Evaluating the role of wild songbirds or rodents in spreading avian influenza virus across an agricultural landscape

Derek D Houston [1,2], **Shahan Azeem** [3], **Coady W Lundy** [1,4], **Yuko Sato** [5], **Baoqing Guo** [5], **Julie A Blanchong** [1], **Phillip C Gauger** [5], **David R Marks** [4], **Kyoung-Jin Yoon** [Corresp., 3,5], **James S Adelman** [Corresp. 1]

[1] Department of Natural Resource Ecology and Management, Iowa State University, Ames, IA, United States

[2] Department of Natural and Environmental Sciences, Western State Colorado University, Gunnison, NY, United States

[3] Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, United States

[4] Animal and Plant Health Inspection Service, Wildlife Services, United States Department of Agriculture, Urbandale, IA, USA

[5] Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, United States

Corresponding Authors: Kyoung-Jin Yoon, James S Adelman
Email address: kyoon@iastate.edu, adelmanj@iastate.edu

Background. Avian influenza virus (AIV) infections occur naturally in wild bird populations and can cross the wildlife-domestic animal interface, often with devastating impacts on commercial poultry. Migratory waterfowl and shorebirds are natural AIV reservoirs and can carry the virus along migratory pathways, often without exhibiting clinical signs. However, these species rarely inhabit poultry farms, so transmission into domestic birds likely occurs through other means. In many cases, human activities are thought to spread the virus into domestic populations. Consequently, biosecurity measures have been implemented to limit human-facilitated outbreaks. The 2015 avian influenza outbreak in the United States, which occurred among poultry operations with strict biosecurity controls, suggests that alternative routes of virus infiltration may exist, including bridge hosts: wild animals that transfer virus from areas of high waterfowl and shorebird densities.

Methods. Here, we examined small, wild birds (songbirds, woodpeckers, etc.) and mammals in Iowa, one of the regions hit hardest by the 2015 avian influenza epizootic, to determine whether these animals carry AIV. To assess whether influenza A virus was present in other species in Iowa during our sampling period, we also present results from surveillance of waterfowl by the Iowa Department of Natural Resources and Unites Stated Department of Agriculture.

Results. Capturing animals at wetlands and near poultry facilities, we swabbed 449 individuals, internally and externally, for the presence of influenza A virus and no samples tested positive by qPCR. Similarly, serology from 402 animals showed no antibodies against influenza A. Although several species were captured at both wetland and poultry sites, the overall community structure of wild species differed significantly between these types of sites. In contrast, 83 out of 527 sampled waterfowl tested positive for influenza A via qPCR.

Discussion. These results suggest that even though influenza A viruses were present on the Iowa landscape at the time of our sampling, small, wild birds and rodents were unlikely to be frequent bridge hosts.

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[1]Department of Natural Resource Ecology and Management, Iowa State University, Ames, IA, United States of America

[2]Department of Natural and Environmental Sciences, Western State Colorado University, Gunnison, CO, USA

[3]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, United States of America

[4]United States Department of Agriculture - Animal and Plant Health Inspection Service - Wildlife Services, Urbandale, IA, United States of America

[5]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, United States of America

*Corresponding Authors

Email: adelmanj@iastate.edu (JSA); kyoon@iastate.edu (KJY)

Abstract

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sites, the overall community structure of wild species differed significantly between these types of sites. In contrast, 83 out of 527 sampled waterfowl tested positive for influenza A via qPCR.

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Introduction

Avian influenza (AI) is caused by Type A influenza viruses that exist naturally in wild bird populations and can cross the wildlife-domestic animal interface, sometimes causing widespread epizootics in domestic poultry [1]. Such events can prove extremely costly to the commercial poultry industry and enhance the potential for zoonotic spillover into humans [2,3]. Clinical manifestations of avian influenza virus (AIV) infection can vary and the viruses are classified as highly or low-pathogenic strains (HPAIV and LPAIV, respectively) based on virulence in poultry, with H5 and H7 subtypes being the most common HPAIVs [4–12]. In the spring of 2015, a HPAIV strain of H5N2 subtype caused the most detrimental and costly outbreak in the United States [13–15]. This epizootic event had a devastating impact on the regional commercial poultry industry, particularly in Iowa where over 30 million chickens were destroyed with an estimated economic impact of at least $1.2 billion [16–18]. In some cases, initial introduction of AIV from wild bird populations into domestic flocks has been attributed to migratory waterfowl, but in others it has been introduced via human activities or other unknown factors [8]. Given the

destructive impacts of HPAI outbreaks it is important to better understand modes of AIV transmission.

Migratory waterfowl and shorebirds are natural reservoirs for AIV [19–25; but see also 26]. These birds often exhibit few clinical signs of infection, but they can carry and shed the virus along migratory pathways [20,27–31], and thus are generally considered to be vital reservoirs for AIV [32]. AIV can infect other species, including terrestrial bird populations (i.e., songbirds, woodpeckers, etc.) and domestic poultry [26,27,33]. Such infections typically do not result in severe disease outbreaks, but HPAI outbreaks can emerge in domestic flocks if LPAIV strains mutate into HPAIV strains, if multiple LPAIV strains reassort and become HPAIV strains [4,7,8,10,34], or if domestic poultry are infected with HPAIV from elsewhere [35]. It remains unclear how AIV is transmitted into domestic bird populations, especially considering that most poultry farms now enforce strict biosecurity protocols to prevent outbreaks facilitated by human activities (although in practice, compliance may be inconsistent), waterfowl rarely inhabit commercial poultry farms in areas where some outbreaks have occurred, and disease outbreaks spread regionally among domestic populations even after migratory bird movements have ended [8]. Further complicating the issue of AI outbreaks, prior studies have shown that annual prevalence of AIV can be cyclical in wild waterfowl, suggesting that re-emergence of the disease is a threat even after isolated outbreaks have subsided [36,37].

Given the severity of the 2015 AIV outbreak, along with Iowa's close proximity to multiple migratory pathways (Iowa is administratively classified under the Central flyway, but birds from the Mississippi and Atlantic flyways pass through some parts of the state), and intense egg production in the state, this region is at high risk for AIV and is thus an

98 important area in which to study potential means of AIV transmission. With uncertainty
99 about the mechanisms of AIV transfer into domestic poultry and the high possibility of AIV
100 re-emergence, we sought to examine alternate conduits of AIV transmission from wildlife
101 reservoirs into domestic poultry farms. Specifically, we performed surveillance on small,
102 wild birds (i.e., non-waterfowl) and mammals as potential bridge hosts for AIV transfer
103 from wetlands to commercial poultry operations [38–44].

104 We focused on wild bird (non-waterfowl) and small mammal species for the
105 following reasons. First, modern poultry production often occurs in confinements without
106 large openings, effectively barring waterfowl from entering. Second, as part of the
107 biosecurity measures concerning introduction of AIV from waterfowl, many of these
108 facilities are located away from large water bodies such as lakes or ponds commonly used
109 by migratory waterfowl. Third, the most abundant wildlife residing in poultry farms and
110 feed mills are small songbirds and rodents [45,46], some of which have been shown
111 capable of carrying AIV experimentally or through surveillance programs [25,26,38–40,42].
112 Fourth, these small wild birds and mammals are capable of travel between poultry barns
113 and wetlands where waterfowl stop during migration, serving as potential bridge hosts
114 that may augment the risk of poultry epizootics [43,44,47,48]. Given the severity of the
115 2015 AI epizootic in North America and the potential for these small animals to act as
116 conduits between AIV-contaminated wetlands and commercial poultry facilities [43,44], it
117 is important to understand the roles, if any, that non-waterfowl wild birds and mammals
118 play in spreading the virus.

119 Although some prior studies have reported AIV in atypical reservoir or vector
120 species, such as songbirds and small mammals [49,50], such work often omits critical

121 considerations about AIV biology and salient sampling locations [51]. First, prior studies of
122 AIV in small terrestrial birds have focused almost exclusively on successful infection of
123 these species (i.e., detecting AIV *inside* an animal) [49]. This type of study, while
124 informative, neglects an important aspect of AIV biology: these viruses can persist outside
125 of the body [52] and could be transmitted mechanically (i.e., on the *outside* of an animal)
126 [45,53]. As such, the ability of small, wild birds to transfer AIV from conventional wildlife
127 reservoirs (e.g., waterfowl) into commercial poultry facilities may be underestimated.
128 Second, persistence of AIV outside of an avian host leaves open the possibility that other
129 animals, such as rodents, could also transport AIV, either internally or externally [40,41].
130 Third, prior studies of AIV in songbirds or mammals have often included habitat types with
131 little or no potential for interaction among species of concern (i.e., waterfowl), thus missing
132 or diluting the most important sampling locations [54–57; but see also 42,43,46,54–56]. In
133 contrast, ideal sampling should focus on habitats where potential bridge hosts, including
134 small mammals and birds, are most likely to interact with known AIV reservoirs like
135 migratory waterfowl and shorebirds (e.g., wetlands and marshes) and to interact with
136 poultry or their feed (e.g., commercial poultry operations, or feed-mills that serve those
137 operations) [43,44,58–60]. Hence, the actual role of small birds and mammals in spreading
138 AIV has not been definitively evaluated, particularly in the United States, even though these
139 species have the potential to carry AIV biologically and mechanically.
140 While surveillance among these types of species will help determine their potential
141 to carry AIV, successful bridge species must also have the potential to visit both wetland
142 sites and poultry facilities [43,44]. As such, assessing the risk of small birds and mammals
143 as potential bridge species requires some consideration of community structure (i.e., the

144 types and abundances of species present) at different types of sites [43,44]. For instance, if
145 a given species is found to carry AIV, but inhabits wetlands exclusively and never visits
146 poultry facilities, that species is unlikely to successfully facilitate AIV transmission from
147 wild to domestic animals.

