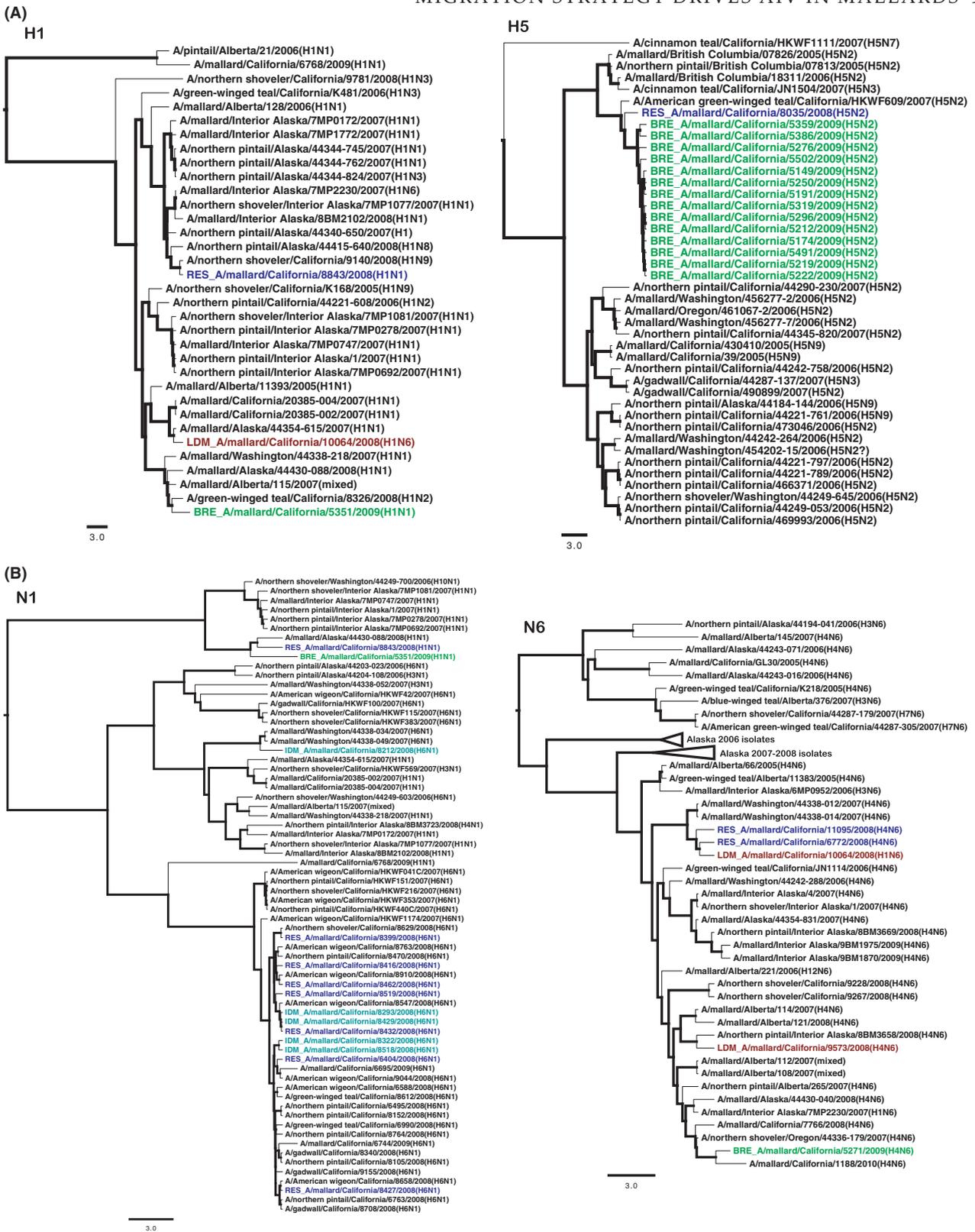


Fig. 4 Phylogenetic relationships of A) HA and B) NA genes from mallards in California and regions of migratory connectivity in North America. Sequences are coloured according to resident (RES: dark blue), intermediate-distance migrant (IDM: teal blue), long-distance migrant (LDM: red), breeding season mallards (BRE: green) or reference sequences from North America (black). Phylogenetic trees were generated with a Bayesian Markov chain Monte Carlo (MCMC) coalescent approach. Nodes supported by posterior probabilities higher than 0.95 are highlighted in bold. Scale bar corresponds to years.

