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Seroprevalence of avian influenza in Baltic common eiders (*Somateria mollissima*) and pink-footed geese (*Anser brachyrhynchus*)

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ABSTRACT

Blood plasma was collected during 2016–2018 from healthy incubating eiders (*Somateria mollissima*, n = 183) in three Danish colonies, and healthy migrating pink-footed geese (*Anser brachyrhynchus*, n = 427) at their spring roost in Central Norway (Svalbard breeding population) and their novel flyway through the Finnish Baltic Sea (Russian breeding population). These species and flyways altogether represent terrestrial, brackish and marine ecosystems spanning from the Western to the Eastern and Northern part of the Baltic Sea. Plasma of these species was analysed for seroprevalence of specific avian influenza A (AI) antibodies to obtain information on circulating AI serotypes and exposure. Overall, antibody prevalence was 55% for the eiders and 47% for the pink-footed geese. Of AI-antibody seropositive birds, 12% (22/183) of the eiders and 3% (12/427) of the pink-footed geese had been exposed to AI of the potentially zoonotic serotypes H5 and/or H7 virus. AI seropositive samples selected at random (n = 33) showed a low frequency of serotypes H1, H6 and H9. Future projects should aim at sampling and isolating AI virus to characterize dominant serotypes and virus strains (PCR). This will increase our understanding of how AI exposure may affect health, breeding and population viability of Baltic common eiders and pink-footed geese as well as the potential spill-over to humans (zoonotic potential).

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1. Introduction

Influenza viruses are negative-sense single-stranded RNA viruses within the family of *Orthomyxoviridae* (Alexander, 2008). It holds Influenza A, B, C and D. Influenza A, B and C cause disease in humans, while only Influenza A viruses infect birds including waterfowl (Giwa et al., 2020; Lycett et al., 2019). The classification of Influenza A is based on profiling of surface proteins hemagglutinin (HA) and neuraminidase (NA), which divide the viruses into 18 and 11 subtypes, respectively (Giwa et al., 2020; Lycett et al., 2019). Waterfowl are considered a main reservoir of Avian Influenza (AI) A viruses with both low (LPAI) and high (HPAI) pathogenic subgroups; the latter causing disease outbreaks in both domestic and wild birds (Su et al., 2017; Beerens et al., 2020). So far, only H7 and H5 have been associated with HPAI, but they are not always pathogenic or cause clinical disease (Alexander, 2007). HPAI occurrence has far exceeded LPAI in flocks of wild birds although relative frequencies vary on a regional scale (Alexander, 2007; Pohlmann et al., 2019). AI appears to have the ability to infect most bird species, with the highest isolation rates found in waterfowl and birds from the *Anatidae* family which are more frequent hosts than any other family of birds (Stallknecht and Shane 1988).

Pink-footed geese and common eiders are long-distance migratory bird species that carry AI and thus represent a potential for spreading the virus over wide geographical ranges (Harris et al., 2010; Hoye et al., 2011; Pasick et al., 2007). Moreover, the finding of influenza A in seals shows the virus' potential to cross wide species barriers (Bodewes et al., 2013; Krog et al., et al., 2015).

Eiders occurring in Denmark are part of the Baltic/Wadden Sea flyway population, and has declined by 36% over the past decades (Christensen et al., 2013; Helcom, 2013; Waltho and Coulson, 2015). In addition, pink-footed geese, likely due to environmental and climatic changes, have developed novel flyways into the Russian Arctic through Finland. Therefore, these two species are particularly appropriate for the monitoring of AI in wild *Anatidae* in the Baltic. The aim of the present study was therefore to investigate AI seroprevalence including occurrence of subtypes H5 and H7 in Baltic common eiders and pink-footed geese. These two species enable a broad coverage of Baltic terrestrial, brackish and marine ecosystems spanning from the Western to the Eastern and Northern parts of the area. Samples from three Danish eider colonies and migrating pink-footed geese were selected for study. The geese were sampled when they arrived from Denmark at their spring roost in Central Norway (Svalbard breeding population) and at their new flyway in the Finnish Baltic Sea (Russian breeding population). This is the basis for assessing environmental AI exposure in two Baltic key-species.

