

1 **Prevalence of Avian Influenza Virus in Synanthropic Birds**
2 **Associated with an Outbreak of Highly Pathogenic Strain EA/AM**
3 **H5N1**

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34 Abstract

35 The 2022 – 2023 highly pathogenic avian influenza (HPAI) virus outbreak of H5N1 Eurasian
36 lineage goose/Guangdong clade 2.3.4.4b is the largest in North American history and has
37 significantly impacted wild bird populations and domestic poultry across the United States.
38 Synanthropic birds may play an important role in transmitting the virus laterally to other wild
39 bird species and domestic poultry. Understanding the prevalence of HPAI H5N1 in different
40 avian orders may help inform management decisions and potential risk factors for both wild and
41 domestic bird populations. Following the confirmation of infection of HPAI H5N1 in domestic
42 poultry at two commercial premises in IN, USA, we sampled and tested 266 synanthropic avian
43 species within the Columbiformes and Passeriformes orders and found no detection of the virus
44 at either location. Additionally, laboratories within the National Animal Health Laboratory
45 Network were queried for influenza Type A rRT-PCR assay test results from morbidity and
46 mortality events in wild birds, consisting of 10,786 birds tested across eight orders and 1,666
47 avian influenza virus detections. Query results were assessed by taxonomic groups for viral
48 prevalence and suggested that the virus most often was observed in predatory and scavenging
49 birds. Although detections were found in non-predatory synanthropic birds including the orders
50 Columbiformes, Galliformes, and Passeriformes, the risk of transmission from and between these
51 groups appears comparatively low, with apparent prevalence rates of 0.0090, 0.0688, and 0.0147,
52 respectively. The highest prevalence was observed in raptors (0.2514), with prevalence rates in
53 exclusively scavenging *Cathartidae* reaching up to 0.5333. There is strong evidence that
54 consumption of infected tissues is a key pathway for transmission of avian influenza viruses.
55 Understanding the impact of the 2022 – 2023 HPAI outbreak in wild bird populations can
56 provide pertinent information on viral transmission, disease ecology, and risk to humans and
57 agriculture.

58 Introduction

59 The outbreak of highly pathogenic avian influenza (HPAI) H5N1 Eurasian lineage
60 goose/Guangdong (Gs/GD) clade 2.3.4.4b virus (hereafter H5N1) throughout 2022 and 2023 is
61 the largest in North American history and has impacted wild bird populations and domestic
62 poultry significantly across the continent. The first known infection of H5N1 in North America
63 occurred in a wild great black-backed gull (*Larus marinus*) from Newfoundland and Labrador
64 Province, Canada, in November 2021 [1]. In January 2022, H5N1 was reported in apparently
65 healthy wild waterfowl from NC and SC, USA, and since has been detected in wild birds in 49
66 United States (U.S.) states [2]. As HPAI H5Nx subtypes continue to circulate throughout Eurasia
67 and the Americas [3,4], the migratory nature of wild birds introduces the risk of recombination
68 and reassortment and the introduction of new strains into North America [5,6,7]. Understanding
69 the prevalence in wild bird species can help inform management decisions for wild bird
70 populations and the commercial poultry industry.

71 The avian orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls,
72 and terns) act as the primary reservoir hosts of avian influenza (AI) viruses in the wild [8,9].
73 While waterfowl play a significant role in the transmission of AI viruses due to their gregarious,
74 migratory nature and their potential for significant viral shedding, evidence has shown that they
75 often present as asymptomatic and survive viral infection [10,11]. Significant research has been
76 conducted on AI viruses in Anseriformes and Charadriiformes, which predominantly replicate AI
77 viruses in the intestinal and respiratory tract and can readily transmit AI viruses by the oral –

78 fecal route to other avifauna that share water resources [6,12]. While methods of viral
79 transmission are well understood in these orders, less is known about the role alternative hosts
80 play in transmitting AI viruses across the landscape during an HPAI virus outbreak.
81 Understanding the viral prevalence of different orders may help identify areas with greater risk
82 of HPAI virus infection to alternative avian hosts, threatened and endangered species, and
83 domestic poultry.

84 Although the previous North American outbreak of HPAI Eurasian H5 viruses in 2014 – 2015
85 caused mortality in some wild bird species, the impact was less severe than the 2022 – 2023
86 outbreak. Between December 2014 and June 2015, 98 birds tested positive for HPAI viruses out
87 of approximately 7,084 wild birds sampled: 75 from apparently healthy waterfowl, 16 from
88 mortality events involving snow geese (*Chen caerulescens*) and ringed-necked ducks (*Aythya*
89 *collaris*), and seven captive raptor mortalities [13]. Conversely, with over 7,400 confirmed HPAI
90 H5Nx detections in the USA since January 2022 in over 150 wild bird species across numerous
91 avian orders, the impact of this outbreak on wild bird populations is much greater [2].

92 Domestic poultry populations in the USA also have been substantially impacted following the
93 initial detection of HPAI H5N1 in a commercial turkey facility in IN in February 2022.
94 Detections in domestic poultry (commercial and backyard flocks) have occurred alongside wild
95 bird detections throughout the course of the 2022 – 2023 outbreak with confirmed infections in
96 47 states [14]. Initial genetic sequencing conducted by the U.S. Department of Agriculture
97 (USDA) National Veterinary Services Laboratories (NVSL) suggests that most poultry
98 detections have wild bird origins with a minority occurring by lateral transmission [15]. Thus,
99 the concern from the commercial poultry industry is high and has triggered increased
100 surveillance in wild birds since the beginning of the outbreak in 2022. Commercial poultry
101 assessments investigating routes of transmission following previous HPAI outbreaks have
102 identified high risk factors such as poor biosecurity practices and the movement of people,
103 equipment, and domestic birds [16]. Exact mechanisms of H5N1 transmission from wild birds to
104 poultry throughout 2022 – 2023 are speculative, but bridge hosts, which are non-maintenance
105 host species that can transmit pathogens from reservoir species to domestic poultry through
106 shared resources (e.g., water, crops, feed sources), could play a vital role [17,18]. Synanthropes,
107 or species that are ecologically associated with human populations and regularly utilize
108 anthropogenically modified environments, may act as bridge hosts [19]. Synanthropic species,
109 such as those from the families *Columbidae* and *Passeriformes*, are often found in and around
110 poultry facilities and thus are suspected as potential routes of HPAI virus transmission, as they
111 could act to transport viruses between infected commercial premises. Expanding the lens to other
112 synanthropic species and their potential role in transmission, particularly considering the 2022 –
113 2023 outbreak, can help better focus management resources and mitigate viral spread.

