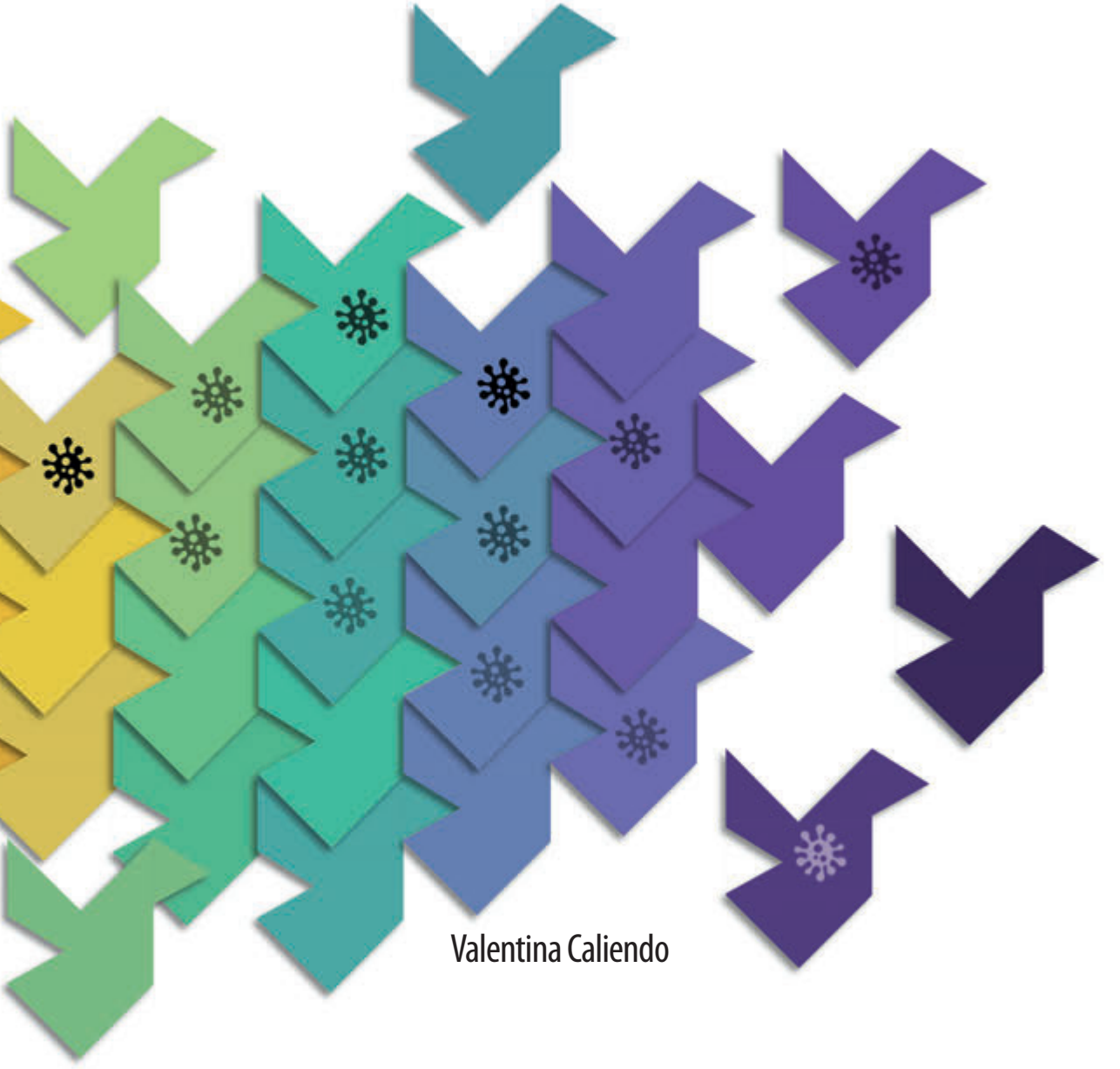
Pathogenesis and epidemiology of the Goose/Guangdong lineage of highly pathogenic avian influenza in wild birds



Valentina Caliendo

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Pathogenesis and Epidemiology of the Goose/ Guangdong Lineage of Highly Pathogenic Avian Influenza in Wild Birds

Pathogenese en epidemiologie van de Goose/ Guangdong lineage van hoogpathogene vogelgriep bij wilde vogels

Thesis

to obtain the degree of Doctor from the
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by command of the
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The public defence shall be held on

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by

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Chapter 1: General Introduction

Overall introduction

Highly pathogenic avian influenza (HPAI) is a major detriment to healthy and profitable poultry production worldwide as well as a potential threat for zoonotic outbreaks. The original reservoir of avian influenza are wild birds. However, the viruses occurring in wild birds cause no or mild disease in poultry and are therefore classified as low pathogenic avian influenza (LPAI) viruses. Recently, the dynamics of the HPAIV infection have dramatically changed due to the fact that the viruses have started using wild birds as a new environmental niche. This new system provides a new route of virus transmission into poultry farms, resulting in an increased risk of HPAI virus infection.

An important gap in our knowledge about avian influenza is how HPAI virus, which originates from poultry, has become adapted to the wild bird reservoirs. The immediate goal of the work described in this thesis is to understand the key factors that determine the adaptation of HPAI virus in wild birds. Hence, we investigated the potential for some HPAI viruses (e.g. H5N8 clade 2.3.4.4) to be maintained in wild bird populations and spread over long distances by observing the responses of certain wild bird species to the virus infection, including clinical signs and route of viral excretion, and by investigating which organs are infected by the virus. The ultimate goal of this project is to reduce the incidence of HPAI virus infection in poultry, wild birds, and humans.

LPAI viruses in wild birds and poultry

Avian influenza (AI) virus belongs to the family Orthomyxoviridae and genus Alphainfluenzavirus (1,2). Genetically, AI virus is a negative sense single-stranded RNA virus, composed of six gene segments that code for the polymerase complex (polymerase basic 2 [PB2], polymerase basic 1 [PB1] and polymerase acidic [PA]), the nucleoprotein (NP), the matrix proteins (M1 and M2), and the nonstructural proteins (NS1 and NS2). Influenza virus subtypes are classified based on two surface glycoproteins, the haemagglutinin (HA) and neuraminidase (NA), encoded by the HA gene and the NA gene respectively (1-5). To date, 16 HA subtypes (H1 – H16) and 9 NA subtypes (N1 – N9) have been isolated in birds (1). Mutations and reassortments of the viral genome occur frequently, and they are the primary determinants of genetic diversity in influenza viruses (3,6,7).

Wild waterbirds, in particular waterfowl such as Anseriformes (mainly ducks, geese, and swans) and Charadriiformes (mainly gulls, terns and waders), are the main reservoir of AI viruses (1,7,8). Migratory waterbirds are primarily responsible for annual AI virus dissemination and for the occurrence of novel strains through reassortment (1,9-13). Wild birds are often vectors for viral incursion in poultry farms, with serious consequences for poultry industry (7, 14-17).

The pattern of disease associated to AI virus infection is different in waterbirds compared to poultry. Wild ducks and geese infected with LPAI virus typically do not show signs of disease (6,7). Epithelial cells are the main target of infection in these birds and the virus

mainly infects the intestinal epithelium, and less frequently the respiratory epithelium (6). Infection with LPAI viruses in poultry typically produces limited clinical signs, and the virus preferentially targets epithelium of the respiratory tract (6,7). The primary transmission route of AI between birds is the fecal–oral route (9). Avian influenza viruses are transmitted when waterfowl forage in water contaminated with feces from infected birds (1,9). Dabbling ducks filter feed to strain food on the surface of the water, allowing effective fecal-oral transmission (1). Viral particles in feces and freely within the environment (predominantly water) are relatively stable. Virions may retain infectivity in duck feces for at least 30 days at 4 °C and for seven days at 20 °C, and in surface waters for more than seven months (18-28).

HPAI viruses in poultry

Low pathogenic avian influenza viruses, especially H5 and H7 subtypes, are able to mutate into a highly pathogenic (HP) pathotype in poultry (6,7). In conventionally housed poultry, the mutation from LPAI virus to HPAI virus gives the virus a selective advantage, at least temporarily. The presence of a multi-basic cleavage site (the insertion of several nucleotides coding for basic amino acids at the cleavage site of the HA protein) is the criterion to classify AI virus as HP (1,7,8). The multi-basic cleavage site enables the viral HA to be cleaved (activated) by ubiquitous furin-like proteases with subsequent systemic virus replication. Intravenous inoculation in six-week-old chickens is used for the determination of the intravenous pathogenicity index (IVPI), that also categorizes the pathogenicity of AI viruses (6).

Infection with HPAI viruses of the H5 and H7 subtypes is associated with severe systemic disease in galliform poultry (6,7). HPAI viruses can infect endothelial cells of multiple organs, such as lung, liver, heart, kidney, spleen, pancreas, bursa, and brain (6). HPAI viruses are endotheliotropic in Galliformes and infection manifests with edema formation and hemorrhages, coagulation disturbances, and induction of acute pro- inflammatory cytokines (cytokine storm), which may lead to acute mortality (6,8).

HPAI viruses have evolved in poultry and become endemic in certain countries. Traditionally, the viruses spread via the transport of infected poultry, infected poultry products, or HPAIV-contaminated materials (1). Until 1996, HPAI viruses had been detected once in wild birds, but this was not associated with poultry outbreaks (1).

Emergence of the GS/GD lineage

In 1996 the Goose/ Guangdong (GS/GD) lineage of H5 HPAI viruses emerged in Asia. The emergence of the GS/GD lineage was associated with marked increase of HPAI outbreaks (1). While most of these outbreaks have been controlled relatively quickly, HPAIV H5N1 has been circulating in poultry continuously since 1997 (1).

In addition to this continued circulation, HPAI H5 virus was also unusual in the unprecedented scale and geographical spread of the outbreaks, the transmission to a wide variety of mammalian species including humans and the introductions into wild birds (1,10,11).

Regional land use and poultry husbandry systems in southeast Asia contributed to the spillover of HPAIV from poultry to wild waterfowl, and consequently made possible for the virus to be transported long distance along migratory flyways to previously unaffected areas (10,11). The introduction of HPAIV into wild birds and the subsequent spread throughout Asia, the Middle East, Africa, North America and Europe has put a new focus on the role of wild birds in the geographical spread of avian influenza (1, 10, 11).

The GS/GD HPAI H5 viruses diversified into multiple genetic clades. Several of these clades have spread intercontinentally via wild birds: clade 1 in 2004, clades 2.2 and 2.2.1 from 2005 to 2007, clade 2.3.2 from 2008 to 2010, and clade 2.3.4.4 from 2014 to 2019 (1,10,34-40). In mid-October 2020, novel clade 2.3.4.4 HPAI H5N8 viruses were detected in Europe, causing the most devastating HPAIV outbreak ever recorded (1,36).

The transmission of GS/GD HPAI H5 viruses is associated with avian migrations, but the routes for viral dispersion, as well as the species involved, are still under investigation (10). At a global scale, the spread of GS/GD HPAI H5 lineage follows a seasonal pattern with a high density of outbreaks from autumn to early spring (i.e., October to March) that is associated with the patterns of migration of wild birds (1,10). The wild bird species involved in the global spread of HPAIVs are rapidly increasing (10). In 2014/2015, only four wild bird species were found positive for HPAIV in Europe. Subsequently, 56 species were found positive in Europe in 2016/2017 (10). Several species (e.g., Eurasian wigeon [*Mareca penelope*], Eurasian teal [*Anas crecca*], and northern pintail [*Anas acuta*]) have migratory routes that correspond to the observed pattern of virus spread, have been found infected with H5 HPAI virus at different locations along their respective migratory routes, and can be infected with and excrete H5 HPAI virus without showing detectable clinical signs (1,10,41-44).

In infected wild birds, GS/GD HPAIV H5 viruses are recovered mainly in oropharyngeal swabs rather than in cloacal swabs, suggesting respiratory transmission, and they predominantly infect epithelial cells of the respiratory tract (6). Systemic viral spread may lead to infection of parenchymal cells of other organ systems with associated multi-organ necrosis and inflammation (6). Clinical signs associated with HPAIV infection in wild birds vary greatly, and range from absence of signs to neurological signs, and to sudden death (7). The knowledge of protective effect and duration of HPAI H5-specific antibody detection in wild birds is limited, but seroconversion or increased H5 antibody titers are associated with survival in ducks (1, 29-33).

National surveillance programs, aimed to test wild birds for the presence of virus (or serum antibodies), represent early warning systems for the presence of HPAI viruses (1,31,37). Surveillance in wild birds is also a mechanism to monitor the zoonotic potential of newly circulating HPAIVs, as zoonotic infections from wild birds to mammals and humans may occur (6,7,36).

Thesis outline

The dynamics of GS/GD H5 HPAI viruses in wild birds are rapidly evolving and there is an urgent need to adopt a new approach in dealing with these changes. This thesis aims to

provide new information on the pathogenesis and epidemiology of GS/GD H5 HPAI viruses in wild birds, that will support decision making for HPAI control.

Chapter 2 (chapter 2.1- 2.4) explores the pathogenesis of HPAI H5 viruses that circulated during the 2014/2015, 2016/2017 and 2020/2021 outbreaks in key host wild bird species. The degree and duration of protection in wild birds from previous HPAI virus infection is poorly understood. In **chapter 2.1** tufted ducks (*Aythya fuligula*) and mallards (*Anas platyrhynchos*) were serially infected first with 2014 H5N8 and after 9 months with 2016 H5N8. Clinical signs, viral excretion, water contamination and serological response were monitored during the experiment.

The mass mortality of wild birds associated to the 2016/2017 outbreak in the Netherlands was an indication that the virus was able to rapidly infect a large number of wild birds in close proximity. HPAI viruses have been described to mainly infect epithelial cells of the respiratory system of wild birds; **chapter 2.2** investigated whether a change in viral tropism had occurred that could explain the high number of infected wild birds.

The 2020/2021 outbreak was also devastating for wild birds in the Netherlands. New wild bird species, such as the barnacle goose (*Branta leucopsis*), experienced a high number of infections and mass mortality. **Chapter 2.3** investigated the pathogenesis in this new host and provided possible explanations for this event. During the 2020/2021 HPAI outbreak, common buzzards (*Buteo buteo*) were also reported infected and dead in large numbers. The characteristics of HPAI virus infection in this raptor species were investigated in **chapter 2.4** in view of their possible role as bio-sentinels for HPAIV presence in wild bird populations.

Chapter 3 explores the evolving epidemiology of HPAI in wild birds. During the 2020/2021 outbreak, mass mortality of wild birds in the Netherlands was once again reported. **Chapter 3.1** investigated the species most affected and the spatial-temporal pattern of the outbreak. In 2021 HPAI virus was also detected in Canada, having originated from European wild birds. The possible dynamics, routes and wild bird species linked to the spread from Europe to Canada were reviewed in **chapter 3.2**. Wild birds under rehabilitation in recue centers are also at risk of HPAI virus infection. **Chapter 3.3** reconstructed the events leading to an outbreak in a bird rescue center and provided advice on prevention of future incidents.

Finally, the findings presented in chapter 2 and 3 are evaluated in the summarizing discussion (**chapter 4**).

Chapter 2: Pathogenesis of the GS/GD HPAI H5 viruses in wild birds

Chapter 2.1

Long-term protective effect of serial infections with H5N8 highly pathogenic avian influenza virus in wild ducks. 2022

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Submitted

Abstract

Highly pathogenic avian influenza viruses (HPAIVs) of the Goose/Guangdong (Gs/Gd) lineage are an emerging threat to wild birds. In the 2016-2017 H5N8 outbreak, unexplained variability was observed in susceptible species, with some reports of infected birds dying in high numbers, and others of apparently subclinical infections. This experimental study was devised to test the hypothesis that previous infection with a less virulent HPAIV (i.e., 2014 H5N8) provides long-term immunity against subsequent infection with a more virulent HPAIV (i.e., 2016 H5N8). Therefore, two species of wild ducks—the more susceptible tufted duck (*Aythya fuligula*) and the more resistant mallard (*Anas platyrhynchos*)—were serially inoculated: first with 2014 H5N8, and after 9 months with 2016 H5N8. For both species, a control group of birds was first sham-inoculated, and after 9 months inoculated with 2016 H5N8. Subsequent infection with the more virulent 2016 H5N8 caused no clinical signs in tufted ducks that had previously been infected with 2014 H5N8 (n = 6), but caused one death in tufted ducks that had been sham-inoculated (n = 7). In mallards, 2016 H5N8 infection caused significant body weight loss in previously sham-inoculated birds (n = 8) but not in previously infected birds (n = 7).

Importance

This study showed that ducks infected with a less virulent HPAIV developed immunity that was protective against a subsequent infection with a more virulent HPAIV 9 months later. Following 2014 H5N8 infection, the proportion of birds with detectable influenza-specific antibody declined from 100% (8/8) in tufted ducks and 78% (7/9) in mallards after 1 month to 33% (2/6) in tufted ducks and 29% (2/7) in mallards after 9 months. This finding will help predict the expected impact that an HPAIV outbreak may have on wild bird populations, depending whether they are immunologically naïve or have survived previous infection with HPAIV.

Introduction

Highly pathogenic avian influenza viruses (HPAIVs) of the Goose/Guangdong lineage are an emerging threat to wild birds (1-11). Since the emergence of the H5 A/goose/Guangdong/1/96 (Gs/Gd) lineage in 1996, HPAIVs have successfully adapted and circulated widely in several wild bird species (3,8). Wild waterfowl now constitute an important vector for HPAIVs and their global spread. Wild Anseriformes offer HPAIVs the great evolutionary advantage to travel via their migratory routes and the opportunity to change their genetical pool by reassorting with circulating low pathogenic avian influenza viruses (LPAIVs) (7,12). Examples of these successful mechanisms are the numerous global incursions of the subtype H5N8, clade 2.3.4.4, in 2014-2015, 2016-2017 and 2020-2021, which to date are responsible for the highest number of HPAIV outbreaks in wild birds. During the H5N8 outbreak in 2014-2015, the virus spread long-distance from Asia to Europe and North America via infected migratory birds (1,2,6). Epidemiological analysis and experimental infection studies showed that wild ducks (including Eurasian wigeons *Anas penelope* and mallards *Anas platyrhynchos*) can be infected with 2014 H5N8 virus without clinical or pathological evidence of disease (13-20). Two years later, the 2016-2017 H5N8 outbreak also spread intercontinentally along the wild bird migratory pathways, and caused a large and widespread highly pathogenic avian influenza (HPAI) epidemic in Europe. In the Netherlands alone, more than 13,600 wild birds were reported dead and up to 5% of the wintering populations of tufted ducks (*Aythya fuligula*) and Eurasian wigeons (more than 2,500 birds for each species) may have died (9). The 2020-2021 outbreak also caused extensive mortality in wild birds and for the first time in geese. In the Netherlands, Barnacle geese (*Branta leucopsis*) were the most affected species (1,2). During these outbreaks, HPAIV H5N8 was isolated from apparently clinically healthy free-living wild ducks (mainly Eurasian wigeons and mallards) with some birds also presenting HPAI H5 virus-specific antibodies (7,16,17).

It is not known how come there were so many differences in outcome within a single species in the 2016-2017 outbreak; particularly for the Eurasian wigeon, there were both events of high, HPAI-related, mortality as well as events of live, HPAIV-positive, but otherwise apparently healthy, birds (9,16). In the field, the fact that apparently healthy birds have serum antibodies against avian influenza viruses (AIVs) is an indication that birds can survive HPAIV infections. However, it is not understood what determines that some wild ducks die from infection, but others do not. Experimental studies comparing the pathogenesis of infection with 2014 H5N8 versus 2016 H5N8 showed that 2016 H5N8 had an augmented virulence for two duck species (13, 18). Experimental studies have shown the effect of short-term protection after serial HPAIV infection in Pekin ducks (*Anas platyrhynchos domesticus*) and mallards (19). However, it is not known whether previously infected birds can survive subsequent challenges after long intervals, for example between two consecutive autumn migrations. It is also not known whether this is valid for all bird species, or there are differences in outcome between species that are highly susceptible to disease (e.g., tufted duck), and less susceptible species (e.g., mallard).

