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Pathogen Surveillance in Swallows (family *Hirundinidae*): Investigation into Role as Avian Influenza Vector in Eastern Canada Agricultural Landscapes

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ABSTRACT: First detected in Atlantic Canada in December 2021, highly pathogenic avian influenza virus (HPAIV) subtype H5N1 clade 2.3.4.4b, A/Goose/Guangdong/1/96 lineage, has caused massive mortality in wild birds and domestic poultry in North America. Swallows (*Hirundinidae*), abundant in North American agricultural ecosystems, have been proposed as possible (bridge) species for HPAIV transmission between wild and domestic birds. We aimed to seek evidence of the potential role of swallows in bridging AIV infection between wild bird reservoirs and poultry flocks in eastern Canada. During a wide-scale outbreak of HPAIV in wild birds and poultry farms across eastern Canada, 200 samples were collected from swallow breeding sites in the Canadian provinces of New Brunswick, Nova Scotia, Ontario, and Quebec, June–August 2022. Samples came from Barn Swallow (*Hirundo rustica*; n=142), Tree Swallow (*Tachycineta bicolor*; n=56), and Cliff Swallow (*Petrochelidon pyrrhonota*; n=2) nests. All samples tested negative for AIV, suggesting that HPAIV and low pathogenic AIV (LPAIV) strains were probably not circulating widely in swallows during the 2022 breeding season in eastern Canada; thus swallows may present a low risk of transmitting AIV. Within a management context, these findings suggest that removing nests of Barn Swallows, a species at risk in Canada, from the exterior of biosecure domestic poultry facilities may not significantly reduce risks of HPAI transmission to poultry.

Key words: Bridge host, environmental surveillance, highly pathogenic avian influenza virus, poultry, RT-PCR, species at risk.

Highly pathogenic avian influenza virus (HPAIV) subtype H5N1 clade 2.3.4.4b of the A/Goose/Guangdong/1/96 lineage has been circulating in North America since December 2021 when it was first detected in Atlantic Canada (Caliendo et al. 2022). The virus has been transmitted from wild birds to domestic flocks (i.e., poultry) and back, causing massive mortality in wild and domestic birds (Harvey et al. 2023). Commercial poultry operations in North America employ strict biosecurity measures to prevent the entry of pathogens (US Department of Agriculture Animal and Plant Health Inspection Service [USDA APHIS] 2016; Canadian Food Inspection Agency 2018), raising questions as to how the H5N1 virus critically infiltrated commercial facilities in every province in southern Canada.

One proposed infiltration mechanism is through an intermediate wild bird bridge host, such as birds with affinity for agricultural areas and buildings, which may transmit the virus from reservoir hosts to commercial poultry. Swallows (family *Hirundinidae*) are common on Canadian farms (Burns et al. 2012), frequently using agricultural habitats and structures for feeding and nesting, including structures on poultry barns. Swallow species also co-occupy multiple habitats, including wetlands and smaller waterways, which are also used by known avian influenza virus (AIV) reservoirs such as waterfowl (Caron et al. 2014). This co-occupation of multiple habitats makes them a suitable species to facilitate pathogen transmission at the wild bird–domestic poultry interface as either mechanical vectors (e.g., transportation of nesting materials gathered from nearby areas [Kilgore and Knudson 1977]), or as biological vectors of AIV (e.g., as

carriers of the virus), including HPAIV (Zhong et al. 2019).

Wild birds, particularly waterfowl, are known to shed AIVs through feces (Webster et al. 1978; Hénaux and Samuel 2011), and although AIV can be shed via oral secretions (Achenbach and Bowen 2011), there is concern that swallows might shed enough virus through their feces to transmit AIV to poultry via nests located on barns. This is of particular concern because Barn Swallows (*Hirundo rustica*) and Cliff Swallows (*Petrochelidon pyrrhonota*) regularly defecate over the side of their nests; this can create small piles of feces building up directly beneath their nesting site. These fecal piles therefore are the largest concern for AIV transmission in an agricultural setting. Environmental sampling of birds, including wild bird feces, has been applied extensively during pathogen surveillance activities globally (Hood et al. 2021), but this approach is relatively novel in the current context of the HPAIV outbreak in Canada on agricultural lands.