114 Known broadly for their synanthropic behavior, species in the order Columbiformes (doves and
115 pigeons) often have been the subject of AI virus research [19] and investigated as potential
116 bridge hosts in transmitting AI viruses between migratory birds and poultry or between poultry
117 facilities during disease outbreaks [20]. Experimental infections of rock doves (*Columba livia*;
118 often referred to as pigeons) have shown their role in AI virus transmission is likely via fomite or
119 mechanical routes, and when they do shed virus, the quantities and time frames of shedding are
120 limited [19,21]. While the risk for transmission to domestic poultry is low, there is evidence that
121 some AI virus strains can spread from Columbiformes to other avian species and cause infection
122 [19].

123 The order Passeriformes contains several families of birds that demonstrate synanthropic
124 behavior, including *Corvidae* (crows, jays, magpies, and ravens), *Fringillidae* (finches),
125 *Hirundinidae* (swallows), *Icteridae* (blackbirds and grackles), *Passeridae* (Old World sparrows),
126 *Sturnidae* (starlings), and *Turdidae* (robins and thrushes) [19]. Many species within these
127 families commonly are found on farms and have the potential to act as bridge hosts.
128 Susceptibility to AI viruses has been shown both experimentally and in the wild in several
129 Passeriformes species. In their review evaluating AI virus infection rates in wild birds globally,
130 Caron, Cappelle, and Gaidet [22] calculated a 0.0206 prevalence rate for all Passeriformes tested;
131 however, evidence of AI virus susceptibility differs between species.

132 Many *Corvidae* species are omnivorous, opportunistic foragers, and keen scavengers that
133 commonly are attracted to carcasses accessible on farms. Studies evaluating both natural and
134 experimental infections of HPAI viruses in *Corvidae* suggest they may play an important
135 ecological and epidemiological role in HPAI H5 viruses. In South Korea in 2003 – 2004, H5N1
136 was detected in Korean magpies (*Pica pica sericea*) found dead at a poultry facility [23], and
137 investigations of large-billed crow (*Corvus macrorhynchos*) mortalities closely associated with
138 an H5N1 domestic poultry outbreak in Japan in 2004 demonstrated their susceptibility to
139 infection [24]. Experimental inoculation of house crows (*Corvus splendens*) with H5N1 crow
140 and chicken virus isolates caused clinical signs and mortalities in 66.7% and 50% of study
141 animals, respectively [25], suggesting the potential for virus transmission between crows and
142 poultry. Rooks (*Corvus frugilegus*) experimentally inoculated with HPAI H5 all seroconverted
143 and shed virus with a 25% mortality rate [26]. Furthermore, an assessment of risk factors
144 predicting H5N1 infections on poultry farms in Bangladesh identified house crows as the
145 greatest risk factor for virus dispersal [27]. While susceptibility to H5N1 has been demonstrated
146 in several cases, more research is warranted to determine the role *Corvidae* play in virus
147 transmission.

148 Non-*Corvidae* species in the Passeriformes order often are colloquially referred to as songbirds,
149 but distinct differences between them have important implications for HPAI virus susceptibility
150 and transmission. Of species in the *Fringillidae* family, house finches (*Haemorhous mexicanus*)
151 commonly display synanthropic behavior, yet the few assessments of their susceptibility to AI
152 viruses have found low prevalence rates suggesting the risk of transmission is low [19]. While
153 the insectivorous diet of *Hirundinidae* species could decrease their likelihood of interacting with
154 poultry or shared resources [19], their global abundance and occupancy on farms stresses the
155 importance of understanding their role in AI virus ecology [19]. Studies have demonstrated
156 swallows' susceptibility to AI viruses [28,29] and potential to act as bridge hosts [30,31]. Several
157 *Icteridae* species are a common presence on farms, including the common grackle (*Quiscalus*
158 *quiscula*), red-winged blackbird (*Agelaius phoeniceus*), and brown-headed cowbird (*Molothrus*
159 *ater*) [19]. Results of AI virus transmission in *Icteridae* species are mixed, and more research is
160 needed to better understand the role they play in spillover to poultry. Within the *Passeridae*
161 family, sparrows are susceptible to many AI viruses of which they can shed high levels and
162 transmit to poultry [19]. Two studies that experimentally inoculated (1) tree sparrows
163 (*Spizelloides arborea*) with four HPAI H5Nx virus strains [32] and (2) house sparrows (*Passer*
164 *domesticus*) with HPAI H5N1 [21] found both species to be highly susceptible. European
165 starlings (*Sturnus vulgaris*), the most common, widespread synanthrope in the *Sturnidae* family,
166 often flock to farms for food resources and nesting sites in groups so large that even small
167 amounts of viral shedding by individuals collectively could cause AI virus spillover to poultry

168 [19,33,34]. Starlings sampled and tested for AI viruses across 14 studies showed a 0.018
169 prevalence rate, but their role in transmission may be strain-dependent [19]. Within the *Turdidae*
170 family, AI viruses were detected in American robin (*Turdus migratorius*) and Swainson's thrush
171 (*Catharus ustulatus*) at rates of 0.0376 and 0.0377, respectively, during a surveillance study
172 conducted in passerines across the USA [35]. While an experimental study inoculated American
173 robins with HPAI H5Nx viruses and found 0.8800 prevalence [36]. Ultimately, songbird
174 susceptibility to AI viruses is variable, and more work is needed to evaluate the spillover risk to
175 poultry.

176 The order Galliformes (pheasants, turkeys, peafowl, and quail) often exhibit synanthropic
177 behavior and evidence has shown that many species in this family are susceptible to and can shed
178 AI viruses [19]. Many Galliformes that have been studied are domesticated and raised in
179 backyard or gamebird farms, and less is understood about the contact frequency between wild
180 and domestic individuals and AI virus dynamics in wild Galliformes. Galliformes have the
181 potential to act as bridge hosts, as agricultural areas may attract wild individuals searching for
182 food resources or conspecifics [19]. A serosurvey in Italy of 219 free-living pheasants
183 (*Phasianus colchicus*) found a 0.1230 prevalence rate but detected no antibodies to low-
184 pathogenic avian influenza (LPAI) virus H5 subtypes [37]. A similar study of hunter-harvested,
185 wild-captured bobwhite quail (*Colinus virginianus*) in TX, USA, found 1.4% positive and 7.6%
186 suspect for AI viruses [38].