148 Our objective in this study, conducted in the wake of the 2015 AIV outbreak in the
149 United States, was to evaluate the potential of small wild birds and rodents to serve as
150 bridge species for AIV transmission. To do so, we assessed the prevalence of AIV in a
151 variety of wild birds and rodents (internally or externally) using qPCR and serology, and
152 compared species communities captured at sites near wetlands vs. commercial poultry
153 facilities across Iowa, USA. To determine whether influenza A viruses were present on the
154 Iowa landscape more generally during our sampling, we compared our results with those
155 from sampling efforts in waterfowl performed under a separate effort by the Iowa
156 Department of Natural Resources and U.S. Department of Agriculture Wildlife Services.
157

158 # Materials and Methods

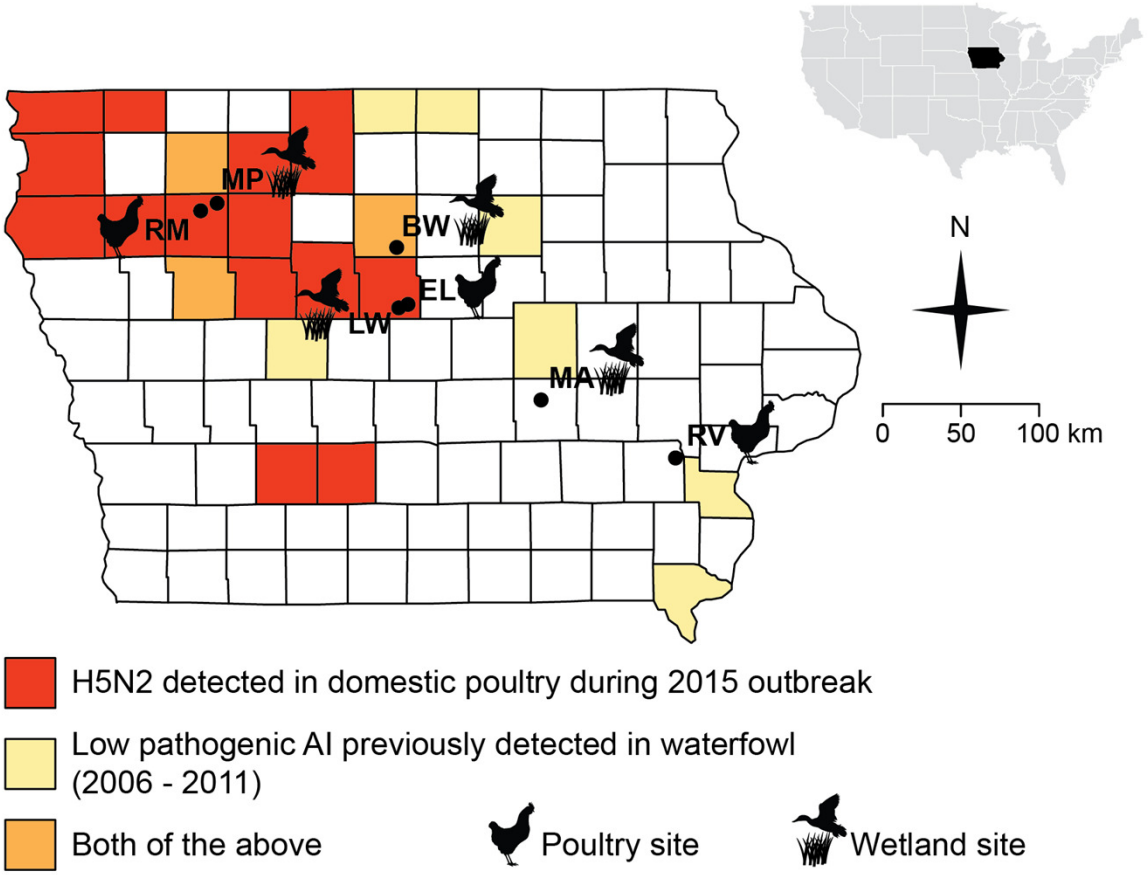
159 ## Field Sampling, Small Birds and Mammals

160 The Iowa State University Animal Care and Use Committee approved all procedures
161 for the handling of specimens and samples (Protocol 9-15-8094-W). Field collections and
162 captures were approved under the following state and federal permits: Iowa Department of
163 Natural Resources Scientific Collecting Permit (SC1133 to JSA), United States Geological
164 Survey Bird Banding Lab Master Banding Permit (23952 to JSA). We obtained samples
165 from wild birds and small mammals at seven sites distributed across Iowa, USA (Fig. 1;

Table 1). Samples were collected during and after the fall migration of 2015 (October 23 – December 14), and during spring migration of 2016 (March 22 – May 10), when the movements of waterfowl would be most likely to bring AIV to the region and when ambient temperatures are low enough to allow persistence of AIV in the environment [61,62]. Sampling sites (Fig. 1) were chosen based on their proximity to areas that experienced HPAI outbreaks in 2015 or where prior monitoring (2006-2011) [63] detected AIV in waterfowl (John Baroch, USDA-APHIS-WS, personal communication with KJY). Four of the sites were wetlands (Big Wall Lake, Little Wall Lake, Malcom, Marathon Poland Park), three of which are located in counties where HPAI outbreaks occurred in 2015, and the remaining three sites were commercial properties (Ellsworth, Rembrandt, Riverside), two of which were in counties that experienced HPAI occurrence during 2015. Precise choices of sampling sites were constrained by cooperation with local landowners and poultry producers concerned with the additional surveillance our sampling would represent on their property. Waterfowl were observed at all wetland sites during the fall and spring sampling periods (by DDH and CL). The time spent sampling each site averaged 4.9 days in the fall and 3.1 days in the spring, with time spent sampling per day ranging from 8 to 12 hours.

For capture, we targeted small bird and mammal species that spend significant time on the ground, where they were most likely to interact with waterfowl or wading birds. Small wild birds were captured using mist nets deployed near bird feeders that had been placed at sites between two and five days prior to sampling. At wetland sites, nets were placed within 100m of water; at poultry farms, nets were placed as close to buildings as cooperators would permit, with a range of 10-200m. In addition, some samples of invasive

189 avian species (house sparrows [*Passer domesticus*] and European starlings [*Sturnus*
190 *vulgaris*], N=9) were obtained by lethal collection via air rifle. Small mammals were
191 trapped using folding, metal live-traps (H.B. Sherman Traps, Inc., Product #: LFA,
192 Tallahassee, FL, USA). We deployed 200 traps at each site. Mammal traps were placed on
193 the ground within 100m of water at wetland sites and between 10-200m of buildings at
194 poultry facilities. Traps were baited with peanut butter, which was wrapped in waxed
195 paper and frozen prior to deployment. Traps were placed at dusk. On nights with projected
196 overnight temperatures below 40°F, traps were lined with cotton balls for any trapped
197 animals to use for insulation.
198 At both wetland and commercial poultry sites, we began netting birds and checking
199 mammal traps between 6-8am and continued through the daylight hours, weather
200 permitting. Nets and traps were closed if rain or snow became heavy, but left open in light
201 drizzle or flurries. In such cases, nets were checked and any captured animals were
202 removed every 5-10 minutes.
203



204

205 **Fig. 1: Sampling sites were chosen in counties where the 2015 H5N2 outbreak**
206 **occurred (red, orange) or along a diagonal band where prior surveillance had found**
207 **AIV in waterfowl (yellow, orange).** Sampling localities visited for this study (during Fall
208 2015 and Spring 2016) are marked with black circles and names are abbreviated as
209 follows: Big Wall Lake (BW), Ellsworth (EL), Little Wall Lake (LW), Malcom (MA), Marathon
210 Poland Park (MP), Rembrandt (RM), Riverside (RV).

