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Viral shedding of clade 2.3.4.4 H5 highly pathogenic avian influenza A viruses by American robins

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Abstract

American robins (*Turdus migratorius*) are commonly associated with farmsteads in the United States and have shown previous evidence of exposure to an H5 avian influenza A virus (IAV) near a poultry production facility affected by a highly pathogenic (HP) H5 virus in Iowa, USA during 2015. We experimentally infected American robins with three clade 2.3.4.4 HP H5 viruses (H5N2 and H5N8). A total of 22/24 American robins shed virus, and all three strains were represented. The highest virus titres shed were $10^{4.3}$, $10^{4.3}$ and $10^{4.8}$ PFU/ml, associated respectively with viruses isolated from poultry, a captive gyrfalcon (*Falco rusticolus*), and a Northern pintail (*Anas acuta*). Of those birds that shed, viral shedding was initiated 1 or 2 days post-infection (DPI) and shedding ceased in all birds by 7 DPI. This study adds an additional synanthropic wildlife species to a growing list of animals that can successfully replicate and shed IAVs.

KEYWORDS
American robin, Avian influenza A virus, Biosecurity, Clade 2.3.4.4, Experimental infection, H5N2, H5N8, Highly pathogenic, Outbreak, Passerine, *Turdus migratorius*

1 | INTRODUCTION

During 2015, the US poultry industry was negatively impacted by clade 2.3.4.4 highly pathogenic (HP) avian influenza A viruses (IAV), especially in the Midwestern states. Through both mortality from HP avian IAV infection and culling of infected and potentially infected birds, these viruses were responsible for the deaths of millions of poultry in this region (Shriner, Root et al., 2016).

Although aquatic birds are considered as primary avian IAV reservoir hosts (Halvorson, 2008), increasing attention has been associated with the potential of passerines in IAV ecology during recent years. For example, some workers recently suggested that passerines are influenza reservoirs and important species in the epidemiology of influenza (Fuller et al., 2010). However, others found no evidence suggesting that passerines are natural reservoirs for IAVs (Slusher et al., 2014). Regardless of their potential roles as reservoirs, American robins could act as potential IAV bridge hosts if they are competent for replication of the virus in question or can mechanically transmit the virus and come into direct or indirect contact with maintenance hosts (e.g., waterfowl) and poultry (Caron, Cappelle, Cumming, de Garine-Wichatitsky, & Gaidet, 2015).

Some recent and more dated reports of relatively small surveys for IAV exposures in American robins have been reported in the literature. Following wildlife epidemiological investigations of some HP avian IAV-affected farms in Iowa, two American robins (*Turdus migratorius*) were assessed to be antibody positive to an H5 IAV at one of the affected premises (Shriner, Root et al., 2016). In addition, a single American robin from a wildlife refuge in northwestern Minnesota had antibody to an unidentified IAV during a survey conducted at an earlier time period (Slusher et al., 2014). Furthermore, molecular evidence (PCR) of IAV infection was reported in 3.8% of 133 American robins sampled during 2005-2008 in the United States. (Fuller et al., 2010). However, during an earlier survey in the 1970s, zero of six American robins sampled provided evidence of IAV infection in a region of Canada (Boudreault, Lecomte, & Hinshaw, 1980).

Although the investigations mentioned above suggest that American robins can exhibit a serological response to or a molecular

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signature of (likely) multiple IAV subtypes, they do not provide any information associated with the level of virus shedding that may ensue following infections from HP IAVs. Because of this, as well as the recent documentation of antibody-positive American robins on a HP IAV-affected poultry farm in the United States (Shriner, Root et al., 2016) and our common observations of American robins at poultry facilities, the objective of this study was to assess the replication competence of American robins experimentally infected with clade 2.3.4.4 HP IAVs and to relate this information to biosecurity at poultry farms.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty-four American robins wild-caught in Larimer County, CO were used in the experimental infection studies. The birds were group-housed in four large cages for a minimum of 2 weeks prior to being transferred to a BSL-3 facility during which time a blood sample was collected from each individual. Within the BSL-3 facility, birds were housed in bird cages (four per cage) within HEPA-filtered cage racks, one bird cage per isolator cage. Two cages placed in two individual isolator units were used to house birds for each of the three viruses (see below). The cages were equipped with perches and multiple food and water bowls. The birds were maintained with meal worms, moistened dry kitten food and fresh fruit (strawberries, raspberries and blueberries). The animal methods used in this study were approved by the Institutional Animal Care and Use Committees of the National Wildlife Research Center and Colorado State University.

