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Age and season predict influenza A virus dynamics in urban gulls: consequences for natural hosts in unnatural landscapes

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Abstract. Gulls are ubiquitous in urban areas due to a growing reliance on anthropogenic feeding sites, which has led to changes in their abundance, distribution, and migration ecology, with implications for disease transmission. Gulls offer a valuable model for testing hypotheses regarding the dynamics of influenza A virus (IAV) – for which gulls are a natural reservoir in urban areas. We sampled sympatric populations of Ring-billed (*Larus delawarensis*), Herring (*L. argentatus*), and Great Black-backed Gulls (*L. marinus*) along the densely populated Atlantic rim of North America to understand how IAV transmission is influenced by drivers such as annual cycle, host species, age, habitat type, and their interplay. We found that horizontal transmission, rather than vertical transmission, played an outsized role in the amplification of IAV due to the convergence of gulls from different breeding grounds and age classes. We detected overlapping effects of age and season in our prevalence model, identifying juveniles during autumn as the primary drivers of the seasonal epidemic in gulls. Gulls accumulated immunity over their lifespan, however short-term fluctuations in seroprevalence were observed, suggesting that migration may impose limits on the immune system to maintain circulating antibodies. We found that gulls in coastal urban habitats had higher viral prevalence than gulls captured inland, correlating with higher richness of waterbird species along the coast, a mechanism supported by our movement data. The peak in viral prevalence in newly fledged gulls that are capable of long-distance movement has important implications for the spread of pathogens to novel hosts during the migratory season as well as for human health as gulls increasingly utilize urban habitats.

Key words: *avian influenza; gull; immunity; pathogen; prevalence; transmission; urban; virus.*

Introduction

Gulls are one of the most ubiquitous migratory species in urban areas worldwide due to a growing reliance on human sources of food (Burger 1981, Belant 1997, Belant et al. 1998, Washburn et al. 2013). The proliferation of anthropogenic feeding sites including landfills, animal farms, fisheries, and wastewater treatment plants (Belant et al. 1998, Plaza and Lambertucci 2017, Shlepr et al. 2021), as well as reductions in traditional food sources, have contributed to changes in gull abundance, distribution, and migration ecology over the last century (Drury and Nisbet 1972, Burger 1981, Anderson et al. 2016, Giroux et al. 2016). The distribution of gulls is becoming centered on human activity, as the distance between breeding and non-breeding sites is reduced, often in an age-dependent manner (Drury and Nisbet 1972, Belant and Dolbeer 1993, Tyson et al. 1999, Clark

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et al. 2016, Anderson et al. 2019). These changes are increasingly recognized as a potential problem for pathogen spread and public health. Gulls congregate and disperse between human-induced feeding sites, thereby aiding the spread of pathogens between anthropogenic, freshwater, and marine ecosystems (Nelson et al. 2008, Plaza and Lambertucci 2017). Gulls therefore epitomize the growing challenge associated with human–wildlife interactions in the Anthropocene and offer a valuable model for testing hypotheses regarding the dynamics of emerging infectious diseases.

Gulls play a unique role in the ecology of influenza A virus (IAV) due to their demonstrated capacity to spread the virus at local, regional, and global scales. Gulls are the primary reservoirs of the hemagglutinin (HA) subtypes H13 and H16 (Fouchier et al. 2005, Olsen et al. 2006), known to spillover and infect marine mammals (Hinshaw et al. 1986, Groth et al. 2014) and even poultry (Laudert et al. 1993). They are also permissive hosts for all known subtypes (H1–H16) detected in birds (Arnal et al. 2015) and are readily infected with strains infectious to poultry and humans, including highly pathogenic (HP) forms of the H5 subtype (Ellis et al. 2004, Brown et al. 2006) and H9N2 (Wille et al. 2011*b*). On a global scale, gulls contribute to the intercontinental movement and reassortment of IAV lineages due to exchange of virus among dense gull colonies at northern latitudes and long-distance overland and pelagic movements (Krauss et al. 2007, Wille et al. 2011*a*, *b*, Dusek et al. 2014, Verhagen et al. 2020).

There has been a lack of studies tracking the epidemiology of IAV in gulls against the backdrop of increasing urbanization, a trend that is occurring worldwide and at a rapid pace during the Anthropocene (Schmidt et al. 2020). The goal of this study was to understand how infection is influenced by temporal and spatial dynamics that vary over the course of a gull's lifespan in urban habitats. Addressing this question will improve the understanding of whether infection dynamics in urbanized gulls are different than in waterfowl and shorebird hosts, which have less frequent human interaction. We revisit the often-cited notion that juveniles are the key drivers in IAV dynamics (Olsen et al. 2006, Hoye et al. 2011, van Dijk et al. 2014*a*). Once juvenile gulls leave the breeding colony they may stage for migration in nearby areas and mix with other age classes as well as other species before undertaking migration. The challenges of capturing gull chicks increase after they fledge from the nest, resulting in a paucity of information on how IAV disperses throughout the annual cycle. These annual patterns of migration and mixing among gulls have the potential to shape infection dynamics throughout an individual's lifespan, which is now largely spent in urban areas.

The northern Atlantic coast of North America is home to several gull species, the most common of which are the Ring-billed (*Larus delawarensis*), Herring (*L. argentatus*), and Great Black-backed Gulls (*L. marinus*). These species overlap in their distributions during all or part of the year, providing a multispecies system in which to study influenza dynamics. During the non-breeding season, all three species congregate (Tyson et al. 1999, Clark et al. 2016), whereas Herring and Great Black-backed Gulls breed on some of the same offshore islands (Ronconi and Wong 2003). The Herring Gull is widespread throughout the Northern Hemisphere, having expanded its range southward from Canada in the mid-20th century (Kadlec and Drury 1968, Burger 1979). This expansion partially displaced the smaller Ring-billed Gull (Wetherbee et al. 1972), which is found throughout much of North America and is now recognized as one of the most abundant bird species on the planet (Callaghan et al. 2021). Herring Gulls, in turn, co-occur and compete with the larger and more recent arrival, the Great Black-backed Gull (Rome and Ellis 2004), whose range bridges North America and Europe.

Urbanization of gulls is not a challenge unique to the North Atlantic, but represents a human–animal interface that is generalizable worldwide. Influenza studies focused on waterfowl have demonstrated that prevalence tends to decline with age, whereas seroprevalence increases, suggesting the accumulation of immunity over time (e.g. Farnsworth et al. 2012, Spivey et al. 2017). However, the predictive effect of age is likely to interact with other ecological factors such as season, host species, and habitat type, dynamics that have been largely overlooked despite being especially relevant for gulls. In this study, we first tested the relationship of age as a continuous and categorical variable against (1) acquired immunity and (2) viral prevalence, rather than simply defining age in binary terms (juvenile vs. adult) as is customary in waterfowl studies. Second, we tested the influence of seasonal stage, gull species, and habitat type against the same measures of infection to evaluate how ecological conditions modify the predominant effect of age. Ultimately, we used our findings to build a model of infection with the goal of predicting where and when emergence events could happen in an increasingly urban-adapted wild bird reservoir.

