ECOLOGY LETTERS

*Ecology Letters*, (2016) doi: 10.1111/ele.12629

LETTER

# Transmission of influenza reflects seasonality of wild birds across the annual cycle

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**Abstract**

Influenza A Viruses (IAV) in nature must overcome shifting transmission barriers caused by the mobility of their primary host, migratory wild birds, that change throughout the annual cycle. Using a phylogenetic network of viral sequences from North American wild birds (2008–2011) we demonstrate a shift from intraspecific to interspecific transmission that along with reassortment, allows IAV to achieve viral flow across successive seasons from summer to winter. Our study supports amplification of IAV during summer breeding seeded by overwintering virus persisting locally and virus introduced from a wide range of latitudes. As birds migrate from breeding sites to lower latitudes, they become involved in transmission networks with greater connectivity to other bird species, with interspecies transmission of reassortant viruses peaking during the winter. We propose that switching transmission dynamics may be a critical strategy for pathogens that infect mobile hosts inhabiting regions with strong seasonality.

**Keywords**

Avian influenza, biological rhythms, bird migration, host contact structure, influenza A virus, migratory cycle, seasonality, transmission networks, viral flow, zoonotic disease.

*Ecology Letters* (2016)

## INTRODUCTION

Studies of a range of host-pathogen systems indicate that the annual cycle represents a myriad of challenges for survival of pathogens transported by migratory animals (Altizer *et al.* 2011; Hall *et al.* 2014; Martinez-Bakker & Helm 2015). Fluctuations in host density, species composition, contact structure and immunity, as well as environmental conditions are among the many ecological barriers that pathogens encounter during the annual cycle. IAV, an RNA orthomyxovirus with a segmented genome, is uniquely resilient to ecological change within and outside the primary host – wild birds of the orders Anseriformes and Charadriiformes. These birds host the widest diversity of IAV subtypes and are considered a reservoir for low pathogenic IAVs, in which the virus replicates without causing debilitating disease (Webster *et al.* 1992). Migratory birds from seasonal habitat alternate between reproductively active states and non-breeding states leading to annual life-history cycles that are spatially and temporally separated (Silk *et al.* 2014). An important yet unresolved question in IAV ecology is how influenza overcomes barriers to transmission, triggered by the cyclic isolation and connection of hosts at alternating habitats.

Sampling wild birds over multiple annual cycles provides an opportunity to test the mechanisms by which IAV overcomes seasonal challenges to transmission. After amplification due to the summer birth pulse (Stallknecht & Brown 2008), autumn migration represents a stage when IAV is dispersed via wild birds, encountering novel environments and host phenotypes to which viruses may or may not be well-suited. Mixed species flocks that congregate at staging (Wallensten *et al.* 2007; Ramey *et al.* 2011) and wintering sites (Ferro *et al.* 2010; Hill *et al.* 2012b) may act as a transmission barrier for viruses maladapted to exploit a novel host phenotype. Viruses that are successful at replicating inside host cells, once shed into the environment face degradation depending on UV, temperature, salinity and pH conditions (Stallknecht & Brown 2009). Conditions that determine IAV persistence in the external environment may fluctuate widely along the migration route, presenting a challenge for the survival of IAV in novel habitats.

In this study, we test the prediction that transmission dynamics vary relative to the changing biotic and abiotic challenges to IAV spread due to the seasonal migration of wild birds throughout the annual cycle. We postulate that transmission (or 'viral flow') in wild birds can be measured across three axes that represent transmission dynamics within, between and outside the host. Firstly, within-host: IAVs replicate by exchanging gene segments between two viruses infecting the same cell (reassortment) or all segments from one virus (full complement). Secondly, host-to-host: viruses spread either between host species of the same phenotype (intraspecies) or different species phenotypes (interspecies). Thirdly, outside host: virus dispersal is restricted spatiotemporally and limited to the same season (intraseason) or dispersal across different seasons of the annual cycle (interseason).

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Using data from over 4 years of North and Central American wild bird surveillance with a focus on breeding sites in Alaska, we employed network analysis of viruses to test our predictions. Networks offer a novel solution for modelling the spread of pathogens capable of switching genes horizontally between two pathogens (reassortment), a process that confounds characterisation by phylogenetic trees that model evolution from a single, common ancestor (Chan *et al.* 2013). Additionally, phylogenies are not easily resolved into transmission pairs, and while networks may not equate to actual transmission events, they link individuals in the same epidemiological cluster (Wertheim *et al.* 2014). Representation of a transmission event as nodes (wild bird host) linked by edges (viral transmissions) makes networks uniquely suited to quantifying the frequency and importance of IAV transmission parameters throughout the annual cycle.

Understanding how transmission dynamics vary seasonally may prove instructive for mitigating the spread of IAV and spillover to domestic animals. Using a network approach, we empirically tested some of the major tenets of how IAVs circulate during the wild bird annual cycle stemming from four decades of surveillance. The hypotheses tested were fourfold: (1) over-wintering of IAVs at breeding grounds occurs but is limited (Breban *et al.* 2009; Kleijn *et al.* 2010); (2) IAVs transmit between birds of different species on the breeding grounds (Chen & Holmes 2009; Pearce *et al.* 2011); (3) dispersal of IAVs occurs due to the autumn migration of birds and arrival on the wintering grounds (Wallensten *et al.* 2007; Tian *et al.* 2014); and (4) diffusion of IAVs occurs latitudinally along flyways but is constrained longitudinally (Lam *et al.* 2012). Our study indicated that the success of IAV to circulate in migratory birds hinges on switching transmission dynamics to reflect the changing life history of the host in seasonal environments.

## MATERIALS AND METHODS

### Sampling wild birds

The study site was located at the Minto Flats State Game Refuge, interior Alaska (64.90, − 148.85), a wetland complex that serves as a major breeding, moulting and staging area for over 23 waterfowl species (Mallek & Groves 2009; B. Meixell, pers. obs). During 2008–2010, we sampled a total of 14004 individual birds on 20253 occasions (including recaptures), representing seven dabbling duck (*Anatini*), five diving duck (*Aythyini*), three sea duck (*Mergini*), and one goose (*Anserini*) species (Table S1). The northern pintail (*Anas acuta*) was the most numerous duck sampled, followed by the mallard (*Anas platyrhynchos*) reflecting local abundance (Mallek & Groves 2009). Sampling began at spring breakup when birds arrived to nest in May. Different methods (rocket nets, baited swim-in traps, drive traps, decoy traps) were used to capture the broad range of species across the life-history stages of breeding, and ducks were hunter-shot during September. Live-captured birds were usually sampled < 1 h after capture and hunted ducks were usually sampled < 6 h post-mortem.