211 Three samples were obtained from each animal captured. First, individuals were
212 swabbed externally (e.g., feet, feathers/fur) with single-use, sterile polyester fiber-tipped
213 synthetic swabs, which were placed into individually labeled tubes containing 2 mL of
214 brain heart infusion (BHI) medium, which has been demonstrated to be optimal for AIV

recovery [64]. Next, internal samples were taken with oropharyngeal and cloacal/anal swabs, and the two internal swabs from each individual were pooled into a single labeled tube containing 2 mL of BHI. Both oropharyngeal and cloacal/anal swabs were taken because it has been demonstrated that different species exhibit different levels of AIV in these swabs, suggesting variation among species, or virus strains, in potential transfer [65]. Lastly, blood samples were taken using heparinized microhematocrit tubes following venipuncture of the brachial vein (wing) using 26 or 27 gauge needles for birds, or the saphenous vein (leg) using a 23 gauge needle for mammals (after removing leg hair using electric clippers). Blood samples were immediately transferred to individually labeled 0.7 mL microcentrifuge tubes, free of any additional anticoagulant. All BHI and blood samples were chilled during transport, and were usually back in the laboratory within 8 hours of collection. All individuals were released at their point of capture after processing was completed; and birds were banded prior to their release. Recaptured individuals identified by bands (birds) or by the presence of a shaved patch of fur (mammals), were immediately released without resampling. Upon arrival at the laboratory, all blood samples were immediately spun in mini-centrifuges for 10 minutes to separate red blood cells from plasma, then plasma was transferred to new individually labeled microcentrifuge tubes using a 100 µL Hamilton syringe. All blood, plasma, and BHI samples were stored at -20˚C until they were transferred in an Iowa State University vehicle, still frozen, to the Veterinary Diagnostic Laboratory (VDL) at Iowa State University for further processing. Transfer to the VDL occurred one week after the end of each sampling season, each of which spanned eight weeks, so individual samples were frozen for 1-9 weeks before transfer.

238

239 Field Sampling, Waterfowl

240 From August 2015 through January 2016, a separate team of researchers from the Iowa
241 Department of Natural Resources (DNR) and the United States Department of Agriculture
242 (USDA) Wildlife Services (WS) collected 527 samples of waterfowl, both from hunter-
243 collected carcasses and by live-trapping. Data presented here are done so in accordance
244 with USDA data transfer policy. Oropharyngeal and cloacal swabs were collected and
245 pooled by individual (making one sample per individual) to assess the presence of virus via
246 qPCR (see below), but blood samples were not drawn to assess serology. Samples were
247 collected from several Iowa counties: Adair, Appanoose, Cerro Gordo, Hancock, Harrison,
248 Jackson, Johnson, Louisa, Lucas, Marshall, Union, Wayne, and Winneshiek (Fig. 2). As with
249 samples for small birds and mammals, swabs from waterfowl were placed into BHI
250 medium and stored on ice until freezing at -20˚C before lab processing. Samples were
251 collected, processed and shipped following the protocols previously described [66,67].

252

253 Laboratory Testing, Small Birds and Mammals

254 Sample processing

255 All samples were processed as soon as possible upon arrival to the veterinary
256 diagnostic laboratory, or otherwise stored at -80˚C. All swab tubes containing BHI medium
257 were vortexed for 60 seconds and an aliquot of BHI medium from each tube was dispensed
258 into 96-well plates for RNA extraction. No additional processing other than aliquoting
259 plasma samples was required prior to testing for the virus or anti-AIV antibodies.

260

261 **PCR assay for influenza A virus**

262 We extracted viral RNA from each swab sample via magnetic bead based separation
263 technology using an Ambion® MagMAX™-96 Viral RNA Isolation Kit (Life Technologies,
264 Carlsbad, CA) following the protocol provided by the manufacturer. The procedure was
265 performed in a KingFisher® 96 automated magnetic particle processer (ThermoFisher
266 Scientific, Prussia, PA) as per manufacturer's instructions. Extracted viral RNA was eluted
267 in 50-µL elution buffer.

268 We used a commercially available one-step real-time multiplex RT-PCR kit
269 (VetMAX™-Gold AIV Detection Kit; Life Technologies, Austin, TX), designed to target viral
270 matrix and nucleoprotein genes, to amplify influenza viral RNA. The USDA approves this kit
271 for AIV surveillance testing. The PCR reaction was set up in a 25 µL volume containing 12.5
272 µL of 2X multiplex RT-PCR buffer, 1.0 µL nuclease-free water, 1.0 µL of influenza virus
273 primer probe mix, 2.5 µL of multiplex RT-PCR enzyme mix and 8.0 µL of RNA template (i.e.,
274 extract) or controls. Xeno™ RNA Control supplied with the kit was included as an internal
275 control for RNA purity to assess possible PCR inhibition from samples. Influenza Virus-
276 Xeno™ RNA Control (1000 copies/µL) included in the kit was used as a positive
277 amplification control (PAC). An AIV isolate [A/Turkey/WI/68 (H5N9)], which was obtained
278 from the USDA, was used as an AIV Matrix PCR extraction control. Nuclease-free water was
279 used as a no amplification control. Thermocycling was performed in a 7500 Fast PCR
280 System (Applied Biosystems, Foster City, CA) under the following conditions: reverse
281 transcription at 48 °C for 10 minutes, reverse transcriptase inactivation/initial

denaturation at 95 °C for 10 minutes, and 40 cycles of amplification and extension (95 °C for 15 seconds and 60 °C for 45 seconds) [68].

We analyzed the PCR data using "Manual Cycle Threshold (C_T)" and default baseline cycle 3-15. The AB AIV master detector threshold was determined by multiplying the delta Rn of PAC at cycle 40 by 0.05. Amplification plots were viewed to ensure that positive controls crossed the threshold and that negative controls did not. AIV RNA and Xeno™ RNA control were detected by using FAM™ and VIC™ dyes, respectively. Samples with C_T values ≤ 40 were recorded as positive for influenza A viral RNA, whereas samples with C_T values > 40 were recorded as negative as per manufacturer's instructions.

Serology

Plasma samples were tested on a USDA-approved IDEXX AI MultiS-Screen Ab Test kit (IDEXX Laboratories, Inc. Westbrook, ME) for influenza A virus antibodies. In brief, samples were vortexed before transferring them to test plates, 100 µL of undiluted negative and positive kit controls each, as well as 100 µL of 1:10 diluted house controls or plasma samples were dispensed into appropriate wells of the plate, and plates were incubated for 60 minutes. Each plate was washed 3-5 times with the wash solution that was supplied with the kit, after which 100 µL of anti-AI horseradish-peroxidase conjugate was dispensed into each well and the plate was incubated for another 30 minutes. After incubation, the plates were washed again, then 100 µL of substrate solution was dispensed into each well and the plates were incubated for another 15 minutes. The reaction was stopped by addition of 100 µL of stop solution and absorbance (i.e., optical density) was read at a 650 nm wavelength. All incubation steps were conducted at ambient temperature.

305 Each plate was validated by noting the absorbance of the negative and positive
306 control means, and, if validated, the Sample/Negative (S/N) ratio was calculated for each
307 sample. In accordance with manufacturer recommendations, if the S/N ratio was < 0.5, the
308 sample was classified as positive for antibodies to influenza A virus. If the S/N ratio was ≥
309 0.5, the sample was classified as negative for antibodies to influenza A virus. We note that if
310 a more conservative cutoff value of 0.6 is applied, our results do not change.
311

312 # Laboratory Testing, Waterfowl

313 Waterfowl samples were tested at the United States Geological Survey National
314 Wildlife Health Center (NWHC) in Madison, Wisconsin. The NWHC is one of the National
315 Animal Health Laboratory Network facilities and is certified by the USDA National
316 Veterinary Services Laboratories. Samples were tested following the national wild bird
317 surveillance program protocols, including qPCR matrix tests and additional qPCR to probe
318 for H5 and H7 if matrix tests proved positive. Detailed descriptions of the national wild bird
319 surveillance diagnostic testing protocols have been described previously [66,69].
320

321 ## Analyses

322 When estimating disease prevalence, frequentist statistical approaches assume
323 perfect detection in the laboratory assays. Because this is unlikely, we estimated the
324 prevalence of AIV infection and exposure in potential bridge species using a Bayesian
325 approach that incorporates estimates of assay sensitivity and specificity [70] in R v.3.1.3
326 [71] We used the following values for assay sensitivity and specificity, obtained from the
327 manufacturers, with a possible range from 0-1: ELISA sensitivity = 0.820, ELISA specificity

= 1.00; qPCR sensitivity = 0.984, qPCR specificity = 0.991. We report only raw results for waterfowl sampling because these data are included for the sole purpose of illustrating that influenza A viruses were present in Iowa around the time of our sampling. As such, our goal was not to provide any estimate of prevalence in these species.

Communities of small birds and mammals captured (number and abundances of different species) were compared between wetland and poultry sites using the vegan package v.2.3-5 in R [72]. Briefly, community similarity was calculated between each site using Bray-Curtis distance estimation and analyzed using permutation-based ANOVA (PERMANOVA). This analysis was conducted including all species and for birds only. Analyses were not performed on mammals alone, as so few species were captured. In these analyses, we assume, as have several prior studies, that the animals captured reflect an accurate sample of the community present. We note, however, some species present were likely not captured, particularly high-flying avian species [73].