Methods

Sampling at breeding and non-breeding sites

Gulls were sampled between 2010 and 2013 at three breeding colonies with active banding and monitoring efforts (Fig. 1). All gulls except 1-week-old chicks were banded with federal and plastic field-readable bands. Our first site, Appledore Island (part of the Isles of Shoals in the Gulf of Maine (42°59′N, 70°36′W)), is a breeding colony for hundreds of Herring and Great Black-backed Gulls (Savoca et al. 2011) that have been studied since 2004. Over 5 days in both June and July of 2013, Herring Gull chicks were captured by hand at approximately 1 and 5 weeks old. Oropharyngeal and cloacal swab samples were collected with sterile polyester swabs (Puritan Medical Products, Guilford, ME, USA),

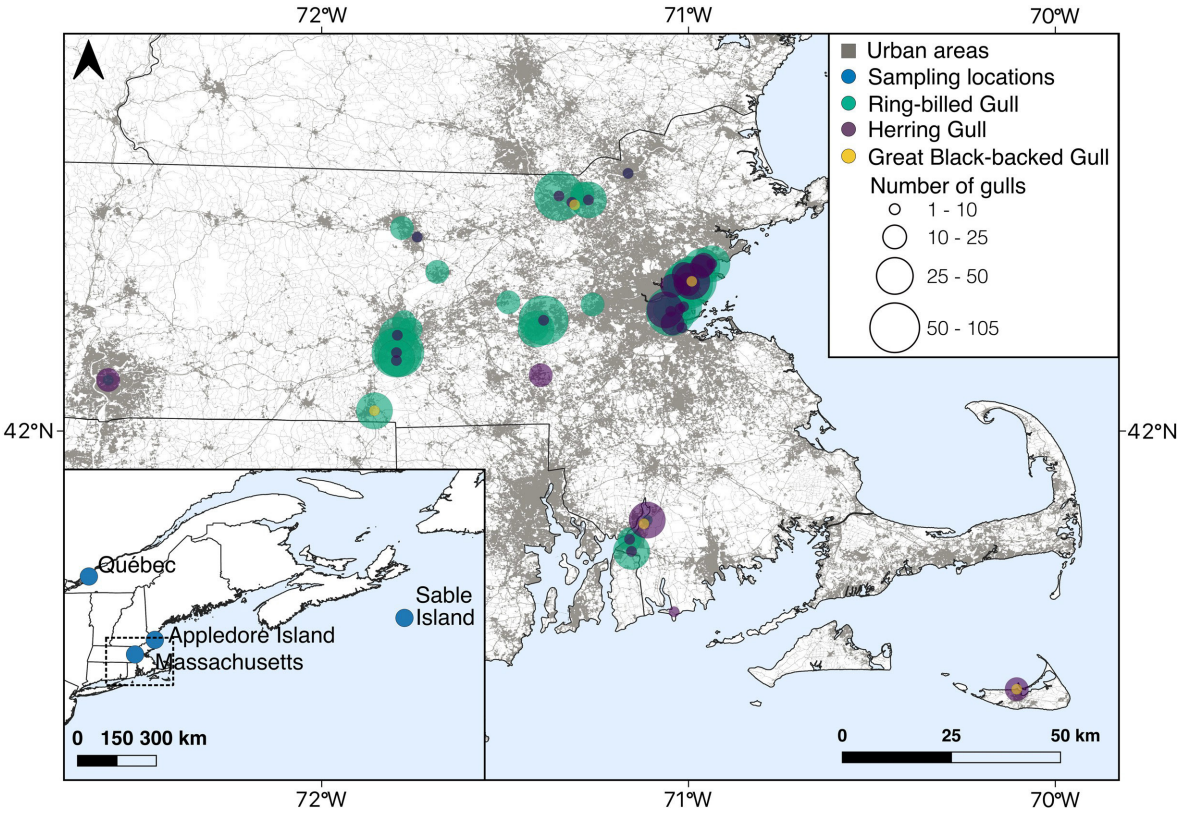

FIG. 1. Map of locations (inset, blue markers) where Ring-billed, Herring, and Great Black-backed Gulls were sampled from 2010 to 2014. In Massachusetts, circle size indicates number of gulls sampled at each capture site. Massachusetts sites are shown in relation to urban areas based on the 2011 National Land Cover Database (Homer et al. 2015).

placed in separate tubes of viral transport medium (VTM; Remel Inc., Lenexa, KS, USA), and kept on ice for no more than 3 h before being stored in a liquid nitrogen shipper. Blood was collected from the cutaneous ulnar vein in the wing from both 1-week-old (100-200 μL) and 5-week-old (1 mL) chicks. Samples were allowed to clot at ambient temperature, centrifuged, and the sera were collected and stored in a liquid nitrogen shipper.

Our second site, the ^Ile Deslauriers colony in the St. Lawrence River near Montreal, Quebec (45° 43^{0}N, 73°25^{0}W), supports over 40,000 breeding pairs of Ring-billed Gulls (Giroux et al. 2016), which have been stud-ied since 2009. In mid-May 2010, adult Ring-billed Gulls were captured using a net launcher. Oropharyngeal and cloacal swabs were collected and stored in the same tube at 4°C for a maximum of 6 h until transfer to 80°C prior to testing.

Our third site, Sable Island, Nova Scotia (43°56^{0}N, 59°54^{0}W), is a remote barrier island that serves as a breeding site for approximately 850 pairs of Herring Gulls and 450 pairs of Great Black-backed Gulls (Ronconi et al. 2016). From late May to mid-August 2013, we captured adult and sub-adult Herring and Great Black-backed Gulls using nest traps and hand-captured Great Black-backed Gull chicks. We collected oropharyngeal and cloacal swabs, which were stored in separate tubes of VTM and kept on ice until transfer to liquid nitrogen shippers up to 6 h later.

From 2012 to 2014, Herring, Great Black-backed, and Ring-billed Gulls were captured in Massachusetts during the non-breeding period of the annual cycle (Fig. 1; Appendix S1: Table S1). Gulls were trapped from October to April using a net launcher (Clark et al. 2014). Sites were primarily in parking lots of commercial areas, wastewater treatment plants, landfills, or beaches and were confirmed to be urban based on GIS analysis of the 2011 National Land Cover Database (Homer et al. 2015). Trapping locations in Massachusetts were divided into two broad habitat types based on distance from the ocean: urban coastal (≤5 km) or urban inland (>5 km). In view of the high winter site fidelity and persistence exhibited by Herring and Ring-billed Gulls within and between seasons, as observed from satellite transmitter data (Clark et al. 2016), we assumed infrequent movement of gulls between coastal and inland habitats and considered these as distinct, non-overlapping habitat types. Gulls were banded with a federal band and plastic or metal field-readable band. A subset of gulls also received patagial tags as part of a separate study (Clark et al. 2016). Age (juvenile, sub-adult, or adult) was determined based on plumage characteristics (Pyle 1997).

Oropharyngeal and cloacal swabs were collected and stored in separate tubes of VTM on ice until transfer to a 80°C freezer within 5 h. We also collected 1 mL of blood, which was stored on ice and allowed to clot. Blood samples were centrifuged and the sera were collected and stored at 80°C.

Characterizing seasonal stages and movement using banding data

To characterize seasonal stages for gull populations in eastern North America, we obtained records from the US Geological Survey Bird Banding Laboratory (BBL) of all the banded Great Black-backed, Herring, and Ring-billed Gulls that were resighted from 2012 to 2016. From these records, we only used gulls that were resighted at least once in Massachusetts as well as any resights of those individuals outside the state. As some locations were only accurate to a 10 min block, an 18.5 km buffer around the state of Massachusetts was used to clip locations. We then assessed potential biases in resight data due to high reporting by observers in densely populated regions (i.e., urban Massachusetts) and low reporting in sparsely populated regions (i.e., rural Nova Scotia) using QGIS v.2.8 software (QGIS Development Team 2015). All resights were mapped, overlaid with a 0.1° graticule, and assigned to a grid cell to evaluate the distribution of resights according to space and time (month) stratification. We determined the 99% quantile from summary statistics of each cell (resights/month), which was then used to randomly downsample to remove data from over-represented time–space strata. To visualize the resulting data, a heat map of resight latitude (2.5° increments) was plotted by Julian date (10 day increments). Changes in the spatiotemporal distribution of gulls were used to define the stages of the seasonal cycle and the corresponding start and end Julian date of each season: autumn (251–330), winter (331–80), spring (81–140), and summer (141–250; Appendix S1: Fig. S1).

To assess migratory connectivity between the three breeding colonies and non-breeding sites in our study, we mapped resights of gulls that were banded on Appledore Island (2009–2013), Sable Island (2011–2013), and ^Ile Deslauriers (2009–2013) and subsequently resighted between 2009 and 2014. These data, mostly reports of field-readable bands, provided us with a larger set of resights specific to our study than was available from the BBL database. For each resight with a reported band number, we determined age class at resight using age at and time since banding. We then calculated monthly mean distance from banding location to resight location by age class and species to evaluate movement patterns over the annual cycle.