This experimental study was devised to complement field observations and to provide an explanation for the dynamics of the infection survival rate during the 2014-2015 and 2016-2017 H5N8 outbreaks in wild birds. The hypothesis was that a previous infection with a less virulent HPAIV (i.e., 2014 H5N8) provides a long-term immunity against a subsequent

infection with a more virulent HPAIV (i.e., 2016 H5N8). To test this hypothesis, two groups of wild ducks (either mallards or tufted ducks) were serially inoculated, first with 2014 H5N8, and after 9 months with 2016 H5N8. For both species, a control group of birds was first sham-inoculated, and after 9 months inoculated with 2016 H5N8. We used species that differed in their susceptibility to disease from HPAIV infection, with the mallard being less susceptible and the tufted duck being more susceptible. We had planned to include the Eurasian wigeon as another less susceptible species, but could not source sufficient birds. The timing of the inoculations was planned to correspond with the autumn peak of AIV infections, to better mimic the field dynamics of the outbreaks. We hypothesized that all mallards, both sham-inoculated and 2014-H5N8 inoculated, and possibly 2014-H5N8-inoculated tufted ducks would survive the infection with 2016 H5N8. Conversely, we hypothesized that the sham-inoculated tufted ducks would not survive the infection with 2016 H5N8, because they lacked protective immunity.

Results

Inoculation with HPAIV 2014 H5N8 (first inoculation). At the first inoculation, all 17 inoculated birds became infected, 100% (17/17) based on RT-PCR and 94% (16/17) based on virus isolation (Table 1).

Common name (taxonomic name)	Group	Virus	No. of birds	No. of birds with clinical signs	No. of birds excreting from pharynx		No. of birds excreting from cloaca	
					Virus isolation	PCR	Virus isolation	PCR
Mallard (*Anas platyrhynchos*)	H5-mallards	H5N8 2014	9	0	8	9	3	9
Mallard (*Anas platyrhynchos*)	H5-H5-mallards	H5N8 2016	6	0	0	3	0	2
Tufted duck (*Aythya fuligula*)	H5-tufted ducks	H5N8 2014	8	8	8	8	2	8
Tufted duck (*Aythya fuligula*)	H5-H5-tufted ducks	H5N8 2016	7	0	2	6	0	2
Mallard (*Anas platyrhynchos*)	Sham-mallards	Sham	8	0	Np	Np	Np	Np
Mallard (*Anas platyrhynchos*)	Sham-H5-mallards	H5N8 2016	7	0	7	7	2	7
Tufted duck (*Aythya fuligula*)	Sham-tufted ducks	Sham	7	0	Np	Np	Np	Np
Tufted duck (*Aythya fuligula*)	Sham-H5-tufted ducks	H5N8 2016	6	6	6	6	4	6

Table 1 Health status and virus excretion of 32 ducks experimentally inoculated with HPAIVs H5N8 (2014 H5N8 and 2016 H5N8). Np, not performed.

There was no significant loss of body weight of the infected tufted ducks ('H5-tufted ducks') compared to the control tufted ducks ('sham-tufted ducks'). One H5-tufted duck presented with transient, excessive eye blinking at 24 hr p.i.. There was significant loss of body weight (paired t-test, $p < 0.002$) of the infected mallards ('H5-mallards') compared to the control mallards ('sham-mallards') (Figure 1).

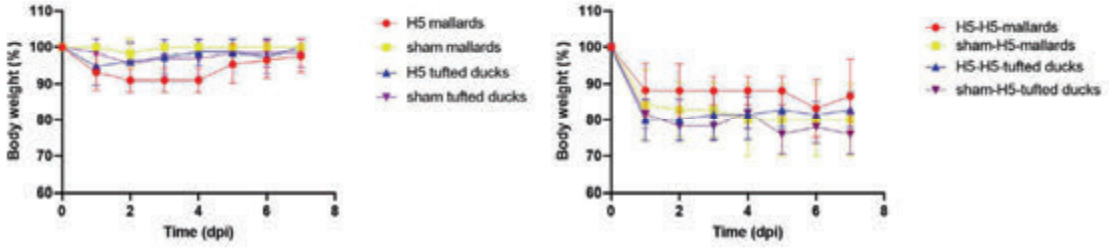

Figure 1 Body weight loss after inoculation with HPAIV 2014 H5N8 or sham inoculation (A) and after inoculation with HPAI 2016 H5N8 (B) in mallards and tufted ducks. After inoculation ducks were weighed and mean and standard deviation to the relative weight loss compared to the body weight at the day of inoculation was calculated.

Pharyngeal and cloacal excretion of infectious 2014 H5N8 between H5-mallards and H5-tufted ducks were not statistically different (Kruskal-Wallis test, AUC 0-7 dpi) (Table 2).

Host	Virus	Pharyngeal swabs			Cloacal swabs			Water		
		AUC (Mean ± SE)	Median (dpi)	Peak (dpi)	AUC (Mean ± SE)	Median (dpi)	Peak (dpi)	AUC (Mean ± SE)	Median (dpi)	Peak (dpi)
H5-mallards	H5N8 2014	7 ± 1.9	4.5	2	3.7 ± 0.2	4	3	4.1 ± 0	4.5	3
H5-H5-mallards	H5N8 2016	3.5 ± 0	0	0	3.5 ± 0.1	0	2	3 ± 0	0	1
H5-tufted ducks	H5N8 2014	4.1 ± 0.5	4	1	3.6 ± 0.2	0	3	12.8 ± 0	4	2
H5-H5-tufted ducks	H5N8 2016	4.1 ± 2.1	4	1	3.5 ± 0	0	0	3.8 ± 0	1.5	1
Sham-H5-mallards	H5N8 2016	16 ± 2.1	5	3	3.5 ± 0.1	5	2	3.5 ± 0	0	0
Sham-H5-tufted ducks	H5N8 2016	12 ± 1.8	4	3	4.3 ± 0.5	4.5	3	8.1 ± 0	4	3

Table 2 Level and duration of virus excretion of HPAIVs H5N8 (2014 H5N8 and 2016 H5N8) from the pharynx and cloaca in mallards and tufted ducks based on virus isolation, as well as virus contamination of drinking water. AUC, area under the curve summarizes infectious virus excretion from day 0 to 7 post inoculation; SE, standard error; dpi, days post inoculation. The

minimal detection limit of virus isolation was log 0.5 TCID50/ml and minimal area under the curve from day 0 to 7 post inoculation was 3.

For both groups, pharyngeal excretion exceeded cloacal excretion according to virus isolation and RT-PCR (paired t-test, p< 0.001) (Figure 2).

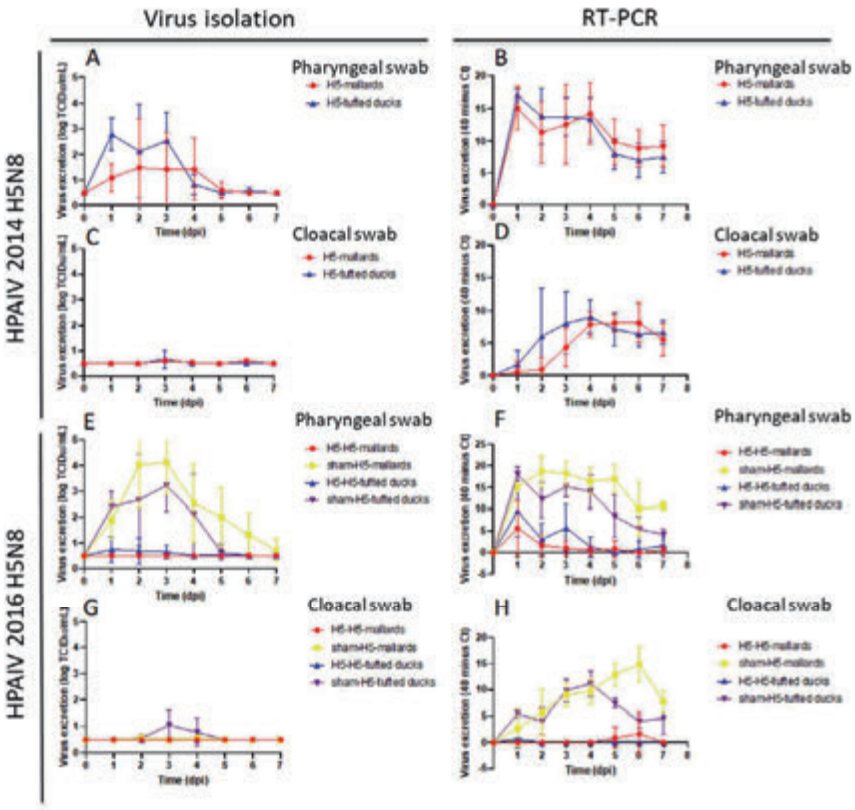

Figure 2 Virus excretion in mallards and tufted ducks of: HPAIV 2014 H5N8, via the pharynx based on virus isolation (A), and virus detection by RT-PCR (B); via the cloaca based on virus isolation (C), and virus detection by RT-PCR (D); and HPAIV 2016 H5N8, via the pharynx based on virus isolation (E), and virus detection by RT-PCR (F); via the cloaca based on virus isolation (G), and virus detection by RT-PCR (H). Symbols indicate mean values and error bars.

Virus concentration in drinking water samples of the H5-tufted ducks exceeded that of the H5-mallards, according to virus isolation (t-test, p< 0.02) but not to RT-PCR (Figure 3). However, the different drinking behaviors that the two species manifested during the

experiment (mallards generally used more water than tufted ducks, both for drinking and preening their feathers) may have affected this evaluation.

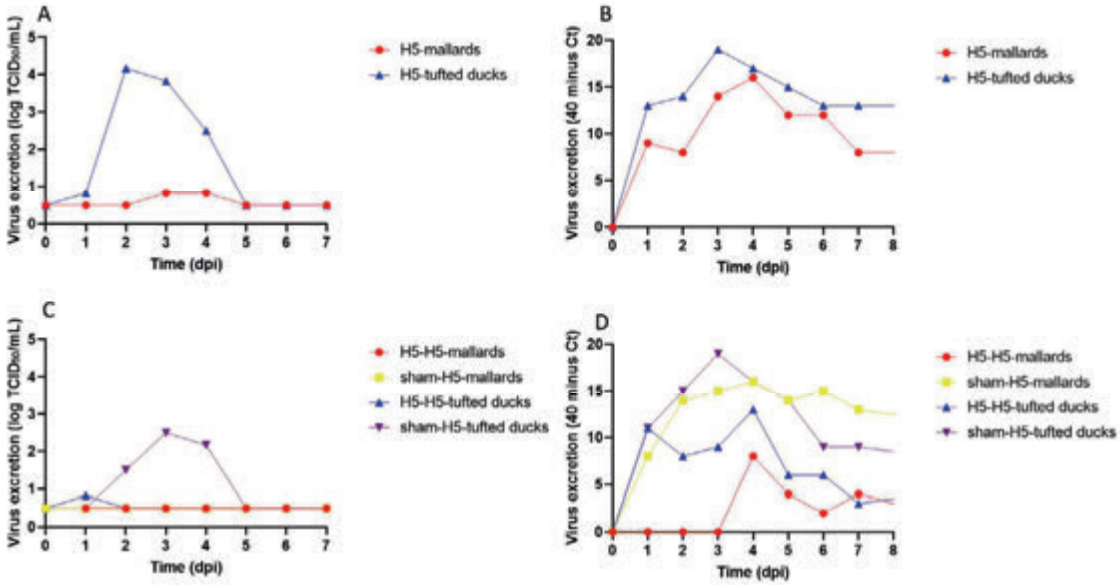

Figure 3 Virus contamination of drinking water for mallards and tufted ducks after inoculation with HPAIV 2014 H5N8, based on virus isolation (A) and virus detection by RT-PCR (B); and after inoculation with HPAIV 2016 H5N8, based on virus isolation (C) and virus detection by RT-PCR (D).

At 1 month p.i., 87% (7/8) H5-mallards and 100% (8/8) H5-tufted ducks had detectable NP-specific antibodies (Table 3). Just before the second inoculation, at 9 months p.i., these percentages had decreased to 33% (2/6) for H5-mallards and 28% (2/7) for H5-tufted ducks.

Inoculation with HPAIV 2016 H5N8 (second inoculation). After inoculation, 100% (13/13) control birds (seven sham-H5-mallards and six sham-H5-tufted ducks) became infected, based on both RT-PCR and virus isolation; 50% (3/6) of the H5-H5-mallards became infected based on RT-PCR and 33% (2/6) based on virus isolation; 85% (6/7) of the H5-H5-tufted ducks became infected based on RT-PCR and 28% (2/7) based on virus isolation (Table 1).

There was no significant loss of body weight of H5-H5-tufted ducks compared to the sham-H5-tufted ducks (Figure 1). One of the sham-H5-tufted ducks presented with general weakness and died overnight at day 4 p.i. Post-mortem examination macroscopically revealed multifocal areas of necrosis in the pancreas. Histologically, the necrotic areas colocalized with influenza virus antigen expression in pancreatic acinar cells (Figure 4).

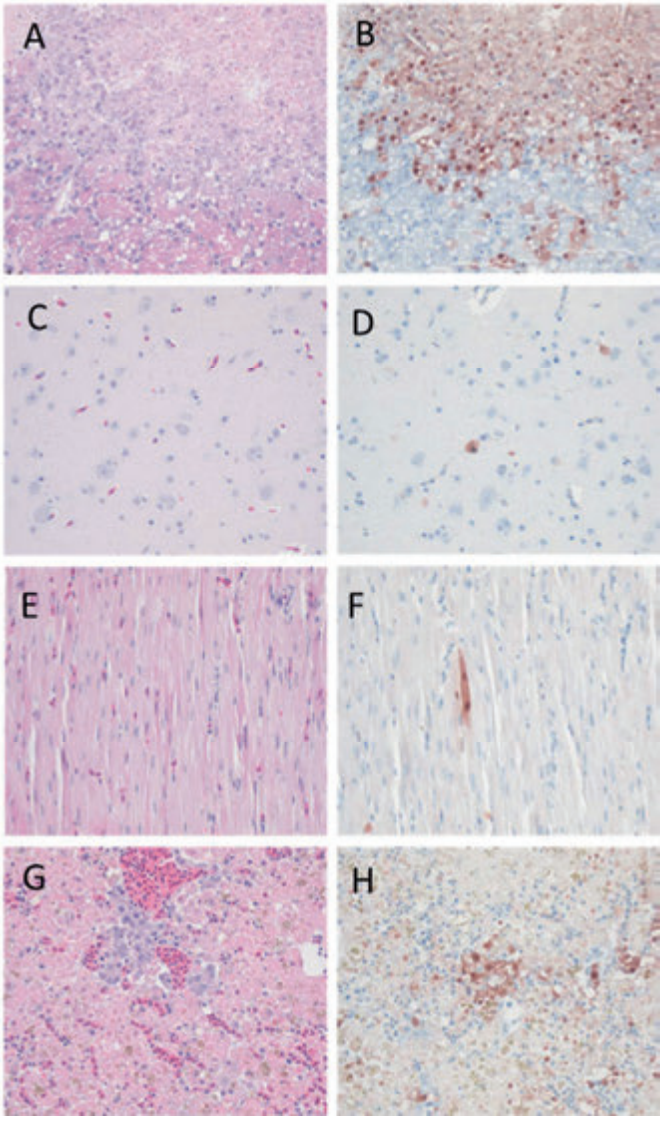

Figure 4 Tissue sections stained by hematoxylin and eosin (left column) or by immunohistochemistry (right column) from a sham-H5-tufted duck found dead at 4 days post infection with HPAIV 2016 H5N8. Pancreas: severe necrosis (A) and abundant expression of influenza antigen (B); brain: mild inflammation (C) and moderate expression of influenza antigen (D); heart: mild inflammation (E) and moderate expression of influenza antigen (F); liver: necrotizing hepatitis (G) and abundant expression of influenza antigen (H).

The liver had histological evidence of necrotizing hepatitis with abundant virus antigen expression in hepatocytes. Mild virus antigen expression was also present in brain (neurons), heart (myocytes), lungs and air sacs (epithelial cells). Virus antigen expression was not observed in the intestine. Virus was detected in tissues of all the main organs by virus isolation and RT-PCR, indicating that the virus had spread systemically (Table 4).

Organ	RT-PCR (40-Ct)	Virus titration (log $TCID_{50}$/ml)
Brain	19	5.1
Trachea	14	6.5
Air sac	18	4.8
Lung	12	5.5
Heart	12	5.1
Stomach	19	5.5
Jejunum	18	5.5
Colon	16	5.5
Pancreas	16	7.5
Liver	12	7.5
Spleen	16	5.5
Kidney	20	6.1

Table 4 Amount of HPAIV 2016 H5N8 present in tissues of a sham-H5-tufted duck that died at day 4 p.i., based on RT-PCR and virus titration.

There was a significant loss of body weight (paired t-test, $p< 0.01$) of sham-H5-mallards compared to the H5-H5-mallards, without any other clinical signs of disease. However, both groups of mallards, in particular H5-H5-mallards, had established a strong dominance hierarchy (dominant birds had priority access to food and water compared to subordinate birds) that may have interfered with body weight evaluation.

Pharyngeal excretion significantly differed between groups (one-way ANOVA, AUC 0-7 days p.i., $p<0.001$) (Figure 2). The mean quantity of virus excreted per group from 0 to 7 days p.i. was highest for sham-H5-mallards, followed by sham-H5-tufted ducks, H5-H5-tufted ducks, and was lowest for H5-H5-mallards, according to virus isolation and RT-PCR. Pharyngeal excretion was statistically higher for sham-H5-mallards vs H5-H5-mallards (t-test, $p<0.04$) and for sham-H5-tufted ducks vs H5-H5-tufted ducks (t-test, $p<0.02$). Pharyngeal excretion exceeded cloacal excretion in the different groups. Cloacal excretion was scarce and there was no statistically significant difference in cloacal excretion between groups, according to virus isolation. Cloacal excretion was scarce for H5-H5-mallards and H5-H5-tufted ducks, and cloacal excretion was statistically higher for sham-H5-mallards vs H5-H5-mallards (t-test, $p<0.0001$) and for sham-H5-tufted ducks vs H5-H5-tufted ducks (t-test, $p<0.0001$), according to RT-PCR. Concentration of virus in drinking water was scarce

for the different groups except that for sham-H5-tufted ducks, according to virus titration; virus concentration in the water was statistically higher for sham-H5-tufted ducks vs H5-H5-tufted ducks (t-test, $p<0.04$), according to the RT-PCR.

After the second inoculation, all the birds had detectable serum NP-specific antibodies at 15 days p.i.. All survivor birds were euthanized at the end of the experiment. Macroscopic and histologic examination of their organs did not show any abnormal findings, and thus no further tests were performed.

Discussion

This study showed that ducks infected with a less virulent HPAIV (2014 H5N8) developed a long-term immunity that was protective against a subsequent infection with a more virulent HPAIV (2016 H5N8). This finding is consistent with previously demonstrated long-term protective effect of LPAIV infection and short-term protective effect of HPAIV infection (19,20); and further demonstrates that long-term protection applies to HPAIV reinfections in relevant wild duck species.

Prior infection with less virulent 2014 H5N8 protected against clinical signs from more virulent 2016 H5N8 infection nearly one year later. After inoculation with 2014 H5N8, both mallards and tufted ducks became infected and excreted infectious virus. In accordance with our hypothesis, all mallards, both sham-inoculated and 2014-H5N8 inoculated, as well as 2014-H5N8-inoculated tufted ducks survived the infection with 2016 H5N8. The 2014-H5N8-infected mallards showed detectable clinical signs of disease (i.e., weight loss), and one of the 2014-H5N8-infected tufted ducks showed clinical signs of disease, consisting in mild neurological signs. Post inoculation with the more virulent 2016 H5N8, control mallards showed weight loss and control tufted ducks manifested increased mortality, although less than we had hypothesized, with only 14% (1/7) mortality rate. This incongruence could be due to the fact that birds in our study were all in good health pre-infection, and may have had a greater chance of survival when compared to free-range wild birds (12).

Pharyngeal viral excretion was higher than cloacal excretion, in accordance with previous experimental studies in these and other wild bird species (13-15). Infectious virus was excreted up to five days p.i., and likely transferred from the pharynx to drinking water. This fits with the idea that water can be an important source of infection of HPAIV for birds (13).

Antibody response in ducks is still only partially understood, and there is evidence that ducks only develop poor antibody responses and lack hemagglutination-inhibition-antibody responses to natural and experimental AIV infections (21). Studies on gulls and ducks showed that serum antibody levels did not correlate with protection against LPAIV infection (20,22). Although the humoral defense is unlikely to be the primary protection against AIV infection in ducks, the anti-NP-responses following infection in mallards may serve for protection against the virus (directly or via opsonisation to activate other parts of the immune system) (23). In our study we screened for NP antibodies because NP ELISAs are often used to assess the serological response against AIVs in multiple bird species and frequently replace the hemagglutination inhibition test for research and diagnostic purposes in wild birds and poultry (24-29). After inoculation with 2014 H5N8, presence of NP-specific antibodies was not associated with previous infection, nor with reinfection outcome. One month p.i. with 2014 H5N8, 88% of ducks had detectable NP-specific antibodies; however, this

percentage was much lower at 9 months p.i., when only 33% of inoculated mallards and 28% of infected tufted ducks presented serum antibodies against avian influenza virus. After inoculation with 2016 H5N8 there was no difference in survival for previously infected birds with and without NP-specific antibodies. This result is in line with the experimental study of Verhagen et al. 2015 (20), and further demonstrates that the value of this serological test in wild birds is limited in time, because of the short window of detection of NP-specific antibodies.

