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Transmission dynamics of highly pathogenic avian influenza virus at the wildlife-poultry-environmental interface: A case study

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ABSTRACT

Avian influenza viruses (AIVs) regularly circulate between wild and domestic bird populations. Following several high-profile outbreaks, highly pathogenic AIVs (HPAIV) with zoonotic potential have been the subject of increasing attention. While we know that HPAIV is transmitted between domestic birds, wildlife, and the environment, little is known about persistence and spillover/back at these interfaces. We integrated the test results of samples collected on and around an infected domestic poultry premise (IP) where H5N1 HPAIV was confirmed in a flock of poultry in 2022 in Southern Ontario, Canada to explore the transmission cycle of AIVs in wildlife and the environment. We sampled a captive flock of Mallards (*Anas platyrhynchos*) that resided on site, sediment samples collected from water bodies on site, and examined samples collected through surveillance within a 100 km radius of the IP from live wild ducks and sick and dead wildlife. We found serologic evidence of H5 exposure in the captive mallards that resided on site despite no evidence of morbidity or mortality in these birds and no PCR positive detections from samples collected at two different timepoints. Genetic material from the same H5N1 HPAIV subtype circulating in the domestic birds and from low pathogenicity avian influenza viruses were detected in wetlands on site. The results of live and sick and dead surveillance conducted within a 100 km radius confirmed that the virus was circulating in wildlife before and after IP confirmation. These results suggest that biosecurity remains the most critical aspect of minimising spillover/back risk in a virus that has been shown to circulate in asymptomatic wild birds and persist in the surrounding environment.

1. Introduction

Avian influenza outbreaks come at enormous economic and ecological cost. In Canada as of April 10th, 2024, an ongoing outbreak of HPAIV has affected more than 11 million domestic birds since it began in late 2021 and has cost hundreds of millions of dollars in disease management and containment costs and in lost revenue [1]. At a global scale, HPAIV outbreaks harm the global poultry industry, lead to job losses,

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and result in population declines and deaths among wild birds and susceptible mammalian species [2]. Some HPAIVs can infect humans and the first cases of chicken-human transmission of H5N1 occurred in China in 1997. Of eighteen human patients, six died [3]. Between 2003 and 2021, the WHO identified 862 cases of avian influenza A(H5N1) in humans, 455 of which resulted in death, constituting a case-fatality rate of more than 50 % [4]. While the extent of bird-human transmission is limited, the lethality of AIV in humans and the potential for ongoing spillovers to result in sustained human-human transmission of avian influenza are of mounting concern [5–8].

While improved diagnostic techniques and reporting infrastructure have increased our ability to detect HPAIV outbreaks, the increase in their frequency and intensity is in part due to changes in agricultural practices that have led to increased densities of poultry populations, and their proximity to wild birds [9,10]. Despite efforts to control the spread of HPAIVs (e.g. improved biosecurity and monitoring), the viruses have continued to circulate and evolve, resulting in recurrent outbreaks [10,11].

Wild migratory birds are known reservoirs of LPAIV which have spilled over to domestic flocks, where the virus can evolve to become HPAIV [12]. However, increasingly frequent detections of HPAIVs in wild birds have raised questions about their role in the maintenance and spread of HPAIVs [10,12,13]. More specifically, recent work suggests that HPAIVs are maintained through bidirectional viral exchange between domesticated and wild birds [3,14]. Further, bidirectional viral exchange facilitates the adaptation of HPAIVs to wild birds, and promotes dispersal and spread through migration, thus helping to explain the shifting epidemiology of HPAIVs towards asymptomatic infection in wild birds, and unprecedented rates of spread across the globe [3,14–17]. In particular, H5Nx viruses belonging to the A/Goose/Guangdong/1/96-derived HA clade 2.3.4.4 have steadily increased their geographic and host ranges, becoming atypically persistent in wild bird populations [17–20].

The potential for environmental compartments such as wetlands to act as reservoirs for low- and high-pathogenicity AIVs is also an area of growing concern. Waterborne transmission of AIV has been well-established [21–23], and depending on temperature and salinity, LPAIV and HPAIV have been shown to be viable in pond, river, and seawater for between ten and more than sixty days [24–30]. Additionally, PCR testing and targeted genomic sequencing of wetland sediment has been used to detect and genotype multiple hemagglutinin (HA) and neuraminadase (NA) AIV subtypes, including low- and high-pathogenicity viruses [31,32]. Interestingly, empirical studies have found that even low levels of environmental transmission can act as a "persistence mechanism", by which AIVs can continue to circulate within populations too small to support an epidemic by direct transmission alone [33]. Theoretical studies have supported this claim, suggesting that locations themselves, in addition to individuals, may be infectious, resulting in the (re-)occurrence of outbreaks in the absence of direct transmission by animals [34]. From a management perspective, it has also been shown that neglecting to consider environmental transmission may limit our ability to accurately predict the magnitude and duration of AIV epidemics [34].

Here, we integrate the results of samples collected during a defined 221-day timeframe on and around an infected domestic poultry premise (IP) where 2.3.4.4b H5N1 HPAIV was confirmed in 2022 in Southern Ontario, Canada. Specifically, we describe: (1) AIV RT-PCR results from sick and dead birds and mammals within a 100 km radius of the IP, (2) AIV RT-PCR results from samples collected during live wild bird surveillance conducted within a 100 km radius of the IP, (3) AIV RT-PCR and serological results from samples collected from a captive flock of Mallards (*Anas platyrhynchos)* maintained in wetlands on the domestic premises, and (4) AIV targeted genomic sequencing results from water and sediment samples collected from wetlands on the domestic premises. By comparing these data across sectors during a similar timeframe, we aim to highlight patterns and differences in viral presence and exposure.

2. Methods

2.1. Site and context

Due to privacy considerations, sampling dates and locations will be referred to in relative terms. H5N1 HPAIV was confirmed in fall on a commercial poultry premises by the Canadian Food Inspection Agency's (CFIA) National Centre for Foreign Animal Disease (NCFAD). The day of HPAIV confirmation on premises is referred to as Day 0 and the facility is then referred to as an 'infected premises' (IP). Following detection, the flock of domestic poultry was depopulated by the CFIA in accordance with the Health of Animals Act (Fig. 1).

The IP in question had wetlands on site including a series of five ponds where approximately 15,000 "premise mallards" were raised for commercial sale (Fig. S1). In this paper, we define "premise mallards" as phenotypically indistinguishable, but behaviorally distinct from wild mallards (*Anas platyrhynchos*). The poultry barn was approximately 100 m from the nearest pond. Premise mallards were fed daily on the shoreline of that same pond. For context, each year a breeding stock of approximately 500 premise mallards was maintained indoors over the winter and the remaining birds were processed for commercial sale. Eggs were collected from the breeding stock and incubated at a local hatchery, and ducklings were reared in captivity for 4–6 weeks before being released on site. After egg collection, the breeding stock was processed for commercial sale. The premise mallards were fed twice daily, and although flighted, were maintained on-site through animal husbandry practices. Therefore, at the time of H5N1 HPAIV detection in the commercial poultry flock, there were approximately 15,000 premise mallards, hatched that same year, maintained outdoors in and around the ponds on site. Premise mallards did not have any direct contact with the poultry or access to the barn where the commercial poultry were housed. Following confirmation of 2.3.4.4b H5N1 HPAIV in the domestic birds, premise mallards were depopulated over the course of three months (Fig. 1).

2.2. Sick and dead wild bird and wild mammal sampling within 100 km of the IP

Sick and dead wild birds in the region were submitted to the Canadian Wildlife Health Cooperative (CWHC) as part of Canada's Interagency Agency Surveillance Program for Avian Influenza Viruses in Wild Birds [15]. Sick and dead wild mammals were also received by the CWHC during this time frame. The relevant data for the region within 100 km of the IP were extracted in March 2023 from a database export provided by the CWHC.