Results

Samples from a total of 449 wild birds and small mammals were obtained from four wetland sites and three domestic poultry farms distributed across Iowa (Fig. 2, Tables 1 and 2). None of these 449 individuals tested positive for influenza A virus by qPCR from external or internal swabs (95% confidence interval of prevalence: 0.005% – 0.83%; note that because our analyses account for less than 100% sensitivity, confidence intervals do not include 0). Serology was possible on blood samples from 402 animals, none of which showed antibodies against influenza A virus (95% confidence interval of prevalence: 0.01% - 1.21%). Our sample sizes were more modest for any one species and as such, estimates of

351 prevalence for specific species are considerably less precise. For qPCR estimates, these
352 would range from 0.03 % - 3.75 % for the most abundant species (dark-eyed juncos—note
353 "*Peromyscus sp.*" included more individuals, but of two difficult-to-distinguish species
354 lumped together, *P. maniculatus* and *P. leucopus*) to 1.39 % - 85.2 % for the least abundant
355 species (any of those with only 1 individual). Similarly, serology-based estimates would
356 range from 0.04 % - 4.61 % for dark-eyed juncos to 1.37 % - 91.6 % for those species with
357 only one individual captured.
358
359 **Table 1. List of sampling localities during fall 2015 and spring 2016.** Numbers of
360 animals sampled from each locality are listed for each season. The first number represents
361 the sample size included in qPCR analysis; the number in parentheses represents the
362 sample size included for serology. Discrepancies reflect individuals from which blood
363 samples were not taken due to escape or insufficient blood draw. Recaptured animals were
364 immediately released and not sampled a second time.

	Fall 2015		Spring 2016		
Sampling locality	Birds	Mammals	Birds	Mammals	Total
Big Wall Lake [w]	23 (21)	25 (15)	25 (24)	10 (9)	83 (69)
Ellsworth [p]	27 (25)	22 (19)	29 (29)	10 (10)	88 (83)
Little Wall Lake [w]	19 (18)	33 (18)	21 (20)	1 (0)	74 (56)
Malcom [w]	18 (17)	21 (21)	27 (26)	11 (7)	77 (71)
Marathon Poland Park [w]	14 (14)	16 (16)	42 (42)	2 (2)	74 (74)
Rembrandt [p]	0 (0)	0 (0)	21 (18)	0 (0)	21 (18)
Riverside [p]	0 (0)	0 (0)	31 (30)	1 (1)	32 (31)
Total	101 (95)	117 (89)	196 (189)	35 (29)	449 (402)

[p] denotes a domestic poultry farm; [w] denotes a wetland site

Overall species community composition of species captured showed differences between wetland-adjacent and poultry-adjacent sites (PERMANOVA, all species: $F_{1,5} = 5.36$, $p = 0.026$; bird species only: $F_{1,5} = 3.94$, $p = 0.025$). However, there was overlap in community composition, with 14 out of 39 species captured at both types of sites (Table 2), including six of the 10 most commonly captured species (three bird and three mammal species).

Surveillance of waterfowl conducted by the USDA WS and Iowa DNR from August of 2015 through January of 2016 show that avian influenza was present on the Iowa landscape during our sampling (Fig. 2, Table 3). Of 527 samples collected from waterfowl, 83 tested positive for avian influenza A virus by matrix qPCR, with 20 testing positive for H5 subtypes and none testing positive for H7 subtypes by additional, specific qPCRs (Table 3). Virus was not isolated from any of these samples. Positives were spread among 8 out of 13 counties sampled across the state (Fig. 2).

384

385 **Table 2. Potential AIV bridge species sampled by species and site type (poultry-**
386 **adjacent or wetland-adjacent).** Individuals were sampled at four wetland sites and three
387 domestic poultry farms in Iowa during the Fall 2015 and Spring 2016 sampling seasons.
388 Numbers in parentheses indicate the percentages of all individuals captured at a given site
389 type that belong to a given species. All individuals were swabbed externally (feet,
390 feathers/fur) and internally (oropharyngeal, anal/cloacal) to test for the presence of AIV.
391 We were able to collect blood samples from the majority of individuals (402/449) to test
392 for presence of anti-AIV antibodies.

Species		Num. Individuals, Poultry Sites (%)	Num. Individuals, Wetland Sites (%)	Num. Individuals, Total (%)
Birds				
dark-eyed junco*	*Junco hyemalis*	22 (15.6%)	75 (24.4%)	97 (21.6%)
house sparrow	*Passer domesticus*	44 (31.2%)	0 (0%)	44 (9.8%)
song sparrow	*Melospiza melodia*	0 (0%)	22 (7.1%)	22 (4.9%)
American tree sparrow*	*Spizelloides arborea*	1 (0.7%)	20 (6.5%)	21 (4.7%)
American robin*	*Turdus migratorius*	13 (9.2%)	7 (2.3%)	20 (4.5%)
red-winged blackbird	*Agelaius phoeniceus*	0 (0%)	13 (4.2%)	13 (2.9%)
northern cardinal	*Cardinalis cardinalis*	0 (0%)	11 (3.6%)	11 (2.4%)
common grackle*	*Quiscalus quiscula*	1 (0.7%)	9 (2.9%)	10 (2.2%)
black-capped chickadee	*Poecile atricapillus*	0 (0%)	6 (1.9%)	6 (1.3%)
European starling	*Sturnus vulgaris*	5 (3.5%)	0 (0%)	5 (1.1%)
fox sparrow	*Passerella iliaca*	0 (0%)	5 (1.6%)	5 (1.1%)
blue jay	*Cyanocitta cristata*	0 (0%)	4 (1.3%)	4 (0.9%)
chipping sparrow	*Spizella passerina*	4 (2.8%)	0 (0%)	4 (0.9%)
white-throated sparrow	*Zonotrichia albicollis*	4 (2.8%)	0 (0%)	4 (0.9%)
eastern phoebe*	*Sayornis phoebe*	2 (1.4%)	1 (0.3%)	3 (0.7%)
rusty blackbird*	*Euphagus carolinus*	1 (0.7%)	2 (0.6%)	3 (0.7%)
white-crowned sparrow*	*Zonotrichia leucophrys*	2 (1.4%)	1 (0.3%)	3 (0.7%)
American goldfinch *	*Spinus tristis*	1 (0.7%)	1 (0.3%)	2 (0.4%)
brown-headed cowbird*	*Molothrus ater*	1 (0.7%)	1 (0.3%)	2 (0.4%)
brown thrasher*	*Toxostoma rufum*	1 (0.7%)	1 (0.3%)	2 (0.4%)
rock pigeon	*Columba livia*	2 (1.4%)	0 (0%)	2 (0.4%)
swamp sparrow	*Melospiza georgiana*	0 (0%)	2 (0.6%)	2 (0.4%)
wood thrush	*Hylocichla mustelina*	2 (1.4%)	0 (0%)	2 (0.4%)
Baltimore oriole	*Icterus galbula*	1 (0.7%)	0 (0%)	1 (0.2%)
Brewer's blackbird	*Euphagus cyanocephalus*	0 (0%)	1 (0.3%)	1 (0.2%)

brown creeper	*Certhia americana*	0 (0%)	1 (0.3%)	1 (0.2%)
downy woodpecker	*Picoides pubescens*	0 (0%)	1 (0.3%)	1 (0.2%)
eastern bluebird	*Sialia sialis*	0 (0%)	1 (0.3%)	1 (0.2%)
golden-crowned kinglet	*Regulus satrapa*	0 (0%)	1 (0.3%)	1 (0.2%)
Harris's sparrow	*Zonotrichia querula*	0 (0%)	1 (0.3%)	1 (0.2%)
ring-necked pheasant	*Phasianus colchicus*	1 (0.7%)	0 (0%)	1 (0.2%)
white-breasted nuthatch	*Sitta carolinensis*	0 (0%)	1 (0.3%)	1 (0.2%)
mammals				
deer mouse*	*Peromyscus sp.*	3 (2.1%)	109 (35.4%)	112 (24.9%)
house mouse*	*Mus musculus*	19 (13.5%)	1 (0.3%)	20 (4.5%)
northern short-tailed shrew*	*Blarina brevicauda*	5 (3.5%)	6 (1.9%)	11 (2.4%)
meadow vole*	*Microtus pennsylvanicus*	2 (1.4%)	2 (0.6%)	4 (0.9%)
Norway rat	*Rattus norvegicus*	4 (2.8%)	0 (0%)	4 (0.9%)
long-tailed weasel	*Mustela frenata*	0 (0%)	1 (0.3%)	1 (0.2%)
Totals		141	308	449

* species occurs at both poultry and wetland sites.

393

394 **Table 3. Waterfowl sampled by USDA-WS/IADNR indicate that Influenza A was**

395 **present on the Iowa landscape during 2015-2016.** Samples reflect pooled cloacal and

396 oropharyngeal swabs collected from both live-captured and hunter-harvested individuals

397 from 13 Iowa counties between August 2015 to January 2016.