Viral screening and isolation

Oropharyngeal and cloacal swabs were screened for active IAV shedding. Swabs from all sites were tested separately, except for those from the ^Ile Deslauriers colony, which were combined by individual gull. Swabs were thawed on ice and quickly vortexed. RNA was extracted from 50 μL of VTM per swab using the MagBind Viral DNA/RNA 96 Kit (Omega Bio-Tek Inc., Norcross, GA, USA) on a semiautomated KingFisher Purification System robot (ThermoFisher Scientific, Waltham, MA, USA). Swabs from the ^Ile Deslauriers colony were clarified by centrifugation and 50 μL of sample was spiked with an exogenous internal control. RNA was extracted with the MagMax™-96 Total RNA Isolation Kit using the MagMax 96-well robotic system (Applied Biosystems/Ambion, Austin, TX, USA). One-step real-time RT-PCR was used to detect influenza RNA using qScript XLT 1-Step RT-qPCR ToughMix (Quanta BioSciences, Gaithersburg, MD, USA) and primers targeting the matrix gene (Spackman et al. 2002). The RT-PCR assays for the ^Ile Deslauriers samples were modified to also detect the exogenous control (Weingartl et al. 2010). Screening was performed for the ^Ile Deslauriers samples using the SmartCycler system (Cepheid, Sunnyvale, CA, USA) and for all other samples using the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Samples with a cycle threshold (Ct) value <45 were considered positive. Individual gulls were considered positive for IAV if the Ct value of at least one swab was less than 45.

To amplify virus, 100 μL of VTM from samples identified as PCR positive were inoculated into the allantoic cavity of 9-to 11-day-old embryonating specific pathogen free chicken eggs (Charles River Laboratories, Wilmington, MA, USA) and incubated at 37°C for 72 h or until embryo death, as detected by daily candling. RNA was extracted from the amnio-allantoic fluid (AAF) and screened using the matrix real-time (r)RT-PCR to evaluate successful viral culture. For AAF samples that amplified at 30 cycles or less, whole-genome sequencing was attempted at the J. Craig Venter Institute in Rockville, MD, as described by Nelson et al. (2007), and all sequences were deposited into GenBank (Appendix S1: Table S2). The HA and neuraminidase (NA) subtypes were determined by BLASTn of the sequence against isolates in GenBank and identifying the subtype match that showed the highest percentage identity.

Seroprevalence of anti-influenza antibodies

To test for the presence of influenza antibodies in serum, we used an enzyme-linked immunosorbent assay kit (IDEXX AI MultiS-Screen, Westbrook, ME, USA) following the manufacturer's instructions. Each sample was run in duplicate using 10 μL of serum per well, diluted 1:10 with sample buffer. Positive and negative controls were run on each plate. Absorbance values were measured with an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA) and the mean absorbance value for each sample was used to calculate the sample to negative ratio. Following the manufacturer's

guidelines, samples with a ratio <0.5 were considered positive for influenza antibodies.

Phylogenetic origins of virus

To assess the origins of each gene segment by hemisphere (North American vs Eurasian), a simple BLASTn search was performed. The top 10–50 hits were used to broadly assign segments according to this binary classification. The phylogenetic origins of the HA gene segment were analyzed to identify the most recent common ancestor. Two sequences were generated during the study, both of the H13 subtype. For comparative analysis, all HA sequences of the H13 subtype were obtained from the Influenza Virus Resource (NCBI database) on September 20, 2019. Only full-length segments of H13 sampled during the period 1976–2019 were considered. To facilitate filtering out duplicate sequences, taxa names were edited to remove apostrophes and brackets and all hyphens were replaced with underscores. Sequences containing misreads (NNNs) were deleted to improve the quality of alignments and subsequent trees. Sequences were then aligned using MUSCLE v3.8.31 software (Edgar 2004) and inspected visually in Jalview 1.8 to remove InDels and taxa containing premature termination codons. In total, 398 taxa were included in the final dataset.

Phylogenetic trees were reconstructed using Markov chain Monte Carlo (MCMC) sampling analyses performed using the *BEAST* v1.10.4 package (Drummond et al. 2012) in combination with the BEAGLE library. Phylogenies were reconstructed using a General Time Reversible nucleotide substitution model with gamma distribution of substitution rates, a Gaussian Markov random field skyride coalescent model and an uncorrelated lognormal clock. Four independent MCMC runs of 200 million chains were performed for each segment. Runs were combined to ensure that an effective sample size of at least 200 was achieved and that the maximum clade credibility tree was determined. Lineages of the phylogenetic tree were visually inspected and classified according to bird migratory flyway (BirdLife International 2021). This classification scheme was used to determine the geographic origins of the HA segment and evaluate the potential for intercontinental viral flow between North America and Europe.

Assessing predictors of IAV dynamics

Changes in viral shedding and antibody prevalence were explored in relation to gull age at several temporal resolutions. Using a mean hatch date of June 10 (Savoca et al. 2011), we calculated days since hatch (DSH) for all gulls captured on Appledore Island and in Massachusetts for which we had both viral shedding and serology data. For the purposes of our analysis, we assumed that sub-adults were in their second cycle. Adults were combined into one group at the beginning of their fourth cycle. We binned DSH into 50-day periods, ensuring that each bin contained at least 30 individuals. To evaluate the more conventional binary measure of age, we binned sub-adults and adults into after hatch year (AHY) while juveniles were considered hatch year (HY). Chicks were not included in the juvenile age class. We then tested these three age variables as predictors of infection dynamics in the Massachusetts gulls: (1) DSH as a continuous variable, (2) age class as a categorical variable (juvenile, sub-adult, adult), and (3) age class as a binary variable (HY, AHY). We created two sets of binomial generalized linear models (GLMs) with a logit link, one with viral shedding and one with seroprevalence as the dependent variable. Models were ranked using Akaike information criteria corrected for small sample size (AICc) with the *MuMIN* package (Barton 2020).

To further assess patterns of viral prevalence and seroprevalence in the Massachusetts gulls, we used GLMs with DSH, seasonal stage (autumn, winter, spring), habitat type (coastal, inland), species (Herring Gull, Ring-billed Gull), sampling year (2012/13, 2013/14), and either serology or shedding status (positive, negative) as potential effects. Great Black-backed Gulls were excluded due to small sample size ($n = 7$). All possible additive combinations of variables were used to create a candidate set of models ($n = 64$; Appendix S1: Tables S3, S4). Models were ranked using AICc and a top model set was created by eliminating models greater than D6 AICc from the model with the lowest AICc. We then applied the nesting rule to remove overly complex models (Richards et al. 2011). We assessed differences in viral and antibody prevalence between groups using post hoc tests with the *multcomp* package (Hothorn et al. 2008). For comparison, we repeated this process with the binary and categorical age variables in place of DSH and included an interaction with seasonal stage.

Shedding route was examined for all IAV-positive Massachusetts gulls and then the subset of birds that were also seropositive by comparing viral prevalence in cloacal and oropharyngeal swabs using chi-square tests. We then explored whether age class was predictive of shedding route (cloaca, oropharynx, both, or neither) using a multinomial logistic model. We also used the Ct value as a proxy of infection intensity and compared between shedding routes and age classes using analysis of variance (ANOVA) and *t*-tests. All analyses were conducted in R v.3.6.1 (R Core Team 2018).

Results

Sampling and migration ecology

We captured and sampled 2,069 gulls throughout the annual cycle between 2010 and 2014. During the breeding season, we sampled 439 Herring Gull chicks on Appledore Island; 18 Great Black-backed Gull chicks, 34 adult and sub-adult Great Black-backed Gulls, and 12 adult and sub-adult Herring Gulls on Sable Island;

and 69 adult Ring-billed Gulls on ^Ile Deslauriers. During two non-breeding seasons in Massachusetts, we captured a total of 1,497 gulls, including 311 Herring Gulls, 1,169 Ring-billed Gulls, and seven Great Black-backed Gulls (Appendix S1: Table S1). Gulls banded on the three breeding colonies in our study exhibited migratory overlap according to resight data, with some individuals reported in Massachusetts (Fig. 2). Resights of the three species broadly overlapped throughout the winter and occurred all along the northern Atlantic coast of North America and as far inland as the Great Lakes. Juvenile Herring Gulls tended to move farther than other age classes from autumn to spring, with some migrating to the Gulf of Mexico, while adults tended to stay closer to their respective breeding colony. Resights of Great Black-backed and Ring-billed Gulls revealed less differentiation in migratory distance between age classes than for Herring Gulls (Fig. 2). Juveniles of all three species tended to move away from the colony in the post-breeding dispersal period and were farther from the colony in spring than the other age classes.