2. Materials and methods

2.1. Field sampling

During 2016–18, we collected blood plasma from healthy incubating eiders ($n = 183$) in three Danish colonies, as well as from healthy migrating pink-footed geese ($n = 427$) at their spring roost in Central Norway and at their novel Finnish flyway. Together, these species and flyways represent terrestrial, brackish and marine ecosystems spanning from the Western to the Eastern and Northern parts of the Baltic Sea. The pink-footed geese, belonging to the Svalbard and Russian breeding populations, were caught using canon nets and sampled for their blood during their spring migration in Norway (Ekne and Skogn in Levanger) and Finland (Tyrnävä in the Oulu area) (Madsen et al., 1999; Hoye et al., 2011) (Fig. 1). Weighing was carried out using a spring scale to an accuracy of 50 g, sex determination was based on cloacal examination, and age determination was based on feather characteristics according to Elder (1955) and Gundersen et al. (2017). Female eiders were sampled during their incubation in April and May in the Danish colonies Hov Røn, Agersø and Christiansø (Fig. 1). Eiders

were caught with self-made 3 m long rackets in the vicinity of their nests in the early incubation period (Hov Røn and Agersø) while eiders at Christiansø were taken directly from their nests by hand in the phase of late incubation (Garbus et al., 2019a). Blood was taken from the brachial vein and transferred to a BD Vacutainer® Lithium Heparin and BD Vacutainer® EDTA tubes. Safety measures (e.g. gloves) were taken by the personnel and no subsequent disease was reported. Vacutainers were centrifuged within 0.5–2 h at 2500 rpm for 10 min (~839G) and the supernatant plasma was transferred to a sterile Eppendorf® tube. Samples were frozen at -20°C until further AI analyses. Body mass of individual birds was recorded with a Pesola Spring balance with 10 g accuracy. An overview of sample numbers, year and species is provided in Tables 1 and 2.

2.2. Permits, legislation and animal welfare

Wild birds and their nests, eggs and chicks are protected according to Danish law (Wildlife Management and Hunting Act; LBK nr. 735 af 14/06/2013). Permission to handle nesting female eiders and collect blood samples was therefore obtained from the Nature Agency and the Danish Ministry of Environment and Food (permission no. 2011/561–17, NST-304–0008, 2017–15-0201–01205). Similar permits were obtained from the Finnish Animal Welfare Committee ESAVI (ESAVI/1924/2018) and from the Norwegian Animal Welfare Committee Mattilsynet (FOTS ID 13979).

2.3. Serological analyses of avian influenza

All plasma samples were analysed for AI-specific antibodies using a commercial ELISA Kit (Influenza A Virus Antibody Test Kit, Idexx Montpellier SAS, France) which detects AI-specific antibodies by blocking an enzyme-labelled specific antibody in the conjugate. This blocking format was required since other available test formats do not detect antibodies from wild waterfowl species. Analyses were carried out according to the manufacturer instructions. All positive samples were then subjected to a HI (Hemagglutination-inhibition) test to detect antibodies against H5 and H7. Additionally, 33 randomly selected ELISA-positive samples were tested by HI test for antibodies against H1, H6 and H9 which have previously been found in wild waterfowl (Hanson et al., 2005; Latorre-Margalef et al., 2014). HI tests were performed according to OIE standards (OIE 2019). Briefly, plasma in a twofold dilution was coated with the respective antigen and incubated with erythrocytes from SPF chickens after 30 min. Titers were assessed by determining the highest dilution of complete inhibition of the test antigen by agglutination. The data are shown in Table S1.

2.4. Statistical analyses

In order to make full use of the dataset, we constructed linear models to investigate how sampling location, age and sex affected weight and seroprevalence of pink-footed geese. First, we fitted a linear model with body weight of individuals as a function of four factors: sampling location, age, sex and seroprevalence. We also fitted a linear logistic model where the seroprevalence was a function of three factors: sampling location, age and sex. Then, we used the function stepAIC to eliminate predictor variables that did not significantly contribute to a better fit of the model (Table S2) and subsequently used the coefficient estimates in the results section. All statistical analyses were performed in the statistical software program R (R Core Team, 2019).

3. Results

3.1. Biometrics

Biometrical information for pink-footed geese and common eiders included in the present study is shown in Table 1. All common eiders



Fig. 1. Sampling sites for common eiders (Hov Røn, Agersø and Christiansø) and pink-footed geese (Levanger and Oulu) spanning from the Western to the Eastern and Northern part of the Baltic Sea.

Table 1
Weight, age group, sex distribution and AI prevalence of pink-footed geese caught during spring (April–May) 2016–2018 in Norway and Finland.

| Country | Area | Year | n | Weight (g) | | | | Age | | Sex M/F | AI prevalence (%) | | | |
|---------|----------|------|-----|------------|-----|------|------|-------|----------|---------------------|-------------------|----|-------|-----------|
| | | | | Mean | SD | min | max | Adult | Juvenile | | F | M | Adult | Juvenile |
| Norway | Levanger | 2016 | 174 | 3105 | 275 | 2250 | 3800 | 173 | 1 | 101/73 | 54.8 | 45 | 49 | 0 (n = 1) |
| Norway | Levanger | 2017 | 243 | 3411 | 304 | 2550 | 4300 | 235 | 8 | 146/89 ^a | 51.7 | 45 | 46 | 75 |
| Finland | Oulu | 2018 | 10 | 3235 | 283 | 2800 | 3650 | 10 | 0 | 5/5 | 40 | 60 | 50 | – |

– Juveniles not caught.