We obtained a cloacal swab from each bird and where possible a tracheal swab was collected. Samples from nesting birds were obtained by swabbing fresh droppings when flushed from the nest. Polyester-tipped applicators were used to collect swabs, placed in viral transport media (VTM: Remel, KS) and kept on ice until transfer to liquid nitrogen vapour shippers (− 150 °C) up to 3 h later.

### Annual cycle analysis

Upon capture, birds were fitted with an aluminium federal tarsus band; ducklings that were too small for bands were marked with individually coded web tags. Birds with existing markers were recorded as recaptured birds. We obtained the recovery/recapture data for birds banded at Minto Flats from the U.S. Geological Survey Bird Banding Laboratory. Of the 11777 ducks banded at Minto Flats between 2006 and 2011, a total of 1506 (12.8%) bands were reported between 2006 and 2014, mostly representing hunter-shot ducks. Band returns from six duck species (a focus of subsequent network analysis) were mapped using QGIS 2.0 (Open Source Geospatial Foundation Project. http://qgis.osgeo.org) to describe connectivity of Minto Flats with other regions in North America (Fig. 1a, Fig. S1).

To characterise the migration chronology of ducks breeding at Minto Flats, the mean latitude of capture/recapture was plotted against Julian date to derive four stages of the annual cycle (Fig. 1b): summer (30 April–28 August), autumn (29 August–6 November), winter (7 November–19 February) and spring (20 February–29 April). Summer was further categorised into pre-fledge (30 April–31 July) and post-fledge (1–28 August) to account for changes in waterfowl mobility and behaviour that may influence transmission. Pre-fledge encompassed nesting, brood-rearing and moulting stages of the breeding season when mobility was lowest and birds were generally dispersed in small groups; post-fledge broadly encompassed the transition from flightless to flight-capable when birds congregated in large flocks prior to autumn migration.

### Influenza screening

Viral RNA was extracted using the MagMax-96 Viral Isolation Kit (Ambion Inc. Foster City, CA). RNA was screened using a two-step real-time Reverse Transcriptase – Polymerase Chain Reaction (rRT-PCR) targeting the matrix gene (Runstadler *et al.* 2007). PCR assays were run on an ABI 7500 real-time PCR System (Applied Biosystems, Foster City, CA). PCR-positive samples (Ct value < 45) were subjected to a H5- and H7-specific rRT-PCR to identify potentially highly pathogenic samples (Wang *et al.* 2008). To amplify virus, positive VTM (100 μL) was inoculated into the allanotic cavity of 9–11-day-old embryonating specific pathogen-free chicken eggs (Charles River, CT) and incubated at 37 °C for 72 h or until embryo death, as detected by daily candling. RNA was extracted from the allantoic fluid and the matrix rRT-PCR repeated. Up to three sequential passages were attempted to determine if virus was present (Eisfeld *et al.* 2014). Whole-genome sequencing was performed at the J. Craig Venter Institute in Rockville, MD as described by Nelson *et al.* (2007) and all sequences deposited into Genbank. Subtyping

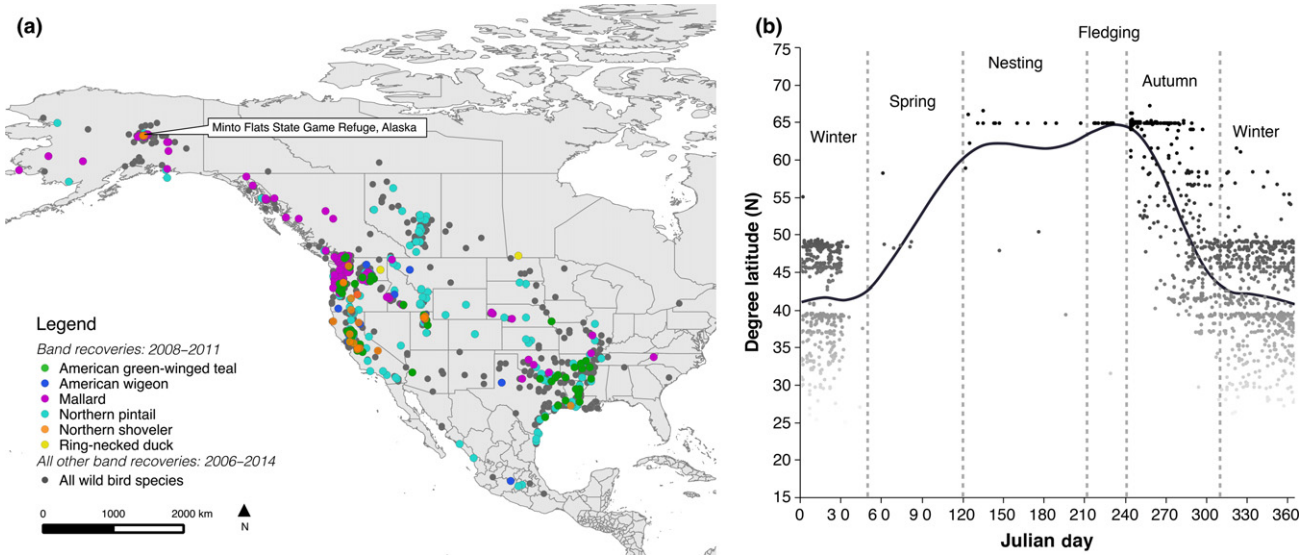


**Figure 1** Spatial and temporal distribution of wild birds banded at Minto Flats State Game Refuge, Alaska from 2006–2014 and recovered in North America based on 1506 band recoveries. (a) Spatial distribution of band recoveries indicates movement spanning the Pacific, Central, Mississippi and Atlantic flyways (all markers). Band recoveries reported between 2008–2011 from duck species included in the network analysis are colour coded. (b) Temporal distribution of band returns (2006–2014) indicates approximate arrival of birds at Minto Flats for nesting (May) and departure for autumn migration (September). Dots indicate date of band recovery relative to latitude (low: light grey to high: dark grey) and black line indicates mean timing and location for the six duck species.

of virus was confirmed by BLASTn of the sequence against isolates in Genbank and identifying the subtype match that showed highest percentage identity.

### Network analysis

The most intensive sampling effort at Minto Flats occurred in 2009 and 2010, yielding a large number of IAV sequences ($n$ = 512) conducive to network analysis. Isolates from 2008 obtained at Minto Flats ($n$ = 33) were also included to allow characterisation of interannual transmissions. Sequences from all wild avian hosts sampled in North and Central America were downloaded from the Influenza Research Database (IRD: Squires *et al.* 2012) on 6 Feb 2014, including full-length, whole-genome sequences collected between 2008 and 2011. Single day resolution was required for network analysis; therefore, sequences that lacked information about the exact day of collection were removed. A total of 1787 sequences were included in the dataset, including 545 whole-genome virus sequences from Minto Flats (Fig. S1, Table S2). Sequences were isolated from 35 bird species, representing seven families (Fig. S2).