Influenza prevalence in relation to age

We detected no active IAV shedding in either the 1-or 5-week-old chicks on Appledore Island (Fig. 3a) based on rRT-PCR. Similarly, all adult and sub-adult gulls (0/46) and chicks (0/18) sampled on Sable Island were negative, however, we did detect shedding in 5.8% (4/69) of adult gulls on ^Ile Deslauriers at the beginning of the breeding season. On Appledore Island, influenza antibodies were detected in 15.0% (31/207) of 1-week-old chicks in June and in none of the 5-week-old chicks (0/218; Pearson chi-square (χ^2) = 33.036, df = 1, $P < 0.001$; Fig. 3b). Within the June sampling period, seroprevalence significantly declined between the first 2 days and last 2 days ($\chi^2 = 6.877$, df = 1, $P < 0.01$). Combining the Appledore and Massachusetts gulls, we examined prevalence in gulls that were approximately 7- to 1,200+ days old. We found that viral prevalence was not significantly correlated with DSH (Pearson correlation $R = 0.36$, $P = 0.34$; Fig. 3a), however, there was a general negative trend when chicks were excluded from the analysis. Seroprevalence and DSH, conversely, exhibited a strong positive correlation ($R = 0.91$, $P < 0.001$; Fig. 3b). When we compared DSH to the binary and categorical age variables, the GLM with DSH had more support for both viral shedding and seroprevalence (Table 1).

Predictors of viral and seroprevalence

In the Massachusetts gulls, we detected an overall viral prevalence of 8.7% (130/1487) and antibody prevalence of 54.0% (756/1400). A GLM of viral prevalence with DSH, seasonal stage, and habitat had the most support (Table 2). We found that, for every day older, the log odds of a gull actively shedding IAV decreased by 0.0006 (Table 3). Viral prevalence was highest in autumn (14.0%, 95% confidence interval [CI] 10.7–18.1%) and declined significantly by winter (7.2%, 95% CI 5.7–9.1%; Tukey's test, $P < 0.01$; Table 3). Shedding was lowest in spring (5.2%, 95% CI 2.3–10.9%), although the differences were not significant compared with autumn ($P = 0.15$) and winter ($P = 0.99$). Habitat also had explanatory power in the model, with gulls captured near the coast (9.8%, 95% CI 7.9–12.1%) more likely to be shedding virus than inland gulls (7.5%, 95% CI 5.7–9.8%; $P < 0.05$, Table 3). The most supported GLMs with categorical or binary age variables instead of DSH also included seasonal stage, age class, and habitat (Appendix S1: Tables S5, S6). Considering categorical age, juveniles (13.7%, 95% CI 10.2–18.1%) were significantly more likely to be shedding IAV than adults (6.8%, 95% CI 5.4–8.6%; Tukey's test, $P < 0.05$; Appendix S1: Table S7). Sub-adult prevalence was intermediate (10.3%, 95% CI 6.2–16.5%) and did not significantly differ from juveniles ($P = 0.85$) or adults ($P = 0.29$). There was a general negative trend in viral prevalence from autumn to spring within each age class (Fig. 3c).

The most supported GLM of seroprevalence included DSH, seasonal stage, and sampling year (Table 2). In contrast with shedding prevalence, the log odds of a gull being seropositive increased by 0.002 for every 1 day older (Table 3). Seroprevalence peaked in the winter (61.5%, 95% CI 58.2–64.7%) and was significantly higher than in spring (53.8%, 95% CI 44.9–62.4%, $P < 0.05$), but not autumn (34.5%, 95% CI 29.5–39.8%; Tukey's test, $P = 0.27$). Seroprevalence was slightly higher in the second year of sampling (2013/14; 55.8%, 95% CI 52.4–59.1%) compared with the first year (2012/13; 51.3%, 95% CI 47.0–55.6%; $P < 0.05$). The most supported models with categorical or binary age instead of DSH also included sampling year as well as an interaction between age class and seasonal stage (Appendix S1: Tables S5, S6). Seroprevalence in adults (69.1%, 95% CI 66.0–72.0%) was significantly higher than in juveniles (13.5%, 95% CI 9.94–18.1%; Tukey's test, $P < 0.001$; Appendix S1: Table S7) and sub-adults (39.0%, 95% CI 31.2–47.5%; $P < 0.001$). Sub-adults also had significantly higher seroprevalence than juveniles ($P < 0.001$). The winter peak in seroprevalence was similar across age classes, although it was more pronounced for juveniles and sub-adults than adults, which showed consistently high seroprevalence (Fig. 3d).

Temporal changes in infection and immunity

For the Massachusetts gulls with both serology and virology data, we looked at the change in infection status by age class and seasonal stage (Fig. 4a). The relative proportions of each infection status did not significantly vary across seasons for sub-adults (Pearson chi-square (χ^2) = 3.963, df = 6, $P = 0.68$) and adults ($\chi^2 = 7.209$, df = 6, $P = 0.30$), but did for juveniles ($\chi^2 = 35.738$, df = 6, $P < 0.001$). From autumn to winter, there was a decrease in juveniles that were both virus positive and