187 Feeding methods of avian scavengers and predators provide the opportunity for contact with
188 HPAI virus-infected carcasses or prey. Susceptibility to HPAI viruses is high, and exposures and
189 infections have been detected in Accipitriformes (hawks and eagles), Cathartiformes (New
190 World vultures), Falconiformes (falcons), and Strigiformes (owls) [19]. Bertran et al. [39]
191 confirmed both HPAI and LPAI virus transmission to Gyr-Saker hybrid falcons (*Falco rusticolus*
192 x *Falco cherrua*) through the experimental ingestion of infected chickens. While conducting
193 passive surveillance following the HPAI H5Nx outbreak in the USA in 2014 – 2015, Ip et al.
194 [10] found raptors (hawks, eagles, and owls) to be particularly susceptible to HPAI H5 viruses,
195 with an overall positivity rate of 52.4%. Hall et al. [40] found American kestrels (*Falco*
196 *sparverius*) to be highly susceptible to H5N1 with 100% mortality rate of experimentally
197 inoculated birds. However, other studies have noted low prevalence in raptor species. Findings in
198 an examination of raptors in OK, USA, found only 0.0160 prevalence in red-tailed hawks (*Buteo*
199 *jamaicensis*) [41]. Raptors that specifically scavenge or prey upon aquatic birds were screened
200 for influenza A antibodies at wildlife rehabilitation centers in MN and VA, USA [42]. They
201 found evidence of AI virus exposure in bald eagles (*Haliaeetus leucocephalus*; 5.1%), negligible
202 evidence of exposure in peregrine falcons (*Falco peregrinus*; 0.2%), great horned owls (*Bubo*
203 *virginianus*; 1.2%), and Cooper's hawks (*Accipiter cooperii*; 1.0%), and zero evidence of
204 exposure in vultures, concluding that bald eagles likely would be affected by HPAI viruses
205 should one be detected in waterfowl. Regardless, there is strong historical evidence of
206 susceptibility to highly pathogenic and other AI viruses in these orders and understanding their
207 prevalence throughout the 2022 – 2023 outbreak can help add to the body of knowledge and
208 provide management insight [43,44].

209 Our investigation focuses on synanthropic species submitted for HPAI testing as part of
210 morbidity/mortality (M/M) investigations and commercial poultry facility sampling events
211 during the 2022 – 2023 H5N1 outbreak. For the purposes of our study, synanthropic refers to
212 terrestrial wild bird species that are (1) non-reservoir hosts of AI viruses, (2) associated

213 ecologically with human populations, and (3) regularly utilize anthropogenically modified
214 environments [19]. Waterfowl and other aquatic, coastal, and pelagic orders such as
215 Pelecaniformes, Anseriformes, and Charadriiformes are excluded from this evaluation.

216 The objectives of this study were (1) to assess the presence of HPAI viruses in synanthropic
217 birds captured around H5N1-positive commercial poultry premises in response to the initial
218 detection in domestic poultry in the USA and (2) to evaluate the prevalence of AI viruses in
219 synanthropic bird orders during an HPAI outbreak. To address the first objective, we initiated a
220 surveillance project to sample synanthropic bird species around HPAI-affected commercial
221 poultry premises and tested for the presence of HPAI. To address the second objective, we
222 evaluated data from the National Animal Health Laboratory Network (NAHLN) on wild bird
223 species submitted for AI virus diagnostic testing as part of morbidity/mortality (M/M)
224 investigations. In this study, we report results from the targeted surveillance project, compare
225 prevalence rates of AI viruses in several avian orders submitted from M/M investigations from
226 February 2022 to March 2023, and provide the total number of HPAI H5Nx positive birds
227 confirmed by the NVSL from avian orders of interest.

228 **Materials and Methods**

229 **Targeted Surveillance**

230 Synanthropic bird species were sampled at two adjacent commercial domestic turkey farms with
231 confirmed HPAI H5N1 in Dubois County, IN, USA, in February 2022. Samples were collected
232 in accordance with the USDA Wild Bird Avian Influenza Surveillance Field Procedures Manual
233 (Summer FY2022 to Winter FY2023) and within the guidelines and regulations set forth by the
234 U.S. Fish and Wildlife Service (USFWS) under permit number MB124992. All samples were
235 collected with the permission of the farm owners. Sampling of wild birds began approximately
236 two weeks following virus detection and the initiation of poultry depopulation. A clean and dirty
237 line was established on both premises, requiring all people, vehicles, supplies, and equipment to
238 be fully cleaned and disinfected prior to crossing from the dirty side to the clean side. Traps were
239 deployed to target European starlings, house sparrows, and rock doves. Five trap designs were
240 used: custom three-hole wooden nest box traps composed of vertically stacked Sherman traps (H.
241 B. Sherman Traps, Inc., Tallahassee, FL, USA); custom made PVC single hole nest box traps
242 with PVC caps and a single catch trap door (Van Ert Enterprises, Decatur, IA, USA); custom
243 portable single-axle trailer drop-in starling decoy traps; baited walk-in traps with funnels; and
244 decoy, walk-in pigeon traps (Tomahawk Live Trap, Hazelhurst, WI, USA). Traps were set within
245 the perimeter of the infected farms on the clean side of the line and placed around poultry barns,
246 grain bins, feed silos, other farm structures, and suspected avian movement corridors on the
247 edges of natural or agriculturally modified habitat. Traps were set every morning on each site
248 and checked within 24 hours for a total of 18 days. Traps were baited with commercial bird seed,
249 dry cat food, and corn. Traps were disinfected with Virkon™ S (LANXESS, Pittsburgh, PA,
250 USA) before transferring to a new location.

251 All captured species were identified by field biologists. Upon capture, birds were immediately
252 euthanized via cervical dislocation and subsequently sampled. Oropharyngeal and cloacal swabs
253 (Harmony Lab and Safety Supplies, Grove Garden, CA, USA) were collected from all captured
254 birds. Both swabs were pooled into a single tube containing 1.5 mL of PrimeStore® Molecular
255 Transport Medium (MTM; EKF Diagnostic, Barleben, Germany) and were shipped to the

256 Veterinary Diagnostic Laboratory at Colorado State University within three days to maintain
257 sample integrity. Nucleic acids were extracted from the samples following standard extraction
258 protocols, and a general influenza Type A rRT-PCR assay targeting the conserved region of the
259 avian influenza matrix gene was performed [45,46]. Prevalence rates were calculated for each
260 species sampled and tested.