All swallow species are protected in Canada under the *Migratory Birds Convention Act, 1994* (Department of Justice Canada 2017). Barn Swallows are additionally a species at risk in Canada and are protected under the *Species at Risk Act* (Department of Justice Canada 2023). These birds flourish in agricultural landscapes where they have been documented nesting under barn awnings, inside poultry barns, and within agricultural storage buildings (Burns et al. 2012; Caron et al. 2014). Barn Swallows may potentially represent a biosecurity risk on farms if they make their nests inside barns, therefore the abovementioned legislation establishes permitting guidelines that are required for the movement or removal of Barn Swallow nests. These nest-removal guidelines, coupled with the fact that Barn Swallows can be located on poultry barns where nests are at risk of deliberate destruction to reduce pathogen transmission potential in the current HPAIV outbreak, have motivated HPAIV surveillance for Barn Swallows nesting in and around agricultural landscapes. Although the role of swallow species in the transmission of HPAIV is plausible, there has been no definitive evidence to demonstrate that swallows play a role in the spillover of HPAIV into domestic poultry operations. Thus, in order to balance the needs of agriculture to secure poultry premises from wild birds with species-at-risk concerns, there is a requirement to assess the role swallows play as HPAIV reservoirs to help inform nest-removal policies and permitting decision making. In the context outlined above, the overall objective of this work was to determine how prevalent AIV was in fresh feces of swallows in eastern Canada.

Samples for avian influenza virus testing were collected from three swallow species in the Canadian provinces of Ontario, Quebec, New Brunswick, and Nova Scotia, from June to August 2022 (Barn Swallows [n=142], Tree Swallows [*Tachycineta bicolor*; n=56], and Cliff Swallows [n=2]; Table 1; Fig. 1). The sampling period was during an outbreak of HPAIV in numerous wild bird species and poultry farms across eastern Canada (Canadian Food Inspection Agency 2023). We sampled recent deposits of Tree Swallow and Barn Swallow feces below active nests from birds collected in Ontario and Nova Scotia, and from Cliff Swallows in Nova Scotia. This included nests on several types of man-made structure, including active barns. Additionally, samples were collected from the interior surface of active Barn Swallow nests in Ontario and Quebec, and cloacal swabs from Tree Swallows in Ontario.

Samples were collected using sterile mini-tipped nylon flocked swabs (Copan Diagnostics, Murrietta, California, USA) and stored in 3 mL of virus transport medium (Copan Diagnostics). Samples from recently deposited feces were prioritized to reduce the potential for degradation of viral RNA prior to preservation. Feces were determined to be recently deposited if the sampler observed an individual bird defecating, or if fecal deposits under an active nest appeared to be moist while the rest of the surrounding area was dry (e.g., no recent precipitation). For samples collected from the interior of Barn Swallow nests, moist areas of the nest were swabbed to target potential AIVs shed through fecal droppings

TABLE 1. Number of fecal samples collected from swallows (family *Hirundinidae*) in eastern Canada (Nova Scotia, New Brunswick, Ontario, and Quebec) during the breeding season in 2022 and tested for avian influenza viruses using a heminested RT-PCR approach.

	Species			
Canadian province	Barn Swallow (*Hirundo rustica*)	Tree Swallow (*Tachycineta bicolor*)	Cliff Swallow (*Petrochelidon pyrrhonota*)	Total
New Brunswick	14	0	0	14
Nova Scotia	37	10	2	49
Ontario	88	47	0	135
Quebec	3	0	0	3
Total	142	57	2	201

within the nest, secretions from the cloaca, or secretions from the respiratory tract deposited on the nests during nest building. For each fecal deposit, up to five swabs were pooled in a single tube of viral transport medium.