The CWHC conducts opportunistic surveillance for AIV on samples submitted from carcasses, often by members of the public, as described previously [15]. From each bird, an oropharyngeal and cloacal swab were collected and pooled in a 3 mL Universal Transport Medium (Copan Italia S.p.A., Brescia, Italy) and from each mammal an oral and rectal swab were collected and pooled as above. Vials were kept cold until transport to the diagnostic laboratory. The data were filtered to include carcasses originating within a 100 km radius of the IP in the eight weeks preceding and following Day 0. We included mammals in this analysis in addition to birds given their susceptibility to HPAIV [35] to provide a more comprehensive understanding of viral presence on the landscape within the vicinity of the IP. Sample data were aggregated by week for descriptive purposes.

2.3. Live apparently healthy wild bird sampling within 100 km of the IP

We queried the Environment and Climate Change Canada (ECCC) AIV surveillance dataset for live wild birds captured and sampled for AIV as part of Canada's Interagency Agency Surveillance Program for Avian

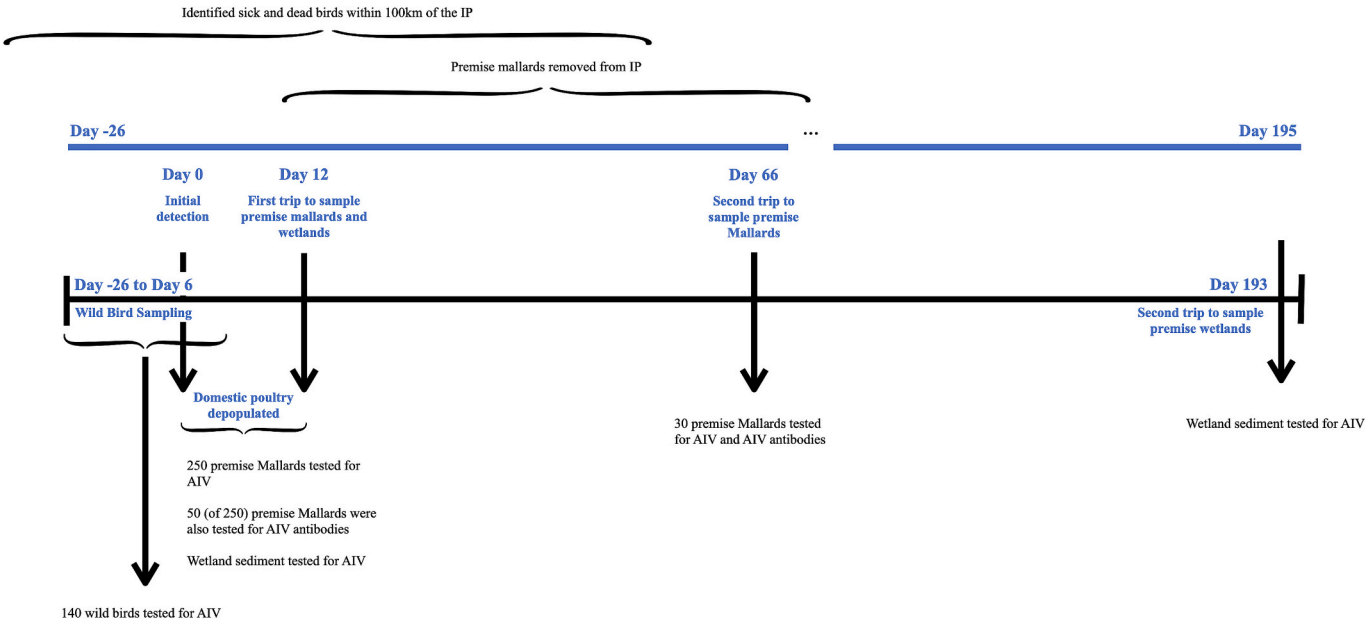

Fig. 1. Timeline of events in relation to the detection of Highly Pathogenic Avian Influenza Virus (HPAIV) on the infected premise (IP). The date of the detection at the IP is referred to as 0, with days before and after represented as integers.

Influenza Viruses in Wild Birds [15] that were within 100 km of the IP prior to or shortly after Day 0. Live wild birds were sampled opportunistically in conjunction with ongoing ECCC banding programs. Birds were recorded as either "hatch-year" (for the purposes of this paper, this is defined as birds hatched in the same calendar year as sampling occurred) or "after hatch year" (hatched in a previous calendar year) determined based on wing feather characteristics (i.e., wear, and shape) [36]. From each bird, one oropharyngeal and one cloacal swab was collected, and both swabs were pooled in a 3 mL UTM-RT vial. Vials were kept cold until transport to the diagnostic laboratory.

2.4. Premise mallard sampling

On Day 12, oropharyngeal and cloacal swabs were collected and pooled in 3 mL UTM-RT vials from 250 premise mallards. Swabs were submitted to the Animal Health Laboratory (AHL) at the University of Guelph, Ontario, Canada on the day of sampling. Blood samples were collected from a subset of 50 birds. Decapitation was performed and blood collected from the jugular vein into a sterile collection container. Concurrent depopulation efforts by the property owner necessitated this approach to minimize stress and handling time and PPE requirements on the infected premises restricted alternative methods. Blood samples were transferred into individual vials and either centrifuged in the field by ECCC staff or at the AHL. In both cases, serum was separated and stored at −20 °C until testing. On Day 66, swabs and blood samples were similarly collected from 30 premise mallards. Sampling and euthanasia of premise mallards was conducted under the authority of a scientific permit from ECCC (SC-OR-2022-00085).

2.5. Sediment sampling

On Day 12, sediment samples were collected from five ponds on the premises one meter into the water from the shore at three locations around each of the five ponds (i.e. six samples from each pond; Fig. S1). Collection protocols were based on Kuchinski et al. (2024). Sediment samples from each site were collected concurrently and stored in two 60 mL sterile urine collection containers for a total of 120 mL per sample and left to settle for thirty to sixty seconds. If the sample was insufficient ($\lessapprox$10 mL), water was removed from the sample before re-sampling. Containers were then re-sealed and placed in a Ziploc bag. GPS coordinates, environmental characteristics (pH, salinity, temperature), and site characteristics (sample depth, distance from shore, sediment type, presence/absence of waterfowl, species/abundance of waterfowl, presence/absence of bird faeces, type of wetland, and estimated depth of water) were recorded at each site using a water probe and observation key. Sediment samples were kept cool and sent to the National Wildlife Research Centre (NWRC) in Ottawa for pre-processing and storage, before being sent to the British Columbia Centre for Disease Control (BCCDC) for testing [32].

On Day 193 sediment samples were collected from the same ponds, at the same locations. While the previously collected samples were collected and stored in two separate 60 mL containers, these samples were consolidated into a single 120 mL container for ease of storage. These samples were sent to the National Wildlife Research Centre for pre-processing and storage, before being sent to the British Columbia Centre for Disease Control (BCCDC).

2.6. Nucleic acid extraction and PCR of swab samples

Pooled cloacal and oropharyngeal swabs were submitted to the AHL at the University of Guelph. Total nucleic acids were extracted using the MagMAX-96 Viral RNA Isolation Kit in a MagMAX Express-96 (Thermo Fisher) with Armored RNA Enterovirus (Asuragen) as an internal control. RNAs were reverse-transcribed and amplified using Ag Path-ID One-Step RT-PCR Kit (Thermo Fisher). Reverse transcription, PCR amplification and detection were carried out in a Light Cycler 480 (Roche) with primers targeting influenza A virus matrix gene under thermocycling conditions as described previously [37]. Samples with cycle threshold (Ct) values below 36 were considered positive and samples with Ct values of 36 and higher were considered inconclusive. If no amplification was detected the sample was considered negative. Non-negative samples were tested with H5 and H7 specific tests and were subsequently sent to the National Centre for Foreign Animal Disease (NCFAD) in Winnipeg, Manitoba, Canada for confirmatory testing.