261 **Morbidity and Mortality Investigations**

262 Morbidity and mortality (M/M) investigations were conducted across numerous species of birds
263 that appeared sick, moribund, or dead due to suspected exposure to HPAI H5N1 within the
264 conterminous U.S. and Alaska throughout the 2022 – 2023 outbreak. Tracheal and cloacal
265 swabs, whole carcasses, or tissue samples were collected opportunistically by state agencies,
266 federal agencies, or rehabilitation facilities. Sampling methodologies may have differed
267 depending on the collecting state, agency, or facility in terms of the number of birds sampled at
268 each M/M event and type(s) of samples collected. Samples were submitted to labs in the
269 NAHLN for diagnostic testing, which included a general influenza Type A rRT-PCR assay for
270 all samples, and any samples with a resulting non-negative cycle threshold (Ct) value were
271 further tested using an H5 rRT-PCR subtyping assay [45,46].

272 We queried all laboratories in the NAHLN and provided a standardized spreadsheet to be
273 completed with a list of species across multiple taxonomic groups. We focused on groups that
274 most commonly exhibit synanthropic behavior but excluded known reservoir hosts and other
275 waterfowl species. Labs recorded the number of each species tested from 1 February 2022 to 31
276 March 2023, and the resultant number of non-negative samples as determined by the general
277 influenza Type A rRT-PCR assay. Responses were compiled to calculate the prevalence of AI
278 viruses in each sampled species, and species were grouped by order and family. Known captive
279 and domestic birds were excluded from the dataset.

280 **Confirmatory Testing of HPAI H5 Detections**

281 Lastly, we report the total number of H5Nx positive samples for synanthropic orders of interest
282 from the wild bird HPAI detection dataset [2]. These samples had previously undergone
283 confirmatory testing at the NVSL, which included an rRT-PCR assay targeting Eurasian lineage
284 Gs/GD H5 clade 2.3.4.4b (SEPRL; Real-Time RT-PCR Assay for the Detection of
285 Goose/Guangdong lineage Influenza A subtype H5, clade 2.3.4.4; NVSL-WI-1732), as well as
286 an N1 subtyping rRT-PCR assay (SEPRL; Real-Time RT-PCR Assay for the Detection of
287 Eurasian-lineage Influenza A Subtype N1; NVSL-WI-1768). Samples submitted to the NVSL for
288 confirmatory testing included those submitted as part of M/M investigations as well as samples
289 collected from apparently healthy birds as part of targeted surveillance programs. Samples
290 submitted from birds belonging to the orders Anseriformes, Charadriiformes, Pelecaniformes,
291 Suliformes, and Gruiformes were removed from the dataset.

292 **Results**

293 **Targeted Surveillance**

294 Samples were obtained from a total of 266 wild synanthropic birds across eight species from two
295 adjacent commercial turkey farms with confirmed HPAI H5N1 in Dubois County, IN (Table 1).
296 None of the 266 individuals tested positive for influenza A virus by rRT-PCR from pooled

297 cloacal and oral swabs, resulting in zero prevalence of AI virus in the sample. Samples were
298 obtained from the families *Columbidae* (44), *Icteridae* (81), *Passeridae* (89), and *Sturnidae* (52).

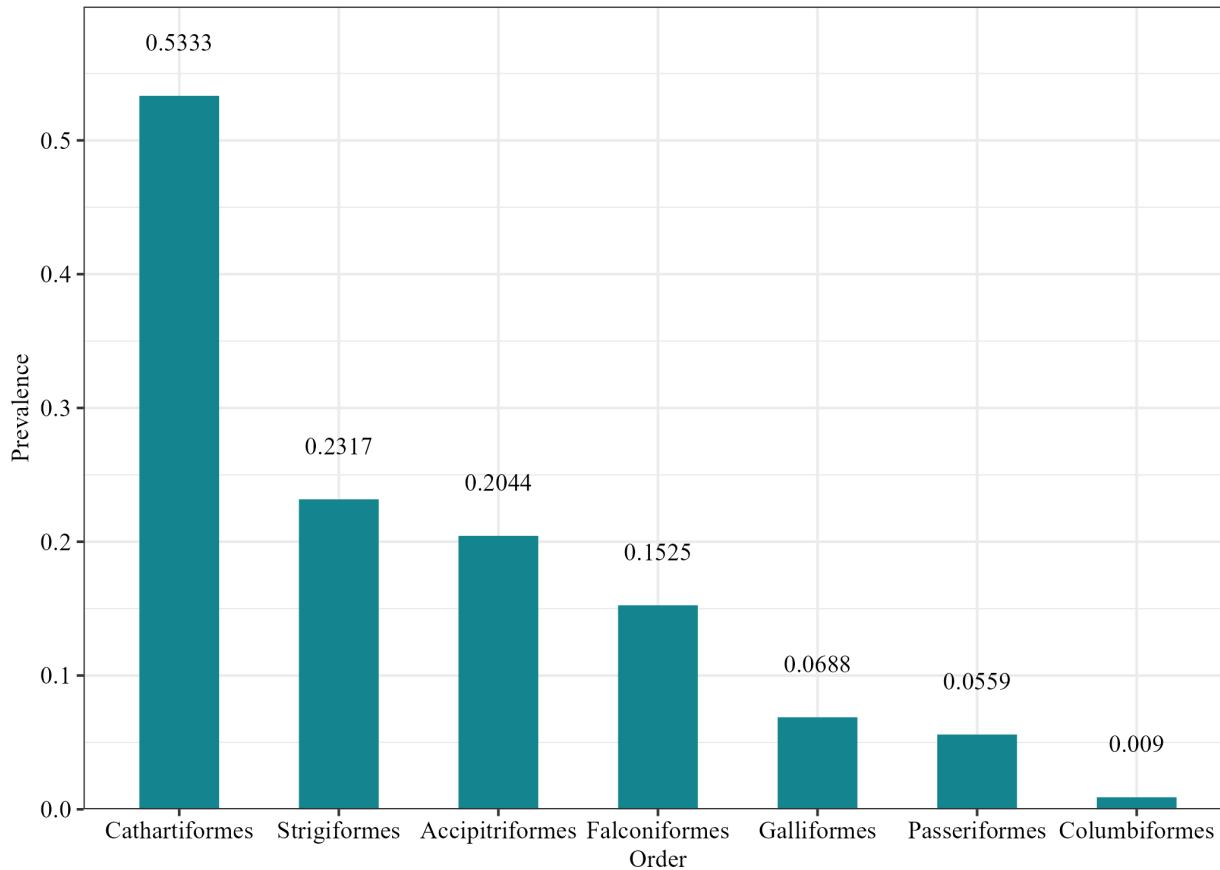
299 Table 1. Number of synanthropic bird species sampled and prevalence of avian influenza virus at
300 HPAI – affected commercial farms in Dubois Co, IN.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
<i>Columbidae</i>	Eurasian collared-dove	1	0	0
	Mourning dove	3	0	0
	Rock dove	40	0	0
		44	0	0
<i>Icteridae</i>	Brown-headed cowbird	51	0	0
	Common grackle	17	0	0
	Red-winged blackbird	13	0	0
		81	0	0
<i>Passeridae</i>	House sparrow	89	0	0
		89	0	0
<i>Sturnidae</i>	European starling	52	0	0
		52	0	0
Total		266	0	0

