Crossroads of highly pathogenic H5N1: overlap between wild and domestic birds in the Black Sea-Mediterranean impacts global transmission

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Abstract

Understanding transmission dynamics that link wild and domestic animals is a key element of predicting the emergence of infectious disease, an event that has highest likelihood of occurring wherever human livelihoods depend on agriculture and animal trade. Contact between poultry and wild birds is a key driver of the emergence of highly pathogenic avian influenza (HPAI), a process that allows for host switching and accelerated reassortment, diversification, and spread of virus between otherwise unconnected regions. This study addresses questions relevant to the spillover of HPAI at a transmission hotspot: what is the nature of the wild bird–poultry interface in Egypt and adjacent Black Sea-Mediterranean countries and how has this contributed to outbreaks occurring worldwide? Using a spatiotemporal model of infection risk informed by satellite tracking of waterfowl and viral phylogenetics, this study identified ecological conditions that contribute to spillover in this understudied region. Results indicated that multiple ducks (Northern Shoveler and Northern Pintail) hosted segments that shared ancestry with HPAI H5 from both clade 2.2.1 and clade 2.3.4 supporting the role of Anseriformes in linking viral populations in East Asia and Africa over large distances. Quantifying the overlap between wild ducks and H5N1-infected poultry revealed an increasing interface in late winter peaking in early spring when ducks expanded their range before migration, with key differences in the timing of poultry contact risk between local and long-distance migrants.

1. Introduction

Avian influenza viruses (AIV) are RNA viruses (Orthomyxoviridae) that are widespread in nature, primarily replicating in a variety of waterfowl or persisting in aquatic environments (Webster et al. 1992; Swayne, Senne, and Beard 1998). Wild waterfowl are well-known reservoirs for the majority of low pathogenic (LPAI) subtypes and their migratory cycles help to circulate and propagate these viruses over large geographic scales (Webster et al. 1992). Periodic emergence of highly pathogenic avian influenza (HPAI) occurs mostly in anthropogenic settings, specifically in poultry operations where virulent strains evolve without going extinct due to the high density and turnover of birds (Lebarbenchon et al. 2010; Joseph et al. 2017; Dhingra et al. 2018). In 1996, HPAI H5N1 emerged in poultry markets in Guangdong province, southern China (Gs/Gd lineage) causing mortality in poultry and humans and triggering concerns about pandemic transmission (Webby and Webster 2001; Li et al. 2004). After a decade of circulation of HPAI H5N1 clade 2.2.1 in Asia, Africa, and the Middle East, the emergence of clade 2.3.4.4 H5Nx resulted in an unprecedented global expansion of the virus with the first documented introduction into North America (Lee et al. 2015; Hill et al. 2017). The long-distance movement of clade 2.3.4.4 viruses (Tian et al. 2015; Lycett et al. 2016; Lee et al. 2017) provided compelling evidence that migratory birds are important hosts in driving HPAI transmission and highlighted a lack of information regarding the mechanisms for wild-domestic bird spillover that vary widely across the globe.

Understanding where and when wild birds and poultry interact is important in Asia and Africa where numerous HPAI lineages circulate endemically in poultry. While it is characteristic for spillover to occur where poultry density is high and biosecurity is lacking (Dhingra et al. 2018), the interface varies from country to country depending on geography, natural resources, local agricultural practices, and the ecology of wild birds. Studies conducted in Central and East Asia suggest that overlap between wild birds and backyard poultry on the wintering grounds can be predictive of northward dispersal during the spring migration (Newman et al. 2012; Tian et al. 2015). Comingling of domestic ducks and waterfowl in rice fields or wetlands in Asia is one of the most visible examples of how infection can spread between wild and domestic populations (Hulse-Post et al. 2005; Gilbert et al. 2006). However, this is unlikely to be representative of the wild–domestic bird interface in Africa and the Middle East where rice agriculture is limited due to arid conditions and less rainfall (Xiao et al. 2007). The overlap between wild birds and poultry takes many forms and requires granularity of data at the scale of the virus and host to adequately characterize an important goal for understanding interfaces outside of Asia.

Egypt represents a country that is central to the evolution and spread of HPAI by acting as a source and sink for viruses circulating globally (Hosny et al. 1980; Zhou et al. 2016; Selim et al. 2017; Fusaro et al. 2019), as well as supporting established lineages of the virus in poultry (Watanabe et al. 2012; Kayali et al. 2014). Within the African continent, Egypt was the first country where detection of HPAI occurred. Since initial detection of clade 2.2.1 in 2005 from a Common Teal (*Anas crecca*) (Saad et al.

2007), the virus has become endemic in poultry (Naguib et al. 2019). Over a decade later, Egyptian poultry remains an abundant and susceptible host population in which HPAI H5 virus circulates year-round (Abdelwhab et al. 2016) resulting in diversification of the virus into clade 2.2.1, and sub-clades 2.2.1.1a and 2.2.1.2 (Smith et al. 2015). In 2016, the recently emerged HPAI H5N8 (clade 2.3.4.4.) was detected in a Common Coot (*Fulica atra*) and a Common Teal in an Egyptian live bird market (Selim et al. 2017). The virus was closely related to HPAI H5 circulating in Russian wild birds sampled in the Uvs-Nuur Lake region based on genetics of the HA and NA segments (Yehia et al. 2018) highlighting the importance of migratory birds in repeatedly connecting Egyptian and European viral lineages over long distances. The complexity of poultry agriculture in Egypt, including markets that sell live poultry and hunted wild birds as well as backyard farming of mixed poultry (ducks, chickens, geese, pigeons) has created a large and under-studied wild–domestic interface (El-Zoghby et al. 2013).

While there is large potential for overlap between migratory waterfowl and domestic poultry in Egypt, little is known about the movements of wild birds or the degree and seasonality of poultry contact risk. Egypt is located at the intersection of the Black Sea-Mediterranean and East African-West Asian Flyways making it key habitat for a wide variety of waterfowl that connect Africa and Eurasia (Scott and Rose 1996; Boere, Galbraith, and Stroud 2006). Within this context, we hypothesize that contact between wild birds and poultry peaks on the wintering ground due to the density of poultry at overwintering habitat, a process consistent with transmission dynamics in Asia. We further hypothesize that the precise timing of contact risk is governed by species-specific migration strategies of ducks that can differ widely. We tracked three waterfowl species in Egypt: Common Teal, Northern Shoveler (*Anas clypeata*), and Common Shelduck (*Tadorna tadorna*) with satellite telemetry and conducted a spatiotemporal analysis of the wild–domestic interface in the region. The aims of our study were to: (1) evaluate the potential of wild birds to spread IAV within and beyond the Black Sea-Mediterranean and East African-West Asian Flyways (henceforth collectively referred to as the 'Black Sea-Mediterranean Flyway'); (2) characterize local and migratory movements of wild birds in this region; and (3) investigate overlap between wild birds and poultry outbreaks and detect seasonal or species-dependent patterns in contact risk.

2. Methods

2.1 Capture, sampling, and marking

We captured birds in Egypt during January and November 2009 and in Turkey in February 2010 to coincide with the overwintering period of these migratory species. In Egypt, birds were captured at Lake Manzala near Port Said (31.279°N, 32.173°E) and on Lake Qarun near Faiyum (29.475°N, 30.665°E). Lake Manzala represents the largest of Egypt's Mediterranean wetlands located on the Nile Delta of lower Egypt. Lake Qarun is the third largest lake in Egypt and is situated 80 km southwest of Cairo. In Turkey, birds were captured in the Kizilirmak Delta (41.662°N, 36.014°E), the largest and most intact wetland on the Black Sea coast. All sites are recognized as 'Important Bird Areas'

according to Birdlife International ([BirdLife 2020](#)) because of the large number of waterbirds they attract.