The influenza A virus matrix gene was targeted using a one-step reverse transcriptase PCR (RT-PCR) followed by a heminested PCR reaction for increased sensitivity to detect low viral loads, compared to conventional real-time RT-PCR screening approaches. We isolated RNA from 140 µL of each sample using the Qiagen Viral RNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and stored these at −80 °C until analysis. The first PCR was performed using the NEB OneTaq One-Step RT-PCR Kit (New England Biolabs, Whitby, Canada) in 25-µL reactions using 12.5 µL of 2X reaction mix, 1 µL of 25X enzyme mix, 1 µL of 10 mM FluSc_F2 (5'-CTTCTRACHGAGGTCGAAACG-3'), which is a modified version of the established M52C primer (Fouchier et al. 2000), 1 µL of 10 mM M253R (5'-AGGGCATTTTGGACAAAKCGTCTA-3'), 1.5 µL of nuclease-free water, and 8 µL of RNA. Thermal cycling parameters were 48 °C for 30 min, 95 °C for 1 min, 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 30 s, followed by 68 °C for 5 min and 4 °C for 10 min. The heminested PCR was performed using DreamTaq Green PCR Master Mix (ThermoScientific, Waltham, Massachusetts, USA) in 25-µL reactions using 12.5 µL of 2X DreamTaq master mix, 0.5 µL of 10 mM FluSc_F2, 0.5 µL of 10 mM FluSc_R2 (5'-AAANCGTCTACGYTGCAGTC-3'), 9 µL of nuclease-free water, and 2.5 µL of input from the original RT-PCR. Thermal cycling parameters were 95 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 7 min and 4 °C for 10 min. The RNA isolated from a pooled oropharyngeal and cloacal swab sample from a wild bird previously determined to contain a high RNA load was used as a positive

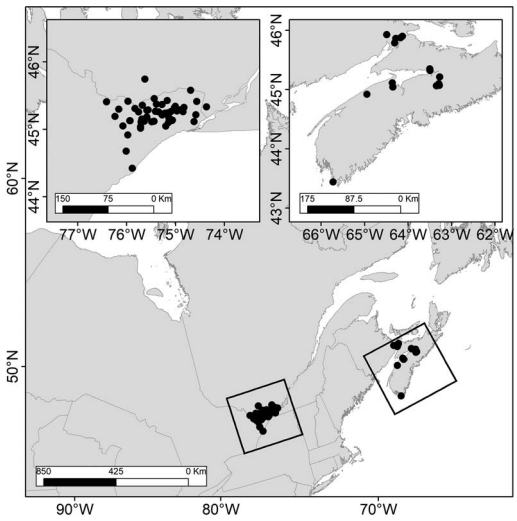

FIGURE 1. Distribution of sampling locations where fecal samples were collected from swallows (family *Hirundinidae*) in eastern Canada (provinces of Nova Scotia, New Brunswick, Ontario, and Quebec) during the breeding season (June–August) in 2022.

control in each batch of samples tested, after diluting the RNA to a concentration that resulted in detectable amplification in both the one-step and the heminested reactions. The products from the original one-step and heminested reactions were run on 1.5% TAE agarose gels containing SYBR Safe (Invitrogen, Waltham, Massachusetts, USA) and visualized under blue light on a Gel Doc EZ Imager (Bio-Rad Laboratories, Inc., Hercules, California, USA). All gel images were processed using Image Lab (version 6.1.0 build 7, Bio-Rad).

We collected 200 samples, with all samples testing negative for AIV. At the time of this study (2022), AIV infection was at 5.4% prevalence in the more than 11,000 birds tested in Canada, with a range of 0–8.4% in wild live birds (Giacinti et al., 2023). If we assume that AIV infection was randomly distributed in swallow populations at a relatively low prevalence of 1.5% (within the range of observed prevalence in North America in 2022), the minimum sample size required to detect the presence of AIV infection with a perfect diagnostic test would be 199 (Duncan and Humphry 2023). Therefore, the number of samples that we tested was deemed sufficient for detecting active infections, even if the virus was circulating on the lower range of the observed prevalence at the time. Thus, 6–8 mo after the virus was first detected in a wild bird in North America (Caliendo et al. 2022), and although other species were experiencing prevalence levels as high as 8.4%, we did not detect the virus in nesting swallows. These results suggest that AIV, including HPAIV and LPAIV strains, was not appreciably circulating in swallows during the 2022 breeding season in eastern Canada.