301

302 **Morbidity and Mortality Investigations**

303 Out of the 48 labs queried in the NAHLN, 32 labs (67%) provided AI virus diagnostic testing
304 data broken down by individual species. Of these labs, a total of 10,786 birds were tested and
305 1,666 AI virus detections were observed (prevalence of 0.1545; see Table S1 in the
306 Supplementary Material for a comprehensive list of all species tested). Prevalence rates were
307 highest in Cathartiformes followed by Strigiformes, Accipitriformes, Falconiformes,
308 Galliformes, Passeriformes, and Columbiformes (Figure 1).



309 Figure 1. Prevalence of avian influenza A virus. Detections of AI viruses in avian orders
310 submitted to the NAHLN as part of morbidity/mortality investigations from 1 February 2022 to
311 31 March 2023.

312 **Pigeons, Doves: Order Columbiformes, Family *Columbidae***

313 Table 2. Avian influenza A virus detections in *Columbidae* morbidity/mortality submissions as
314 reported by diagnostic laboratories in the NAHLN from 1 February 2022 to 31 March 2023.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
<i>Columbidae</i>	Mourning dove	92	2	0.0217
	Rock dove	244	2	0.0082
	Other <i>Columbidae</i> spp.	122	0	0
Total		443	4	0.0090

315
316 Out of the 443 samples collected from the family *Columbidae*, four tested positive for AI
317 viruses, resulting in a prevalence of 0.0090 (Table 2). Mourning doves (*Zenaida macroura*) and
318 rock doves accounted for 76% of *Columbidae* samples and all AI virus detections, with a slightly
319 higher prevalence rate in mourning doves (0.0217) than rock doves (0.0082).

320 **Songbirds: Orders Passeriformes and Piciformes**

321 Table 3. Avian influenza A virus detections in songbird morbidity/mortality submissions as
322 reported by diagnostic laboratories in the NAHNL from 1 February 2022 to 31 March 2023.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
<i>Bombycillidae</i>		21	0	0
<i>Cardinalidae</i>		16	0	0
<i>Fringillidae</i>	American goldfinch	19	1	0.0526
	Pine grosbeak	4	1	0.2500
	Other <i>Fringillidae</i> spp.	23	0	0
<i>Hirundinidae</i>		47	2	0.0426
	Tree swallow	19	2	0.1053
	Violet-green swallow	4	3	0.7500
	Other <i>Hirundinidae</i> spp.	12	0	0
<i>Icteridae</i>		35	5	0.1429
	Boat-tailed grackle	1	1	1
	Common grackle	95	1	0.0105
	Red-winged blackbird	9	1	0.1111
	Other <i>Icteridae</i> spp.	12	0	0
<i>Mimidae</i>		120	3	0.0250
<i>Oriolodae</i>		12	0	0
<i>Paridae</i>		7	0	0
<i>Parulidae</i>		28	0	0
<i>Passerellidae</i>	Dark-eyed junco	32	0	0
	Other <i>Passerellidae</i> spp.	19	1	0.0526
		11	0	0
<i>Passeridae</i>	Other <i>Passerellidae</i> spp.	30	1	0.0333
	House sparrow	165	1	0.0061
<i>Picidae</i>		15	0	0
<i>Sittidae</i>		4	0	0
<i>Sturnidae</i>		72	0	0
<i>Thraupidae</i>		3	0	0
<i>Troglodytidae</i>		14	0	0
<i>Turdidae</i>	American robin	170	1	0.0059
	Other <i>Turdidae</i> spp.	80	0	0
		250	1	0.0040
<i>Tyrannidae</i>		12	0	0
<i>Vireonidae</i>		2	0	0
Total		882	13	0.0147

323

324 A total of 889 samples were obtained from the orders Passeriformes and Piciformes, 13 of which
325 tested positive for AI viruses, resulting in a total prevalence of 0.0150 (Table 3). Of the families
326 tested, AI virus was detected in *Fringillidae*, *Hirundinidae*, *Icteridae*, *Passerellidae*, *Passeridae*,

327 and *Turdidae*. Prevalence was highest in *Hirundinidae* (0.1429), with five total detections in
328 swallow species (*Tachycineta bicolor* and *Tachycineta thalassina*). *Fringillidae* had a prevalence
329 of 0.0426, with one detection each in an American goldfinch (*Spinus tristis*) and pine grosbeak
330 (*Pinicola enucleator*). While prevalence was highest in the pine grosbeak (0.2500), the sample
331 size was small with only four birds tested. *Passerellidae* had a prevalence of 0.0333, with one
332 detection in a dark-eyed junco (*Junco hyemalis*). *Icteridae* yielded a prevalence of 0.0250, with
333 one detection each in a boat-tailed grackle (*Quiscalus major*), a common grackle, and a red-
334 winged blackbird. Lowest prevalence rates were observed in the families *Passeridae* (0.0061),
335 with one house sparrow detection, and *Turdidae* (0.0040), with one American robin detection.