Mallards were our model for less susceptible species, and a comparison for elaborating on the dynamics of the 2016 H5N8 outbreak in the Eurasian wigeon population. All mallards survived the two infections and control mallards excreted more virus p.i. than control tufted ducks. The mallard excretion pattern of 2014 H5N8 was similar to those described by Keawcharoen et al. (2008) for 2005 H5N1 and van den Brand et al. (2018) for 2014 H5N8 (14,15). In concurrence with those studies, this study found that control mallards excrete a relatively high quantity of infectious virus and thus may be suitable vectors of HPAI H5 viruses. However, after reinfection with HPAIV H5N8, mallards did not excrete any infectious virus. This result may have an important consequence in the field because it excludes a significant role for previously infected mallards (and possibly other duck species) in spreading infectious HPAIVs. Given the increasing frequency of new HPAIV outbreaks, and the long-term protective effect of previous HPAIV infections, older mallards may not contribute to the persistence of HPAIV in the bird population. This also implies that shorter-lived wild waterbird species, and thus with a larger proportion of juvenile birds in the population, may be more important as reservoirs of HPAIV than longer-lived birds. Wild mallards are relatively short-lived (around 1-3 years) on account of heavy hunting pressure, while Eurasian wigeons and tufted ducks are relatively longer-lived species (around 3 and 10 years, respectively) (30-32).

Tufted ducks were our model for more susceptible species, based on both field and experimental data. Unusually high mortality of tufted ducks was reported during the 2016 H5N8 outbreak, as well as the earlier 2005-2006 H5N1 outbreak (9,11). Experimental studies showed that tufted ducks, and diving ducks more generally, develop fatal disease after infection with HPAIV H5N1 (15); however, infection with 2014 H5N8 in common pochards (Aythya ferina, a diving duck species) was asymptomatic (14). Our study showed that, under experimental conditions, previous infection protects tufted ducks 100% from clinical signs including body weight loss and mortality, compared to sham-inoculated tufted ducks that were clinically affected. This could explain why susceptible species (e.g. tufted ducks and Eurasian wigeons) that are infected in repeated HPAIV outbreaks have disparate outcomes: some remain apparently healthy because they have been infected in previous years, while others die because they are immunologically naïve.

Based on this observation, we could predict the impact of future HPAIV outbreaks on wild bird populations: previously infected bird populations (i.e., survived infection with a less virulent HPAIV) will have higher chances of surviving a subsequent infection with a more virulent HPAIV in the following influenza outbreak. The effect of this implication is suggested in the trend of the current 2020-2021 HPAIV H5 outbreak. During this outbreak, high mortality in duck populations was not reported, which could be related to the long-term protective effect of their exposure to previous HPAIVs (e.g., 2016 H5N8). Conversely, goose species like the Barnacle goose (Branta leucopsis), that were not reported infected during

previous HPAIV outbreaks, had unusually high deaths related to the infection in 2020-2021 (1,2), possibly because they lacked protective immunity from previous exposures. However, we cannot exclude the possibility that geese are inherently more susceptible to severe disease with 2020 H5 viruses.

Experimental and field studies showed that prior infections with both homologous and heterologous AIV can prevent re-infection, reduce the duration and extent of AIV shedding, or result in a higher infective dose required for subsequent infections (19,20,33,34). Pre-exposure to LPAIVs can also provide some level of protective immunity against a subsequent HPAIV infection (19,34). We would expect, however, that exposure to HPAI H5 exposure provides a stronger protective effect than to a LPAI H5, and that exposure to other LPAI virus subtypes provides an even lower protective effect.

In conclusion, this study showed that ducks infected with a less virulent HPAIV developed a long -term immunity that was protective against a subsequent infection with a more virulent HPAIV nearly one year later. This finding will help to understand and potentially predict the expected impact that an HPAIV outbreak may have on bird populations, depending whether they are previously exposed or naïve to HPAIV infections. This study also showed that serum antibodies post-HPAIV infection have a short window of detection, which should be taken in account during surveillance and assessment of outbreaks.

Materials and Methods

Virus preparation. The two HPAIVs used in this study were 2014 H5N8 clade 2.3.4.4 group A (A/Eurasian wigeon/Netherlands/emc-1/2014) isolated from the feces of a non-symptomatic wild Eurasian wigeon and 2016 H5N8 clade 2.3.4.4 group B (A/Eurasian wigeon/Netherlands/4/2016) isolated from a dead wild Eurasian wigeon. Full length HA and NA sequences and full genome sequences for these two virus isolates were obtained by Sanger sequencing and sequences were deposited in a public database (http://www.gisaid.com, EPI_ISL_168746 and EPI_ISL_255912). The viruses were propagated by two passages in Madin-Derby canine kidney (MDCK) cells. The harvested supernatant had a titer of 1 x 10^7 median tissue culture infectious dose ($TCID_{50}$)/ml and was diluted with phosphate-buffered saline (PBS) to 1 x 10^6 $TCID_{50}$/0.1 ml. These viruses were chosen because they both circulated in wild birds during the correspondent outbreaks and were expected to reproduce the field infections more realistically. All experiments with HPAIVs (2014 H5N8 and 2016 H5N8) were performed under Biosafety Level 3 conditions.

Animals. Two species of ducks were inoculated experimentally: one species of diving duck (tufted duck, *Aythya fuligula*) and one species of dabbling duck (mallard, *Anas platyrhynchos*). All ducks used for the infection experiments were captive bred. Birds were 4–5 months of age at time of first inoculation. Blood samples, cloacal swabs, and pharyngeal swabs were collected from all ducks one week before inoculation. Sera were analyzed, as described below, by using a commercially available influenza A virus antibody ELISA kit for the detection of antibodies against nucleoprotein (IDEXX) according to the manufacturer's instructions (16). Swabs were tested by reverse transcription–PCR (RT-PCR) (14,15). Prior to inoculation, no duck had anti-nucleoprotein antibody, and all tested negative by reverse transcription-polymerase chain reaction (RT-PCR) targeting the matrix gene.

Ducks were first inoculated with 2014 H5N8, and after 9 months with 2016 H5N8. For both species, a control group of birds was first sham-inoculated, and after 9 months inoculated with 2016 H5N8. The time between the two inoculations was chosen to approximate the time period between two consecutive autumns, when infection usually takes place in the field in Europe. Drinking water was sampled after the inoculations to check if birds could infect each other by contact with water.

Experimental design. For the infection with 2014 H5N8 (first inoculation), nine mallards and eight tufted ducks were housed in two negatively pressurized isolator units (Table 1). The method of inoculation was a standard method established by the Delta-Flu consortium. Each bird in these two groups (infected group, H5-mallards and H5-tufted ducks) was inoculated intra-choanally with 1×10^6 $TCID_{50}$ HPAIV 2014 H5N8 in 0.1 ml. At the same time, eight mallards and seven tufted ducks were sham-inoculated intra-choanally with 0.1 ml of PBS (naïve group, sham-mallards and sham-tufted ducks). Each day, a qualified veterinarian assessed all birds for clinical signs of disease. Body weights, water samples, and cloacal and pharyngeal swabs were collected daily for the first 7 days and every 2 days thereafter. After inoculation, ducks were weighed and mean and standard deviation to the relative weight loss compared to the body weight at the day of inoculation was calculated. Pharyngeal and cloacal swabs were collected using sterile cotton swabs and each placed in 1 ml virus transport medium (14,15). Each drinking bucket held a volume of 5 l, and water in the container was replaced daily. From each drinking bucket just before replacing water, 1 ml of water was collected in a sterile 2 ml tube containing 1 ml of virus transport medium. In order to test for the presence of infectious virus, to ensure that the birds would not carry infectious virus with them upon transfer to the BSL2 enclosure (after approval from the Erasmus University Biosafety Committee and in adherence to the Biosafety in Microbiological and Biomedical Laboratories-BMBL 4th edition), on days 9, 11 and 13 post inoculation (p.i.) we collected swab samples from the feathers and feet of the birds, in addition to collecting cloacal and pharyngeal swabs. All the swabs collected on days 9, 11 and 13 p.i. tested negative for virus at virus isolation. On day 15 p.i., after it was confirmed that the infected group had stopped shedding infectious virus, the birds were moved to an indoor BSL2 enclosure. All ducks were clinically inspected monthly. During inspection, blood samples were collected to monitor the presence of anti-nucleoprotein antibody, and cloacal and pharyngeal swabs were collected to monitor virus excretion. All ducks tested negative for virus during monthly checks.

During the time between the two inoculations, three mallards and one tufted duck from the H5-infected groups, and one mallard from the sham-inoculated group, died for causes unrelated to HPAIV infection (trauma, aspergillosis, egg bound).

Nine months after infection with 2014 H5N8, six mallards and seven tufted ducks from the infected group, and seven mallards and six tufted ducks from the naïve group were inoculated intra-choanally with 1×10^6 $TCID_{50}$ HPAIV 2016 H5N8 in 0.1 ml (second inoculation). The birds were monitored and sampled using the established methods. On day 15 p.i. all survivor birds were euthanized. The study was approved by an independent animal experimentation ethical review committee, approved by the Dutch government (Stichting DEC consult) (permit number AVD1010020186744, protocol 18-6744-01).

RT PCR and virus titrations. RNA isolation and RT-PCR were performed as described (14,15). Briefly, RNA from swabs and tissue suspensions was isolated by using a MagNaPure

LC system with the MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, the Netherlands). Real-time RT-PCR assays were performed on an ABI Prism 7500 Sequence Detection System machine (Applied Biosystems, Foster City, CA, USA) by using the TaqMan EZ RT-PCR Core Reagents Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) according to the manufacturer's instructions. For each run the samples were prepared and processed in parallel with several negative and positive control samples. Virus titers were determined by serial 10-fold dilution of the homogenized tissue samples and swabs on MDCK cells, as described (14,15). Virus titrations were performed in triplicate.

Antibody test. After the first inoculation, blood samples were taken monthly, and tested for serum antibodies. NP-specific antibodies were detected by using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (Idexx A Ab Test; Idexx Laboratories BV, Hoofddorp, the Netherlands). Idexx bELISA is a high throughput method for NP antibody quantitation in various host species (24,25,28,29). The test uses mouse-derived monoclonal antibodies to compete with serum antibodies for binding to the antigen-labelled test kit and performs well in multiple avian species (24,25). Applying the test per manufacturer's recommendations results in good performance with 84% sensitivity and 100% specificity (35). Samples were tested according to the manufacturer's instructions (16, 35). A sample was considered NP positive when the signal-to-noise ratio (i.e., ratio of the mean optical density [ODx] of the sample/ODx of the negative control) was 0.5 or lower.

Pathologic examination and immunohistochemical testing. Autopsies and tissue sampling were performed for all the birds, either ill from HPAIV infection or euthanized at the end of the experiment. After fixation of tissue samples in 10% neutral-buffered formalin and embedding in paraffin, two sequential tissue sections were processed for histology with hematoxylin eosin staining or for immunohistochemistry with a monoclonal antibody against nucleoprotein of influenza A virus as the primary antibody for detection of influenza viral antigen (14,15). The following tissues were examined: brain, trachea, lung, air sac, proventriculus, duodenum, pancreas, liver, jejunum, ileum, cecum, colon, spleen, kidney, heart, and adrenal gland.

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Ethics approval and consent to participate: The study was approved by an independent animal experimentation ethical review committee, approved by the Dutch government (Stichting DEC consult) (permit number AVD1010020186744, protocol 18-6744-01).

Chapter 2.2

Enterotropism of highly pathogenic avian influenza virus H5N8 from the 2016/2017 epidemic in some wild bird species.

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Abstract: In 2016/17, H5N8 highly pathogenic avian influenza (HPAI) virus of the Goose/Guangdong lineage spread from Asia to Europe, causing the biggest and most widespread HPAI epidemic on record in wild and domestic birds in Europe. We hypothesized that the wide dissemination of the 2016 H5N8 virus resulted at least partly from a change in tissue tropism from the respiratory tract, as in older HPAIV viruses, to the intestinal tract, as in low pathogenic avian influenza (LPAI) viruses, allowing more efficient faecal-oral transmission. Therefore, we determined the tissue tropism and associated lesions in wild birds found dead during the 2016 H5N8 epidemic, as well as the pattern of attachment of 2016 H5N8 virus to respiratory and intestinal tissues of four key wild duck species. We found that, out of 39 H5N8-infected wild birds of 12 species, four species expressed virus antigen in both respiratory and intestinal epithelium, one species only in respiratory epithelium, and one species only in intestinal epithelium. Virus antigen expression was association with inflammation and necrosis in multiple tissues. The level of attachment to wild duck intestinal epithelia of 2016 H5N8 virus was comparable to that of LPAI H4N5 virus, and higher than that of 2005 H5N1 virus for two of the four duck species and chicken tested. Overall, these results indicate that 2016 H5N8 may have acquired a similar enterotropism to LPAI viruses, without having lost the respirotropism of older HPAI viruses of the Goose/Guangdong lineage. The increased enterotropism of 2016 H5N8 implies that this virus had an increased chance to persist long term in the wild waterbird reservoir.

Key words: avian influenza, H5N8, H5N1, wild birds, tropism, virus histochemistry, immunohistochemistry, pathology, low pathogenic, highly pathogenic

Introduction

Avian influenza causes major economic damage to the poultry industry, as well as welfare issues to the poultry involved. For example, the global highly pathogenic avian influenza (HPAI) virus epidemic of the subtype H5N8 in 2014/15 led to the death or culling of over 50 million birds in seven countries in Asia, Europe, and North America (1,2,3). In addition, the ability of HPAI viruses to cross the species barrier and cause severe disease in humans, other mammals, and wild birds poses a more general threat to human and animal health (4,5).

The Goose/Guangdong lineage of H5 HPAI virus (originating from H5N1 HPAI virus A/Goose/Guangdong/1/1996) has persisted in poultry populations in parts of South-East Asia at least since 2003, and has circulated between poultry and wild birds, allowing continual virus evolution as well as reassortment of H5 HPAI virus with other avian influenza (AI) viruses (1,3). Descendants of the HPAI H5 Goose/Guangdong lineage are able to survive long enough in migrating wild birds to spread from South-East Asia as far as West Europe, North America, and—perhaps—South Africa (2). The adaptation of HPAI virus to wild birds provides an additional route of virus incursion into poultry holdings, and expands the geographic range over which HPAI virus poses a threat to human and animal health (1).

Low pathogenic avian influenza (LPAI) viruses are endemic in wild birds of the orders Anseriformes and Charadriiformes. LPAI viruses replicate in intestinal epithelial cells and are excreted mainly from the cloaca (6,7,8,9,10). These enterotropic viruses are transmitted by the faecal-oral route, including via the water bodies on which these birds reside [8]. In contrast, the H5 HPAI viruses that spread via migratory birds and caused epidemics in 2005/06 and 2014/15 were respirotropic and were excreted mainly from the pharynx. Specifically, experimentally infected ducks of several species showed evidence of virus replication in epithelial cells of the respiratory tract, but not of the intestinal tract (11,12,13,14,15). The scant cloacal shedding detected in some of those birds was attributed to virus replication in the liver, pancreas, or both, which are in contact with the intestinal lumen via bile and pancreatic ducts, respectively (11). Since then, H5 HPAI viruses spread again from Asia to Europe in 2016/17, causing the biggest and most widespread epidemic on record in wild and domestic birds in Europe (3,16).

It is unknown whether the H5 HPAI viruses from 2016/17 have become better adapted to replication in and transmission among wild birds, and so allowed the virus to spread so widely in Europe and to infect so many wild birds. We hypothesized that the dissemination of H5 HPAI virus in wild birds in Europe in 2016/17 resulted, at least in part, from a reversal of virus tropism from the respiratory tract to the intestinal tract, thus allowing more excretion from the cloaca and more efficient faecal-oral transmission via contaminated water. This would allow the phenotype of H5 HPAI virus to resemble that of LPAI virus, as a type of convergent evolution. To test this hypothesis, we reviewed literature of HPAI cases prior to 2016 to determine tissue tropism; determined tissue tropism and associated lesion in wild birds that died during 2016/2017 epidemic; and showed the pattern of virus attachment of 2016 H5N8 virus to respiratory and intestinal tissues of wild ducks, compared with older HPAI viruses.

Materials and Methods

Study design

This study consisted of two parts. In the first part, we examined the carcasses of 39 wild birds that were found dead during the 2016/17 H5N8 HPAI epidemic in The Netherlands and that tested positive for H5N8 HPAI virus (Table 1), in order to characterize the pathology and cell type tropism of this virus infection in different organs. We were particularly interested to determine whether the H5N8 HPAI virus had more tropism for the digestive tract of wild birds than that of HPAI viruses from previous epidemics, based on published accounts. For the literature review, we retrieved articles in English published between 2004 and 2018 from the PubMed database (www.ncbi.nlm.nih.gov/pubmed/). Key words used were "avian influenza", "H5N1", "H5N8", "wild birds", "immunohistochemistry" and "IHC".

Our null hypothesis was that the 2005/06 HPAI H5N1 and 2016/17 HPAI H5N8 viruses have similar tropism for the intestinal tract of wild birds; the alternate hypothesis was that these two subtypes have a different tropism.

In the second part, we performed virus histochemical analysis of four avian influenza viruses in order to compare the pattern of attachment of these viruses in the respiratory and intestinal tracts of five bird species. We were particularly interested to determine whether the 2016/2017 H5N8 HPAI virus attached better to the digestive tract of four key wild duck species (Eurasian wigeon, *Mareca penelope*; mallard, *Anas platyrhynchos*; tufted duck, *Aythya fuligula*; common pochard, *Aythya ferina*) compared to the 2014/15 HPAI H5N8 and 2005/06 HPAI H5N1 viruses. We included a common LPAI virus as a representative virus with a clear tropism for the digestive tract of mallards, and included tissues of the chicken as a representative poultry species.

Pathology and immunohistochemistry of naturally infected wild birds

The carcasses of 39 wild birds had been collected in the provinces of Flevoland, Gelderland, Noord and Zuid Holland (The Netherlands) in November and December 2016. All the birds tested positive for H5N8 2016 by real-time reverse-transcription PCR (RRT-PCR) assays in oropharyngeal and/or cloacal swabs as described previously (7).

The postmortem examinations and tissue sampling were performed according to a standard protocol. The following tissues, when available, were examined: brain, lungs, air sacs, pancreas, liver, stomachs (proventriculus and ventriculus), small intestine (jejunum, ileum), large intestine (cecum, colon), kidney, adrenal gland, spleen and heart. The tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissues were sectioned at 3 µm and stained with hematoxylin and eosin for histopathological analysis or stained with a monoclonal antibody against nucleoprotein of influenza A virus for immunohistochemical detection of influenza viral antigen, as described previously (8).

Virus histochemistry

The following four viral strains were used as input viruses: 2008 LPAIV H4N5 (A/Mallard/Netherlands/13/08), 2014 HPAIV H5N8 (A/Eurasian wigeon/Netherlands/emc-1/2014), 2016 HPAIV H5N8 (A/Eurasian wigeon/Netherlands/19/2016), 2005 HPAIV H5N1 (A/Turkey/Turkey/1/05). The viruses were individually passaged in Madin-Darby canine kidney (MDCK) cells. After 2-3 days, the supernatant was harvested and cleared of

cell debris by low speed centrifugation for 20 min at 1455 g. The viruses were individually concentrated by centrifugation of the cleared supernatants in filter tubes (Amicon Ultra-15 100K filter-tubes, Millipore, UFC9100024, Darmstadt, Germany) for 40 min at 4000 x g at 4 oC. The concentrated virus was inactivated by dialysing against 0.1 % formalin for 3 days at room temperature (RT). After inactivation, the virus solution was dialysed against phosphate-buffered saline solution (PBS) and complete inactivation was confirmed by passaging on MDCK cells. Virus was labelled by adding an equal volume of 0.1 mg/ml of fluoresceinisothiocyanate (FITC) (Sigma-Aldrich, Saint Louis, MO) in 0.5 M bicarbonate buffer (pH 9.5) for 1 hour at RT while constantly stirring. Labelled virus was dialysed against PBS in order to lose all unbound FITC. The concentration of the different virus suspensions used for virus histochemistry was standardized at 50 hemagglutination units/100 µl (HAU) using hemagglutination assay.

Tissue sections of the following species were used: tufted duck (n=3), common pochard (n=2), Eurasian wigeon (n=3), mallard (n=3), and domestic chicken (n=2). These tissues came from the Erasmus MC tissue bank, and were from healthy animals that showed no abnormalities or histological lesions. From the respiratory tract, tissues selected were trachea, primary bronchus, secondary bronchus, tertiary bronchus or parabronchus, air capillaries and air sacs. From the digestive tract of same birds, tissues selected were duodenum, jejunum, ileum and colon.