Network analysis was implemented using the SeqTrack algorithm in the Python programming language as described by Jombart *et al.* (2011) but with modifications to make the network host-oriented, approximating transmissions between wild birds. Transmissions defined here are akin to viral flow, but due to the absence of contact and epidemiological data for each host, represent plausible viral movements and are not definitive. The SeqTrack algorithm requires that each sequence has a unique identifier and is time-stamped (date of collection). When identifying the source of a virus using SeqTrack, two rules were applied: (1) the source should occur prior to the isolate being considered, and (2) the source should have the highest pairwise identity (PWI) with the isolate being considered. As IAVs have a segmented genome, we modelled viral segments as independent entities. We used the first rule of SeqTrack to identify possible sources for each of the eight viral segments (Fig. 2a and b). To account for the possibility that the order of sampling (collection date) may not reflect order of infection, sources could occur up to 6 days after the sink isolate (Fig. 2c). This period corresponds with the mean shedding duration (~ seven whole days) less one day for incubation, based on experimental studies of North American dabbling ducks (Table S3).

Rather than using a 99% cut-off (see: Reeves *et al.* 2011), we used a clustering technique to establish a data-driven threshold above which a transmission event was considered plausible. A machine-learning algorithm called Affinity Propagation (Frey & Dueck 2007) was used to cluster the sequences for each segment based on PWI values calculated using Clustal Omega. Each cluster had a minimum PWI value; we then used the minimum of all PWIs as our chosen threshold value. The mean, maximum and minimum PWI for each segment is presented in Fig. S3. One graph was yielded for each segment (8 total), where the nodes represented individual isolates, and directed edges represented hypothesised transmission events.

The eight transmission graphs were then summed together to identify transmission pathways involving all eight gene segments (Fig. 2a). The second rule of SeqTrack was applied to the directed edges, removing transmissions that were below the maximum pairwise identity (Fig. 2d). This resulted in the 'full complement' transmission graph, where all eight segments

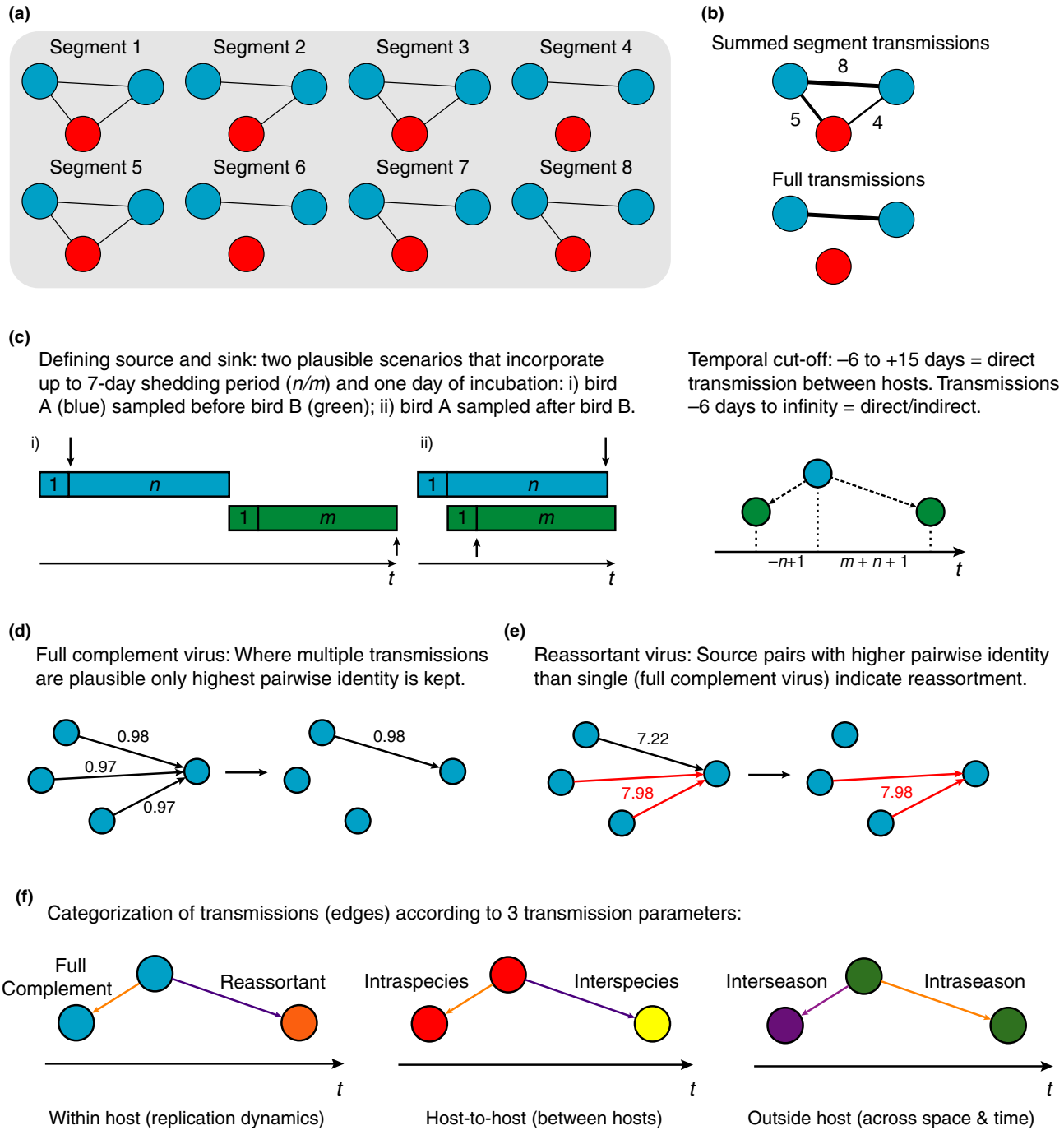


**Figure 2** Depiction of network analysis using influenza sequences from wild birds (nodes) to define transmissions (edges): (a) identification of possible segments that fall above the computed threshold value, (b) identification of full transmissions by summing up edges and removing those that did not involve eight segments, (c) incorporation of host shedding period to determine the timeframe for transmission, (d) rule of highest pairwise identity applied to define only one plausible transmission, (e) source pairs to identify reassortants and (f) use of host/ecological metadata to inform edge classification.

were hypothesised to move together. Any edges not present between two virus isolates (nodes) for all eight transmission graphs (i.e. fewer than eight segments transmitted) and isolates whose source edges were at the lowest 10% of PWI scores were candidates for reassortant transmission. For these, two sources were searched for within the network, whereby pairs of sources together revealed highest PWI with complementary segments of the sink node. If the summed PWI across segments for the two viruses was greater than the single-source search, we identified the isolate as a reassortant (Fig. 2e). This method only accounted for double reassortants, as we reasoned that co-infection of the same host cell with $\geq 3$ viruses had a low probability relative to infection with one or two viruses. The network structure is represented in Fig. S4 to illustrate how edges are connected across the four annual cycles (2008–2011).