336 **Crows, Ravens, Jays, and Magpies: Order Passeriformes, Family Corvidae**

337 Table 4. Avian influenza A virus detections in *Corvidae* morbidity/mortality submissions as
338 reported by diagnostic laboratories in the NAHLN from 1 February 2022 to 31 March 2023.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
	American crow	301	30	0.0997
	Common raven	106	25	0.2358
	<i>Pica</i> spp.	42	6	0.1429
	Fish crow	24	5	0.2083
	Other <i>Corvidae</i> spp.	59	0	0
<i>Corvidae</i>				
	Total	532	66	0.1240

339

340 Of the 531 *Corvidae* tested, 66 were positive for AI viruses, resulting in a total prevalence of
341 0.1240 (Table 4). Prevalence was highest in common ravens (*Corvus corax*; 0.2358), followed
342 by fish crows (*Corvus ossifragus*; 0.2083), magpies (*Pica* spp; 0.1429), and American crows
343 (*Corvus brachyrhynchos*; 0.0997).

344 **Raptors: Orders Accipitriformes, Cathartiformes, Strigiformes, and Falconiformes**

345 Table 6. Avian influenza A virus detections in raptor morbidity/mortality submissions as
346 reported by diagnostic laboratories in the NAHLN from 1 February 2022 to 31 March 2023.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
	Bald eagle	1150	294	0.2557
	Broad-winged hawk	120	6	0.0500
	Coopers hawk	279	22	0.0789
	Golden eagle	69	3	0.0435
	Red-shouldered hawk	179	20	0.1117
	Red-tailed hawk	747	193	0.2584
	Rough-legged hawk	16	8	0.5000
	Sharp-shinned hawk	55	4	0.0727
	Swainson's hawk	19	4	0.2105

	Hawk (unidentified)	78	10	0.1282
	Eagle (unidentified)	13	2	0.1538
	Other <i>Accipitridae</i> spp.	43	0	0
<i>Accipitridae</i>		2768	566	0.2044
	Black vulture	495	336	0.6788
	California condor	80	3	0.0375
	Turkey vulture	186	73	0.3925
	<i>Cathartidae</i> (unidentified)	34	12	0.3529
<i>Cathartidae</i>		795	424	0.5333
	American kestrel	101	3	0.0297
	Merlin	46	4	0.0870
	Peregrine falcon	148	46	0.3108
	Prairie falcon	1	1	1.0000
	<i>Falconidae</i> (unidentified)	9	5	0.5556
	Other <i>Falconidae</i> spp.	82	0	0
<i>Falconidae</i>		387	59	0.1525
<i>Pandionidae</i>	Osprey	82	4	0.0488
	Barred owl	320	23	0.0719
	Eastern screech-owl	96	3	0.0313
	Great horned owl	610	234	0.3836
	Long-eared owl	11	1	0.0909
	Short eared owl	3	1	0.3333
	Snowy owl	28	9	0.3214
	<i>Strigidae</i> (unidentified)	108	10	0.0926
	Other <i>Strigidae</i> spp.	36	0	0
<i>Strigidae</i>		1213	281	0.2317
<i>Tytonidae</i>	Barn owl	61	0	0
	Total	5306	1334	0.2514

347

348 Of the 5,306 raptor samples submitted for testing, 1,334 were positive for AI viruses, resulting in
 349 a total prevalence of 0.2514 (Table 6). Prevalence was highest in the *Cathartidae* family
 350 (0.5333), followed by *Strigidae* (0.2318), *Accipitridae* (0.2044), *Falconidae* (0.1525), and
 351 *Pandionidae* (0.0488). With a sample size of one, the prairie falcon (*Falco mexicanus*) had the
 352 highest prevalence (1.000) of all raptor species. The next highest prevalence rates were from
 353 black vultures (*Coragyps atratus*; 0.6788), unspecified *Falconidae* (0.5556), and rough-legged
 354 hawks (*Buteo lagopus*; .05000). Of the remaining *Cathartidae*, prevalence rates in turkey
 355 vultures (*Cathartes aura*; 0.3925) and unspecified *Cathartidae* (0.3529) were higher than that of
 356 California condors (*Vultur gryphus*; 0.0375). In the *Accipitridae* family, prevalence was highest
 357 in red-tailed hawks (0.2584), followed by bald eagles (0.2557), Swainson's hawks (*Buteo*
 358 *swainsoni*; 0.2105), unspecified eagles (0.1538), unspecified hawks (0.1282), and red-shouldered
 359 hawks (*Buteo lineatus*; 0.1117). The prevalence rates of the remaining *Accipitridae* species
 360 tested were below 0.1000. Following the prairie falcon and unspecified *Falconidae*, peregrine
 361 falcons had a prevalence of 0.3108. The remaining *Falconidae* species had prevalence rates

362 below 0.1000. Osprey (*Pandion haliaetus*), the only species within *Pandionidae*, had a
363 prevalence rate of 0.0488. Prevalence in the *Strigidae* family was highest in great horned owls
364 (0.3836), followed by short-eared owls (*Asio flammeus*; 0.3333) and snowy owls (*Bubo*
365 *scandiacus*; 0.3214). Barred owls (*Strix varia*), eastern screech-owls (*Megascops asio*), long-
366 eared owls (*Asio otus*), and unidentified *Strigidae* all had prevalence rates below 0.1000.
367

368 **Pheasants, Turkeys, and Quail: Order Galliformes**

369 Table 5. Avian influenza A virus detections in Galliformes morbidity/mortality submissions as
370 reported by diagnostic laboratories in the NAHLN from 1 February 2022 to 31 March 2023.

Family	Species	Number of Birds Sampled	Influenza Type A rRT-PCR Detections (N Positive)	Prevalence
	Greater sage grouse	5	1	0.2000
	Pheasant (unidentified)	674	61	0.0905
	Ring-necked pheasant	233	31	0.1330
	Ruffed grouse	15	1	0.0667
	Wild turkey	451	29	0.0643
	Other <i>Phasianidae</i> spp.	52	0	0
<i>Phasianidae</i>		1430	123	0.0860
<i>Odontophoridae</i>	Quail (unidentified)	757	3	0.0040
	Total	3617	249	0.0688

371
372 Out of the 3,617 Galliformes species submitted for testing, 249 tested positive for AI viruses,
373 resulting in a total prevalence of 0.0688 (Table 5). Prevalence rates within the *Phasianidae* and
374 *Odontophoridae* families were 0.0860 and 0.0040, respectively. Of the species tested within
375 *Odontophoridae*, prevalence was highest in greater sage grouse (*Centrocercus urophasianus*;
376 0.2000), followed by ring-necked pheasant (*Phasianus colchicus*; 0.1330), unspecified pheasants
377 (0.0905), ruffed grouse (*Bonasa umbellus*; 0.0667), and wild turkey (*Meleagris gallopavo*;
378 0.0643).