2.2 | Viruses and experimental infection

The viruses used in this study were A/turkey/Minnesota/9845-4/2015 (H5N2), A/gyrfalcon/Washington/41088-6/2014 (H5N8), and A/Northern pintail/Washington/40964/2014 (H5N2), which will be referred to as the turkey, gyrfalcon, and NOPI viruses hereinafter. American robins were inoculated orally (75% volume) and nasally (25% volume) with approximately $10^{5.3}$ of the turkey virus (n = 8), $10^{5.7}$ of the NOPI virus (n = 8), and $10^{6.0}$ of the gyrfalcon virus (n = 8). Following inoculation, the 24 birds were sampled daily from 1–10 days post-infection (DPI). Daily sampling included an oral and cloacal swab and general health observations of each bird. Swabs were placed in 1 ml of BA-1 viral transport media and stored at −80°C prior to analyses. All robins were bled and euthanized on 14 DPI. Blood samples were centrifuged to collect serum.

2.3 | Laboratory assays

Oral and cloacal swab samples were tested by plaque assay as employed during a previous study (Achenbach & Bowen, 2011). Each swab sample was dispersed into 1 ml of viral transport medium and virus titres are therefore described as PFU/ml, with a limit of detection for both sample types of 10 PFU/ml. Serology was conducted with the FlockCheck® Avian Influenza MultiS-Screen Antibody Test Kit (IDEXX Laboratories, Inc, Westbrook, ME) and results were based on the manufacturers' cut-off value (sample-to-negative [S/N] ratio of <0.5) as well as an alternative cut-off value (S/N ratio of <0.7) prior to the initiation of the study (Shriner, VanDalen, Root, & Sullivan, 2016).

3 | RESULTS

Testing of pre-inoculation sera indicated that none of the robins were classified as seropositive based on the cut-off suggested by the manufacturer of the ELISA kit (<0.5), but that six robins were suspect positive based on an alternative threshold optimized for waterfowl (0.7; Table 1). Nonetheless, the six birds mentioned above shed virus following experimental inoculation (Table 1). A total of two individual robins did not shed detectable levels of one of the three viruses during the experimental sampling period (Robins 1 and 3; Table 1).

All 24 robins survived to the end of the experiment and none exhibited any clinical signs of disease, regardless of the inoculated virus. American robins shed each of the three viruses tested, but not all individuals shed. For example, six of eight birds shed the gyrfalcon virus, while eight of eight birds shed the turkey and NOPI viruses (Table 1). Most birds initiated shedding on 1 DPI, while others initiated shedding on 2 DPI (Table 1). The highest viral titres shed by the oral route were $10^{4.3}$, $10^{4.8}$ and $10^{4.3}$ PFU/ml for the turkey, NOPI and gyrfalcon viruses, respectively (Table 1). A single American robin inoculated with the gyrfalcon virus shed virus by the cloacal route (Robin 2). Cloacal shedding in this individual had a maximum titre of $10^{3.5}$ PFU/ml and lasted from 2–4 DPI. Of interest, this bird exhibited cloacal shedding 1 DPI prior to when it initiated oral shedding.

Viral shedding lasted a maximum of 6 days for the three viruses (Table 1). In four individuals, oral shedding ceased on a given day but resumed subsequently. For example, one American robin inoculated with the turkey virus shed orally on 1 DPI and 3-6 DPI (Robin 12; Table 1). In general, each bird produced its highest oral titre during the first day it began shedding. However, exceptions to this trend were noted for birds infected with each of the viruses tested. For example, one robin inoculated with the gyrfalcon virus shed its highest oral titres during 3-4 DPI (Robin 11; Table 1). A different bird, infected with the turkey virus, produced its highest oral titre on 5 DPI, which was the last day it shed virus (Robin 13; Table 1). However, the titres shed during several other DPI were very close to the maximum level observed on 5 DPI. A similar trend was noted for a robin infected with the NOPI virus, as this individual shed virus at the highest levels during 3–4 DPI, the last two days it shed virus following its inoculation (Robin 23; Table 1). Because all birds stopped shedding virus by 7 DPI and only one bird shed by the cloacal route, plaque assays were not conducted on oral swab samples collected after 8 DPI or on cloacal swab samples collected after 5 DPI.