Three-µm-thick formalin-fixed paraffin-embedded sections of each tissue were deparaffinized in xylene and hydrated using graded alcohols and incubated overnight with FITC-labelled viruses at a concentration of 50 HAU/100 µl. To enable visualization by light microscopy, FITC was detected with a peroxidase-labeled rabbit anti-FITC antibody (DAKO, Glostrup, Denmark). The signal was amplified using a tyramide amplification system (Perkin-Elmer, Boston, MA). Peroxidase was revealed with 3-amino-9-ethylcarbazole (Sigma-Aldrich) resulting in a bright red precipitate. Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Whitehouse Station, NJ). Omission of the FITC-labelled virus was used as a negative control.

The slides were assessed with light microscopy to estimate the abundance of viral attachment to epithelial cells and scored as follows: : attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++). Finally, the median score was determined for each species at the different anatomical sites. Sections were examined without knowledge of the identity of the birds.

Results

Influenza virus antigen expression and associated lesions in naturally infected wild birds

The 39 HPAI-virus-positive carcasses of 12 wild bird species, plus unspecified ducks (Table 1), were examined for influenza virus antigen expression. We were particularly interested in virus antigen expression in epithelial cells of digestive and respiratory tracts, because these correspond to virus excretion from cloaca and pharynx, respectively.

Species	No. of birds	Positive RRT-PCR for H5N8 virus in: pooled CL and OP swabs	separate CL and OP swabs	separate CL swab only	separate OP swab only
Tufted duck *Aythya fuligula**	7	1	3	0	3
Common pochard *Aythya ferina**	1	Nd	1	0	0
Great crested grebe *Podiceps cristatus**	1	Nd	1	0	0
Eurasian teal *Anas crecca**	1	Nd	1	0	0
Eurasian wigeon *Mareca penelope**	10	3	7	0	0
Mallard *Anas platyrhynchos*	2	Nd	2	0	0
Duck (unspecified species)	10	10	Nd	Nd	Nd
Greylag goose *Anser anser**	1	Nd	1	0	0
Great black backed gull *Larus marinus**	1	Nd	1	0	0
Lesser black backed gull *Larus fuscus*	1	1	Nd	Nd	Nd
Black-headed gull *Chroicocephalus ridibundus**	1	Nd	1	0	0
Eurasian buzzard *Buteo buteo*	2	1	0	0	1
Eurasian magpie *Pica pica*	1	1	Nd	Nd	Nd

CL, cloacal
OP, oropharyngeal
Nd, not done
*Previously reported in Poen et al 2018 [7]

Table 1: Detection of HPAIV H5N8 in cloacal and oropharyngeal swabs from carcasses of wild ducks.

Four wild bird species (Eurasian wigeon; tufted duck; black-headed gull, *Chroicocephalus ridubundus;* Eurasian magpie, *Pica pica*) expressed influenza virus antigen in epithelial cells of both digestive tract and respiratory tract (Table 2). One species (great black-backed gull, *Larus marinus*) and unspecified ducks expressed influenza virus antigen in epithelial cells of respiratory tract only, and one species (greylag goose, *Anser anser*) expressed influenza virus antigen in epithelial cells of digestive tract only. Besides epithelial cells, other cell types in digestive and respiratory tracts that expressed influenza virus antigen were endothelial cells and neurons.

Number of birds expressing influenza virus antigen in a cell type of an organ

Species	No of birds	Respiratory tract						Gastro-intestinal tract								
		Lung			Air sac			Proventriculus			Small intestine			Large intestine		
		EP	E	N	EP	E	N	EP	E	N	EP	E	N	EP	E	N
Tufted duck *Aythya fuligula*	7	1	4	0	0	1	0	1∞	2	2	1∞	4	1	0	4	0
Common pochard *Aythya farina*	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Great crested grebe *Podiceps cristatus*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eurasian teal *Anas crecca*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eurasian wigeon *Mareca Penelope*	10	5	6	1	2	5	0	2∞	7	0	3∞*	8	1	1*	8	0
Mallard *Anas platyrhynchos*	2	0	0	0	1	0	0	0	1	0	1	1	1	0	1	0
Duck (unspecified species)	10	8	0	0	8	0	0	0	3	0	0	2	1	0	3	1
Greylag goose *Anser anser*	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Great black backed gull *Larus marinus*	1	0	1	0	1	1	0	0	0	0	0	1	0	0	1	0
Lesser black backed gull *Larus fuscus*	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Black-headed gull *Chroicocephalus ridibundus*	1	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1
Eurasian buzzard *Buteo buteo*	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Eurasian magpie *Pica pica*	1	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0
Total	39	16	13	1	15	8	0	6	14	2	6	17	6	2	18	2

Ep, epithelial cell; E, endothelial cell; N, neuron

∞ one bird expressed influenza virus antigen in the epithelial cells of both proventriculus and small intestine
*one bird expressed influenza virus antigen in the epithelial cells of both small and large intestine

Table 2: Expression of AIV antigen in cell types of different organs of the respiratory and gastro-intestinal tracts.

Besides tissues in digestive and respiratory tracts, tissues in other organs also expressed influenza virus antigen (see Additional file 1). The degree to which this occurred reflects the capacity of the virus to spread systemically.

Grossly, the main pathological changes consisted of multifocal necrosis in liver (7 birds) and pancreas (6 birds); sub-pericardial hemorrhages (10 birds); and multifocal pulmonary consolidation (6 birds) (see Additional file 2). Histologically, lesions were detected in the liver (16 birds), brain (12 birds), pancreas (8 birds), kidney (8 birds), lungs (8 birds), heart (8 birds), and intestine (7 birds). Lesions were characterized by multifocal necrosis in liver and pancreas; necrosis and inflammation in brain, intestine, and kidney; hemorrhages in lungs; and hemorrhages and necrosis in heart (Figure 1 and 2; see Additional file 3).

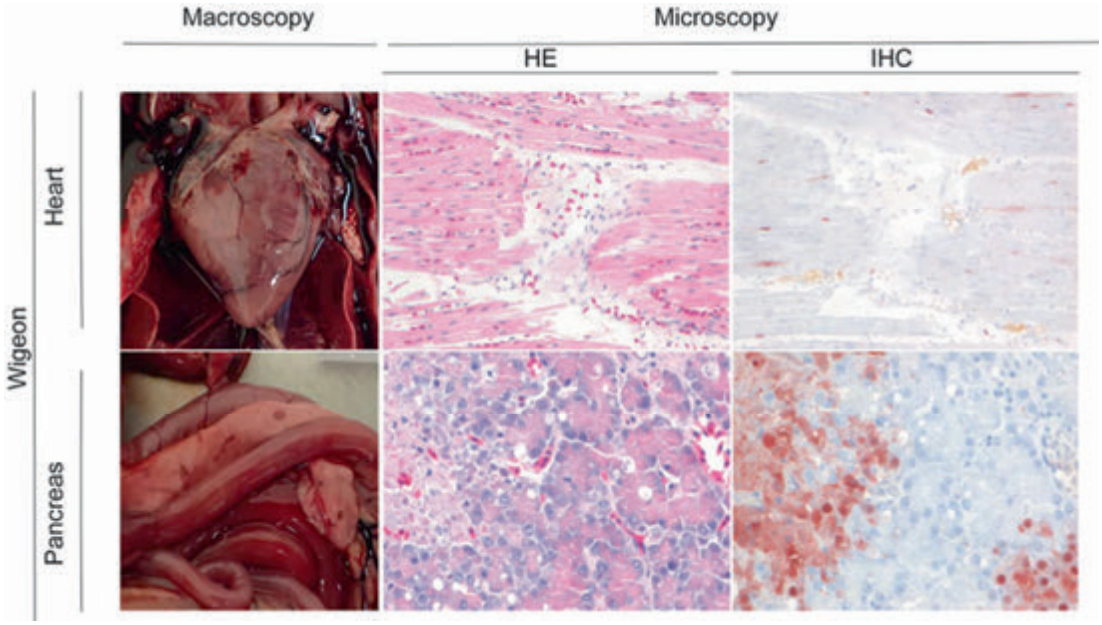

Figure 1: Macroscopy, histological lesions and virus antigen expression in tissues of wild birds.

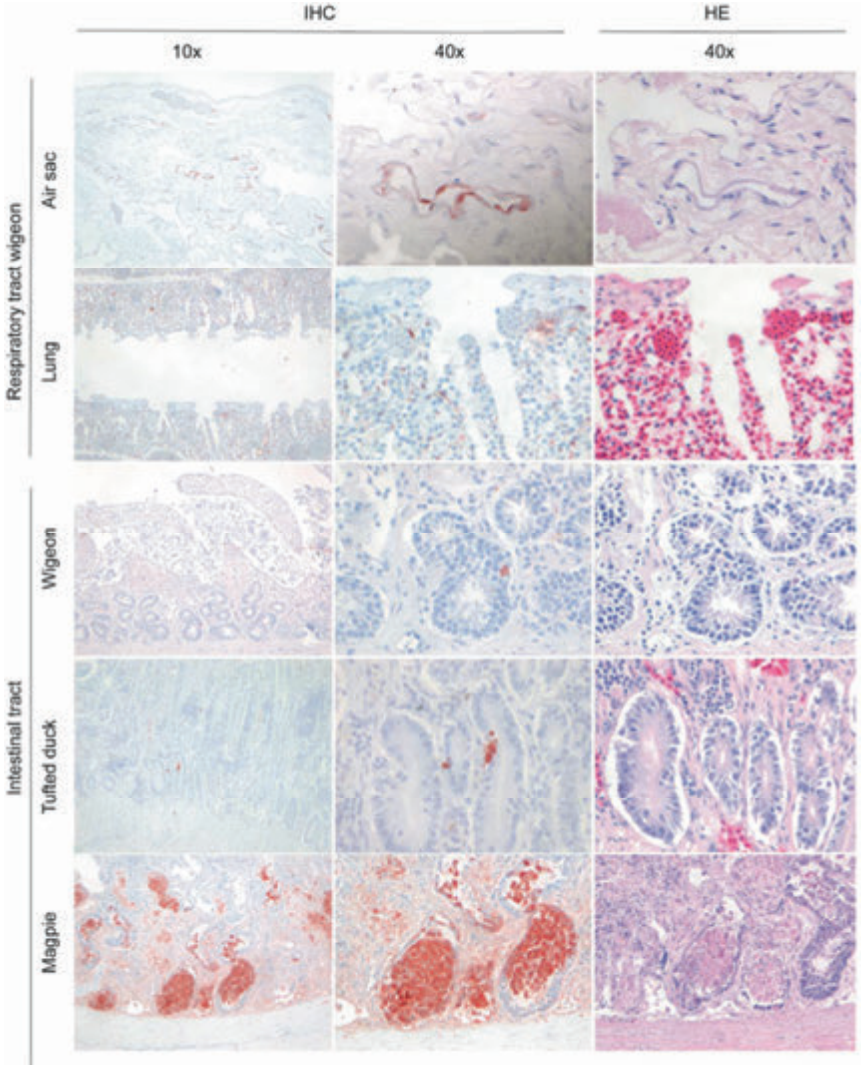

Figure 2: Histological lesions and virus antigen expression in tissues of wild birds.

Pattern of virus attachment to epithelia of digestive and respiratory tracts

In intestinal epithelia, the level of attachment varied per virus and per host species (Table 3). In overall comparison among viruses, H5N1 had lower attachment to intestinal epithelia than 2014 H5N8, 2016 H5N8 and H4N5. In overall comparison among host species, virus attachment to intestinal epithelia was low in tufted duck and Eurasian pochard, intermediate in Eurasian wigeon, and high in chicken and mallard. For a given virus and a given host species, the level of attachment among different parts of the intestine (duodenum, jejunum, ileum, colon) was comparable.

		Avian influenza viruses			
Species	Tissues	H4N5	2014 H5N8	2016 H5N8	H5N1
Mallard	Duodenum	++	+	++	++
	Jejunum	++	++	++	++
	Ileum	++	+	++	+
	Colon	++	+	++	+
Pochard	Duodenum	nd	±	±	±
	Jejunum	±	±	±	-
	Ileum	-	±	-	-
	Colon	±	±	-	-
Tufted Duck	Duodenum	±	±	±	±
	Jejunum	±	±	±	-
	Ileum	±	±	-	-
	Colon	±	±	-	±
Wigeon	Duodenum	+	±	±	±
	Jejunum	+	+	+	±
	Ileum	+	+	+	±
	Colon	+	+	+	±
Chicken	Duodenum	++	++	++	±
	Jejunum	++	++	++	±
	Ileum	++	++	++	±
	Colon	+	+	+	±

Mean abundance of attachment was scored as follows: attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++).
nd, not detected

Table 3: Pattern of attachment of avian influenza viruses to the epithelial cells of the intestinal tract.

The level of attachment of the different viruses to intestinal epithelia differed per host species. In the mallard, 2016 H5N8 had high attachment to intestinal epithelia, comparable to that of H4N5. It was higher than the level of attachment of H5N1 and 2014 H5N8, because of improved attachment to ileum and colon. In the Eurasian wigeon, both 2014 H5N8 and 2016 H5N8 had moderate attachment to intestinal epithelia, just lower than that of H4N5. These three viruses had higher attachment than that of H5N1, because of improved attachment to jejunum, ileum, and colon. In the tufted duck and Eurasian pochard, both 2014 H5N8 and 2016 H5N8 had low attachment to intestinal epithelia, comparable to that of H4N5. These three viruses had slightly higher attachment than that of H5N1, because of improved attachment to jejunum, ileum, and/or colon. In the chicken, both 2014 H5N8 and 2016 H5N8 had high attachment to intestinal epithelia, comparable to that of H4N5. These three viruses

had markedly higher attachment than H5N1, because of improved attachment to all parts of the intestine.

In respiratory epithelia, the level of virus attachment differed per tissue, but not per virus or host species (Table 4). With a few exceptions, virus attachment to trachea, primary bronchi, secondary bronchi, and air sacs was high regardless of virus and host species. In contrast, virus attachment to parabronchi, atria, and air capillaries was low regardless of host species, except for the chicken, where attachment to parabronchi was moderate (2014 H5N8) to strong (other three viruses).

		Avian influenza viruses			
Species	Tissues	H4N5	2014 H5N8	2016 H5N8	H5N1
Mallard	Trachea	++	++	++	++
	Primary bronchus	++	++	++	++
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	±
	Air capillaries	+	±	±	+
	Air sac	++	++	++	++
Pochard	Trachea	++	++	++	+
	Primary bronchus	++	++	++	+
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	±
	Air capillaries	±	±	±	+
	Air sac	++	++	++	++
Tufted Duck	Trachea	++	+	++	++
	Primary bronchus	++	++	++	++
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	+
	Air capillaries	+	±	±	+
	Air sac	++	+	++	++
Wigeon	Trachea	++	++	++	++
	Primary bronchus	++	++	++	++
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	±
	Air capillaries	±	±	±	+
	Air sac	++	++	++	++
Chicken	Trachea	++	++	++	++
	Primary bronchus	++	++	++	+
	Secondary bronchus	++	++	++	+
	Parabronchus atria	++	+	++	++
	Air capillaries	±	-	±	+
	Air sac	++	++	++	++

Mean abundance of attachment was scored as follows: attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++).
nd, not detected

Table 4: Pattern of attachment of avian influenza viruses to the epithelial cells of the respiratory tract.

A review of natural and experimental HPAI virus infections revealed 18 articles in first search. Of these 18 articles, 15 examined the gastro-intestinal tract but failed to detect HPAI virus antigen expression in gastro-intestinal epithelium. Of these 15 articles, 7 articles did not report any virus antigen expression at all in the gastro-intestinal tract, and 8 articles reported the presence of virus antigen expression in non-epithelial tissues: vascular endothelium or parasympathetic ganglia in the submucosal and muscular plexi of the gastro-intestinal tract. In the remaining three articles, influenza virus antigen expression [for, respectively, H5N1/2005; A/

chicken/Vietnam/14/2005 (H5N1); A/swan/Germany/R65/06(H5N1)] was detected in the gastro-intestinal epithelium of three species: Eurasian magpie, Canada goose (Branta canadensis), and whooper swan (Cygnus cygnus) [17,18,19] (see Additional file 4 and Additional file 5).

Discussion

The null hypothesis that H5N1 and 2016 H5N8 have similar tropism for the intestinal tract was rejected based on both virus antigen expression and virus attachment studies. Based on virus antigen expression, intestinal epithelium expressed 2016 H5N8 antigen in four of twelve bird species (5 of 29 individual birds, excluding 10 unspecified ducks) that were naturally infected with 2016 H5N8, compared to the more limited virus antigen expression in the intestinal epithelium of wild birds infected with earlier viruses of the Goose/Guangdong lineage (this study). Based on virus attachment, 2016 H5N8 attached better than H5N1 to intestinal epithelium in three of four duck species tested. Based on these results, we accept the alternate hypothesis that 2016 H5N8 is more enterotropic than H5N1.

Enterotropism of HPAI virus is a novel phenomenon in wild birds. According to our review, infection of epithelial cells of the gastro-intestinal tract was reported in only three or four species (Eurasian magpie, Canada goose, and mute and/or whooper swan), none of them ducks. In contrast to HPAI virus, LPAI virus is well known to be enterotropic in wild birds. In fact, the epithelium of intestine and cloacal bursa were the only tissues that expressed LPAI virus antigen in naturally infected mallards and black-headed gulls (8,9). An important difference between the two pathotypes of AI virus is that LPAI virus infection is not known to cause any intestinal lesions in wild birds, while 2016 H5N8 infection caused marked necrosis and inflammation of the intestinal mucosa (this study) (10). Two caveats regarding virus antigen detection in intestinal mucosa are that intestinal mucosa autolyses rapidly after death of a bird, making it more difficult to detect virus antigen, and that, historically, intestinal tissues have not been sampled extensively. To improve the sensitivity of influenza virus detection in intestinal mucosa, carcasses should be cooled to 4^{o} C, samples of intestine should be collected and fixed in formalin as soon as possible after death, and the intestinal mucosa should be sampled at multiple levels, from duodenum to colon. A useful technique to increase the amount of intestinal mucosa to be scanned for virus antigen expression is the so-called ‘Swiss role’ technique, which allowed a 7-cm-long segment of mallard intestine to be embedded in one paraffin block prior to making tissue sections (20).

In addition to gaining enterotropism, 2016 H5N8 retained respirotropism. Infection of the respiratory tract is considered to be the main source of HPAI virus excretion from the

oropharynx in wild birds (11). In the seven birds in our study that expressed influenza virus antigen in gastro-intestinal epithelium, five also expressed it in respiratory epithelium. This situation is intermediate between respirotropic H5N1 infection and enterotropic LPAI virus infection (9,11).

Besides in respiratory tract and intestinal tract, 2016 H5N8 antigen also was expressed in other tissues, including in particular brain, liver, lung, heart, and pancreas. Infection in most of these tissues was associated with both necrosis and inflammation, and it is likely that these 2016 H5N8-associated lesions were fatal to the birds in our study. The character and severity of these lesions were similar to those caused by H5N1 infection, with the exception of intestinal lesions, which are not present in H5N1 infection (18). However, this does not necessarily mean that 2016 H5N8 and 2005 H5N1 are comparable in virulence. It is very difficult to estimate the virulence of HPAI virus infection in wild birds from field data, since we do not know how many wild birds were infected, and which proportion of infected birds died. Therefore, the case fatality rate (proportion of infected individuals that died) is unknown both for H5N8 and 2005 H5N1.

Based on experimental infections in different species of ducks, 2014 H5N8 from the USA and from the Netherlands is less virulent than H5N1 (12,15). However, the more recent 2016 H5N8 shows an increased virulence compared to 2014 H5N8 and it is able to produce severe disease in waterfowl both in natural and experimental settings (16,22). The reasons that caused this increase in virulence are still unclear but, from a clinical and pathological point of view, this severity of the disease is likely due to a more systemic involvement.

From the results of this study, we can conclude that 2016 H5N8 has a tropism for both digestive tract and respiratory tract of at least four wild bird species (Eurasian wigeon, tufted duck, black-headed gull, Eurasian magpie) based on virus antigen detection in naturally infected birds. This means that 2016 H5N8 mirrors the enterotropism of LPAIV, without having lost the respirotropism of older viruses of the Goose/Guangdong lineage, like H5N1. How the H5 viruses of the Goose/Guangdong lineage will evolve in wild birds in future is not clear. One possibility is that they become completely enterotropic, like LPAIV in mallards and black-headed gulls. Another possibility is that they retain tropism for both digestive and respiratory tracts. It is conceivable that such a dual tropism provides maximum flexibility for the virus to adapt to multiple species of wild and domestic birds, depending on the ecological niche where the virus happens to be.