^a Sex not recorded in eight animals.

Table 2
Avian influenza (AI) in eiders and pink-footed geese sampled in the Baltic 2016–2018. Juv: juvenile, F: female, M: male.

| Species | Age group | Sex | Country | Area | Year | Season | n | Seropositive (%) | H5 and/or H7 (%) | H5 + 7 (%) | H5 (%) | H7 (%) |
|-------------------|-----------|-----|---------|-------------|------|--------------|-----|------------------|------------------|------------|--------|---------|
| Eiders | Adult | F | Denmark | Christiansø | 2016 | Spring | 21 | 7 (33) | 1 (5) | 0 | 0 | 1 (5) |
| | Adult | F | Denmark | Christiansø | 2017 | Spring | 28 | 20 (71) | 3 (11) | 0 | 3 (11) | 0 |
| | Adult | F | Denmark | Agersø | 2018 | Spring | 29 | 18 (62) | 2 (7) | 1 (3) | 1 (3) | 2 (7) |
| | Adult | F | Denmark | Hov Røn | 2018 | Spring | 82 | 41 (50) | 12 (15) | 4 (5) | 9 (11) | 7 (9) |
| | Adult | F | Denmark | Christiansø | 2018 | Spring | 23 | 15 (65) | 4 (17) | 1 (4) | 3 (13) | 2 (9) |
| | | | | | | Total | 183 | 101 (55) | 22 (12) | 6 (3) | 16 (9) | 12 (7) |
| Pink-footed geese | Juv/adult | F/M | Norway | Levanger | 2016 | Spring | 174 | 85 (49) | 3 (2) | 1 (1) | 3 (2) | 1 (0.6) |
| | Juv/adult | F/M | Norway | Levanger | 2017 | Spring | 243 | 112 (46) | 8 (3) | 0 | 8 (3) | 0 |
| | Juv/adult | F/M | Finland | Oulu | 2018 | Spring | 10 | 5 (50) | 1 (10) | 0 | 1 (10) | 0 |
| | | | | | | Total | 427 | 202 (47) | 12 (3) | 1 (0.2) | 12 (3) | 1 (0.2) |

(n = 183) were adult females. Their weight varied highly from early to late phase of incubation, and since our samples constitute a mix, weight statistics are not included herein. The sampled population of pink-footed geese (n = 427) were dominated by adults (98%) and males (60%). During this study, no birds were found dead or showed any clinical signs of respiratory disease, such as sneezing, coughing, or other related signs or symptoms of active avian influenza infection such as loss of coordination, swollen head and conjunctivitis. Furthermore, no decrease in egg production was observed during 2016–2018 and only two deformed eggs were observed (one “dwarf” egg and one blue coloured eggshell) which is not an uncommon observation in eider colonies.

Body weight of sampled pink-footed geese differed between sampling locations/years (Table S2). According to the selected linear model estimates (mean \pm 95% CI), body weight in Finland sampled in the period 2016–2018 was 3066 \pm 166 g while that sampled in Steinskjer was 2923 \pm 56 g and 3236 \pm 53 g in 2016 and 2017, respectively. On average, juvenile birds were 438 \pm 174 g lighter than adults and males were 212 \pm 51.4 g heavier than females. Interestingly, the body weight of AI seropositive individuals was 125 \pm 50 g higher than that recorded for seronegative individuals.

3.2. Seroprevalence

AI Seroprevalence in pink-footed geese and common eiders is shown in Tables 1 and 2. Seroprevalence ranged between 33 and 71% for eiders and fluctuated among years and colonies. For pink-footed geese, it was more homogenous, ranging 46–50% between locations and year of sampling. There was no effect on AI seroprevalence with respect to sampling location, age or sex (Table 1).

Overall, the prevalence of antibodies against hemagglutinins H5 and H7 were low for both pink-footed geese and common eiders (Table 2). The specific serotype was not detected in 77 of the 101 seropositive common eiders (76%) and in 182 of the 202 seropositive pink-footed geese (90%). Of 183 eiders analysed, 12% (n = 22) were seropositive for antibodies against H5 and/or H7 while the number for the 427 pink-footed geese was only 3% (n = 12). H5/H7 co-seropositivity was rare in pink-footed geese (0.2%; i.e. one individual), while it occurred in 3% (six individuals) of common eiders. The highest seroprevalence of H5 and/or H7 was found among 23 adult female common eiders sampled at Christiansø in 2018 where the number was 17% (four individuals) (Table 2).

