MASTER THESIS

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Investigating avian influenza A infections in a predator-prey system, resident tawny owls (*Strix aluco*) and migrating passerine birds in central Norway

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Abstract: English

Climate change is changing migration patterns of transitory species, which in turn changes pathogen spread. Northern regions are warming at a faster rate than the rest of the planet and consist of less ice than before, which are making previously un-colonisable areas more accessible for pathogens. Continued habitat destruction is also affecting the environment, putting humans and animals in closer proximity than ever before, facilitating spill-over risk of pathogens from humans to animals and vice versa. Investigating the prevalence of pathogens in northern ecosystems, where wildlife and humans lives close together, is therefore important due to a changing climate and from a One Health perspective.

Pathogens associated with migrating species pose a threat for resident wildlife species, e.g., through transfer via the food chain. To investigate the prevalence and exposure route of an important pathogen, type A Avian Influenza Viruses (AIVs), into ecosystems in central Norway, this thesis focused on a predator/prey system of migrating passerine birds (the prey and potential reservoirs) and the top predator the tawny owl (*Strix aluco*), a resident species. For pathogen detection, swabs with viral transport media were used to take samples from both the cloaca and the oropharyngeal region of juveniles and adult *S. aluco* (adults n = 43; juveniles n = 28) and passerine birds (adults n = 30; juveniles n = 32). For pathogen detection, RNA/DNA extraction and qPCR was completed for each swab. The results showed no evidence of active AIV infections in any *S. aluco*, nor any passerines sampled. This is the first dedicated study of AIV in *S. aluco* and passerines in central Norway. It provides critical baseline knowledge from a One Health perspective, especially in light of recent (autumn 2020) AIV outbreaks in Europe, as well as managing infectious diseases in wildlife.

This extended summary is presenting an extended edition of the research article: “Resident tawny owls (*Strix aluco*) and migratory passerine birds in central Norway show no signs of avian influenza A virus in 2021 despite severe outbreaks in Europe”. Journal chosen for submission is BMC Veterinary research.

Abstrakt: Norsk

Klimaendringer har vist seg å endre migrasjonsmønstre hos migrerende arter, noe som igjen kan påvirke utbredelse av patogener. Nordlige områder har en raskere temperaturøkning enn resten av planeten, og arealene med isdekke reduseres. Dette fører til at områder som tidligere ikke ga muligheter for kolonisering, blir mer attraktive for patogener. Arealbeslag og ødeleggelse av habitat fører mennesker og dyr nærmere hverandre og dette øker risikoen for overføring av patogener fra mennesker til dyr, og omvendt. På bakgrunn av endringer i klima, og fra et En Helse-perspektiv, er det derfor viktig å undersøke forekomsten av patogener i nordlige økosystemer, hvor dyreliv og mennesker lever tett sammen..

Patogener knyttet til migrerende arter utgjør en trussel for lokale arter, for eksempel gjennom smittespredning via næringskjeden. I denne oppgaven er utbredelse og eksponeringsveiene for fugleinfluensa A virus (AIVs) i økosystemer i Midt-Norge studert. Hovedfokuset i oppgaven er å undersøke predator-/byttedyrsystemet mellom spurvefugler (bytte og mulige AIV reservoarer) og en toppredator, kattugle (*Strix aluco*), som er en stasjonær art i Trøndelag. For påvisning av patogen ble svabre med viralt transportmedium brukt til å ta prøver fra både kloakk og orofaryngeal-regionen til unger og voksne *S. aluco* (voksne n = 43; unger n = 28) og spurvefugler (voksne n = 30; unger n = 32). For patogendeteksjon ble RNA/DNA-ekstraksjon og qPCR utført på hver svaber. Resultatene viste ingen tegn til aktive AIV-infeksjoner hos verken *S. aluco* eller hos de spurvefuglene som ble testet. Studiet representerer den første undersøkelsen av AIV hos *S. aluco* og spurvefugl i Midt-Norge. Det gir grunnleggende kunnskap fra et En Helse-perspektiv, spesielt i lys av nylige (høst 2020) AIV-utbrudd i Europa, samt at det gir kunnskap om håndtering av smittsomme sykdommer hos ville dyr.

Sammendraget representerer en utvidet utgave av forskningsartikkelen: «Resident tawny owls (*Strix aluco*) and migratory passerine birds in central Norway show no signs of avian influenza A virus in 2021 despite severe outbreaks in Europe». Tidsskrift valgt for innsending av artikkel er BMC Veterinary research.

Preface

I acknowledge my supervisor Courtney Alice Waugh, and co-supervisor Ingvild Buran Kroglund for helping me carry out my master's thesis. Further, I acknowledge the cooperation with the One Health course at Nord University and all the students helping with sample collection and laboratory work. I would also thank Erik Fagervik Gaden for contributing to field, photos, and creating a map of the study area. A great thanks to Jan-Erik Frisli and the monitoring program of *S. aluco* in central Norway in association with Birdlife Norway, and the Norwegian Veterinary Institute for analysing the *S. aluco* samples.

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Extended summary

Introduction

Due to climate change, bird migration flyways are changing, which results in pathogens spreading to new areas (Hasle et al., 2009; Hoye et al., 2011; Weber & Stilianakis, 2007). Hence the cold climate in the northern areas, there are fewer species habituating on these grounds which makes competition lower in these regions (Hartley, 2019). However, northern areas are warming at an alarming rate and are consisting of less ice and snow, making northern territories more preferable for new migratory species and pathogens (Samal, 2019a). This article and extended summary address this prominent problem by looking into the prevalence of Avian Influenza A viruses (AIVs) in a predator-prey system between the tawny owl (*Strix aluco*) and their prey of passerine birds, in central Norway. The article presents a short version of the thesis written for publication in BMC Veterinary research, while the extended summary elaborates on the background, results, and discussion in a broader context. The extended summary will also elaborate more on AIV infections that has been discovered in Norway thus far and discuss ideas for further research and management.

The article is formed after the journal of choice, BMC Veterinary research. This is an open access journal with focuses on infectious diseases and host-pathogen interactions in animals. The journal also focuses on studies on zoonotic and emerging infections, which makes this baseline article highly suited for the journal.

One Health and Avian Influenza A viruses

The health of people, animals and the environment are closely linked to one another, and continued habitat destruction is putting humans and animals in closer proximity than ever before (World Health Organization, 2012). The One Health concept has grown in significance in recent years, with a systematic response to global health threats linked to climate change, decline in biodiversity, antimicrobial resistance, and of course emerging and endemic infectious diseases in focus (Nguyen-Viet et al., 2022). It is estimated that 60% of all human viruses can be transmitted from animals to humans through food, water, or the environment (World Health Organization, 2012). Birds are the main reservoir and carrier of AIVs, but the virus has zoonotic (epizootic) potential and can in certain cases transfer to humans and other mammals. However, AIV transmission to humans and mammals is not that common (Alexander, 2007; Liu et al., 2017) , which makes AIVs zoonotic behaviour quite interesting.

AIVs are divided into subtypes which are based on the two surface proteins of the virus: hemagglutinin (HA) and neuraminidase (NA). There are 16 HA and 9 NA subtypes, which has been detected in wild birds, domestic poultry, and mammals (Alexander, 2000). The two main subtypes of AIVs are the highly pathogenic avian influenza (HPAI) and the low pathogenic avian influenza (LPAI). The main difference between these main subtypes is that LPAI has local replication, were the HA protein of LPAI can only be cleaved by trypsin-like protein which is primarily found in the respiratory and the enteric tracts (Suarez & Schultz-Cherry, 2000). Further, LPAI have three surface proteins (hemagglutinin, neuraminidase, and matrix 2 protein) which can induce a protective immune response in the host through antibody production (Hoye et al., 2011). HA proteins on HPAI on the other hand, can be cleaved by endogenous proteases, which are found in most cells of the body. This results in a systemic replication and infection, which alters the pathogenicity (Suarez & Schultz-Cherry, 2000).

There are documented incidences of pathogen transfer from wild birds to domestic birds, to farm animals, and to humans (Liu et al., 2017). One of the latest zoonotic incidence (2021) in mainland China resulted in two human fatalities and three hospitalizations from a HPAI infection caused by the subtype H5N6 (Lee, 2022). All of them had been working closely to domestic poultry (*Gallus gallus*). In November 2021, UK had their largest outbreak of HPAI subtype H5N1 in domestic poultry flocks (Department of Environment, 2022). In January (2022), one person was infected with a subtype of H5, believed to be H5N1, but the person did not need medical assistance at a hospital (Roberts, 2022). Further, there are also documented marine mammal die offs due to AIV infections (Kim, 2018; Shin et al., 2019), an example of how AIV can transmit to mammals and cause mass-mortalities.

Epidemiology and Avian Influenza A viruses

Avian influenza A viruses (AIVs) have a wide host range, were both wild birds and poultry work as reservoirs and carriers of the virus (Kaleta & Taday, 2003; Samal, 2019b). In Scandinavia, most of the studies on AIV prevalence are on poultry (*G. gallus*) (Adlhoch et al., 2021; Brun, 2005) or wild marine bird species (Verhagen et al., 2021), with only a few studies on detection of pathogens in raptors (Krone et al., 2018; Lee et al., 2019; Samal, 2019a). This could have created a sampling bias, where it seems like some wild bird species are less likely to be exposed to AIV, but it may just be a lack of sampling efforts. AIVs have historically been associated with marine bird species like *Anseriforms*, *Charadriiforms* and *Passeriformes*, were the most consistent reservoir of AIVs is ducks, geese, and gull species (Adlhoch et al., 2020; Alexander, 2006). The virus transfers through the faecal-oral route and

under the right conditions, AIV in excrement can survive several days in sediments and lake water (Hoye et al., 2011). These birds are therefore specially exposed to AIVs through their foraging strategy, were they are in constant contact with potentially infected sediments or water when they feed (Samal, 2019a).

Antibody studies on AIVs shows that there are many bird species exposed to AIVs during their lifetime, yet their infections are not associated with clinical disease nor mortality (Samal, 2019b). These incidences are due to LPAI, and usually occurs in populations were AIV is endemic (Samal, 2019b). However, species that are not endemic to the infection, could suffer lethal consequences. Raptors, for instance, are usually not associated with AIV infections, but in recent years they have experienced increasing incidents of HPAIs infections associated with neurological disease and death around the globe (Krone et al., 2018; OIE, 2021; Samal, 2019a; Swayne, 2017). Most of the raptors studied for diseases have been captive or breed birds (Alexander, 2000), but in recent years pathogen prevalence and host-pathogen effects in wild raptor species have been investigated (Krone et al., 2018; Lee et al., 2019).

Unlike aquatic birds, raptors do not have a documented history of AIV infections (Swayne, 2017), so symptoms, mortality rates and exposure routes are still under investigation. Raptors can potentially become infected by preying on other bird species; systemic infection caused by HPAI causes neurological symptoms and thus they are easier prey. Since it could take weeks until an infected bird dies of HPAI (Lu et al., 2003; Swayne, 2017), the raptor may be more likely to feed on infected prey which are easier to catch.

In a study conducted by Shearn-Bochsler et al (2019) it was stated that HPAI clade 2.3.4.4 H5N2 and H5N8 viruses caused severe systematic disease in infected raptors. They detected mortality in six raptor species, were all of them had injuries by necrosis in heart, pancreas, lung and the brain (Shearn-Bochsler et al., 2019). The same clade of AIV was found in white-tailed eagles (*Haliaeetus albicilla*) (2016, 2017) in Germany which showed mild to severe neurological symptoms (limber neck, movement in circles, ataxia, and torticollis). The same year, *H. albicilla* was found positive for HPAI H5N8 in both Finland and Denmark (Krone et al., 2018). Raptors infected with HPAI seem likely to suffer from physiological symptoms due to necrosis, and it seems possible to predict HPAI infection due to behaviour.