2.7. Sequencing of bird specimens

PCR positive samples with Ct values <30 were directly processed for Nanopore sequencing as described previously [15]. Samples that could not be sequenced directly were processed for virus isolation and samples

that yielded isolates were sequenced as described previously [35].

2.8. cELISA methods

Serum was tested for the presence of anti-NP, anti-H5 and anti-H7 antibodies using in-house developed competitive enzyme-linked immunosorbent assay (cELISA) methods by NCFAD as described previously [35,38,39].

2.9. Hemagglutination inhibition

Hemagglutination inhibition (HI) assays were carried out on samples that tested positive on the H5 cELISA to determine the presence of anti-hemagglutinin antibodies. Serum samples were heat inactivated and hemadsorbed using chicken red blood cells prior to testing. A/Fancy chicken/NL/FAV-0033/2021 (clade 2.3.4.4b H5N1) was used as an antigen (4HA units) in the HI assay. HI antibody titers were determined as the reciprocal of the highest sample dilution resulting in complete inhibition of the chicken red blood cells from hemagglutination.

2.10. Nucleic acid extraction, RT-PCR, and sequencing of environmental samples

Sediment specimens were analyzed using the laboratory methods and bioinformatic tools described in Kuchinski et al. (2024). Briefly, total RNA was extracted from two grams of sediment then screened for the presence of influenza A virus genomic material using an RT-qPCR assay that targets a conserved region of the M segment. Sequencing libraries were constructed from specimens that tested positive by RT-qPCR, during which each fragment of genomic material was tagged with unique molecular identifiers on both ends. These libraries were pooled, then influenza A virus genomic material was captured and amplified using a custom panel of hybridization probes covering all 8 genome segments of all subtypes circulating in avian, swine, and human hosts. Raw sequencing data was processed using HopDropper v1.0.0 (https://github.com/KevinKuchinski/HopDropper) to discard chimeric artefacts, resolve unique molecular identifiers, and generate consensus sequences for both ends of each genomic fragment recovered. These fragment end consensus sequences were analyzed with FindFlu v1.0.1 (https://github.com/KevinKuchinski/FindFlu) to identify high-confidence influenza A virus fragments, merge consensus sequences from both fragment ends into a single fragment sequence, and determine the genome segment from which each fragment originated. For HA and NA fragments FindFlu also determined subtype, and for H5 fragments it further determined lineage and clade.

2.11. Phylogenetic analysis

H5N1 whole-genome sequences were genotyped according to Signore et al. [40]. Briefly, individual viral segments were assigned to sub-groups based on clustering within phylogenetic trees. The concatenation of sub-group labels for each segment from a given virus determined its genotype (e.g., a virus with segments belonging to the following sub-clades: PB2: “G”, PB1: “F”, PA: “D”, HA: “5”, NP: “B”, NA: “1”, M: “A”, NS: “A”, would be assigned genotype “GFD5B1AA”). Viruses from the IP and those with the same genotype collected in the vicinity were used in phylogenetic analyses. Individual viral segments (PB2, PB1, PA, HA, NP, NA, M and NS) were trimmed of regions flanking the open reading frames and concatenated.

Concatenated whole-genome sequences were aligned using MAFFT v7.525 [41]. The resulting 13,136 nucleotide long multiple sequence alignment was used to build a maximum likelihood phylogenetic tree with IQ-TREE v2.3.3 [42]. The tree was estimated with the best fitting model of nucleotide substitution as determined by ModelFinder [43], and node support for the resulting tree was assessed by 5000 ultrafast bootstrap replicates [44]. The bootstrap consensus tree was rooted using augur-refine from the Augur bioinformatics toolkit with the options “–root best”, “–stochastic-resolve” and “–divergence-units mutations”. The host status (live or dead) and origin (wild or domestic) were reconstructed as discrete characters across the refined tree using augur-traits from the Augur bioinformatics toolkit.

H5 fragments recovered from sediment were locally aligned to the bird-origin sequences composing the phylogenetic tree using BLASTN v2.5.0 [45]. Each fragment’s best-matching bird-origin sequence was determined by alignment bitscore, then that bitscore was added to that bird-origin sequence’s total bitscore allocation. In cases where fragments had multiple best matches (due to short fragment length and fragments derived from conserved genomic regions), their bitscores were fractionally allocated and evenly distributed among all their best matches. The resulting total bitscore allocations provided a series of single values representing the similarity of each bird-origin sequence in the phylogenetic tree to the H5 fragments recovered from the environment. These single values reflected the number of recovered fragments having their best match to a bird-origin sequence, the number of nucleotide mismatches between a bird-origin sequence and their best-matching fragments, indiscriminate matching between bird-origin sequences and fragments due to short fragment lengths, and indiscriminate matching between bird-origin sequences and fragments due to fragments originating from conserved genomic regions.

2.12. Analyses

Descriptive summaries were carried out in R version 4.2.2. Confidence intervals were calculated using the exact method.

3. Results

The virus detected in the domestic poultry was found to be a reassortant H5N1 (clade 2.3.4.4b) with gene segments PB2, PB1, PA and NP of North American lineage and remaining gene segments of Eurasian origin that was previously detected in a wild Blue-Winged Teal in Manitoba, Canada (genotype GFD5B1AA).

3.1. Sick and dead wildlife within 100 km radius of the IP

In the eight weeks preceding and following Day 0, 110 sick or dead wildlife (90 wild birds and 20 wild mammals) were found within 100 km of the IP and submitted to the CWHC for AIV diagnostics (Fig. 2). Of the 21 carcasses that tested positive on matrix PCR, 16 were confirmed H5N1 HPAIV positive including 12 Canada Geese (*Branta canadensis*; 2 HY, 7 AHY, 3 unknown age), 3 Turkey Vultures (*Cathartes aura*; 1 AHY, 2 unknown age), and 1 juvenile Red Fox (*Vulpes vulpes*; Fig. 2). Based on whole genome sequencing, all 16 had gene segments PB2, PB1, PA and NP of North American lineage and remaining gene segments of Eurasian origin. Of these, 12 were the same genotype detected on the IP (genotype GFD5B1AA) [40] including 8 Canada Geese (2 HY, 4 AHY, 2 unknown age), all 3 Turkey Vultures, and the juvenile Red Fox. Genotype GFD5B1AA viruses were detected in carcasses found on average 51 km (range: 19–92; median: 45) from the IP and ranging from 18 days prior to 10 days after.

3.2. Apparently healthy live wild birds sampled within 100 km radius of the IP

Between Day −26 and Day 6, samples were collected from 140 wild ducks, including 110 wild Mallards (*A. platyrhynchos)*, 25 Wood Ducks (*Aix sponsa)*, 4 Blue-Winged Teal (*Spatula discors)*, and 1 Green-Winged Teal (*Anas carolinensis)*, at three wetland and pond sites within 100 km of the IP. At the time of sampling, all individuals appeared healthy and were released following sampling. Of the 140 wild birds swabbed, 77 (55 %, 95 % CI: 0.46–0.63) tested positive on RT-PCR for the matrix AIV gene (Table 1). Of these, 53 were Mallards (4 AHY, 49 HY), 21 Wood