Forty-eight randomly selected AI positive samples, comprising 23 common eiders and 25 pink-footed geese all being H5/H7 seronegative, were screened for antibodies against H1, H6 and H9. This showed that 30% of the eiders (seven individuals) and 24% of the geese (six individuals) were positive for one or more of these serotypes.

4. Discussion

4.1. AI prevalence and serotypes

In this study, the overall AI seroprevalence was around 50% for both pink-footed geese and common eiders. This proportion is similar to previous studies of 1550 eiders and 319 pink-footed geese sampled in the North Atlantic (Hall et al., 2015; Hoye et al., 2011). The overall prevalence in this study, ranging from 2 to 17% for H5/H7, indicates that these two serotypes are fairly common in the two flyway populations. Specific serotype could however not be determined in the majority of samples. Serotypes H1, H6 and H9 were tested in a subset of samples to allow for any indication of dominant serotypes among these three, but revealed a low prevalence overall. Common serotypes in waterfowl generally include H2, H3 and H4, and these may have been involved in the overall high AI seropositivity found in our study (Hanson et al., et al., 2005; Latorre-Margalef et al., 2014). Answers are nevertheless pending as to which specific AI serotypes predominate in

the two flyway populations included herein. Investigations using direct virus demonstration and molecular methods are therefore recommended, as well as alignments and comparisons between ELISA-based seroprevalence and PCR-based methods which can inform on past and/or present AI exposure including specific serotype profiling. Previous studies using RT-PCR have shown active infections of 5.4% in European and Scandinavian eiders (n = 37) and 2.1% in pink-footed geese (n = 285) (Munster et al., 2007). In a study of North Atlantic eiders (n = 1550), AI seroprevalence was 61% while RT-PCR only detected active infection in 0.4%, reflecting the ability of serology to report on both active and past infections. This means, that in general, significantly lower proportion of birds actively carry and shed the virus than what the seroprevalence reflects (Hall et al., 2015). Similar seroprevalence are reported for ducks and migratory waterfowl across European ecosystems and species over the past decades (Astorga et al., 1994; de Marco et al., 2003, 2004; Gronosova et al., 2008).

4.2. AI disease dynamics

Dynamics of AI in waterfowl, such as eiders and geese, are sensitive to a number of parameters such as age, season, sampling location, species and immunity. In the present study, AI seropositive pink-footed geese presented with a higher mean weight compared to seronegative birds. We did not achieve data on the specific age of the birds, however, the reason for higher mean weight is likely that seropositive individuals are older thus heavier. Likelihood of encountering AI and developing subsequent seroconversion and humoral protection detectable by serology is likely higher the older the bird becomes, particularly if seroconversion persists (Hill et al., 2016; Wilson et al., 2013). This hypothesis is untested, but in mature birds, antibodies may persist for a year, while maternal antibodies persist for only up to 5 weeks which may explain higher AI mortality in young geese (Dirsmith et al., 2018; Hoye et al., 2011; Pasick et al., 2007; Verhagen et al., 2015).

Waterfowl are the most important AI reservoir species. They are exposed to AI throughout their life-span, but despite this exposure, clinical illness is rarely observed (Haider et al., 2017). Seasonal variation in AI seroprevalence has been shown in pink-footed geese sampled along their flyway from the Netherlands to Svalbard where seropositivity declined within a year (Hoye et al., 2011). Other studies have also shown that AI vary among years and geographic location which is also reflected in the year and colony differences ranging 33–71% for the 3 Baltic eider colonies herein (Endo and Nishiura 2018; Wilson et al., 2013). Another important factor for AI spread and exposure, for ducks especially, is faecal transmission, e.g. transmission via fecally contaminated water bodies. This has been suggested as a central characteristic making waterfowl (ducks in particular) important reservoirs of influenza A viruses (Webster et al., 1978).

By definition, LPAI represents low pathogenicity in waterfowl. However, highly pathogenic H5N8 subtype are detected in Europe and it is important to survey for its presence in Baltic pink-footed geese and eiders due to their increased susceptibility posed by novel flyway migration routes and declining populations (Grund et al., 2018; Helcom 2013). Furthermore, high seroprevalence found in the present study is in a sense ‘good news’ as they inform that the animals have survived past infections and now may carry protective antibodies against HPAI H5N1 (Arnold et al., 2018; Berhane et al., 2014; Koethe et al., 2020). Other factors, such as colony composition and size, also affect AI seroprevalence (Torrontegi et al., 2019). But colony composition and size are also known to be affected by other stressors, such as pollution and climate change which challenges an in-depth understanding of how AI epizootiology affects migratory waterfowl (Sonnie et al., 2012).