Species of interest

The tawny owl (*S. aluco*), is a resident apex predator, which makes it sensitive to local environmental changes (Badry et al., 2020). Its abundance is relatively high over Europe,

except for Ireland and Iceland; and Grong, Trøndelag (central Norway) is the northern boundary of the distribution range (Badry et al., 2020; Olsen, 2007). This makes it an excellent sentinel species for monitoring pathogen prevalence worldwide. *S. aluco* has a wide habitat niche, they can be found nesting in urban areas close to humans, but prefers farmlands with patched forests (Badry et al., 2020), which makes them a focus for One Health. They easily nest in human made nestboxes, which gives researchers access to many individuals and makes *S. aluco* suitable for monitoring pathogens in local terrestrial ecosystems.

S. aluco's health and habituation are important to monitor, since this species is sensitive to environmental change like climate change, pollutants, invasive species, and pathogens. There has been documented different pathogens in *S. aluco* all over Europe; *Chlamydia psittaci* (Italy, 2020, Germany, 2001) (OIE, 2022; Stokes et al., 2021), two subtypes of herpesvirus FHV-1 and StHV-1 (UK, 2004) (Zsivanovits et al., 2004), and AIV (Russia, 2017, Denmark, 2020, and Sweden, 2021) (OIE, 2022). Disease transfer of herpesvirus is depending on species behaviour, were it seems that the main transfer is through ingestion of prey species (Zsivanovits et al., 2004) or intraspecific from adults to young (Žlabravec et al., 2021). However, since *S. aluco* usually feeds on small rodents over smaller birds when this is possible, it seems like it is not that exposed to herpesviruses compared to other raptors (falcons, eagles, and hawks) (Žlabravec et al., 2021; Zsivanovits et al., 2004). As for *Chlamydia spp*, they transmit trough air contamination, feathers, and excrements (Stokes et al., 2021).

The species are also sensitive to anticoagulant rodenticides and toxic metals (lead and mercury). Anthropogenic uses such as mining, metal production and farming poses threats when it comes to use and production of lead and rodenticides (Badry et al., 2020). Raptors are therefore exposed secondarily to these pollutants through their prey (especially rodents). From 1986 to 2004, *S. aluco* in central Norway was monitored for organochlorines and brominated flame retardants (Yoccoz et al., 2009). According to the results, it was only the p,p′-DDE (1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene) that had effect on the owls fitness, were it affected the thickness of the eggshells (Yoccoz et al., 2009). Pollution concentrations in the ecosystem in this time period was too low to affect reproduction and survival of the population, probably since *S. aluco* consumes prey at a lower trophic level compared to other raptors (Yoccoz et al., 2009). However, *S. aluco* is a key sentinel species of environmental health and thus can act as an early warning system for terrestrial ecosystems were wildlife and humans live closely together from a One Health perspective.

S. aluco usually hunt in open landscapes, where they are often observed at open farming fields. These farming fields are also used by other birds such as geese, gulls, ducks, doves and passerines, and domestic poultry which are all possible carriers of AIVs (Adlhoch et al., 2020; Stokes et al., 2021). Due to *S. aluco's* foraging behaviour and preferred habitat, it could be hypothesised that this species therefore may be prone to AIV infection, if it should be circulating in the environment. However, there is a complete lack of empirical data on the pathogen status of *S. aluco* in Norway.

S. aluco is a generalist, that prefers to prey on rodents and different passerine birds (Badry et al., 2020; Ján, 2011). Most of the passerines breeding in Norway are migratory bird species and usually migrate further south in Europe or follow the African-Eurasian flyway to Africa (Hasle et al., 2009; Svensson et al., 2011). In the last couple of years there has been an increasing number of HPAI infected passerines in central Europe (mostly northern Germany, Denmark, and northern Netherlands) (Adlhoch et al., 2021; Department of Environment, 2022), this despite passerines not being historically counted as a reservoir for AIV infections (Adlhoch et al., 2021). From EFSAs (European food safety authority) report on AIV infections trough 2021, 47 wild bird species were reported infected with AIVs in Europe; 19/305 waterfowl species, 7/43 raptor species, and 21/73 other bird species (Adlhoch et al., 2021). Among documented passerines infected in Europe in 2021 was nine sparrows, two magpies, a starling and one *Turdidae* species (Adlhoch et al., 2021), all in central Europe (from UK to Russia).

AIV has been reported to be spread across countries by migratory species working as vectors (Hoye et al., 2011; Samal, 2019b; Weber & Stilianakis, 2007), for example introduction of HPAI in *G. gallus* in Rogaland (Norway) was most likely transported through wild birds from Russia (Moldal, 2021). There is also evidence that colonially nesting birds are more likely to spread diseases during reproduction compared to solitary breeders (Lemus et al., 2010). This due to the degree of interaction between colonially nesting birds versus solitary breeders, whereas colonial breeders are usually social mixing through mating, nesting, and parental care (Arroyo et al., 2001; Ritchie et al., 2021). During the 2021 migration season, both colonial and solitary breeders of passerine birds was tested for AIV infections. *S. aluco*, on the other hand, is a solitary species, but arrival of new emerging diseases such as HPAIs could potentially cause severe ecological effects on the species, given the sensitivity of raptors to HPAI associated disease.

One of the most common passerines for *S. aluco* to feed on is the fieldfare (*Turdus Pilaris*) (personal communication, Østnes, 2020, Nord University), a common species in central Norway belonging in Turdidae (Bozó, 2019; Olsen, 2007). *T. pilaris* is a migratory species that are mostly wintering in the Western Palearctic (Svensson et al., 2011). Amongst the other passerines sampled, the european greenfinch (*Carduelis chloris*) and the brambling (*Fringilla montifringilla*) are also migrating further south in the winter months. *F. montifringilla* usually migrates to southern Europe and northern Africa, whilst *C. chloris* migrates to the north of Europe and England (Svensson et al., 2011). As for the great tit (*Parus major*), blue tit (*Cyanistes caeruleus*) and yellowhammer (*Emberiza citronella*), there are only a few individuals that are migrating further south during the winter times. All depending on temperatures, food supply and habitat access.

When starting this project, no active AIV infections had been detected in bird species in Norway, and only seroprevalence was found in a couple of waterfowl species in central Norway and at Svalbard (Hoye et al., 2011; Lam et al., 2020). At this time, seroprevalence studies on white-tailed eagle (*H. albicilla)* and northern goshawk (*Accipiter gentilis*) sampled in 2016-2017 had all been negative (Lee et al., 2019), which means that the *H. albicilla* and *A. gentilis* in central Norway had not been in contact with AIV before. Even though it is more common for aquatic bird species to host AIV, this study focused on the terrestrial S. *aluco* habituating the same areas as reservoir waterfowl species (e.g., geese and gulls). Infection and health status of *S. aluco* could also give information about the health of the ecosystem nearby human settlements. In this way, it's possible to monitor prevalence of infectious diseases that could have an economic impact, as well as health impact on humans and other species.

Pathogen transfer and sampling

AIVs is usually spread through faecal contamination but could also transfer through airborne transmission and feed. Contaminated surfaces e.g., soil, water, skin, and feathers could also trigger transmission (Samal, 2019a). Sexually transmitted diseases (STDs) are associated with pathogens found in the avian cloaca and could be passed between partners during copulation. During breeding season, almost all pathogens from the avian gut could potentially be transmitted through the cloaca ((Benskin et al., 2009; Lombardo, 1998). There are only a few studies done on AIV transmission during copulation (ducks, quail, and turkeys) (Cardona et al., 2021; Hegyi et al., 2009), but since AIV transmits through the faecal route (cloaca), there is to believe that breeding behaviour could trigger AIV transfer during breeding season. However, transmission of AIVs through infected males' semen to naïve female is still

unclear. The risk of transmission during copulation is nevertheless high due to direct contact between the female and male's faecal route.

In regard to infectious disease dynamics, *T. pilaris* are a special case. As well as they are migratory species, *T. pilaris* also usually nest in colonies (Kleven et al., 2019). Colonial nesting species tend to be more prone of pathogen exposure due to the degree of interaction between different individuals in the population. Hence, social monogamy is quite common among passerine birds (Arroyo et al., 2001; Ritchie et al., 2021). Pathogens do not spread through copulation when monogamy is maintained (Lombardo, 1998), but due to *T. pilaris* behaviour with social mixing there is a higher probability for pathogen transfer between successful males and females during the breeding seasons. Further, social monogamy results in that one clutch most likely has several fathers (extra-pair paternity) (Haas, 1985; Kleven et al., 2019). This could affect nestlings' immune system and exposure effects of pathogens due to antibody transfer between female and her young (Samal, 2019a). Since nestlings in the same nest could have different fathers, every *T. pilaris* nestling in each nest was sampled.

Secondly, *T. pilaris* prefer feeding on small invertebrates living in soil, especially earthworms (Shukshina, 2020). Correlation between pathogens in the avian gut and pathogen found at the foraging grounds and in food is associated with species foraging behaviour. Ground-foraging species are in danger of ingesting food contaminated with AIV by birds and other animals' droppings (Samal, 2019a). *T. pilaris* can therefore be exposed to and infected by pathogens on their feeding grounds and can then transmit infections across ecosystems and land boarders due to their migration routes. The yellowhammer (*Emberiza citronella*) are also a ground foraging species, and in the summertimes, it feeds on insects e.g., caterpillars, earthworms, beetles, and snails (Stoate et al., 1998). However, this species does not usually migrate outside of Norway (Svensson et al., 2011). As for the other passerines sampled in this study, they are primarily granivorous (feeds on seeds and grains), but in the summertimes they feed on flying insects or spiders (Svensson et al., 2011; Wilson et al., 1999). Due to foraging behaviour and colonial nesting, *T. pilaris* was therefore a focus prey species in this study.

Birds are vulnerable to pathogenic infections at all life stages, from embryo to adult. Even though the egg represents a physical barrier between embryo and the external environment, some pathogens can penetrate the eggshell and infect avian embryos (Benskin et al., 2009). LPAI has not yet been confirmed to access the embryo and cause internal lesions of the egg (Hegyi et al., 2009). However, it is possible that LPAI viruses causes lesions in the

reproductive tract of the female. If AIV is received during copulations but does not cause an infection in the female, it may clear from the cloaca with the faeces. If it enters the oviduct and triggers an infection, the virus could settle for a longer time period and may contaminate the eggs externally during egg laying (Hegyi et al., 2009).

The main colonisation of microbes and viral particles of nestlings starts right after they hatch (Lucas & Heeb, 2005). If the eggshell is contaminated, the chick could still be in danger of AIV transmission after hatching. Nestlings are also exposed to pathogens through other nest materials, like moulting feathers from mother bird, food, and adult saliva (Benskin et al., 2009). Food and leftovers stored in the nest, combined with close interactions with siblings and parents, are all factors that increases risk of pathogen transmission after hatching.