The implications of increased tropism for the digestive tract is that relatively more virus is excreted from the cloaca and contaminates the water bodies on which wild waterbirds reside. Indirect transmission via contaminated water, up to several weeks after the infected birds have left the water body, is a key factor for the maintenance of LPAI in wild waterbirds (23). If indirect transmission via contaminated water becomes a major route of transmission for H5N8 or other viruses of the Goose/Guangdong lineage, they have an increased chance to persist indefinitely in the wild waterbird reservoir.

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pathology staff of Wageningen Bioveterinary Research for necropsy and sample collection of dead wild birds.

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Additional Material

		Number of birds expressing influenza virus antigen in a cell type of an organ											
		Nervous system				Digestive system				Other systems			
		Brain		Peripheral nerve		Pancreas		Liver		Adrenal gland		Heart	
Species	No of birds	E	N	E	N	E	EP	E	H	CC	N	E	M
Tufted duck *Aythya fuligula*	7	3	0	2	1	0	1	0	4	2	1	0	3
Common pochard *Aythya farina*	1	0	0	0	0	0	0	0	0	0	0	0	0
Great crested grebe *Podiceps cristatus*	1	0	0	0	0	0	0	0	0	0	0	0	0
Eurasian teal *Anas crecca*	1	0	0	0	0	0	0	1	0	0	0	0	0
Eurasian wigeon *Mareca Penelope*	10	4	3	4	0	3	7	4	7	1	0	4	3
Mallard *Anas platyrhynchos*	2	0	1	0	1	0	0	0	2	0	1	0	0
Duck (unspecified species)	10	0	0	0	0	0	0	0	0	0	0	0	0
Greylag goose *Anser anser*	1	1	0	1	0	0	1	0	1	0	1	0	1
Great black backed gull *Larus marinus*	1	0	0	0	0	0	0	0	0	0	0	0	0
Lesser black backed gull *Larus fuscus*	1	1	0	0	0	0	0	0	0	1	0	0	0
Black-headed gull *Chroicocephalus ridibundus*	1	0	1	1	0	1	1	0	1	0	0	0	1
Eurasian buzzard *Buteo buteo*	2	0	1	0	0	0	1	0	1	0	0	0	1
Eurasian magpie *Pica pica*	1	1	1	0	0	0	1	0	1	0	0	0	0

Ep, epithelial cell; E, endothelial cell; N, neuron; H, hepatocyte; CC, chromaffin cell; M, myocytes.

Additional file 1: Expression of avian influenza virus antigen in cell types of other organs than those of respiratory and gastro-intestinal tracts.

Species	No. of birds with gross lesions in the:										
	No of birds	Gross lesions (Organs)?	Air sac‡	Heartπ	Intestine	Kidney	Liver#	Lung§	Pancreas¶	Proventriculus∞	Unknown
Tufted duck *Aythya fuligula*	7	Y (H,L,Lu,P)	0	3	0	0	1	3	2	0	4
Common pochard *Aythya farina*	1	N	0	0	0	0	0	0	0	0	
Great crested grebe *Podiceps cristatus*	1	N	0	0	0	0	0	0	0	0	
Eurasian teal *Anas crecca*	1	N	0	0	0	0	0	0	0	0	
Eurasian wigeon *Mareca Penelope*	10	Y(H,L, Lu, P,Pr)	0	4	0	0	5	3	4	2	3
Mallard *Anas platyrhynchos*	2	Y(A,H)	1	1	0	0	0	0	0	0	
Duck (unspecified species)	10	UK	NR	NR	NR	NR	NR	NR	NR	NR	10
Greylag goose *Anser anser*	1	N	0	0	0	0	0	0	0	0	
Great black backed gull *Larus marinus*	1	N	0	0	0	0	0	0	0	0	
Lesser black backed gull *Larus fuscus*	1	UK	NR	NR	NR	NR	NR	NR	NR	NR	1
Black-headed gull *Chroicocephalus ridibundus*	1	Y(H,I)	1	1	0	0	0	0	0	0	
Eurasian buzzard *Buteo buteo*	2	Y(H,L)	0	1	0	0	1	0	0	0	1
Eurasian magpie *Pica pica*	1	UK	NR	NR	NR	NR	NR	NR	NR	NR	1

A,air sac; H, heart; I, intestine; K, kidney; L, liver; Lu, lung; N, no; NR, not recorded P, pancreas; Pr, proventriculus; UK, unknown; Y, yes

‡Airsacculitis, characterized by mild, multifocal to diffuse opacity and thickening of the air sacs.

πSubpericardial hemorrhage

#Diffuse hepatic necrosis.

§Multifocal pulmonary consolidation.

¶Multifocal pancreatic necrosis, consisting of red or gray foci of 1–4 mm in diameter affecting up to 25% of the pancreas.

∞Hemorrhages

Additional file 2: Frequency and distribution of gross lesions associated with virus antigen expression in carcasses of wild birds.

Species	No of birds	Histological lesions (Organs)?	No. of birds with histological lesions in the: Air sac‡	Brainω	Heartπ	Intestineω	Kidney^{H}	Liver#	Lung§	Pancreas¶	Proventriculus∞	Unknown
Tufted duck *Aythya fuligula*	7	Y(A,B,H,I,K,L, Lu,P,Pr)	2	3	3	3	1	3	3	2	1	4
Common pochard *Aythya farina*	1	Y(L)	0	0	0	0	0	1	0	0	0	
Great crested grebe *Podiceps cristatus*	1	N	0	0	0	0	0	0	0	0	0	
Eurasian teal *Anas crecca*	1	Y(L)	0	0	0	0	0	1	0	0	0	
Eurasian wigeon *Mareca Penelope*	10	Y(B,H,I,K,L, Lu, P,Pr)	0	5	4	2	4	6	4	4	1	3
Mallard *Anas platyrhynchos*	2	Y(B,L)	0	1	0	0	0	2	0	0	0	
Duck (unspecified species)	10	UK	Na	na	na	na	Na	na	na	Na	na	10
Greylag goose *Anser anser*	1	Y(B,L)	0	1	0	0	0	1	0	0	0	
Great black backed gull *Larus marinus*	1	Y(L,Lu)	0	0	0	0	0	1	1	0	0	
Lesser black backed gull *Larus fuscus*	1	UK	Na	na	na	na	Na	na	na	Na	na	1
Black-headed gull *Chroicocephalus ridibundus*	1	Y(B,I,L)	0	1	0	1	0	1	0	0	0	
Eurasian buzzard *Buteo buteo*	2	Y(B,H,L,P)	0	1	1	0	0	1	0	1	0	
Eurasian magpie *Pica pica*	1	Y(I,P,Pr)	0	0	0	1	0	0	0	1	1	

A,air sac; B, brain; H, heart; I, intestine; K, kidney; L, liver; Lu, lung; N, no; P, pancreas; Pr, proventriculus; na, not available; Y, yes

‡ Airsacculitis, characterized by multifocal aggregates of lymphocytes and plasma cells in the interstitium . ω Necrotizing encephalitis with gliosis.
Π Hyperaemia, multifocal hemorrhages and necrosis of cardiomyocytes
ω Necrosis and inflammation of the intestinal mucosa.
^{H} Necrosis and interstitial nephritis.
Multifocal necrosis of hepatocytes.
§ Hyperemia, edema and hemorrhage.
¶ Moderate to severe, multifocal to confluent acinar necrosis
∞ Inflammatory lymphoplasmacellular infiltration.

Additional file 3: Frequency and distribution of histological lesions associated with virus antigen expression in carcasses of wild birds.

Antigen in epithelium of digestive tract present	Species	Infection type	Virus	Location of viral antigen in digestive tract	Cell infection recorded	References
Y	Magpie	N	H5N1	Intestine	Intestinal epithelium	Kwon 2005 [17]
Y	Canada goose	E	A/ chicken/Vietnam/ 14/2005 (H5N1)	Proventriculus, small intestine and cecum	Epithelium, parasympathetic ganglia and mesenteric plexi, occasional scattered smooth muscle and vascular endothelial cell	Pasick 2007 [18]
Y	Whooper swan	N	A/swan/Germany/ R65/06(H5N1)	Proventriculus	Epithelium	Teifke 2007 [19]
N	Bar-headed goose, Canada goose	N	H5N1	None		Ellis 2004 [24]
N	Eastern Zhejiang white geese	E	A/Bar-headedGoose/Qinghai/0510/05 (H5N1)	None		Zhou 2006 [25]
N	Mallard, northern pintail, common teal, redhead, wood duck, laughing gulls	E	A/Whooper Swan/Mongolia/244/05 (H5N1), A/Duck Meat/Anyang/01 (H5N1)	Small intestines	Parasympathetic ganglia in the submucosal and muscular plexus	Brown 2006 [26]
N	Pekin duck	E	A/Thailand PB/6231/04(H5N1)	Proventriculus		Pantin-Jacwood 2007 [27]
N	Pekin duck	E	A/Crow/Thailand/ 04(H5N1)	Proventriculus, intestine		Pantin-Jacwood 2007 [27]
N	Pekin duck	E	A/Egret/HK/7572/ 02(H5N1)	Proventriculus, intestine		Pantin-Jacwood 2007 [27]
N	Call duck	E	A/chicken/Yamaguchi/7/04(H5N1)	None		Yamamoto 2007 [28]
N	Tufted duck	E	A/turkey/Turkey/1/05(H5N1)	None		Londt 2008 [29]

N	Mute swan	E	A/Cygnuscygnus/Germany/R65/2006(H5N1)	Proventriculus, intestine	Vascular endothelium	Kalthoff 2008 [30]
N	Tufted ducks, Eurasian pochards, mallards common teals, Eurasian wigeons, gadwalls	E	A/turkey/Turkey/1/2005 (H5N1)	None		Keawcharoen 2008 [11]
N	Tufted ducks	N	H5N1	Proventriculus		Brojer 2009 [31]
N	Canada goose	E	A/chicken/Vietnam/14/05(H5N1)	Proventriculus, duodenum, ceca		Neufeld 2009 [32]
N	Mute swan, greylag goose, mandarin duck	E	A/chicken/Korea/IS/06(H5N1)	Small and large intestine	Vascular endothelium	Kwon 2010 [33]
N	Pekin duck	E	A/duck/Sleman/BBVW-59832226/2007(H5N1)	None		Wibawa 2013 [34]
N	Coot	N	H5N8 2014	Intestine		Kim 2014 [35]
N	Eurasian wigeon, common pochard mallard, common teal	E	A/chicken/Netherlands/emc-3/2014 (H5N8)	None		Van de Brand 2018 [12]
N	Pekin ducks, muscovy duck	E	DE14¬H5N8A	Proventriculus		Grund 2018 [22]
N	Pekin ducks, muscovy duck	E	DE16¬H5N8B	Proventriculus, duodenum		Grund 2018 [22]

Y yes, n no, N natural, E experimental,

Additional file 4: Literature review of articles in English from the PubMed database.

Literature reporting influenza virus antigen expression in the gastro-intestinal epithelium

Common name	Scientific name
Bar-headed goose	*Anser indicus*
Black swan	*Cygnus atratus*
Call duck	*Anas platyrhynchos*
Canada goose	*Branta canadensis*
Coot	*Fulica atra*
Laughing gull	*Larus atricilla*
Mandarin duck	*Aix galericulata*
Muscovy duck	*Cairina moschata*
Mute swan	*Cygnus olor*
Northern pintail	*Anas acuta*
Pekin duck	*Anas platyrhynchos domesticus*
Redhead	*Aythya americana*
Trumpeter swan	*Cygnus buccinator*
Whooper swan	*Cygnus cygnus*
Wood duck	*Aix sponsa*

Additional file 5: Common and scientific name of additional bird species mentioned in Additional file 4

Chapter 2.3

Tropism of highly pathogenic avian influenza virus H5 from the 2020/2021 epidemic in Eurasian wigeon and Barnacle goose

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Abstract: Highly pathogenic avian influenza (HPAI) outbreaks have become increasingly frequent in wild bird populations and have caused mass mortality in many wild bird species. The 2020/2021 epizootic was the largest and most deadly ever reported in Europe, and many new bird species tested positive for HPAI virus for the first time. This study investigated the evolution of tropism of HPAI virus in wild birds. We tested the pattern of virus attachment of 2020 H5N8 virus to in-testinal and respiratory tissues of key bird species; and characterized pathology of naturally in-fected Eurasian wigeons (Mareca penelope) and barnacle geese (Branta leucopsis). This study determined that 2020 H5N8 virus had high level of attachment to the intestinal epithelium (en-terotropism) of dabbling ducks and geese and retained attachment to airway epithelium (respi-rotropism). Natural HPAI 2020 H5 virus infection in Eurasian wigeons and barnacle geese also showed a high level of neurotropism, as both species presented with brain lesions that co-localized with virus antigen expression. We concluded that the combination of respirotropism, neurotropism, and possibly enterotropism contributed to the successful adaptation evolution of 2020/2021 HPAI H5 viruses in successfully adapting to wild waterbird populations.

Keywords: avian influenza; HPAI; H5N8; Eurasian wigeon; Barnacle goose; enterotropism; neurotropism

Introduction

Highly pathogenic avian influenza (HPAI) outbreaks have become increasingly frequent in wild bird populations and have caused mass mortality in many wild bird species (1-9). Recently circulating HPAI H5 viruses descending from the A/Goose/Guangdong/1/1996 (GsGd) lineage, principally HPAI H5N8 and H5N1 viruses belonging to clade 2.3.4.4, were responsible for the latest outbreak events (6-9).

In Europe, outbreaks coincide with the autumn migrations of wild birds, a time when many long-distance migratory waterbirds congregate to overwinter or stop-over around water-rich areas (10). The two most deadly HPAI epizootics occurred in Europe during the avian influenza seasons in 2016/2017 and in 2020/2021 (11,12). In the Neth-erlands, over 13000 wild birds were reported dead during the 2016/2017 epizootic; duck species such as Eurasian wigeons (Mareca penelope) and tufted ducks (Aythya fuligula) accounted for the highest rates of mortality [11]. The 2020/2021 epizootic was the largest and most deadly ever reported in Europe; geese, in particular barnacle goose (Branta leucopsis) and graylag goose (Anser anser), accounted among the highest rates of mortality (6-9). Furthermore, like the above-mentioned barnacle goose, several new bird species were reported infected with HPAI virus for the first time. Temporally, the virus continued to circulate within Europe well after the periods of autumn migration and wintering, and it was regularly detected in wild birds during the following 2021 summer (9). During the first months of the 2020/2021 epizootic, H5N8 was the most frequently detected virus subtype; however this virus subtype locally reassorted with low patho-genic avian influenza viruses (LPAIVs), so that many different virus subtypes (i.e., H5N1, H5N2, H5N3, H5N4, H5N5, H5N8) were subsequently identified (6-9, 13).

Both experimental studies and field observations identified wild ducks, and in particular dabbling species like Eurasian wigeons and mallards, as the most suitable vectors for the long-distance spread of HPAI viruses (1,2.14,15). Wild ducks can be in-fected with HPAI virus without presenting any clinical signs (15). During the 2020/2021 epizootic, only a relatively small number of infected Eurasian wigeons and mallards (Anas platyrhynchos) were reported dead in Europe during surveillance (6-9). In some cases, birds of these species tested positive after being professionally hunted, and re-ported as otherwise apparently healthy. These findings indicate a relatively lower vir-ulence of H5 viruses in some duck species and may support the theory of increased adaptation and replication of HPAI virus in these species. Viral adaptation may relate to an increased viral enterotropism in these hosts, as is found for low pathogenic avian influenza viruses that are enzootic in some waterbird species. A previous study already showed the increased enterotropism of 2016 H5N8 (16).

In this study we investigated whether 2020 H5N8 virus followed the evolutionary trend of its predecessor, 2016 H5N8 virus, of increased enterotropic potential. We hy-pothesized that enterotropism is likely to be maintained, even enhanced, especially in bird species like the mallard and Eurasian wigeon that are hypothesized as being vectors for long-distance viral dispersion. Furthermore, enterotropism could be also evident in new host species, like graylag goose and barnacle goose, with the advantage of virus perpetuation in these globally abundant host populations and their movements.

Our study is based on the hypothesis that the 2020 HPAI H5N8 virus was more enterotropic than the 2016 HPAI H5N8 virus, both in established and in new host spe-cies. To investigate this hypothesis, we determined the pattern of attachment of 2020 H5N8 virus to intestinal and respiratory tissues of key species of wild ducks and geese, as well as chickens, and then compared it with the patterns of attachment of older HPAI viruses such as H5N1, 2014 H5N8, and 2016 H5N8 viruses. Furthermore, we examined wild Eurasian wigeons and barnacle geese that died during the 2020/2021 epizootic and determined virus distribution, tissue tropism and associated lesions in their major or-gans. Because the barnacle goose is a new host species for HPAI virus, associated pathological findings in this species have not yet been described. Because HPAI vi-rus-infected geese have been reported to show mainly neurological clinical signs (e.g. tremors, incoordination, torticollis) (7), we were particularly interested in investigating whether the virus was neurotropic in these birds.

Materials and Methods

Study design

Our study consisted of two parts. First, we performed virus histochemical analysis of four HPAI viruses to compare the pattern of attachment of these viruses in the res-piratory and intestinal tracts of five bird species. We were particularly interested to determine whether 2020 H5N8 virus attached similarly to 2016 H5N8, 2014 HPAI H5N8 and 2005 H5N1 viruses to the digestive tract of three wild duck species (Eurasian wig-eon; mallard; tufted duck) and one key wild goose species (Graylag goose). Second, we examined the carcasses of 14 wild birds (5 Eurasian wigeons and 9 Barnacle geese) that were found dead during the 2020/21 H5N8 HPAI epidemic in the Netherlands and that tested positive for H5 HPAI virus, to characterize the virology, pathology, and cell type tropism of this virus infection in different organs. The Eurasian wigeons were freshly dead at the time of necropsy; the barnacle geese were at different stages of autolysis, ranging from freshly dead to mildly autolyzed. We were particularly interested to de-termine whether 2020 H5N8 HPAI virus had similar tropism as 2016 H5N8 HPAI virus, from the 2016/17 epidemic, for the digestive tract of wild birds. The techniques of in-vestigation were similar to Caliendo et al 2020 [16], and they are described below.

Virus histochemistry

The following four virus isolates were used: 2005 HPAI virus H5N1 (A/Turkey/Turkey/1/05), 2014 HPAIV H5N8 (A/Eurasian wig-eon/Netherlands/emc-1/2014), 2016 HPAI virus H5N8 (A/Eurasian wig-eon/Netherlands/19/2016), and 2020 HPAI virus H5N8 (A/Eurasian Wig-eon/Netherlands/3/2020). The viruses were individually passaged in Madin-Darby ca-nine kidney (MDCK) cells. After 2-3 days, the supernatant was harvested and cleared of cell debris by low speed centrifugation for 20 min at 1455 x g. The viruses were indi-vidually concentrated by centrifugation of the cleared supernatants in filter tubes (Amicon Ultra-15 100K filter-tubes, Millipore, UFC9100024, Darmstadt, Germany) for 40 min at 4000 x g at 4 oC. The concentrated virus was inactivated by dialysing against 0.1 % formalin for 3 days at room temperature (RT). After inactivation, the virus solution was dialysed against phosphate-buffered saline solution (PBS) and complete inactivation was confirmed by passaging twice on MDCK cells. Virus was labelled by adding an equal volume of 0.1 mg/ml of fluoresceinisothiocyanate (FITC) (Sigma-Aldrich, Saint Louis, MO) in 0.5 M bicarbonate buffer (pH 9.5) for 1 hour at RT while constantly stir-ring. Labelled virus was

dialysed against PBS in order to lose all unbound FITC. The concentration of the different virus suspensions used for virus histochemistry was standardized at 50 hemagglutination units/100 µl (HAU) using hemagglutination assay with turkey red blood cells.

Tissue sections of the following species were used: tufted duck (n=3), Eurasian wigeon (n=3), mallard (n=3), graylag goose (n=3) and chicken (Gallus gallus domesticus) (n=2). These tissues came from the Erasmus MC tissue bank, and were from healthy animals that showed no abnormalities or histological lesions. From the digestive tract of the same birds, tissues selected were duodenum, jejunum, ileum and colon. Sections of jejunum and ileum of graylag goose were not available for virus histochemistry because of limitations of sampling due to high workload during HPAI outbreak. From the res-piratory tract, tissues selected were trachea, primary bronchus, secondary bronchus, tertiary bronchus or parabronchus, air capillaries and air sacs.