### Constructing a host-centric network

A host-centred network was constructed by removing (weighting as zero) any implausible transmissions from the network. Edges were removed that connected (1) the same longitudinally resampled bird, and/or (2) the same bird connected via an edge due to shedding from both the cloaca and oropharynx. In addition, when the time difference between two nodes (time delta) associated with an edge was − 6 to + 6 (corresponding with 7-day shedding duration less one day for incubation), the directionality of viral flow was ambiguous and the role of nodes as a source/sink could not be resolved. Within the network, time-ambiguous edges were represented twice as both a source and sink, and therefore weighted by 0.5. Henceforth, nodes represented a wild bird host that was either a 'source' (initial host) or 'sink' (recipient host) and edges represented viral transmissions.

### Classifying nodes and edges

Metadata associated with viral sequences from North American wild birds available in IRD included host species, collection date, viral subtype and latitude and longitude of collection (age was rarely reported outside Minto Flats). We used metadata to classify edges according to viral replication dynamics (full complement/reassortment), host contact structure (intraspecies/interspecies), and seasonal dynamics (intraseason/interseason). Viral replication strategy was determined using the network to identify reassortants (two source nodes) and full complement (one source node). Reassortant transmissions were represented by two edges given that co-infection with two different viruses is required, whereas full complement transmission was represented by a single edge. Second, we categorised transmissions based on edges that connected nodes between the same bird species ('intraspecies') or different bird species ('interspecies'). Third, edges were categorised as 'intraseasonal' if the source and sink nodes occurred within the same season of the same annual cycle (i.e. autumn 2009 to autumn 2009) or 'interseasonal' if source and sink nodes occurred in two different seasons (i.e. autumn 2009 to autumn 2010). Sample size (counts of nodes and edges) for each category generated by network analysis is presented in Table S4. To explore the possibility that biased sampling of bird species, geographic locations and seasons may impact the construction of the network and subsequent counts of nodes and edges, sensitivity analyses were performed (Fig. S5a, b and c).

### Statistical analysis

To identify whether transmission parameters differed across the annual cycle, generalised linear models (GLM) were performed for each of four stages (pre-fledge, post-fledge, autumn, winter). Spring was excluded as the small number of whole-genome sequences available were all from shorebirds at Delaware Bay, representing a bias in sampling for the spring season. Three transmission parameters were tested that were defined by binomial outcomes: (1) full complement vs. reassortment; (2) intraspecies vs. interspecies; and (3) intraseason vs. interseason. Transmission parameter was treated as the response variable, and the model was constructed with an underlying binomial distribution (link function = logit). Fixed effects included season (ordinal variable: pre-fledge, post-fledge, autumn, winter) and subtype (nominal variable: hemagglutinin H1–H12 and neuraminidase N1–N9). To assess the geographic dispersal of virus, the absolute difference in degrees between source and sink locations of sampled ducks was quantified for latitude (latitude delta) and longitude (longitude delta).

The importance of each variable to the fit of the GLM was determined by ranking the total effect size of each parameter. Variable importance estimates assessed whether variability of the dependent/response factor was a function of variability associated with each parameter (Saltelli 2002). This process relied on Monte Carlo resampling of observed values due to the assumption of non-uniformity of duck sampling across space and time.

To characterise changes in transmission parameters relative to the annual cycle, we conducted an exploratory curve-fitting analysis. A series of linear, exponential and peak regression models were used to fit the predictor variable (four stages of the annual cycle) and response variable (each of the three transmission parameters). Bayesian information criterion (BIC) was used to rank models in terms of best fit. All statistics were conducted using JMP Pro 11.0 (SAS Institute Inc. Cary, NC).

## RESULTS

### Breeding sites are a mixing ground for overwintering and regionally sourced viruses

Analysis of influenza transmissions at Minto Flats indicated intense amplification of locally sourced virus from the same breeding season (86.37%, 412/477) visualised by large clusters of viruses with source and sink dates that overlapped (Fig. 3a). Reassortment accounted for 27.25% (130/477) of virus at Minto Flats over the full course of the summer breeding season. Interannual persistence of IAVs at Minto Flats was estimated at 4.65% (22/473), consisting of (1) intact virions (full complement: 4/473) persisting for a maximum of 324 days; and (2) viral genes (reassortment: 18/473) persisting for a maximum of 366 days.

Import of viruses from 10 states, provinces or countries across North and Central America also contributed to the viral pool at Minto Flats. California (7/473), Wisconsin (6/473), Ohio (3/473), New Brunswick (2/473), Minnesota (1/473), Illinois (1/473), North Dakota (1/473), Arkansas (1/473), Mississippi (1/473) and Guatemala (1/473) acted as a source of IAV genes, suggesting introduction from a wide range of latitude and longitudes. IAVs that were imported into Minto Flats were significantly associated with reassortment (d.f. = 1, $\chi^2 = 104.67$, $P < 0.001$), interspecies transmission (d.f. = 1, $\chi^2 = 264.13$, $P < 0.001$) and originated primarily from the prior autumn and winter (Fig. 3a).

Overlap occurred between regions that seeded Minto Flats and the 11 states, provinces or countries seeded by Minto Flats. California (14/492), Minnesota (9/492), Wisconsin (6/492), Alberta (3/492), Missouri (3/492), Illinois (2/492),

