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Serologic Evidence for Influenza A Virus Exposure in Three Loon Species Breeding in Alaska, USA

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ABSTRACT: Limited information exists about exposure to influenza A viruses (IAVs) in many wild waterbird species, including loons. We analyzed serum samples from breeding adult Pacific (*Gavia pacifica*), Red-throated (*Gavia stellata*), and Yellow-billed (*Gavia adamsii*) loons sampled at three locations along the coast of Alaska, US from 2008 to 2017 to gain a better understanding of the potential role loons play in IAV ecology. We screened loon sera for IAV antibodies using three tests—blocking enzyme-linked immunosorbent assay (bELISA), agar gel immunodiffusion (AGID), and hemagglutination inhibition (HI)—and examined patterns in seroprevalence among species and sampling locations. We found evidence of IAV infection in all loon species and at all breeding locations, although concordance was imperfect among serological tests. Diagnostic tests yielded seroprevalence estimates of 24% (42/172) with bELISA, 8% (5/60) with AGID, and 6% (4/70) with HI. The IAV subtypes to which loon sera reacted using HI were consistent with those detected in waterfowl and gulls at other locations in Alaska, suggesting that loons may be exposed to IAV maintained in sympatric waterbirds. Our study provided evidence that loons inhabiting Alaska were exposed to IAV. However, given imperfect concordance among serologic tests, and relatively low seroprevalence as compared to other avian taxa exposed to IAV in Alaska, they make poor IAV surveillance targets.

Key words: AGID, antibodies, avian influenza, bELISA, divers, influenza A virus, loons, serology.

Wild waterbirds, particularly waterfowl (order *Anseriformes*) and gulls and shorebirds (*Charadriiformes*), are recognized as the natural reservoir for influenza A viruses (IAVs; Stallknecht et al. 2007). While relatively large numbers of samples from birds of these taxonomic groups have been tested for the presence of IAV or antibodies thereto, comparatively fewer samples from less-abundant waterbird groups such as loons (family *Gaviidae*), grebes (*Podicipedidae*), rails (*Rallidae*), and herons (*Ardeidae*) have been tested for IAV or for antibodies to IAV (Stallknecht et al. 2007). Given recent evidence for prior exposure to IAVs of loons inhabiting the Arctic Coastal Plain of Alaska, US (Van Hemert et al. 2019), we aimed to further explore IAV antibody prevalence in this taxon by testing samples collected in other areas of the state and in additional years. Our objectives were to assess: 1) antibody prevalence in three species of loons sampled at three different breeding areas in Alaska, 2) antibody prevalence in juvenile birds that had not yet dispersed from areas where they hatched, 3) the presence of subtype-specific antibodies to IAV, and 4) the consistency of three routinely used serological assays for detecting IAV-specific antibodies in loon sera. Our results provided baseline information on exposure of loons to IAV, specifically, exposure of birds breeding along the northern and western coasts of Alaska.

We sampled adult Red-throated (*Gavia stellata*), Pacific (*Gavia pacifica*), and Yellow-billed (*Gavia adamsii*) loons at three general areas in Alaska from 2008 to 2017 as part of studies investigating loon breeding ecology (Table 1). These three geographical areas included the Beaufort Sea Coast (BSC), Chukchi Sea Coast (CSC), and Yukon-Kuskokwim Delta (Fig. 1). We also sampled Red-throated (n=62) and Pacific Loon (n=2) chicks along the CSC. All three study sites are characterized by low-lying tundra-graminoid vegetation and nearly continuous wetland complexes.

We captured loons during the breeding season each year from mid-June through mid-August using bow-net nest traps (Salyer

TABLE 1. Detection of influenza A virus antibodies using blocking enzyme-linked immunosorbent assay from sera collected from adult Red-throated (*Gavia stellata*), Pacific (*Gavia pacifica*), and Yellow-billed (*Gavia adamsii*) loons sampled along the Beaufort Sea Coast, Chukchi Sea Coast, and Yukon-Kuskokwim Delta in Alaska, USA, during 2008–17.

