Veterinary Research

RESEARCH ARTICLE

Open Access

Avian influenza virus circulation and immunity in a wild urban duck population prior to and during a highly pathogenic H5N1 outbreak

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 clade 2.3.4.4b viruses were first detected in St. John's, Canada in late 2021. To investigate the patterns of avian influenza virus (AIV) infection and immune responses subsequent to the arrival of H5N1, we sampled the wild urban duck population in this area for a period of 16 months after the start of the outbreak and compared these findings to those from archived samples. Antibody seroprevalence was relatively stable before the outbreak (2011–2014) at 27.6% and 3.9% for anti-AIV (i.e., NP) and H5-specific antibodies, respectively. During the winter of 2022, AIV-NP and H5-specific antibody seroprevalence both reached 100%, signifying a population-wide infection event, which was observed again in late February 2023 following a second H5N1 incursion from Eurasia. As expected, population-level immunity waned over time, with ducks seropositive for anti-AIV-NP antibodies for approximately twice as long as for H5-specific antibodies, with the population seronegative to the latter after approximately six months. We observed a clear relationship of increasing antibody levels with decreasing viral RNA loads that allowed for interpretation of the course of infection and immune response in infected individuals and applied these findings to two cases of resampled ducks to infer infection history. Our study highlights the value of applying both AIV surveillance and seroprevalence monitoring to provide a better understanding of AIV dynamics in wild populations, which may be crucial following the global dissemination of clade 2.3.4.4b H5Nx subtypes to assess the threats they pose to both wild and domestic animals, and to humans.

Keywords Highly pathogenic avian influenza virus, H5N1, serology, resident and migratory ducks, immunity

Handling editor: Kate Sutton.

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Introduction

Wild birds are the reservoir hosts of avian influenza viruses (AIVs), with waterfowl being one of the main reservoir groups and vectors by which AIVs are spread, along with gulls, shorebirds, and seabirds [1–4]. Low pathogenic avian influenza virus (LPAIV) infection of waterfowl rarely results in overt disease symptoms, with birds usually clearing the infection within a matter of days. Dabbling ducks (Anatinae) infected with highly pathogenic avian influenza virus (HPAIV) H5Nx subtypes, similar to LPAIV infection, can be minimally affected while shedding large quantities of virus, with mild disease symptoms and delayed local movements in some cases [5–8]. While many species of diving ducks (Aythyinae) also appear to be minimally affected, some such as tufted ducks (*Aythya fuligula*) have been shown to be particularly prone to experience symptomatic HPAIV H5Nx infections and can exhibit severe infection outcomes and high rates of mortality [6, 9, 10]. Recently, higher mortality in dabbling ducks due to HPAIV infections has been observed, representing a new pattern in one of the main reservoir hosts [10–12].

HPAI clade 2.3.4.4 H5Nx viruses have been circulating with increasing frequency in wild birds in Eurasia and Africa since 2005 [9, 13–15], with the first incursion of A/goose/Guangdong/1/1996 (GsGd) lineage H5N8 clade 2.3.4.4 viruses into North America taking place in 2014 [16]. This virus, and a reassortant H5N2, did not persist and become established in North American wild bird populations. However, new incursions of clade 2.3.4.4b viruses starting in late 2021 have resulted in extensive reassortment with North American lineage LPAIVs, widespread circulation of H5Nx viruses throughout North and South America within a wide array of avian hosts, and multiple spillover events into mammals [17–20]. These HPAIVs now seem to be part of the endemic viral population in wild birds in the Americas, Eurasia, and Africa.

AIV surveillance in wild birds has been a global focus for decades and has contributed to understanding viral dynamics and identifying circulating strains in different regions and species. However, this has not been without challenges. Non-gallinaceous birds infected with LPAIVs are usually asymptomatic and test positive for viral RNA for only a very short period, generally 5–11 days [21–25], providing a narrow sampling window for the detection of active infections. An increasing number of serological studies have helped address this shortcoming, by which past AIV infection can be documented via detection of anti-AIV antibodies in the peripheral circulation for a period of months [24, 26–28]. A combined approach of AIV infection surveillance and serology can therefore help capture AIV dynamics in more detail and over a longer time frame, allowing interpretation of both active and past infections in populations [8, 29, 30].

A HPAI H5N1 clade 2.3.4.4b virus was identified in a great black-backed gull (GBBG, *Larus marinus*) that died in November 2021 in St. John's, Newfoundland and Labrador, Canada, and was found to be closely related to viruses circulating in northwestern Europe in the spring of 2021 [31]. Shortly after this first detection, the virus was identified in an exhibition farm in the area that housed primarily domestic fowl, which resulted in mass mortality [31]. Sampling of wild urban ducks in the area began about a week later and active H5N1 infection was detected in the duck population in late December 2021.

The aim of this study was to thoroughly investigate patterns of AIV infection and immunity in this duck population over a period of approximately 16 months after the first arrival of H5N1 clade 2.3.4.4b to North America in November 2021. We accomplished this goal by employing a combination of AIV surveillance to understand when infection was occurring and serology, specifically of general anti-AIV-NP as well as H5-specific antibodies, to understand immune responses. This work focused on how immunity changed over time while epidemiological information provided context and timing of infection(s) and bird movements.

Materials and methods

Bird capture and sampling

Wild ducks were caught either by hand or bait trapping at several locations in or near the city of St. John's, Newfoundland, including Bowring Park (47.528862°, −52.745943°), Commonwealth Pond (47.500765°, −52.789646°), Kenny's Pond (47.591366°, −52.715759°), Kent's Pond (47.589212°, −52.722767°), Mundy Pond (47.551419°, −52.741791°), Quidi Vidi Lake (47.579076°, −52.699627°), and Topsail Pond (47.524388°, −52.903371°). Sampling occurred in the fall and early winter months from 2011 to 2014, and at 11 timepoints through 2022 and 2023 during the ongoing HPAIV outbreaks (see Additional file 1). Bird age was determined using plumage aspect and cloacal characteristics [32, 33]. Age categories included hatch year (HY), after hatch year (AHY), second year (SY), and after second year (ASY). Hatch year birds that have not yet fledged are denoted as local (L). All birds were banded with a metal leg band issued by the Canadian Wildlife Service Bird Banding Office.

Capture efforts targeted primarily mallards (*Anas platyrhynchos*), American black ducks (*A. rubripes*), northern pintails (*A. acuta*), and occasionally hybrid ducks that were a combination of American black ducks, mallards, and/or feral domesticated ducks (*Anas* spp.). Additional species were sampled opportunistically in 2022 and 2023, specifically American wigeon (*Mareca americana*),

Eurasian wigeon (*M. penelope*), and lesser scaup (*Aythya affinis*). As there were a limited number of ducks that were AIV RNA-positive at the time of capture, we included AIV surveillance and serology data from several seabird species originating from other work to explore a larger dataset for an analysis on the relationship between RNA load and antibody levels. These species were also impacted by outbreaks of HPAI H5N1 that occurred during the summer of 2022. Data for 100 seabirds sampled at breeding colonies in eastern Newfoundland were included, with Atlantic puffins (*Fratercula arctica*), black-legged kittiwakes (*Rissa tridactyla*), and common murres (*Uria aalge*) sampled between June and August 2022 and in June 2023, and northern gannets (*Morus bassanus*) sampled in July 2022.