Three-µm-thick formalin-fixed paraffin-embedded sections of each tissue were deparaffinized in xylene and hydrated using graded alcohols and incubated overnight at 4°C with FITC-labelled viruses at a concentration of 50 HAU/100 µl. To enable visuali-zation by light microscopy, FITC was linked to a peroxidase-labeled rabbit anti-FITC antibody (DAKO, Glostrup, Denmark). The signal was amplified using a tyramide am-plification system (Perkin-Elmer, Boston, MA). Peroxidase was revealed with 3-amino-9-ethylcarbazole (Sigma-Aldrich) resulting in a bright red precipitate. Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Whitehouse Station, NJ). Omission of the FITC-labelled virus was used as a negative control.

The slides were assessed with light microscopy to estimate the abundance of viral attachment to epithelial cells and scored as follows: attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++). Finally, the median score was determined for each species at the different anatomical sites. Sections were examined without knowledge of the identity of the birds.

Virology, pathology and immunohistochemistry of naturally infected wild birds

The carcasses of 14 wild birds (5 Eurasian wigeons and 9 Barnacle geese) had been collected in the Netherlands in November and December 2020. Pharyngeal and cloacal swabs were collected from each bird for virus diagnostics, using sterile cotton swabs placed in 1 ml of virus transport medium. All the birds tested positive for HPAIV H5 2020 by real-time reverse-transcription PCR (RRT-PCR) assays in oropharyngeal and/or cloacal swabs as described previously (12). At postmortem examination, the following tissues, when available, were exam-ined: brain, lungs, air sacs, pancreas, liver, stomachs (proventriculus and ventriculus), small intestine (duodenum, jejunum at 5,15,35 cm along its lenght, ileum, ileocaecal junction), large intestine (cecum, colon), kidney, adrenal gland, spleen and heart. Du-plicate tissue sections were collected for virus detection and kept at -80 °C until analysis, or were fixed in 10% neutral-buffered formalin and embedded in paraffin. Organs of barnacle geese were not available for virology. For virus detection, tissue samples were first weighed and then homogenized with a FastPrep 24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution and centrifuged briefly before dilu-tion in lysis buffer for RNA extraction (14,15). Extracted total RNA of tissue samples, pharyngeal and cloacal swabs were tested for the presence of influenza A virus matrix gene-fragment; viruses in pharyngeal and

cloacal swabs were further subtyped using real-time RT-PCR targeting fragments of the H5 and N8 genes (17). Briefly, RNA from the tissue and swab suspensions was isolated using a MagNaPure LC System with the MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, the Netherlands). RT-PCR were performed on an ABI 7700 machine (Applied Biosystems, Foster City, CA, USA) using a TaqMan Fast Virus 1-Step Master Mix (Applied Biosys-tems). The oligonucleotides (5′-CTT-CTR-ACC-GAG-GTC-GAA-ACG-TA-3′) and (5′ -TCT-TGT-CTT-TAG-CCA-YTC-CAT-GAG-3′) and the labeled probes (5′ 6-FAM-TCA-GGC-CCC-CTC-AAA-GCC-GAG-A-BHQ-3′) and (5′ 6-FAM-TCA-GGC-CCC-CTC-AAA-GCC-GAA-A-BHQ-3′) were used for detection of the matrix segment of influenza A viruses. Samples were considered virus positive if the cycle threshold (Ct) value was <40 (14,15,17). For histopathology and immunohisto-chemistry, tissues were sectioned at 3 µm and stained with hematoxylin and eosin for histopathological analysis or stained with a monoclonal antibody against nucleoprotein of influenza A virus for immunohistochemical detection of influenza viral antigen, as described previously (14,15).

Results

Pattern of virus attachment to epithelia of digestive and respiratory tracts

In intestinal epithelia, 2014 H5N8, 2016 H5N8, and 2020 H5N8 had overall higher attachment than H5N1(Table 1).

Species	Tissues	Avian influenza viruses			
		2005 H5N1	2014 H5N8	2016 H5N8	2020 H5N8
Mallard	Duodenum	++	+	++	++
	Jejunum	++	++	++	++
	Ileum	+	+	++	++
	Colon	+	+	++	++
Eurasian wigeon	Duodenum	±	±	±	±
	Jejunum	-	±	±	±
	Ileum	-	±	-	±
	Colon	-	±	-	±
Tufted Duck	Duodenum	±	±	±	±
	Jejunum	-	±	±	-
	Ileum	-	±	-	-
	Colon	±	±	-	-
Graylag goose	Duodenum	±	±	±	+
	Jejunum	Nd	Nd	nd	nd
	Ileum	Nd	Nd	nd	nd
	Colon	±	±	±	±
Chicken	Duodenum	++	++	++	++
	Jejunum	++	++	++	++
	Ileum	++	++	++	++
	Colon	+	+	+	+

Mean abundance of attachment was scored as follows: attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++). nd, not done.

Table 1. Pattern of attachment of avian influenza viruses to the epithelial cells of the intestinal tract.

Species	Tissues	Avian influenza viruses			
		2005 H5N1	2014 H5N8	2016 H5N8	2020 H5N8
Mallard	Trachea	++	++	++	++
	Primary bronchus	++	++	++	++
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	±
	Air capillaries	+	±	±	±
	Air sac	++	++	++	++
Eurasian wigeon	Trachea	+	++	++	++
	Primary bronchus	+	++	++	++
	Secondary bronchus	++	++	++	++
	Parabronchus atria	±	±	±	±
	Air capillaries	+	±	±	±
	Air sac	++	++	++	++
Tufted Duck	Trachea	++	+	++	++
	Primary bronchus	++	++	++	++
	Secondary bronchus	++	++	++	±
	Parabronchus atria	+	±	±	±
	Air capillaries	+	±	±	±
	Air sac	++	+	++	++
Graylag goose	Trachea	++	++	++	++
	Primary bronchus	nd	Nd	nd	nd
	Secondary bronchus	++	++	++	++
	Parabronchus atria	++	+	+	+
	Air capillaries	+	+	++	+
	Air sac	++	++	++	++
Chicken	Trachea	++	++	++	++
	Primary bronchus	+	++	++	++
	Secondary bronchus	+	++	++	++
	Parabronchus atria	++	+	++	++
	Air capillaries	+	-	±	+
	Air sac	++	++	++	++

Mean abundance of attachment was scored as follows: attachment to < 1% of epithelial cells (-), attachment to ≥1 and < 10% of epithelial cells (±), attachment to ≥10% and < 50% of epithelial cells (+), and attachment to ≥50% of epithelial cells (++). nd, not done.

Table 2. Pattern of attachment of avian influenza viruses to the epithelial cells of the respiratory tract.

In comparison among host species, virus attachment to intestinal epithelia was low in tufted duck, intermediate in Eurasian wigeon and graylag goose, and high in chicken, mallard. The level of attachment of 2020 H5N8 per host species was: in mallard, similar to 2016 H5N8 and higher than H5N1 and 2014 H5N8; in Eurasian wigeon, similar to 2014 H5N8, and higher than H5N1 and slightly higher than 2016 H5N8; in tufted duck lower than 2014 H4N8, 2016 H5N8 and H5N1; in graylag goose, slightly higher than 2014 H5N8, 2016 H5N8 and H5N1, because of the higher attachment to the duodenum; in chicken, all the viruses had the same, high level of attachment.

In respiratory epithelia, the virus attachment of each virus was generally high in all species, especially for trachea, primary and secondary bronchus (Table 2).

The level of attachment of 2020 H5N8 was lower than 2016 H5N8 in secondary bronchi of tufted duck and air capillaries of graylag goose; and higher than 2016 H5N8 in the air capillaries of chicken.

Virology, influenza virus antigen expression and associated lesions in naturally infected wild birds

For Eurasian wigeons, pharyngeal and cloacal swabs were positive in all five tested birds (Table 3).

Samples	Eurasian wigeons					Barnacle geese								
	W1	W2	W3	W4	W5	G1	G2	G3	G4	G5	G6	G7	G8	G9
Virus	H5N8	H5N1	H5N8	H5N8	H5N8	H5N8	H5N8	H5N8	H5N8	H5N8	H5N8	H5	H5N8	H5N8
Pharyngeal swab	22	30	28	33	23	np	27	29	33	np	33	np	22	27
Cloacal swab	30	27	26	24	27	28	29	np	28	30	29	34	28	28
Lung	25	32	23	31	27	nd	nd	nd	nd	nd	nd	nd	nd	nd
Liver	29	30	35	34	22	nd	nd	nd	nd	nd	nd	nd	nd	nd
Heart	23	30	23	32	27	nd	nd	nd	nd	nd	nd	nd	nd	nd
Jejunum	27	30	23	30	22	nd	nd	nd	nd	nd	nd	nd	nd	nd
Brain	28	34	24	32	28	nd	nd	nd	nd	nd	nd	nd	nd	nd

*Cycle threshold (Ct) value, cut-off value is 40; np, not present; nd, not done.

Table 3. RT-PCR results in swabs and organs of H5N8-infected wild Eurasian wigeons (Mareca penelope) and barnacle goose (Branta leucopsis).

Viral RNA was detected in all the organs tested (i.e., lung, liver, heart, jejunum, brain), meaning that the virus had spread systemically.

For barnacle geese, cloacal swabs were more frequently positive than pharyngeal swabs (88% cloacal swab, positive/tested 8/9; 66% pharyngeal swab, positive/tested 6/9), but, when both positive, they overall contained a similar amount of virus.

Grossly, the main pathological changes consisted of multifocal necrosis in liver and pancreas; pin-point hemorrhages in the brain; sub-pericardial hemorrhages; and mul-tifocal pulmonary. Histologically, lesions were detected in the liver, brain, kidney, lung, heart (Figure 1, Table 4 and Table 5).

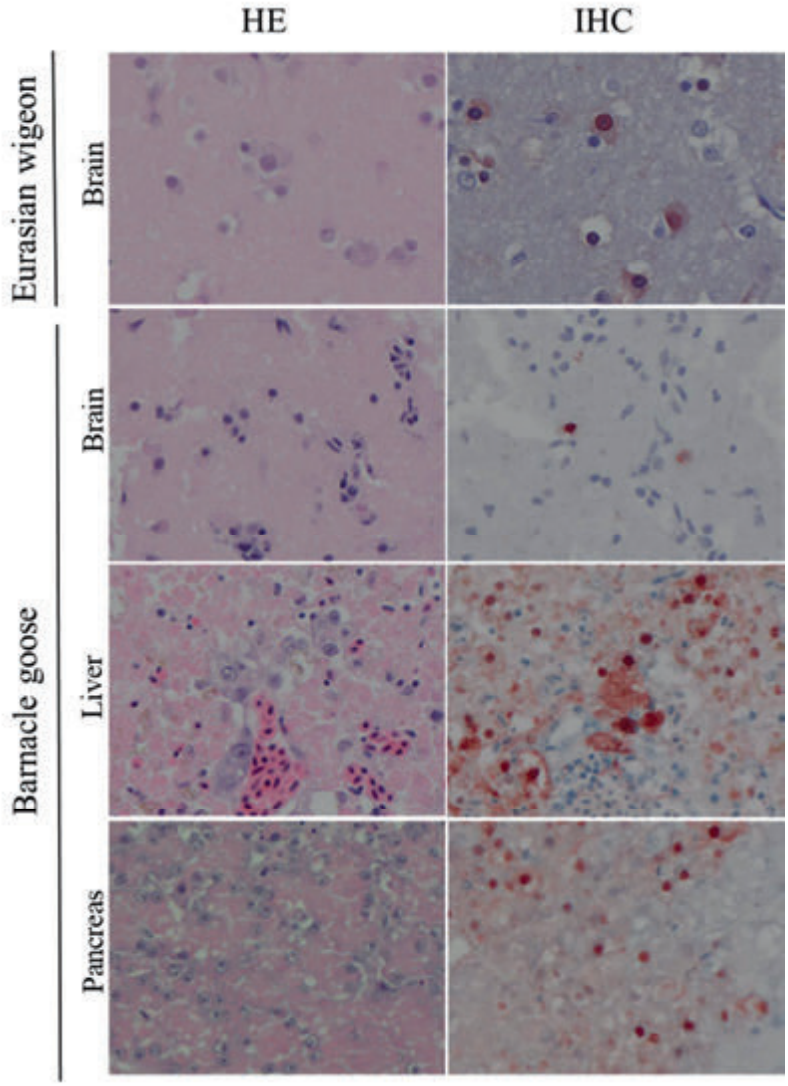

Figure 1. Histological changes and influenza virus antigen expression in tissues of H5-infected Eurasian wigeons (Mareca penelope) and Barnacle geese (Branta leucopsis). Tissue sections on the left column are stained with hematoxylin and eosin (HE). Serial tissue sections in the right column are stained for influenza virus antigen by immunohistochemistry (IHC). In all tissues there is necrosis and inflammation associated with virus antigen expression.

		Number of birds with gross (G) and histological (H) lesions in the:															
		Respiratory system				Digestive system						Other systems					
		Lung		Air sac		Intestine		Pancreas		Liver		Brain*		Heart		Kidney	
Species	No of birds	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H
Eurasian wigeon	5	3	0	0	0	0	0	1	0	0	1	1	3	1	1	0	0
Barnacle goose	9 (*available for 4 birds)	3	2	0	0	0	0	5	3	0	1	3	4	2	0	0	1

Table 4. Frequency and distribution of gross and histological lesions in carcasses of wild birds.

		Number of birds expressing influenza virus antigen in a cell type of an organ															
		Respiratory system						Digestive system						Other systems			
		Lung			Air sac			Intestine			Pancreas		Liver		Brain*		Heart	
Species	No of birds	EP	E	N	EP	E	N	EP	E	N	E	EP	E	H	E	N	E	M
Eurasian wigeon	5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1
Barnacle goose	9 (*available for 4 birds)	2	0	0	0	0	0	0	0	0	0	2	0	1	0	4	0	0

Ep, epithelial cell; E, endothelial cell; N, neuron; H, hepatocyte; M, myocytes.

Table 5. Expression of avian influenza virus antigen in cell types of different organs of wild birds.

In liver and pancreas, lesions were characterized as multifocal, mild to moderate necrosis; these necrotic foci co-localized with presence of viral antigen. In the brain, le-sions were characterized as multifocal encephalitis with foci of gliosis, neuronal de-generation, and necrosis; abundant viral antigen was present in the nucleus and cyto-plasm of several neurons in these lesions. In kidney, lesions were characterized as mild interstitial nephritis, with no presence of viral antigen. In the heart, lesions were char-acterized as multifocal to focally extensive myocardial necrosis; few myocardial cells in these lesions presented viral antigen. Mild to moderate, inflammatory changes and infiltration with mononuclear cells were observed in lung, and few epithelial cells in these lesions presented viral antigen. There were no histological lesions and no de-tectable viral antigen in the gastro-intestinal tract of the examined birds.

Discussion

The high number of birds and the increasing number of wild bird species infected during recent HPAI outbreaks suggest that HPAI H5 viruses are adapting to wild birds. During past epizootics, domestic birds had played an important role for the Gs/Gd lin-eage by acting as main reservoir where virus evolution could take place (1,2). Gradually the virus adapted better to wild birds and now appears to be maintained in wild pop-ulations independently of domestic bird populations (6-9). One of the factors behind this adaptation may be the newly acquired tropism of HPAI viruses for the intestinal tract of their hosts (16). Enterotropism is a mechanism more commonly seen for LPAIVs, but also recently reported for HPAI H5N8, and that allow fecal-oral virus transmission in wild birds (18). The findings of this study partially support our hypothesis that 2020 H5N8 virus has higher tropism for the intestinal tract of wild birds than 2016 H5N8 virus. First, 2020 H5N8 virus had slightly higher level of attachment to the intestinal epithelium of Eurasian wigeon and graylag goose. In mallards the level of attachment for the intestinal tract of 2020 H5N8 virus was unchangingly high compared to 2106 H5N8 virus. The tufted duck is a less likely long-distance vector for HPAIV, and 2020 H5N8 virus had a low level of attachment to the intestinal epithelium of this species.

Second, cloacal swabs were consistently positive for viral RNA in naturally infected Eurasian wigeons and barnacle geese, and more reliable in detecting the infection in barnacle geese. In addition, the jejunum of the five infected Eurasian wigeons was consistently positive for virus RNA by RT-PCR. These findings contrast with previous studies, where cloacal excretion of HPAI virus infected birds was usually uncommon and, if present, lower than pharyngeal excretion (14-16). These findings support the possibility of HPAI virus replication in the intestinal tract of wild birds.

However, a finding against our hypothesis was the lack of virus antigen expression and associated histological lesions in the intestinal epithelium of naturally infected Eurasian wigeons and barnacle geese. Virus antigen expression had been previously reported in the gastro-intestinal epithelium of Eurasian wigeon and graylag goose naturally infected with 2016 H4N8 (16). Therefore, we could not confirm 2020 H5N8 virus replication in the intestinal tract of these birds.

Eurasian wigeons and geese have similar biological traits that may be relevant for the epidemiology of HPAI. Eurasian wigeons have already been described as long-distant vector; HPAI virus infection in barnacle and graylag geese is instead a more recent and less studied event. Although migratory goose species were not previously found to disperse LPAIVs (19), this question should be revisited for HPAI viruses given the novel involvement of barnacle geese and graylag geese in recent HPAI epizootics.

Another common trait between Eurasian wigeons and geese is their feeding biol-ogy. Apart from dabbling, Eurasian wigeons use a diverse feeding strategy that includes foraging by grazing on land (20,21). During migration, wigeons make use of a great va-riety of wetland habitats, including wet farm fields, and often feed on grass, leaves, stems and roots. The use of water-rich land for resting and feeding is also common feature in geese, as these birds regularly congregate and feed on improved agricultural pastures (22,23). During these events, a high density of birds makes use of limited sur-face space. The close contact, and the fact that birds contaminate the grass that they eat with potentially infected feces, may increase

chances of AIV infection via the orofecal route in flocks of Eurasian wigeons as well as geese.

Respirotropism is retained for 2020 H5N8 virus, based on patterns of virus at-tachment and virus antigen expression observed in this study. The combination of these two mechanisms (i.e., respirotropism and enterotropism) could give HPAI viruses an evolutionary advantage over LPAIVs that are mainly excreted via the orofecal route.

This study also described the virology and pathology of HPAI virus infection in barnacle geese, a new host species for the virus. Infection with HPAI H5 viruses in both Eurasian wigeons and barnacle geese was characterized by a high level of neurotropism. Both species presented with brain lesions associated with virus antigen expression. Most likely, this also represented the cause of death for the birds. Brain lesions were also compatible with neurological signs (i.e., incoordination, body tremors, torticollis) shown by many birds, and described by field operators during the 2020/2021 HPAI surveillance (7). The high neurotropic potential of HPAI viruses has been reported in domestic species and it is more often reported also in wild birds (24-27). Pronounced neurotropism is a maintained trait for HPAI H5N8 clade 2.3.4.4b viruses (16,26).

The mortality of waterbirds from the neurotropism of 2020 HPAIV H5N8 could serve in reaching yet more new host species. Infected waterbirds with neurological signs are more visible and trigger hunt response of their predators; they are easier prey to catch and more likely to be eaten (28,29). In 2020/21 newly HPAI virus infected species were predatory birds like great skua (Stercorarius skua) and golden eagle (Aquila chrysaetos) (8-9). Also, for the first time since the HPAI H5N1 epidemic in 2005/2006, during the 2020/2021 HPAI H5 epidemic wild mammalian carnivores were reported HPAI virus infected; European foxes (Vulpes vulpes), gray seals (Halichoerus grypus), and harbor seals (Phoca vitulina) were found dead, probably from contact or ingestion of infected wild birds (8-9). Because of the relatively limited history of HPAI in wild birds, it is possible that virulence levels have not yet been optimized for transmissibility by natural selection (30,31); and the continuously evolving dynamics of HPAI in wild birds may bring new directions for virulence and tropism of HPAI virus in wild birds.

In conclusion, HPAI H5 viruses from the 2020/2021 epidemic expressed a diverse repertoire of tissue tropism, including respirotropism and neurotropism, as well as a high level of in-vitro enterotropism, in infecting wild ducks and geese. Because of the constant evolution shown by HPAI viruses, continued monitoring of the future out-breaks is vital to better understand the new dynamics between virus and its hosts.