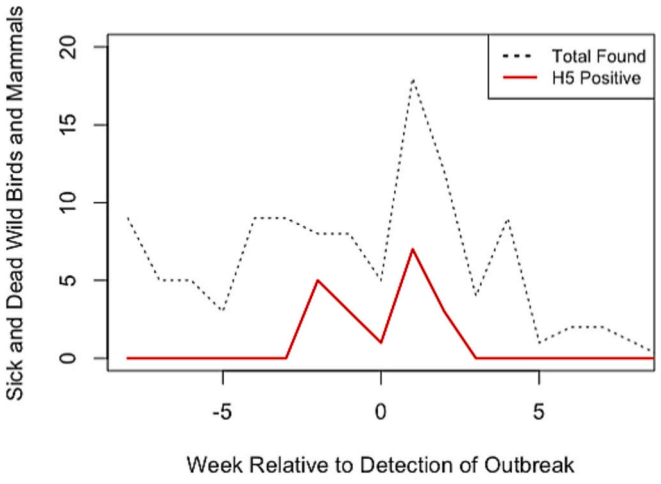

Fig. 2. The number of sick and dead wildlife that were found and submitted to the Canadian Wildlife Health Cooperative (black dashed line) within 100 km of a domestic premises in the eight weeks prior to and following the date of detection of highly pathogenic avian influenza on that premises (i.e., Day 0). The number of sick and dead wildlife that were H5 positive on RT-PCR at the National Centre for Foreign Animal Disease is shown by the red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Ducks (1 AHY, 20 HY), 2 Blue-Winged Teal (2 HY), and 1 Green-Winged Teal (1 AHY). An additional 38 (27 %, 95 % CI: 0.20–0.35) wild bird samples tested suspicious on RT-PCR for the matrix AIV gene (Table 1) including 33 Mallards (4 AHY, 29 HY), 3 Wood Ducks (1 AHY, 2 HY), and 2 Blue-Winged Teal (2 HY). A total of 44 (31 %, 95 % CI: 0.24–0.40) tested positive on RT-PCR for H5, including 39 Mallards, 2 Wood Ducks, and 3 Blue-Winged Teal. An additional 23 (16 %, 95 % CI: 0.11–0.23) wild bird samples tested suspicious on RT-PCR for H5 (Table 1) including 21 Mallards (3 AHY, 18 HY), 1 Blue-Winged Teal (1 HY), and 1 Green-Winged Teal (1 AHY). Only a subset of samples that tested positive or suspicious on matrix or H5 PCR were further subtyped and sequenced due to lab capacity and prioritization. The NCFAD produced 11 whole or partial genome sequences from the H5 positive samples. Of these, 4 samples had complete genome sequence and were all found to be the same genotype detected on the IP (GFD5B1AA). These samples were taken ~37 km from the IP all from the same wetland and ranging from 9 days prior to 6 days after; no sick and dead wildlife were submitted from this wetland during the timeframe of this study, 4 sick and dead wildlife were submitted from the nearest city, but all were negative for AIV. These viruses, combined with those collected from sick/dead birds, share a high degree of sequence similarity. The pairwise identity of all genotype GFD5B1AA sequences presented here is 98.8 % across the (13,136 bp) genome, with no two genomes differing by more than 32 nucleotide mutations (Fig. 4). A diversity of non-H5 LPAIV subtypes were detected among the samples for which this information was available (Table 1).

Table 1
Avian Influenza RT-PCR and serology diagnostic results for premise mallards sampled on an infected domestic poultry premise (IP) in southern Ontario in 2022 following confirmation of highly pathogenic avian influenza (HPAIV) (day of IP confirmation = Day 0) and RT-PCR diagnostic results for apparently healthy wild birds sampled within a 100 km radius of the IP. Diagnostic results are from the National Centre for Foreign Animal Disease (NCFAD) and the Animal Health Laboratory; where results disagree NCFAD results are used.

			Day −26 to Day 6	Day 12	Day 66
			Wild dabbling ducks	Premise mallards	Premise mallards
RT-PCR	Sample Size		140 (127 HY[a], 13 AHY[b])	250 (250 HY[a], 0 AHY[b])	30 (30 HY[a], 0 AHY[b])
	Matrix [N positive (%); N suspicious (%)]	HY[a]	71 (0.56); 33 (0.26)	60 (0.24); 112 (0.45)	0 (0); 0 (0)
		AHY[b]	6 (0.46); 5 (0.38)	–	–
		Overall	77 (0.55); 38 (0.27)	60 (0.24); 112 (0.45)	0 (0); 0 (0)
	H5 [N positive (%); N suspicious (%)]	HY[a]	39 (0.31); 19 (0.15)	0 (0); 0 (0)	0 (0); 0 (0)
		AHY[b]	5 (0.38); 4 (0.31)	–	–
		Overall	44 (0.31); 23 (0.16)	0 (0); 0 (0)	0 (0); 0 (0)
	H7 [N positive (%); N suspicious (%)]	HY[a]	0 (0); 0 (0)	0 (0); 0 (0)	0 (0); 0 (0)
		AHY[b]	0 (0); 0 (0)	–	–
		Overall	0 (0); 0 (0)	0 (0); 0 (0)	0 (0); 0 (0)
	Subtypes Identified		H3N6 ($n = 1$) H4N6 (n = 3) H5N1[c] ($n = 9$) H5Nx ($n = 58$) H12N1 (n = 1) Unknown ($n = 43$)	H3N8 ($n = 5$) H8N4 (n = 1) H12N5 ($n = 3$) Unknown ($n = 163$)	–
cELISA	Sample Size		–	50[d] (50 HY[a]; 0 AHY[b])	30 (30 HY[a]; 0 AHY[b])
	NP cELISA N positive (%)		–	48 (0.96)	20 (0.67)
	H5 cELISA N positive (%)		–	48 (0.96)	0 (0)
	H7 cELISA N positive (%)		–	0 (0)	2 (0.07)
Hemagglutination Inhibition	Sample Size		–	39[e] (39 HY[a]; 0 AHY[b])	–
	N positive (%)		–	39 (100 %)	–

[a] Hatch Year.
[b] After Hatch Year.
[c] The same genotype detected on the infected premise.
[d] Only a subset of birds that were swabbed had blood samples collected.
[e] Only a subset of samples positive on H5 cELISA had sufficient serum remaining to undergo hemagglutination inhibition.

3.3. Premise mallards

Of the 250 premises mallards swabbed on Day 12, 60 (24 %; 95 % CI: 0.19–0.30) and 112 (45 %; 95 % CI: 0.39–0.51) tested positive and inconclusive, respectively, on RT-PCR for the matrix gene. All 250 samples were negative for H5 and H7. The NCFAD produced nine whole or partial genome sequences from these samples, all of which were LPAIV (Table 1). Of the 50 premise mallards blood-sampled, 48 (96 %; 95 % CI: 0.86–100.00) tested positive for anti-NP and anti-H5 antibodies (Table 1; Fig. S2). Of 39 H5 positive samples with sufficient volume remaining, 100 % tested positive on HI (Table 1).

Of the 30 premises mallards swabbed on Day 66, all tested negative on matrix, H5, and H7 RT-PCR and 20 (67 %; 95 % CI: 0.47–0.83) tested positive for anti-NP antibodies (Table 1; Fig. S2). No blood samples tested positive for anti-H5 antibodies and 2 tested positive for anti-H7 antibodies (Table 1; Fig. S2).

3.4. Sediment samples from premise ponds

Environmental characteristics for the ponds can be found in Table 2. Genomic material from diverse AIVs was detected in the ponds on the IP. Six haemagglutinin (HA) and six neuraminidase (NA) subtypes were detected (Fig. 3). This included detections of genomic material from the HA segments of HPAI H5 viruses, specifically Clade 2.3.4.4 of the goose/Guangdong lineage, the same clade/lineage responsible for the outbreak on the IP. To assess their origin, the fragmentary H5 sequences recovered from the ponds were aligned to HA segment sequences recovered from the poultry involved in the IP and HPAI-positive local wildlife (Table 3, Fig. 4). This indicated that the poultry were the most likely source of the H5 fragments, based on the number of fragments having their best match to these bird-origin sequences as well as the strength of those alignments. Genomic material from HP H5s constituted a small minority of the total AIV genomic material recovered, however; within specimens where it was detected, HP H5 constituted only 1.4 % to 6.7 % of genomic fragments originating from HA segments.