Observations of wild bird movements

Observations and remarks regarding the patterns of arrival of migratory individuals and timeline and movements of non-resident species were primarily made directly while working in the field, with additional support provided by local experts, other birders in the region, sightings posted to birding social media pages, and submissions to eBird [34].

Bird banding and encounter data

To provide further support of this wild urban duck population being comprised of primarily resident individuals, we obtained encounter data (reporting of a bird band) from any location for all dabbling and diving ducks banded within a 20-km radius of St. John's from 1 January 2010 to 17 May 2023 from the Canadian Wildlife Service Bird Banding Office.

Sampling periods

For analysis purposes, samples collected between 2011 and 2014 were grouped into sampling seasons. Sampling occurred across multiple months between September and March of each period, and are referred to as the 2011–2012, 2012–2013, 2013–2014, and 2014–2015 seasons. Samples collected after H5N1 was first detected in 2021 were grouped as follows: winter-spring 2022 (January to May 2022), summer-fall 2022 (July to September 2022), and then separately for samples collected in the months of February, March, and April 2023. Specific details about when each sample was collected can be found in Additional file 1.

Serology

Two to three millilitres of blood were drawn from the brachial wing vein of each captured individual. Serum was separated from clotted blood by centrifugation at 3000 × *g* for ten minutes and subsequently stored at −20 °C for future analysis. For 19 samples collected at the start of the outbreak and 28 archived samples, AIV competitive enzyme-linked immunosorbent assays (cELISAs) were performed at the National Centre for Foreign Animal Disease (NCFAD) laboratory as previously described [35]. All other samples were tested using the IDEXX AI MultiS Screen Ab test (IDEXX Canada, Product # 99-12119) as per the manufacturer's instructions, which detects antibodies against influenza A nucleoprotein (NP) [36], with 12 samples initially tested at the NCFAD later re-tested using this assay. A sample to negative control ratio (S/N) of < 0.5 was considered positive for influenza antibodies. As some studies have employed a S/N ratio of < 0.7 for positivity [27, 36, 37], this value is shown on relevant figures for comparison purposes. Samples from the 2011–2012 period were no longer available, therefore the previously published data were used [38]. All sera positive for anti-AIV-NP antibodies were subsequently tested at the NCFAD for antibodies specifically against subtype H5 using an in-house developed cELISA that detects antibodies against all clades of subtype H5 [39].

Swab samples and RNA extraction

Oropharyngeal and cloacal swabs were collected from all individuals from 2022 to 2023 and the paired swabs were pooled into a single tube of Multitrans viral transport medium (Starplex Scientific, Product # S160-100) and represent a single sample per individual. Samples were stored in a cooler on ice and an aliquot was removed for RNA extraction within six hours, and samples were subsequently stored at −80 °C. RNA was extracted from 140 µL of each sample using the Qiagen Viral RNA Mini Kit (Qiagen, Product # 52906) as per the manufacturer's instructions and stored at −80 °C until further analysis.

Screening for influenza A viruses

Real-time RT-PCR was performed using AgPath-ID™ One-Step RT-PCR reagents (Applied Biosystems, Product # 4387424) on either a StepOnePlus or 7500 Fast Real-Time PCR System (Applied Biosystems). All samples were screened for the presence of the influenza A virus (IAV) matrix gene and subsequent positives were screened for the H5 subtype of the haemagglutinin gene. RT-qPCR primers and probes, and cycling conditions detailed in [40] were used, with some modifications. For the initial RT-qPCR targeting the IAV matrix gene, 25 µL reactions were prepared using 12.5 µL of 2X RT-PCR buffer, 1 µL of 25X RT-PCR enzyme mix, 0.25 µL of 20 µM F25 (5′-AGATGAGTCTTCTAACCGAGGTCG-3′), 0.25 µL of 20 µM R124 (5′-TGCAAAAACATCTTCAAGTCTCTG-3′), 0.25 µL of 20 µM R124M (5′-TGCAAAGACACTTTCCAGTCTCTG-3′), 0.25 µL of 6 µM double-quenched probe

F64P (5′-[FAM]-TCAGGCCCC[ZEN]CTCAAAGCCGA-[IB]-3′) (IDT Inc., Canada), 1.67 µL of AgPath Detection Enhancer (Applied Biosystems, Product # A44941), 0.83 µL of nuclease-free water, and 8 µL of RNA. Cycling was performed in standard mode, with parameters as follows: 45 °C for 20 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, and 60 °C for 1 min at which time fluorescent signal was detected. A standard curve of IAV RNA as well as no-template controls were included during each run. Thresholds were determined automatically by the instrument's software based on the standard curve, and this threshold was applied after manual confirmation to determine the cycle threshold (Ct) values for each sample. Samples that yielded the characteristic amplification curve and had a $Ct \leq 45$ were interpreted as positive [41–43], while those that yielded the characteristic amplification curve but did not surpass the threshold were interpreted as inconclusive and denoted as having a $Ct > 45$.

All samples that yielded an amplification curve for the IAV matrix RT-qPCR were subsequently screened for the H5 subtype using primers, probes, and cycling conditions detailed in [44], with some modifications. The 25 µL reactions were prepared using 12.5 µL of 2X RT-PCR buffer, 1 µL of 25X RT-PCR enzyme mix, 0.25 µL of 20 µM H5_1456-NA_F (5′-ACGTATGACTATCCACAA TACTCA-3′), 0.25 µL of 20 µM H5_1456-EA_F (5′-ACG TATGACTACCCGCAGTATTCA-3′), 0.125 µL of 20 µM H5_1685_R (5′-AGACCAGCTACCATGATTGC-3′), 0.125 µL of 20 µM H5_1685M_R (5′-AGACCAGCTATC ATGATTGC-3′), 0.25 µL of 6 µM double quenched probe H5_1637P (5′-[FAM]-TCAACAGTG[ZEN]GCGAGT TCCCTAGCA-[IB]-3′) (IDT Inc., Canada), 2.5 µL of nuclease-free water, and 8 µL of RNA. Cycling was performed in standard mode, with parameters as follows: 45 °C for 20 min, 95 °C for 10 min, followed by 45 cycles of 94 °C for 10 s, 57 °C for 40 s at which time fluorescent signal was detected, and 72 °C for 5 s. Any samples that yielded the characteristic amplification curve were interpreted as positive for H5.