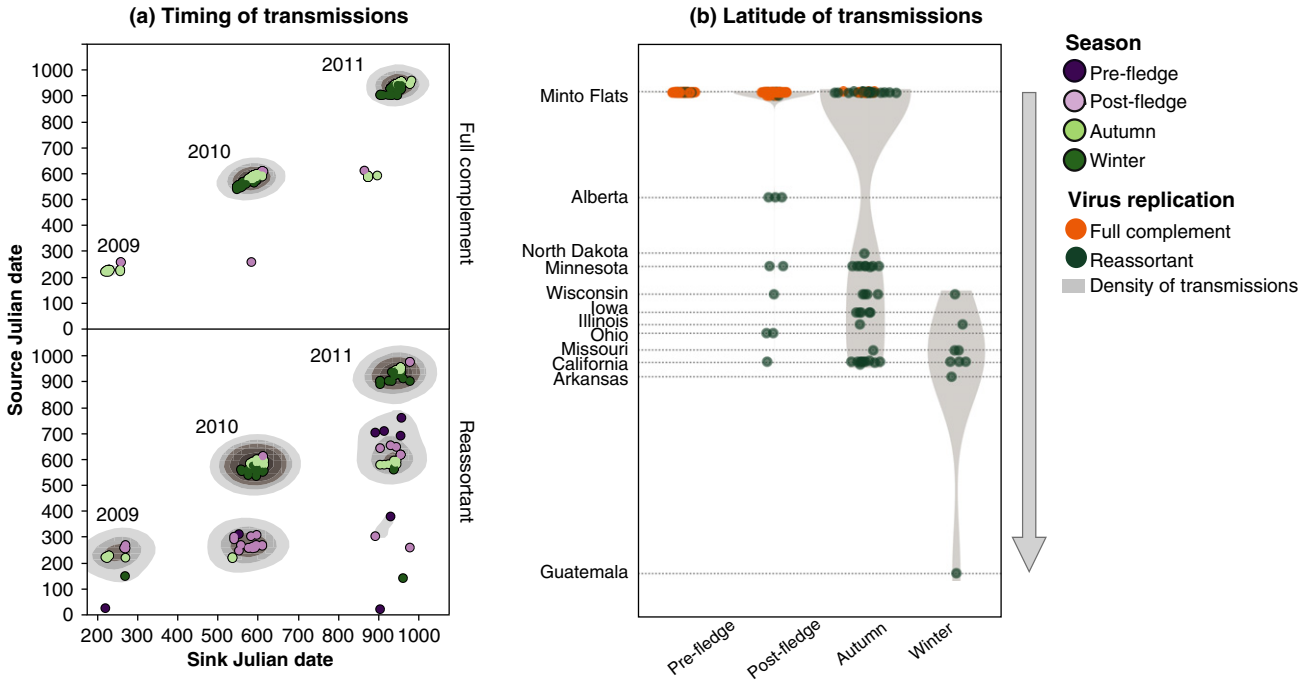


**Figure 3** The distribution of transmissions at Minto Flats, Alaska according to (a) time and (b) latitude. Temporal analysis (a) depicts the density of transmissions among ducks at Minto Flats (grey concentric circles) during 2009–2011. The majority of full complement transmissions (top panel) were sourced locally from Minto Flats and involved ducks with source and sink dates that overlapped (i.e. transmission within the same breeding season). However, reassortant viruses at Minto Flats (lower panel) were also seeded from virus shed by ducks up to two years earlier, originating primarily in autumn and winter from prior annual cycles. Data from 2008 is included but not depicted as this year was a source, but not sink of interannual transmissions. Spatial analysis (b) indicates the latitudinal span of locations that were sourced by virus originating at Minto Flats. The majority of viruses introduced into lower latitudes were detected as reassortants.

Arkansas (1/492), Iowa (1/492), North Dakota (1/492), Ohio (1/492) and Guatemala (1/492) were seeded by Minto Flats viruses (Fig. 3b). IAVs from Minto Flats that infected wild birds at later stages of the annual cycle were significantly associated with reassortment (d.f. = 1, $\chi^2 = 76.61$, $P < 0.001$), interspecies transmission (d.f. = 1, $\chi^2 = 8.30$, $P < 0.004$) and primarily seeded the autumn and winter following the summer (Fig 3a). Overall, Minto Flats was a larger source of IAV transmissions ($n = 42$) than a sink ($n = 24$), indicating asymmetric viral flow from high to low latitudes.

### Interseasonal transmission peaks during winter

To assess seasonal connectivity of influenza among North American wild birds, four stages of the annual cycle: pre-fledge, post-fledge, autumn and winter, were compared. During summer (pre- and post-fledge), transmissions were latitudinally restricted (above 35° N) but shifted southward as the annual cycle progressed, visualised by plotting the source and sink latitude of transmissions and examining the 90% ellipse (encompassing 90% of the data points in the heat map). The ellipse shifted from diagonal to vertical orientation as the annual cycle advanced from pre-fledging to winter (Fig. 4). Viruses that circulated during winter originated from a much wider range of latitudes (10–65° N) compared to the summer.

Transmission sources were significantly associated with the same season as the sink (d.f. = 3, $\chi^2 = 25.50.19$, $P < 0.001$, Fig. 4). For example, IAVs infecting birds during pre-fledge (early breeding) were typically sourced by a bird from the same season, a trend also apparent for post-fledge and autumn. However, interseasonal transmissions increased significantly as the seasons progressed through the annual cycle (d.f. = 3, $\chi^2 = 384.33$, $P < 0.001$). The winter had the largest number of transmissions sourced from other stages of the annual cycle (75.93%, 224/295), representing the peak in interseasonal transmission. Despite representing a smaller number of transmissions overall (37.42%, 736/1967), interseasonal viral flow was evident for all four stages of the annual cycle investigated (excluding spring).

### Within- and between-host transmission parameters shift with the annual cycle

To investigate the relative importance of transmission parameters throughout the annual cycle, host range and virus replication mode were compared across the four seasons. Intraspecies transmissions prevailed as more common during pre-fledge (69.97%, 247/353) and post-fledge (63.51%, 355/559). However, the species specificity of transmissions evident early in the annual cycle declined to 40.39% (267/661) during autumn, reaching a low of 31.06% (91/293) in winter. The number of interspecies transmissions was significantly affected by stage of the annual cycle (d.f. = 3, $\chi^2 = 165.61$, $P < 0.001$) increasing progressively from pre-fledge to winter (Fig. 4).

Virus replication parameters also varied significantly across the annual cycle (d.f. = 3, $\chi^2 = 290.90$, $P < 0.001$).