Species		Total Num. Sampled	Num. positive for Influenza A (matrix qPCR)	Num. positive for H5 qPCR	Num. positive for H7 qPCR
American green-winged teal	*Anas carolinensis*	17	1	0	0
American wigeon	*Anas americana*	3	0	0	0
blue-winged teal	*Anas discors*	63	9	1	0
mallard	*Anas platyrhynchos*	206	70	17	0
northern pintail	*Anas acuta*	7	0	0	0
northern shoveler	*Anas clypeata*	11	3	2	0
redhead	*Aythya Americana*	1	0	0	0
wood duck	*Aix sponsa*	219	0	0	0
Totals		527	83	20	0

398

399

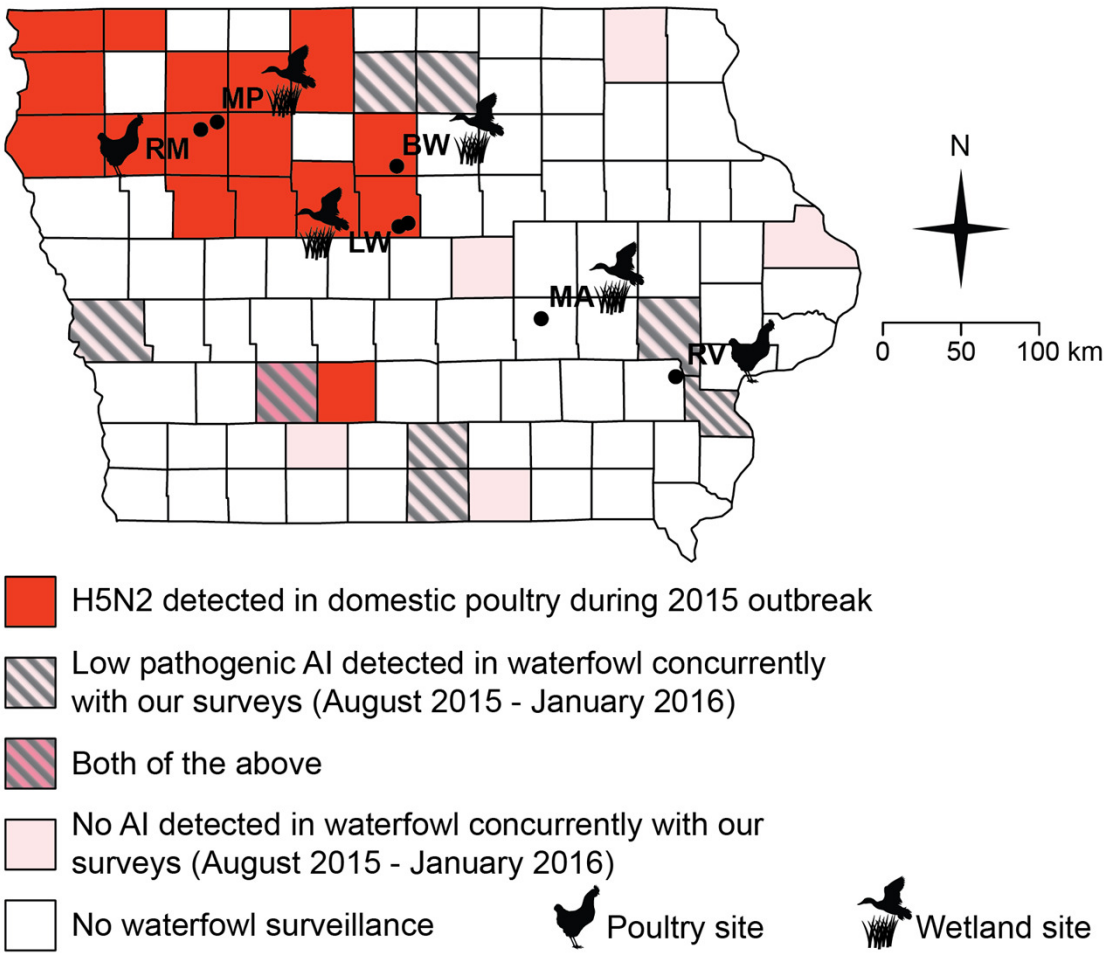


Fig. 2: All but one of our sampling sites fell within counties impacted by the 2015 H5N2 outbreak or adjacent to counties where low pathogenic AIV was detected in waterfowl between August 2015 and January 2016. Black circles and abbreviated site names (see Fig. 1) indicate locations where we sampled small birds and mammals during this study (October-December, 2015; March-May 2016).

Discussion

We found no evidence for low- or high-pathogenic AIV in small wild birds or mammals across a predominantly agricultural landscape in two migratory seasons

following an AIV epizootic. None of the 449 individuals we sampled carried AIV internally or externally based on our qPCR results. Moreover, no Influenza A-specific antibodies were detected in the 402 serological samples, suggesting these animals had not been recently exposed. It remains unclear how long anti-AIV antibodies persist in small mammals and birds. However, results from wild geese surveillance suggest that although antibody levels can wane across seasons, they remain detectable for at least 3-6 months [74,75]. Given that the HPAI outbreaks occurred in summer 2015, we expected to detect antibodies in wild birds or mammals at least during our fall 2015 sampling if these animals had been exposed. That we did not detect AIV in any of our samples via qPCR or anti-AIV antibodies in serology suggests that infection was highly unlikely in small birds and mammals in Iowa at the time of sampling, consistent with most prior surveillance in these types of animals [25,38,40,42,76].

The lack of AIV positive samples among small birds and rodents cannot be explained by a complete absence of AIV in Iowa during our sampling (Fig. 2). Because surveillance by state and federal agencies detected AIV in waterfowl during the fall of 2015, we can be confident that some amount of virus was present in the state. However, because this surveillance was not conducted in a randomized design, estimates of overall prevalence for AIV in the state's waterfowl would not be robust. Moreover, we must note that surveillance for AIV in waterfowl did not completely overlap (spatially or temporally) with our sampling of small birds and rodents (Fig. 2). As such, it is possible that AIV was only present briefly in a select few locations in Iowa, which contributed to our lack of positive samples in small birds and rodents. However, the fact that 8/13 sampled counties showed positive samples from waterfowl suggests that this is unlikely. Additionally, because

433 several waterfowl positives were located in counties adjacent to our small bird and rodent
434 sampling (Fig. 2), it is unlikely that a complete absence of the virus on the Iowa landscape
435 drove the patterns presented here.

436 Although some species of small birds and mammals are found at both wetland sites
437 and poultry facilities, the overall community structure of these small birds and mammals
438 differs between these types of sites. Taken together with our disease surveillance results,
439 this suggests that on the whole, small, wild birds and mammals are unlikely to play major,
440 ongoing roles in transporting AIV from waterfowl to domestic poultry. However, these
441 community data provide an important set of potential species for further surveillance in
442 the future. Specifically, species that were found at both wetland and poultry sites (Table 2)
443 are those with the most potential to act as bridge species [43,44]. Future sampling, with
444 capture techniques targeted toward these species, could improve estimation of AIV
445 prevalence in these animals and our understanding of their role as bridge species. We also
446 note that because we have analyzed communities from samples across the state, it is
447 possible that our estimates of co-occurrence at poultry and wetland sites would vary
448 within specific sub-regions. Moreover, sampling techniques like bird point counts could
449 provide additional information about broader avian communities and may reveal new
450 potential bridge species [43,44]. We suggest that future surveillance efforts in the Midwest
451 U.S. take such community ecology into account if conducting surveillance in small birds and
452 rodents.

453 If wild songbirds and small mammals were major sources of AIV transmission to
454 domestic flocks from wetland sites, we would expect these animals to be exposed
455 regardless of whether or not outbreaks were ongoing in commercial operations. However,

456 our data do not suggest that this is the case: we found no evidence of viral RNA or
457 antibodies despite a ~16% LPAIV infection rate of waterfowl in the region at the time we
458 collected our samples. Indeed, when researchers examine wild populations of small birds
459 and mammals, they typically have only detected AIV in a small proportion of individuals
460 sampled, and detection was more likely during active AIV outbreaks [25,38,40,42].
461 The pattern of small birds and mammals exhibiting low levels of AIV during an
462 epizootic holds true in other surveillance efforts performed during the 2015 HPAI outbreak
463 in the U.S. Jenelle et al. [25] found very low levels of AIV by PCR in non-waterfowl in
464 Minnesota, U.S., just north of the areas of Iowa hit hardest during the AI outbreak.
465 Specifically, they isolated HPAIV from one Cooper's hawk, that they postulated had likely
466 been infected via a prey item [25]. They also sampled three chickadees that exhibited
467 erratic behavior, but the virus was not isolated, despite HPAI viral RNA being detected in
468 one of them [25]. Despite these detections, they found no HPAIV in Minnesota waterfowl at
469 the height of the 2015 outbreak in the Midwest [25]. Additionally, after the outbreak had
470 subsided, Grear and colleagues [76] sampled small, wild birds and mammals at three sites
471 in WI during the fall of 2015, finding no animals positive by qPCR and only two deer mice
472 (*Peromyscus sp.*) with antibodies against AIV (both at poultry farms) out of a total of 284
473 animals sampled. Finally, sampling at Iowa poultry facilities during the 2015 outbreak,
474 Shriner et al. [77] found only one of 648 peridomestic birds and mammals to be qPCR
475 positive for AIV. Combining these results with ours, collected after the HPAI outbreak had
476 subsided, it is plausible that the AIV detections reflect the virus crossing the wildlife-
477 domestic interface into wild birds from infected domestic populations. This is not