Samples positive for the matrix gene that tested negative for H5 were presumed to be LPAIVs. For these samples, a 686-bp fragment of the HA2 region of the haemagglutinin gene was amplified using the NEB OneTaq® One-Step RT-PCR Kit (New England Biolabs, Product # E5315S) and sequenced to determine the HA subtype. 25 µL reactions were prepared using 12.5 µL of 2X OneTaq One-Step Reaction Mix, 1 µL of 2X OneTaq One-Step Enzyme Mix, 1 µL of 10 µM HA-1134F (5′-GGRATGRTHGAYG-GNTGGTAYGG-3′), 1 µL of 10 µM Bm-NS-890R (5′-ATA TCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′), 1.5 µL of nuclease-free water, and 8 µL of RNA. Cycling parameters were as follows: 48 °C for 60 min, 95 °C for 5 min, 7 cycles of 94 °C for 15 s, 42 °C for 30 s, and 68 °C for 3 min, then 35 cycles of 94 °C for 15 s, 58 °C for 30 s, and 68 °C for 3 min, followed by a final extension at 68 °C for 7 min. PCR products were subjected to electrophoresis for visualization, and amplicons were purified using AMPure XP beads (Beckman Coulter) and subjected to Sanger sequencing at The Hospital for Sick Children (Toronto, Canada).

Classification of individual infection status

We used antibody levels (S/N ratios), AIV RNA load, and epidemiological data, specifically the known dates of population-wide infection, to classify each individual based on their infection status. Currently infected individuals were those with detectable viral RNA. Recently infected individuals represented those that were negative for AIV RNA but were sampled within six months of the population-wide infection events or had elevated antibody levels ($S/N < 0.7$). They were subdivided into categories of being infected one, three, or six months previously based on the epidemiological patterns, or recently infected if the time between infection and sampling was unknown. Individuals having low antibody levels ($S/N > 0.7$) were classified as being either naïve or having antibody levels that had waned.

Statistical analysis and data visualization

R v4.1.0 [45] was used to perform data manipulation, statistical analysis, and data visualization. To test whether antibody levels declined over time at different rates for anti-NP and anti-H5 antibodies, we used a generalized linear model with a binomial response and a logit link function. S/N ratios were used as the response, with the week of the year as a linear covariate and a week by anti-NP/anti-H5-specific antibody interaction. A significant interaction term meant the rates of decline for the two types of antibodies were different. In order to test whether antibody levels had changed since the time of last infection, a simple linear model was used, with S/N ratios as the response, and time since last infection (1, 3 or 6 months) as a linear covariate. A p-value < 0.05 was considered as significant for all tests. R packages used included cowplot v1.1.1 [46], data.table v1.14.2 [47], ggpattern v1.1.1-0 [48], ggplot2 v3.4.0 [49], and readxl v1.4.2 [50].

Results

Samples collected and used in the study

A total of 217 serum samples were collected from ducks between 2011 and 2014. After the first cases of HPAI H5N1 in the province in late 2021, 83 paired swab and serum samples were collected from ducks between January 2022 and April 2023. In total, 300 duck sera are

included in this study from 298 individuals, with two ducks recaptured and resampled in 2023. Paired swab and serum samples from 100 seabirds that were sampled during the summers of 2022 and 2023 were also included for an analysis on the relationship between viral RNA load and antibody levels, providing a larger dataset than available solely from the ducks.

Movement patterns of banded ducks

In total, 1045 ducks were banded within the St. John's area (20-km radius) between 1 January 2010 to 17 May 2023. Of these banded birds, 176 were reported as being encountered at least once, with 172 (97.7%) being reported in St. John's, two reported elsewhere on the island of Newfoundland, one reported in Labrador, and one reported in Nova Scotia. Of all 176 encounters, 104 were reported dead/by hunters, 70 by recapture or resightings, and two were unspecified. This provides additional support that the wild urban duck population comprises primarily resident individuals that spend their entire lives in the same local region.

Changes in population seroprevalence over time

Before the incursion of HPAI H5N1 into the region in November 2021, the overall mean AIV-NP seroprevalence was 27.6% (range 17.6–52.6%) for the sampling seasons of 2011–2015. Antibodies specifically against H5 were markedly lower at a mean seroprevalence of 3.9% (range 2.2–5.6%) between 2012 and 2014 (Figure 1), which reflects the fact that there were no HPAI H5Nx viruses circulating in this region during this time period and that LPAIV H5 strains circulate in this population at low prevalence [38]. In the winter-spring period of 2022, one to four months after the arrival of HPAI H5N1, AIV-NP and H5-specific seroprevalence reached 90.9% (20/22) and 81.8% (18/22), respectively. This indicates that after the introduction of H5N1 to the region, most of the population was infected with this virus. During the summer-fall period of 2022, seroprevalence decreased to