AIV contamination of the ponds decreased substantially between the first and second visits (Fig. 4). During the first visit (Day 12), AIV genomic material was detected in sediment from all five ponds, and for all but one of the ponds, it was detected in 66 % to 100 % of sub-locations sampled. During the second visit (Day 193), however, there was almost no AIV contamination: detections occurred in only one sub-location of one pond. HP H5 contamination also decreased between the first and second visit (Fig. 3). During the first visit, HP H5 genomic material was detected in three ponds in 33 % to 66 % of sub-locations sampled. No HP H5 genomic material was detected in any of the ponds during the second visit.

4. Discussion

We present a unique case study of an H5N1 HPAIV outbreak on a domestic poultry premises in Ontario, Canada. We focus on examining the presence of avian influenza virus in premise mallards, the wetlands they used, and wildlife within a 100 km radius as a case study of the transmission of AIV at the wildlife-poultry-environmental interface.

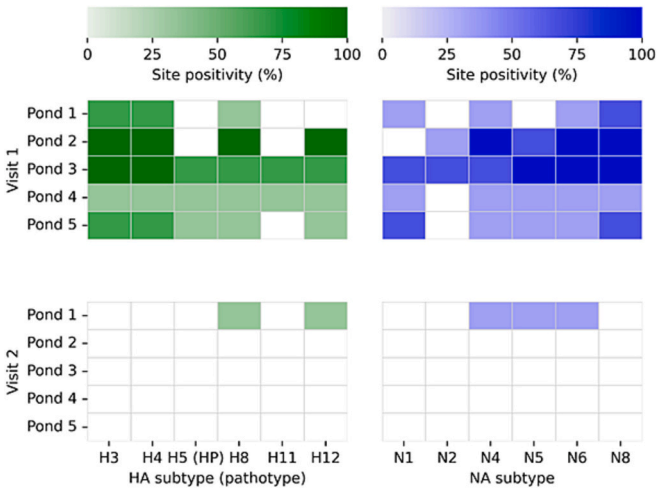

Fig. 3. Avian influenza virus genomic material was recovered from five ponds on the infected premise. Two visits were made six months apart, during which superficial sediment specimens were collected from three sub-locations within each pond. Genomic material from the haemagglutinin (HA) and neuraminidase (NA) genome segments was recovered and subtyped using a custom targeted genomic sequencing method. Heatmap shading depicts the percentage of sub-locations within the indicated pond that were positive for the indicated HA or NA subtype.

The detection of the same genotype of H5N1 HPAIV in dead wild birds and mammals within 100 km of the infected premise (IP) confirms that this virus was in circulation in wildlife more broadly in the area preceding and following confirmation on the IP (Fig. 2). Five days prior to the first identification of this genotype in wild birds in Ontario, this virus was detected more than 1300 km from the IP in a dead Blue-Winged Teal in Winnipeg, Manitoba, Canada, before being detected a month later on a domestic premise in the same region of Winnipeg (A. Signore and Y. Berhane unpublished data).

We also confirmed the presence of this genotype of H5N1 HPAIV in healthy wild dabbling ducks within a 100 km radius of the IP (Table 1). The detection of several LPAIVs in the dabbling ducks presented an increased risk for virus reassortment. High Ct values in many samples from these ducks resulted in inconclusive RT-PCR results, possibly due to low viral loads shed from asymptomatic individuals or late-stage shedding. Despite the challenges they pose for isolation and sequencing, these samples offer crucial insights into the viral diversity across the landscape, particularly in these healthy individuals likely to spread the virus over large distances and are important to prioritize for sequencing where possible.

The premise mallards were maintained on-site at high densities for extended periods. Notably, while none of the premise mallards were found to be shedding H5 AIV when they were sampled on Day 12,

Table 2
Sediment sample collection, site characteristics.

Pond	Day	pH	Salinity (ppm)	Temperature (°C)	Sample Depth (cm)	Sample Distance from Shore (m)	Waterfowl Present
1	12	7.55	187	15.7	30	1	Y
	193	7.35	146	6.3	31	2	N
2	12	7.53	199	15.2	30	<1	Y
	193	8.52	173	6.6	28	>1	Y
3	12	9.38	95	14.7	30	<1	Y
	193	8.09	96	6.7	20	>1	Y
4	12	8.75	144	14.8	20	<1	Y
	193	8.15	154	6.8	27	>1	Y
5	12	8.97	116	15.2	20	<1	Y
	193	8.06	153	7.5	25	>1	N

Table 3
Genetic similarity between H5 fragments recovered from ponds on the infected premise and the H5 segment sequence recovered from infected poultry on that premise. Environmental specimens were collected during two visits from five ponds on the infected premise. H5 fragments were recovered during only site visit 1. Two replicate specimens were collected from three sub-locations at each pond. The fragment depth of coverage column indicates the number of sequencing read pairs describing each fragment. The percent sequenced column indicates the percentage of the total bases in the fragment (fragment length) that were successfully sequenced (an A,T,G, or C base call). The percent identical column indicates the percentage of aligned positions where the fragment and H5 segment sequence from the poultry contained the same A, T, G, or C base call. Similarly, the percent ambiguous column indicates the position of aligned positions with N or degenerate nucleotide base calls. The fragment coverage column indicates the percentage of fragment positions that aligned to the H5 segment sequence from the poultry. The poultry sequence coverage (sequenced) column indicates the percentage of H5 segment sequence positions covered by the fragment alignment, whereas the reference sequence coverage (total) column indicates the percentage of H5 segment sequence positions covered by the fragment (including unaligned positions in the fragment, e.g. unsequenced middle regions of longer fragments). These metrics were calculated against all four H5 segment sequences recovered from infected poultry on the premise, and the median values are reported in this table.

Pond	Replicate	Fragment ID	Fragment length	Fragment depth of coverage	Percent sequenced	Percent identical	Percent ambiguous	Fragment coverage	Poultry sequence coverage (sequenced)	Poultry sequence coverage (total)
3 A	1	Fragment 2	317	486	100	100	0	100	18.6	18.6
3 A	1	Fragment 59	113	44	100	100	0	100	6.6	6.6
3C	2	Fragment 250	712	158	80.2	97.9	1.9	81.7	34.2	41.8
3C	2	Fragment 385	386	202	100	100	0	100	22.7	22.7
3C	2	Fragment 723	249	341	100	100	0	100	14.6	14.6
4C	1	Fragment 1451	195	184	100	100	0	100	11.4	11.4
4C	1	Fragment 1708	157	49	100	100	0	100	9.2	9.2
4C	2	Fragment 197	229	51	100	100	0	100	13.4	13.4
4C	2	Fragment 342	189	87	100	100	0	100	11.1	11.1
5 A	1	Fragment 6	204	895	100	100	0	100	12	12