Birds were captured with leg nooses (monofilament loops attached to wooden sticks connected with nylon cord in lines of 50–100 nooses) or with mist nets, whoosh nets, or clap traps. Upon capture, we recorded sex, age, weight, culmen, flat wing, and diagonal tarsus. Cloacal and oral swabs were collected on rayon-tipped swabs (MicroPur™, PurFybr Inc., Munster, IN). Swabs were preserved in cryovial tubes containing viral transport media (VTM), and samples were stored on ice for up to 8 h before storage in a −80 °C freezer prior to analysis.

A subset of captured birds that appeared in good condition based on weight and physical appearance were marked with either: 9.5 or 12 g platform terminal transmitters (PTTs: Microwave Telemetry, Columbia, MD, USA); or 22 or 30 g GPS solar-powered PTTs. The PTTs were attached to each bird with a Teflon® backpack harness (Bally Ribbon Mills, Bally, PA, USA), with packages weighing <3.1 percent of the birds' body mass. Birds were released near capture locations within 3 h of processing. PTTs were programmed to record six to twelve locations per day.

In Egypt, a total of ninety-two ducks were captured including: thirty-three Common Teal (*Anas crecca*), twenty-nine Northern Shoveler (*Anas clypeata*), twenty-three Common Shelduck (*Tadorna tadorna*), five Northern Pintail (*Anas acuta*), and two Mallard (*Anas platyrhynchos*). A subset of forty-five ducks (twenty-three Common Teal, eleven Common Shelduck, and eleven Northern Shoveler) were outfitted with transmitters to track their migratory movements. In Turkey, a total of eighty-one ducks were captured including: seventy-two Common Teal, two Northern Shoveler, two Northern Pintail, two Mallard, two Eurasian Wigeon (*Anas penelope*), and one Common Pochard (*Aythya ferina*). Of these birds, twenty were fitted with satellite PTTs: twelve Common Teal, two Northern Pintail, two Eurasian Wigeon, two Northern Shoveler, one Common Pochard, and one Mallard.

All applicable institutional and/or national guidelines for the care and use of animals were followed. Capture permits were obtained from the relevant authorities in Egypt (Egyptian Environmental Affairs Agency) and Turkey (Samsun Province Directorates of Agriculture). The US Geological Survey Animal Care and Use Committee and the University of Maryland Baltimore County Institutional ACUC approved the procedures used for capture, handling, and marking (Protocol EE070200710).

2.2 Influenza Screening and Phylogenetics

Viral RNA was extracted from swab samples using the MagMax-96 Viral Isolation Kit (Ambion Inc.) and screened using real-time reverse transcription-polymerase chain reaction (rRT-PCR) targeting the matrix (M) segment ([Spackman and Suarez 2008](#)). PCR-positive samples (C_t value $\leq$ 40) were used to generate amplicons for direct sequencing or as template for generation of cDNA ([Hoffmann et al. 2001](#)). Due to limited quantities of RNA or cDNA as starting template, only primers targeting the hemagglutinin (HA), neuraminidase (NA), nonstructural (NS), or M segments were used to amplify sample for Sanger sequencing. Sequence reads were assembled and contigs were submitted to the GISAID database (accession numbers listed in [Supplementary Table S1](#)). The majority of HA and NA sequences were <1,000 nucleotides, and none represented full-length open reading frames (ORFs). In contrast, most samples yielded M and NS sequences that were complete or nearly full-length ORFs.

To identify the strains and genotypes of viruses, sequences were analyzed against all viruses in the Global Initiative on Sharing All Influenza Data (GISAID) using BLASTn and the top 200 hits from each search were used as reference taxa. Alignments were performed using default settings in MUSCLE v.3.8.31 ([Edgar 2004](#)). The size of taxon sets for each segment were as follows: H1: $n = 323$, H7: $n = 329$, H10: $n = 346$, N1: $n = 262$, N7: $n = 278$, N9: $n = 182$, M: $n = 332$, NS: $n = 230$. Maximum-likelihood phylogenetic trees were constructed using RAxML v8.2.12 ([Stamatakis 2006](#)) and employing the GTRGAMMA substitution model. A general time reversible model was chosen to account for the complexity of the evolutionary process that governs how influenza viruses mutate. The best ML tree was selected from 1,000 independent searches and bootstrap values indicated support for each bifurcation. The closest ancestral strain was used to infer the migratory Flyway from which each viral segment was associated.

To identify the most recent common ancestor of the putative HPAI segments, the phylogenetic origins of two internal segments: M and NS were analyzed. Datasets included samples from Egyptian waterfowl in this study as well as reference sequences from GISIAD down-sampled according to geography and subtype. Search criteria included complete sequences from avian hosts for which the collection year was known. Data were down-sampled to 85–100 taxa per geographic region (six categories: Egypt, Sub-Saharan Africa, Black Sea-Mediterranean Flyway, East Atlantic Flyway, Central Asian Flyway, and the East Asian Flyway) and stratified by subtype (HA-NA) to ensure a wide representation of subtypes. Trees were reconstructed using samples collected between 1959 and 2019 to ensure accurate rooting for the tree topology, but only sequences from 2000 onward were included in the phylogeographic analysis. This allowed us to analyze IAV evolution over a more recent time period that also represented the bulk of data. The size of the taxon sets was M: $n = 595$ and NS: $n = 591$.

Phylogenies were reconstructed using a GTR nucleotide substitution model with gamma distribution of substitution rates, a GMRF Skyride coalescent model and an uncorrelated lognormal clock ([Supplementary Figs S1 and S2](#)). Analysis was performed using BEAST 1.8.4 ([Drummond et al. 2012](#)). Four independent Markov chain Monte Carlo (MCMC) runs of 200 million chains were performed for each segment. Runs were combined to ensure an effective sample size (ESS) of at least 200 was achieved and the maximum clade credibility tree was determined. A subset of the last 500 trees from the posterior distribution was used to generate an empirical tree for performing the subsequent phylogeographic analysis, an approach which reduced computational time and burden.

To evaluate the diffusion of virus between migratory Flyways, a discrete trait model was performed based on six geographic regions relevant to the study population: Egypt, Sub-Saharan Africa, Black Sea-Mediterranean Flyway, East Atlantic Flyway, Central Asian Flyway, and the East Asian Flyway. This allowed Egypt to be evaluated as a source or sink of IAV within a global context and compare with results from satellite telemetry tracking of Egyptian waterfowl. A Bayesian stochastic search variable selection (BSSVS) was used to summarize the diffusion rates and Bayes factors were estimated using SPREAD v1.0 ([Bielejec et al. 2011](#)). Rates were considered statistically supported when BF >3.0, strong support when BF > 10, and decisive support when BF >100 ([Jeffreys 1961](#)).

2.3 Utilization distributions of ducks

We analyzed telemetry data for two periods: winter and spring migration. A total of twenty-four birds (26,946 locations) with transmitter signal lasting >31 days were used in the analyses

(Supplementary Table S2). This included three species: Common Teal, Common Shelduck, and Northern Shoveler. The winter period extended from the time of capture until the bird migrated from the area. The spring period extended from the time after the bird left the wintering area until the bird arrived at a breeding area or signal loss (due to PTT failure, transmitter loss, or mortality). Using the telemetry data, utilization distributions (UD) in three dimensions were generated for each duck species (Newman et al. 2012). Each UD spanned 14 days to encompass the 7 days asymptomatic period estimated from experimental studies of waterfowl (Brown, Stallknecht, and Swayne 2008; Fereidouni et al. 2009; Kwon, Thomas, and Swayne 2010) and the estimated 7 days when the virus could persist in freshwater (Stallknecht and Brown 2009). For both the winter and spring, we extracted the 99 percent contour intervals of the UDs to represent wild bird home ranges. For winter, 19 birds marked in Egypt (5 Common Teal, 7 Common Shelduck, and 7 Northern Shoveler) had sufficient locations for spatial analyses (Supplementary Table S2). Birds marked in Turkey were not used for the winter analysis due to the relatively late capture of these individuals. Marked birds with incomplete migrations were excluded from the spring analysis, resulting in sufficient data for Common Teal ($n = 3$, two marked in Turkey and one marked in Egypt) and Common Shelduck ($n = 3$, all marked in Egypt), however insufficient data was available for Northern Shoveler.