Location	Loon species	Years sampled	% Positive (*n* positive/*n* tested)
Beaufort Sea Coast	Red-throated	2010, 2012–14	23 (3/13)
	Pacific	2012–16	10 (5/48)
	Yellow-billed	2012–14	3 (1/29)
Chukchi Sea Coast	Red-throated	2008–10	45 (15/33)
	Pacific	2008–10	54 (15/28)
	Yellow-billed	2017	14 (1/7)
Yukon-Kuskokwim Delta	Pacific	2016	14 (2/14)

1962) or suspended dive nets (Uher-Koch et al. 2016). Some Red-throated Loon chicks (n=13) were captured 2–4 times within 1–12 d (mean=4.7 d between captures). From each bird, we collected up to 6 mL of whole blood by jugular or tarsal venipuncture. We allowed the blood to clot for a minimum of 2 h and then centrifuged whole blood at 3,000 × G for up to 15 min. Serum was transferred to cryovials and stored at –20 C in the field

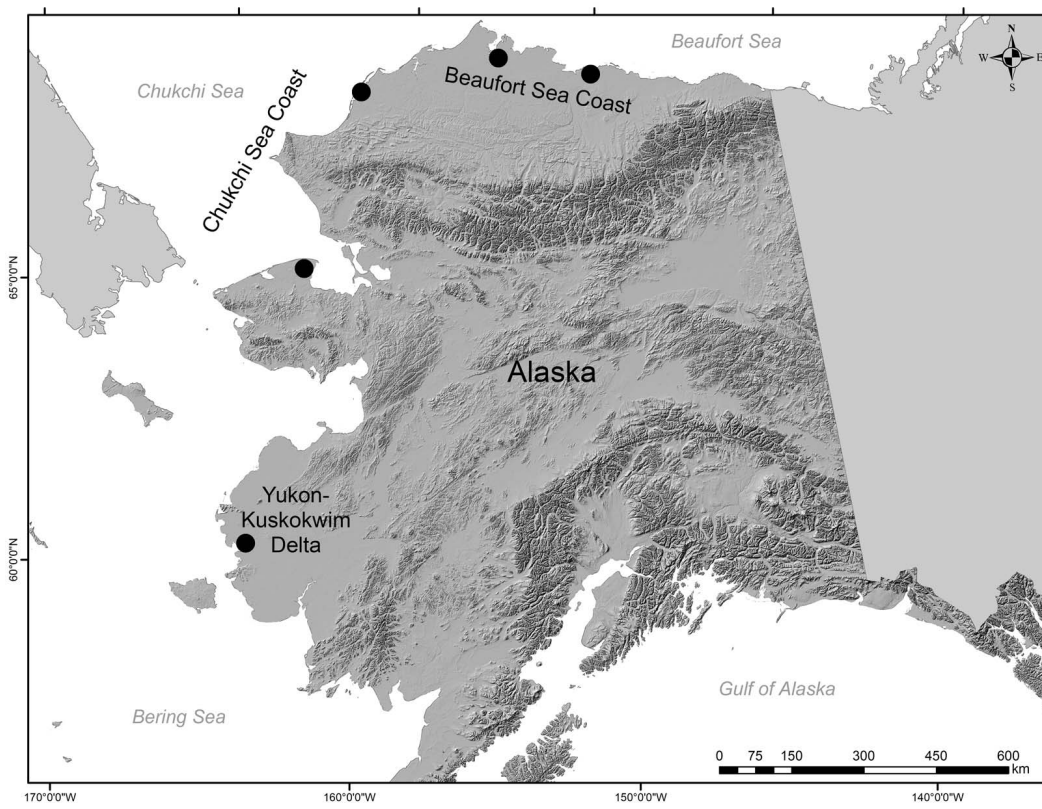

FIGURE 1. General locations (black circles) in Alaska, USA, from which sera samples were collected during 2008–17 to assess exposure of Red-throated (*Gavia stellata*), Pacific (*Gavia pacifica*), and Yellow-billed (*Gavia adamsii*) loons to influenza A virus.

and then at –80 C prior to laboratory analyses.