Table 3 Estimates of evolutionary divergence within avian influenza subtypes detected in California, 2008–2009. Divergence is determined by averaging the number of substitutions per site over all sequence pairs within each subtype. Standard error estimates (SE) were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the maximum composite likelihood model. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011).

Subtype	Evolutionary divergence $\times 10^{-2}$ (SE)	Host group			
		RES	IDM	LDM	BRE
H1	4.35 (± 1.08)	■	■	■	■
H3	1.38 (± 0.40)	■	■	■	■
H4	7.00 (± 1.64)	■	■	■	■
H5	0.69 (± 0.20)	■	■	■	■
H6	1.58 (± 0.43)	■	■	■	■
H10	2.98 (± 0.77)	■	■	■	■
H11	2.13 (± 0.54)	■	■	■	■
H12	N/A	■	■	■	■
N1	2.54 (± 0.57)	■	■	■	■
N2	29.81 (± 1.58)	■	■	■	■
N5	N/A	■	■	■	■
N6	1.89 (± 0.44)	■	■	■	■
N7	2.70 (± 0.63)	■	■	■	■
N8	1.27 (± 0.38)	■	■	■	■
N9	2.89 (± 0.63)	■	■	■	■

IDM, intermediate-distance migrants; LDM, long-distance migrants; RES, residents.

wintering grounds in California. Comparison of viral subtype diversity before and after the arrival of migrants during winter was not attempted in this study but may better address this question. The high viral diversity in California, compared to northern breeding grounds in Alaska sampled within the same annual cycle (Hill *et al.* 2012), may result from mixing of virus imported by birds of different geographic origin, and also different species. Of interest, local-born mallards (BRE) sampled during the summer revealed the lowest viral diversity in our study. The low population numbers and single origin of mallards compared to wintering birds may have contributed to a lower viral diversity.

Our study identified migratory mallards as agents in the movement of a limited range of virus subtypes along the Pacific Flyway. Isolates from LDM (H1, H4 and N6) were closely related to Alaskan strains, supporting infection of migrants on northern breeding grounds followed by introduction of virus into California. This southward pattern of gene flow was also apparent for IDM mallards. Virus from IDM (N1 and, N2) clustered within the Washington clade, consistent with their origin from breeding sites along the Pacific NW. Migration along the Pacific Flyway has been implicated in the introduction of HA and NA seg-

ments of Asian lineages occurring in California (H6, N8, N1 and N2: Bahl *et al.* 2009; Pearce *et al.* 2009; zu Dohna *et al.* 2009). Our findings refine this hypothesis by highlighting LDM mallards from Alaska and IDM mallards from the Pacific Rim may act as agents for the southward dispersal of AIV. Mallards are considered one of the most robust host species and are a candidate for long-distance dispersal of both high and low pathogenic AIV (Keawcharoen *et al.* 2008; Fereidouni *et al.* 2009). However, whether introduction of AIV from northern breeding grounds occurred in a single, uninterrupted dispersal event or involved infection of successive hosts or species at stopover sites remains unresolved.

Virus introduced by LDM mallards was not detected in the wintering waterfowl population or the following breeding season in mallards. One explanation is that migrants may be at the tail-end of infection, resulting in lower viral titres and reduced transmissibility. Mallards infected with low pathogenic AIV shed virus relatively briefly based on studies conducted in the field (8 days: Latorre-Margalef *et al.* 2009) and experimental settings (12 days: Jourdain *et al.* 2010). Alternatively, virus imported from Alaskan breeding grounds may have lower fitness in local-born hosts and the California environment. Environmental persistence is an important determinant of AIV transmission (Brebant *et al.* 2009; Roche *et al.* 2009; Rohani *et al.* 2009), and the physico-chemical properties of wetlands combined with freeze-thaw cycles in winter (Lebarbenchon *et al.* 2011) may be a constraint on the persistence of virus imported into California. Our conclusion that migrants imported virus that did not readily circulate is consistent with a recent study of AIV from waterfowl and seabirds within the Pacific Flyway that found virus from Alaska and California was strongly spatially structured during 2006–2008 (Girard *et al.* 2012). However, our study focused on a single migration cycle that may not have coincided with an introduction event that triggered infection of wintering birds, and therefore, longer-term studies are needed to clarify the frequency with which these events may occur.