Serologic responses were noted in seven of eight birds inoculated with the turkey virus, seven of eight birds inoculated with the

TABLE 1 Oral shedding and serological responses of American robins (*Turdus migratorius*) experimentally infected with clade 2.3.4.4 highly pathogenic H5N2 and H5N8 avian influenza A viruses

			Days postinfection								
Cage	Robin number[a]	Virus	1	2	3	4	5	6	7	8	Serology[f]
1	**8**	Turkey[b]	3.6	1.9	<1	<1	<1	<1	<1	<1	+
1	9	Turkey	3.4	<1	<1	<1	<1	<1	<1	<1	–
1	**13**	Turkey	2.9	2.8	2.9	2.3	3.0	<1	<1	<1	+
1	15	Turkey	4.3	1.5	<1	<1	<1	<1	<1	<1	+
2	10	Turkey	3.7	2.8	<1	<1	<1	<1	<1	<1	+
2	**12**	Turkey	2.2	<1	2.5	3.4	2.1	1.5	<1	<1	+
2	14	Turkey	2.3	2.8	1.6	2.1	<1	<1	<1	<1	+
2	18	Turkey	3.5	1.7	2.1	1.6	<1	<1	<1	<1	+
3	21	NOPI[c]	3.4	<1	<1	<1	<1	2.7	<1	<1	+
3	22	NOPI	<1	2.6	2.0	3.4	2.5	<1	<1	<1	+
3	23	NOPI	2.8	2.6	3.5	3.6	<1	<1	<1	<1	+
3	24	NOPI	2.0	<1	<1	<1	<1	<1	<1	<1	+
4	**16**	NOPI	4.8	1.5	<1	<1	<1	<1	<1	<1	+
4	17	NOPI	<1	2.6	3.1	2.7	3.7	<1	<1	<1	+
4	19	NOPI	3.6	1.8	<1	<1	<1	<1	<1	<1	+
4	**20**	NOPI	3.6	2.6	<1	2.3	<1	2.4	<1	<1	sp
5	2	Gyr[de]	<1	<1	3.6	2.8	2.9	1.7	<1	<1	+
5	**5**	Gyr	3.5	2.5	2.6	3.0	<1	<1	<1	<1	+
5	6	Gyr	3.3	2.7	2.4	3.4	3.1	1.9	<1	<1	+
5	7	Gyr	3.4	<1	<1	1.5	<1	<1	<1	<1	–
6	1	Gyr	<1	<1	<1	<1	<1	<1	<1	<1	–
6	3	Gyr	<1	<1	<1	<1	<1	<1	<1	<1	–
6	4	Gyr	2.8	2.4	<1	<1	<1	<1	<1	<1	sp
6	11	Gyr	<1	2.8	4.3	4.1	2.3	<1	<1	<1	+

Note. [a]Numbers shown in bold represent birds that had pre-experiment serum samples with S/N ratio averages of <0.70 (see methods and results). [b]A/turkey/Minnesota/9845-4/2015 (H5N2). [c]A/Northern pintail/Washington/40964/2014 (H5N2). [d]A/gyrfalcon/Washington/41088-6/2014 (H5N8). [e]This American robin also shed by the cloacal route during 2-4 DPI. [f]ELISAs are based on sera collected during 14 DPI. + (positive) = S/N ratio <0.5, sp (suspect positive) = S/N ratio 0.5–0.7, – (negative) = S/N ratio >0.7.

NOPI virus and five of eight birds inoculated with the gyrfalcon virus. One bird inoculated with the NOPI virus, which had a pre-experiment S/N ratio of <0.6, produced a post-experiment S/N ratio of <0.6.

4 | DISCUSSION

A total of six American robins experimentally inoculated in the current study had pre-experiment S/N ratios of <0.7 (Table 1). Considering this assay has not been comprehensively evaluated on robin sera, the interpretation of these data should proceed with caution. Nonetheless, some of the birds from the current study had S/N ratios consistent with that of a confirmed antibody positive robin from an outbreak poultry farm (Shriner, Root et al., 2016). Regardless of these potentially suspect positive serological results, all robins exhibiting pre-experiment S/N ratios <0.7 successfully replicated and shed their respective viruses and five of six of the birds had post-experiment S/N ratios of <0.5 (Table 1). The two American robins that did not shed virus, both of which were inoculated with the gyrfalcon virus, had pre-experiment S/N ratio values of 0.83 and 0.84.

American robins are the most populous and have the largest distribution of any thrush in North America (Vanderhoff, Pyle, Patten, Sallabanks, & James, 2016). As such, they can be a common part of the fauna associated with farmsteads. This species is known to have very malleable nest site requirements and will build nests associated with a variety of objects, including building ledges (Howell, 1942). Anthropogenic changes to landscapes, such as those found in suburban areas, can provide productive feeding grounds and suitable nesting sites, which are favourable to this species (Howell, 1942). Thus, considering its distribution, abundance and its ability to thrive in anthropogenically modified habitats, it is conceivable that American robins could come into contact with IAVs associated with domestic animals, including poultry, which could lead to viral shedding and potential transmission of these viruses in certain situations.