2.4 Analysis of the duck–poultry transmission interface

We obtained data on HPAI H5N1 virus outbreaks in poultry that coincided and extended beyond the satellite tracking period of marked wild ducks (January 2005–October 2012) from the FAO EMPRES-i Database (http://empres-i.fao.org). Outbreaks were defined as reported disease events within a specific location that ranged from 1 to multiple infected bird hosts. This 8-year time frame was selected to characterize the long-term pattern of outbreaks in a region where under-reporting is a known challenge and therefore minimize the potential for false negative associations between outbreaks and wild bird movements. Additionally, this time frame allowed us to analyze outbreak data relevant to the emergence of HPAI H5N1 in 2005. Outbreaks were selected based on overlap with minimum convex polygons (MCPs) calculated for all ducks in the study. This represented the largest domain where an outbreak could have involved wild birds from our study. The MCPs were created with Home Range Tools extension in ArcMap 9.3.1 (ESRI, CA, USA) and the Aggregate Polygons tool was used to expand the MCP to encompass the extent of all home ranges. We quantified the number of outbreaks within the UDs compared with the number of outbreaks outside the UD (but inside the MCP) according to 14-day periods. Using 1-sample, exact binomial tests in SAS 9.3 (SAS Institute Inc., Cary, NC), we determined whether the proportion of outbreaks inside the UD differed significantly from outside the UD compared to expected values ("null proportion"). The null proportion was based on the proportion of the UD area compared to the MCP area. We performed tests for each 14-day period and values of $P < 0.05$ were considered significant.

3. Results

3.1 Detection of viral segments with highly pathogenic ancestry

In Egypt, a total of eighty-one ducks were tested for AIV of which eleven (13.6%) were positive (Supplementary Table S3)

including Northern Shoveler, Northern Pintail, and Common Shelduck. Positive birds did not present with clinical symptoms during handling. In Turkey, eighty-one birds were tested of which one unmarked Northern Pintail was positive (1.2%). We found evidence of shared ancestry with HPAI H5N1 in the internal and surface protein segments of viruses shed by Egyptian ducks. Of the eleven viruses that were sequenced, four contained segments (NA, M, or NS) related to HPAI H5N1 based on maximum likelihood phylogenetic analysis (Fig. 1). The N1 and NS segments were most closely related to viruses from clade 2.3.4 HPAI H5 infecting domestic and wild birds in East Asia between 2005 and 2010 (Supplementary Fig. S3). In contrast, the M segment was most closely related to clade 2.2.1 circulating in Egyptian poultry (Supplementary Fig. S3). One virus (SM-049) consisted of segments related to both clade 2.2.1 and 2.3.4 indicating the potential for reassortment between HPAI lineages (Fig. 1). For all the putative HPAI H5N1 segments detected, the HA subtype could not be determined due to lack of swab samples; therefore, the possibility that these are HPAI H5 cannot be excluded or confirmed.

3.2 Molecular dating of highly pathogenic ancestry

To investigate the timing and source of HPAI H5N1 ancestry, the internal segments were analyzed using Bayesian methods. Molecular dating indicated that a potential reassortment event between July 2007 and November 2008 (95% HPD, posterior: 0.87) involving HPAI H5N1 from Egyptian poultry (Supplementary Fig. S4). This time period coincided with the overwintering of waterfowl in Egypt and occurred a few months prior to our sampling effort, supporting the possibility of spillover across the domestic–wild bird interface. Therefore, the virus (A/Northern Shoveler/Egypt/SM-049/2009) may have emerged due to reassortment between HPAI H5N1 that circulated endemically in Egyptian poultry (clade 2.2.2) and LPAI infecting wild birds. All other internal segments ($n = 3$) with putative HPAI H5N1 ancestry were related to clade 2.3.4 that emerged in East Asia between July 2005 and September 2006 (95% HPD, Supplementary Fig. S4). The HPAI H5N1 origin of these reassortment events was supported by relatively weak posterior probabilities (0.02–0.38) indicating ambiguity owing to a lack of sequence data. Shared ancestry with clade 2.3.4 HPAI H5N1 was consistent for three viruses sampled from Egyptian ducks (Supplementary Fig. S4). As a result, we infer that the evolutionary history of these viral segments was highly similar, potentially involving the same viral ancestor, however the precise origin cannot be estimated.