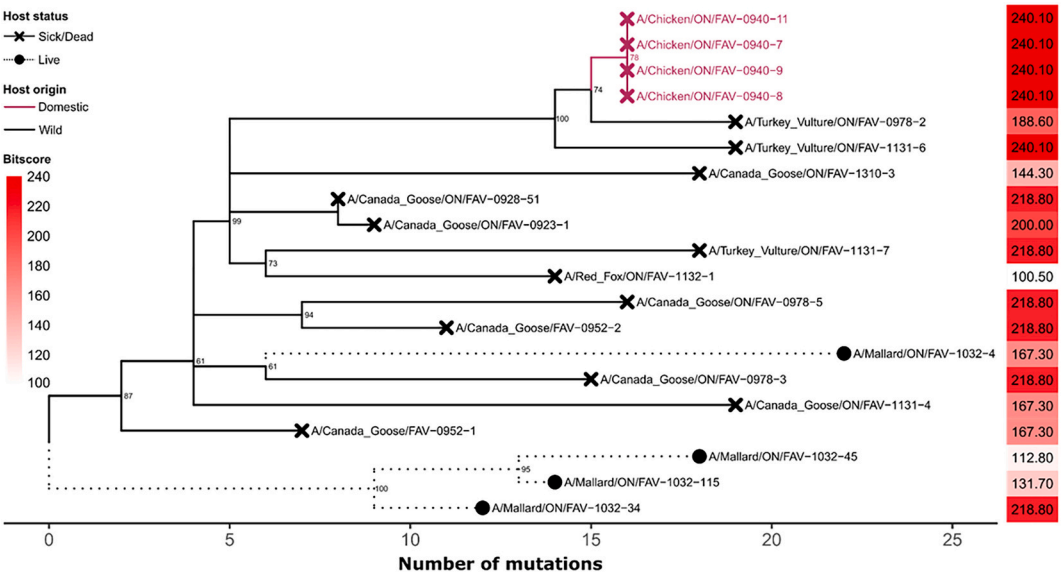

Fig. 4. Phylogenetic relationships between HPAI H5N1s collected from the infected premise and local wildlife surveillance. Phylogenetic tree was constructed using all 8 segments of the IAV genome. Genomes recovered from live hosts are depicted by circular leaves while those recovered from dead hosts are depicted with "X" leaves. The tree branch line-type represents the viral host status when sampled (sick/dead or live) and branch color represents host origin (either wild or domestic). Ancestral states for host origin and status are reconstructed across all nodes of the tree. Fragmentary HPAI H5 sequences recovered from ponds on the premise were aligned to HA segment sequences from the bird-origin HPAI genomes composing the tree, and the best match(es) for each fragment were determined based on alignment bitscores. Each bird-origin HPAI genome in the tree was allocated a score based on the number of pond fragments for whom it was their best match as well as the identity and length of its alignments with those fragments. These scores are indicated in the heatmap next to the tree. The branch length scale bar indicates number of mutations across all 8 segments of the IAV genome.

several LPAIV subtypes were detected (Table 1). This is consistent with sediment results which exhibited extensive LPAIV subtype diversity indicating a high level of environmental contamination (Fig. 4). Furthermore, on day 12 a majority of premise mallards (96 %) had antibodies against H5 AIV, suggesting recent exposure. However, the lack of active H5 shedding detected in premise mallards (Table 1), the small amount of H5 genomic material in the sediment (Fig. 4), and the high percent inhibition (PI%) on H5 cELISA (Fig. S2) together suggest

that exposure to H5 AIV in these birds was recent, but preceding the date of sampling (i.e., Day 12), and most likely prior to or concurrent with virus detection in domestic birds (i.e., Day 0). These results emphasize the value of combining viral and serological testing to more comprehensively understand virus exposure and dynamics in wild bird populations, extending insights beyond mere detection of active infection within the limited timeframe of viral shedding.

Although the cELISA cannot distinguish between LPAIV and HPAIV H5 exposure, the rare detection of LPAIV H5 viruses in wild birds in Ontario prior to this study, combined with the presence of H5 HPAIV virus in the ponds used by these birds, suggests that the premise mallards were likely exposed to H5 HPAIV. Despite this exposure, the premise mallards remained healthy, exhibiting neither clinical signs nor unusual mortality. This is important to consider in the context of wild bird health, and which species may harbour the virus, and show no obvious signs of infection. Similar observations were reported by Teitelbaum et al. (2023), who found that North American wintering mallards infected with highly pathogenic avian influenza (HPAI) exhibited no significant changes in movement behavior or body condition, suggesting that infected mallards can continue to contribute to the maintenance and potential spread of HPAI without apparent symptoms [46].

Previous LPAIV infection in mallards has been found to confer at least partial immunity to subsequent infection with LPAIV or HPAIV, depending on the subtype, resulting in lower viral loads and shorter periods of shedding [47,48]. However, these premise mallards were all hatch year birds and beyond the period when maternal antibodies would be expected to be present. Therefore, pre-existing immunity would be expected to be limited. Between Day 12 and Day 66, we found a decrease in the proportion of premise mallards testing positive for anti-NP antibodies by approximately a third, while the proportion of premise mallards testing positive for anti-H5 antibodies decreased to zero. These findings suggest differential persistence of NP and H5 antibodies in hatch year birds and raise questions about the longevity of H5 antibodies, and the level of protection conferred following first exposure. The inability to conduct repeated sampling on the same individuals due to the sampling requirements on the IP is a limitation of this study. There is no indication that the sampling methodology would lead to differential risk of exposure between premise mallards sampled on Day 12 and Day 66. Furthermore, considering the behavior, proximity, and density of premise mallards, these birds were considered to constitute a single epidemiological unit with consistent exposure.

AIV contamination in the ponds on the infected premise decreased dramatically between the first and second visit (Days 12 and 193). This suggests that the premise mallards were primarily responsible for the AIV contamination in the ponds we detected, and thus, that the accumulation of AIV genomic material in the pond sediment stopped due to removal and depopulation of the premise mallards or seasonal changes in their access to the ponds. An important corollary of this finding is that AIV genomic material may not persist long-term in the environment, despite previous reports that virions may remain infectious in various controlled experiments under laboratory and in situ conditions [23,26,27,30]. Furthermore, the existing environmental contamination did not appear to exert infection pressure sufficient to maintain antibody production in the premise mallards such that decreasing environmental exposure contributed to waning antibody levels in the premise mallards between Day 12 and Day 66.

This study also demonstrated the potential utility of environmental sampling and targeted genomic sequencing for AIV surveillance. Current and previous infections with various AIV subtypes were detected in the premise mallards by molecular and serological testing all those HA and NA subtypes were detected in sediment from the premise ponds, along with additional HA and NA subtypes that had not been detected in the premise mallards. Some of these additional subtypes had been detected in wild birds preceding the outbreak, while others had not been detected by any poultry testing or wildlife surveillance. Since genomic sequences from all the bird specimens were not available for comparison, it cannot be concluded that the AIV genomic material recovered from the premise ponds originated from the same viral lineages infecting the premise poultry and local wild birds. However, these results do show that broad AIV diversity can be monitored by environmental genomic surveillance at interfaces between livestock, wildlife, and humans. Furthermore, the above finding that AIV genomic material does not persist in the sediment in the absence of a persistent deposition source, indicates that environmental detections are contemporary and meaningful risk signals.

Our findings suggest that, even at high density with likely exposure to high viral loads, phenotypically wild mallards can survive infection with the H5 HPAIV genotype, which has caused morbidity and mortality in both wild birds and mammals and has been identified on over 20 domestic premises in Canada at the time of this study. However, the longevity of H5 antibodies appears to be brief in hatch-year birds following initial exposure, with potential implications for risk projections upon re-exposure for wild bird populations that experienced extensive mortality following first exposure. Additionally, our results raise questions about the role of environmental compartments as reservoirs and sources of transmission, especially in the absence of persistent virus deposition by wild birds. These results suggest that biosecurity remains the most critical aspect of minimising spillover/back risk in a virus that has been shown to circulate in asymptomatic wild birds and in the surrounding environment.

Further research is essential to gain a more detailed understanding of transmission dynamics at the wildlife-poultry-environmental interface, to enhance management practices, and to thereby reduce the risk of infection to domestic flocks given the persistence of HPAIVs on the landscape. Regardless of the specific transmission dynamics, the artificially dense and prolonged congregation of premise mallards significantly heightened the spillover risk for the domestic flock and potentially increased the risk to regional populations of wild birds and mammals.

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CRediT authorship contribution statement

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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References

[1] Canadian Food Inspection Agency (CFIA), Status of ongoing avian influenza response by province. https://inspection.canada.ca/animal-health/terrestrialanimals/diseases/reportable/avian-influenza/hpai-in-canada/status-of-ongoing-avianinfluenza-response/eng/1640207916497/1640207916934, 2024 (accessed 2024).