The current study adds American robins to the list of passerines that can replicate and shed various HP IAVs. However, considering that the three HP IAV strains used in the current study are closely related, the likelihood of American robins shedding other IAVs cannot be predicted at this time. Thus, shedding of IAVs by robins may not be ubiquitous to all strains and subtypes. Most American robins that shed virus during the current study did so by the oral route. However, a single robin infected with the gyrfalcon virus shed by the cloacal route for multiple days. Of interest, molecular evidence of IAVs has been previously reported from approximately five of 133 cloacal swabs collected from this species (Fuller et al., 2010). Thus, perhaps the paucity of cloacal shedding observed in the current study is largely due to the use of closely related strains for inoculations, as the field data presented by others (Fuller et al., 2010) suggest that cloacal shedding may be more common in this species than observed herein. In comparison, mallards (*Anas platyrhynchos*) inoculated with two of the viruses (NOPI and gyrfalcon) used in the current study replicated viruses in multiple tissues and shed virus by the oral and cloacal routes (Pantin-Jackwood et al., 2016).

Some peridomestic bird species, such as European starlings (*Sturnus vulgaris*), can form very large groups during certain times of the year. Thus, if this species were to shed an IAV, even in small amounts, the sheer number of birds that might use a farm-oriented resource (e.g., spilled feed or a small water source) could collectively deposit an infectious dose at the resource in question. This is unlikely to be the case for American robins, as it is highly improbable for this species to approach the flock sizes that can be produced by European starlings. Considering this aspect of their behavioural ecology, American robins may not pose the same level of threat when infected with IAVs as birds that form large flocks.

Compared to certain other common farm-side bird species, such as house sparrows and European starlings, the foraging habits of robins are less likely to put them into close contact with poultry in most instances. For example, unlike granivorous birds, American robins are primarily consumers of invertebrates and fruit (Vanderhoff et al., 2016) and are not attracted to spilled feed or to poultry feed within the interior of a barn for foraging purposes. Thus, small water sources near poultry facilities are likely the most parsimonious transmission vehicle to this species if IAV infected waterfowl are present nearby (Figure 1). In addition, horizontal ledges associated with poultry buildings, which are potential avian nesting sites (Shriner, Root et al., 2016), likely represent one of the few reasons American robins would utilize a poultry building. Alternatively, as an omnivorous species with invertebrates representing a large part of its diet, American robins could be attracted to poultry farms with high insect burdens. Although the ubiquity of the following observation has yet to be brought to bear, HP avian IAVs have been detected in select insect species near an infected poultry farm (Sawabe et al., 2006). Thus, simply removing attractants, such as reducing water puddles and providing fewer suitable nest sites associated with poultry barns (Shriner, Root et al., 2016), could help to reduce potential IAV trafficking risk posed by American robins. Due to the limited number of reports of IAV detections in terrestrial invertebrates at this time, it is unclear if reducing insect burdens near poultry farms would produce a substantial robin-associated biosecurity benefit. Furthermore, if it is possible for American robins to acquire an IAV infection following the ingestion of a contaminated invertebrate, this scenario would appear more likely to occur at a poultry farm already affected by an IAV. Of interest, ingestion of IAV-exposed freshwater snails (*Physa* sp.) failed to transmit the virus to mallards in an experimental setting (Oesterle et al., 2013).

As a common backyard bird species, American robins are highly regarded by many individuals. They also provide the ecological service of seed dispersal of numerous woody plant species (Vanderhoff et al., 2016) through regurgitation and defecation of seeds away from parent plants (Meyer & Witmer, 1998). We have commonly observed American robins on poultry farms and they have also been commonly observed in and near crop fields (Beecher, Johnson, Brandle, Case, & Young, 2002). In a study, which excluded several bird species from crop fields (including American robins), various insect species were found at higher densities in test plots where birds were excluded (Tremblay, Mineau, & Stewart, 2001). Thus, insect control could represent an additional value American robins produce to natural and human-modified landscapes in some situations.

FIGURE 1 Photographs of a hen and a drake mallard (*Anas platyrhynchos*; top) and an American robin (*Turdus migratorius*; bottom) utilizing the same small waterbody. This scenario represents a possible transmission mechanism of avian influenza A viruses from waterfowl to American robins through the ingestion of virus-laden water from a common water source [Colour figure can be viewed at wileyonlinelibrary.com]

Although the current study suggests that some American robin individuals can shed relatively high titres (up to $10^{4.8}$ PFU/ml of a wild bird virus) of HP clade 2.3.4.4 avian IAVs, their foraging and behavioural ecology suggests that they may pose somewhat less of a threat to poultry production than certain other wildlife species that can shed HP IAVs. Thus, avoiding items that could attract this species, such as water sources and nesting substrates, may be sufficient for limiting their use of buildings and grounds associated with poultry production facilities and may reduce biosecurity concerns from this common thrush of North America when other appropriate biosecurity measures are in place at facilities.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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