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Strength in numbers: Avian influenza A virus transmission to poultry from a flocking passerine

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Abstract

The effects of flock size of European starlings (*Sturnus vulgaris*) was experimentally manipulated to assess the potential of influenza A virus (IAV; H4N6) transmission from a flocking passerine to bobwhite quail (*Colinus virginianus*) through shared food and water resources to mimic starling intrusions into free-range and backyard poultry operations. Of the three starling flock sizes tested ($n = 30$, $n = 20$ and $n = 10$), all successfully transmitted the virus to all or most of the quail in each animal room (6/6, 6/6 and 5/6) by the end of the experimental period, as determined by seroconversion and/or viral RNA shedding. Although starlings have been shown to be inconsistent shedders of IAVs and when they do replicate and subsequently shed virus they typically do so at low to moderate levels, this study has provided evidence that relatively small flocks (i.e., 10 or possibly a smaller number) of this species can collectively transmit the virus to a highly susceptible gallinaceous bird species. Future work should assess if starlings can transmit IAVs to additional poultry species commonly found in backyard or free-range settings.

KEYWORDS
avian influenza A virus, Bobwhite quail, *Colinus virginianus*, common resources, European starling, flocking bird, poultry, quail, *Sturnus vulgaris*, transmission, water

1 | INTRODUCTION

Although wild waterfowl and shorebirds are thought to be the primary natural hosts of avian influenza A viruses (IAVs) (Halvorson, 2009), some attention has started to focus on the potential role that other avian species, such as passerines, could play in IAV epidemiology (Slusher et al., 2014). European starlings (*Sturnus vulgaris*) are common peridomestic birds in the United States that often occur in large flocks during certain times of the year. Further, this species is commonly found near various agricultural production facilities (Depenbusch et al., 2011), including facilities associated with poultry production (Burns et al., 2012). While there is some published evidence suggesting this species can shed certain IAVs (Hall et al., 2016; Nemeth et al., 2010; Qin et al., 2011), assessing their ability to transmit IAVs to poultry remains largely untested, especially in terms of the probability of transmission associated with/as a function of different flock sizes of virus-shedding passerines.

Eurasian H5 viral RNA was detected in lung tissue of a European starling collected at a poultry farm that had been affected by a highly pathogenic (HP) IAV in Iowa (Shriner, Root, et al., 2016). However, experimental inoculations of European starlings with three clade 2.3.4.4 HP H5 IAVs did not result in detectable viral shedding nor mortality in these birds (Bosco-Lauth et al., 2019). Nonetheless, experimental inoculations of starlings (presumably *S. vulgaris*) with an H7N7 IAV (A/Chicken/Vic/1/85) isolated from poultry in Australia resulted in high mortality rates in deliberately infected and contact birds (Nestorowicz et al., 1987). Of interest, similar results were obtained when starlings were inoculated with a

related H7N7 (A/Starling/Vic/5156/85) IAV isolated from a starling (Nestorowicz et al., 1987). At relatively high inoculation doses of HP H5N1 viruses, European starlings shed moderate levels of virus in oropharyngeal swabs and exhibited 100-percent survival during the challenge period (Boon et al., 2007). Similarly, European starlings challenged with A/chicken/Hong Kong/220/97 (H5N1) survived a 14-day experimental period with no morbidity or mortality observed (Perkins & Swayne, 2003).

Experimental challenge of European starlings with a low pathogenic H3N8 IAV resulted in oral shedding of viral RNA in 35 of 36 of birds tested (Nemeth et al., 2010). Inoculation with an H7N9 IAV (A/Anhui/1/2013) resulted in productive infections in some European starlings inoculated at a relatively high dose, with one bird shedding up to 10^6 based on RNA equivalents (Hall et al., 2016). Evidence of IAV genetic material has been detected in digestive and tracheal samples from European starlings collected in Ohio (Qin et al., 2011) and from a cloacal swab (only one individual was sampled) collected from this species in Slovenia (Račnik et al., 2008). Some European starlings challenged with LP H2N3 and H4N2 shed the viruses from the oral and cloacal routes (Qin et al., 2011). Many other challenge studies of European starlings have been conducted. As described in a recent review paper, IAV challenge studies of European starlings have produced highly variable results (Shriner & Root, 2020).