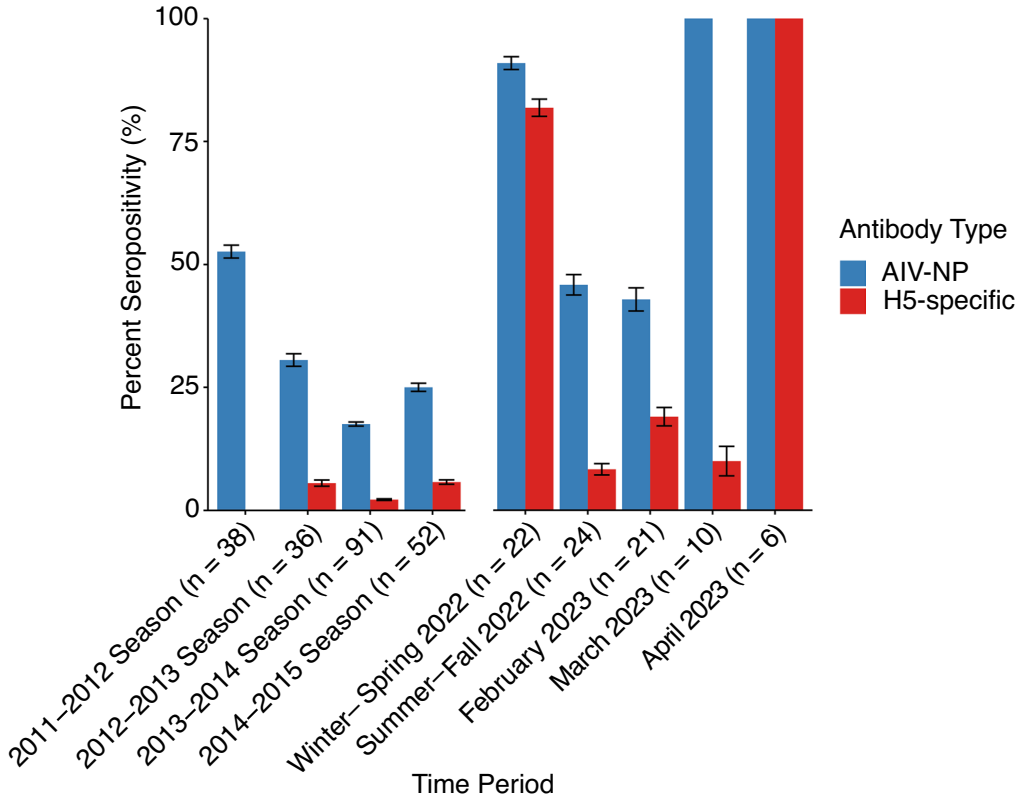

Figure 1 AIV-NP and H5-specific seropositivity in the urban duck population over time. Sampling occurred in the fall and early winter months between 2011 to 2014, and then at 11 timepoints through 2022–2023 during the ongoing HPAI H5N1 outbreak. AIV seropositivity data for samples from 2011–2012 were previously published [38] and the H5-specific ELISA was not performed with these since they were no longer available. Only samples that were positive for AIV antibodies were tested for H5-specific antibodies. Mean seropositivity with standard error bars is shown.

45.8% (11/24) and 8.3% (2/24) for AIV-NP and H5-specific antibodies, respectively. Therefore, H5 seropositivity essentially returned to the baseline levels observed before the arrival of H5N1. In February 2023, just over one year after the original incursion, AIV-NP seroprevalence was approaching similar levels as observed over 2011–2014 at 42.9% (9/21), while H5-specific seroprevalence had increased to 19% (4/21). Approximately three weeks later in March 2023, AIV-NP seroprevalence rose to 100% (10/10) and H5 RNA was detected in four (40%) of these ducks. Only one of the ducks (10%) was seropositive for H5-specific antibodies at this time. Seven weeks later, at the end of April 2023, all six individuals sampled were seropositive for both AIV-NP and H5-specific antibodies (Figure 1).

To further understand how immunity in the population changed over time, we investigated the seroprevalence ($n = 75$) over 64 weeks, specifically from 28 January 2022 through 25 April 2023, and also tested for AIV infection over this period (Figure 2). After the initial incursion of the clade 2.3.4.4b H5N1 virus and the population-wide infection event, seroprevalence decreased substantially over time. By approximately six months later, all ducks were seronegative for H5-specific antibodies, while half were still anti-AIV-NP antibody seropositive. AIV-NP antibodies were elevated for approximately twice as long as H5-specific antibodies ($\chi^2 = 4.97$, df = 9, $p = 0.0005$) over this period. A change in AIV-NP seropositivity occurred between weeks 32 and 37, corresponding to July and August 2022, when several individuals tested positive for non-H5 AIVs (H9Nx, H11Nx, and two additional strains of unknown HA subtype). This resulted in a slight increase in seropositivity that aligned with detection of LPAIVs through the summer of 2022 to February 2023 (Figure 2). In March 2023, H5-subtype viral RNA was again detected, and all birds sampled were AIV-NP seropositive, with only one of these individuals seropositive for H5-specific antibodies at this time. By the end of April 2023, all individuals sampled were seropositive for both AIV-NP and H5-specific antibodies, indicating that an H5 subtype virus had again spread through the population. Overall, using a combination of AIV surveillance and strain subtyping, serology, and epidemiology we were able to construct a robust timeline of AIV

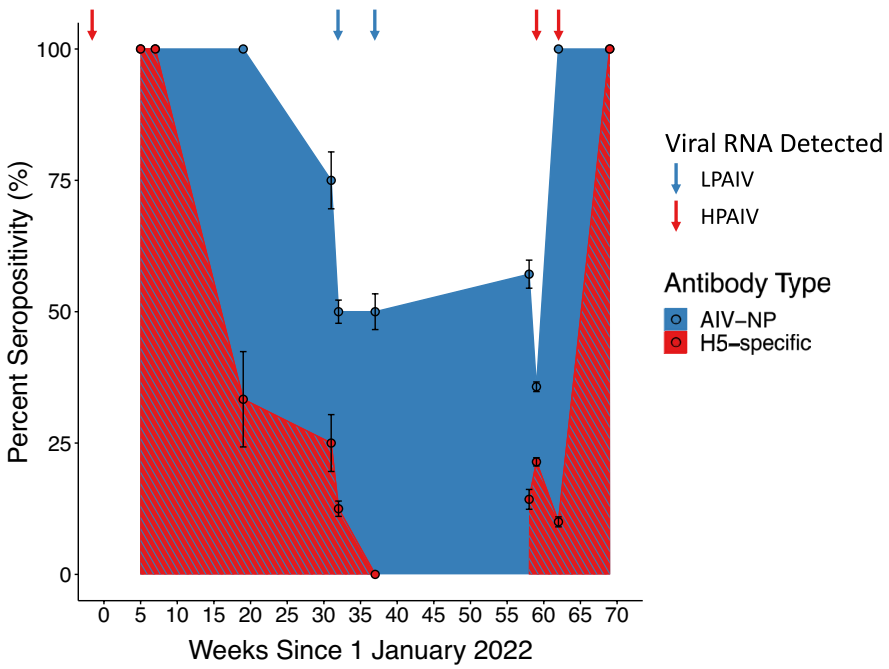

Figure 2 Changes in AIV-NP and H5-specific seropositivity over the course of 16 months after the arrival of H5N1. Sampling began on 28 January 2022 and continued until 25 April 2023. Arrows above the plot correspond to AIV detections in individuals sampled at that time point, with red arrows denoting when HPAIV (H5N1) was detected and blue arrows denoting LPAIV(s) was detected. Mean seropositivity along with standard error bars is shown. A hatched pattern is used for H5-specific seroprevalence to indicate that it overlays the AIV-NP seroprevalence. The two seronegative individuals sampled in February 2022 were removed from this week-by-week analysis as they were believed to have not been present at the time of the population-wide infection event (see Discussion). Additionally, the six individuals sampled on 23 August 2022 were also removed from this analysis as they were presumed to be primarily migratory individuals, coinciding with the large influx of individuals during this post-breeding migration period.