4.3. Considerations

The present study shows an overall seroprevalence of AI of approximately 50% for both pink-footed geese and common eiders of

Scandinavia and the Baltic, of which up to 17% of the birds were H5 and/or H7 seropositive. All antigenic subtypes of influenza A (Hemagglutinins H1-16 and Neuraminidase N1-9) spread via migratory waterfowl, like Baltic eiders and pink-footed geese, but display variable pathogenicity (Garamszegi and Møller, 2007; Hubálek 2004). Most infections are caused by LPAI viruses and remain subclinical or only cause mild disease (Alexander, 2008), while infections by HPAI, such as H5N1-Asia, leads to high mortality rates (Kalthoff et al., 2010). It is, however, unclear to what extent exposure of Baltic eiders and geese to AI may affect mortality and population dynamics of the flyways. The overall high AI seroprevalence is interesting as it adds to knowledge concerning health stressors in eiders and pink-footed geese – species already exposed to additional and additive natural and anthropogenic stressors in one of the most contaminated seas of the World, the Baltic Sea (Cederqvist et al., 2019). Important contaminants include for example polychlorinated biphenyls (PCBs) and lead (Pb) which are known to increase DNA lesions and reduce viability and reproduction of eiders (Fenstad et al., 2016; Garbus et al., 2018a, 2018b, 2019b; Lam et al., 2020).

Over the years, AI has received a lot of attentions because of its potential to cause high mortality in birds and spill-over to humans (Bailey et al., 2018). Currently, a number of influenza A subtypes have a zoonotic potential as seen for H5N1 (1997 and 2003 in Asia), H7N7 (2003 in Holland) and H7N9 (2016–17 in China) outbreaks (Naguib et al., 2019; Su et al., 2017). Previously reported low active infection rates, commonly between 1 and 6% in populations with similar high overall seroprevalence to our study, suggest that active infections with H5 or H7 subtypes are uncommon in the two species tested herein, for which seroprevalence (most likely represented by previous infections) was three and 12 %, in eiders and pink-footed geese, respectively (Bailey et al., 2018). Virus isolation and PCR-based methods are needed to resolve whether virus subtypes found in pink-footed geese and eiders pose a zoonotic risk to biologists, hunters and other people in frequent contact with these birds. It is highly difficult for these people, through observation alone, to differentiate between infected and non-infected wild birds, and they should take precautions when working with (Baltic) waterfowl to lower the risk of virus transmission.

5. Conclusions

We found an AI seroprevalence of 55% in eiders and 47% in pink-footed geese. Analyses determined that a different serotypes were involved, many unknown, but including H5 and H7. PCR-based analyses and virus isolation from live, infected or dead birds are required to determine serotypes more specifically. This will further increase the understanding of the health and potential stressors for Baltic eiders and pink-footed geese, as well as AI zoonotic potential risk towards humans.

CRediT authorship contribution statement

Su Shiung Lam: Writing - review & editing. **Rune Skjold Tjørnløv:** Writing - review & editing, Software, Data curation. **Ole Roland Therkildsen:** Writing - review & editing, Funding acquisition, Investigation. **Thomas Kjær Christensen:** Writing - review & editing, Methodology. **Jesper Madsen:** Writing - review & editing, Investigation. **Tobiafs Daugaard-Petersen:** Writing - review & editing. **Jose Maria Castaño Ortiz:** Writing - review & editing. **Wanxi Peng:** Writing - review & editing. **Maël Charbonneau:** Writing - review & editing. **Esteban Iglesias Rivas:** Writing - review & editing. **Svend-Erik Garbus:** Writing - review & editing, Investigation. **Peter Lyngs:** Writing - review & editing, Investigation. **Ursula Siebert:** Writing - review & editing. **Rune Dietz:** Writing - review & editing, Funding acquisition. **Kristina Maier-Sam:** Writing - review & editing, Methodology, Investigation. **Michael Lierz:** Writing - review & editing, Methodology, Investigation. **Ingunn M. Tombre:** Writing - review & editing, Investigation. **Emilie Andersen-Ranberg:** Writing - review &

editing, Software, Data curation. **Christian Sonne:** Project administration, Conceptualization, Methodology, Funding acquisition, Writing - original draft, Writing - review & editing, Data curation.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

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