All sera samples (n=172) were first screened for antibodies to IAV by using a commercially available blocking enzyme-linked immunosorbent assay (bELISA; IDEXX Laboratories, Westbrook, Maine, USA) following the manufacturer's instructions. We considered the threshold of sample result to negative control (S/N) ratio <0.5 as positive for IAV antibodies per the manufacturer's recommendations and analyzed 15% of samples in duplicate for quality control and assurance. We then screened subsets of samples for antibodies to IAV using agar gel immunodiffusion (AGID; n=62) and hemagglutination inhibition (HI; n=70) assays using standard procedures (Swayne et al. 2008; Pedersen 2014). For AGID, we used standard avian influenza immunodiffusion positive and negative reference serum from the US Department of Agriculture, National Veterinary Services Laboratory (Ames, Iowa, USA) and interpreted precipitation bands as negative or positive (which included both strong positive and weak positive samples). For HI, we tested sera using antigens representing all subtypes of IAV previously identified in wild birds inhabiting North America (H1–H14, H16; Van Hemert et al. 2019). We interpreted samples with HI titers <1:40 to be positive for subtype-specific IAV antibodies. For both AGID and HI assays, we tested samples with a range of S/N values, including sera inferred as positive (<0.5) and negative (≥0.5) for IAV antibodies by bELISA. Some samples collected during 2012–16 along the BSC were included in a previous investigation (n=87 bELISA, n=20 HI; Van Hemert et al. 2019), whereas we present results for samples collected at other locations and other years (Uher-Koch 2019).

Using bELISA, we detected antibodies to IAVs in 24.4% (42/172) of loon samples (Table 1). Seropositive samples were identified in all species and sampling areas, with point estimates by species or location ranging from 3% (1/29) in Yellow-billed Loons sampled at the BSC to 54% (15/28) in Pacific Loons sampled along the CSC (Table 1). No sera from loon chicks were seropositive by bELISA upon initial capture or at recapture. Duplicate analyses of samples by bELISA (n=40) were 100% consistent in identifying seropositive sera; however, results for bELISA, AGID, and HI showed imperfect concordance (Tables 2, 3). Only one sample of the 62 tested was seropositive in all three assays. Five samples were identified as seropositive for IAV antibodies by AGID, and all five were also positive with the bELISA (Table 3). Three of four sera testing positive for IAV antibodies by HI were also positive by bELISA (Table 3). One sample was negative by bELISA and AGID but yielded HI titers <1:40 to H1 and H11 HA subtype IAVs. We used HI to identify antibodies reactive to five IAV HA subtypes (H1, H4, H5, H9, H11) in sera from four Pacific Loons. Sera from two of the four Pacific Loons reacted to antigens of multiple IAV subtypes (H4 and H5; H1 and H11). No Red-throated or Yellow-billed Loon samples were seropositive via HI.

In this study, three different serological tests provided evidence that loons breeding in Alaska were infected with IAV, and these results are consistent with those from limited screening of loon samples collected elsewhere that suggest that this taxon may commonly be exposed to IAV (Zakstelskaya et al. 1975; Iftimovici et al. 1980; Senne 2007). The detection of H1, H4, H5, H9, and H11 antibodies in Pacific Loon sera was also consistent with viral subtypes previously detected in waterfowl on the Yukon-Kuskokwim Delta (Reeves et al. 2013) and ducks, geese, and gulls at other locations in Alaska (Hill et al. 2016; Reeves et al. 2018). Loon seroprevalence point estimates in our study (3–54%) were generally lower than seroprevalences of waterfowl species sampled in Alaska, but they did overlap some waterfowl species: 36% in Black Brant (*Branta bernicla nigricans*) and 51% in Long-tailed Ducks (*Clangula hyemalis*; Wilson et al. 2013). These findings suggest that loons may serve as a common spillover host, capable of becoming infected with IAVs maintained in sympatric waterbirds. However, it is unclear if loons can maintain IAVs in the absence of previously