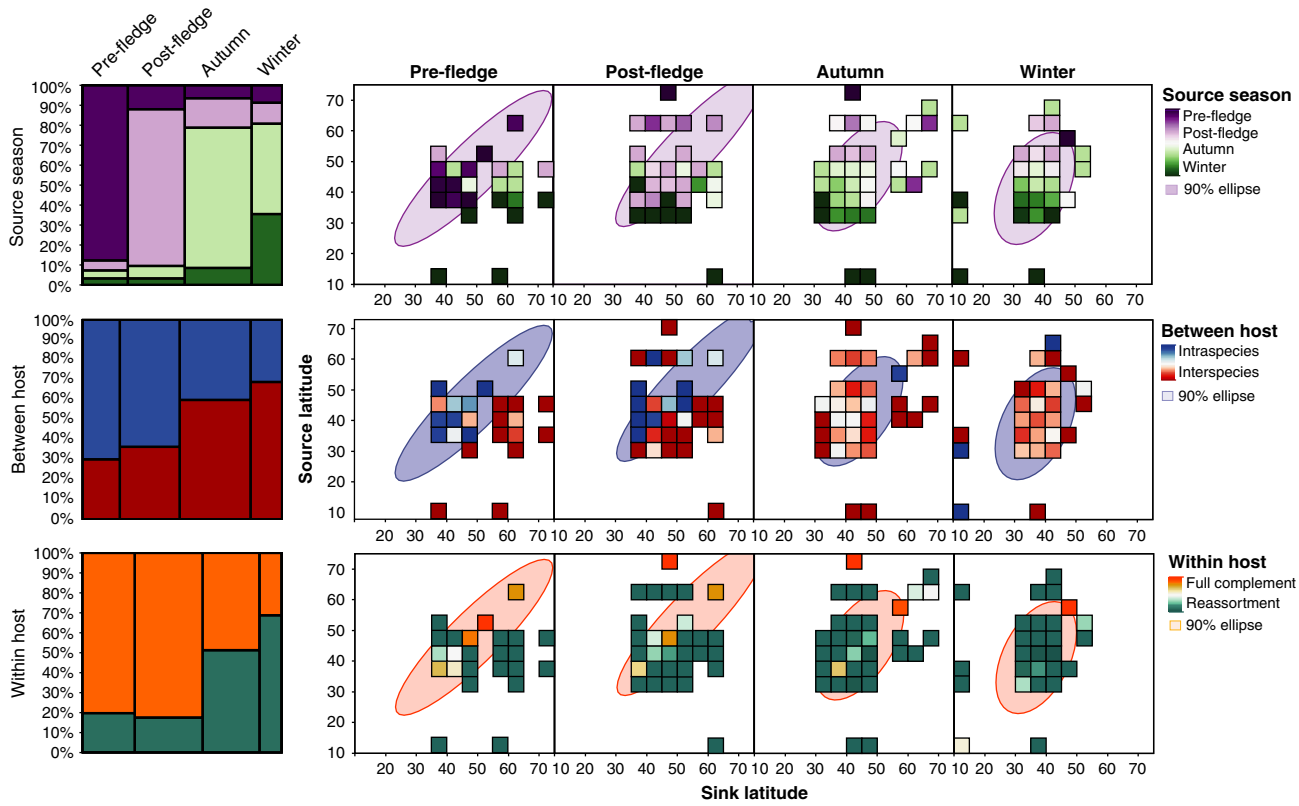


**Figure 4** Spatial distribution of transmission throughout the annual cycle of North American wild birds (2008–2011). Mosaic charts (left panels) indicate the proportion of transmission types and heatmaps (right 4 panels) indicate the predominant type of transmission for each season. The 90% ellipse (shaded) indicates where transmissions are concentrated. A strong interseasonal signal of transmissions is apparent. Virus that circulates during pre- and post-fledge originates locally during the same season as indicated by the diagonal orientation of the ellipse. In contrast, virus that circulates during winter originates from a much wider range of latitudes (10–65° N) as indicated by the vertical orientation of the ellipse. Transmission shifts from intra- to interspecies transmissions and full complement to reassortant as the annual cycle progresses.

Transmission of full complement virus prevailed during all four stages, but as viral flow moved south coinciding with autumn, reassortment accounted for an increasing majority of transmissions (Fig. 4). Reassortment of virus significantly increased as the annual cycle progressed from post-fledge to autumn (d.f. = 1, $\chi^2 = 44.84$, $P < 0.001$) and autumn to winter (d.f. = 1, $\chi^2 = 44.42$, $P < 0.001$), reaching a peak of 68.84% (137/199) in winter.

Overall, the percentage of interseasonal, interspecies and reassortant transmissions increased from breeding to winter but a linear increase was not identified as the best fit for the transition. Curve-fitting indicated that the increase was best modelled by either an exponential distribution (interspecies: BIC = − 11.58, $R^2 = 0.956$; reassortment: BIC = − 7.71, $R^2 = 0.932$) or Lorentzian peak distribution (interseasonal: BIC = − 25.02, $R^2 = 0.999$) indicating transitions between seasons of the annual cycle were an abrupt rather than gradual process.

### Diffusion of virus is unrestricted by latitude or longitude

The drivers of geographic dispersal of IAV, both latitudinal and longitudinal, were assessed at the scale of North and Central America. The combination of reassortant and interspecies transmissions was identified as achieving the greatest geographic dispersal of IAV (Fig. 5). Transmission of full complement virus between wild birds was geographically localised, as visualised by the 90% ellipse occupying a narrow, diagonal plane (Fig. 5). For reassortant transmissions, the sources and sinks of virus were more geographically separated, depicted by a larger 90% ellipse in the latitudinal and longitudinal heat maps (Fig. 5).

Latitudinal and longitudinal dispersal were strongly correlated early in the annual cycle during pre-fledge (Spearman's $\rho = 0.802$, $P < 0.001$) and post-fledge (Spearman's $\rho = 0.838$, $P < 0.001$), but had a stronger longitudinal component during winter (Spearman's $\rho = 0.430$, $P < 0.001$), indicating that later stages of the annual cycle were linked with diffuse viral flow. Our banding data (Fig. 1a) support this by demonstrating that waterfowl from Minto Flats diverge in their migratory pathway, flying either south (along the Pacific Flyway) or south-east (along the Central/Mississippi flyway).

### Subtypes are equally represented in interseasonal transmissions

Subtypes of HA and NA were investigated to assess differences in the frequency of interseasonal transmission, with the exception of H13 and H16, which are gull-associated and under-represented in our study. HA subtype had a weakly significant effect on interseasonal transmission (d.f. = 11,