Because of their tendency to form large flocks and attraction to various agricultural production facilities, the European starling is an obvious candidate to evaluate for its transmission potential for IAVs. Further, based upon some of these behavioural traits, as well as from observations of this species entering poultry barns, European starlings have been suggested as a priority species for IAV testing (Burns et al., 2012). Nonetheless, when starlings do shed various IAVs, they typically do so at low levels that may be insufficient for a single individual to initiate intra- or interspecific transmission. However, flocks of IAV infected starlings might produce a different outcome. Therefore, the objective of this study was to determine if European starlings have the capacity to transmit IAVs to poultry by manipulating starling flock sizes of deliberately inoculated birds.

2 | METHODS

European starlings were live captured in colony traps in Weld County, Colorado. Bobwhite quail (*Colinus virginianus*; hereinafter referred to as 'quail') were purchased from a commercial vendor. Generically speaking, quail have been previously shown to be a well-suited recipient species in IAV transmission studies (Bosco-Lauth et al., 2016; Root et al., 2017). Additionally, the transmission scenario we mimicked could be informative to both poultry and wild game bird settings. All birds were banded for individual identification and bled for pre-experiment antibody assessments prior to the initiation of the experiment. The pre-experiment antibody assessments were used to compare with those obtained post-experiment and as a screening/exclusion tool to assess if any of the birds used in this study may been exposed to IAV previously. Following a $\geq$ 2-week quarantine/acclimation period, star-
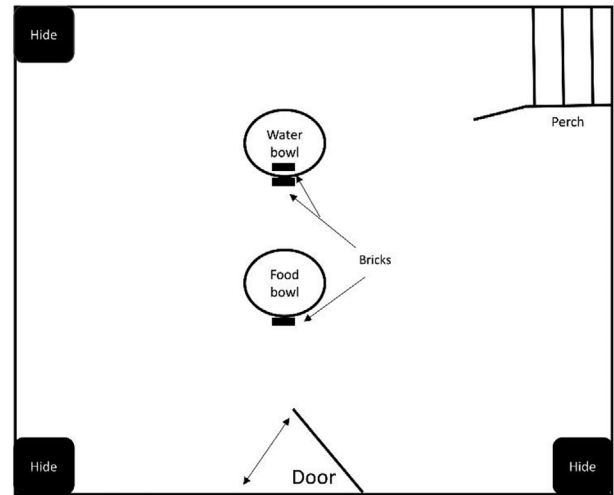

FIGURE 1 Layout of BSL-2 animal rooms that included three hides, a large perch, and shared food and water bowls. Bricks were placed at the perimeter of both bowls so birds could more easily eat or drink. A brick was also placed within the water bowl so birds could more readily remove themselves from these bowls if needed. The room size was approximately 13.76 square meters

lings were assigned to one of three flock size treatments ($n = 10$, $n = 20$ and $n = 30$) and moved to one of three independent BSL-2 animal rooms. Because we have successfully inoculated European starlings and Bobwhite quail with H4N6 previously, no control animals were used (Ellis et al., 2021; Pepin et al., 2012). Animal methods were approved by the USDA NWRC institutional animal care and use committee.

Each animal room was outfitted with perches, a food bowl, a water bowl and three hides located in three of the four corners of each room (Figure 1). The layout of the rooms was designed to encourage the shared use of food and water and provided quail hides to escape to when workers entered the animal room (Figure 1). The food bowl in each room contained multiple feed types (Purina® Layena® Pellets, Purina® Layena® Crumbles and Purina® Game Bird Flight Conditioner, Purina Animal Nutrition, LLC, Arden Hills, MN). The water bowl in each room contained approximately 5.5 L of water. On 7 days post-inoculation (DPI)/7 days post-contact (DPC), a poultry waterer was added to each room. Life water (e.g. water treated to remove chlorine and other chemicals) was used for all watering devices from 1 DPI through the morning of 10 DPI/DPC when water devices were refilled with tap water and this source of water was used for the remainder of the experiment.

Upon transfer to the BSL-2 animal rooms, all European starlings in each of the three flock size treatments were inoculated with an H4N6 (A/Mallard/CO/P70F1-03/08(H4N6)) IAV that was originally isolated from a wild bird (Root et al., 2014). Each bird was inoculated with approximately 10^6 EID_{50} of virus by the nasal, choanal and ocular routes in a 100 μl vehicle (approximately one-third of the volume by each route) on two occasions separated by multiple hours (i.e., each starling received a total of approximately 2×10^6 EID_{50}). The evening of the same day, six IAV naïve quail were introduced to each animal

room. Oral swab samples were collected from European starlings on 2 and 4 DPI and from quail on 4 and 6 DPC. Swabs were placed in cryovials containing BA-1 viral transport media (Shriner et al., 2012) and were frozen at −80°C until further analyses were conducted. In addition to the pre-experiment blood samples, blood samples were collected from starlings on 14 DPI and from quail on 21 DPC; all birds were euthanized the day their final blood samples were obtained. Water samples were collected in each animal room daily from 1 to 10 DPI.