3.3 Low pathogenic viruses and geographic origins

We were able to identify three LPAI virus subtypes: H1N1, H7N7, and H10N9 circulating in the duck population. The HA segments of LPAI subtypes were primarily acquired locally within Egypt or the Black Sea-Mediterranean Flyway (Fig. 1). In contrast, the NA segments primarily originated from the East Asian or East Atlantic Flyway. Phylogenetic analysis of internal segments (M, NS) using Bayesian methods suggested these viruses from Egyptian wild ducks had diverse evolutionary histories involving multiple Flyways across Eurasia, distinctly different from a signal of sustained, local transmission (Fig. 2). Rarely did viral segments have the same common ancestor as indicated by the lack of clustering among these viruses for the NS and M phylogenetic trees. This pattern suggested that while these birds were sampled within 7 days from the same or adjacent wetlands within the Nile delta, their viruses had diverse origins
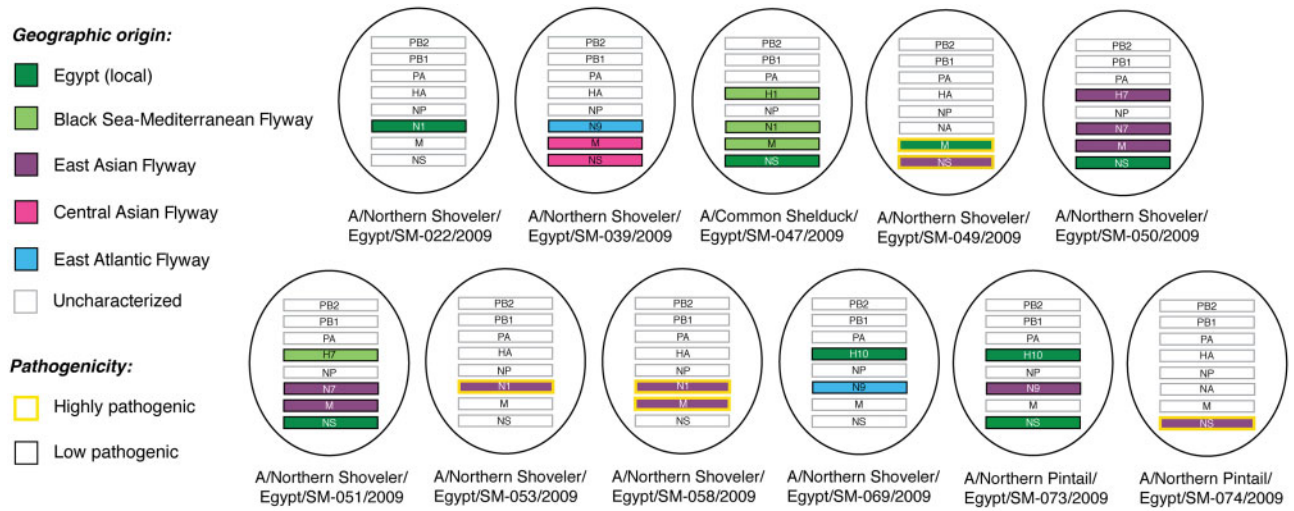
Figure 1. Genotypes for the 11 influenza A viruses detected in wild waterfowl, Egypt (January 2009). Segments are color coded according to geographic origin and pathogenicity (high or low pathogenic) based on phylogenetic tree reconstructions (Supplementary Fig. S3): hemagglutin (H1, H7, H10), neuaraminidase (N1, N7, N9), matrix M, and nonstructural (NS) segments.
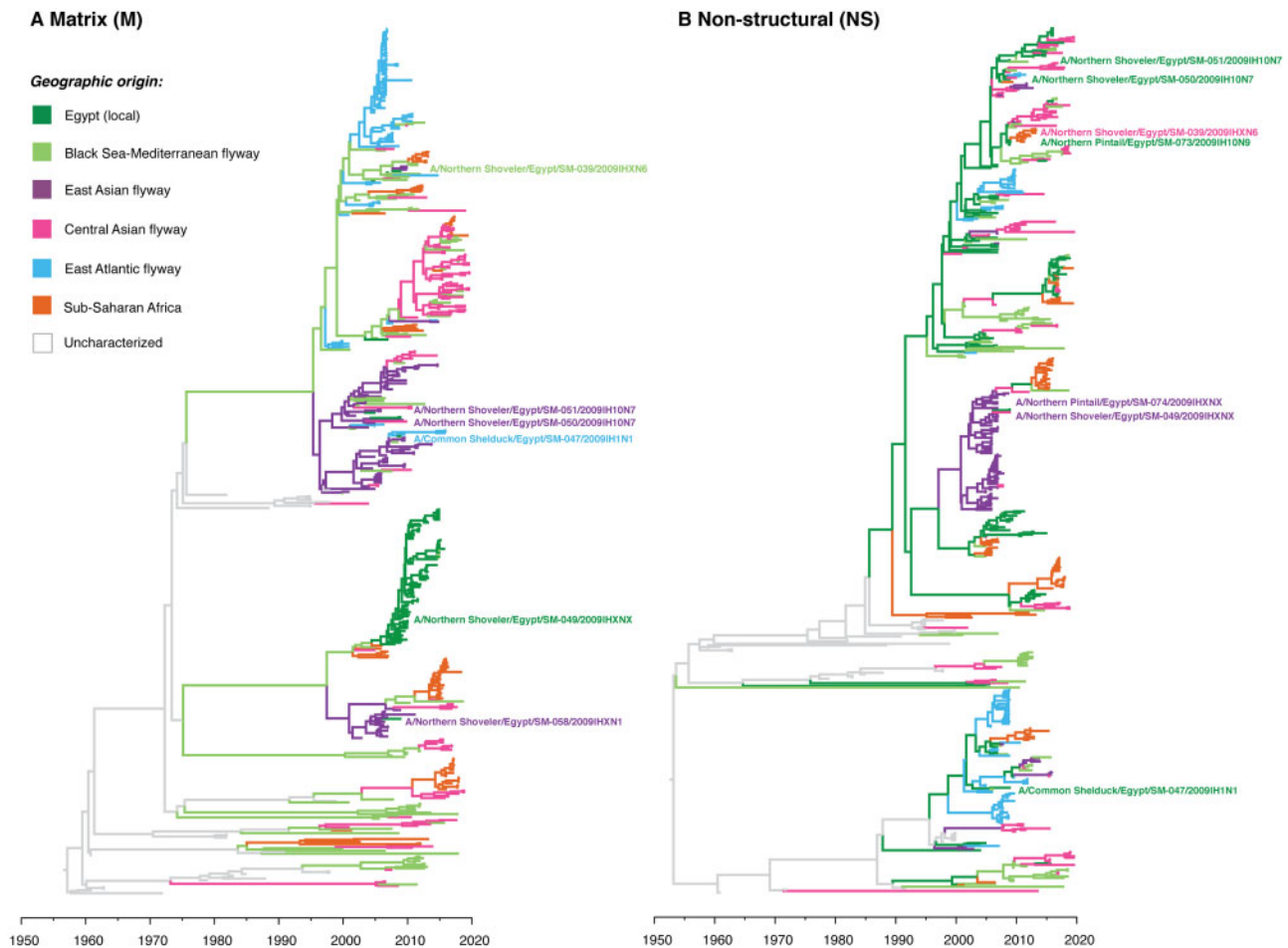

Figure 2. Bayesian phylogenetic trees depicting the ancestral state reconstruction of geographic locations using a discrete trait model for the matrix (M: $n = 595$) and nonstructural (NS: $n = 591$) protein segments. Viruses sampled from Egyptian wild ducks during this study (January 2009) are indicated. Viral sequences collected before 2000 (gray branches) contribute to the tree topology but were not included in the phylogeographic analysis.
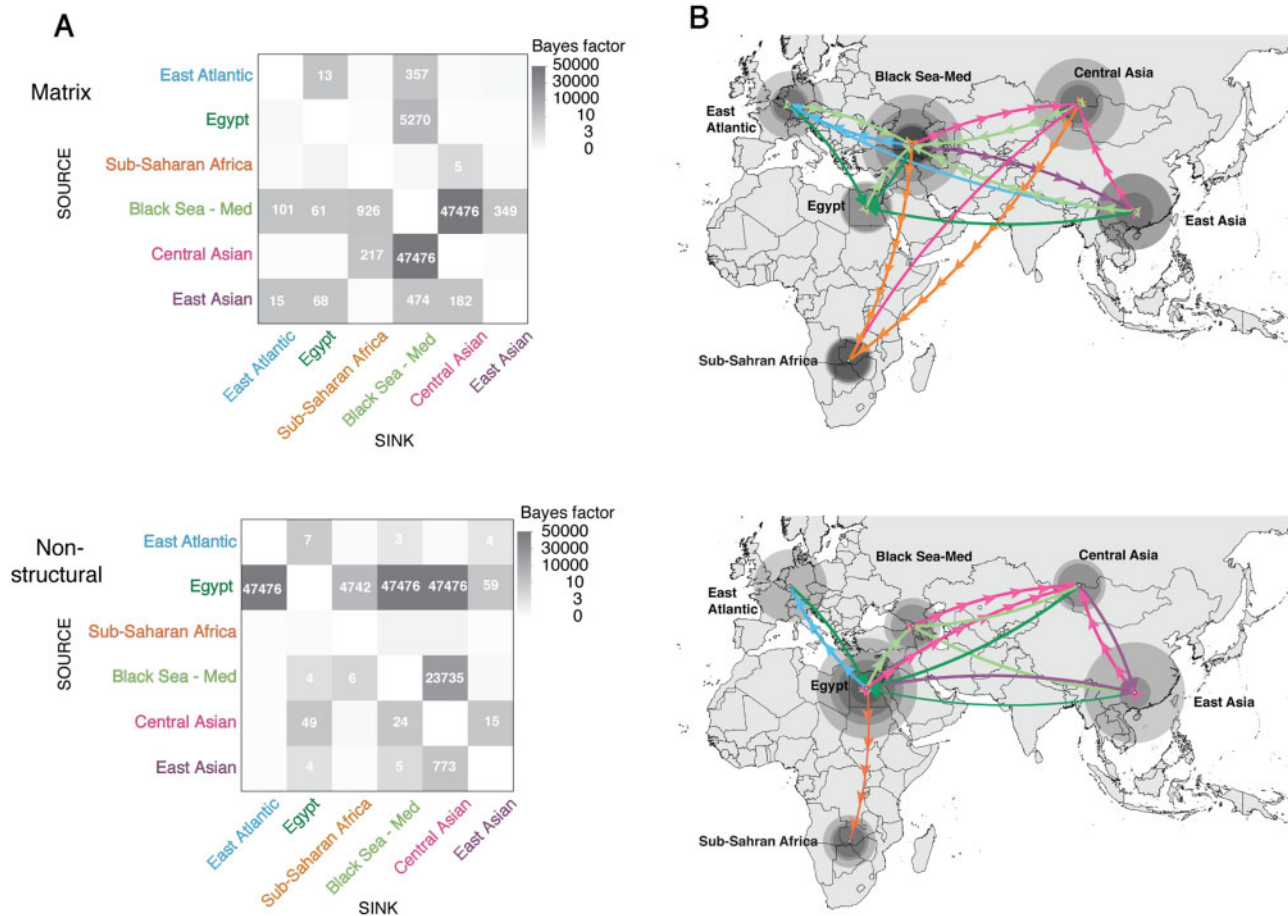
Figure 3. (A) Viral diffusion between geographic locations shown on a matrix where the color of the cell corresponds to strength of support (Bayes factors) for viral movement between each pairwise Flyway/location. The green box indicates virus diffusion from Egypt to the Black Sea-Mediterranean, the same directionality as the satellite tracked wild birds in this study. (B) Egypt and the Black Sea-Mediterranean relative to global diffusion dynamics. Size of polygons around each location is proportional to the number of lineages that maintain that location. Lines are colored by the destination or 'sink' location. Westward movements are depicted by lines with an upward curvature, eastward movements are depicted by lines with a downward curvature.