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Chapter 2.4

Pathology and virology of natural highly pathogenic avian influenza H5N8 infection in wild Common buzzards (Buteo buteo)

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Abstract: Highly pathogenic avian influenza (HPAI) in wild birds is a major emerging disease, and a cause of increased mortality during outbreaks. The Common buzzard (Buteo buteo) has a considerable chance of acquiring the infection and therefore may function as bio-sentinel for the presence of virus in wildlife. This study aimed to determine the virus distribution and associated pathological changes in the tissues of Common buzzards that died with HPAI H5 virus infection during the 2020-2021 epizootic. Eleven freshly dead, HPAI H5 virus-positive Common buzzards were necropsied. Based on RT-PCR, all birds were systemically infected with HPAI H5N8 virus, as viral RNA was detected in cloacal and pharyngeal swabs and in all 10 selected tissues of the birds, with mean Ct values per tissue ranging from 22 for heart to 32 for jejunum. Based on histology and immunohistochemistry, the most common virus-associated pathological changes were necrotizing encephalitis (9/11 birds) and necrotizing myocarditis (7/11 birds). The proventriculus of two birds showed virus-associated necrosis, indicating tropism of this virus for the digestive tract. Our advice is to collect at least a miniset of samples including brain, heart, liver, and spleen, as these tissues were positive both by RT-PCR and for virus-antigen-associated lesions.

Key words: avian influenza, H5N8, wild birds, pathology, Common buzzard, Buteo buteo

Introduction

Highly pathogenic avian influenza (HPAI) is a major emerging disease, and a cause of mass mortality in wild birds during outbreaks (1-15). The 2020-2021 epizootic of HPAI H5 virus in Europe was the biggest on record in both wild birds and poultry. Based on the animal disease notification system of the affected countries, over 1,000 detections of HPAI H5 virus were reported in wild birds (4). The subtype H5N8 was the first and most widespread genotype circulating during the first months of the epizootic (i.e., October-November 2020) (3,4). Nevertheless, the virus reassorted multiple times, so that sixteen distinct genotypes co-circulated. Early surveillance showed that the first incursions in Europe of the HPAI H5N8 virus occurred in the fall of 2020; however, the epizootic season extended into winter-spring-summer of 2021, with the outbreaks mainly clustered in two peaks: in November 2020 and March 2021 (4). The highest number of detections in wild birds occurred in waterfowl species (order Anseriformes). The second highest number of detections occurred in raptors (orders Accipitriformes, Strigiformes, and Falconiformes); nine different raptor species were found positive, and Common buzzard (Buteo buteo, order Accipitriformes) accounted for the highest number of detections in raptors (4). These birds are infected predominantly by ingesting infected prey. Medium-sized raptor species like the Common buzzard, that can hunt and feed on sizeable birds and ingest a high quantity of infected meat, are considered to be at high risk of becoming infected with HPAIV, and to die of related disease (10,11). In this context, the investigation of Common buzzard mortality for HPAI virus could be used in addition to waterfowl mortality as a passive-surveillance system for early warning and duration of the presence of the virus in wild bird populations.

Wild raptors also have been affected by severe HPAI disease during earlier HPAIV epizootics (7-12,15). Studies on those birds showed that HPAI viruses in wild raptors are primarily neurotropic, and that infected raptors usually present with neurologic signs due to encephalitis, often resulting in death (8,10,15). The condition of some of the carcasses in those reports was suboptimal due to a combination of autolysis, scavenging and freezing, all factors that may impede detailed pathological assessment, in particular identification of specific cell types and evaluation of autolysis-prone tissues such as enteric epithelium.

Despite the high number of reported infected Common buzzards, we are not aware of any studies on the pathogenesis of infection with recent HPAI H5 viruses in this species. This is particularly relevant because, since the 2005-2006 HPAI H5N1 outbreak, HPAI H5 viruses have evolved rapidly and currently are thought to persist longer in wild bird populations (1-6). Updated knowledge of the virology and pathology of infections with more recently circulating viruses is necessary in order to better understand the current pathogenesis of HPAI in Common buzzards; to provide updated guidance regarding the key tissues to collect for HPAI diagnosis at autopsies; and to compare the presence of virus in cloacal and pharyngeal swabs, routinely tested for HPAIV surveillance, with the presence of virus in the main tissues.

To determine the virus distribution and associated pathological changes in the tissues of Common buzzards that died with HPAI H5 virus infection in the Netherlands during the 2020-2021 HPAI epizootic, a collaboration was established among citizen scientists, animal organizations and researchers, aiming to retrieve and examine freshly dead Common buzzards. Eleven Common buzzards were examined and necropsied. All the birds tested positive for HP H5N8. This study documents the virological and pathological findings associated with 2020-2021 HPAI H5N8 infection in Common buzzards and discusses its implications.

Results

Serology. All birds tested negative for antibodies against avian influenza virus NP.

Virology. Pharyngeal and cloacal swabs were positive for HPAI H5N8 virus by RT-PCR, with comparable viral RNA levels in pharyngeal and cloacal swabs (Table 1). Sequencing and molecular characterization of HPAI H5N8 virus, detected in the pharyngeal swab of Common buzzard B9, is publicly accessible (http://www.gisaid.com, A/Common Buzzard/Netherlands/4/2020, isolate EPI_ISL_1575129). Based on virus culture in MDCK-cell culture, three birds in total tested positive in pharyngeal (B9 and B11), and cloacal (B3 and B9) swabs.

All tissues tested were virus-positive by RT-PCR (Table 1), indicating a systemic spread of the infection (n.b., Ct values give an indication of the viral RNA amount in each tissue). Although their Ct values were relatively close together, the tissues with the higher content of viral RNA were, in descending order: heart, brain, air sac, spleen, and lung; the tissues with the lower content of viral RNA were, in descending order: liver, pancreas, colon, kidney, jejunum.

RT-PCR values* (virus titers)**												
Samples	**Common buzzards**											
	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	**Ct-mean (±SD)**
Pharyngeal swab	28 (n)	30 (n)	28 (n)	33 (n)	23 (n)	32 (n)	31 (n)	36 (n)	20 (4.8)	29 (n)	25 (4)	28 (±4)
Cloacal swab	32 (n)	27 (n)	26 (1.8)	24 (n)	27 (n)	33 (n)	23 (n)	26 (n)	22 (2.8)	25 (n)	21 (n)	26 (±3)
Lung	np	32	25	25	25	32	31	31	29	20	17	28 (±7)
Air sac	31	25	np	24	29	34	30	14	19	22	19	26 (±8)
Liver	35	np	34	24	25	31	np	29	20	28	23	30 (±7)
Spleen	35	24	31	30	31	32	31	29	25	28	21	27 (±4)
Heart	24	16	23	31	28	32	31	16	22	18	11#	22 (±6)
Kidney	37	np	np	29	26	31	34	26	26	30	20	31 (±5)
Jejunum	34	np	32	np	27	35	34	26	21	35	20	32 (±7)
Colon	np	34	33	30	31	27	34	30	19	28	23	31 (±5)
Pancreas	30	34	np	26	24	36	35	29	20	32	32	31 (± 6)
Brain	25	32	21	25	32	31	34	24	24	26	18	26 (±4)

Table: RT-PCR results and virus titers in swabs and organs of H5N8-infected wild Common buzzards (Buteo buteo). *Cycle threshold (Ct) value, cut-off value is 40; ** all titers are given in $TCID_{50}$ log 10; # this value is likely an outlier; n, negative (<0.5 for virus titers); np, not present, SD, standard deviation.

Gross pathology. Birds did not present external lesions, and were in moderate to good state of nutrition. The crop of the birds was empty (an indication that they had not fed at least for 24 hours prior death), and four birds had scarce food remains from a recent meat meal in the ventriculus. At internal examination only two birds presented gross abnormalities. When present, the main lesions were multifocal, well-delimited hemorrhages in the heart and in the brain of one bird. The liver of a different bird appeared swollen and with rounded margins. The other organs appeared grossly normal.

Histopathology and Immunohistochemistry. Several organs, and in particular the brain, heart, liver, and spleen, showed histological lesions that colocalized with moderate to abundant presence of viral antigen (Figure 1). The brain of 81% (9/11) of the birds showed multifocal encephalitis with foci of gliosis, neuronal degeneration, and necrosis; abundant viral antigen was present in the nucleus and cytoplasm of several neurons. The heart of 63% (7/11) of the birds showed marked, multifocal to focally extensive myocardial necrosis; few myocardial cells presented viral antigen. The liver and the spleen of 54% (6/11) of the birds showed multifocal, mild to moderate hepatocellular and splenic necrosis, respectively; viral antigen was associated with areas of necrosis. The kidneys of 18% (2/11) of the birds showed mild interstitial nephritis, with no presence of viral antigen. The pancreas of 18% (2/11) of the birds showed multifocal necrosis with no presence of viral antigen. The ovary of 18% (2/11) of the birds showed moderate infiltration with mononuclear cells; few epithelial cells presented viral antigen. The proventriculus of 18% (2/11) of the birds showed necrotic lesions; few necrotic cells, most likely epithelial cells, presented viral antigen. Mild to moderate, inflammatory changes and infiltration with mononuclear cells were observed in the nasal passage, trachea, lung, and air sac of 18% (2/11) of the birds; few epithelial cells in these organs presented viral antigen.

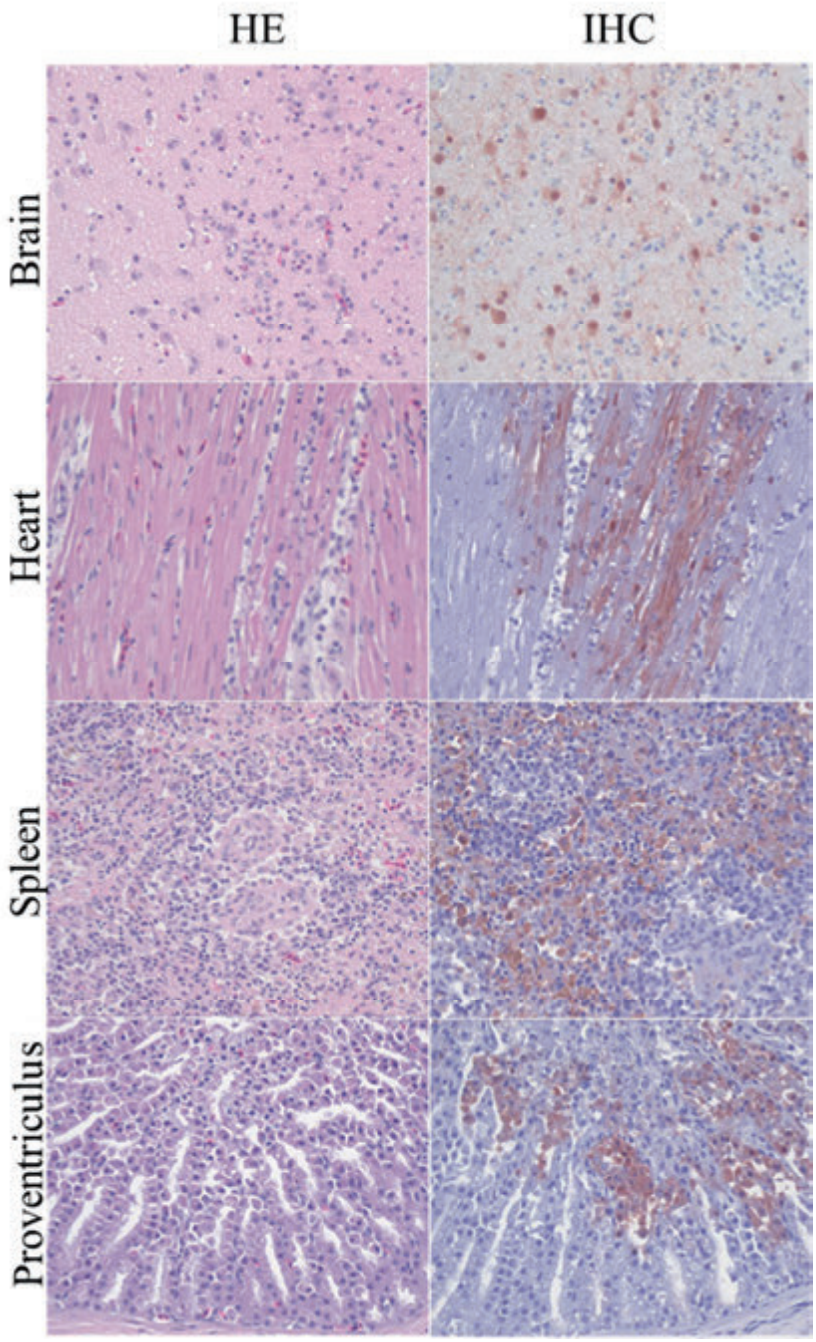

Figure: Histological changes and influenza virus antigen expression in tissues of H5N8-infected Common buzzards (Buteo buteo). Tissue sections on the left column are stained with hematoxylin and eosin (HE). Serial tissue sections in the right column are stained for influenza virus antigen by

immunohistochemistry (IHC). In all tissues there is necrosis and inflammation associated with virus antigen expression.

Discussion

This study describes the virological and pathological findings of Common buzzards infected with the 2020-2021 HPAI H5N8 virus. These analyses showed that the main lesions were HPAI virus-associated inflammation and necrosis in multiple tissues including brain and heart, confirming HPAI as cause of death or severe disease.

The Common buzzard presents with several characteristic traits that make it a valuable bioindicator of HPAIV presence in wildlife. It is a medium-sized raptor, present almost throughout Europe. In the Netherlands , its population has been stable since 1970 with an estimated maximum winter population of 30,000-50,000 individuals (16). The Common buzzard is mainly a resident bird, which generally inhabits woodlands but is adaptable to wetlands (16,17). Its feeding behavior as an opportunistic predator and scavenger has the potential to expose it to HPAIV-infected prey. Given these predisposing biological traits, it is not unexpected that Common buzzards accounted for the highest number of HPAI virus detections in raptors during the 2020-2021 epizootic.

Previous studies showed that HPAI viruses in raptors are highly neurotropic and cause severe neurological disease (8,10,15,19, 20). This study also supports those findings, as the most consistent lesion in Common buzzards was viral encephalitis, with confirmed presence of viral antigen in affected neurons. In addition to the nervous system, all the tissues tested of the Common buzzards were positive for virus based on RT-PCR and showed infection-related, histological lesions, indicating that HPAI H5N8 virus infection in the Common buzzard causes systemic disease.

This study showed that HPAI H5N8 virus is also highly cardiotropic, as the myocardium of the Common buzzards contained the highest amount of virus based on RT-PCR (Table 1), and virus-associated, severe histological lesions in 63% (7/11) birds . In addition, 54 % (6/11) of the Common buzzards showed virus-associated lesions in the liver and spleen.

The Common buzzard is considered to be infected via the oral route by ingesting HPAIV-infected preys. Transmission of HPAIV from ingesting infected chicken meat has been experimentally confirmed in raptors (18). Interestingly, the proventriculus of two birds in our study showed necrotic lesions with viral antigen. This finding further supports the oral route of infection, although we cannot exclude the possibility that the proventriculus was infected via the hematogenous route. It also provides new records of HPAIV enterotropism in wild birds. The adaptation to the intestinal tract is a mechanism recently reported for HPAI H5N8 virus, that may allow a more efficient fecal-oral transmission in wild birds (5).

Real time PCR (RT-PCR) is the preferred test for HPAI virus detection for active and passive bird surveillance (9). In this study, cloacal and pharyngeal swabs had comparable RNA-levels, and both were adequate for the detection of the virus. The tissue analysis by RT-PCR showed that heart, brain, and air sac had highest viral RNA concentrations compared to other organs. Although not confirmed by a quantitative real time PCR, the results obtained by RT-PCR are well supported by histopathology and immunohistochemistry. Our advice for diagnostic pathologists is to collect at least a miniset of samples including brain, heart, liver and spleen, as these tissues are relatively easily sampled and were positive by both RT-PCR

and for virus-antigen-associated lesions. For virus diagnosis of Common buzzards found dead (but without the interest or possibility to perform pathological examination), it is enough to collect pharyngeal and cloacal swabs, because they were positive by RT-PCR with Ct values that were comparable to those in most tissues (with exception of heart, that had higher Ct values).

We did not detect antibodies against avian influenza virus NP in the sera of the Common buzzards in this study. Most of the birds (8/11) were juveniles in their first year of life, and likely they did not have protective antibodies from previous infections, as this was the first time in their lives that they experienced a HPAI epizootic. The absence of antibodies indicates also that the Common buzzards died acutely soon after infection, similarly to experimentally infected raptors that did not seroconvert before early death (20). All the birds in our study were females. Females are larger than males (adult female weigh about 15% more than adult males), thus it is possible that female raptors are easier to find during surveillance or that there are sex-associated differences in feeding patterns.

This study showed that HPAIV infection in Common buzzards produced severe systemic disease, and subsequent acute death based on the stage of the pathological changes and absence of serum antibodies. Cloacal and pharyngeal swabs were comparable in detecting the infection. Many organs contained viral RNA; with heart, brain and air sac containing the highest amount of viral RNA. The proventriculus of two birds showed virus-associated lesions, implying a possible adaptation of the virus to the gastro-intestinal tract.

Materials and Methods

Birds. In November 2020, during the first peak of the 2020-2021 HPAIV epizootic, 11 wild Common buzzard carcasses were presented at Erasmus MC, Rotterdam, for pathological and virological investigations. These birds were found by citizen scientists; nine birds were found dead, and two were still alive but subsequentially were euthanized after showing severe neurological signs of disease, such as torticollis and body tremors. The carcasses were refrigerated, and autopsies were performed at Bio Security Level 3 settings within 24 hours after retrieval.

Necropsy. The birds appeared freshly dead and in good state of preservation. All the birds were female based on the presence of an oviduct and ovaries in the coelomic cavity; eight were juveniles (first year) and three were adults (second year) based on their plumage. Pharyngeal and cloacal swabs were collected for virology from each bird, using sterile cotton swabs placed in 1 ml of virus transport medium. Tissue samples from nasal turbinate, air sac, trachea, lung, heart, liver, spleen, kidney, pancreas, stomach, jejunum, duodenum, colon, and brain were collected for virology and kept at -80 °C until analysis. Duplicate samples of the same tissue were collected for histopathology and immunohistochemistry and fixed in 10% neutral-buffered formalin until analysis. Blood from the heart was collected in a plain 2 ml tube, and centrifuged for 15 min at 1500 g.

Serology. Sera were tested for nucleoprotein (NP)-specific antibodies with a commercial blocking enzyme-linked immunosorbent assay (bELISA) (Idexx A Ab Test ; Idexx Laboratories BV, Hoofddorp, The Netherlands). Samples were tested according to the manufacturer's instructions. A sample was considered NP positive when the signal-to-noise

ratio (i.e., ratio of the mean optical density [ODx] of the sample/ODx of the negative control) was 0.5 or lower.

Virology. Tissue samples were first weighed and then homogenized with a FastPrep 24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution and centrifuged briefly before dilution in lysis buffer for RNA extraction [10]. Extracted total RNA of tissue samples, pharyngeal and cloacal swabs were tested for the presence of influenza A virus matrix gene-fragment; pharyngeal and cloacal swabs were further subtyped using real-time RT-PCR targeting fragments of the H5 and N8 genes. Samples were characterized as HPAI H5 virus by detection of a multi-basic cleavage site upon Sanger sequencing of the HA gene [9]. Full genome sequence of the virus isolated from the pharyngeal swab of Common buzzard 9 (B9), was obtained by Sanger sequencing. In order to assess the excretion of infectious virus, pharyngeal and cloacal swabs were also tested by virus culture. Virus titers of the pharyngeal and cloacal swabs were obtained with triplicate 10-fold serial dilutions in confluent layers of Madin-Darby canine kidney (MDCK) cells [10].

Histopathology and Immunohistochemistry. Tissues samples were embedded in paraffin, sectioned at 4 μm, and stained: with hematoxylin and eosin (HE) for presence of histopathological changes; with an immunohistochemical test, using monoclonal antibody against nucleoprotein of influenza A virus as a primary antibody, for detection of influenza viral antigen [10].

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Chapter 3: Epidemiology of the GS/GD HPAI H5 viruses in wild birds

Chapter 3.1

Impact on wild bird populations of 2020-2021 highly pathogenic avian influenza epidemic in The Netherlands

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Abstract: The European 2020/2021 HPAI epizootic was particularly relevant because record numbers of HPAI-related infections and mortalities of wild birds were registered. To quantify deaths among wild species groups with known susceptibility for H5 during the 2020/2021 outbreak in the Netherlands, from 1 October 2020 to 30 September 2021, daily mortality data of wild birds were collected and recorded by the AI-impact consortium, a cooperation among Dutch ornithologists, virologists, and animal health organizations. A total of 16,631 wild birds of 160 species were reported dead. Waterbirds accounted for 70%, and land birds for the remaining 30% of the total mortality reports. Anseriformes represented 50% of the total mortality reports. The species with the highest numbers of reported dead and infected individuals were within the Anseriformes group, in primis barnacle goose (*Branta leucopsis*). During the outbreak, wild bird mortalities clustered in two peaks, the first in November 2020 and the second in April and May 2021. Monitoring live and dead wild birds is an essential tool for the surveillance of the evolving dynamics of HPAI. Next to surveillance for HPAI, we advocate that implementing recording of wild bird mortality and the involvement of citizen scientists should be encouraged and endorsed systematically also at international level.