S. aluco, lay their eggs over 5 to 7 days (Olsen, 2007), which makes up an age difference of a week between the oldest and the youngest nestling. The oldest nestling would most likely been exposed first to several pathogens through food and nest materials before the youngest sibling. Weight is therefore an indicator on the nestling's age. Further, siblicide is a normal phenomenon in *S. aluco*, so usually the smallest nestling will be eaten by the older siblings (Simmons, 2002). Thus, the oldest nestling gives the most powerful image of pathogen exposure amongst the nestlings sharing the same nestbox. In this study, we therefore sampled only the heaviest *S. aluco* nestling to limit stress to the younger nestlings.

Objectives

Prevalence of pathogens in birds are affected by both exposure factors (e.g., life-history and demographic effects, environment, and population dynamics) and susceptibility (immunity) (Benskin et al., 2009). Due to globalisation and climate change, pathogens are spreading over larger distances than before, which means that correlation between immunity and pathogen infection will be of great concern in the future. An understanding of the changing spread of avian pathogens could serve as a useful model for prevalence of other diseases transmitting from birds to other species and taxa, for instance when there are several infections spreading in similar ways (e.g., bacterial infections and viral infections spread with migration species) (Benskin et al., 2009; Hasle et al., 2009; Weber & Stilianakis, 2007).

By the use of viral transport medium swabs (VTMs), *S. aluco* and several passerine bird species have been investigated for the occurrence of AIVs. Such data can give us an important baseline information about the health of the ecosystem nearby human settlements in central Norway and allow us to continue annual sampling to document any changes in AIV

prevalence. In this way, it's possible to monitor the prevalence of AIV from a One Health perspective.

In this project I investigated the prevalence of AIVs in *S. aluco* using swab samples from adult females and their nestlings. Further, I investigated the prevalence of AIVs in adult and nestling *T. pilaris*, and adult passerines, to check for AIV in migrating species, as well as a possible exposure source for infectious diseases in *S. aluco*. The project delivers novel knowledge about pathogen prevalence in central Norway via the following:

1 Detection of AIVs in *S. aluco*.
I hypothesized that AIVs are present in free-living populations of *S. aluco*, due to current (autumn 2020) AIV outbreaks in domestic and wild birds in Europe and Norway.

2. Detection of AIVs in migrating passerine birds.
Since there is a growing concern of AIVs in passerine birds in Europe I hypothesized that passerine birds in central Norway also could be infected.

3 Finding correlation between pathogen-infection in *S. aluco* and their preys passerine birds.
I hypothesized that *S. aluco* could be exposed to infectious diseases through their food source. Since *T. pilaris*, which are a migratory bird, are one of the most common passerines for *S. aluco* in central Norway to feed on, I hypothesized that this species might be one of the main sources of pathogen infections for *S. aluco*.

Method

Ethical statement

S. aluco were sampled in collaboration with Birdlife Norway's monitoring project of breeding *S. aluco* in central Norway. In collaboration with an experienced FELASA C certified ornithologist, the owls were safely removed from their nesting boxes and sampled via non-destructive methods as outlined below, before being returned to the nest boxes. The *S. aluco* population in Trøndelag has been monitored for 40 years, and the adults and juveniles have had no severe effects from the annual measurements (wingspan, weight, feather sampling, and ring-marking), and the adults couple returns to the same nesting box yearly. The passerines were also collected by FELASA C certified ornithologists from Nord University. Swab and feather sampling were approved by the Norwegian Food Safety Authority (FOTS ID: 27025/23120).

Study area

This study was carried out in a cultural landscape in the north-eastern parts of Trondheimsfjorden (64°N, 11°E) in central Norway, this includes Steinkjer, Inderøy, Verdal, Ytterøy, Levanger, Snåsa and Grong (Figure 1). The study area represents the northern boundary of the *S. aluco's* distribution range in Europe (Badry et al., 2020). A total of 150 boxes were visited in April-May 2021. Since *S. aluco* prefer nesting in mixed habitats, i.e., where there is open landscape for hunting during the evenings/night and forest for cover during daytime, most of the nestboxes were located nearby farming lands.

T. pilaris were sampled from Levanger and Verdal, where most of the nests were found at Grafmarka in Levanger (63.73°N, 11.33°E) (Figure 1). There was no need to visit a lot of different nesting areas for *T. pilaris* considering that females and males in the same areas have migrated from different wintering places. *T. pilaris* prefer foliage and mixed forests with dense and moist wood floor (Kleven et al., 2019), which describes the study area. Nestlings were picked from their nests, where in total 9 nests were visited. The other passerines (Table 1) were caught at Ørin field station in Verdal (63.79°N, 11.44°E) (Figure 1).

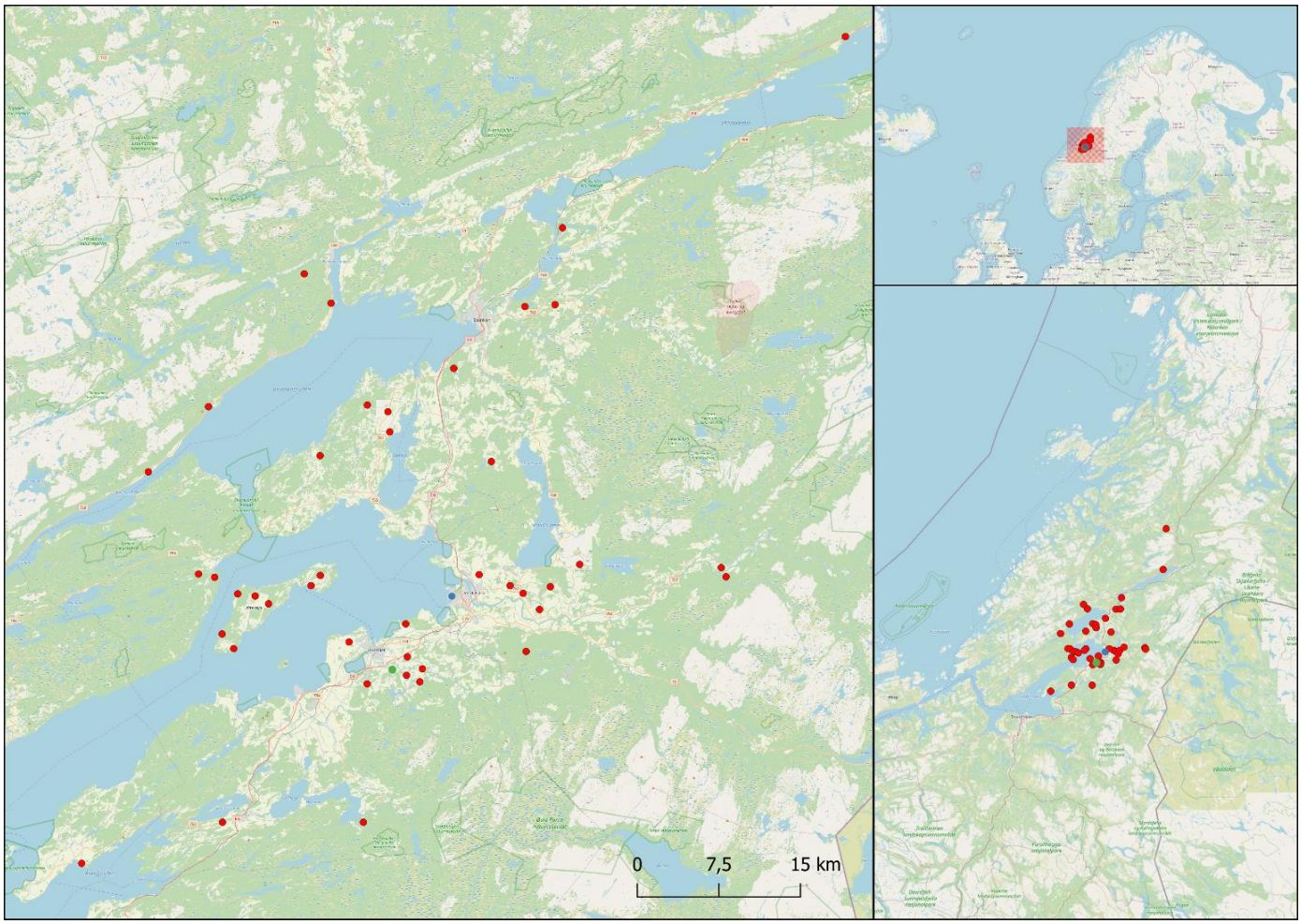

Figure 1: In the left pane is an overview of the study area. Red pins describe *S. aluco's* nesting boxes, green pin describe Grafmarka where the *T. pilaris* were sampled in June, and the blue pin describes Ørin field station where rest of the passerines were sampled in October. In the top-right pane is a map of Scandinavia (Norway, Sweden, Denmark and Finland). The red mark indicates northern Trøndelag where the birds were sampled. In the low-right pane is a closer look up of the study area.

Catching and handling S. aluco

In collaboration with Birdlife Norway and their monitor program on *S. aluco* in Trøndelag, I managed to access all the adults and juveniles from their nesting boxes. The boxes are made by hobby-ornithologists (Figure 2A).

Adults

A total of 43 samples from cloaca and beak were collected from adult *S. aluco's*. The females were sampled during breeding season (late April to early May 2021). During this period, the female incubates the eggs and is therefore available for capture. The owls were caught by placing hand-nets over the peephole of their nestbox and then gently tapping the nestbox so

they would exit into the net (Figure 2A). After sampling, the owl is placed in a bag to reduce stress before being placed back in its box. After it is returned the peephole is covered for a couple of minutes so the owl can calm down and continue incubation.

Nestlings

A total of 28 samples from cloaca and beak were collected from the nestlings. The nestlings were sampled during the first 2-3 weeks after hatching (during May and early June 2021). At this time, nestlings have developed a proper digestive system, with a large beak and cloaca, but are still too small to jump the nest. If one wait until the nestling's are 4 weeks, they are difficult to find and catch as they have started to leave the nestboxes. The nestlings were gently plucked from the nesting box, where they were stored in a tight bag during transportation between the nestbox and the ground. During the handling time of the nestlings, the adult female was either caught with the hand net as described above and held in a bag to reduce stress, or she flew away and returned when we left. After sampling, the nestlings were placed back to their nest again.

AIV sampling

The respiratory mucosa (hereafter beak) and cloaca of n = 43 adult and n = 28 nestling *S. aluco* were swabbed using VTM swabs (Copan diagnostic). Separate swabs were used for the cloaca and the beak. For the nestlings, samples were only taken from the heaviest individual of each clutch. Biometrics that were taken was the wingspan for the adults, and every owl, including nestlings, were ring marked and weighed (Supplementary table 1).

Samples from the beak were taken by running the swab from the back of the throat, along the palate, to the end of the palate (Figure 2B). This were repeated 3 times. During beak sampling, the person holding the owl was holding the beak open, so it was easy to access with the swab. Samples from cloaca was taken by inserting the swab carefully inside the cloaca until the tip of the swab was completely submerged, and then twisting it gently 3 times (Figure 2C). To detect the cloaca the individual was laid on its back with the legs held up high (Figure 2C). Then the cloaca was detected by moving from underneath the tail, up towards the stomach until the cloaca was spotted. It was easier to detect the cloaca of nestlings than adults because they were still early in their feather development. Handling time of each individual was under 20 minutes, including catching, sampling and placing the owl back in the nest box.

A  B  C 

Figure 2: A) Catching *S. aluco* by the use of hand net which are covering the peep hole on the nesting box. B) Respiratory mucosa samples from adult *S. aluco*. C) Cloacal sample from adult *S. aluco*. One person is holding the owl, while another is taking the samples.