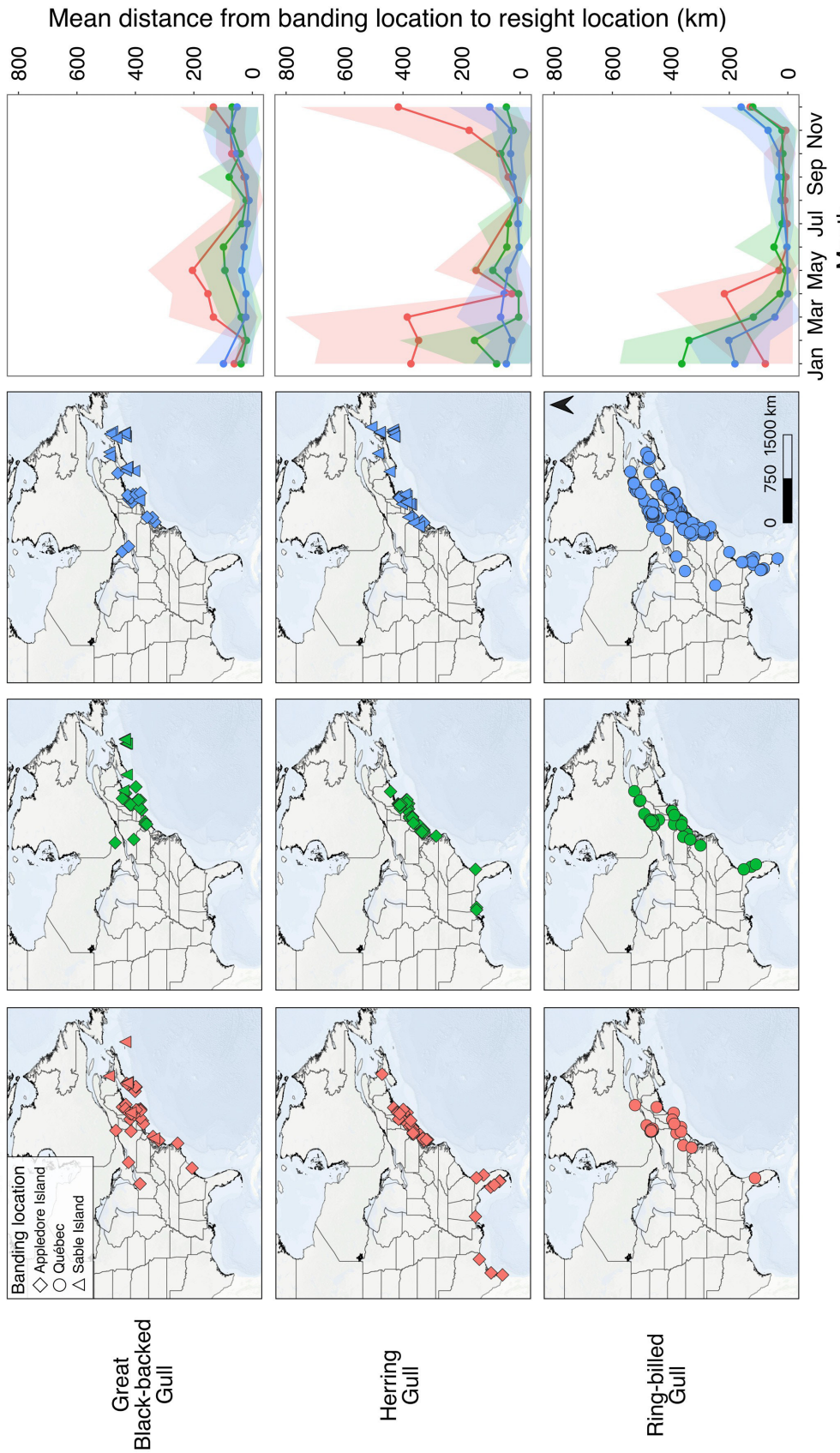

FIG. 2. Band resight locations by species and age class for gulls banded on Appledore Island (2009–2013; diamonds), Sable Island (2011–2013; triangles), and 'Ile Deslauriers in Quebec (2009–2013; circles) that were resighted from 2009 to 2014. Right column shows mean distance from banding location to resight location by month. Shading represents standard deviation.

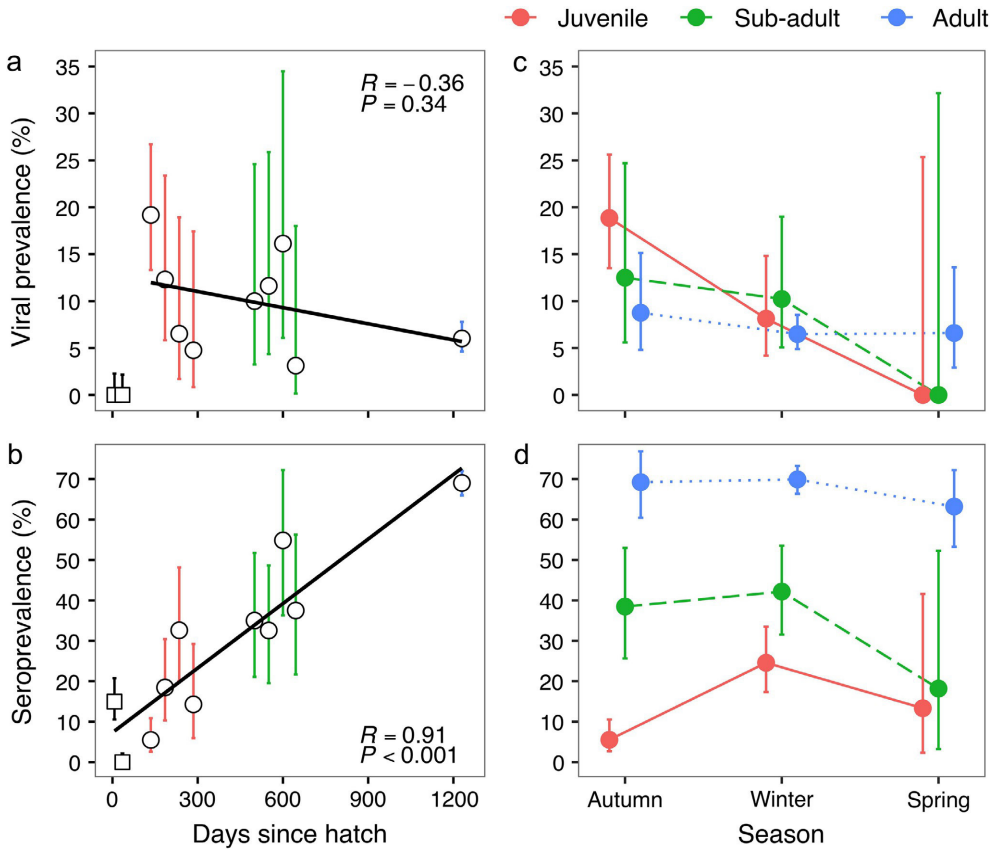

FIG. 3. Correlation between days since hatch and (a) viral prevalence or (b) seroprevalence for Herring Gull chicks captured on Appledore Island in 2013 (open squares) and Herring and Ring-billed Gulls captured in Massachusetts, 2012–2014 (open circles). Chicks were sampled at approximately 1 week old and 5 weeks old. Juveniles and sub-adults were binned into 50-day periods. Adults were combined into one group representing gulls at the beginning of their fourth cycle. Solid black lines represent best-fit regression lines. Error bars represent 95% confidence intervals. (c) Viral prevalence and (d) seroprevalence by seasonal stage and age class for Herring and Ring-billed Gulls sampled in Massachusetts, 2012–2014 (closed circles).

TABLE 1. Comparison of age variables for predicting influenza A virus prevalence and seroprevalence in Herring and Ring-billed Gulls sampled in Massachusetts.

Model	K	logLik	AICc	ΔAICc	w_i
Viral prevalence					
DSH	2	378.09	760.20	0.00	0.81
Age	3	378.94	763.89	3.69	0.13
HY	2	380.57	765.14	4.94	0.07
Null	1	386.67	775.34	15.14	0.00
Sero-prevalence					
DSH	2	799.24	1602.48	0.00	0.64
Age	3	798.81	1603.63	1.15	0.36
HY	2	822.63	1649.27	46.79	0.00
Null	1	956.17	1914.35	311.87	0.00

Notes: Generalized linear models were ranked by AICc. Age, categorical age class (juvenile, sub-adult, adult); DSH, days since hatch (assumed June 10 hatch date); HY, binary age class (hatch year, after hatch year); K, number of parameters; logLik, log likelihood; w_i, model weight.

seronegative and an increase in birds that were both virus negative and seropositive (Fig. 4a). Small sample sizes in the spring season may have limited our ability to analyze changes in these proportions from winter to spring. For 15 individuals captured twice over the course of the study, we found evidence of seroconversion ($n = 1$) as well as seroreversion ($n = 2$; Fig. 4b). One adult maintained a positive serostatus across 16.5 months, while another adult captured twice within 2.5 weeks went from positive to negative serostatus. The two recaptured juveniles were seronegative on both capture dates.

Shedding intensity and route

There were no differences in shedding intensity among age classes for the cloacal ($F_{2,71} = 0.556$, $P = 0.58$) and oropharyngeal swabs ($F_{2,58} = 1.467$, $P = 0.24$), however we found that adults shed more viral particles via the cloaca than the oropharynx ($P < 0.05$; Appendix S1:

TABLE 2. Top model sets from generalized linear models predicting influenza A virus prevalence and seroprevalence in Herring and Ring-billed Gulls sampled in Massachusetts.

Model	*K*	logLik	AICc	ΔAICc	w_i
Viral prevalence					
Season + DSH + Habitat	5	371.23	752.50	0.00	0.73
Season + DSH + Species	5	373.40	757.83	4.33	0.08
Season + DSH	4	374.58	757.19	4.69	0.07
Season + Habitat	4	375.11	758.25	5.75	0.04
Null	1	386.67	775.34	22.84	0.00
Seroprevalence					
Season + DSH + Year	5	790.44	1590.92	0.00	0.82
Season + Year	3	794.11	1594.23	3.31	0.16
Null	1	956.17	1914.35	323.43	0.00

Notes: Models were ranked by AICc, subset to the Δ6 AICc top models, then the model nesting rule was applied. The null model is shown for reference. DSH, days since hatch (assumed June 10 hatch date); Habitat, coastal or inland; Species, Herring Gull or Ring-billed Gull; *K*, number of parameters; logLik, log likelihood; Season, seasonal stage (autumn, winter, spring); w_i, model weight; Year, sampling year (2012–2013, 2013–2014).