indicative of long-distance diffusion with frequent exchange of viral segments along the way.

3.4 Regional phylogeographic analysis of viral diffusion

The role of Egyptian wild birds and poultry in the spread of virus between 2000 and 2019 was evaluated relative to regional diffusion using a phylogeographic model of the NS and M internal segments. The pattern of geographic diffusion differed substantially between the two segments (Fig. 3a), indicating largely independent and stochastic evolutionary processes and a lack of co-carriage across M and NS. Egypt and the Black Sea-Mediterranean Flyway emerged as important for connecting viruses between Asia (Central and East Asian Flyways) and Europe (East Atlantic: Fig. 3b). The role of Egypt as a source of virus that seeded the Black Sea-Mediterranean (BF = 47476), Europe (BF = 47476), Central Asia (BF = 47476), Sub-Saharan Africa (BF = 4743), and East Asia (BF = 59) was demonstrated by the NS segment (Fig. 3a).

Additionally, the prominent role of the Black Sea-Mediterranean Flyway as a source of virus for Central Asia (BF = 47476), Sub-Saharan Africa (BF = 926), East Asia (BF = 349), East Atlantic (BF = 101), and Egypt (BF = 61) was demonstrated by the M segment (Fig. 3a). Frequent viral flow from Egypt to the Black

Sea-Mediterranean Flyway (i.e. northward) was well supported by both segments; however, viral flow in the reverse direction (i.e. southward) was less consistently supported (M: BF = 61, NS: BF = 4.3). The Black Sea Mediterranean and Central Asian Flyways were involved in bidirectional viral flow (M: BF = 47476, NS: BF = 24–23735). In contrast, Sub-Saharan Africa and the East Atlantic Flyway were more isolated and less frequently sources or sinks for Asian-origin virus (Fig. 3a).

3.5 Waterfowl migration

Four of the 7 Northern Shoveler marked in Egypt migrated along the Black Sea-Mediterranean Flyway performing medium-distance migrations (Fig. 4). Two birds (91250, 91252) migrated to Turkey. Another individual (91237) flew across Syria and eastern Turkey to eastern Azerbaijan before the signal was lost. A fourth individual (91238) migrated along a similar route, crossing the Caspian Sea with its transmission ending in northern Kazakhstan. The remaining 3 Northern Shoveler stayed in northern Egypt, moving locally between the Damietta and Rosetta arms of the Nile River suggesting these individuals may be resident (Fig. 4).

Five of the 7 Common Shelduck marked in Egypt migrated along the east coast of the Mediterranean Sea performing local or medium-distance migrations (Figs 4 and 5). One bird (46123)
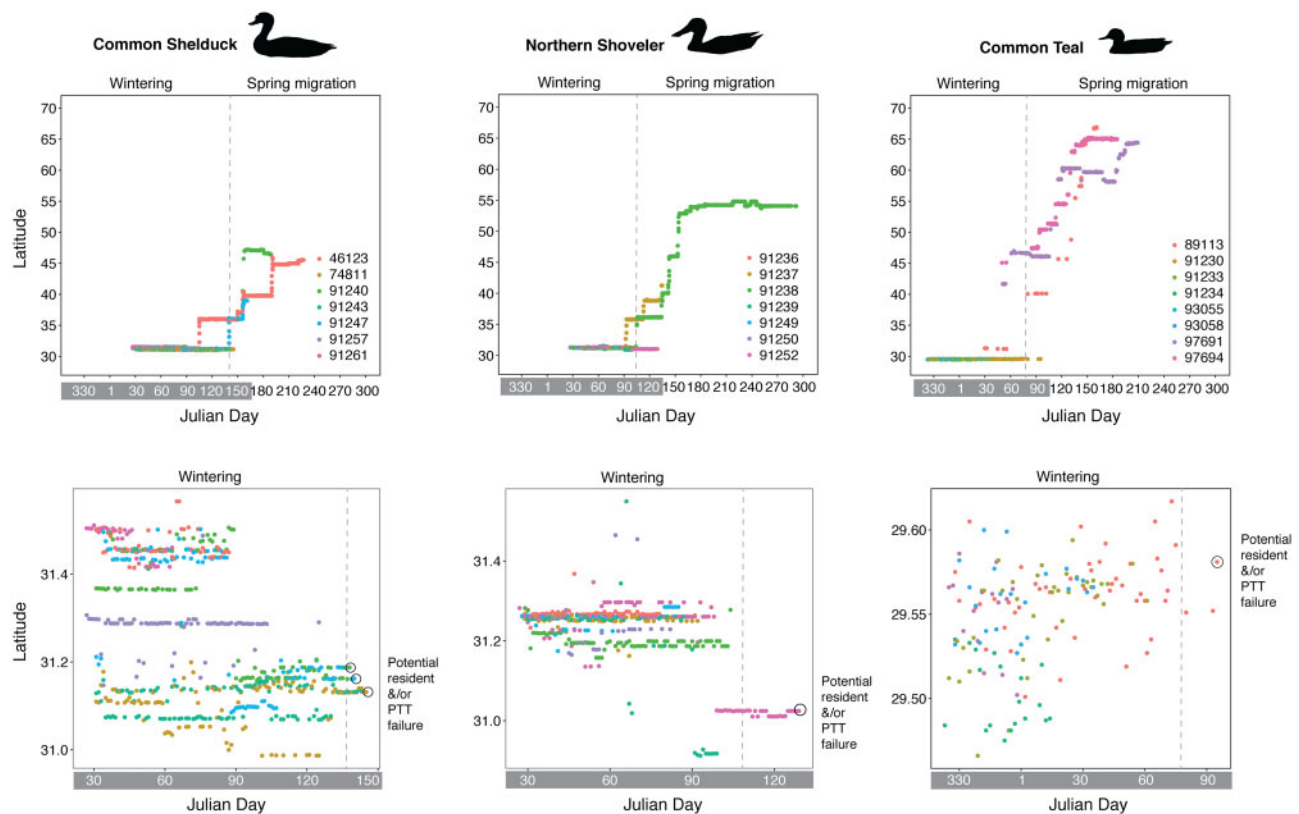
Figure 4. The top panel indicates the locations of 24 waterfowl marked in Egypt and Turkey and tracked with satellite transmitters over the course of the study 2009–2010. For each species, the wintering and spring periods are delineated with a dashed line indicating the median day of departure from the wintering grounds. The gray bars indicate the temporal overlap between the upper and lower panels. The bottom panels indicate birds that remained on the wintering grounds after the start of the median date of spring migration and were considered potential nonmigratory residents as indicated via circled points.

crossing Syria into the Caspian Sea and into western Kazakhstan, while another bird (91240) migrated across the Black Sea into southwestern Russia near the Sea of Asov. One bird (91247) migrated to and remained in Turkey. Two birds (74811, 91243) migrated as far as Turkey before their signals were lost. Their median initiation date (20 May) was the latest of all 3 species, suggesting that they remained longer in their wintering area (Fig. 4, Supplementary Table S4). The remaining 3 Common Shelduck stayed in northern Egypt moving locally between Damietta and Port Said before their signals ceased (Fig. 4).