379 **National Veterinary Services Laboratories**

380 Table 7. Prevalence in avian orders with confirmed HPAI EA H5 detections as determined by
381 rRT-PCR assay targeting Eurasian lineage Gs/GD H5 clade 2.3.4.4b at the NVSL from 1
382 February 2022 to 31 March 2023.

Order	H5 2.3.4.4b rRT-PCR Detections
Accipitriformes	840
Cathartiformes	671
Falconiformes	61
Galliformes	30
Passeriformes	149
Strigiformes	370
Total	2,121

383 A total of 2,121 samples from our synanthropic species of interest were confirmed as the
384 Eurasian lineage Gs/GD H5 clade 2.3.4.4b subtype at the NVSL between 1 February 2022 and
385 31 March 2023 (Table 7). Detections from orders Anseriformes, Charadriiformes,
386 Pelecaniformes, Suliformes, and Gruiformes were excluded from our dataset. Of the remaining
387 orders, approximately 92% of the samples (1,942) originated from raptors: 840 Accipitriformes,
388 671 Cathartiformes, 61 Falconiformes, and 370 Strigiformes. Detections also were confirmed in
389 149 Passeriformes and 30 Galliformes.

390 Discussion

391 Targeted Surveillance

392 Based on rRT-PCR results, we did not detect any AI viruses (HPAI or other) in the 266 wild
393 birds we sampled at two commercial poultry premises with confirmed poultry detections of
394 H5N1 in Dubois County, IN. A total of three commercial poultry premises in Dubois County
395 were confirmed positive for H5N1 during February 2022, and anecdotal reports confirm flocks
396 of migrant European starlings and mixed blackbird species in the area. It is possible that the virus
397 was present in wild bird species around these premises; however, factors in our sampling
398 methods may have negatively impacted the ability to detect AI viruses. First, surveillance began
399 after the commercial facilities were quarantined and poultry were euthanized, potentially
400 preventing the capture of wild birds that may have been utilizing poultry barns. Further, the
401 approximate two-week delay between H5N1 confirmation at the premises and the initiation of
402 wild bird surveillance might have contributed to the lack of detections. Other studies similarly
403 noted that such a delay may have contributed to a lack of HPAI virus detections [18,47]. Second,
404 our study did not investigate non-infected farms, but sampling at non-infected farms in
405 conjunction with infected farms could provide a more comprehensive view of disease ecology
406 and host population dynamics in the area [48]. Third, our low sample size, approximately 130
407 birds per farm, may have influenced the ability to detect any AI viruses in captured species.
408 Similar limitations in the surveillance of synanthropic birds on HPAI infected farms have been
409 noted in previous investigations [18]. Enhanced surveillance with a sufficient sample size of wild
410 birds in known areas of HPAI virus detections in poultry is essential to understand disease
411 ecology and the role potential bridge hosts play in transmission [49,50]. Conducting future
412 sampling concurrent with poultry depopulation activities, minimizing the delay between the
413 confirmation of HPAI and initiation of wild bird sampling, and investigating populations at
414 uninfected farms all could provide a more comprehensive picture of wild bird – poultry
415 transmission risk and directionality.

416 While this investigation suggests that synanthropic species minimally contribute to the spread of
417 HPAI to poultry, there are inherent limiting factors that may have underrated the perceived risk
418 of transmission. Synanthropic birds may die quickly once infected and their probability of
419 capture is lower than that of healthy individuals, resulting in a potential underestimation of
420 disease prevalence [18,47]. Further, as passerine species tend to be smaller in size than raptors or
421 waterfowl species, moribund passerines may have a lower detection probability due to a smaller
422 distribution of feathers and bones or quick removal by scavengers or predators [49,51]. Wobeser
423 and Wobeser [52] found approximately 70% of small bird carcasses experimentally placed were
424 removed within 24 hours by natural means and noted the presence of several scavenging species
425 during that time frame. Although rates of carcass removal are site specific and variable, evidence

426 indicates the probability of detecting a species is negatively correlated with both the length of
427 time post mortality and the size of the birds.

428 Full length viral genome sequence analyses of 1,369 HPAI H5N1 detections in wild birds,
429 commercial poultry, and backyard flocks from December 2021 to April 2022, suggest that at
430 least 85% of U.S. HPAI virus detections in poultry premises and non-poultry flocks are
431 consistent with wild bird origin, while approximately 15% of detections are consistent with
432 lateral transmission (poultry to poultry) [15]. This suggests that wild birds are major contributors
433 to the spread of HPAI H5N1 to poultry, and environmental contamination or direct transmission
434 from a variety of wild bird species are potential sources. Further research is needed to understand
435 the transmission pathways from wild birds to poultry.

436 Conducting risk assessments and determining wild bird activity on farms can be used to increase
437 biosecurity and protect domestic poultry populations [53]. Knowledge of the wild bird – poultry
438 interface, species of concern, and the space where interspecific interactions occur is critical in
439 developing biosecurity methods to decrease contact and risk of AI virus transmission [31].
440 Understanding the disease ecology and risk of viral transmission could aid producers in
441 minimizing the risk to poultry by reducing attractants and contact between wild birds and poultry
442 on farms. Although AI viruses previously have been detected experimentally in passerine
443 species, including five out of the eight species sampled during targeted surveillance, both
444 targeted sampling and M/M investigations throughout the ongoing 2022 – 2023 H5N1 outbreak
445 in the USA show low prevalence in this order [2]. More research is needed to determine which
446 wild bird species may be involved in viral transmission to domestic poultry.

447 **Morbidity/Mortality Investigations**

448 The 2022 – 2023 outbreak of HPAI H5N1 was widespread in wild avifauna, with virus
449 detections across the conterminous U.S. and Alaska in synanthropic orders Accipitriformes,
450 Cathartiformes, Falconiformes, Galliformes, Passeriformes, and Strigiformes. Prevalence rates of
451 AI virus detections from 1,666 M/M samples from 1 February 2022 to 31 March 2023, tested at
452 the NAHLN were highest in vultures (0.5333) followed by owls (0.2318), eagles and hawks
453 (0.2044), falcons (0.1525), corvids (0.1240), pheasants and grouse (0.0860), songbirds (0.0147),
454 doves (0.0090), and quail (0.0040). Confirmatory testing by the NVSL of over 2,100 samples
455 across the same orders and timeframe suggests that HPAI H5N1 was the predominant strain
456 circulating and causing morbidity and mortality in wild bird populations in the USA.