TABLE 3. Top model results from logistic regression of influenza A viral and seroprevalence in Herring and Ring-billed Gulls sampled in Massachusetts.

	Estimate	SE	*z*-Value	*P*-value
Viral prevalence				
Intercept	1.0866	0.2556	4.251	<0.0001
DSH	0.0006	0.0002	2.866	0.0042
Winter	0.7764	0.2492	3.116	0.0018
Spring	0.8102	0.4436	1.826	0.0678
Inland	0.5690	0.2228	2.554	0.0107
Seroprevalence				
Intercept	2.4160	0.1986	12.167	<0.0001
DSH	0.0022	0.0002	14.373	<0.0001
Winter	0.2436	0.1606	1.517	0.1293
Spring	0.3035	0.2415	1.257	0.2088
2013/14	0.3821	0.1259	3.035	0.0024

Notes: Autumn, 2012–2013, and coastal were the reference levels for the categorical variables. DSH, days since hatch (assumed June 10 hatch date).

Fig. S2a). Overall, viral shedding prevalence was not significantly different for cloacal vs. oropharyngeal swabs ($v^2 = 1.284$, df = 1, $P = 0.26$). For 68 gulls, the oropharyngeal swab was positive, while the cloacal swab was negative, whereas the opposite was true for 54 individuals. Eight gulls were positive for both swabs, while the remaining ($n = 1{,}357$) were negative for both swabs (Appendix S1: Fig. S2b). From the multinomial model, we found that the probability of shedding from the cloaca compared with not shedding at all was 60% more likely for juveniles compared with adults (Appendix S1: Fig. S2c, Table S8). Furthermore, seropositive gulls that were also virus positive were equally likely to shed via the cloaca ($n = 27$) or oropharynx ($n = 26$; $v^2 = 0.00$, df = 1, $P = 1.00$).

Phylogenetic origins of virus and reassortment

We were able to isolate two viruses from the gull population, from one oropharyngeal swab and one cloacal swab. Both were low pathogenic subtypes, H13N6 and H13N8, collected from juvenile Ring-billed Gulls sampled during the early winter (November 9 and December 19) in Massachusetts. The viral segments for the two viruses indicated unique geographic origins (Appendix S1: Fig. S3). All eight segments of the H13N6 virus were determined to be of North American origin, while the H13N8 virus was determined to be a reassortant, composed of North American internal segments and Eurasian surface proteins (HA and NA).

To investigate the phylogenetic origins of the HA segments, Bayesian evolutionary reconstruction was performed. The H13N6 virus shared ancestry with viruses circulating in gulls and shorebirds from Delaware Bay to Newfoundland (June 2011 to September 2012: 95% highest posterior density [HPD]), which we termed the 'Northwest Atlantic' lineage (Appendix S1: Fig. S3). More recent descendants of this lineage were detected in Ring-billed Gulls in Minnesota (2017). The H13N8 virus shared ancestry with viruses circulating in Black-headed Gulls (*Chroicocephalus ridibundus*) from the Netherlands (July 2011 to April 2013: 95% HPD). This clade was termed the "Continental Europe" lineage and shared a common ancestor with H13 viruses from the Republic of Georgia and Russia. After detection in Massachusetts during our study, descendants of this lineage were observed in New Jersey and Chile in 2015–2016.

DISCUSSION

Studies of IAV dynamics in wild birds have shown that the age of an individual and length of their lifespan can predict prevalence. Na€ıve individuals, as well as their high turnover in a population, are understood to correlate with higher incidence of infection across both low and HP subtypes of IAV (Hoye et al. 2011, Pybus et al. 2012, Wikramaratna et al. 2014). However, few longitudinal studies have been conducted in nature to explicitly test how these temporal factors are modified by

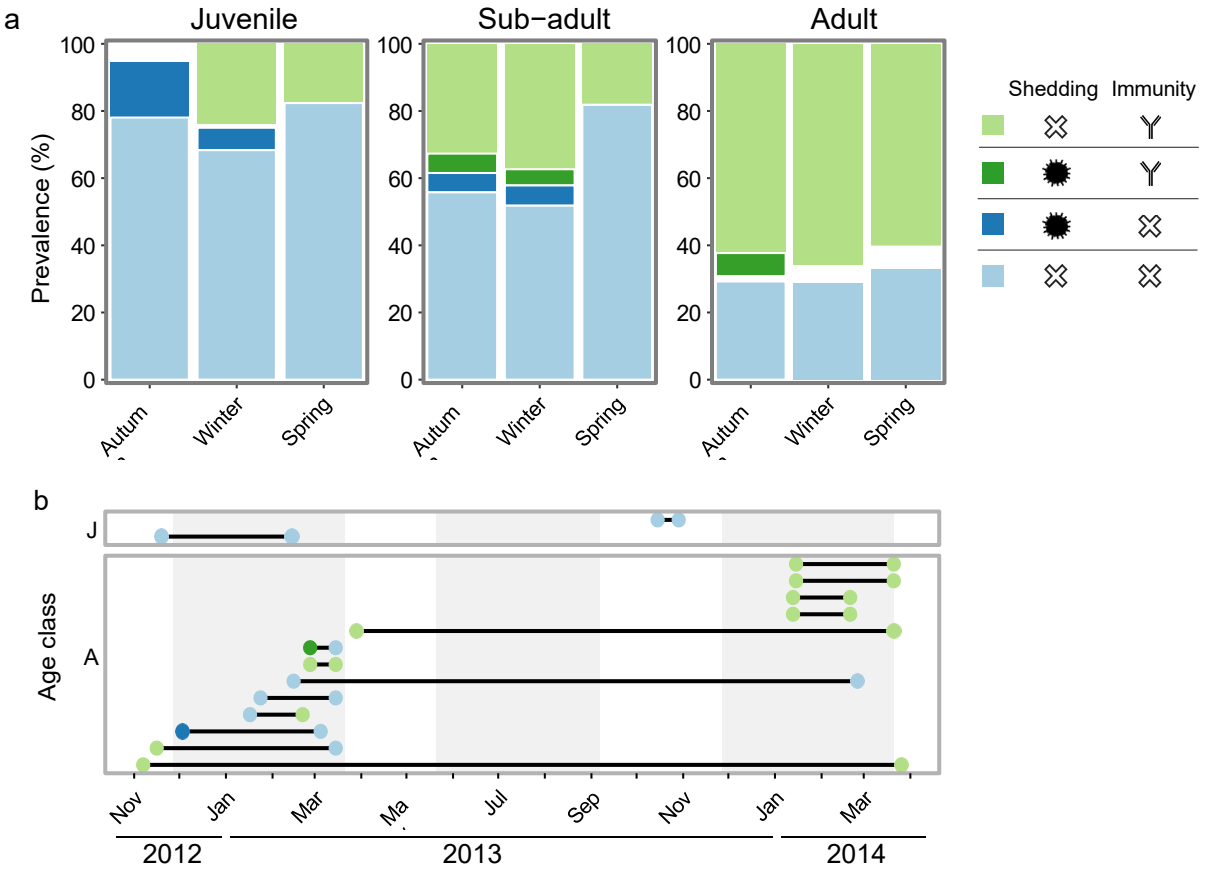

FIG. 4. Influenza A virus infection status in Massachusetts gulls. (a) Changes in viral prevalence (shedding) and seroprevalence (immunity) by age class and seasonal stage. The four categories of infection status include shedding positive (virus symbol), shedding negative (cross symbol), seropositive (antibody symbol) and seronegative (cross symbol). (b) Changes in the presence of anti-IAV serum antibodies in gulls over time for juveniles (J) and adults (A). Only recaptured individuals were included ($n = 15$). Markers are color coded by the four categories of infection status. Vertical gray and white bars indicate seasonal stages.

ecological drivers such as stage of the annual cycle, host species, and habitat type, or their interplay. The Anthropocene has also profoundly impacted the urbanization of landscapes with unknown consequences on the ecology of influenza in gulls. Here we show that both season and age are important determinants of annual epidemics in gulls, suggesting that with a more granular approach to age, we may better predict outbreaks and understand the epidemiology of IAV in the rapidly changing urban landscape.