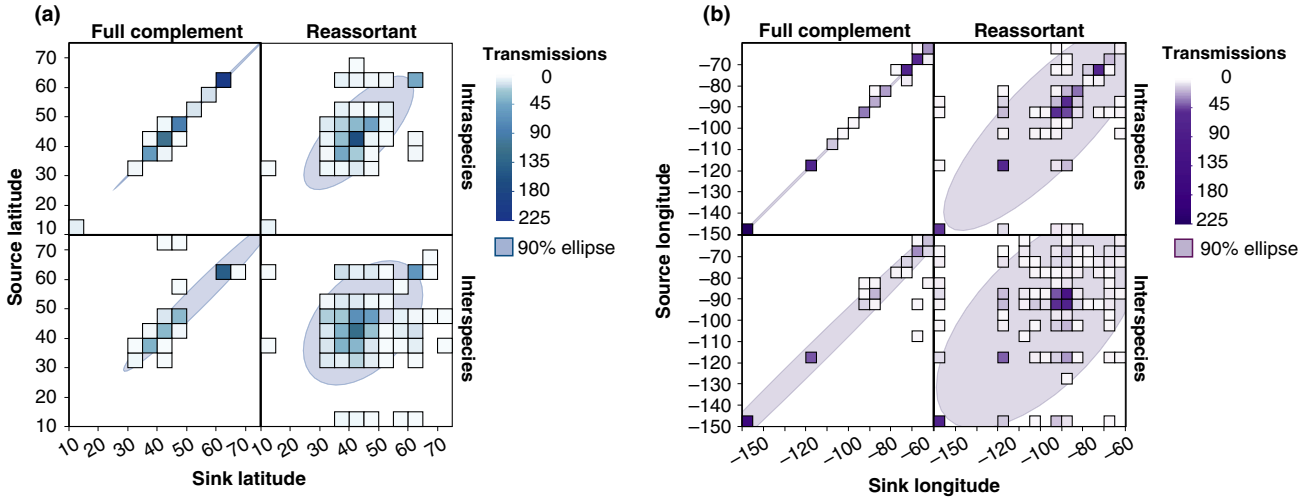


**Figure 5** Spatial pattern of viral transmissions between wild birds in North America, 2008–2011 according to (a) latitude and (b) longitude. Heatmaps indicate the number of transmission events highlighted by colour (blue: latitude, purple: longitude) and the shaded areas (90% ellipse) where transmissions are concentrated. Full complement transmissions are localised both latitudinally and longitudinally, indicated by the source and sink locations overlapping. In contrast, transmissions involving reassortment are more spatially diffuse. The combination of reassortant and interspecies transmission resulted in greatest dispersal both latitudinally and longitudinally.

$\chi^2 = 30.48$, $P = 0.001$) due to the higher propensity of H9 for interseasonal viral flow compared to other subtypes (Fig. 6). NA subtype did not significantly affect interseasonal transmission (d.f. = 8, $\chi^2 = 10.55$, $P = 0.228$), indicating specific NA subtypes were not more commonly involved in interseasonal transmission than others (Fig. 6). Both reassortment (d.f. = 1, $\chi^2 = 289.59$, $P < 0.001$) and interspecies transmission (d.f. = 1, $\chi^2 = 99.52$, $P < 0.001$) were significantly associated with interseasonal transmissions for both HA and NA subtypes.

## DISCUSSION

Wild birds that breed at high latitudes typically move seasonally between habitats, a process that may either link viral pools or present barriers to viral dispersal (Altizer *et al.* 2011). Network analysis of viral sequences from wild birds across North and Central America identified transmission consistent with connectivity of successive seasons throughout the annual cycle, from pre-fledge to winter. Constraints associated with seasonal changes in habitat and host populations may nonetheless act as a barrier to IAV transmission, as indicated by experimental studies that demonstrate RNA viruses are less efficient at replicating after introduction into a novel host population (Dennehy *et al.* 2010). However, our findings highlight a mechanism by which IAV achieves interseasonal transmission involving reassortment of viral genes, accompanied by increased transmission between different bird species. While the majority of waterfowl studies from North America (Stallknecht *et al.* 1991; Krauss *et al.* 2004) and Europe (Munster *et al.* 2007; Wallensten *et al.* 2007) indicate viral flow from high to low latitudes during the autumn, there is substantially less evidence for virus dispersal in the reverse direction during the spring. The spring migration, when birds travel from low to high latitudes, coincides with a decline in prevalence (Wallensten *et al.* 2007; Krauss *et al.* 2010), yielding fewer virus isolates. By considering multiple annual cycles over a 4-year period, our approach compensates for the lack of spring isolates. Assuming all locations along the migratory route have been adequately sampled, this study provides a quantitative estimate of virus flow with evidence for strong north-south transmission, followed by weaker transmission in the opposite direction.

We tested whether within-host replication (full complement/reassortment) and host-to-host transmission (intraspecies/interspecies) parameters were constant or changed relative to the annual cycle and identified marked seasonal variation in IAV transmission. The prevailing transmission strategy switched from intraspecific to interspecific and full complement to reassortment after birds migrated from breeding sites to lower latitudes. Our estimate of relatively high reassortment in autumn (51.49%) is similar to the 56% detected by Wille *et al.* (2013) in mallards sampled during the autumn migration in Sweden. By comparing all seasons, we observed the peak in reassortant (68.84%) and interspecies transmission (69.94%) to occur in winter. Wintering sites concentrate waterfowl from different breeding origins (Hill *et al.* 2012a), facilitating mixing of virus pools and offering an opportunity for IAV to jump between bird species and undergo genomic reshuffling. Studies of wintering sites are consistent in reporting that IAV prevalence in ducks reaches a critical low in late winter, a consequence of the increasing number of birds that develop anti-influenza antibody (De Marco *et al.* 2003; Ferro *et al.* 2010) combined with cell-mediated responses. Reassortment of surface glycoproteins, HA and NA, is crucial for helping IAV to side-step recognition by antibodies generated by hosts previously exposed to IAV (Steel & Lowen 2014). This study highlights the importance of reassortment for enhancing transmission in winter when antigenic shift may be key to avoid extinction.

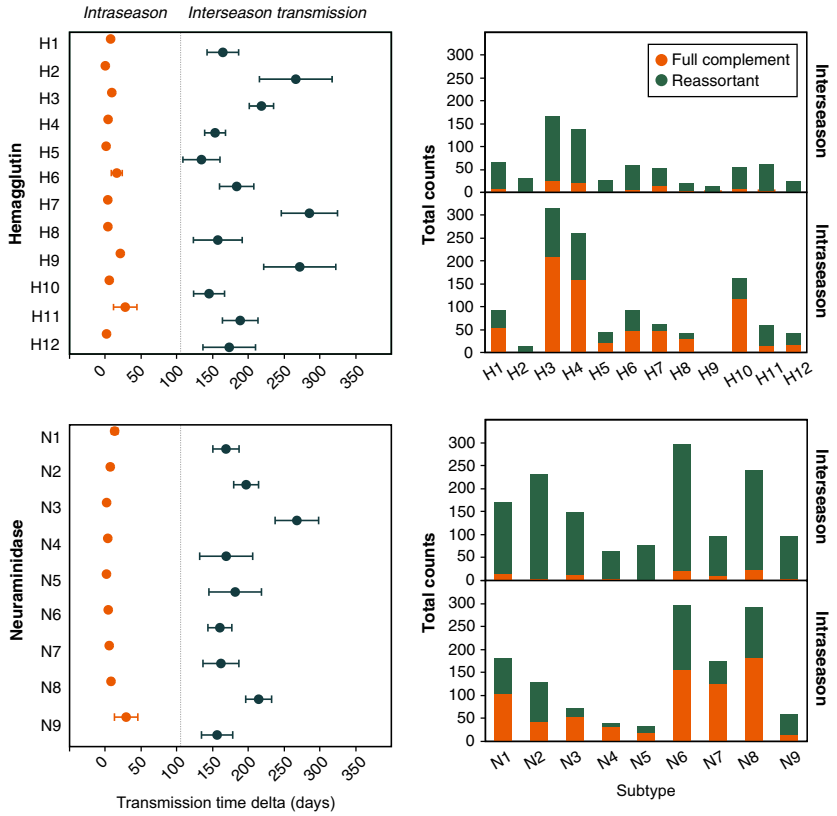


**Figure 6** Frequency of interseasonal and intraseasonal transmissions across hemagglutinin and neuraminidase subtypes in North American wild birds (2008–2011). Round markers (left panels) indicate the mean transmission duration/time delta (days) between source and sink transmissions, with standard error (whiskers) and the dashed line indicates the approximate cut-off between intra- and interseason transmissions. Stacked bar charts (right panels) indicate a lack of subtype effect on the number of interseasonal transmissions but a strong association with reassortant virus.