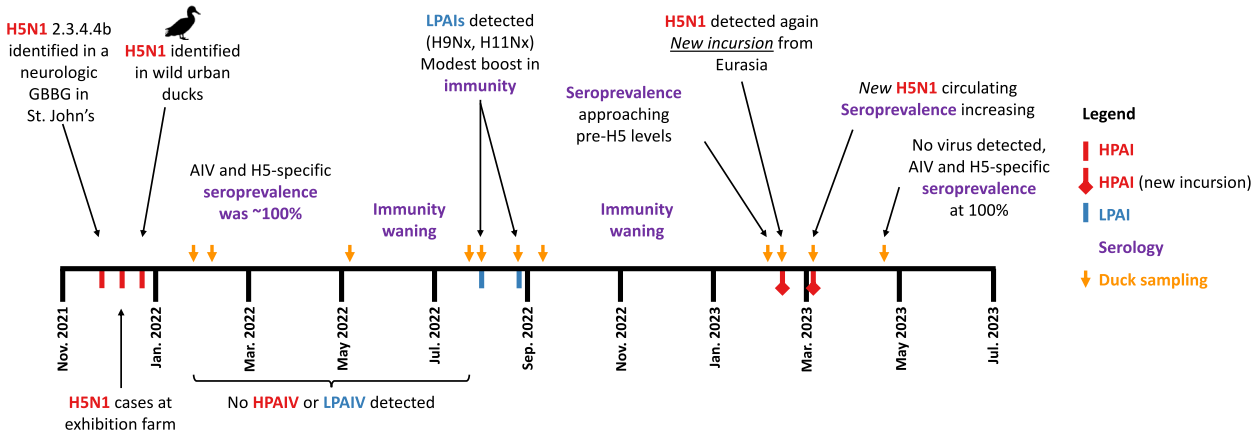

Figure 3 Summary of the AIV infection and immunity timeline since arrival of HPAI H5N1 in the region.

infection and immune response in this population for the 16-month period (Figure 3).

Immune responses in currently infected individuals

There were five individuals that were actively infected that had Ct values < 40, and these showed a negative relationship between antibody levels and viral RNA load (Figure 4). For ducks that were AIV RNA-negative at the time of sampling and where the time since infection was known, specifically those infected one, three, or six months prior to sampling, antibody levels decreased significantly over this time period (Figure 4; $F = 5.71$, df = 1,15, $p = 0.03$).

Including additional AIV prevalence and seroprevalence data obtained from various seabird species further supported the patterns observed in the ducks. Of the combined duck and seabird samples ($n = 176$), 17 individuals (9.7%) were currently infected and showed a clear relationship of increasing antibody levels with decreasing AIV viral RNA load (Figure 5A), an expected immunological response. A generalized linear model was used to highlight this relationship, showing the immune response in infected individuals at a population level (Figure 5B).

Changes in serology of two recaptured ducks

Over the course of sampling between January 2022 and April 2023, two northern pintails were recaptured and resampled, allowing for comparison of antibody levels between the two timepoints. Both individuals were captured and recaptured at the same location, Bowring Park, and were viral RNA-negative at both sampling time points. The first, a male ASY (band # 1196-13442) was first captured on 28 January 2022 and subsequently recaptured on 7 February 2023, totalling 375 days between samples. The second, a female AHY (band # 1196-13448) was first captured on 31 July 2022 and subsequently recaptured on 7 February 2023, totalling 191 days between samples. We do not know if they were infected with AIV(s) between the sampling events, but antibody levels were lower in both individuals at the time of recapture, although to different degrees (Figure 6).

Discussion

In this investigation we used a combination of AIV surveillance, serology, and epidemiology to document AIV infection and immune responses in an urban duck population over a period of 16 months following the incursion of the clade 2.3.4.4b H5N1 virus in late 2021 and compare this to the AIV seroprevalence of the population prior to the arrival of HPAIV. Through repeat sampling of the same population, we investigated changes in antibody levels through two population-wide HPAIV infection events and examined patterns of immune response over the course of AIV infection on individual scales. Using this combined information in the context of a duck population largely composed of non-migratory resident individuals, we were able to generate a robust AIV infection and immunity timeline at a population-wide scale, adding to the growing body of literature about these complex dynamics.

Changes in seroprevalence over time

Over the course of 2011–2014, mean AIV-NP seroprevalence was 27.6%, with some variation between seasons. Sampling over these years typically occurred during the late fall and early winter, during and after typical peak AIV circulation in the region. These data serve to establish a baseline for AIV immunity in this population prior to the incursion of H5N1 into the region. A variety of factors could affect variation in seroprevalence between years, including year-to-year variations among circulating AIV subtypes/strains and population age structure