Catching and handling passerine birds

Adults

A total of 33 samples from cloaca and beak were collected from 30 adult passerine birds. The adult passerines were caught in mist nets in June and October 2021 (Table 1, Table 2). The mist nets were twisted open, and then attached to steel pipes. The steel pipes hold the net up and were placed in the ground. Threads were used to keep the net standing up. The nets are about 3 m tall, 8 m long, and the hole in the net are 15 mm. The nets are oversized which causes the net to form pockets which traps the birds. The nets were placed at open spaced areas in the forest, with around 10 metres apart. Handling the nets and adult birds were done by certified ornithologists.

Nestlings

A total of 32 *T. pilaris* nestlings were sampled in the beginning of June (Table 2). The nestlings were picked from their nests. One person climbed a ladder and laid the nestlings in a bag while transporting them down to the ground. One person was holding the nestling, while another one was taking the samples. Then each nestling was safely placed back in the nest. On average there was 5 nestlings in a nest and each nestling was sampled (Table 2). This due to *T. pilaris* being social monogamous birds (in contrast to *S. aluco*), and it is thus common for the female to breed with several males in the same season (Kleven et al., 2019). This means that the nestlings in the same nest could have different fathers. Since females copulate with different males, they can also be exposed to different parasites, pathogens etc.

Pathogen samples

Mini-dry swabs (Copan diagnostics) were used for the passerine adults and the *T. pilaris* juveniles. Immediately after sample collection, the mini-dry swabs were immersed in 1 ml of

RNA later for storage. Swab samples were taken from the beak and the cloaca of both adults and nestlings. Separate swabs at appropriate size were used for the beak and cloaca. Samples were taken as described for *S. aluco*. Handling time at each nest and with each adult did not exceed 20 minutes.

Table 1: Overview of adult passerine species sampled in October.

	Date	Gender	ID number	Ring numbe	Specie	Beack	Cloaca
	21.10.2021	Adult	21	ES45921	*Parus major*	x	
	21.10.2021	Adult	70	ES45922	*Fringilla montifringilla*		x
	21.10.2021	Adult	85	ES45922	*Fringilla montifringilla*	x	
	21.10.2021	Adult	34	ES45923	*Carduelis chloris*		x
	21.10.2021	Adult	51	ES45923	*Carduelis chloris*	x	
	21.10.2021	Adult	45	HK30417	*Cyanistes caeruleus*	x	
	21.10.2021	Adult	63	8P38160	*Emberiza citrinella*		x
	21.10.2021	Adult	2	8P38160	*Emberiza citrinella*	x	
	21.10.2021	Adult	55	8P38161	*Emberiza citrinella*		x
	21.10.2021	Adult	10	8P38161	*Emberiza citrinella*	x	
	21.10.2021	Adult	93	8P38162	*Emberiza citrinella*		x
	21.10.2021	Adult	33	8P38162	*Emberiza citrinella*	x	
	21.10.2021	Adult	42	ES45923	*Carduelis chloris*		x
	21.10.2021	Adult	60	ES45923	*Carduelis chloris*	x	
	21.10.2021	Adult	80	8P38163	*Emberiza citrinella*		x
	21.10.2021	Adult	88	8P38163	*Emberiza citrinella*	x	
	21.10.2021	Adult	18	EN98883	*Carduelis chloris*		x
	21.10.2021	Adult	69	EN98883	*Carduelis chloris*	x	
	21.10.2021	Adult	90	8P38164	*Emberiza citrinella*		x
	21.10.2021	Adult	78	8P38164	*Emberiza citrinella*	x	
	21.10.2021	Adult	98	HK30094	*Cyanistes caeruleus*	x	
	21.10.2021	Adult	75	ES45924	*Parus major*		x
	21.10.2021	Adult	14	ES45924	*Parus major*	x	
	21.10.2021	Adult	23	8P38165	*Emberiza citrinella*	x	
	21.10.2021	Adult	82	ES45925	*Carduelis chloris*	x	
	21.10.2021	Adult	22	ES45529	*Parus major*	x	

Table 2: Overview of juveniles, their nests, and adults *T. pilaris* sampled in June and October.

Date	Nest	Gender	Number	Ring number	Specie	Beak	Cloaca
05.06.2021	1	Juvenile	1		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	2		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	3		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	4		*Thurdus pilaris*	x	x
05.06.2021	2	Juvenile	5		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	6		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	7		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	8		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	9		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	10		*Thurdus pilaris*	x	x
05.06.2021	3	Juvenile	11		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	12		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	13		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	14		*Thurdus pilaris*	x	x
05.06.2021	4	Juvenile	15		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	16		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	17		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	18		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	19		*Thurdus pilaris*	x	x
05.06.2021	5	Juvenile	20		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	21		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	22		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	23		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	24		*Thurdus pilaris*	x	x
05.06.2021	6	Juvenile	25		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	26		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	27		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	28		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	29		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	30		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	31		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	32		*Thurdus pilaris*	x	x
05.06.2021	no nest	Adult female	1		*Thurdus pilaris*	x	x
05.06.2021	no nest	Adult female	2		*Thurdus pilaris*	x	x
21.10.2021	no nest	Adult female	65	7671104	*Thurdus pilaris*	x	x
21.10.2021	no nest	Adult female	1	7671104	*Thurdus pilaris*	x	

Detecting AIV

For detecting pathogens from the swabs, RNA extraction and Quantitative Polymerase Chain Reaction (qPCR) was done.

RNA extraction

For RNA extraction of all swabs, Thermo Fisher's MagMAX Pathogen RNA/DNA kit was used (supplementary protocol 1). In short, the protocol for low-cell-content samples was followed. All solutions and buffers were prepared beforehand. Two negative controls were added to each 96 well plate to check for accidental contamination. First, the bead mix for extraction was added to each well. All swabs were then vortexed for 1 minute before 50 ul

were extracted and placed at the reaction plate. Then lysis/binding solution was added to each well. The wells were then washed with wash solution 1 and 2 two times each. Between each wash step, a microplate shaker (VWR) was used at moderate speed (5). Then the beads were caught using a magnetic stand for 1 minute. The elution buffer was added to each well for complete bead suspension. The supernatant was then extracted and placed on a clean reaction plate. This plate was stored overnight in the freezer (-20ºC) for analysis.

Quantitative Polymerase Chain Reaction (qPCR) and sequencing

For the qPCR on RNA from the passerine birds, BIO-RAD CFX Connect Real-Time System and the software Bio-Rad CFX Maestro was used. To run the qPCR for avian influenza the VetMAX™-Gold AIV Detection Kit was used (supplementary protocol 2). In short, this kit contained components to make up a Master Mix, which included a specific probe for avian influence virus A (Influenza virus primer probe mix). In given order, 17 uL RT-PCR Master Mix, 8,0 uL test sample and 0,8 uL nuclease-free water was added to each well. Duplicates were made for each sample, as well as duplicates for one positive control (added Influenza Virus-Xeno RNA Control Mix) and one negative control (added Nuclease-free water) to check for accidental cross-contamination. The thermal cycle program had three stages: 1) reverse transcription at 48ºC in 10 min, 2) RT inactivation, initial denaturation at 95ºC in 10 min, and 3) amplification at 95ºC for 15 sec and 60ºC for 45 sec.

RNA/DNA extraction from the *S. aluco* samples was sent to the Norwegian Veterinary Institute at Ås for Avian Influenza Virus (AIV) detection through qPCR and sequencing. The Mx3000 series real time machines were used (supplementary protocol 3).

Results

S. aluco

Swabs from 71 individuals (43 adults and 28 juveniles) were analysed for AIV prevalence. The results showed no AIV detection in samples taken in April to May 2021 from any location. There were no clinical signs of diseases that could be associated with AIV. An adult *S. aluco* usually weigh between 550 g – 750 g (Olsen, 2007). Taking account that there were no clinical signs associated with AIV, and both the nestlings' bodyweight (mean ± sd =399 g ± 53 g) and adults' bodyweight (mean ± sd = 696 g ± 54 g) did not deviate from normal, indicating that the investigated population in Trøndelag currently show no specific health issues. The high nesting success in 2021 for this species, total of 57 successful nesting (each nest with eggs), indicates high access to rodents during the previous winter. However, many clutches were dramatically reduced from 5 eggs to 1 or 2 nestlings (Supplementary table 1).

Passerines

Swabs from 62 individuals; 30 adults of passerine birds, and 32 juveniles of *T. pilaris*, were sequenced for AIVs. No AIV was detected from any sample taken in June nor in October from any of the locations. The birds did not show any clinical signs of diseases that could be associated with AIV (Table 1, Table 2).

Discussion

This study provides the first baseline screening of *S. aluco* and their prey passerine birds in Norway. Despite current HPAI outbreaks in both wild and domestic bird populations in Europe and Norway (autumn 2020) there were no signs of AIV in *S. aluco* nor passerine birds screened in this study. The study shows no evidence of migratory passerines bringing AIV into central Norway, nor that there was a correlation between *T. pilaris* causing AIV infection in *S. aluco.*

Climate change and migration patterns

Emerging diseases and protection of biodiversity are two of the major challenges in the 21st century (Weber & Stilianakis, 2007). Norway has been historically almost free of diseases compared to more tropical regions. Changing climate leading to higher temperatures in the northern regions results in less ice and snow compared to previous years (Cohen et al., 2020). This makes previously un-colonisable areas in the north more accessible for pathogens. There are incidences of species that have changed their migration route and migrated further north than before (Hoye et al., 2011; Lawrence et al., 2022; Redlisiak et al., 2018), as well as new pathogen spreading across the country (Hasle et al., 2009; Madslien et al., 2021). All to believe being connected to a warming climate.

There are still uncertainties around pathogen transmission patterns between species, taxa and across borders. However, researchers believe that migratory birds and poultry industry and trade are the main sources of AIV spreading across the globe (Weber & Stilianakis, 2007). Timing of migration and arrival at the breeding grounds are partly influenced by the conditions on the breeding grounds, as well as the flyways and wintering grounds (Redlisiak et al., 2018). It's known that early arrival of migratory birds at the breeding grounds is correlated with climate indicators as well as reproduction. Climate warming causes an advancement of spring arrival of many migrating birds to northern territories in Europe. The warming patterns are varying, were the more temperate parts (north and south of the globe) are the ones warming the fastest (Redlisiak et al., 2018). The effect of new species arrival and early arrival to the northern region is not well known, and since we still don't have much data to compare with, creating a baseline of the ecosystem's health are therefore crucial in the light of management in the future.

On the other hand, climate is also getting more extreme in the northern regions with late winter storms, cold fonts, and heavy winds, which also modify migration (Overskaug et al., 1999; Weber & Stilianakis, 2007). Migration affects the bird's fitness, where the bird is

running short on resources, which means that a new infection could be devastating for the birds. According to Weber & Stilianakis (2007), infected wild birds should show none or only mild symptoms of disease if they should manage long-distance migration. LPAI infections could give fitness costs to the host, including reduced food intake rates and reduced body mass, which could result in delayed migration (Hoye et al., 2011). However, despite the infection, birds could still manage to migrate long distance, taking to account the timing of infection according to migration. In comparison, bird infected with HPAI would probably not manage long distance migration (Weber & Stilianakis, 2007), and would rather work as vectors over shorter distances. However, since migrating birds are depending on many factors in light of spreading pathogens, the second hypothesis of HPAI spread through poultry trade could be even more efficient.