Identifying when and where reassortment and interspecies transmission of IAV peaks along the migration pathway has implications for surveillance. Reassortment of IAV in nature has been associated with high densities of wild birds at both breeding and wintering grounds (Chen & Holmes 2009). Our study refines this by identifying increasing reassortment of IAV after ducks leave the breeding ground, reaching a peak in winter. Flooded agricultural fields (Ackerman *et al*. 2006) and intensively managed wildlife refuges (Dahl & Johnson 1991) increasingly serve as the principal habitat for waterfowl during the winter, the season when transmission is primarily interspecific and reassortment predominates. Reassortment often precedes transfer of avian influenza genes to domestic animals by allowing bulk changes in the IAV genome that prove advantageous for viral entry and replication in domestic hosts (Steel & Lowen 2014). Eurasian-origin H6N1 (Bahl *et al*. 2009) and more recently HPAI H5N2 (Pasick *et al*. 2015) were first detected in poultry farms in California and the Pacific Northwest following a pattern consistent with movement from northern latitude breeding grounds. Multispecies sampling during the winter is therefore critical as this season represents the highest risk of cross-species transmission among waterfowl, often in habitats where the interface with domestic animals is large.

In contrast to other seasons, the prevailing transmission dynamic during summer involved intraspecific transmission of full complement IAV. The breeding season is associated with the peak in influenza prevalence in wild ducks (Munster *et al*. 2007) due to the birth pulse that annually boosts the number of immunonaive individuals in the population (van van Dijk *et al*. 2013; Wilson *et al*. 2013). In this study, the summer was divided into two stages that distinguished the life-history stages of nesting, brood-rearing and moulting (pre-fledge) from autumn staging (post-fledge) to provide better resolution on infection dynamics relative to changes in host mobility and behaviour. After fledge, a minor increase in interspecies transmission of full complement virus was detected at Minto Flats compared to pre-fledge. Redistribution of birds triggered by staging in late August/early September results in brood groups vacating their natal area and flocking together, changing the contact structure between species prior to migration. During autumn migration and winter, ducks were involved in transmission networks with greater connection to other species, attributable to co-mingling at stopovers or more developed adaptive immune responses as juveniles matured, or likely a combination of both. Exposure to viral strains from birds during breeding may prime the immune system for intraspecific strains, leaving birds primarily susceptible to strains shed by different species later in the annual cycle.

This study supports a model of amplification during the summer sourced by viruses overwintering from the prior breeding season and virus introduced from a wide range of

latitudes. Evidence of low-level environmental persistence of virus at Minto Flats is supported by Ito *et al*. (1995) approximately 20 years earlier at the same site. We did not experimentally test thermostability of viruses implicated in overwintering in Alaska and therefore persistence was deduced rather than directly tested. However, persistence of IAVs in the environment has been demonstrated at high latitudes in Alaska (Lang *et al*. 2008) and the temporal signature of transmission identified in this study was consistent with persistence in an abiotic reservoir, given the minimal change in viral genotype (99.89–100% PWI across eight segments). Consensus exists between field and experimental studies that environmental persistence, even at low levels, plays a critical role in episodic viral transmission between host populations (Breban *et al*. 2009). Our study provides additional insights by indicating that environmental persistence acts on a small number of whole virions (0.85%) during the Alaskan winter, but four times as many overwintering viruses were detected as reassortants. A change in viral genotype, involving integration of gene segments imported from a range of lower latitudes, may be important for enhancing viral fitness in the new breeding population.

Understanding how transmission dynamics change relative to the annual cycle may help to prioritise the timing and location of surveillance in response to introduction of novel strains. Migration of breeding birds into Alaska has been implicated in the periodic introduction of Eurasian-origin IAV including H6 (Bahl *et al*. 2009), H9 (Ramey *et al*. 2015) and most recently, highly pathogenic H5N8 (Lee *et al*. 2015). Sampling hatch-years during autumn staging may offer the best approach for early detection of genes that amplify at high latitudes and ultimately diffuse throughout successive seasons via reassortment, spreading both latitudinally and longitudinally. Phylogenetic studies have identified a pattern of gene flow between breeding and wintering sites (Pearce *et al*. 2009; Lam *et al*. 2012) although our findings suggest movement of IAV may not strictly adhere to diffusion along latitudinal gradients designated by a single flyway (Bahl *et al*. 2013). Dispersal of virus had an increasingly longitudinal component as migratory pathways diverged after breeding and the population followed multiple flyways, providing a mechanism for rapid diffusion of virus spanning east to west. We did not observe a strong effect of subtype on cross-seasonal transmission, suggesting that zoonotic subtypes such as H5 and H7 did not have a higher chance of overcoming seasonal barriers to transmission, relative to other subtypes. Therefore, targeted subtype-specific surveillance in wild birds in North America may overlook subtypes with equivalent capacity to reassort and increase viral diversity.

## ACKNOWLEDGEMENTS

We thank Mike Petrula (Alaska Department of Fish & Game) for leading fieldwork at Minto Flats and the numerous technicians who assisted. We are indebted to Florian Aldehoff for coordinating screening of samples and database management for the entire study. Thanks to Danny Bystrak (Bird Banding Laboratory, U.S. Geological Survey) for supplying banding data. Thanks to Vivien Dugan and David Wentworth at JCVI for viral sequencing. Critical review by John Pearce and Andrew Ramey of the U.S. Geological Survey, and anonymous reviewers helped to improve this manuscript. This study was supported by the U.S. Fish and Wildlife Service (K. Trust, Alaska Region, Migratory Bird Management), Delta Waterfowl Foundation, and the Institute for Wetland and Waterfowl Research, NIH: CEIRS - HHSN272201400008C (current); and HHSN266200700010C (2007-2014) and UAF: INBRE - 5P20RR016466 from the National Center for Research Resources (NCRR) of NIH. Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

## AUTHORSHIP

All authors agree to submission of this manuscript. NH, EM, BM, JR conceived the study. BM, ML, WB performed fieldwork and advised on avian ecology. JR, WB contributed sequence data. EM performed network analysis. NH performed statistics and GIS. NH wrote the manuscript with input from all authors.

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Editor, Bernd Blasius
Manuscript received 24 March 2016
Manuscript accepted 4 May 2016