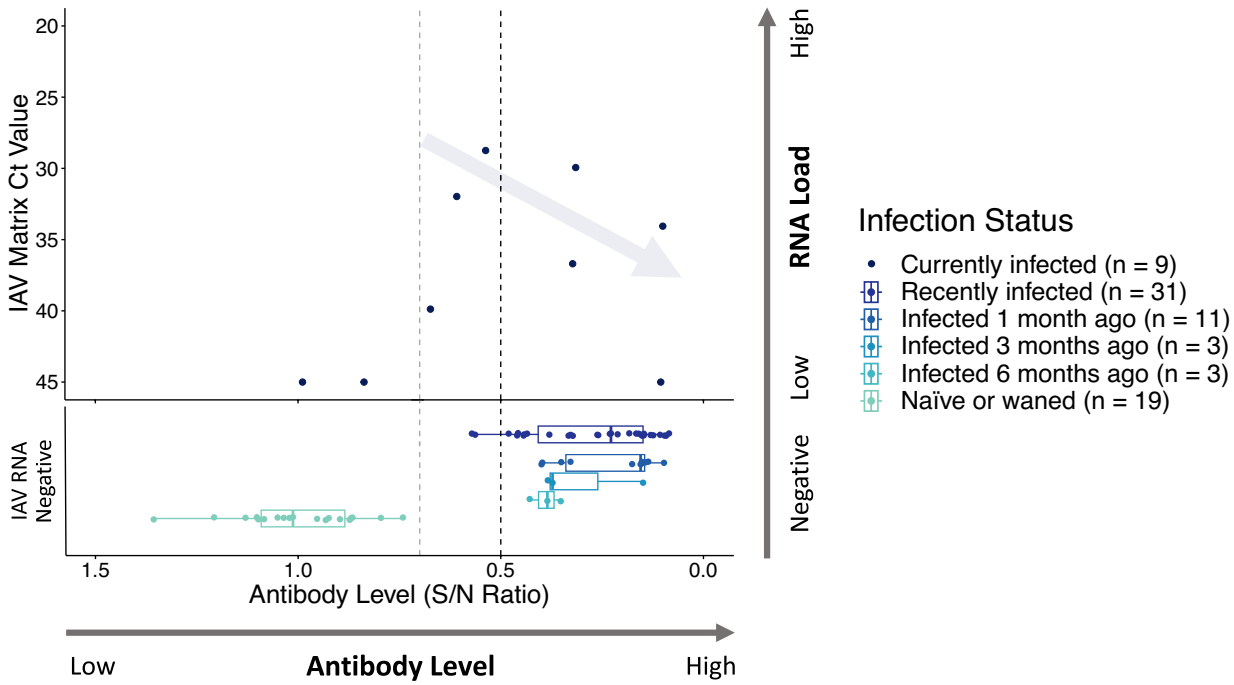

Figure 4 Relationship between viral RNA load and AIV-NP antibody levels for ducks. Each point represents an individual duck ($n = 76$), and colours indicate infection status. Individuals currently infected, shown in the top panel, were those that had detectable viral RNA loads by RT-qPCR. Samples with low RNA loads that provided expected amplification curves but that did not surpass the cycle threshold value are represented as having a Ct value of 45. All individuals that were negative for viral RNA are shown in the lower panel, with the antibody level coloured by the infection status in shades of blue. Recently infected individuals were classified as such if they were viral RNA-negative but had elevated antibody levels. For individuals sampled soon after the population-wide infection event at the start of the outbreak, these recently infected ducks are further separated as being infected one, three, or six months prior. Individuals that were seronegative and had low antibody levels (high S/N ratios) were classified as being naïve or that their antibodies had waned. An arrow denotes the expected immunological response shown by the five currently infected individuals with Ct values < 40, illustrating the observed trend of increasing antibody levels with decreasing viral RNA load. The vertical line at an S/N ratio of 0.5 represents the threshold value used to classify seropositivity, with a line at 0.7 also shown as the threshold occasionally used for this assay in other studies.

and exposure history. AIV prevalence can follow cyclic patterns, with increased prevalence every several years [51–54]. Prevalence was noticeably higher in 2011–2012, however, these individuals were captured by bait-trapping, which may overestimate true AIV prevalence [55], while ducks were captured by hand in the other years.

As expected, AIV seroprevalence increased greatly in the population following the arrival of H5N1 in November 2021, when nearly every duck sampled in January 2022 was seropositive. There was nearly homogenous seroprevalence across all sampled sites and the same banded ducks were observed using multiple urban waterbodies in the area. Given this, along with the extremely high proportion of ducks banded locally only ever being encountered in the same area, we consider the ducks in this urban region at this time to represent a single population. Therefore, there was a population-wide infection event after the arrival of the virus from Eurasia. As AIV-infected waterfowl often exhibit reduced local movements, the large number of infected individuals shedding virus into the local environment could have increased the infection rate at this time, facilitating proficient spread throughout the population to cause the near homogenous seroprevalence [8, 56]. It is possible that the two seronegative individuals sampled in winter-spring 2022, a mallard and an American wigeon, had moved into the area from elsewhere and were therefore not present at the time of the population-wide infection that occurred roughly a month and a half prior to their sampling. Alternatively, although we believe less likely due to an increase of the number of ducks present in February compared to January, they may have been present in the area but were not infected or were infected but did not generate a detectable antibody response.

Using repeated sampling of this population for roughly 16 months we found that AIV-NP and H5-specific seroprevalence changed greatly in the months following the H5N1 incursion. Individuals were seropositive

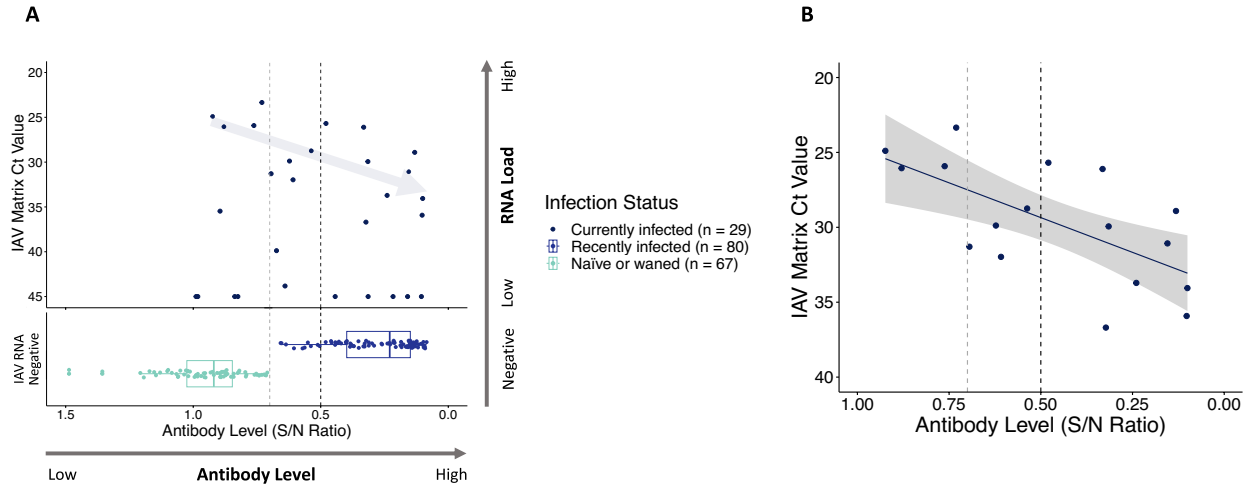

Figure 5 Relationship between viral RNA load and AIV antibody levels for ducks and seabirds. Each point represents an individual and colours indicate infection status. **A** Data from all ducks ($n = 76$) from Figure 4 are included in this plot, along with data for 100 seabirds (42 Atlantic puffins, 16 black-legged kittiwakes, 28 common murres, and 14 northern gannets). Individuals were classified as being currently infected, recently infected, or naïve/waned, using the same classification as in Figure 4. An arrow denotes the expected immunological response shown by the seventeen currently infected individuals, illustrating the relationship between increasing antibody levels with decreasing viral RNA load. These same individuals are shown in **B**, where a generalized linear model (GLM) was fit to show the relationship between antibody level and viral RNA load, with the gray area indicating the 95% confidence interval of the model. The vertical lines at an S/N ratio of 0.5 represent the threshold value used to classify seropositivity, with lines at 0.7 also shown as the threshold occasionally used for this assay in other studies.

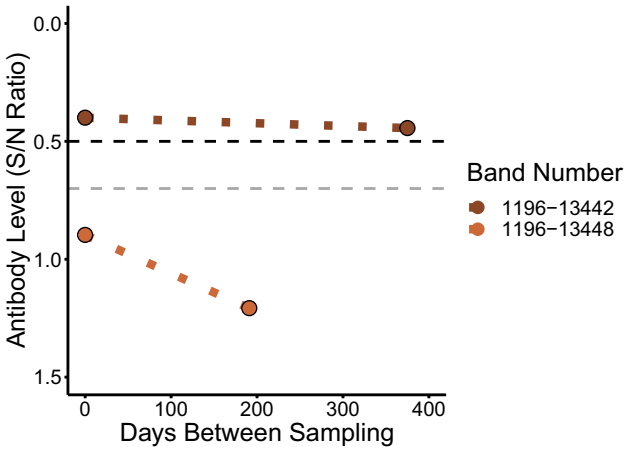

Figure 6 Antibody levels of two northern pintails at two timepoints. The bird with band # 1196-13442 was recaptured after 375 days and the bird with band # 1196-13448 was recaptured after 191 days. Dotted lines connect original capture and recapture values to show the change in antibody levels; these do not represent linear regressions as we do not know if or when they were re-infected with AIV between the sampling events. The vertical line at an S/N ratio of 0.5 represents the threshold value used to classify seropositivity, with a line at 0.7 also shown as the threshold occasionally used for this assay in other studies.