[2] D.J. Alexander, I.H. Brown, History of highly pathogenic avian influenza, Rev. Scient. Techn. l'OIE. 28 (1) (2009) 19–38, https://doi.org/10.20506/rst.28.1.1856.

[3] A.M. Ramey, N.J. Hill, T.J. DeLiberto, S.E.J. Gibbs, M.C. Hopkins, A.S. Lang, R. L. Poulson, D.J. Prosser, J.M. Sleeman, D.E. Stallknecht, X. Wan, Highly pathogenic avian influenza is an emerging disease threat to wild birds in North America, J. Wildl. Manag. 86 (2) (2022), https://doi.org/10.1002/jwmg.22171.

[4] Canadian Food Inspection Agency (CFIA), Avian influenza A (H5N1) 2022 outbreak in Canada. https://ncceh.ca/resources/evidence-briefs/avian-influenza-ah5n1-2022-outbreak-canada, 2022 (accessed 2024).

[5] A. Castillo, R. Fasce, B. Parra, W. Andrade, P. Covarrubias, A. Hueche, C. Campano, C. Tambley, M. Rojas, M. Araya, F. Hernández, P. Bustos, J. Fernández, The first case of human infection with H5N1 avian influenza a virus in Chile, J. Travel Med. 30 (2023) taad083, https://doi.org/10.1093/jtm/taad083.

[6] J.S.M. Peiris, M.D. de Jong, Y. Guan, Avian Influenza Virus (H5N1): A threat to human health, Clin. Microbiol. Rev. 20 (2) (2007) 243–267, https://doi.org/10.1128/CMR.00037-06.

[7] D.A.M. Philippon, P. Wu, B.J. Cowling, E.H.Y. Lau, Avian influenza human infections at the human-animal interface, J. Infect. Dis. 222 (4) (2020) 528–537, https://doi.org/10.1093/infdis/jiaa105.

[8] Y. Yang, M.E. Halloran, J.D. Sugimoto, I.M. Longini, Detecting human-to human transmission of avian influenza A (H5N1), Emerg. Infect. Dis. 13 (9) (2007) 1348–1353, https://doi.org/10.3201/eid1309.070111.

[9] D.J. Alexander, An overview of the epidemiology of avian influenza, Vaccine 25 (30) (2007) 5637–5644, https://doi.org/10.1016/j.vaccine.2006.10.051.

[10] J. Jeong, H.M. Kang, E.K. Lee, B.M. Song, Y.K. Kwon, H.R. Kim, K.S. Choi, J.Y. Kim, H.J. Lee, O.K. Moon, W. Jeong, J. Choi, J.H. Baek, Y.S. Joo, Y.H. Park, H.S. Lee, Y. J. Lee, Highly pathogenic avian influenza virus (H5N8) in domestic poultry and its relationship with migratory birds in South Korea during 2014, Vet. Microbiol. 173 (3–4) (2014) 249–257, https://doi.org/10.1016/j.vetmic.2014.08.002.

[11] J.H. Leibler, J. Otte, D. Roland-Holst, D.U. Pfeiffer, R. Soares Magalhaes, J. Rushton, J.P. Graham, E.K. Silbergeld, Industrial food animal production and Global Health risks: exploring the ecosystems and economics of avian influenza, EcoHealth 6 (1) (2009) 58–70, https://doi.org/10.1007/s10393-009-0226-0.

[12] B. Olsen, V.J. Munster, A. Wallensten, J. Waldenström, A.D.M.E. Osterhaus, R.A. M. Fouchier, Global patterns of influenza a virus in wild birds, Science 312 (5772) (2006) 384–388, https://doi.org/10.1126/science.1122438.

[13] D.J. Alexander, A review of avian influenza in different bird species, Vet. Microbiol. 74 (1–2) (2000) 3–13, https://doi.org/10.1016/s0378-1135(00)00160-7.

[14] R. Bodewes, T. Kuiken, Changing role of wild birds in the epidemiology of avian influenza a viruses, Adv. Virus Res. 100 (2018) 279–307, https://doi.org/10.1016/bs.aivir.2017.10.007.

[15] J.A. Giacinti, A.V. Signore, M.E.B. Jones, L. Bourque, S. Lair, C. Jardine, B. Stevens, T. Bollinger, D. Goldsmith, British Columbia Wildlife AIV Surveillance Program (BC WASPs), M. Pybus, I. Stasiak, R. Davis, N. Pople, L. Nituch, R.W. Brook, D. Ojkic, A. Massé, G. Dimitri-Masson, C. Soos, Avian influenza viruses in wild birds in Canada following incursions of highly pathogenic H5N1 virus from Eurasia in 2021/2022, mBio 15 (8) (2023) e03203–e03223, https://doi.org/10.1128/mbio.03203-23.

[16] M.J. Pantin-Jackwood, M. Costa-Hurtado, K. Bertran, E. DeJesus, D. Smith, D. E. Swayne, Infectivity, transmission and pathogenicity of H5 highly pathogenic avian influenza clade 2.3.4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese, Vet. Res. 48 (1) (2017) 33, https://doi.org/10.1186/s13567-017-0435-4.

[17] R. Xie, K.M. Edwards, M. Wille, X. Wei, S.S. Wong, M. Zanin, R. El-Shesheny, M. Ducatez, L.L.M. Poon, G. Kayali, R.J. Webby, V. Dhanasekaran, The episodic resurgence of highly pathogenic avian influenza H5 virus, Nature 622 (7984) (2023) 810–817, https://doi.org/10.1038/s41586-023-06631-2.

[18] X. Xu, K. Subbarao, N.J. Cox, Y. Guo, Genetic characterization of the pathogenic influenza a/goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong, Virology 261 (1999) 15–19, https://doi.org/10.1006/viro.1999.9820.

[19] S. Sonnberg, R.J. Webby, R.G. Webster, Natural history of highly pathogenic avian influenza H5N1, Virus Res. 178 (2013) 63–77, https://doi.org/10.1016/j.virusres.2013.05.009.

[20] R. Harfoot, R.J. Webby, H5 influenza, a global update, J. Microbiol. 55 (2017) 196–203, https://doi.org/10.1007/s12275-017-7062-7.

[21] V.S. Hinshaw, R.G. Webster, B. Turner, Water-borne transmission of influenza a viruses? Intervirology 11 (1) (1979) 66–68, https://doi.org/10.1159/000149014.

[22] D.D. Markwell, K.F. Shortridge, Possible waterborne transmission and maintenance of influenza viruses in domestic ducks, Appl. Environ. Microbiol. 43 (1) (1982) 110–115, https://doi.org/10.1128/aem.43.1.110-115.1982.

[23] J. Nazir, R. Haumacher, A.C. Ike, R.E. Marschang, Persistence of avian influenza viruses in Lake sediment, duck feces, and duck meat, Appl. Environ. Microbiol. 77 (14) (2011) 4981–4985, https://doi.org/10.1128/AEM.00415-11.

[24] K. Domanska-Blicharz, Z. Minta, K. Smietanka, S. Marché, R. Van Den Berg, H5N1 high pathogenicity avian influenza virus survival in different types of water, Avian Dis. 54 (s1) (2010) 734–737, https://doi.org/10.1637/8786-040109-ResNote.1.

[25] V. Hénaux, M.D. Samuel, Avian influenza shedding patterns in waterfowl: implications for surveillance, environmental transmission, and disease spread, J. Wildl. Dis. 47 (3) (2011) 566–578, https://doi.org/10.7589/0090-3558-47.3.566.

[26] A. Perlas, K. Bertran, F.X. Abad, C.M. Borrego, M. Nofrarías, R. Valle, L. Pailler-García, A. Ramis, M. Cortey, V. Acuña, N. Majó, Persistence of low pathogenic avian influenza virus in artificial streams mimicking natural conditions of waterfowl habitats in the Mediterranean climate, Sci. Total Environ. 863 (2023) 160902, https://doi.org/10.1016/j.scitotenv.2022.160902.