We confirmed the role of RES mallards as AIV reservoirs, facilitating early AIV circulation at lower latitudes. This was best demonstrated by the H5N2 subtype, which was detected at low prevalence in wintering RES mallards yet emerged in BRE mallards as a dominant subtype the following summer. The role of resident birds as a reservoir for AIV has been hypothesized (Stallknecht *et al.* 1990; Stallknecht & Brown 2008; Harris *et al.* 2010) but never demonstrated throughout the annual cycle in the same locale. The prevailing model of AIV circulation has been transmission

between northern breeding grounds and southern wintering sites via migratory birds (Olsen *et al.* 2006; Munster *et al.* 2007). This model fits with experimental evidence that some AIVs are maladapted for warmer temperatures (>32 °C: Brown *et al.* 2009; >28 °C: Brown *et al.* 2007). However, our study identified several subtypes that circulated in both the winter and summer at lower latitudes. Studies characterizing the thermostability of H5N2, H3N8 and other summer-circulating subtypes are needed to assess the possibility that AIV can adapt to exploit permanent host populations at lower latitudes.

Transmission between RES mallards and sympatric dabbling duck species wintering in California was evident from our phylogenetic analyses. Isolates from the American green-wing teal were identified as the parent strain for H4, H5, H10, H11 and N2, N7, N9 viruses shed by RES mallards. Occurrence of this pattern across a wide range of genetic sub-lineages suggests teals were a common source of infection for RES mallards. Green-winged teals are an early migrant in California (Bellrose 1976) that arrive shortly after the northern pintail, providing opportunities for exchange of virus in early winter. Additionally, RES isolates shared ancestry with viruses shed by mallards and northern pintail from Alaska (H10, N1), British Columbia (N2), Alberta (N8) and Washington (H12, N5, N6). Hence, long- and intermediate-distance movement of virus by ducks from northern breeding grounds into the RES population in California may have periodically occurred. The role of mallards and northern pintail as agents for the movement of AIV over long distances has been documented by flyway-scale phylogenetic studies (Lam *et al.* 2012). However, these two species may be overrepresented in sequence databases as surveillance in North America often relies on hunter-shot birds, a biased sample as table birds are preferentially hunted for consumption. Including a wider range of species that are sympatric with mallards on breeding and wintering grounds may elucidate a link between species associations and inter-specific transmission.

Re-evaluating the significance of resident wildlife populations is needed to improve current models of pathogen circulation. Our results highlight that resident mallards can extend the period of transmission at temperate locations, challenging concepts of AIV as a pathogen that is restricted to higher latitudes during summer. Resident waterfowl populations infected with AIV have been identified across migratory flyways in North America (Stallknecht *et al.* 1990; Ferro *et al.* 2010) and are anticipated to play a similar role in the perpetuation of AIV. Changes in the migratory behaviour of animals because of mild winters and year-round

resources are likely to influence pathogen dynamics (Altizer *et al.* 2011). Within the Central Valley, rice agriculture provides a 'buffered' resource for waterfowl that is protected from temperature extremes and desiccation. This dependable food supply supports local-breeding mallards with implications for altering circulation of AIV in the Pacific Flyway. Our results suggest that migrants contribute to the movement of pathogens; however, transmission of imported strains may be constrained by environmental factors or transmissibility of virus. Determining the success of introduced strains to spread beyond the migratory population requires explicit knowledge of the geographic origin of hosts. Through the application of stable isotopes, our study demonstrates how migration strategy of the host can provide a nuanced understanding of pathogen dynamics in wildlife.

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Data accessibility

Accession numbers for AIV sequences: see Table S1. Sequence alignments, capture data and isotope values: DRYAD entry doi:10.5061/dryad.4tf74.

The authors are broadly interested in avian influenza and wild bird ecology. Our research focuses on the Pacific Flyway where avian influenza dynamics are not well understood. Applying ecological tools, including stable isotopes, to address this question is a common area of interest spurring this collaboration.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1. Accession numbers for the nucleotide sequences generated in this study (HA: $n = 55$, NA: $n = 55$).

Fig. S1. Phylogenetic relationships of H3, H4, H6, H10, H11 & H12 genes from mallards in California and regions of migratory connectivity in North America. Sequences are coloured according to resident (RES: dark blue), intermediate-distance migrant (IDM: teal blue), long-distance migrant (LDM: red), breeding season mallards (BRE: green) or reference sequences from North America (black).

Fig. S2. Phylogenetic relationships of N2, N5, N7, N8 & N9 genes from mallards in California and regions of migratory

connectivity in North America. Sequences are coloured according to resident (RES: dark blue), intermediate-distance migrant (IDM: teal blue), long-distance migrant (LDM: red), breeding season mallards (BRE: green) or reference sequences from North America.

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