Swab and water samples were analysed by real-time RT-PCR using standard protocols (SOP-AV-0068: Real-time RT-PCR detection of influenza A virus and avian paramyxovirus type-1) at the Colorado State University Veterinary Diagnostic Laboratory. Two wells were run for each sample when possible. For this study, positive samples were defined as those that amplified RNA in both wells tested (when sufficient samples were available) and produced an average Ct of <38. All serum samples collected during the study were assayed for IAV antibodies using the IDEXX AI MultiS-Screen Ab test. Due to a plate washer error on the first assay of post-experiment serum samples, some starling serological responses are based upon a single well as compared to two wells for all other assays. For the purposes of this study, any bird that had a sample-to-negative (S/N) ratio < 0.7 and showed a significant decrease in pre- versus post-experiment S/N ratios were considered positive. While the manufacturer of this assay suggests an S/N ratio of <0.5 as a cutoff value as positive for validated poultry species, an alternative threshold of <0.7 has been proposed for mallards and other wild bird species because it provides a better balance between sensitivity and specificity (Brown et al., 2009; Shriner, VanDalen, et al., 2016).

3 | RESULTS

All animals (a total of n = 60/60 inoculated European starlings and n = 17/18 quail) survived the experimental period with a single exception. One quail was found dead on 14 DPI. Upon examination, this animal had no obvious trauma attributable to its death. All other animals remained healthy for the duration of the experiment.

Water samples were collected daily in each animal room from 1 to 10 DPI. With few exceptions, all water samples analysed in this study were positive for viral RNA (Table 1). However, one sample was not collected, two samples were insufficient to test in duplicate and a final sample was positive in one plate well, but its duplicate sample produced a negative result. Due to the near constant detection of viral RNA in shared water bowls, we propose that virus contaminated water likely played a significant role in the initiation of transmission to naïve birds in each animal room. Of interest, the lowest Ct values from water samples were collected on 2 DPC from the 20- and 30-flock treatments, while the lowest Ct value from the 10-flock treatment was collected during 10 DPC (Table 1).

Based on serology, widespread transmission occurred to quail in each of the three treatment groups (Figure 2). Although a single quail did not test as serologically positive based on our threshold from serum

TABLE 1 Analysis of water samples collected from animal rooms that housed IAV infected birds. Water samples were routinely collected from 1 to 10 DPI

DPI/DPC[b]	Treatment[a]		
	10	20	30
1	+[c] (34.0)	+ (29.0)	+ (28.6)
2	+ (30.2)[d]	+ (27.7)	+ (27.7)
3	+ (30.7)	+ (28.5)	+ (28.9)
4	+ (30.7)	ND[e]	+ (30.0)[d]
5	+ (31.5)	+ (31.6)	+ (31.9)
6	+ (31.4)	+ (31.7)	+ (31.9)
7	+ (31.5)	+ (30.3)	+ (34.1)
8	+ (33.6)	+ (31.9)	+ (35.2)
9	+ (33.2)	+ (31.5)	+ (35.8)
10	+ (29.9)	+ (33.2)	S[f] (35.6)

[a]The numbers (10, 20 and 30) under the "Treatment" heading refer to size of the European starling flock that was being tested.
[b]DPI = days post-inoculation (European starlings); DPC = days post-contact (quail).
[c]A "+" = sample was assessed to be positive. The number in parentheses is the Ct value associated with the sample.
[d]These two samples are based upon a single well in PCR analysis because the water sample collected was insufficient for two wells.
[e]ND = not done. A water sample was erroneously not collected during that day from that room.
[f]Suspect positive sample. One PCR well was positive and the other was negative.

collected on 21 DPC, its S/N ratio decreased significantly during the experimental period, thereby suggesting that this individual did have a serological response from its exposure. All but two European starlings developed a serological response by 14 DPI (Figure 2). Both birds developed S/N ratios of 0.71, which was just over the defined threshold. One of these starlings was in the 10-flock treatment group and only exhibited a slight decrease in the S/N ratio, while the S/N ratio of the other bird, which was associated with the 30-flock treatment, decreased significantly, thereby suggesting that this bird produced a serological response but did not meet our definition of antibody positive. Of the 74 birds that were considered serologically positive (58 starlings and 16 quail), 68 had S/N ratios < 0.5 and 6 birds had S/N ratios < 0.7. The latter category was only associated with quail (Figure 2).