Common Teal migrated longer distances ($-x = 5{,}993$ km; Supplementary Table S4) and initiated spring migration earlier than the other two species (median date: 21 March). For example, one bird (89113) released from Egypt migrated northeast to the northern limit of the Ob River in Russia (Fig. 4). Four Common Teal (91233, 91234, 93055, 93058) remained near the northeastern and eastern parts of Lake Qarun in Egypt throughout the period during which transmissions were received. One Common Teal (91230) migrated south along the Nile River from Qarun Lake to the Girga area (~500 km south of Cairo) before transmission was lost.

Of the Common Teal marked in Turkey, all four migrated across the Black Sea, to wetlands on either side of the Strait of Kerch where the Black Sea and Sea of Azov meet. From here, their migration paths diverged with two individuals (97691, 97694) moving northeast into the Lower Ob watershed of Russia and the other two individuals (98421, 97693) migrating north to a wetland complex east of Moscow (Fig. 5), where one bird's

signal was lost near the end of the breeding season and the other bird reversed course and returned south to Kazakhstan.

3.6 Duck–poultry interface: effect of season and species

Overlap between wild ducks and poultry outbreaks occurred during both the late winter and the spring migration. However, the onset of the spring migration accounted for a higher degree of overlap between tracked wild ducks and poultry outbreaks along the Black Sea-Mediterranean (Fig. 5). Nineteen birds (5 Common Teal, 7 Common Shelduck, and 7 Northern Shoveler) had sufficient locations for winter spatial analyses (Supplementary Table S1). We excluded 5 Common Teal from analysis because they were captured and marked immediately prior to migration and lacked sufficient winter data. Thus, our winter analyses were restricted to Egypt. During the winter, only a small percentage of the UDs of Common Teal and Common Shelduck were significantly associated with HPAI H5N1 outbreaks in poultry (Fig. 6). For Common Teal, 16.7 per cent (1 of 6) of UDs during the wintering period were significantly associated with poultry outbreaks (Supplementary Table S5). A similar percentage of the Common Shelduck were associated with poultry outbreaks (11.1%: Fig. 6). For the Northern Shoveler, none of their UDs during the winter were associated with poultry outbreaks. For all species, their wintering distributions were centered on coastal habitat away from high poultry density areas located inland. Later in the winter (February–April), the movements of Common Shelduck and Common Teal
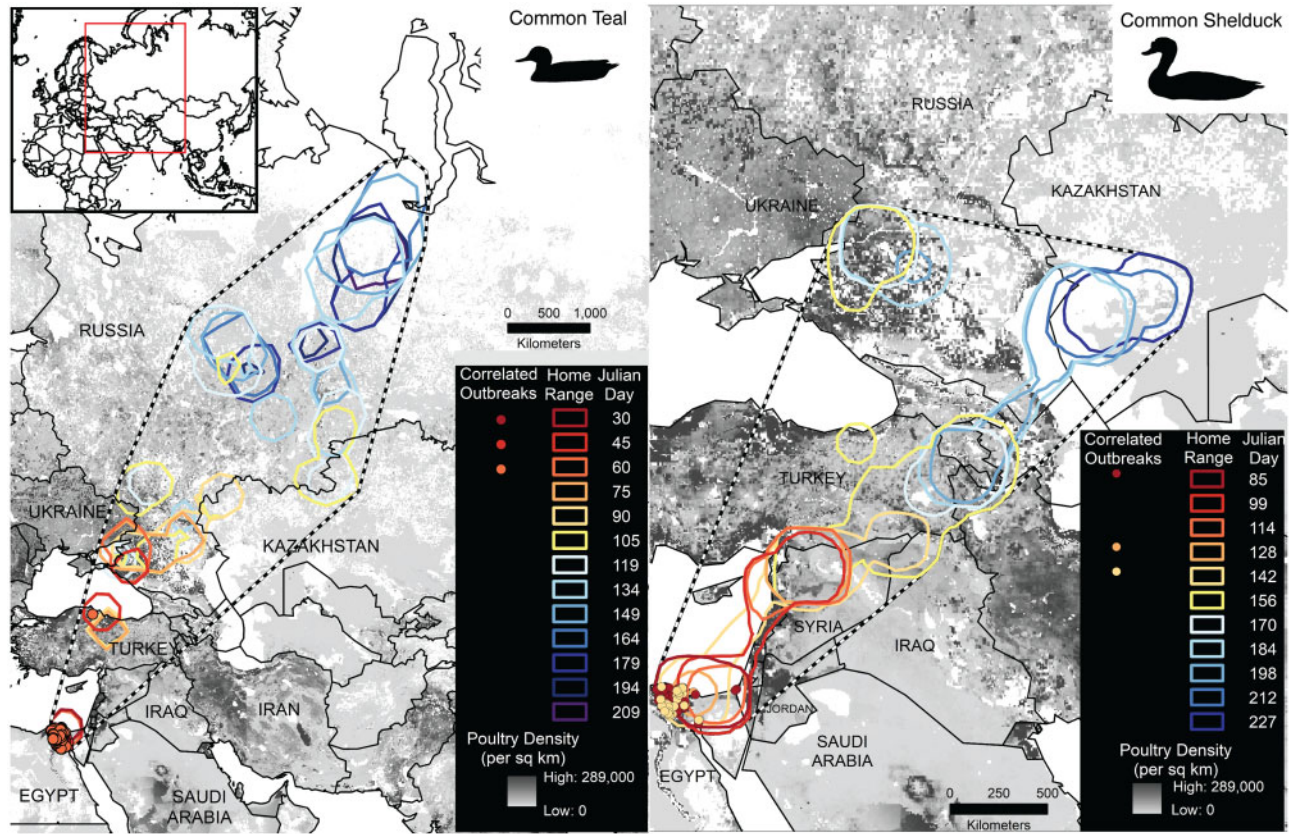
Figure 5. Movements of Common Teal and Common Shelduck in relation to poultry density (gray shading) and highly pathogenic H5N1 outbreaks (round markers). Spring migration is represented by a minimum convex polygon (MCP: dashed line) around all location points for each species. Locations are grouped into 2-week intervals to create biweekly utilization distributions (UDs: colored polygons). Poultry outbreaks that were significantly associated with a UD are indicated by markers color-coded according to time period.
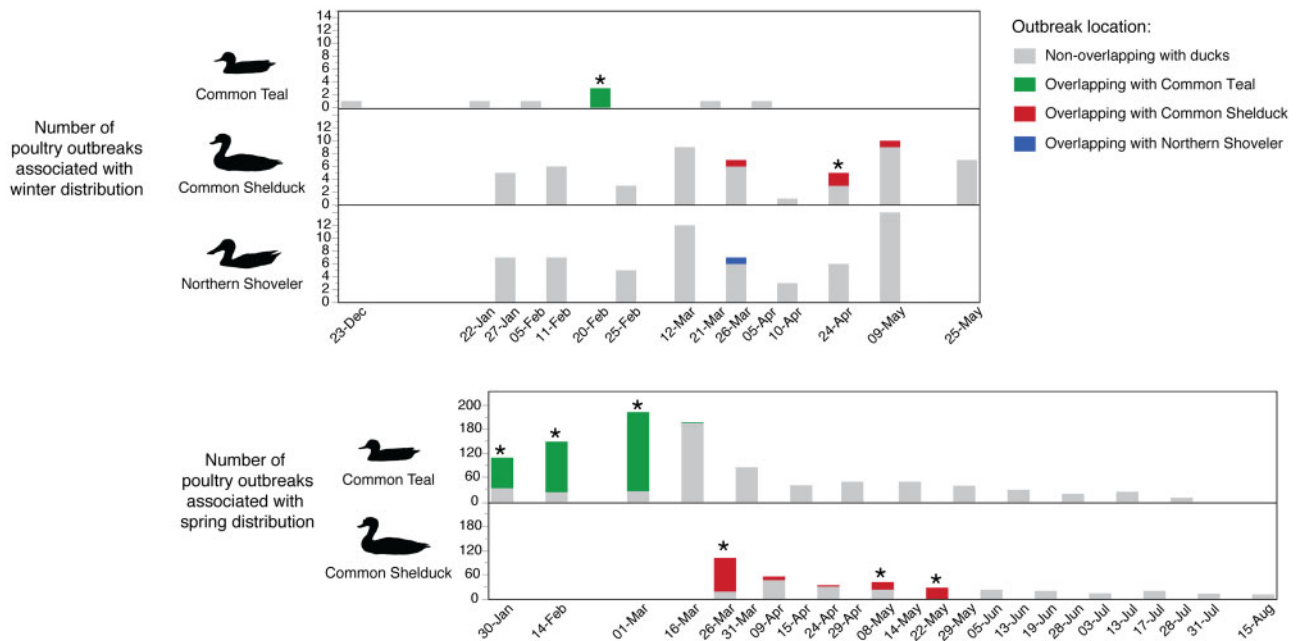