Avian Influenza from a pandemic and economical aspect

Some epizootic AIVs could transmit to humans, causing disease and deaths. There are four different AIV subtypes that we know are capable of infecting humans; H1, H5, H7 and H9. All of them are also pathogens in *G. gallus* (Shortridge et al., 1998). Emerging of H5N1 infections in Hong Kong (1997) was the first evidence of AIV transmission from poultry (*G. gallus*) to humans. This incidence resulted in 6/18 deaths (Shortridge et al., 1998). 16 years later (2013) a new novel AIV strain was found in China, H7N9, which was causing several deaths due to pneumonia and respiratory related complications (Liu et al., 2013). Since the incident in 1997, HPAI H5N1 has spread from Asia to Europe, Africa and the far east through migratory birds and poultry trade (Liu et al., 2013). The same clade is now causing the largest outbreak in domestic poultry flocks in the UK (2021) (Department of Environment, 2022). In 2022, one person in the UK was infected with AIV, believed to be H5N1. Luckily, the person did not need medical assistance at a hospital (Roberts, 2022).

The global pandemic “Swine Flu” caused by a new H1N1 stain of AIV, did most likely originate by reassortment of human and swine influenza virus genome (Khan, 2010). This started in Mexico (2009) and unlike many other AIV stains, H1N1 did not only transmit between swine and human, but also between humans as well. When local people and tourists were getting infected, the virus spread at a global scale, resulting in a pandemic. The disease has infected > 254,206 people in 80 countries, resulting in 625 deaths (Khan, 2010). In Norway there was 900 000 people infected and 29 deaths observed (NHI, 2021). This despite that the humans’ mucous membranes are usually not good receptors for AIV (NHI, 2021), and that it’s rare that HAPI infections transfer between humans. However, when AIV are

transmitted to a new host species, they can evolve new properties and cause greater diseases in the new host. Earlier incidences shows that when AIV do infect humans, the consequences could be mortal. Due to global food trades and human travels across borders, it is crucial to be aware of the risk of pathogen transmission and infection in other countries. Especially when they affect the household industry.

Emerging infectious diseases do have an economic impact, primarily for the poultry industry. In 2021, Norway had the first outbreak of HPAI in a domestic poultry farm, were they had to put out 7500 *G. gallus (NVI, 2021).* England had their largest outbreak of HPAI divided over five different areas in the UK. Every farm with infected poultry had to sanitize every bird in the population (Department of Environment, 2022). In 2021/2022 Italy and France are facing the largest loss of domestic poultry in Europe, were both countries have had experienced a loss of more than 40 poultry farms due to H5N1 outbreaks (Adlhoch et al., 2021; Department of Environment, 2022). Larger incidences, like the H5N1 outbreak in Hong King (1997), approximately 1,5 million birds had to be removed (Shortridge et al., 1998). When the number of infectious birds increases, the economic impacts could be devastating, not only for the farmers, but also for the local and global food production.

Avian Influenza prevalence in Europe

In 2005, Europe experienced the first incidence of AIV, and now in more recent years high pathogenic (HP) subtypes H5 and H7 have been spreading around Europe, causing death of both domestic poultry and waterfowl (Adlhoch et al., 2021; Brun, 2005). The increased spread of HPAI in Europe in the last decades are of increasing concern (OIE, 2021). From October 2021 until April 2022, 32 countries in Europe have had incidences of H5 in both wild birds and domestic poultry. Countries with the highest prevalence of H5N1 in wild birds was Germany (1169), Great Britain (841) and Netherland (426). In Scandinavia, Denmark (113), Sweden (37) and Norway (7) had wild bird species testing positive for H5N1 (Department of Environment, 2022; Granstad, 2022).

Migratory birds belonging to aquatic habitats (*Anseriformes* and *Charadriiformes*) are considered the main reservoirs for AIVs. This is mainly due to their foraging behaviour and the behaviour of the virus. For instance, LPAIV can remain infectious in lake water up to 4 days at 22^{o}C and more than 30 days in lower temperatures e.g., 0^{o}C (Hoye et al., 2011). In 2021, two die-offs related to HPAI infections in marine bird species was observed; 1) the Svalbard's barnacle geese (*Branta leucopsis*) populations, were approximately 12000-13000

birds were found dead along the Solway Coast in Scotland due to H5N1 (Al-Khalaf, 2021; BirdLife, 2022); and 2) 3000 red knots (*Calidris canutus*) was found dead in Germany due to a H5N3 infection (Adlhoch et al., 2021). Further, more recently, poultry have also been stated as a natural reservoir for AIV infection, especially HPAI (Adlhoch et al., 2021; Weber & Stilianakis, 2007).

What's interesting with the new reports from EFSA and the department for Environment, Food and Rural Affairs (EFRA) is that there has been an increased prevalence rate of AIVs in wild bird species that are usually not associated with AIVs. *Passeriformes* are not counted as a reservoir for AIV infections, and there are therefore few publications on AIV prevalence worldwide in this order of birds (Adlhoch et al., 2021; Račnik et al., 2008). Isolation of AIVs from passerine birds are usually done on birds held in quarantine after importation (Perkins & Swayne, 2003). However, according to EFSAs annual rapport on AIV, there has been incidence of several wild passerine species proven positive for HPAI (Adlhoch et al., 2021). Among the passerines, nine sparrows, two magpies, a starling and one *Turdidae* species, was proven positive in 2021 in Europe (Adlhoch et al., 2021). Most of the findings are from passive surveillance or related to disease outbreaks, which could mean that AIV infections could be more common in passerines than first thought.

Further, there has been an increase in HPAI positive related incidences in several raptor species. Raptors found positive for H5H8 in 2020 are listed in "Table 4: Reports of HPAI H5N8 in wild birds in Europe, by species since September, as of 7 am on 07 December 2020, according to ADNS reporting." in EFRAs outbreak assessment #8 (p. 6-9) (Department of Environment, 2020); (supplementary table 2). From this year (2020), *S. aluco* was found positive for H5H8 in Denmark (Department of Environment, 2020). HPAI has also been found in *S. aluco* several places across Europe: Hungary (2022), Russia (2017), and Sweden (2021) (Adlhoch et al., 2021). The reports did not include any clinical signs of the infection. Due to rapid spread of HPAI in Europe, biosecurity has been strengthening, and food and drinking water for poultry must be arrange indoors so wild bird species cannot reach it.

Avian Influenza in Norway

In 2005, The Norwegian Food Safety Authority started a surveillance program for AIVs in wild birds and domestic poultry in Norway (Brun, 2005). The program has been focused on geese, duck, and *G. gallus*, and there has been no active surveillance of raptors nor passerines. From the beginning of the project (2005) until today (2022), there has been an increase in

AIV infected wild birds, and in 2020 Norway had the first incidence of H5N1 in a pink-footed goose (*Anser brachyrhynchus)* (Madslien et al., 2021). Wild birds that have been positive for HPAI in Norway are tested in collaboration with hunting activities. This means that either the bird is found dead or has been shot before sampling to check for diseases. Since passerines and raptors are not normally (nor allowed) to be hunted, they have not been included in the surveillance.

From October 2021 until April 2022, Norway had the largest outbreak of HPAI so far, were H5N1 was found in 7 different species: a rock dove (*Columbia livia*), a goose (no *spp* documented), one mallard (*Anas platyrhynchos*), domestic chicken (*G. gallus)*, mute swans (*Cygnus olor*), eurasian wigeon (*Marea penelope*) and white-tailed eagles (H. *albicilla)* (Granstad, 2022). Most of the individuals was found in western parts of Norway (Rogaland and Vestland). Only the *A. platyrhynchos* and *M. penelope* populations were under active surveillance through hunting, but the other species was found dead by accident (Granstad, 2022). According to the Norwegian Veterinary Institute there has been no reports of abnormal or increased mortality among wild birds anywhere in Norway (Grim Rømo, 2022). However, number of samples from wild birds are still too few to say much about the distribution of AIV in Norway. Our results from 2021 show no evidence of AIV infection in *S. aluco* or passerine birds in the northern part of Trøndelag. Trøndelag is approximately 791 km north of Vestland, which until February 2022 was the northern parts of mainland Norway were there have been wild birds testing positive for active AIV infection.

As a result of this outbreak in 2021-2022, a total of 8 *H. albicilla* have been found positive for HPAI almost all over Norway, including Trøndelag, Nordland and Troms og Finnmark (Granstad, 2022). Most of the *H. albicilla* have been infected with H5N1, but now in March (2022), a new subtype was found in two *H. albicilla*, one in Trøndelag (Hitra) and one in Troms og Finnmark (Tromsø) (Granstad, 2022; NVI, 2022b). There is to believe that the subtype is H5N5, but there are still uncertainties about the N-subtype (Granstad, 2022). That so many *H. albicilla* has been found positive for HPAI in Norway it's unique and raises a lot of questions, like where these stains have arrived from? Researchers believe that H5N1 infection in Norway are coming from East Europe (Russia) (Moldal, 2021). The other subtype is still uncertain.

Whilst climate change is making the northern territories more suitable for migratory birds (Marra et al., 2005), including an increase in geese in this study area, there is no evidence of

active infections in sampled species on the mainland of central Norway. Data from 2019 shows no seroprevalence nor any clinical signs of active infections in *H. albicilla* and northern goshawk (*Accipiter gentilis*) nestlings in Trøndelag (Lee et al., 2019). However, serum samples taken from baltic common eiders (*Somateria mollissima*) and *A. brachyrhynchus* at the multiplicity Levanger in northern Trøndelag, showed an overall LAPI seroprevalence of 50% for both species (Lam et al., 2020). They are both belonging to the Svalbard breeding populations and are often observed migrating to Svalbard together. It has been stated active infections for these populations in the Netherlands but not yet in central Norway (Lam et al., 2020; Munster et al., 2007).

Antibodies of AIV in black-legged kittiwakes (*Rissa tridactyla*) from 2015 and glaucous gulls (*Laurus hyperboreus*) from 2017 has also been detected as far north as Svalbard (78.92° N, 11.91° E) (Lee et al., 2020). *R. tridactyla* migrate at longer distances from Svalbard to their overwintering grounds in the North Atlantic Ocean, compared to *L. hyperboreus* who overwinter in closer proximity south of Svalbard in the Barents Sea, Norwegian Sea and Greenland Sea (Lee et al., 2020). None of them are migrating through the mainland of Norway, but *L. hyperboreus* might overwinter in the most northern parts of the mainlands.

The climate in northern territories of Norway is getting more extreme, which could affect the migration flyway for AIV infected individuals. Migrating species tested positive for active HPAI infections have only been reaching the southern parts of Norway, that we are aware of (Grim Rømo, 2022; NVI, 2022a). Whereas most of the individuals that reached Trøndelag, and Svalbard did not have active HPAI nor LPAI infections (Lam et al., 2020). The middle parts of Norway might still be too far away for migratory birds carrying active diseases, at least HPAI.

Another possible reason for these current outbreaks in the west parts of Norway might be because of the location of the stopover farmlands. The western parts of Norway are surrounded by mountains in the east and a coastline in the west. Due to the mountains, many farming areas are placed near the coastline or at islands along the coast. Which makes excellent stopovers for waterfowl and other migratory species (Hoye et al., 2011). All species found positive for HPAI further north as well, were found in coastal areas (Granstad, 2022). Norway's coastline are excellent stopovers for migratory waterfowl, which are the specie most likely to carrying AIVs. Raptors feeding on aquatic birds, fish and mammals in the

coastal areas could therefore be prone to secondary infection through their food, which could explain the rapid spread of HPAIs among the *H. albicilla*.