TABLE 2. Detection of influenza A virus antibodies in adult Red-throated (*Gavia stellata*), Pacific (*Gavia pacifica*), and Yellow-billed (*Gavia adamsii*) loons sampled in three locations in Alaska, USA, during 2008–17 using blocking enzyme-linked immunosorbent assay (bELISA), agar gel immunodiffusion (AGID), and hemagglutination inhibition (HI).

	% Seropositive (*n* positive/*n* tested)		
Loon species	bELISA	AGID	HI
Red-throated	39 (18/46)	16 (3/19)	0 (0/21)
Pacific	24 (22/90)	6 (2/32)	11 (4/35)
Yellow-billed	6 (2/36)	0 (0/11)	0 (0/14)
Total	24 (42/172)	8 (5/62)	6 (4/70)

recognized waterbird reservoir hosts since loons rarely aggregate throughout their annual cycle.

We acknowledge that a combination of small sample sizes and imperfect concordance among serologic tests for IAV antibodies limits our ability to make rigorous inference regarding patterns of IAV exposure in loons. Discordance between results from bELISA and AGID is not uncommon for IAV serology in wild bird samples and may be a function of the inconsistent performance of AGID (Stallknecht et al. 2007) and higher sensitivity of bELISA (Sullivan et al. 2009; Brown et al. 2010). It is plausible that bELISA may have been more sensitive for the detection of IAV antibodies in loon sera as compared to AGID or HI assays as evidenced by a higher proportion of samples identified as seropositive using bELISA. However, it is unknown which sera are truly seropositive using field samples, and therefore a controlled laboratory study is needed to validate diagnostic testing for loons.

Given that we detected IAV antibodies in Pacific Loon sera using all three assays, whereas there was limited evidence for IAV antibodies in Yellow-billed Loon sera (identified through bELISA only), it is plausible that differences in habitat preferences, population densities or nonbreeding distributions among loon species could have influenced the probability of prior viral exposure (Uher-Koch et al. 2014; McCloskey et al. 2018). Additionally, given higher point estimates for seroprevalence in all three species of loon sampled at the CSC as compared to the BSC, there could have been spatiotemporal variables affecting the exposure of loons to IAVs. Subsequent assessments of antibody prevalence in loons using a validated serologic approach and testing of a sample set controlling for spatiotemporal variability may be useful for identifying relevant ecological

TABLE 3. Summary of concordance among blocking enzyme-linked immunosorbent assay (bELISA), agar gel immunodiffusion (AGID), and hemagglutination inhibition (HI) to detect influenza A virus antibodies among sera from adult Red-throated (*Gavia stellata*), Pacific (*Gavia pacifica*), and Yellow-billed (*Gavia adamsii*) loons collected in Alaska, USA, during 2008–17. Serostatus was defined as either positive or negative. Proportions reflect the number of sera yielding the indicated results for two serologic assays out of the total number assessed using the indicated pair of tests. Dashes represent the same serological test so no concordance could be evaluated.

	AGID positive	AGID negative	HI positive	HI negative
bELISA positive	5/62	26/62	3/70	33/70
bELISA negative	0/62	31/62	1/70	33/70
AGID positive	—	—	1/60	4/60
AGID negative	—	—	3/60	52/60

covariates of IAV exposure. A lack of detectable antibodies to IAV in loon chicks upon initial capture and upon resampling provided no evidence for the exposure of loons to IAVs at Alaska breeding areas. However, sample sizes were small, and therefore we had limited information on which to make inferences. Given the overall low abundance and density of loons, and the remoteness of areas they commonly inhabit throughout the annual cycle, loons may make poor surveillance targets for IAVs despite our finding that they may be commonly exposed to such viruses.

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