[27] A.M. Ramey, A.B. Reeves, B.J. Lagassé, V. Patil, L.E. Hubbard, D.W. Kolpin, R. B. McCleskey, D.A. Repert, D.E. Stallknecht, R.L. Poulson, Evidence for interannual persistence of infectious influenza a viruses in Alaska wetlands, Sci. Total Environ. 803 (2022) 150078, https://doi.org/10.1016/j.scitotenv.2021.150078.

[28] D.E. Stallknecht, V.H. Goekjian, B.R. Wilcox, R.L. Poulson, J.D. Brown, Avian influenza virus in aquatic habitats: what do we need to learn? Avian Dis. 54 (s1) (2010) 461–465, https://doi.org/10.1637/8760-033109-Reg.1.

[29] D.E. Stallknecht, M.T. Kearney, S.M. Shane, P.J. Zwank, Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water, Avian Dis. 34 (2) (1990) 412, https://doi.org/10.2307/1591429.

[30] D.E. Stallknecht, S.M. Shane, M.T. Kearney, P.J. Zwank, Persistence of avian influenza viruses in water, Avian Dis. 34 (2) (1990) 406, https://doi.org/10.2307/1591428.

[31] C.G. Himsworth, J. Duan, N. Prystajecky, M. Coombe, W. Baticados, A.N. Jassem, P. Tang, E. Sanders, W. Hsiao, Targeted resequencing of wetland sediment as a tool for avian influenza virus surveillance, J. Wildl. Dis. 56 (2) (2020) 397, https://doi.org/10.7589/2019-05-135.

[32] K.S. Kuchinski, M. Coombe, S.C. Mansour, G.A.P. Cortez, M. Kalhor, C. G. Himsworth, N.A. Prystajecky, Targeted genomic sequencing of avian influenza viruses in wetland sediment from wild bird habitats, Appl. Environ. Microbiol. 90 (2) (2024), https://doi.org/10.1128/aem.00842-23 e00842–23.

[33] R. Breban, J.M. Drake, D.E. Stallknecht, P. Rohani, The role of environmental transmission in recurrent avian influenza epidemics, PLoS Comput. Biol. 5 (4) (2009) e1000346, https://doi.org/10.1371/journal.pcbi.1000346.

[34] P. Rohani, R. Breban, D.E. Stallknecht, J.M. Drake, Environmental transmission of low pathogenicity avian influenza viruses and its implications for pathogen invasion, Proc. Natl. Acad. Sci. 106 (25) (2009) 10365–10369, https://doi.org/10.1073/pnas.0809026106.

[35] T.N. Alkie, S. Lopes, T. Hisanaga, W. Xu, M. Suderman, J. Koziuk, M. Fisher, T. Redford, O. Lung, T. Joseph, C.G. Himsworth, I.H. Brown, V. Bowes, N.S. Lewis, Y. Berhane, A threat from both sides: multiple introductions of genetically distinct H5 HPAI viruses into Canada via both East Asia-Australasia/Pacific and Atlantic

flyways. Virus, Evolution 8 (2) (2022) veac077, https://doi.org/10.1093/ve/veac077.
[36] S. Carney, Species, Age, and Sex Identification of Ducks Using Wing Plumage, U.S. Department of the Interior, U.S. Fish and Wildlife Service, 1992, p. 144.
[37] H.M. Weingartl, Y. Berhane, T. Hisanaga, J. Neufeld, H. Kehler, C. Emburry-Hyatt, K. Hooper McGreevy, S. Kasloff, B. Dalman, J. Bystrom, S. Alexandersen, Y. Li, J. Pasick, Genetic and pathobiologic characterization of pandemic H1N1 2009 influenza viruses from a naturally infected swine herd, J. Virol. 84 (5) (2010) 2245–2256, https://doi.org/10.1128/JVI.02118-09.
[38] M. Yang, Y. Berhane, T. Salo, M. Li, K. Hole, A. Clavijo, Development and application of monoclonal antibodies against avian influenza virus nucleoprotein, J. Virol. Methods 147 (2) (2008) 265–274, https://doi.org/10.1016/j.jviromet.2007.09.016.
[39] M. Yang, A. Clavijo, J. Graham, T. Salo, K. Hole, Y. Berhane, Production and diagnostic application of monoclonal antibodies against influenza virus H5, J. Virol. Methods 162 (1–2) (2009) 194–202, https://doi.org/10.1016/j.jviromet.2009.08.006.
[40] A. Signore, J. Giacinti, M.E.B. Jones, C.N.G. Erdelyan, A. McLaughlin, T.N. Alkie, S. Cox, S. Lair, C.M. Jardine, B. Stevens, M. Bravo-Araya, N. Pople, M.J. Pybus, T. Hisanaga, W. Xu, J. Koziuk, O. Lung, P. Kruczkiewicz, M. Fisher, J. Wight, I. Rahman, K.E. Hargan, A.S. Lang, D. Ojkic, C. Yason, British Columbia Wildlife AIV Surveillance Program, L. Bourque, T.K. Bollinger, J.F. Provencher, S. Ogilvie, A. Clark, R. MacPhee, H. Eaglesome, S. Gilbert, K. Saboraki, R. Davis, A. Jerao, M. Ginn, C. Soos, Y. Berhane, n.d. Under Review. Phylodynamics of the A(H5N1) avian influenza outbreak in North America reveals the emergence of reassortants with increased fitness. Cell Rep.
[41] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, Mol. Biol. Evol. 30 (4) (2013) 772–780, https://doi.org/10.1093/molbev/mst010.
[42] L.T. Nguyen, H.A. Schmidt, A. von Haeseler, B.Q. Minh, IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies, Mol. Biol. Evol. 32 (1) (2015) 268–274, https://doi.org/10.1093/molbev/msu300.
[43] S. Kalyaanamoorthy, B.Q. Minh, T.K.F. Wong, A. von Haeseler, L.S. Jermiin, ModelFinder: fast model selection for accurate phylogenetic estimates, Nat. Methods 14 (6) (2017) 587–589, https://doi.org/10.1038/nmeth.4285.
[44] D.T. Hoang, O. Chernomor, A. von Haeseler, B.Q. Minh, L.S. Vinh, UFBoot2: improving the ultrafast bootstrap approximation, Mol. Biol. Evol. 35 (2) (2018) 518–522, https://doi.org/10.1093/molbev/msx281.
[45] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T. L. Madden, BLAST+: architecture and applications, BMC Bioinform. 10 (2009) 421, https://doi.org/10.1186/1471-2105-10-421.
[46] C.S. Teitelbaum, N.M. Masto, J.D. Sullivan, A.C. Keever, R.L. Poulson, D.L. Carter, A.G. Blake-Bradshaw, C.J. Highway, J.C. Feddersen, H.M. Hagy, R.W. Gerhold, B. S. Cohen, D.J. Prosser, North American wintering mallards infected with highly pathogenic avian influenza show few signs of altered local or migratory movements, Sci. Rep. 13 (2023) 14473, https://doi.org/10.1038/s41598-023-40921-z.
[47] T.P. Costa, J.D. Brown, E.W. Howerth, D.E. Stallknecht, Effect of a prior exposure to a low pathogenic avian influenza virus in the outcome of a Heterosubtypic low pathogenic avian influenza infection in mallards (Anas platyrhynchos), Avian Dis. 54 (4) (2010) 1286–1291, https://doi.org/10.1637/9480-072210-Reg.1.
[48] S.R. Fereidouni, E. Starick, M. Beer, H. Wilking, D. Kalthoff, C. Grund, Highly pathogenic avian influenza virus infection of mallards with Homo- and Heterosubtypic immunity induced by low pathogenic avian influenza viruses, PLoS One 4 (8) (2009), https://doi.org/10.1371/journal.pone.0006706.