for anti-NP antibodies for approximately twice as long as H5-specific antibodies, with the population being H5-seronegative approximately six months after the incursion. It is well known that waterfowl, and this duck population specifically [38, 57], are frequently infected by LPAIVs, which likely explains the longer period of elevated anti-NP antibodies that are boosted with each subsequent infection. Dabbling ducks using human-dominated landscapes in Atlantic Canada show notably high survival rates and strong annual site fidelity to wintering areas [58], leading to a population with an older age distribution than usual. This older age structure may have contributed to the higher seroprevalence overall and over time, as antibody levels are elevated and persist longer in older individuals [27, 59, 60]. With low seroprevalence of H5-specific antibodies from 2012 to 2014, assumed to be due to occasional circulation of LPAI H5 viruses, most individuals sampled in 2022 were likely infected for the first time with an H5 virus, explaining the shorter period in which these specific antibodies persisted.

In March 2023, H5 viral RNA was again detected in the population. This virus was different than the original virus from the winter of 2022 and represented a new incursion into the region (Wight et al., unpublished data). The appreciable change in both AIV-NP and H5-specific seropositivity observed between weeks 58 and 59 (February 2023) therefore served as a signal of low-level circulation of the new H5 virus in the population prior to detection of active infections. Based on the declining H5-specific antibody seroprevalence since the original population-wide infection event and the lack of H5 viral

RNA detected, H5-specific seropositivity was presumably very low until the time of the second incursion event that occurred sometime in February 2023. By the end of April 2023, all individuals sampled were seropositive for both AIV-NP and H5-specific antibodies, indicative of a second population-wide infection event. Although the population was fully infected a year prior, and approximately half of the population still had elevated AIV-NP antibody levels when the new H5 virus appeared, this was not sufficient to protect against infection and widespread circulation.

We consider there are two plausible routes for transmission of the new H5N1 virus into the wild urban duck population. In February 2023, several species of diving ducks (greater and lesser scaup (*Aythya marila*, *A. affinis*), tufted ducks (*A. fuligula*), ring-necked ducks (*A. collaris*), and red-breasted and common mergansers (*Mergus serrator*, *M. merganser*)) that would normally be using coastal marine habitat at this time of the year [61] took shelter at Quidi Vidi Lake from a harsh winter storm. This mixing of these diving ducks with the urban population could have served as the route of transmission. Alternatively, hundreds of gulls originating from Arctic, European, and mainland North American breeding populations congregate at Quidi Vidi Lake each winter, which is the same location as the first detections of the new H5N1 in the ducks (Additional file 1). Gulls have been identified as important vectors by which Eurasian clade AIVs can enter into North America [62–64], and it is possible they brought the new H5N1 into the region and introduced it into the local duck population.

Re-infection of the population just over a year later could have been due to a variety of factors. The higher virulence and infectivity of HPAIVs compared to LPAIVs likely played an important role in the original and subsequent population-wide infection events [10, 11, 65–67]. Immunological factors such as waning immunity that did not provide protection from re-infection, escape from the immune system due to low cross-reactive antibodies owing to differences between the two viruses, and delay in memory responses that would allow viral infection to occur before a protective response could be mounted, may have also played a role. Several groups have shown that antibody levels decrease over time following infection of AIV-naïve captive ducks with a variety of different AIVs, as expected. However, these homologous and heterologous challenge studies have shown that antibody levels rebound in a matter of days after a second infection and ducks are often protected from clinical disease [23, 24, 68]. Unfortunately, the majority of these studies, including all those on HPAIVs [22, 28, 69–71], have been performed at timescales of weeks, as opposed to months or years, periods that are relevant to timing of bird migration, and therefore it is currently unknown how long HPAIV-specific antibodies remain elevated and how long individuals are protected from subsequent re-infection.

Migratory individuals with lower AIV seroprevalence that arrived in the region as well as AIV-naïve ducks born in the summer of 2022 may have also contributed to the spread of the 2023 H5N1 virus among the population. The success at which these viruses spread throughout the population was also likely influenced by the two H5N1 infection events occurring in early winter, after the typical fall AIV infection peak [3], when antibody levels are waning and low energy stores due to reduced food availability and colder conditions may have made birds more susceptible to infection [21, 43, 72]. Additionally, increased density of birds due to frozen waters may have increased the likelihood of infection during this time. Following the detection of the newly introduced lineage H5N1 in early 2023, several mute swans (*Cygnus olor*) and a number of American black ducks in St. John's were reported dead, while there was no documented mortality of waterfowl in the region when H5N1 initially infected the population in late-2021/early-2022 [73].

Relationship between viral load and antibody levels

Extending beyond population-scale seropositivity, we used S/N ratios as a measure of antibody levels along with AIV RNA load (based on Ct values) to investigate patterns of immune response over the course of infection on an individual scale. As individuals progress along the course of infection and transition from the viremic to immunologic phase, viral RNA decreases while antibody levels begin to increase. With substantial variations by species, virus, and body condition, previous work has found that AIV shedding often peaks between 1 and 8 days post-infection (dpi) and lasts for 5–11 days, although some individuals may shed virus for several weeks [21–24, 43, 68, 74, 75]. The period of viral shedding has also been found to decrease with more frequent infections [59], and therefore there is a very small window in which ducks can be caught and documented with an active infection [25].

Recently infected individuals had a range of antibody levels, with those sampled closer to the date of the population-wide infection having higher levels than those infected many months prior (Figure 4). In contrast to recently infected individuals, no pattern was observed with the timeline of infection and epidemiology or the antibody levels for naïve or waned birds. We did not pursue statistical analyses by age structure or between sexes due to the limited sample size of each group for each sampling event, but there appeared to be no differences

in antibody levels for seronegative individuals by age class, agreeing with previous observations [43].

Immune responses in individuals with active infections

Nine ducks were sampled while actively shedding AIV and there was a negative relationship trend for antibody levels versus viral RNA load. This pattern was further supported by inclusion of additional data from seabirds in order to provide a larger dataset of currently infected individuals and the relationship of increasing antibody levels with decreasing viral RNA load is clear (Figure 5B). This is an expected immunological response and shows that innate and memory immune mechanisms are quickly responding by generating antibodies as individuals are clearing the infection and leaving the viral shedding phase [29]. This also allowed us to infer the phase of infection at an individual level. We are unable to determine previous infection history of each individual as we are interpreting this relationship as a whole population, but factors such as age, infection history, as well as species-level differences would be expected to affect antibody levels on an individual scale [27, 28, 43, 60, 74].