478 unprecedented, as AI outbreaks in African and Asian wild birds have been attributed to
479 spread of the disease from infected domestic poultry [see 62 and references therein].
480 Even though we found no evidence here that small wild birds and mammals
481 contribute to the spread of AIV, we are unable to conclude that they cannot. First, with truly
482 low prevalence of disease, sample sizes required for precise estimation of prevalence are
483 extremely high. As such, the true risk of infection may be higher than estimated here or in
484 other recent studies [25,76,77]. Moreover, at the level of individual species, our (and
485 others' [25,76,77]) sample sizes are often quite modest, meaning that confidence of
486 prevalence for each species is low. We note, however, that our total sample sizes of small
487 birds and mammals are comparable to or exceed those of other studies published in the
488 wake of the 2015 HPAI outbreak that arrive at similar conclusions [25,76,77]. Second, it is
489 possible that when small birds and mammals become infected, they die quickly, becoming
490 evolutionary dead ends for AIV [78–81], and proving difficult to sample. As such, it is
491 possible that any surveillance of these species underestimates true prevalence. Finally,
492 ecological barriers, such as habitat types and distance should help mitigate the spread of
493 AIV by small birds and mammals [43,44,82]. However, it is plausible that such species
494 could still help spread the disease to a degree. Importantly, although the virus may occur at
495 extremely low prevalence in non-waterfowl, among highly abundant species like European
496 starlings, low prevalence could translate a significant number of infected individuals. As
497 such, there remains a real risk that initial outbreaks could be triggered by rare infections
498 and propagated by other means, such as farm-to-farm contact. In addition, species likely to
499 be found at both poultry sites (particularly scavengers) could become infected via food
500 items and then transmit AIV back into waterfowl. For instance, some commercial

operations dispose of dead birds via on-site outdoor composting [17,83]. While proper composting protocols state that care should be taken so that no dead birds are exposed, if wild animals access carcasses before the virus is heat-killed, they could potentially become infected prior to interacting with other wild animals on- or off-site. Jenelle et al. [25] reported HPAI in a Cooper's hawk, which although not typically a scavenger, would regularly prey on birds such as rock pigeons and European starlings that commonly frequent poultry farms and could interact with compost bins. We note, however, that this scenario merely outlines one possibility regarding virus spread once an outbreak in domestic flocks has already begun.

Conclusions

We estimated very low prevalence of AIV in small, wild bird and mammal populations, supporting the hypothesis that these organisms do not play major roles as bridge species in the transmission of AIV in Iowa. The differences in types and abundances of small wild birds and mammals at wetland vs. poultry facilities further support this notion. Therefore, our results suggest that spread of the virus likely relies on alternative routes of transmission and further research on alternative routes of AIV transmission, including human-mediated transfer (on clothing, equipment, etc.), airborne particulates, and contaminated food/water sources is warranted. However, given the cyclical nature of AIV outbreaks, the large numbers of small birds and mammals on Midwestern landscape in general, and the possibility of disease reemergence, continued surveillance of these species, particularly those species likely to appear at both wetland and commercial poultry operations, may yet improve our understanding of virus ecology.

524

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531

532 # References

533 1. Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine. 2007;25:
534 5637–5644. doi:10.1016/j.vaccine.2006.10.051
535 2. Rushton J, Viscarra R, Guerne Bleich E, McLeod A. Impact of avian influenza
536 outbreaks in the poultry sectors of five South East Asian countries (Cambodia,
537 Indonesia, Lao PDR, Thailand, Viet Nam) outbreak costs, responses and potential
538 long term control. World's Poult Sci J. 2005;61: 491–514.
539 doi:doi:10.1079/WPS200570
540 3. Koopmans M, Wilbrink B, Conyn M, Natrop G, Van Der Nat H, Vennema H, Meijer A,
541 van Steenbergen J, Fouchier R, Osterhaus A, Bosman A. Transmission of H7N7 avian
542 influenza A virus to human beings during a large outbreak in commercial poultry
543 farms in the Netherlands. Lancet. 2004;363: 587–593. doi:10.1016/S0140-
544 6736(04)15589-X
545 4. Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human

influenza virus subtypes H2N2 and H3N2. Virology. 1978;87: 13–20. doi:10.1016/0042-6822(78)90153-8

5. Röhm C, Zhou N, Süss J, Mackenzie J, Webster RG. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology. 1996;217: 508–516. doi:10.1006/viro.1996.0145

6. Alexander DJ. A review of avian influenza in different bird species. Vet Microbiol. 2000;74: 3–13. doi:10.1016/S0378-1135(00)00160-7

7. Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, Vasisht N, Steinhauer DA, Daniels RS, Elliot A, Wiley DC, Skehel JJ. The Structure and Receptor Binding Properties of the 1918 Influenza Hemagglutinin. Science (80). 2004;303: 1838–1842. doi:10.1126/science.1093155

8. Thomas JK, Noppenberger J. Avian influenza: A review. Am J Heal Pharm. 2007;64: 149–165. doi:10.2146/ajhp060181

9. Das K, Aramini JM, Ma L-C, Krug RM, Arnold E. Structures of influenza A proteins and insights into antiviral drug targets. Nat Struct Mol Biol. Nature Publishing Group; 2010;17: 530–538. doi:10.1038/nsmb.1779

10. Wille M, Tolf C, Avril A, Latorre-Margalef N, Wallerström S, Olsen B, Waldenström J. Frequency and patterns of reassortment in natural influenza A virus infection in a reservoir host. Virology. 2013;443: 150–160. doi:10.1016/j.virol.2013.05.004

11. Latorre-Margalef N, Tolf C, Grosbois V, Avril A, Bengtsson D, Wille M, Osterhaus ADME, Fouchier RAM, Olsen B, Waldenström J. Long-term variation in influenza A virus prevalence and subtype diversity in migratory mallards in northern Europe. Proc Biol Sci. 2014;281: 20140098. doi:10.1098/rspb.2014.0098

569 12. Lu L, Lycett SJ, Leigh Brown AJ. Reassortment patterns of avian influenza virus
570 internal segments among different subtypes. BMC Evol Biol. 2014;14: 16.
571 doi:10.1186/1471-2148-14-16
572 13. Ip HS, Torchetti MK, Crespo R, Kohrs P, Debruyn P, Mansfield KG, Baszler T, Badcoe,
573 L, Bodenstein B, Shearn-Bochsler V, Killian ML, Pedersen JC, Hines N, Gidlewski T,
574 DeLiberto T, Sleeman JM. Novel Eurasian highly pathogenic avian influenza a H5
575 viruses in wild birds, Washington, USA, 2014. Emerg Infect Dis. 2015;21: 886–890.
576 doi:10.3201/eid2105.142020
577 14. United States Department of Agriculture (APHIS; Veterinary Services). Highly
578 Pathogenic Avian Influenza Response Plan: The Red Book [Internet]. 2015. Available:
579 https://www.aphis.usda.gov/animal_health/emergency_management/downloads/h
580 pai_response_plan.pdf
581 15. Arruda PHE, Stevenson GW, Killian ML, Burrough ER, Gauger PC, Harmon
582 KM,Magstadt DR, Yoon KJ, Zhang J, Madson DM, Piñeyro P, Derscheid RJ, Schwartz KJ,
583 Cooper VL, Halbur PG, Main RG, Sato Y, Arruda BL. Outbreak of H5N2 highly
584 pathogenic avian Influenza A virus infection in two commercial layer facilities:
585 lesions and viral antigen distribution. J Vet Diagnostic Investig. 2016; 1–6.
586 doi:10.1177/1040638716658929
587 16. Decision Innovation Solutions. Economic Impact of Highly Pathogenic Avian
588 Influenza (HPAI) on Poultry in Iowa. 2015.
589 17. Greene JL. Update on the Highly-Pathogenic Avian Influenza Outbreak of 2014-2015.
590 CRS Report #R44114. Washington DC: Congressional Research Service 2015. 2015;
591 1–18. Available: https://www.fas.org/sgp/crs/misc/R44114.pdf

592 18. United States Department of Agriculture (APHIS). [cited 25 Jun 2015]. Available:
593 http://www.aphis.usda.gov/animal-health/aiupdates
594 19. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology
595 of influenza A viruses. Microbiol Rev. 1992;56: 152–79. doi:10.1111/j.1541-
596 0420.2008.01180.x
597 20. Olsen B, Munster V, Wallensten A, Waldenstrom J. Global patterns of influenza A
598 virus in wild birds. Science (80). 2006; 3–7. doi:10.1126/science.1122438
599 21. Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, Fransson T,
600 Rimmelzwaan GF, Beyer WEP, Schutten M, Olsen B, Osterhaus ADME, Fouchier RAM.
601 Spatial, temporal, and species variation in prevalence of influenza a viruses in wild
602 migratory birds. PLoS Pathog. 2007;3: 0630–0638.
603 doi:10.1371/journal.ppat.0030061
604 22. Latorre-Margalef N, Gunnarsson G, Munster VJ, Fouchier RAM, Osterhaus ADME,
605 Elmberg J, Olsen B, Wallensten A, Haemig PD, Fransson T, Bruden L, Waldenström J.
606 Effects of influenza A virus infection on migrating mallard ducks. Proc Biol Sci.
607 2009;276: 1029–1036. doi:10.1098/rspb.2008.1501
608 23. Wilcox BR, Knutsen GA, Berdeen J, Goekjian V, Poulson R, Goyal S, Sreevatsan S,
609 Cardona C, Berghaus RD, Swayne DE, Yabsley MJ, Stallknecht DE. Influenza-A viruses
610 in ducks in northwestern Minnesota: Fine scale spatial and temporal variation in
611 prevalence and subtype diversity. PLoS One. 2011;6: 3–11.
612 doi:10.1371/journal.pone.0024010
613 24. Huang Y, Wille M, Dobbin A, Robertson GJ, Ryan P, Ojkic D, Whitney H, Lang AS. A 4-
614 year study of avian influenza virus prevalence and subtype diversity in ducks of