457 Avian ecology and behavior likely play a major role in the transmission of the virus. Predatory
458 and scavenging species show substantially increased levels of infection when compared to
459 granivorous or insectivorous groups, suggesting that transmission may occur via consumption of
460 infected birds or mammals [40]. The order Accipitriformes had the greatest disease prevalence
461 overall, of which vultures, the only obligate scavenger sampled, had the highest rate of infection.
462 Furthermore, roosting behavior, such as displayed in vulture species, increases sociality between
463 conspecifics and the likelihood of viral transmission, particularly for density-dependent
464 pathogens such as AI viruses that spread fecal – orally [54,55]. Facultative scavenging raptors,
465 such as hawks, eagles, owls, and falcons, consume both carrion and apparently healthy prey,
466 which may explain the lower prevalence rates in these families. Previous research of HPAI
467 susceptibility in raptor species supports these findings. Uno et al. [56] found high levels of HPAI
468 H5N1 infection in kestrels following experimental inoculation or ingestion of infected poultry
469 meat. Furthermore, captive raptor morbidities and mortalities during the 2014 – 2015 outbreak

470 were attributed to ingestion of infected meat [13]. Investigating families based on diet may help
471 explain why *Corvidae*, with frequent scavenging behavior and a higher probability of feeding
472 upon infected animals [57], have a prevalence of 0.1240 compared to approximately 0.0147 in
473 non-omnivorous songbirds. It is possible that ingestion of infected tissue is a key transmission
474 pathway from scavenging species to conspecifics, heterospecifics, or domestic poultry.

475 Although there has been previous concern about high potential rates of infection in Galliformes
476 due to their close association with humans and domestic poultry [58], our observed rates of
477 infection are only slightly higher in Galliformes (0.0688) than songbirds (0.0147) and
478 Columbiformes (0.0090). These groups have similar diets, ecological niches, and contact rates
479 with conspecifics, humans, and domestic animals [58], suggesting that factors influencing AI
480 transmission may go beyond physiology and behavior. Non-predatory species tend to have
481 increased sociality [59]. Thus, lower prevalence rates in these groups suggest that the risk of
482 transmission by direct contact with conspecifics is low. However, as virus was detected in these
483 groups, alternative transmission pathways beyond oral consumption and contact with
484 conspecifics should be considered. While experimental research has shown the potential for AI
485 viruses to be transmitted between species via shared environmental resources such as water
486 sources [60,61], further investigation is needed to understand AI virus transmission across the
487 landscape in free ranging avian populations.

488 Understanding AI virus transmission is critical to protect and manage wild bird populations,
489 especially threatened and endangered species. Raptor species, particularly those with smaller
490 population sizes and geographical ranges (e.g., California condors [*Gymnogyps californianus*]),
491 that scavenge or prey upon other avian species have a higher risk of deleterious population
492 impacts caused by HPAI virus infections [56]. Bertran et al. [39] note that the introduction of
493 HPAI viruses in raptors could negatively impact already threatened species and surveillance may
494 be an invaluable tool to better understand the epidemiology of AI viruses in these populations.
495 An understanding of the increased risk for scavenging species has already been applied to
496 management strategies meant to protect the highly endangered California condor, including
497 vaccination and increased surveillance efforts [62]. Monitoring sensitive species (e.g.,
498 conducting active surveillance or risk assessments) during an HPAI outbreak can offer valuable
499 information to wildlife managers on population dynamics, disease risk, and virus type and
500 distribution. Identifying susceptible species with fragile populations could aid in conservation
501 efforts.

502 Sampling birds as part of M/M investigations may have introduced bias into the dataset as it is
503 more probable to detect disease in these groups than in apparently healthy birds. Further, more
504 charismatic species such as raptors may have had disproportionate detections due to birds being
505 larger, more noticeable, and more publicly valued. However, this methodology allowed for the
506 largest possible dataset, potentially increasing the precision of estimates. The 67% response rate
507 from labs within the NAHLN and the differences in each lab's Laboratory Information
508 Management System taxonomy lists may have restricted the ability to draw comprehensive
509 conclusions on AI virus ecology in different avian orders. Expanding future investigations to
510 include apparently healthy wildlife in conjunction with M/M investigations could provide key
511 insights into the disease ecology of AI viruses and their implications for wildlife, human, and
512 agricultural health.

513 **Conclusions**

514 Active surveillance of wild birds at HPAI infected poultry facilities combined with morbidity
515 and mortality surveillance of synanthropic birds offers an avenue to better understand the
516 ecology of avian influenza viruses and the risks they pose to wildlife, domestic animals, and
517 human health. No virus was detected through active surveillance in the orders Columbiformes
518 and Passeriformes. Further, the lowest prevalence rates from morbidity and mortality
519 investigations were observed in Columbiformes, Passeriformes, and Galliformes. Our results
520 suggest that these orders pose a lower risk of acting as major transmission pathways of AI
521 viruses compared to the orders Cathartiformes, Strigiformes, Accipitriformes, and
522 Falconiformes. The most prevalent viral detections were found in wild predatory, scavenging
523 birds, suggesting that there is strong evidence that the consumption of infected tissue is a key
524 pathway for the transmission of AI viruses in these species. Understanding the factors
525 influencing AI virus transmission is crucial for the development and implementation of superior
526 management strategies.

527 **Data Availability**

528 Data for highly pathogenic avian influenza detections in wild birds confirmed at the NVSL from
529 2022 to 2023 are available at [USDA APHIS | 2022-2023 Detections of Highly Pathogenic Avian](#)
530 [Influenza in Wild Birds](#). The majority of data supporting this research are restricted and not
531 available publicly. Wild bird influenza surveillance data collected between August 2007 and July
532 2023 are available from the Wildlife Services National Wildlife Disease Program (NWDP) of the
533 USDA by contacting the NWDP at nwdpdata@usda.gov.

534 **Conflict of Interest Statement**

535 The authors declare that there is no conflict of interest regarding the publication of this paper.

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547 **Supplementary Materials**

548 Compiled dataset of all families, species, number of birds sampled, and positive detections as
549 determined by a general influenza Type A rRT-PCR assay across all samples submitted as part
550 of morbidity/mortality events that were tested at diagnostic laboratories in the NAHNL from 1
551 February 2022 to 31 March 2023.

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