Ecology

S. aluco are an excellent sentinel species for monitoring pathogen prevalence worldwide in terrestrial ecosystems. The species has a wide habitat niche, and are often nesting in urban areas close to humans or at farmlands with patched forest (Badry et al., 2020). They easily nest in human made nestboxes, and in this study we had access to more than 150 nesting boxes, which also makes *S. aluco* suitable for long time monitoring. *S. aluco* and the passerine birds sampled in this study have given information about the health of the ecosystem nearby human settlements and have contributing to creating baseline knowledge of the AIV status in central Norway (northern Trøndelag).

Since *S. aluco* are a resident species, migratory birds are a possible vector for AIVs into the owl's habitat of the more northern (geographically isolated) regions of Trøndelag, especially considering the current HPAI outbreak in Europe. It is rare that individuals migrate directly between their breeding ground and non-breeding ground (vice versa) without stopping for fuel and rest. The entire flyway is therefore likely to be important in the transmission and maintenance of LPAI during long-distance migrations. For waterfowl, the prevalence of AIV is known to peak during the pre-migratory stages (late summer and early fall) (Hoye et al., 2011; Lam et al., 2020), followed by a rapid decrease during autumn migration when it reaches its wintering grounds (Hoye et al., 2011). Many species aggregate at stopovers or wintering sites, which results in high local densities. Their sites, which in this case are farmlands, might be important for LPAI transmission between other wild bird species and captive birds. Our results for *S. aluco* could have therefore been different if we had tested them in autumn versus spring.

Seasonal migration of passerines and songbirds arrived late in the spring of 2021, and in the same year, *S. aluco* nested earlier than usual (Øien, 2006, 2007, 2008), which could have affected the results. *S. aluco* are a generalists, and usually in the spring, they feed more on amphibians, passerines, and songbirds, and in the winter they feed mostly on rodents (Ján, 2011). This year, *S. aluco* had a successful breeding season, but the clutches were dramatically reduced during their first weeks of living. There are probably several reasons for this (e.g., late arrival of migratory birds which results in less food supply, predation,

pollutants, or diseases), for what we cannot know for sure. However, predation pressure from martens (*martes*) was not unusual compared to earlier years (Øien, 2006, 2007, 2008).

According to Lu et al (2003) study on survival of H7N2 in chickens and the environment, figured that 60% of infectious birds tested positive for H7N2 the first two weeks after infection. Further, 10-20% of the infected birds continued to be positive for another additional 3 weeks (Lu et al., 2003). This is probably due to a later infection of contact birds in the same group. Of the passerines sampled in this study, *T. pilaris*, the european greenfinch (*Carduelis chloris*) and the brambling (*Fringilla montifringilla*) are usually migrating to central Europe during the winter. As for the great tit (*Parus major*), blue tit (*Cyanistes caeruleus*) and yellowhammer (*Emberiza citronella*), there are only few individuals in the population that are migrating to wintering places in September/October (Svensson et al., 2011), depending on habitat and food access in the north. Migrating passerines are arriving to Norway during April/May. Taking to account that an individual would still be carrying AIV infection 2-6 weeks after exposure (Lu et al., 2003), infected *T. pilaris* could potentially infect other individuals the first weeks after arrival (during breeding season). *T. pilaris* sampled in early June were all negative, which indicates that the species are not brining AIV into northern Trøndelag. Migrating passerines tested in October, were also all negative. If our passerines had been infected, they would probably have caught the AIV infection in Norway. However, there could be specie specific responses affecting this infection timeframe. Overall, our negative results indicate that AIV infections are not yet endemic to passerines in central Norway.

Sampling method

The reported frequencies of AIV infected species are dependent on proper data analysis and samplings, were bias data can occur (Swayne, 2017). Some species are easier to take pathogen samples from than others, which could indicate higher prevalence compared to other species that are more difficult to take samples from (Elvira Schettler & Ulrich Wittstatt, 2003). In an environment where birds at lower trophic levels suffer from pathogen infections, it is likely that species higher in the food chain are infected as well (Vlahović et al., 2004). It is therefore important to test for pathogens in a variety of bird species to really be able to map the prevalence of the virus in an area. However, raptors are usually large, shy birds (Olsen, 2007), which makes them hard to catch and handle for pathogen samples.

Sampling wild birds is challenging, for example, there was lower RNA concentrations from the cloacal samples (compared to nasopharyngeal) in both *S. aluco* and the passerines. The bird would protect the cloaca opening while sampling, which makes it hard for the swab to enter the cloacal entirely. One needs also to be careful while sampling inside the cloaca, because if one goes too far it could harm the bird's organ. Smaller swabs or swabbing excrement where it is possible could be a solution for more robust sampling of the cloacal area. Surveillance of seroprevalence via blood sampling is also an option, however, this is considered as an invasive technique and requires more specialised skills from the handler.

While testing for antibodies one does only test for previous infection. Experimental studies done on shorebirds shows that Influenza A specific antibodies was detectable at least 2 months after infection (Hall et al., 2013). However, the time antibodies are produced after infection is relying on the species. There is evidence of maternal antibodies in *G. gallus* which are detected up to 5 weeks after birth, but for quail (*C. coturnix*) and black-legged kittiwakes (*R. tridactyla)* in the same study, detection of antibodies was around 2 weeks (Garnier et al., 2012). All this data is collected through experiments, which have a deadline where they stop continuing measuring antibody levels, which will affect the results.

Lam et al (2020) collected antibody data from a wild pink-footed goose *(A. brachyrhynchus)* population with antibody prevalence and they measured antibody levels in their serum for up to 343 days (Lam et al., 2020). *A. brachyrhynchus* migrates and overwinter in large flocks which could contribute to a longer time span of antibody prevalence. However, this data is probably more reliable while comparing to other wild bird populations. The limitations of seroprevalence are that it gives no information on whether the infection is active or not. For further investigation, it should be an idea to look for both seroprevalence and active infections.

Management

A historic lack of surveillance has led to a lack of baseline data on the number of pathogens shed by wild birds. Selective choice of specific bacteria and viruses are a huge limiting factor, which are creating biased data towards testing for types of pathogens that are suspected to be present, whilst others are being overlooked (Benskin et al., 2009). It is therefore important to annually test for pathogens that are not yet known to be endemic in a population. However, it is costly and logistically challenging to conduct adequate surveillance in wild birds, especially in asymptomatic populations as they are not prioritised.

A possible solution for this might be to expand already existing surveillance programs. This study was funded by a master's scholarship, a PhD scholarship, and the One Health Course (BIO5012) at Nord University, and some of the final analysis (qPCR) was conducted at the Norwegian Veterinary Institute under the government's surveillance funds. Using students to monitor pathogen prevalence could make it possible to keep up surveillance of specific species over several years. An example of this type of surveillance are studies conducted by Student Network for Amphibian Pathogen Surveillance (SNAPS) in Canada, were they are monitoring *Batrachochytrium salamandrivorans* (Bsal) on salamanders (SNAPS, 2022). SNAPS are providing swabs, and students are taking samples of the salamanders, which are send off to the National Wildlife Health Centre for analysis. In Switzerland, they have divided resources from different surveillance programs, and tested for different pathogens (e.g., *Chlamydia spp*) in cooperation with their AIV surveillance program (Adlhoch et al., 2021; Stokes et al., 2021). Possibilities like this will keep the expenses at a moderate level and will create a steady surveillance program for the future years to come.

Conclusion

For further research it is important to conduct long-term surveillance studies and continue monitoring wild bird population in the northern regions. Resident birds in the north are known to have a poorer disease resistance compared to migrator species (Pardal et al., 2017; Westerdahl, 2007), which is correlated with a lower pathogen pressure. Introduction of new pathogens could therefore be lethal for species the infection is not endemic to. The white-tailed eagle (*H. albicilla*) and the Eurasian eagle owl *(Bubo scandiacus)* are both top predators that are habituating and feeding in areas along the Norwegian coastline. Hence, they are already posing a lot of threats due to e.g., pollutions, habitat loss and arrival of new pathogens (Christensen-Dalsgaard et al., 2010; Nygård & Polder, 2012). Since aquatic bird species are usually the main reservoir for AIVs, they could pose a threat for raptors feeding on these birds. It's therefore important with active surveillance of marine raptor species in the future.

So far, there is not documented any active AIV infection in the local *S. aluco* population nor the passerines in Trøndelag. However, there has been antibodies detected in migratory waterfowl tread-passing these areas. It is therefore important with continued monitoring of pathogen prevalence in the north. The susceptibility of different HPAI stains to raptor species cells needs to be investigated align with how a HPAI/LPAI infection alter immune responses in raptor cells that have not been exposed to the virus before. Recently, cell lines form *S.*

aluco blood and feathers has been isolated and cultivated for future *in vitro* experiments (Kroglund, 2022). This method could be taken to use to get a better understanding of these questions.

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Attachments to the extended summary

Supplementary protocol 1: RNA/DNA extraction kit protocol**:** lab protocol swabs.pdf

Supplementary protocol 2: Available qPCR protocol**: qPCR protocol for AIV.pdf**

Supplementary protocol 3: qPCR protocol used by the Norwegian Veterinary Institute**:** detection-avian-influenza-a-virus-matrix-gene-by-rt-taqman-rt-pcr.pdf

Supplementary link 1: Guidelines from the journal of BMC Veterinary Medicine: https://bmcresnotes.biomedcentral.com/submission-guidelines/preparing-your-manuscript [last read 22.03.22]

Supplementary table 1: Table with an overview of every nesting *S. aluco* and juveniles sampled with information about: date, age, sex, wing length, weight, nestlings, eggs and food spotted in the nests: Supplementary table 1_S.aluco.pdf

Supplementary table 2: Reports of HPAI H5N8 in wild birds in Europe, by species since September, as of 7 am on 07 December 2020, according to ADNS reporting**.**

Species	BE	DE	DK	ES	FR	IE	IT	NL	NO	SE	SI	Count
Accipitridae (unidentified) (Accipitridae (incognita))		2										2
Common Buzzard (Buteo buteo)		18	8					3				29
Eurasian Sparrowhawk (Accipiter nisus)		1	1									2
White-tailed Eagle (Haliaeetus albicilla)		1										1
Barnacle Goose (Branta leucopsis)		139	32					7		1		179
Brant Goose (Branta bernicla)		2	1		1							4
Canada Goose (Branta canadensis)	1	2										3
Common Eider (Somateria mollissima)		1	1									2
Common Shelduck (Tadorna tadorna)												1
Egyptian Goose (Alopochen aegyptiaca)	1											1
Eurasian Teal (Anas crecca)		1					1	1				3
Eurasian Wigeon (Mareca penelope)		29	1				2	5				38
Gadwall (Mareca strepera)			1									1
Greater White-fronted Goose (Anser albifrons)	1	1	1					1				4

Species	BE	DE	DK	ES	FR	IE	IT	NL	NO	SE	SI	Count
Greylag Goose (Anser anser)		64	3					11				78
Mallard (Anas platyrhynchos)		21	1				1	3				26
Mute Swan (Cygnus olor)	1	4				3		11			2	21
Pink-footed Goose (Anser brachyrhynchus)	1		1						2			4
Taiga Bean Goose (Anser fabalis)		4	1					2				7
Whooper Swan (Cygnus cygnus)						1						1
Charadriidae (unidentified) (Charadriidae (incognita))		1										1
Common Wood Pigeon (Columba palumbus)	1											1
Eurasian Magpie (Pica pica)	1											1
Common Kestrel (Falco tinnunculus)		4										4
Peregrine Falcon (Falco peregrinus)		6	4	1		2				1		14
Crane (unidentified) (Grus (incognita))		2										2
Eurasian Oystercatcher (Haematopus ostralegus)		1										1