Newfoundland, Canada. Can J Microbiol. 2013;59: 701–708. doi:10.1139/cjm-2013-0507

25. Jenelle CS, Carstensen M, Hildebrand EC, Cornicelli L, Wolf P, Grear DA, Ip HS, Vandalen KK, Minicucci LA. Surveillance for Highly Pathogenic Avian Influenza Virus in Wild Birds during Outbreaks in Domestic Poultry, Minnesota, 2015. Emerg Infect Dis. 2016;22. doi:DOI: 10.3201/eid2207.152032

26. Caron A, Cappelle J, Gaidet N. Challenging the conceptual framework of maintenance hosts for influenza A viruses in wild birds. J Appl Ecol. 2017;54: 681–690. doi:10.1111/1365-2664.12839

27. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci USA. 2006;103: 19368–19373. doi:10.1073/pnas.0609227103

28. Lebarbenchon C, Sreevatsan S, Lefèvre T, Yang M, Ramakrishnan MA, Brown JD, Stallknecht DE. Reassortant influenza A viruses in wild duck populations: effects on viral shedding and persistence in water. Proc Biol Sci. 2012;279: 3967–75. doi:10.1098/rspb.2012.1271

29. Pepin KM, Spackman E, Brown JD, Pabilonia KL, Garber LP, Weaver JT, Kennedy DA, Patyk KA, Huyvaert KP, Miller RS, Franklin AB, Pedersen K, Bogich TL, Rohani P, Shriner SA, Webb CT, Riley S. Using quantitative disease dynamics as a tool for guiding response to avian influenza in poultry in the United States of America. Prev Vet Med. Elsevier B.V.; 2014;113: 376–397. doi:10.1016/j.prevetmed.2013.11.011

30. Fries AC, Nolting JM, Bowman AS, Lin X, Halpin RA, Wester E, Fedorova N, Stockwell TB, Das SR, Dugan VG, Wentworth DE, Gibbs HL, Slemons RD. Spread and persistence

of influenza A viruses in waterfowl hosts in the North American Mississippi migratory flyway. J Virol. 2015;89: 5371–81. doi:10.1128/JVI.03249-14

31. Bengtsson D, Safi K, Avril A, Fiedler W, Wikelski M, Gunnarsson G, Elmberg J, Tolf C, Olsen B, Waldenström J. Does influenza A virus infection affect movement behaviour during stopover in its wild reservoir host? R Soc Open Sci. 2016;3: 150633. doi:10.1098/rsos.150633

32. Reed KD, Meece JK, Henkel JS, Shukla SK. Birds , Migration and Emerging Zoonoses : West Nile Virus , Lyme Disease , Influenza A and Enteropathogens. Clin Med Res. 2003;1: 5–12. doi:10.3121/cmr.1.1.5

33. Sonnberg S, Webby RJ, Webster RG. Natural history of highly pathogenic avian influenza H5N1. Virus Res. Elsevier B.V.; 2013;178: 63–77. doi:10.1016/j.virusres.2013.05.009

34. Lindsay LL, Kelly TR, Plancarte M, Schobel S, Lin X, Dugan VG, Wentworth DE, Boyce WM. Avian influenza: Mixed infections and missing viruses. Viruses. 2013;5: 1964–1977. doi:10.3390/v5081964

35. Capua I, Marangon S. The avian influenza epidemic in Italy, 1999-2000: a review. Avian Pathol. 2000;29: 289–94. doi:10.1080/03079450050118403

36. Hinshaw VS, Wood JM, Webster RG, Deibel R, Turner B. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bull World Health Organ. 1985;63: 711–719. doi:none

37. Curran JM, Ellis TM, Robertson ID, Curran JM, Ellis ATM, Robertson ID. Serological Surveillance of Wild Waterfowl in Northern Australia for Avian Influenza Virus Shows Variations in Prevalence and a Cyclical Periodicity of Infection. Avian Dis.

2015;59: 492–497.

38. Kou Z, Lei FM, Yu J, Fan ZJ, Yin ZH, Jia CX, Xiong KJ, Sun YH, Zhang XW, Wu XM, Gao XB, Li TX. New Genotype of Avian Influenza H5N1 Viruses Isolated from Tree Sparrows in China New Genotype of Avian Influenza H5N1 Viruses Isolated from Tree Sparrows in China. J Virol. 2005;79: 15460–15466. doi:10.1128/JVI.79.24.15460

39. Nemeth NM, Thomas NO, Orahood DS, Anderson TD, Oesterle PT. Shedding and serologic responses following primary and secondary inoculation of house sparrows (Passer domesticus) and European starlings (Sturnus vulgaris) with low-pathogenicity avian influenza virus. Avian Pathol. 2010;39: 411–8. doi:10.1080/03079457.2010.513043

40. Shriner SA, VanDalen KK, Mooers NL, Ellis JW, Sullivan HJ, Root JJ, Pelzel AM, Franklin AB. Low-pathogenic Avian influenza viruses in wild house mice. PLOS One. 2012;7: 1–9. doi:10.1371/journal.pone.0039206

41. Wanaratana S, Amonsin A, Chaisingh A, Panyim S, Sasipreeyajan J, Pakpinyo S. Experimental Assessment of Houseflies as Vectors in Avian Influenza Subtype H5N1 Transmission in Chickens. Avian Dis. American Association of Avian Pathologists; 2013;57: 266–272. doi:10.1637/10347-090412-Reg.1

42. Zhao B, Zhang X, Zhu W, Teng Z, Yu X, Gao Y, Wu D, Pei E, Yuan Z, Yang L, Wang D, Shu Y, Wu F. Novel avian influenza A(H7N9) virus in tree sparrow, Shanghai, China, 2013. Emerg Infect Dis. 2014;20: 7–10. doi:10.3201/eid2005.131707

43. Caron A, Grosbois V, Etter E, Gaidet N, de Garine-Wichatitsky M. Bridge hosts for avian influenza viruses at the wildlife/domestic interface: An eco-epidemiological

framework implemented in southern Africa. Prev Vet Med. Elsevier B.V.; 2014;117: 590–600. doi:10.1016/j.prevetmed.2014.09.014

44. Caron A, Cappelle J, Cumming GS, de Garine-Wichatitsky M, Gaidet N. Bridge hosts, a missing link for disease ecology in multi-host systems. Vet Res. 2015;46: 83. doi:10.1186/s13567-015-0217-9

45. Brown J, Stallknecht D, Lebarbenchon C, Swayne D. Survivability of Eurasian H5N1 Highly Pathogenic Avian Influenza Viruses in Water Varies Between Strains. Avian Dis. American Association of Avian Pathologists; 2014;58: 453–457. doi:10.1637/10741-120513-ResNote.1

46. Leon VA, Fraschina J, Guidobono JS, Busch M. Habitat use and demography of Mus musculus in a rural landscape of Argentina. Integr Zool. 2013;8: 18–29. doi:10.1111/j.1749-4877.2012.00290.x

47. Caron A, Gaidet N, Garine-Wichatitsky M De, Morand S, Cameron EZ. Evolutionary biology, community ecology and avian influenza research. Infect Genet Evol. 2009;9: 298–303. doi:10.1016/j.meegid.2008.12.001

48. Caron A, Garine-wichatitsky M De, Gaidet N, Chiweshe N, Cumming GS. Estimating Dynamic Risk Factors for Pathogen Transmission Using Community-Level Bird Census Data at the Wildlife / Domestic Interface. 2010;15.

49. Slusher MJ. Are passerine birds reservoirs for avian influenza viruses? University of Georgia. 2013.

50. Reperant LA, Rimmelzwaan GF, Kuiken T. Avian Influenza Viruses in Mammals. Rev Sci Tech. 2009;28: 137–159.

51. Hoye BJ, Munster VJ, Nishiura H, Klaassen M, Fouchier RAM. Surveillance of wild

707 birds for avian influenza virus. Emerg Infect Dis. 2010;16: 1827–1834.
708 doi:10.3201/eid1612.100589
709 52. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen
710 TK, Nhuyen TH, Tran TH, Nicoll A, Touch S, Yuen KY. Avian Influenza A (H5N1)
711 Infection in Humans. N Engl J Med. 2005;353: 1374–1385.
712 doi:10.1056/NEJMra052211
713 53. Kaleta EF, Honicke A. Review of the literature on avian influenza A viruses in pigeons
714 and experimental studies on the susceptibility of domestic pigeons to influenza A
715 viruses of the haemagglutinin subtype H7. Dtsch Tierarztl Wochenschr. 2004;111:
716 467–472.
717 54. Peterson AT, Bush SE, Spackman E, Swayne DE, Ip HS. Influenza A virus infections in
718 land birds, People's Republic of China. Emerg Infect Dis. 2008;14: 1644–1646.
719 doi:10.3201/eid1410.080169
720 55. Siengsanan J, Chaichoune K, Phonaknguen R, Sariya L, Prompiram P, Kocharin W,
721 Tangsudjai S, Suwanpukdee S, Wiriyarat W, Pattanarangsan R, Robertson I, Blacksell
722 SD, Ratanakorn P. Comparison of outbreaks of H5N1 highly pathogenic avian
723 influenza in wild birds and poultry in Thailand. J Wildl Dis. 2009;45: 740–747.
724 doi:10.7589/0090-3558-45.3.740
725 56. Fuller TL, Saatchi SS, Curd EE, Toffelmier E, Thomassen H a, Buermann W, DeSante
726 DF, Nott MP, Saracco JF, Ralph CJ, Alexander JD, Pollinger JP, Smith TB. Mapping the
727 risk of avian influenza in wild birds in the US. BMC Infect Dis. 2010;10: 187.
728 doi:10.1186/1471-2334-10-187
729 57. Thinh TV. b, Gilbert M., Bunpapong N., Amonsin A., Nguyen DT., Doherty Jr. PF.,