Figure 6. Spillover risk of highly pathogenic H5N1 between wild birds and infected poultry along the Black Sea-Mediterranean Flyway varies between winter (top panel) and spring (bottom panel). The number of outbreaks associated with the utilization distributions of Common Teal (green), Common Shelduck (red) and Northern shoveler (blue) are shown by color-coded stacked bar charts. Time periods associated with a significant overlap between waterfowl and HPAI H5N1-infected poultry are indicated by an asterisk (*).

more frequently overlapped with poultry outbreaks in Egypt or Turkey (Fig. 5).

During the spring migration, a larger proportion of the utilization distributions of both Common Teal (23.1%) and Common Shelduck (27.3%) significantly overlapped with H5N1-infected poultry (Supplementary Table S6). Six birds (3 Common Teal and 3 Common Shelduck) had sufficient locations for spring migration spatial analyses (Supplementary Table S1). However, the overlap with domestic outbreaks primarily occurred in Egypt or Turkey and not across their migratory path. The number of wild bird outbreaks occurring within the Black Sea-Mediterranean Flyway was low relative to poultry outbreaks. During the spring migration, 20 per cent (1 of 5 migration periods) of UDs for Common Teal were significantly associated with outbreaks in wild birds starting in February, but no other species showed a spatiotemporal overlap with wild bird outbreaks. The region of highest HPAI H5N1 transmission risk between wild birds occurred in Turkey in the early stages of the spring migration.

4. Discussion

Understanding regional differences in spillover risk is of high importance given the variation in poultry production systems, and wild bird migration that are the key drivers of geographic spread of IAV. Within Africa, waterbird movements are poorly characterized and are highly variable among species, confounding efforts to assess their potential role in virus dispersal. Our study demonstrates that migratory connectivity and phenology of wild birds are likely to play a key role in the reassortment, evolution, and dispersal of HPAI between adjacent Flyways providing a mechanism for long-distance spread of gene segments between Africa and Asia.

4.1 Genetic evidence of HPAI reassortment between Flyways

Wild ducks that were sampled during this study in Egypt were shedding virus that shared ancestry with HPAI H5 originating from East Asia (clade 2.3.4) and locally circulating strains infecting Egyptian poultry (clade 2.2.1). Four viruses infecting two different species, Northern Shoveler and Northern Pintail, contained putative HPAI segments that showed evidence of reassorting with wild bird origin LPAI between 2005 and 2008 based on molecular dating. This timeline encompasses multiple years and suggests ambiguity about the exact transmission chain involving wild ducks sampled in our study, particularly for East Asian-origin segments. However, strong support for poultry to wild duck spillover of HPAI clade 2.2.1 occurred locally within Egypt, coinciding with the wintering period of the study population. These results provide support for the role of wild birds in dispersing and mixing clades of HPAI both locally within Egypt and between endemic regions of Asia and Africa, even over large distances.

Shared ancestry with HPAI H5 clade 2.3.4 originating in East Asia was more common among viral segments than shared ancestry with HPAI H5 clade 2.2.1 from Egyptian poultry, suggesting introduction of virus from East Asia due to wild bird migration. The mechanism for east to west viral flow is likely to involve multiple transmission chains across Flyways, rather than direct transmission by a single bird host. As indicated by the migration of marked ducks in our study, host movements were oriented toward northern latitude breeding sites, rather than traversing longitudinally. Studies of HPAI H5

phylogeography of clade 2.2, clade 2.3.2.1, and clade 2.3.4.4 have found that Anseriformes played the major role in connecting Asia and Africa (Fusaro et al. 2019). For our analysis, Asia to Egypt virus dispersal was best explained by movement from the East Asian (source) to the Central Asian (sink) Flyway, and the Central Asian (source) to the Black Sea-Mediterranean (sink) Flyway. These pairwise source–sink relationships had the strongest and most consistent support (across internal segments) , while viral flow from East Asia to Egypt was detected but had lower statistical support.

One plausible explanation for the predominance of clade 2.3.4 ancestry in our study may be due to differences in replication capacity between HPAI H5 clades. Clade 2.3.4 may be better adapted to bind to host cell receptors and undergo replication in waterfowl, compared to clade 2.2.1 which is considered a poultry-adapted lineage (Smith et al. 2009; Bertran et al. 2016). Alternatively, the detection of viral segments that shared ancestry with HPAI H5 clade 2.3.4 in Egyptian ducks may reflect the timing at which birds were sampled in the mid-winter (January 17–23). This period is after the arrival of migratory ducks that breed at mid- and high- latitude 'mixing zones' where clade 2.3.4 is hypothesized to circulate, rather than the spring migration when exposure to local poultry and Egyptian strains is expected to be higher. Lastly, although our analysis attempted to account for sampling bias by including equivalent numbers of viral sequences from each Flyway/region, it cannot be discounted that more intensive surveillance in East Asian countries such as China, Japan, and South Korea may have uncovered greater viral diversity than in adjacent countries of Central Asia where due to lower human population density and less poultry agriculture (Newman et al. 2012), sampling efforts are unlikely to be routine.

4.2 Migratory ducks link adjacent Flyways, while residents amplify virus locally

Our results support that duck migration over medium to long distances may serve to enhance contact with infected wild birds from Asian Flyways at high latitude breeding grounds. The longest distance migrant identified in our study was the Common Teal that bred as far north as Yamalo-Nenetskiy Autonomous District in Northwest Siberia, where multiple Flyways converge and birds of different wintering origins mix after nesting. Connectedness of these circumpolar breeding grounds has been proposed as a primary mechanism for the rapid, global expansion of clade 2.3.4.4 (Lycett et al. 2016). We identified Common Teal shedding virus in Egypt ($n = 2/33$) but were unable to recover sequence due to logistical challenges with cold chain. Of the ducks from which virus was sequenced, the longer distance migrants, Northern Shoveler and Northern Pintail, were associated with virus from the Central and East Asian Flyways, and putative HPAI segments were isolated from both. Owing to the small number of tagged ducks, it was not possible to test predictions that species, migration strategy, or overlap with infected poultry correlated with a higher prevalence of virus or HPAI segments.