Species	BE	DE	DK	ES	FR	IE	IT	NL	NO	SE	SI	Count
Black-headed Gull (Chroicocephalus ridibundus)		5	2									7
European Herring Gull (Larus argentatus)	1	10	5									17
Great Black-backed Gull (Larus marinus)		3										3
Gull (unidentified) (Larus (incognita))		15							1			16
Common Pheasant (Phasianus colchicus)			1									1
Great Crested Grebe (Podiceps cristatus)	1											1
Common Moorhen (Gallinula chloropus)			1									1
Eurasian Coot (Fulica atra)		1										1
Curlew Sandpiper (Calidris ferruginea)		1										1
Eurasian Curlew (Numenius arquata)	2	8	1			1		1				13
Eurasian Eagle-Owl (Bubo bubo)		5										5
Short-eared Owl (Asio flammeus)								1				1
Tawny Owl (Strix aluco)		1										1
	11	**353**	**66**	**1**	**1**	**7**	**4**	**46**	**3**	**2**	**2**	**511**

Article manuscript

Resident tawny owls (*Strix aluco*) and migratory passerine birds in central Norway show no signs of avian influenza A virus in 2021 despite severe outbreaks in Europe

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Abstract

Climate change has led to changes in migration patterns of transitory species, which represents a metamorphosing risk of pathogen spread to previously un-colonisable areas, e.g., northern regions such as Norway. Pathogens associated with migrating species thus pose a threat for resident wildlife species, e.g., through transfer via the food chain. In this study, an important predator/prey system, the top predator the tawny owl (*Strix aluco*) and their prey, passerine birds are being investigated. This can allow us to monitor the prevalence and exposure route of Avian Influenza A viruses (AIVs) into ecosystems in central Norway. For pathogen detection, swabs with viral transport media were used to take samples from both the cloaca and the oropharyngeal region of juveniles and adult *S. aluco* (adults n = 43; juveniles n = 28) and passerine birds (adults n = 30; juveniles n = 32). For pathogen detection, RNA/DNA extraction and qPCR were done on each swab. No AIV was detected in any sample analysed in this study. This is the first dedicated study of AIVs in *S. aluco* and passerines in central Norway. It provides critical knowledge from a One Health perspective, as well as managing infectious diseases in wildlife.

Introduction

In Northern Europe, there are periods with increasingly higher prevalence of avian influenza A viruses (AIVs). This is probably correlated with migration timings and a changing climate, where several northern parts consist of less areas with ice and snow than before [1]. Climate change has already affected migration patterns of several bird species that are known reservoir species [1], for example, geese migration patterns are rapidly changing across Europe [2, 3]. In 2005 Europe experienced the first incidence of AIV, and now in more recent years high pathogenic (HP) subtypes H5 and H7 have been spreading around Europe, causing death of both domestic poultry and wild birds [4, 5]. The increasing spread of HPAI in Europe across the last decades are of concern for both animal and human health [6]. The most recent HPAI outbreaks have reached the most northern parts of mainland Norway, causing widespread deaths in poultry and wild birds [7-11].

Raptors, historically, have not been especially associated with AIV infections, but in recent years they have experienced increasing incidents of HPAIs (highly pathogenic avian influenza) outbreaks around the globe [1, 6, 12, 13]. Therefore, monitoring of raptors in Europe should be increased. The tawny owl (*Strix aluco*) is an excellent sentinel for monitoring AIV prevalence in raptors across Europe. It is an apex predator, which makes it sensitive to environmental changes [14]. Its abundance is relatively high over Europe, except for Ireland and Iceland, where central Norway is the northern boundary of its distribution range [14, 15].

S. aluco has a wide habitat niche, and they can be found nesting in urban areas close to humans, but prefers farmlands with patched forests [14], which makes them important from a One Health perspective also. They easily nest in human made nestboxes, which gives researchers easy access to many individuals and makes *S. aluco* suitable for monitoring pathogens in local terrestrial ecosystems. They are generalist predators, that prefer to prey on

rodents and different passerine birds [14, 15]. Most of the passerines breeding in Norway are migratory bird species and usually migrate further south in Europe or follow the African-Eurasian flyway to Africa [16, 17], thereby acting as potential vectors of infection to raptors in Norway.

In Norway, HPAI has recently been detected in pink-footed geese (2020) (*Anser brachyrhynchus*), mute swan (2021) (*Cygnus olor*), *H. albicilla* (2021) and domestic poultry (2021) (*Gallus gallus*) [8, 10, 18]. *S. aluco* usually hunt in open landscapes, where they are often observed at open farming fields. These farming fields are often used by other birds such as geese, gulls, ducks, doves and passerines, and domestic poultry which are all possible carriers of AIVs [19, 20]. Due to *S. aluco's* foraging behaviour and preferred habitat, this species is likely prone to AIV infection, if it should be circulating in the environment. However, there is a complete lack of empirical data on the pathogen status of *S. aluco* and passerines in Norway.

By the use of swabs in viral transport medium, *S. aluco* and several passerine bird species have been investigated for the occurrence of AIVs. Such data can give us an important baseline information about the health of the ecosystem nearby human settlements in central Norway and allow us to continue annual sampling to document any changes in AIV prevalence. In this way, it's possible to monitor the prevalence of AIV from a One Health perspective.

Results

S. aluco

Swab samples from 71 individuals (43 adults and 28 juveniles) were analysed for AIV. No AIV was detected in samples taken in April to May 2021 from any location. There were no clinical signs of diseases that could be associated with AIV.

Passerines

Swabs from 62 individuals; 30 adults of passerine birds, and 32 juveniles of *T. pilaris*, were tested for AIVs (Table 1, Table 2). No AIV were detected from any samples. The birds did not show any clinical signs of diseases that could be associated with AIV.

Discussion

In this study we screened *S. aluco* and their prey species passerine birds for AIV infections in central Norway. Interestingly, despite ongoing HPAI outbreaks across Europe and in southern Norway during the sampling period, no active AIV infections was detected, nor any clinical signs of disease.

In 2005, The Norwegian Food Safety Authority started a surveillance program for AIV in wild birds in Norway [4]. The program has been focused on geese, duck, and domestic poultry (*G. gallus*), and there has been no active surveillance of raptors nor passerines. From the beginning of the project until today, there has been an increase in AIV infected wild birds, and in 2020 Norway had the first incidence of HPAI H5N1 in pink-footed geese (*A. brachyrhynchus*) [7]. Usually, wild birds that have been positive for HPAI are tested in collaboration with hunting activities. This means that either the bird is found dead or have been shot before sampling to check for diseases. Since passerines and raptors are not normally (nor allowed) to hunt, they have not been in focus of surveillance.

From October 2021 until April 2022, Norway had the largest outbreak of HPAI this far, were H5N1 was found in 7 different species: a rock dove (*Columbia livia*), a goose (no *spp* documented), one mallard (*Anas platyrhynchos*), domestic chicken (*G. gallus)*, mute swan (*Cygnus olor*), Eurasian wigeon (*Marea penelope*) and several white-tailed eagles (H. *albicilla)* [11]. Most of the individuals was found in western parts of Norway (Rogaland and Vestland). Only the *A. platyrhynchos* and *M. penelope* populations were under active surveillance through hunting, but the other species was found dead by accident [11]. In the

same season, a total of 8 H. *albicilla* was found positive for HPAI almost all over Norway, including Trøndelag, Nordland and Troms og Finnmark [11]. Which is a rare case compared to other outbreaks in Europe. According to the Norwegian Veterinary Institute there has been no reports of abnormal, increased mortality among wild birds anywhere in Norway [9]. However, number of samples from wild birds are still too few to say much about the distribution of AIV in Norway. Our results from 2021 show no evidence of AIV infection in *S. aluco* or passerine birds in the northern part of Trøndelag.

Passeriformes are not counted as reservoirs for AIV infections, and there are therefore few publications on AIV prevalence in this order of birds [5, 21]. Isolation of AIV from passerine birds are usually done on birds held in quarantine after importation [22]. However, according to the European Food Safety Authority's (EFSA) annual rapport on AIV, there has been incidence of several passerine species proven positive for HPAI [5]. Among the passerines, nine sparrows, two magpies, a starling and one *Turdidae* species, was proven positive in 2021. What the report also tells us, is that most of the findings are from passive surveillance or related to disease outbreaks. This could mean that AIV infections could be more common in passerines than first thought.

According to Lu et al (2003) study on survival of H7N2 AIV in chickens and the environment, figured that 60% of infectious birds tested positive for H7N2 AIV the first two weeks after infection. Further, 10-20% of the infected birds continued to be positive for another additional 3 weeks [23]. This is probably due to a later infection of contact birds in the same group. Of the passerines sampled in this study, *T. pilaris*, the european greenfinch (*Carduelis chloris*) and the brambling (*Fringilla montifringilla*) are usually migrating to central Europe during the winter. As for the great tit (*Parus major*), blue tit (*Cyanistes caeruleus*) and yellowhammer (*Emberiza citronella*), there are only few individuals in the population that are migrating to wintering places in September/October [17], depending on

habitat and food access in the north. Migrating passerines are arriving to Norway during April/May. Taking to account that an individual would still be carrying AIV infection 2-6 weeks after infection [23], infected *T. pilaris* could potentially infect other individuals the first weeks after arrival (during breeding season). *T. pilaris* sampled in early June were all negative, which indicates that the species are not brining AIV into northern Trøndelag. Migrating passerines tested in October, were also all negative. If our passerines had been infected, they would probably have caught the AIV infection in Norway. However, there could be specie specific responses affecting this infection timeframe. Overall, our negative results indicate that AIV infections are not yet endemic to passerines in northern parts of Trøndelag.

Since *S. aluco* are resident species, migratory birds are a possible vector for AIVs into the owl's habitat of the more northern (geographically isolated) regions of Trøndelag, especially considering the current HPAI outbreak in Europe. It is rare that individuals migrate directly between their breeding ground and non-breeding ground (vice versa) without stopping for fuel and rest. The entire flyway is therefore likely to be important in the transmission and maintenance of, at least, LPAI (low pathogenic avian influenza) during long-distance migrations. For waterfowl, the prevalence of avian influenza is known to peak during the pre-migratory stages (late summer and early fall) [2, 24], followed by a rapid decrease during autumn migration when it reaches its wintering grounds [2]. Many species aggregate at stopovers or wintering sites, which results in high local densities. Their sites, which in Trøndelag are farmlands, might be important for LPAI transmission between other wild bird species and captive birds. Our results for *S. aluco* could have therefore been different if we had tested them in autumn versus spring.

Considering migration and pathogen infections, the climate will also influence migratory individuals. When the climate in the northern territories becomes more extreme, it could

affect the migration flyway for infected individuals. Seroprevalence studies done on baltic common eiders (*Somateria mollissima*) and pink-footed geese (*A. brachyrhynchus)* in Trøndelag showed an overall LPAI seroprevalence of 50% for both species (Lam et al., 2020). Further data from 2019 shows no seroprevalence nor any clinical signs of active infections in white-tailed eagle (*H. albicilla)* and northern goshawk (*Accipiter gentilis*) nestlings in Trøndelag (Lee et al., 2019). More recent studies (2022) show migratory waterfowl testing positive for HPAI only in the southern parts of Norway [8, 9, 11]. As further north, only the *H. albicilla* have been testing positive for HPAI, but with another subtype H5Nx (unidentified) than the rest of the infected birds [9, 11]. The middle parts of Norway might therefore still be too far away for migratory birds carrying active diseases, at least HPAI.