All European starlings showed evidence of infection on at least one occasion based upon the detection of viral RNA from oral swabs (Figure 3). The lowest Ct value obtained from a starling oral swab sample was 23.9. The sample was collected from a starling in the 30-flock treatment group on 4 DPI. Quail exhibited a similar trend. However, two quail were negative for viral RNA on either occasion when oral swabs were collected from them (4 and 6 DPC). Both birds were from the small (10) flock treatment. One of these quail died on 14 DPI; therefore, serological analyses were not performed on this individual. The second quail that did not produce evidence of viral RNA from oral swab samples did show evidence of seroconversion (Figure 3). The lowest Ct
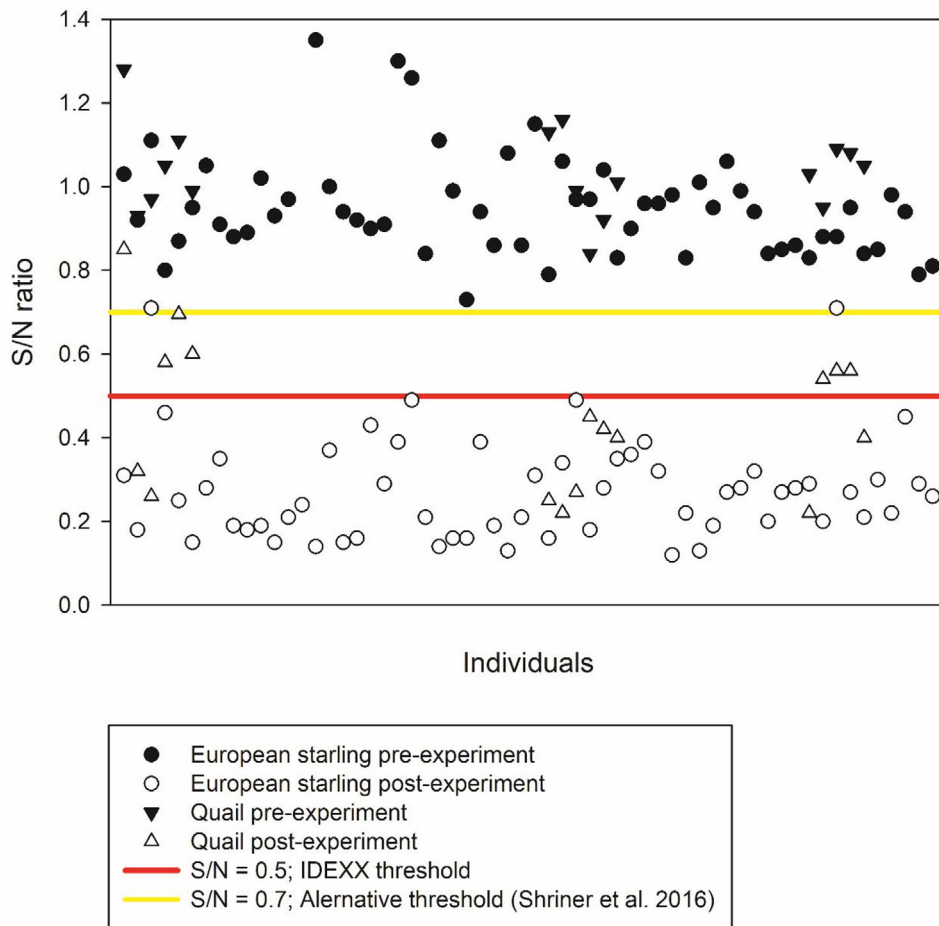
FIGURE 2 Sample-to-negative (S/N) ratios of serum samples collected from bobwhite quail (*Colinus virginianus*; triangles) and European starlings (*Sturnus vulgaris*; circles) pre-experiment and post-experiment (14 DPI for starlings and 21 DPC for quail). Data are not shown for a quail that did not survive until 21 DPC and one pre-experiment outlier (atypically high S/N ratio) from a European starling
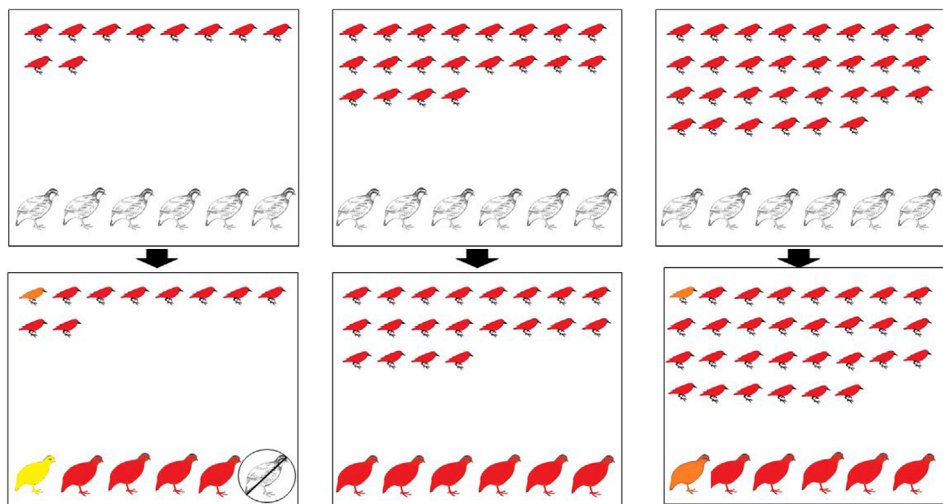