Age-dependent infection and immunity in gulls

Infection in our study was concentrated in younger compared with older individuals, as other studies have shown across avian taxa including both Anseriformes (van Dijk et al. 2014*a*) and Charadriiformes (Verhagen et al. 2014). Our study advances the understanding of the age-dependent patterns of infection in IAV reservoirs by testing the performance of different measures of age. Days since hatch was negatively correlated with viral prevalence when chicks were excluded from the analysis, suggesting that post-fledging, gulls are progressively removed from the susceptible population as they age. Our models indicated that DSH had higher support as a predictor of viral prevalence compared with categorizing age into binary (juveniles, adults) or even tertiary (juveniles, sub-adults, adults) age groups. However, a strictly linear relationship between DSH and viral prevalence was not observed ($R = 0.36$). This may be a consequence of uneven sample size across age groups, especially for the sub-adult gulls for which small sample size and large variation in prevalence occurred. Alternatively, infection may be more episodic, resulting in fluctuations within age groups due to unaccounted variables, rather than a strictly linear relationship.

Complimentary to age-dependent infection, our results showed a strong correlation between DSH and seroprevalence, suggesting the accumulation of adaptive immunity and the ability of gulls to form immune memory. This relationship was strictly linear, an indication that exposure to IAV increases steadily with each

successive annual cycle. Beyond the narrative that young birds have low seroprevalence and adult birds have higher seroprevalence, few studies have contributed to a more resolved understanding of age-structured immunity in natural settings. Studies of Mute Swans (*Cygnus olor*) have shown that exposure to a breadth of subtypes increases with age (Hill et al. 2016), contributing to the higher mortality in juveniles observed with the incursion of novel strains such as highly pathogenic avian influenza H5N1 (Pybus et al. 2012). Introduction of novel strains coupled with the absence of broadly neutralizing immunity in juveniles may translate into higher susceptibility and severe disease for younger gulls. Understanding how the immune profile of gulls changes as a function of age is, therefore, useful for informing predictions of the magnitude and severity of outbreaks that, distinct from waterfowl, are more likely to occur in proximity to densely populated urban areas.

Juveniles drive horizontal transmission

The dense breeding grounds of gulls, coupled with the immuno-na€ıve state of the juveniles' immune systems, would seem prime hotspots for epidemics. Yet we found little evidence to suggest that vertical transmission is commonplace, findings similar to previous studies of wild avian reservoirs (Toennessen et al. 2011, Verhagen et al. 2014). Conversely, the natal site may be a haven of protection for newly hatched chicks due to passive immunity. The presence of seropositive 1-week-old chicks in our study suggests the presence of maternally derived antibodies (MDA) rather than prior infection. Maternal antibody transfer has been detected in other gull colonies up to a prevalence of 66% (Gasparini et al. 2006, Hammouda et al. 2011) as well as in a wide range of wild and captive birds (Garnier et al. 2011, van Dijk et al. 2014*b*, Dirsmith et al. 2018, Kowalczyk et al. 2019). We found that protection was temporary, with antibodies rapidly declining by approximately 2 weeks of age, similar to trends observed in other species (Garnier et al. 2011, Dirsmith et al. 2018). Loss of MDA before juveniles have fledged leaves them immuno-na€ıve when they move away from their natal site, setting the stage for their first infection upon abrupt exposure to other age classes, conspecifics from other colonies, and other avian species.

The large-scale movement during the autumn migration and mixing of immuno-na€ıve juveniles with other gull populations is likely to drive the primary infection peak. We detected some overlapping effects of age and season in our prevalence model, identifying juveniles during autumn as the primary drivers of this seasonal epidemic in gulls. Horizontal transmission appears to be central to the amplification of virus in early autumn, with presumably very little immune selection due to the lack of antibodies in young birds at this time. Positive cases were detected across all age groups in the autumn, suggesting that horizontal transmission involved young and old birds alike, but older birds to a lesser degree. We also found little evidence in our models that this pattern varied between host species. For all ages and species in our study, viral prevalence declined after the autumn peak to a minimum prevalence in spring, a pattern detected in other host systems (van Dijk et al. 2014*a*).

Fluctuations in immunity during the annual cycle

The duration of antibodies is assumed to be long lasting, but longitudinal studies in nature have repeatedly shown that the immune response in avian reservoirs is highly variable. Upon infection, one gull in this study maintained antibodies for up to 16.5 months (although it is also possible that re-infection between sampling events occurred). In Black-headed Gulls, antibodies against H16 were detected up to 11 months after the second inoculation, while H13 antibodies were detected for only 1 month after infection (Verhagen et al. 2015). Antibody titer and duration may depend on HA subtype, but are also influenced by a range of host factors (age, sex, number of prior infections) and ecological pressures (migratory burden, reproductive stage). We found evidence that some immune responses were not long lived, as antibodies waned and dropped below detectable levels in some adults within 18 d. Two adults sero-reverted in late winter, similar to results found in Mallards (*Anas platyrhynchos*) in Alaska (Spivey et al. 2017) and the summer trend in seroreversion in Mallards in Sweden (Tolf et al. 2013). Seroreversion could explain the drop in seroprevalence detected during the spring. A decline in population-wide immunity was observed across all ages, suggesting that the spring migration, or other pressures during this time period, may impose limits on the immune system to maintain circulating antibodies.

Prior infection does not prevent subsequent infection with a different IAV subtype (Verhagen et al. 2015). In addition, antibody titers may be boosted by re-infection with the same or different subtype (Latorre-Margalef et al. 2017). As we did not determine viral or antibody subtype except for two individuals from which viral sequencing identified H13, we cannot determine whether active infections were the same or different subtypes for seropositive gulls. Furthermore, the potential for dominant subtypes to come and go over time (Krauss et al. 2004) and the lack of cross-protective antibodies against antigenically distinct subtypes may explain why sampling year was an important factor in our model of seroprevalence. We suggest that the observed pattern was not due to higher sampling in the second year of the study, as prevalence was not correlated with capture effort binned by week (Pearson correlation; $R = 0.19$, $P = 0.232$).

Adults stay close to home, while juveniles disperse long distances

The peak in viral prevalence in newly fledged gulls has important implications for the spread of pathogens to

new hosts and species during the migratory season (Bogomolni et al. 2008). Juveniles are the most likely to undertake long-distance, exploratory migrations (Drury and Nisbet 1972, Hebert 1998, Camphuysen et al. 2011, Jorge et al. 2011) where they may encounter other gull species (Guinn et al. 2016), shorebirds, and ducks (Krauss et al. 2004). We also detected age-specific migration patterns, with juveniles traveling farther than older birds for two out of the three gull species studied. The patterns of adult dispersal determined by band resightings in this study were consistent with available satellite tracking data of adult gulls (Clark et al. 2016, Anderson et al. 2019, 2020), suggesting that band resights provided a good insight into movement patterns in our gull populations. Juveniles undertaking longer migrations may expand the host contact network and increase the potential for exposure to novel pathogens, against which they are immunologically naɛve. Alternatively, migratory escape may offer animals a way to reduce their disease burden by relocating far from their natal origin (Altizer et al. 2015).

In addition, the inferior foraging abilities of juveniles (Greig et al. 1983, MacLean 1986) is thought to account for their predominance at urban areas and landfills (Kadlec and Drury 1968), potentially increasing pathogen exposure and contact rates between individuals (Becker et al. 2015, Plaza and Lambertucci 2017) instead of escaping the build-up of pathogens close to the breeding grounds (Altizer et al. 2011). However, not all juveniles migrate far from the breeding colony (Drury and Nisbet 1972, Burger 1981, Clark et al. 2016), resulting in a mix of age classes throughout the year at urban anthropogenic feeding sites (Kadlec and Drury 1968, Patton 1988, Belant et al. 1998, Anderson et al. 2019). We captured juveniles throughout the non-breeding period in urban Massachusetts and even recaptured one in the middle of the winter, suggesting that a minority of juveniles over-winter near their breeding grounds. This pattern of residency among adults and a subset of younger birds may influence transmission dynamics, as these gulls may be more likely to stay in urban areas and act as a reservoir. Regardless of migratory tendency, we found that viral prevalence was lowest in spring, suggesting that IAV is not widely circulating in Massachusetts gulls as they head northward toward their breeding colonies.