Serological changes in two recaptured ducks

Based on HPAIV prevalence and epidemiology over the course of this study, it is unlikely that either of the recaptured northern pintails were infected with HPAIV between the two sampling points. However, the male (1196-13442) was likely infected by an LPAIV at some point between the two sampling events as its antibody levels hardly changed between sampling events, roughly one year apart, and it did not have elevated H5-specific antibodies (Figure 6, Additional file 1). Captive infection studies have shown that AIV antibodies persist for several months, however antibodies have not been found to remain elevated to this degree for over a year, even in older individuals [23, 24, 27, 28, 60, 68]. In contrast, the second individual (female, 1196-13448) seems unlikely to have been infected between sampling events. In light of previous findings from captive infection studies, even if this individual became infected soon after initial sampling, elevated antibody levels would likely still have been detected when resampled approximately six months later. Although these data come from only two individuals, using the combined AIV prevalence, seroprevalence, and epidemiological approach helps add to our understanding of AIV dynamics in wild populations. Efforts to resample individuals multiple times from locations with known AIV dynamics and population movements would be of substantial interest in future work to evaluate changes in seroprevalence more thoroughly, particularly on an individual basis, and how this contributes to population level immunity [29, 76].

Summary, conclusions, and future directions

Despite some limitations of this study, such as a limited sample size and that sampling occurred in a single region, our findings significantly add to the understanding of AIV infection dynamics and immunity in one of the most important reservoir host groups. This was enabled by an established understanding of the AIV infection dynamics in this region where this duck population is composed of primarily resident individuals. Using repeated sampling of this population during ongoing HPAIV and LPAIV circulation, we were able to document waning immunity, observe immune activation occurring in infected individuals, and infer infection histories. We found that although population level infection and seroconversion did occur, immunity waned quickly, and the population became susceptible again to the virus. This further supports the high likelihood of this virus becoming endemic with continuous circulation, as it now appears to be, contrasting the low levels of circulation, largely among young recruits and/or naïve birds, as observed for many other microbial pathogens. Our findings have important implications for monitoring the ongoing risk from HPAIVs, as previously infected populations can be reinfected along relatively short time scales, allowing for repeated circulation of the virus. This should be an important consideration for future monitoring efforts as this virus and derived reassortants carried by wild birds may spill over into other hosts such as mammals and poultry, without the typical early warning signs of disease or mortality in wild birds that have often preceded these events.

It will be important to perform similar studies elsewhere to determine if the patterns we observed also occur in other populations, species, and locations, particularly to compare the rate of waning antibody levels after peak AIV prevalence. The determination of whether these antibodies are neutralizing and if and for how long individuals with pre-existing immunity are protected from infection and/or severe disease would also be of significant interest. Additional captive infection studies should be performed to better understand the role of pre-existing and heterotypic immunity to LPAIVs and HPAIVs, particularly along timeframes that are relevant to key aspects of bird biology such as migration. Conducting similar studies on birds other than ducks would help determine host susceptibility, assess the likelihood of experiencing severe disease and mortality, and may help identify species groups most at risk. Wildlife surveillance of infectious diseases is a critical aspect of

preparedness within a One Health framework and is particularly important with respect to HPAIV, which is proving to be a multispecies pathogen that can have impacts far beyond the poultry industry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-024-01397-5.

Additional file 1. Detailed records for samples from ducks that were used in this study. Samples from the 2011–2012 period were no longer available, therefore the previously published data [38] were used and are presented as only anti-NP antibody-positive or -negative.

Acknowledgements
The authors would like to thank all additional individuals who contributed to sampling efforts including A. Bond (2014), S. Duffy (2013), K. d'Entremont (2022), D. Fifield (2011), D. Fife (2023), S. Avery-Gomm (2013), A. Hedd (2023), R. Hoeg (2022), B. Kelly (2022), A. Kroyer (2014), B. Montevecchi (2022), H. Munro (2014), D. Pirie-Hay (2012-2014), P. Ryan (2012, 2013, 2023), K. Studholme (2022), B. Turner (2023), M. Wallace (2023), S. Wallace (2023), and C. Ward (2022).

Author's contributions
Conceptualization: JW, GJR, ASL. Data curation: JW, IR, WX. Formal analysis: JW, GJR. Funding acquisition: GJR, ASL. Investigation: JW, IR, HLW, JTC, SR, GJR, RSR, WX, DZ, TNA, YB, KEH, ASL. Methodology: JW, IR, HLW, GJR, TA, WX, DZ, YB, ASL. Project administration: JW, ASL. Resources: JW, IR, HLW, JTC, SR, GJR, TNA, YB, KEH, ASL. Software: JW. Supervision: ASL. Validation: JW. Visualization: JW. Writing—original draft preparation: JW. Writing—review and editing: JW, IR, HLW, JTC, SR, GJR, RSR, WX, DZ, TNA, YB, KEH, ASL. All authors read and approved the final manuscript.

Funding
This research was supported by funding from Environment and Climate Change Canada core funding and through its Strategic Technology Application of Genomics in the Environment (STAGE) program Genomics Research and Development Initiative (GRDI), and the Natural Sciences and Engineering Research Council of Canada (NSERC) (RGPIN-2022-03791 to ASL). JW, IR, and SR were supported by funding from the Memorial University of Newfoundland School of Graduate Studies. JW was also supported by funding from the NSERC Canadian Lake Pulse Network (NETGP 479720), and an Alexander Graham Bell Canada Graduate Scholarship-Doctoral. HLW was supported by funding from the Memorial University School of Graduate Studies Aldrich Award, a Memorial University Faculty of Medicine Dean's Fellowship (MSc and PhD), a Canadian Institutes of Health Research Banting and Best Canada Graduate Scholarship—Masters, and a Canadian Network on Hepatitis C Virus Doctoral Fellowship. Environment and Climate Change Canada provided materials and reagents to perform the AIV sampling and testing for seabirds.

Availability of data and materials
All metadata and laboratory generated data related to samples from ducks are included in this published article and the additional information file. Additional data, specifically those relating to samples from seabirds, will be made available by the authors upon reasonable request.

Declarations

Ethics approval and consent to participate
This work was carried out under the guidelines specified by the Canadian Council on Animal Care with approved protocols 11-01-AL, 12-01-AL, 13-01-AL, 14-01-AL, 17-05-AL, and 20-05-AL from Memorial University's Institutional Animal Care Committee, biosafety permit S-103 from Memorial University's Institutional Biosafety Committee, and banding and sampling operations under Federal Bird Banding and Scientific Research Permit 10559.

Competing interests
The authors declare that they have no competing interests.

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Received: 26 April 2024 Accepted: 9 September 2024
Published online: 22 November 2024

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