730 Huyvaert KP. Avian influenza viruses in wild land birds in Northern Vietnam. J Wildl
731 Dis. 2012;48: 195–200. Available:
732 http://www.scopus.com/inward/record.url?eid=2-s2.0-
733 84856050162&partnerID=40&md5=85ae3a3043dbb5b70d8bfa6441162304
734 58. Gronesova P, Ficova M, Mizakova A, Kabat P, Trnka A, Betakova T. Prevalence of
735 avian influenza viruses, Borrelia garinii, Mycobacterium avium, and Mycobacterium
736 avium subsp. paratuberculosis in waterfowl and terrestrial birds in Slovakia, 2006.
737 Avian Pathol. 2008;37: 537–543. doi:10.1080/03079450802356953
738 59. Borovská P, Kabát P, Ficová M, Trnka A, Svetlíková D, Betáková T. Prevalence of avian
739 influenza viruses, Mycobacterium avium, and Mycobacterium avium, subsp.
740 paratuberculosis in marsh-dwelling passerines in Slovakia, 2008. Biologia (Bratisl).
741 2011;66: 282–287. doi:10.2478/s11756-011-0016-3
742 60. Cumming GS, Caron A, Abolnik C, Cattoli G, Bruinzeel LW, Burger CE, Cecchettin K,
743 Chiweshe N, Mochotlhoane B, Mutumi GL, Ndlovu M. The ecology of influenza a
744 viruses in wild birds in Southern Africa. Ecohealth. 2011;8: 4–13.
745 doi:10.1007/s10393-011-0684-z
746 61. Handel A, Lebarbenchon C, Stallknecht D, Rohani P. Trade-offs between and within
747 scales: environmental persistence and within-host fitness of avian influenza viruses.
748 Proc R Soc Ser B. 2014;281: 20133051. doi:10.1098/rspb.2013.3051
749 62. Dalziel AE, Delean S, Heinrich S, Cassey P. Persistence of low pathogenic influenza A
750 virus in water: A systematic review and quantitative meta-analysis. PLOS One.
751 2016;11. doi:10.1371/journal.pone.0161929
752 63. Bevins SN, Pedersen K, Lutman MW, Baroch JA, Schmit BS, Kohler D, Gidlewski T,

753 Nolte DL, Swafford SR, DeLiberto TJ. Large-scale avian influenza surveillance in wild
754 birds throughout the United States. PLOS One. 2014;9: 1–8.
755 doi:10.1371/journal.pone.0104360
756 64. Spackman E, Pedersen JC, McKinley ET, Gelb J. Optimal specimen collection and
757 transport methods for the detection of avian influenza virus and Newcastle disease
758 virus. BMC Vet Res. 2013;9: 35. doi:10.1186/1746-6148-9-35
759 65. Costa TP, Brown JD, Howerth EW, Stallknecht DE. Variation in viral shedding
760 patterns between different wild bird species infected experimentally with low-
761 pathogenicity avian influenza viruses that originated from wild birds. Avian Pathol.
762 2011;40: 119–124. doi:10.1080/03079457.2010.540002
763 66. DeLiberto TJ, Swafford SR, Nolte DL, Pedersen K, Lutman MW, Schmit BB, Baroch JA,
764 Kohler DJ, Franklin A. Surveillance for highly pathogenic avian influenza in wild birds
765 in the USA. Integr Zool. 2009;4: 426–439.
766 67. Pedersen K, Swafford S, DeLiberto T. Low pathogenicity avian influenza subtypes
767 isolated from wild birds in the United States, 2006–2008. Avian Dis. 2010;54: 405–
768 410.
769 68. Zhang J, Harmon KM. RNA extraction from swine samples and detection of influenza
770 a virus in swine by real-time RT-PCR. Methods in Molecular Biology. 2014. pp. 277–
771 293. doi:10.1007/978-1-4939-0758-8_23
772 69. DeLiberto T, Swafford S, Van K. Development of a national early detection system for
773 highly pathogenic avian influenza in wild birds in the United States of America. In:
774 Majumdar S. B, Huffman JE, McLean RG, Panah AI, Pietrobon PJ, Keeler SP, et al.,
775 editors. Pandemic influenza viruses: Science, surveillance, and public health.

776 Pennsylvania Academy of Sciences; 2011. pp. 156–175.
777 70. Williams CJ, Moffitt CM. Estimation of fish and wildlife disease prevalence from
778 imperfect diagnostic tests on pooled samples with varying pool sizes. Ecol Inform.
779 2010;5: 273–280. doi:http://dx.doi.org/10.1016/j.ecoinf.2010.04.003
780 71. Team RDC. R: A Language and Environment for Statistical Computing. Vienna,
781 Austria: R Foundation for Statistical Computing; 2015.
782 72. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL,
783 Solymos P, Henry M, Stevens H, Wagner H. vegan: Community Ecology Package
784 [Internet]. 2016. Available: http://cran.r-project.org/package=vegan
785 73. Bonter DN, Brooks EW, Donovan TM. What are we missing with only ground-level
786 mist nets? Using elevated nets at a migration stopover site. J F Ornithol. 2008;79:
787 314–320. doi:10.1111/j.1557-9263.2008.00179.x
788 74. Hoye BJ, Munster VJ, Nishiura H, Fouchier RAM, Madsen J, Klaassen M.
789 Reconstructing an annual cycle of interaction: Natural infection and antibody
790 dynamics to avian influenza along a migratory flyway. Oikos. 2011;120: 748–755.
791 doi:10.1111/j.1600-0706.2010.18961.x
792 75. Samuel, M.D., J.S. Hall, J.D. Brown, D.R. Goldberg, Hon IP, Baranyuk VV. The Dynamics
793 of Avian Influenza in Western Arctic Snow Geese: Implications for Annual and 3
794 Migratory Infection Patterns. Ecol Appl. 2015;25: 1851–1859.
795 76. Grear DA, Dusek RJ, Walsh DP, Hall JS. No evidence of infection or exposure to Highly
796 Pathogenic Avian Influenzas in peridomestic wildlife on an affected poultry facility. J
797 Wildl Dis. 2017;53: 37–45. doi:10.7589/2016-02-029
798 77. Shriner SA, Root JJ, Lutman MW, Kloft JM, VanDalen KK, Sullivan HJ, White TS,

Milleson MP, Hairston JL, Chandler SC, Wolf PC, Turnage CT, McCluskey BJ, Vincent AL, Torchetti MK, Gidlewski T, DeLiberto TJ. Surveillance for highly pathogenic H5 avian influenza virus in synanthropic wildlife associated with poultry farms during an acute outbreak. Sci Rep. 2016;6: 36237. doi:10.1038/srep36237

78. Feare CJ. Role of Wild Birds in the Spread of Highly Pathogenic Avian Influenza Virus H5N1 and Implications for Global Surveillance. Avian Dis. 2010;54: 201–212.

79. Kwon YK, Joh SJ, Kim MC, Lee YJ, Choi JG, Lee EK, Wee SH, Sung HW, Kwon JH, Kang MI, Kim JH. Highly pathogenic avian influenza in magpies (Pica pica sericea) in South Korea. J Wildl Dis. 2005;41: 618–23. doi:10.7589/0090-3558-41.3.618

80. Marjuki H, Wernery U, Yen HL, Franks J, Seiler P, Walker D, Krauss S, Webster RG. Isolation of highly pathogenic avian influenza H5N1 virus from saker falcons (Falco cherrug) in the Middle East. Adv Virol. 2009;2009. doi:10.1155/2009/294520

81. Marchenko AVY, Alekseev AY, Sharshov KA, Petrov VN, Silko NY, Susloparov IM, Tserennorov D, Otgonbaatar D, Savchenko IA, Shestopalov AM. Ecology of Influenza Virus in Wild Bird Populations in Central Asia. Avian Dis. 2012;56: 234–237.

82. Lam TTY, Ip HS, Ghedin E, Wentworth DE, Halpin RA, Stockwell TB, Spiro DJ, Dusek RJ, Bortner JB, Hoskins J, Bales BD, Yparraguirre DR, Holmes EC. Migratory flyway and geographical distance are barriers to the gene flow of influenza virus among North American birds. Ecol Lett. 2012;15: 24–33. doi:10.1111/j.1461-0248.2011.01703.x

83. USDA-APHIS. Epidemiologic and Other Analyses of Indiana HPAI/LPAI-Affected Poultry Flocks: Draft March 4, 2016. Fort Collins, CO; 2016.