In contrast, the more local migrant, Common Shelduck, was linked with virus from the Black Sea-Mediterranean. The tendency toward more localized movement and prolonged overwintering suggests that the Common Shelduck is more likely to amplify local strains. Interestingly, we found evidence of residency among all three duck species. Temperate habitats that experience milder winters are conducive to the presence of sedentary bird populations, a global phenomenon that is known to

be increasing in Africa and Eurasia (Newton 2008; Ambrosini et al. 2016; Rotics et al. 2017). Food provisioning by hunters may also change the migration strategy of wild ducks. In Egypt, specifically on Lake Manzala, hunters lure waterfowl into artificial feeding areas using decoys (Mullié and Meininger 1983). The lengthy hunting season (180 days: September–February) may allow habituation to provisioning and encourage partially migratory species to become less migratory (Brown and Hall 2018). This may lead to increased opportunities for HPAI spillover between poultry and wild birds, outside the peak period of spring migration. However, with so few viruses isolated during this study and none from Common Teal, paired with the limited telemetry data, more data are needed to verify our conclusions about the utility of host traits for predicting virus dynamics.

While we are not able to dismiss the possibility that movements of the monitored individuals were influenced by the tracking devices (Barron, Brawn, and Weatherhead 2010; de Vries 2014; Lameris and Kleyheeg 2017), multiple citizen science reports of unmarked Common Shelduck and Northern Shoveler throughout Turkey and Cyprus during the summer months (eBird 2012) corroborate the short distance migrations observed from Egypt to Turkey. The lack of citizen science data throughout Egypt preempted our ability to use this method to validate our observations of resident birds, though there were numerous reports of resident Common Teal and Northern Shoveler along the West Bank, a wintering grounds which is relatively comparable in latitude to our capture locations in Egypt. Additional clarity may have been reached had our sample size been larger, but this was precluded by the failure of multiple individual birds to provide reliable data. The marking of additional birds could help validate our findings, as could sightings or observations of birds reported by members of the public in an effort to better resolve the movement and habitat preferences of birds throughout these understudied Flyways.

4.3 Overlap between poultry and waterfowl peaks during spring migration

The primary interface between HPAI H5N1-infected poultry and wild ducks in Egypt and Turkey was associated with early spring when ducks expanded their range at the start of migration. In contrast, wintering ducks were more sedentary and movements were constrained to local foraging. Spatial analysis indicated that the winter distributions of ducks were centered on coastal wetlands including the 'Important Bird Areas' of Lake Manzala and Burullus (El Din 1999) distinct from high poultry density areas along the Nile Delta. The interface between wild ducks and HPAI H5N1-infected poultry increased in late winter and peaked during early spring when birds move between habitats to form larger flocks and fed intensively to gain condition for migration. The highest overlap with poultry outbreaks occurred in Egypt and Turkey but not across the migratory path farther northwards. The risk of wild ducks coming into contact with poultry outbreaks declined as they migrated north, correlating with decreasing density of poultry along the Black Sea-Mediterranean from low to high latitudes.

This pattern is consistent with studies that highlight the spring migration plays a prominent role in the long-distance dispersal of HPAI from endemic countries to higher latitudes, relative to poultry trade (Hill et al. 2015; Tian et al. 2015; Lycett et al. 2016). These studies support that transmission of IAV between wild birds can occur efficiently at high latitudes due to the mass migration of breeding birds. Analysis of IAV sequences collected from Egyptian wild ducks in this study suggested the

potential for long-distance spread and frequent exchange of viral segments between migratory Flyways. In addition, bi-directional viral flow between Egypt and the Black Sea-Mediterranean Flyway, and connectivity with the Central and East Asian Flyways implied a mechanism for both autumn and spring dispersal of virus. While telemetry tracks from marked ducks in this study stopped short of the autumn migration, the introduction of IAV from higher latitudes into Egypt is certainly plausible and has been proposed to explain previous HPAI H5 incursion events from Europe in 2005 (Saad et al. 2007) and in 2016 (Selim et al. 2017).

4.4 Migratory behavior of ducks determines timing of poultry contact

Our study identified that contact risk with poultry outbreaks occurred 1–2 months earlier for long distance migrants (Common Teal) compared to local migrants (Common Shelduck) within the Black-Sea Mediterranean Flyway. The Common Teal performed the longest migrations in the study, with one bird flying 7,601 km in 92 days to reach tundra breeding grounds in northwestern Siberia. Heightened contact with infected poultry occurred during February for Common Teal; however, for Common Shelduck, peak overlap occurred over a more sustained period from late March to May. Differences in migratory behavior within and between duck species may contribute to a staggered contact pattern with poultry, such that successive waves of transmission occur during the spring migration, rather than a synchronized pulse. This is likely to be an important factor contributing to the months-long maintenance of HPAI H5 epidemics involving wild birds (Li et al. 2010; Newman et al. 2012). As described above, while we consider this unlikely, the possibility that differences between species could be related to tagging effects or limited sample size should be carefully considered. Collection of additional data via telemetry or reporting of wild bird sightings to public databases would help to assess replicability of these results.

In countries such as Egypt where HPAI has become established, awareness of when and where the wild–domestic interface is greatest may inform how to target surveillance and biosecurity (Naguib et al. 2019). The cost of performing surveillance and enforcing high standards of biosecurity at farms and live bird markets can be prohibitive . Our data supports that the spring migration is a period of high risk for transmission between domestic and wild birds, and that long-distance migrants represent the first wave of interaction beginning in February. Enhanced biosecurity at live bird markets focused on limiting contact between Common Teal and other species during this period may offer prospects for reducing the wild–domestic interface and preventing the first cases of spillover.

4.5 Utility of telemetry and viral genomics for inferring infection dynamics

Both tools identified that Egyptian and Turkish ducks were more connected with the Black Sea-Mediterranean and Asia, rather than Europe and the Atlantic seaboard. However, examination of the viral genome implied a much larger footprint of wild bird-mediated transmission compared to telemetry. The structure of phylogenetic trees can imply close evolutionary links between viruses that in reality are separated by multiple host transmission events. Gaps in geographic coverage of viral sequences contributes to this problem when constructing phylogenetic trees. For example, East Asia is more heavily sampled

than Central Asia owing to differences in human and poultry density that impact the logistics of performing surveillance. The exchange of virus between the Central Asian and Black Sea-Mediterranean Flyway was likely underestimated in our study.

Conversely, while satellite telemetry may offer far more resolution on the spatiotemporal movements of the host during the course of the annual cycle, the data generated may not always be representative. The tracking device may alter the physiology, behavior, and in extreme cases, the survival of individual birds, or alternatively transmissions can be lost due to short battery life, malfunction, or even due to harvesting of birds by hunters for consumption. In the majority of cases, the fate of marked bird remains unknown, as demonstrated by this study. However, applied together and with a critical assessment of each method, satellite telemetry and viral phylogenies have the ability to provide a robust understanding of how and where transmission between wild birds and poultry may occur, especially in understudied regions that are important to the spread of HPAI H5 globally.

Data availability

Viral sequences are publicly available at the GISAID repository. The accession numbers are provided in Supplementary Table S1. The telemetry data reported during the current study are available on Movebank from the corresponding author on reasonable request. Egypt project link: https://www.movebank.org/cms/webapp?gwt_fragment=page=studies, path=study3291889. Turkey project link: https://www.movebank.org/cms/webapp?gwt_fragment=page=studies,path=study3291979.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

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