FIGURE 3 Experimental setup (top) and transmission results (bottom) of an influenza A virus transmission study associated with European starlings (*Sturnus vulgaris*) and bobwhite quail (*Colinus virginianus*) that utilized three starling flock sizes (10, 20 and 30). Birds shown in red in the experimental setup (top) were deliberately infected. The bottom one-half of this figure shows the experimental transmission results. Birds shown in red seroconverted and shed viral RNA, those shown in orange shed viral RNA but did not seroconvert, and those shown in yellow seroconverted but did not shed viral RNA. The single bird with a line through it died prior to 21 DPI. Because of this, the serological status of this bird is unknown. This bird did not meet our definition as positive for viral RNA in oral swabs on either occasion it was sampled

value obtained from a quail oral swab sample was 21.1. The sample was collected from a quail associated with the animal room containing the 20-flock treatment group and was collected on 4 DPI. This quail oral swab sample had a lower Ct value than any starling and/or water sample tested during the study.

4 | DISCUSSION

In this study, we assessed the general premise that the risk of IAV transmission to poultry may be associated with flock size was evaluated for European starlings. A recent publication indicated that indirect transmission of IAV to starlings from water contaminated by IAV-infected mallards is possible if not highly probable (Ellis et al., 2021). If large numbers of starlings become infected by drinking from and bathing in a small waterbody previously contaminated with virus by waterfowl, these same starlings could, in turn, travel to one or more livestock facilities where they could then environmentally transmit an IAV by depositing virus within feeders or waterers in facilities that do not exclude them. This transmission scenario could be facilitated by the fact that starlings are often attracted to poultry facilities for food resources. The current study aimed to test whether flocks of starlings could transmit IAV to a poultry species through shared resources or less likely through direct contact.

In general, the bulk of the quail in each animal room seroconverted by 21 DPC. The ranges of the S/N ratios of the quail associated with the three-flock treatments at 21 DPC were 0.22–0.56 (10-flock), 0.22–0.45 (20-flock) and 0.26–0.85 (30-flock). The medians of S/N ratios at 21 DPC were 0.54 (10-flock; $n = 5$), 0.34 (20-flock; $n = 6$) and 0.59 (30-flock; $n = 6$). It is uncertain why the 20-flock treatment was the only treatment in which all quail yielded S/N ratios of < 0.5. Further, the reason why one quail in the 30-flock treatment did not produce an antibody response is unclear but could be based on individual condition, immunological histories, immune function and/or individual behaviour. Based upon the number of infected European starlings in this flock treatment, the quail in this animal room were undoubtedly exposed to more virus than were birds in the other animal rooms. Nonetheless, in a previous study associated with the interspecific transmission of an IAV, some quail (*Coturnix* sp.) showed evidence of shedding viral RNA but did not seroconvert by the end of the study period (Root et al., 2017). Furthermore, one quail in the study mentioned above showed no evidence of exposure even though both quail that it was co-caged with shed viral RNA or shed viral RNA and seroconverted (Root et al., 2017).