In addition to our direct sampling of swallow nests and fecal deposits, 25 combined oral and cloacal swab samples from sick or dead Bank Swallows (*Riparia riparia;* $n=2$), Barn Swallows ($n=9$), Cliff Swallows ($n=5$), and Tree Swallows ($n=9$) were collected during wildlife disease surveillance activities led by the Canadian Wildlife Health Cooperative (CWHC) in 2022 and 2023 in New Brunswick, Newfoundland and Labrador, Northwest Territories, Ontario, Prince Edward Island, Saskatchewan, and Yukon Territory, Canada. All of these samples also tested negative for AIV (Giacinti et al. 2023). Swallows are small, which can lead to detection issues in the wild when birds are sick or die; however, given the extensive swallow monitoring in Canada (as outlined in the method efforts), lack of detection is unlikely to be an issue for this group of birds in the study region. These data further support that swallows were not significant reservoirs for the virus in the 2022 HPAIV outbreak. It is noteworthy that HPAIV H5N1 was detected in a Tree Swallow collected through wildlife disease surveillance in Alaska, US in 2022 (USDA APHIS 2023), indicating that swallow species are susceptible to infection by AIVs. Taken together, although swallows can be infected with HPAIV, surveillance of samples collected from the environment and sick or dead birds in Canada suggests that these species were not severely impacted in 2022. Our study also suggests that swallows are probably not a primary transmission route for AIVs, at least in the agricultural areas examined, and by extension, the presence of these birds around biosecure domestic poultry facilities probably poses little transmission risk.

These findings are especially encouraging for the Barn Swallow, a species at risk in Canada that has suffered substantial population declines in North America (Put et al. 2021). We recognize that the lack of oral swabs in this study means that an active infection might have been missed. Thus, oral swabs should be considered for all future work, as collecting both oral and cloacal swabs is preferable for detecting active infections in migratory birds. Although our work suggests that risk of AIV transmission from swallows is likely very low, future work pairing serological with fecal and oral swab testing during the nesting and brood-rearing periods would confirm whether swallows had previously been exposed to AIVs, in addition to detecting active infection (e.g., see Samuel et al. 2015). The short time window associated with active virus shedding by wild birds may limit the ability to detect AIVs through molecular testing of swab samples, as the virus is typically shed over only 1–10 d (Ellis et al. 2021).

Without serological testing, the time window for detecting virus exposure is short; however, it can be challenging to collect a large enough blood sample for serological testing because of the swallows' small body size (e.g., the species discussed can range from mean 10–30 g, with a safe sample (approximately 1%) being 0.10–0.30 mL of blood).

Importantly, our work suggests that removing Barn Swallow nests (which is illegal without proper permitting) located on the exterior of biosecure domestic poultry facilities may not be necessary to mitigate the risk of HPAIV transmission to domestic poultry. Previous research has shown that a wide variety of species can be infected with AIV (Harvey et al. 2023). These viruses naturally circulate in wild bird populations, particularly in waterfowl such as ducks and geese (Hénaux and Samuel 2011). Other species, including House Sparrows (*Passer domesticus*) and European Starlings (*Sturnus vulgaris*), might potentially act as bridge hosts in agricultural landscapes (Boon et al. 2007; Brown et al. 2009). Biosecurity measures preventing wildlife from entering poultry barns probably remain the best way to prevent transmission of AIVs. More research is required to understand AIV vectors in agricultural ecosystems better, and to identify potential bridge hosts to ascertain which species, including nonavian taxa, may play a role in AIV transmission at the wildlife–domestic poultry interface.

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