Viral reassortment in urban coastal habitat

Gulls are considered to play an outsized role in the long-distance, interhemispheric movement of IAV compared with waterfowl, and studies have repeatedly shown a high degree of reassortment in virus hosted by gulls (Wille et al. 2011*a*, *b*, Hall et al. 2013, Dusek et al. 2014, Huang et al. 2014). Consistent with this notion, an interhemispheric reassortant was identified as one of the two viruses that were successfully isolated and sequenced from juvenile Ring-billed Gulls in Massachusetts. The H13N6 virus was determined to be entirely North American in origin, while the H13N8 virus was a reassortant, composed of North American internal segments and European surface proteins (HA and NA). That this reassortant virus was detected in urban Massachusetts highlights the potential for gulls to disperse virus far from their European source, particularly juveniles in view of their long-distance migration. The exact mechanism for interhemispheric viral flow is likely to involve multiple transmission chains, rather than direct transmission by a single gull. Further studies are needed to understand whether urbanization of gull habitat accelerates the redistribution of viral segments, as we lacked the statistical power to evaluate this further.

We found that gulls in coastal habitats had higher viral prevalence than gulls captured inland, possibly correlating with higher richness of waterbird species along the coast. Virus circulation may be amplified where gulls overlap with other migratory species that rely on coastal habitat as a stopover or wintering site. For instance, arctic breeding species that winter along the coast may be in contact on the breeding grounds with species that winter in Europe (Dusek et al. 2014). The three gull species that were the focus of our study converged spatially along the coast, rather than inland, indicating that opportunities for interspecies transmission between gull species from different geographic origins may be concentrated at the shoreline (see also Clark et al. 2016, Anderson et al. 2019). We propose that coastal habitat is likely to support a higher turnover and species richness of hosts relevant to transmission of gull-origin virus, a process that is also favorable to reassortment involving co-infection by multiple viruses. The interhemispheric reassortant virus identified in this study was recovered from Revere Beach, an urban area of the Atlantic coast with proximity to Boston, while the non-reassortant was identified inland. A future direction for surveillance is to understand whether urbanization, particularly along the continental margins, is associated with reassortant hotspots that contribute to hemispheric mixing of virus.

Implications for surveillance

Based on our observed patterns of virus and antibody prevalence across ages and seasons, we developed a model of influenza dynamics, representative of gulls in urban landscapes (Fig. 5). The implications for surveillance include targeted sampling of the sites where juvenile gulls first congregate on the mainland after leaving the colony, as this is where most virus will be detected. Despite our nearly year-round sampling of this regional population of gulls, we did not have any samples from late August to mid-October and potentially missed the largest spike in viral prevalence and infection intensities soon after fledging (Brown et al. 2012, Verhagen et al. 2015). Sampling juveniles during this period may be challenging due to their mobility. Mainland resights of gulls banded on island colonies could provide target sampling locations to complete the picture of exactly

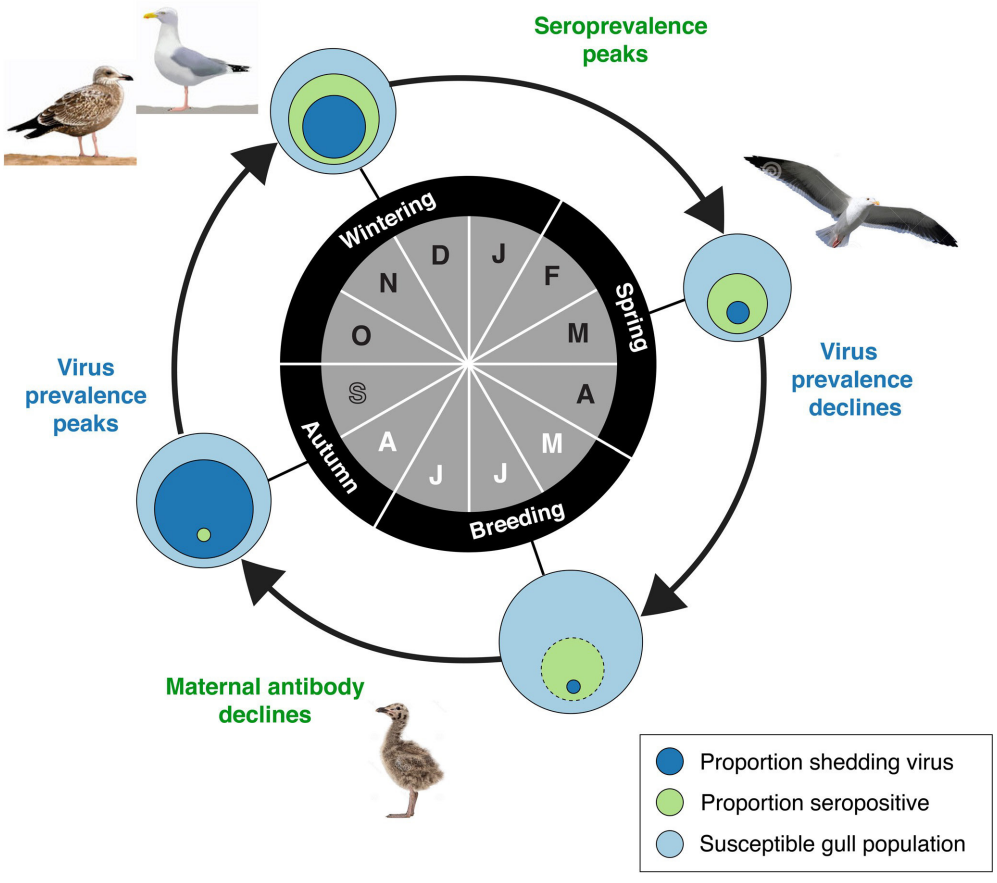

FIG. 5. Proposed chronology of influenza A virus prevalence and seropositivity in juvenile gulls throughout the annual cycle. Circle markers indicate the relative proportion of juveniles actively shedding virus (dark blue), seropositive (light green), and susceptible (light blue). Months during which gulls were captured and sampled in Massachusetts (black letters) and on breeding colonies (white letters) are indicated.

when and where fledglings are becoming infected. During this same time frame, infection also spiked in subadults, which were lumped into one age class for our study. When possible, we suggest aging to annual cycle based on plumage to separate second and third year birds. This will allow a more continuous assessment of prevalence with age, as our DSH models begin to demonstrate, although obtaining adequate sample sizes may be a challenge.

Furthermore, our shedding route findings suggest that both cloacal and oropharyngeal swabs should be collected during sampling, which will help to avoid negatively biasing viral prevalence results (Froberg et al. 2019). We found that very few positive individuals were actively shedding from both the cloaca and oropharynx. Juveniles were more likely to shed via the cloaca than adults, who shed from the cloaca and oropharynx at equal rates, but overall prevalence and infection intensities were similar between swab types. We were also able to amplify and sequence viruses from both swab types, suggesting that neither swab type was preferable over the other.

While tracking gulls over the course of the annual cycle is challenging, their tendency to congregate in urban areas makes sampling relatively easy. Given the scale of the human footprint, non-urban gulls should also be sampled as they may still come into contact with and congregate at anthropogenic sites away from urban areas, creating other opportunities for IAV transmission. Future surveillance efforts across the urbanization spectrum will help to further expand our understanding of how anthropogenic (Murray et al. 2019, Geffroy et al. 2020), demographic, and environmental factors drive IAV dynamics throughout the annual cycle. As gulls increasingly adapt to rapidly expanding urban landscapes in the Anthropocene, these insights will be key to informing global conservation efforts and protecting animal and human health.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/eap./full

Open Research

Data (Ineson 2021) are available through Figshare: https://doi.org/10.6084/m9.figshare.14174852