For further research it is important to conduct long-term surveillance studies and continue monitoring wild bird population in the northern regions. Resident birds in the north are known to have a poorer disease resistance compared to migrator species [25, 26], which is correlated with a lower pathogen pressure. Introduction of new pathogens could therefore be lethal for species the infection is not endemic to. So far, there is not documented any active AIV infection in the local *S. aluco* population nor the passerines in Trøndelag. However, there has been antibodies detected in migratory waterfowl, as well as one HPAI infected *H. albicilla*, in these areas. It is therefore important with continued monitoring of pathogen prevalence in the north.

Method

Study area

This study was carried out in a cultural landscape in the north-eastern parts of Trondheimsfjorden (64°N, 11°E) in central Norway (figure 1). The study area represents the northern boundary of the *S. aluco's* distribution range in Europe [14]. A total of 150 boxes were visited in April-May 2021. *S. aluco* prefer nesting in mixed habitats, i.e., where there is open landscape for hunting during the evenings/night and forest for cover during daytime. Most of the nestboxes were therefore located nearby farming lands.

The passerines were also sampled in central Norway. For fieldfare (*Turdus pilaris*) most of the nests were found at Grafmarka in Levanger (63.73°N, 11.33°E) (figure 1). Nestlings were picked from their nests, and a total of 9 nests where visited. The other passerines (table 1) were caught at Ørin field station in Verdal (63.79°N, 11.44°E) (figure 1).

Figure 1: In the left pane is an overview of the study area. Red pins describe *S. aluco's* nesting boxes, green pin describe Grafmarka where the *T. pilaris* were sampled in June, and the blue pin describes Ørin field station where rest of the passerines were sampled in October. In the top-right pane is a map of Scandinavia (Norway, Sweden, Denmark and Finland). The red mark indicates northern Trøndelag where the birds were sampled. In the low-right pane is a closer look up of the study area.

Catching and handling S. aluco

Adults

A total of 43 adult female *S. aluco* were sampled at both the cloaca and beak. The females were sampled during breeding season (late April to early May 2021). During this period, the female incubates the eggs and is therefore available for capture. The owls were caught by placing hand-nets over the peephole of their nestbox and then gently tapping the nestbox so they would exit into the net (figure 2A). After sampling, the owl is placed in a bag to reduce

stress before being placed back in her box. After it is returned, the peephole is covered for a couple of minutes.

Nestlings

A total of 28 nestling was sampled at both cloaca and beak. The nestlings were sampled during the first 2-3 weeks after hatching (during May and early June 2021). At this time, nestlings have developed a proper digestive system, with a large beak and cloaca, but are still too small to jump the nest. The nestlings were gently plucked from the nesting box, where they were stored in a tight bag during transportation between the nestbox and the ground. After sampling, the nestlings were placed back to their nest again. Only the heaviest nestling was sampled.

AIV sampling

The respiratory mucosa (hereafter beak) and cloaca of n = 43 adult and n = 28 nestling *S. aluco* were swabbed using viral transport medium (VTM) swabs (Copan diagnostic). Separate swabs were used for the cloaca and the beak.

Samples from the beak were taken by running the swab from the back of the throat, along the palate, to the end of the palate (Figure 2B). This was repeated 3 times. During beak sampling, the person holding the owl was holding the beak open, so it was easy to access with the swab. Samples from cloaca were taken by inserting the swab carefully inside the cloaca until the tip of the swab was completely submerged, and then twisting it gently 3 times (Figure 2C). To detect the cloaca the individual was laid on its back with the legs held up high (figure 2C). Then the cloaca was detected by moving from underneath the tail, up towards the stomach until the cloaca was spotted. Handling time of each individual was under 20 minutes, including catching, sampling and placing the owl back in the nestbox.

Figure 2: A) Catching *S. aluco* by the use of hand net which are covering the peep hole on the nesting box. B) Respiratory mucosa samples from adult *S. aluco*. C) Cloacal sample from adult *S. aluco*. One person is holding the owl, while another is taking the samples.

Catching and handling passerine birds

Adults

A total of 62 samples from cloaca and beak were collected (30 adults and 32 juveniles). The adult passerines were caught in mist nets between June and October 2021 (Table 1, Table 2) Handling the nets and adult birds was done by certified ornithologists.

Nestlings

T. pilaris nestlings were sampled in June (Table 2). The nestlings were picked from their nests. One person was holding the nestling, while another one was taking the samples. Then each nestling was safely placed back in the nest. On average there were 5 nestlings in a nest and each nestling was sampled (Table 2). Each nestling was sampled in the nest due to *T. pilaris* being social monogamous birds (in contrast to *S. aluco*). It is thus common for the female to breed with several males in the same season [27], which means that they can also be exposed to different parasites, pathogens etc.

Pathogen samples

Mini-dry swabs (Copan diagnostics) were used for the passerine adults and the *T. pilaris* juveniles. Immediately after sample collection, the mini-dry swabs were immersed in 1 ml of RNA later for storage. Swab samples were taken from the beak and the cloaca of both adults and nestlings. Separate swabs at appropriate size were used for the beak and cloaca. Samples were taken as described for *S. aluco*. Handling time at each nest and with each adult did not exceed 20 minutes.

Table 1: Overview of adult passerine species sampled in October.

	Date	Gender	ID number	Ring numbe	Specie	Beack	Cloaca
	21.10.2021	Adult	21	ES45921	*Parus major*	x	
	21.10.2021	Adult	70	ES45922	*Fringilla montifringilla*		x
	21.10.2021	Adult	85	ES45922	*Fringilla montifringilla*	x	
	21.10.2021	Adult	34	ES45923	*Carduelis chloris*		x
	21.10.2021	Adult	51	ES45923	*Carduelis chloris*	x	
	21.10.2021	Adult	45	HK30417	*Cyanistes caeruleus*	x	
	21.10.2021	Adult	63	8P38160	*Emberiza citrinella*		x
	21.10.2021	Adult	2	8P38160	*Emberiza citrinella*	x	
	21.10.2021	Adult	55	8P38161	*Emberiza citrinella*		x
	21.10.2021	Adult	10	8P38161	*Emberiza citrinella*	x	
	21.10.2021	Adult	93	8P38162	*Emberiza citrinella*		x
	21.10.2021	Adult	33	8P38162	*Emberiza citrinella*	x	
	21.10.2021	Adult	42	ES45923	*Carduelis chloris*		x
	21.10.2021	Adult	60	ES45923	*Carduelis chloris*	x	
	21.10.2021	Adult	80	8P38163	*Emberiza citrinella*		x
	21.10.2021	Adult	88	8P38163	*Emberiza citrinella*	x	
	21.10.2021	Adult	18	EN98883	*Carduelis chloris*		x
	21.10.2021	Adult	69	EN98883	*Carduelis chloris*	x	
	21.10.2021	Adult	90	8P38164	*Emberiza citrinella*		x
	21.10.2021	Adult	78	8P38164	*Emberiza citrinella*	x	
	21.10.2021	Adult	98	HK30094	*Cyanistes caeruleus*	x	
	21.10.2021	Adult	75	ES45924	*Parus major*		x
	21.10.2021	Adult	14	ES45924	*Parus major*	x	
	21.10.2021	Adult	23	8P38165	*Emberiza citrinella*	x	
	21.10.2021	Adult	82	ES45925	*Carduelis chloris*	x	
	21.10.2021	Adult	22	ES45529	*Parus major*	x	

Table 2: Overview of juveniles, their nests, and adults *T. pilaris* sampled in June and October.

Date	Nest	Gender	Number	Ring number	Specie	Beak	Cloaca
05.06.2021	1	Juvenile	1		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	2		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	3		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	4		*Thurdus pilaris*	x	x
05.06.2021	2	Juvenile	5		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	6		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	7		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	8		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	9		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	10		*Thurdus pilaris*	x	x
05.06.2021	3	Juvenile	11		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	12		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	13		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	14		*Thurdus pilaris*	x	x
05.06.2021	4	Juvenile	15		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	16		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	17		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	18		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	19		*Thurdus pilaris*	x	x
05.06.2021	5	Juvenile	20		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	21		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	22		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	23		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	24		*Thurdus pilaris*	x	x
05.06.2021	6	Juvenile	25		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	26		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	27		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	28		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	29		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	30		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	31		*Thurdus pilaris*	x	x
05.06.2021		Juvenile	32		*Thurdus pilaris*	x	x
05.06.2021	no nest	Adult female	1		*Thurdus pilaris*	x	x
05.06.2021	no nest	Adult female	2		*Thurdus pilaris*	x	x
21.10.2021	no nest	Adult female	65	7671104	*Thurdus pilaris*	x	x
21.10.2021	no nest	Adult female	1	7671104	*Thurdus pilaris*	x	

Detecting AIV
RNA extraction

Thermo Fisher's MagMAX Pathogen RNA/DNA kit was used for RNA extraction of all swabs (supplementary protocol 1). In short, the protocol for low-cell-content samples was followed. All solutions and buffers were prepared beforehand. Two negative controls were added to each 96 well plate to check for accidental contamination. First, the bead mix was added to each well. All swabs were then vortexed for 1 minute before 50 ul were extracted and placed at the reaction plate. Then lysis/binding solution was added to each well. The wells were then washed with wash solution 1 and 2 two times each. Between each wash step, a microplate shaker (VWR) was used at moderate speed (5). Then the beads were caught using a magnetic stand for 1 minute. The elution buffer was added to each well for complete bead suspension. The supernatant was then extracted and placed on a clean reaction plate. This plate was stored overnight in the freezer (-20^{o}C) for analysis.

Quantitative Polymerase Chain Reaction (qPCR) and sequencing

For the qPCR on RNA from the passerine birds, BIO-RAD CFX Connect Real-Time System and the software Bio-Rad CFX Maestro was used.

To run the qPCR for avian influenza the VetMAX™-Gold AIV Detection Kit was used (supplementary protocol 2). In short, this kit contained components to make up a Master Mix, which included a specific probe for avian influence virus A (Influenza virus primer probe mix). In given order, 17 uL RT-PCR Master Mix, 8,0 uL test sample and 0,8 uL nuclease-free water was added to each well. Duplicates were made for each sample, as well as duplicates for one positive control (added Influenza Virus-Xeno RNA Control Mix) and one negative control (added Nuclease-free water) to check for accidental cross-contamination. The thermal cycle program had three stages: 1) reverse transcription at 48^{o}C in 10 min, 2) RT inactivation, initial denaturation at 95^{o}C in 10 min, and 3) amplification at 95^{o}C for 15 sec and 60^{o}C for 45 sec.

RNA/DNA extraction from *S. aluco* was sent to the Norwegian Veterinary Institute at Ås for Avian Influenza Virus (AIV) detection through qPCR and sequencing. The Mx3000 series real time machines were used (supplementary protocol 3).

Ethical statement

S. aluco were sampled in collaboration with a monitoring project of breeding *S. aluco* in central Norway. In collaboration with an experienced FELASA C certified ornithologist, the owls were safely removed from their nesting boxes and sampled via non-destructive methods as outlined in this study, before being returned to the nest boxes. The passerines were also collected by FELASA C certified ornithologists from Nord University. Swab sampling were approved by the Norwegian Food Safety Authority (FOTS ID: 27025/23120).

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