In parallel with quail, most starlings in the three animal rooms showed evidence of seroconversion. The ranges of the S/N ratios of the starlings associated with the three flock treatments were 0.20–0.71 (10-flock), 0.12–0.49 (20-flock) and 0.13–0.71 (30-flock). The medians of S/N ratios at 14 DPI were 0.28 (10-flock; $n = 10$), 0.28 (20-flock; $n = 10$) and 0.21 (30-flock; $n = 10$). It is unclear why two birds yielded high S/N ratios at the end of the experimental period. The reason(s) may be similar to those proposed above for quail. However, in terms of the 30-flock treatment, the increased flock size of this treatment likely increased the probability of outliers. Nonetheless, this would not be the case for the 10-flock treatment.

Overall, oral swabs from quail produced the lowest Ct values (e.g. 21.1 and 22.1) that were detected across all sample types and animal rooms. As the recipient species, this observation provides additional support to previous studies that have suggested that quail are highly susceptible to various IAVs.

IAV has been occasionally isolated and/or evidence viral nucleic acids have been detected from wild-caught European starlings (Lipkind et al., 1982; Shriner, Root, et al., 2016). A summary of IAV detections in passerines has been reviewed elsewhere (Slusher et al., 2014). Based upon surveys of New Zealand poultry farms, poultry waterers have been suggested as an indirect means of IAV transmission between wild birds and poultry in some situations (Zheng et al., 2010). Similarly, in surveys conducted in France, large numbers of wild birds, including starlings, within close proximity to duck breeding facilities was suggested as a potential risk factor for IAV introduction (Duvauchelle et al., 2013). We believe that the shared water sources in our animal rooms were likely the key vehicle from which quail were exposed to IAV in the current study. Notably, a recent study provided evidence that starlings become infected with IAVs following their exposure to relatively small water bodies used by IAV-infected mallards (*Anas platyrhynchos*) (Ellis et al., 2021).

Interspecific transmission in the experimental system described herein likely occurred via oral secretions from starlings. Previous studies that evaluated oral and cloacal swabs in European starlings following LP IAV challenge indicated that a very small percentage of individuals shed via the cloacal route, and when cloacal shedding did occur, it was at very low quantities (Ellis et al., 2021; Nemeth et al., 2010). However, a different study reported higher levels of shedding by the cloacal as compared to the tracheal routes (Qin et al., 2011).

Data from the current study suggests that transmission was not density dependent at the three densities that we mimicked, but density undoubtedly plays a role in successful transmission at a level lower than was measured for the tested strain of IAV. Nonetheless, at the lowest starling density that was tested ($n = 10$), two of the six recipient quail did not produce evidence of viral RNA shedding from oral swab samples (Figure 3). In comparison, all quail associated with higher densities of starlings (20 and 30) shed viral RNA on both occasions that they were sampled. Taken together, this trend suggests the minimum number of infected starlings needed produced an infectious dose to successfully transmit IAV to quail through environmental contamination reflects a number smaller than was tested herein. Of interest, quail were often observed sitting in the food bowls, frequently all six birds. This suggests that even if a small number of quail were infected from environmental contamination by starlings, the gregarious behaviour of the quail could have facilitated intraspecific transmission. Further, quail may have become infected through feed previously contaminated by starlings.

No direct interactions of starlings and quail were observed during this study, as both species tended to flock with conspecifics. When workers entered animal rooms for sampling or animal care, quail typically moved to one or more of the hides in the room (Figure 1).

In contrast, starlings typically took to flight during these instances. However, based upon remote observations as well as faecal deposition in select areas of the animal room, starlings undoubtedly spent a large portion of their time resting upon the perch in the room (Figure 1). In contrast, quail were never observed using the perch present in each of the three animal rooms. Although no direct interspecific interactions were observed during this study, time spent observing the birds was limited. Thus, while not directly observed, due to their similar sizes, it is quite possible starlings and quail acquired sustenance while in close proximity to each other.

The results of this study indicate that free-range and backyard poultry producers should endeavour to take measures to reduce farm characteristics that attract starlings to their facilities. For example, strategic placements of watering devices in areas that eliminate their use by wild birds could help reduce potential transmission to poultry when birds breach the facility. Recommendations to reduce wildlife attractants, prevent wildlife access and provide wildlife deterrents at farms have been published elsewhere (Shriner, Root, et al., 2016). Future studies should assess if European starlings can transmit IAVs to other backyard poultry species such as chickens. Further, considering all the flock sizes used in the current study produced transmission to quail, additional studies utilizing smaller flock sizes would be useful to assess the minimum number of starlings needed to initiate transmission in poultry flocks.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Study data are included in the article and/or are available upon request.

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