Keywords: avian influenza; HPAI; wild birds; poultry; H5N8; H5N1; barnacle goose; the Netherlands

Introduction

Since the emergence of goose/Guangdong/1/96-lineage (Gs/GD/96) H5 virus in 1996, highly pathogenic avian influenza (HPAI) outbreaks have frequently occurred in wild birds worldwide (1-4). In Europe, large outbreaks of HPAI took place in 2005/2006, 2014/2015, 2016/2017, 2020/2021, and currently in 2021/2022 (1-6). Long-distance migratory Anatidae (ducks, geese, and swans) have been responsible for the intercontinental spread of H5 HPAI viruses (1-4). Long-distance migratory species can be vectors of H5 viruses from wintering grounds in Asia to wintering grounds in Europe in a two-step process via breeding grounds in northern Russia (1-4). Wild birds infected with HPAI virus can present with a broad spectrum of clinical manifestations, ranging from absence of clinical signs to acute death (4).

The European 2020/2021 HPAI epizootic was particularly relevant because record numbers of HPAI-related infections and mortalities of wild birds were registered (5,6). In the Netherlands, a similar scenario had previously occurred during the HPAI outbreak in 2016/2017, when more than 13,000 wild birds were reported dead (7-10). The first incursion of H5 HPAI virus in the Netherlands in 2020 was reported in nine live Eurasian wigeons (*Mareca penelope*) on 16 October, coinciding with the autumn migration of these birds to the Netherlands (11). This bird species was possibly involved in the incursion of the virus into the country. On 17 October 2020, wild bird mortality started as two mute swans (*Cygnus olor*) were found dead and infected with HPAI H5N8 clade 2.3.4.4b virus (12). Since then, an unusual mass mortality of waterbirds, especially geese, was recorded at specific locations. Because a high proportion of sampled wild dead birds tested positive for highly pathogenic avian influenza, there was strong evidence that wild bird mortality was correlated to HPAIV infection. Captive birds were also infected, and the first introduction of HPAIV was recorded in a commercial poultry farm on 29 October 2020 in Altforst, Gelderland. HPAI is a serious issue for the poultry industry (4-6). During the 2020/2021 outbreak, 3,777 detections were reported in poultry birds in Europe (5,6). The presence of wild birds nearby poultry farms is often associated to the incursion of HPAI virus in the holding (4).

To quantify deaths among wild species groups with known susceptibility for H5 during the 2020/2021 outbreak in the Netherlands, from 1 October 2020 to 30 September 2021, daily mortality data of wild birds were collected and recorded by the AI-impact consortium, a cooperation among Dutch ornithologists, virologists, and animal health organizations.

The aims of this study were to 1) identify the wild bird species that were more substantially affected by HPAI-related mortality during the 2020/2021 in the Netherlands; 2) identify the spatial-temporal pattern of wild bird mortality during the outbreak, also in relation to viral incursion in poultry farms.

Methods

The methods for this study were similar to the ones used by Kleyheeg et al. 2017 (7). Briefly, from 1 October 2020 to 30 September 2021, wild bird mortality data were collected and classified by species, date, and location of finding. Dead birds were reported to the AI-Impact consortium by the national competent authorities, as part of the national infectious disease surveillance and by citizen scientists, invited to report sightings of dead birds on the web-platforms of the AI-Impact members (https://dwhc.nl/dode-vogels-melden/, https://www.sovon.nl/nl/content/vogelgriep, https://www.nvwa.nl/onderwerpen/vogelgriep-

preventie-en-bestrijding). Reports were classified per bird species, date of and location of finding. Double-counts were excluded as much as possible. As per similar studies, it is highly likely that the number of reported carcasses underestimates the actual number of deaths between 10%-25% (7).

Bird mortalities were reported per avian group, such as Anatidae (i.e., geese, swans, ducks), other water birds (i.e., gulls, grebe, herons, cormorant, waders, rallids), raptors and other land birds. Individual bird species were also analyzed, whether because experienced particularly high mortality within their avian group (i.e., barnacle goose *Branta leucopsis*, graylag goose *Anser anser*, mute swan, and common buzzard Buteo buteo, or because they had experienced high mortality in the previous 2016/2017 outbreak (i.e., Eurasian wigeon, tufted ducks *Aythya fuligula*, peregrine falcon *Falco peregrinus*, great black-backed gull *Larus marinus*) and we were interested in comparing these species mortality trends between the two outbreaks. The number of deaths per avian groups between October 2020-September 2021 were compared with those occurring in the same timeframe from 2010-2011 to 2015-2016 in the public database of Sovon (Dutch center for Field Ornithology, Nijmegen, the Netherlands) as per Kleyheeg et al. (7).

A limited proportion of carcasses were tested for HPAI virus by real-time reverse transcription on oropharyngeal and cloacal swabs, as previously described (9,10).

Results

A total of 16,631 wild birds of 160 species were reported dead. Waterbirds accounted for 70%, and land birds for the remaining 30% of the total mortality reports (Table 1). Anseriformes represented 50% of the total mortality reports. The species with the highest numbers of reported dead and infected individuals were within the Anseriformes group, in primis barnacle goose, then greylag goose and mute swan; common buzzard was the species with the highest numbers of reported dead and infected individuals within the raptor group. When calculating the potential effects that the outbreak had for wintering bird populations, it was estimated that 0.4 %- 0.5% of barnacle geese, 0.07%- 0.1% of greylag geese, 0.7%-1% of mute swan, and 1%-2% of common buzzards have died (Table 1). Higher estimated mortality rates were registered in species with smaller populations, such as the black-backed gull and the peregrine falcon that respectively lost 2%-3% and 5%-6% of their wintering populations. Relative number of deaths are presented in Figure 1.

Avian group and species		*Maximum estimated winter population, x 1000	No. carcasses (mortality rate)	No. carcasses HPAI positive/no. tested
Anatidae			7901	361/628
	Geese		4802	234/332
	Barnacle goose	780-820	3435 (0.4%-0.5%)	147/171
	Goose unidentified		607	35/59
	Greylag goose	510-580	390 (0.07%-0.1%)	30/59
	Swans		996	60/136
	Mute swan	38-46	305 (0.7%-1%)	38/93
	Swan unidentified		629	19/54
	Ducks		2103	67/160
	Eurasian wigeon	860-940	125 (0.01%-0.02%)	20/29
	Tufted duck	180-240	45 (0.02%-0.03%)	1/19
Other waterbirds			4068	19/162
	Gulls		1074	7/61
	Great black-backed gull	5.4-6.5	137 (2%-3%)	1/1
	Grebes		62	0/2
	Herons		250	0/33
	Cormorants		234	2/14
	Waders		1045	9/49
	Rallids		327	0/2
Raptors			1011	42/155
	Common buzzard	30-50	365 (1%-2%)	27/91
	Peregrine falcon	0.5-0.8	27 (5%-6%)	4/5
Other land birds			3651	5/40
	Corvids		271	1/24
Total			16631	427/985

Table 1. Reported bird species, winter population size estimates, number of carcasses, and RT-PCR test results for wild birds sampled during the 2020/2021 HPAI outbreak in the Netherlands. *Data from Sovon (Dutch Centre for Field Ornithology, Nijmegen, the Netherlands).

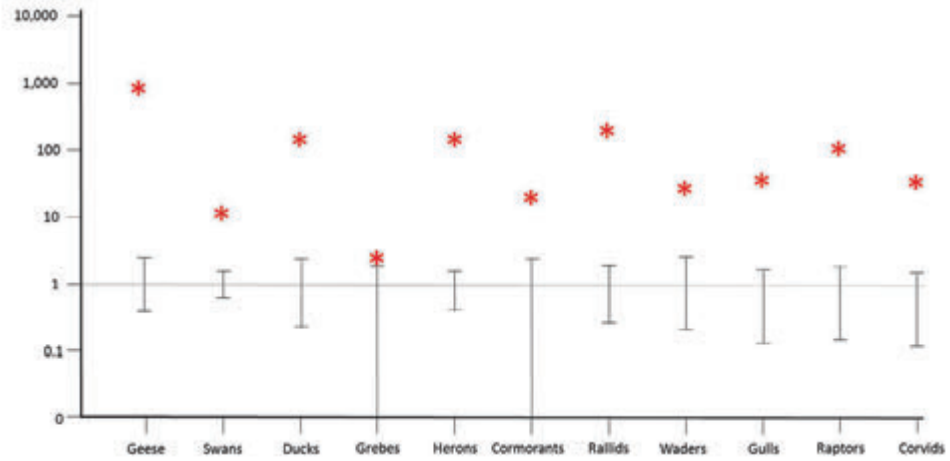

Figure 1. Relative number of deaths among wild birds in the Netherlands, during the 2020/2021 HPAI outbreak. Number of reported deaths during the outbreak (*red asterisks) is shown relative to the normalized number of deaths reported over the same period from 2011-2012 to 2015-2016 (average is 1, error bars indicate maximum and minimum mortality; data from Sovon, Dutch Centre for Field Ornithology, Nijmegen, the Netherlands). The y-axis is on a log-scale, so that reported deaths among geese during 2020/2021 was 1.000 times greater than in the previous years.

Temporally, the 2020/2021 outbreak started in October 2020 and HPAI H5 viruses were regularly detected up to September 2021. For barnacle geese, the first HPAIV positive bird was sampled on 27 October 2020 in Friesland, in the municipality of Moddergat (53° 24′ 7″ N, 6° 4′ 57″ E), and the first mass mortality event was on 30 October 2020 in the municipality of Ameland (53° 27′ 0″ N, 5° 46′ 0″ E). During the 2020/2021 outbreak wild bird mortalities clustered in two peaks, the first in November 2020 and the second in April and May 2021 (Figure2). The virus was still detected in October 2021, but that date was considered the start of the new HPAI 2021/2022 outbreak. In February 2021, the Netherlands were hit by a cold spell with temperatures in certain areas dropping to -15.4 Celsius. A consequence of this event was an unusual higher mortality of certain bird species, such as common kingfisher (*Alcedo atthis*) and Eurasian woodcock (*Scolopax rusticola*). A possible HPAIV infection was ruled out for these species. During the cold spell, several water areas usually visited by waterfowl were frozen. This event possibly influenced the movements of different waterfowl species, by bringing HPAIV-infected birds into contact with non-infected birds and leading to higher bird-to-bird contact, thereby increasing HPAIV prevalence in the following months.

Geographically, around the first peak of the outbreak (from October 2020 to March 2021) the mortality of wild birds was concentrated in the north-west coast of the Netherlands, with some inland clusters around water-rich agricultural areas (Figure 3). Friesland, especially the municipality of Noardeast-Fryslân (53° 20′ 0″ N, 6° 3′ 0″ E) and the Frisian Islands, was the province with the largest hotspots for mortalities, with mortality events registering over hundred dead barnacle geese in the same location. Around the second peak of the outbreak (from April to June 2021) the mortalities moved to the north-east coast. Groningen,

especially the municipalities of Oldambt (53° 9′ 0″ N, 7° 2′ 0″ E) and Delfzijl (53° 20′ 0″ N, 6° 55′ 0″ E), was the province with the largest hotspots for mortalities, with mortality events registering over fifty dead barnacle geese in the same location. Around summer (from July to September 2021) mortality events were more inland, in the south-west of the country. Sixteen commercial poultry farms in the Netherlands were also infected with HPAI H5 viruses during the 2020/2021 outbreak (Figure 3).

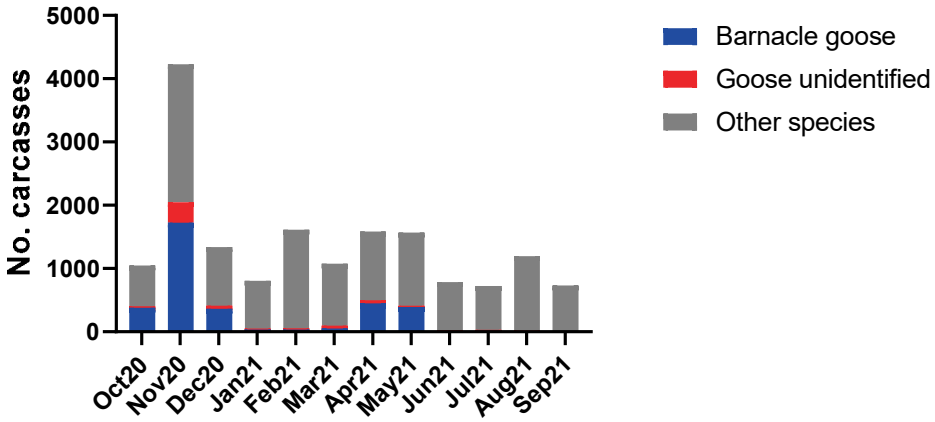

Figure 2. Temporal pattern of wild bird deaths during the 2020/2021 HPAI outbreak in the Netherlands.

Figure 3. Spatial overview of the reported cumulative number of wild bird deaths (circles) and infected poultry farms (red triangles) in the Netherlands during: A) October, November, December 2020; B) January, February, March 2021; C) April, May, June 2021; D) July, August, September 2021.

Discussion

Highly pathogenic avian influenza dynamics in wild birds are constantly evolving. The 2020/2021 outbreak was, to date, the most devastating outbreak in Europe and presented with record numbers of HPAI-related infections and mortalities of wild birds (5,6). In the

Netherlands between 1 October 2020 and 30 September 2021, mortality of certain wild bird groups was higher than the average mortality estimates in previous years (i.e., compared to the same timeframe between 2011 and 2016, years when major wild bird mortalities from outbreaks of HPAIV did not occur).

Geese species, such as the barnacle goose, accounted for the highest number of mortalities and infections. During the outbreak, high rates of infection in geese were also reported in Germany (13). Duck species, although consistently tested positive for HPAIV, had relatively low mortality. This is a different scenario compared to the 2016/2017 HPAI outbreak, when duck species, such as Eurasian wigeon and tufted duck experienced the highest number of mortalities (7). Because Eurasian wigeons and tufted ducks are long lived species, it is possible that infected birds that survived the 2016/2017 outbreak were still immunologically protected during the 2020/2021 outbreak, and so were able to survive re-infection. Eurasian wigeon was the first species detected HPAIV positive during the 2020/2021 outbreak in the Netherlands (11). Eurasian wigeons have been repeatedly involved in epizootics in Eurasia, including in 2014-2015 and 2016-2017, and are considered prime candidates for carrying HPAIV over long distances (1,2,4,10,11,14). Also, during the first stages of an outbreak they are one of the first species to be detected HPAI virus positive, often asymptomatically (4). A recent study in Italy during the 2020/2021 outbreak confirmed that hunted Eurasian wigeons had high prevalence of infection but stayed clinically healthy when recaptured weeks apart (15).

Contrarily, the role of barnacle geese in the 2020/2021 HPAIV outbreak is unprecedented. Barnacle geese are one of the most abundant geese species in the Netherlands (16,17). They are gregarious, herbivores birds with a preference for coastal grassland and water-rich agricultural fields (18,19). Barnacle geese overlap within their territories with other herbivorous birds, such as Eurasian wigeon and mute swan. During the 2020/2021 outbreak in the Netherlands, mass mortalities of barnacle geese started on 30 October 2020 and peaked in November 2020. Because this postdated two weeks the first detection of HPAIV in wild birds, it is unlikely that barnacle geese were the vectors of viral incursion in the Netherlands. A more likely scenario was that barnacle geese fist contracted the infection via direct or indirect contact with other infected bird species, such as Eurasian wigeons or mute swans. The large number of geese and their gregarious behavior facilitated the viral intraspecies transmission by direct or indirect contact (e.g., via contaminated grass, contaminated water). Because barnacle geese in the Netherland comprise of both migratory and resident populations, it is possible that infected, resident geese dispersed the virus to naïve (sub-)populations via their local movements within the country, for example during the spring/breeding season. Geographically, this explains the two different hotspots for the two peaks of mortalities, that coincided with overwintering areas of north-west coast of the Netherlands in October, November December 2020, and with breeding areas in the north-east coast in April, May, June 2021.

The great number of infected geese carcasses in the wild was a determinant for interspecies viral transmission for hunting or scavenger bird species. Raptors, for example, are exposed to infection by ingesting infected prey (20). During the outbreak, eight different raptor species were found to be infected, and the highest number of infections and mortilities occurred in buzzards. Because of the relatively small populations of certain raptor species, such as the peregrine falcon, HPAI may represent a new threat for bird conservation.

Clinically, the infection in wild birds was characterized by mainly neurological signs such as incoordination, body tremors, and torticollis, that were associated with brain lesions and a high level of neurotropism (20,21).

The abundant circulation of HPAI virus in new host species indicates that the virus has well adapted to certain wild birds. Temporally, during the 2020/2021 outbreak the virus was recovered from Dutch wild bird populations for over one year, indicating that it can be spread and maintained long-term in those populations (5,6). This is a new feature compared to the 2016/2017 outbreak, when viral circulation was mainly limited to autumn and winter (7). A consequence of the unusual persistence of the virus over summer was that naïve, newly hatched birds, in particular juvenile Anseriformes, such as mute swans and greylag geese, and raptors, such as white-tailed eagle (*Haliaeetus albicilla*), were exposed and died because of the infection during spring/summer 2021 (5). From the spring of 2021, and for the first time since the 2005/2006 HPAI H5 virus outbreak, the virus was also detected in several carnivore species, such as European foxes (*Vulpes vulpes*), gray seals (*Halichoerus grypus*), and harbor seals (*Phoca vitulina*) most likely infected via contact or ingestion of infected wild birds (5,6). Because of these new events, avian influenza infection should be systematically included as a differential diagnosis in the assessment of the health status of these species. The fact that the 2020/2021 H5 virus can naturally infect mammalian species creates concerns about its increased zoonotic potential (5,6). During the outbreak, six HPAIV subtypes were identified (H5N1, H5N2, H5N3, H5N4, H5N5, H5N8) (5,6). The most frequently detected subtypes were HPAI H5N8 virus during the first part of the outbreak, and HPAI H5N1 virus from the second part the outbreak (5,6). This was a different presentation compared the 2016/2017 outbreak in the Netherlands, when only one subtype (H5N8) was detected (7). The greater subtype variety of the more recent outbreak is an indication that the virus could successfully reassort locally with low pathogenic avian influenza viruses circulating at the same time in the wild bird hosts.

The high number of infected dead birds around wetlands, as well as in more urbanized areas, posed a biosecurity issue for the risk of viral spill-over to other wild birds as well as poultry. The AI-impact consortium, together with the competent health authorities, provided a decisional tree for the clean-up of carcasses, aiming to reduce the environmental contamination from infected birds (Supplementary Material, Appendix 1). The main advice was to prioritize the clean-up of areas regularly used by scavengers and where birds congregate in high numbers.

Because HPAI virus remained present at high levels in the wild bird population from October 2020 to September 2021, commercial poultry farms were at constant risk of viral incursion. Sixteen commercial poultry farms were infected with HPAI H5 virus during the 2020/2021 outbreak. The locations of these farms partially matched with high concentrations of waterbirds in the Netherlands, suggesting that infection of farmed birds is enhanced by high presence of waterbirds. To prevent and mitigate the risk of viral spread from wild birds to commercial poultry, new technology such as the use of automated laser are useful in keeping wild birds away from nearby farms (22). However, in the long-term, the modern poultry farming industry will have to make important transformative changes to address issues, such as extremely high number of farms, number of birds per farm, and density of farms, that, together with high environmental presence of HPAIV, make for a substantial risk of virus incursion especially where poultry farms and wild waterbirds rich areas overlap.

The Netherlands is an important staging area for migratory wild birds across Eurasia and to America (7,14). Data from wild bird surveillance in the Netherlands made possible to link the transatlantic spread of HPAI virus from Europe to Canada (14). Monitoring live and dead wild birds is an essential tool for the surveillance of the evolving dynamics of HPAI (4, 22). For the constant monitoring of wild bird mortality during the 2020/2021 HPAI outbreak, citizen scientists were a fundamental resource and made possible to obtain a wider impression of the actual scale of mortality in wild birds, otherwise limited to the data of the official surveillance. Next to surveillance for HPAI, we advocate that implementing recording of wild bird mortality and the involvement of citizen scientists should be encouraged and endorsed systematically also at international level.

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Chapter 3.2

Transatlantic spread of highly pathogenic avian influenza H5N1 by wild birds from Europe to North America in 2021

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Abstract: Highly pathogenic avian influenza (HPAI) viruses of the A/Goose/Guangdong/1/1996 lineage (GsGd), which threaten the health of poultry, wildlife and humans, are spreading across Asia, Europe, Africa and North America but are currently absent from South America and Oceania. In December 2021, H5N1 HPAI viruses were detected in poultry and a free-living gull in St. John's, Newfoundland and Labrador, Canada. Our phylogenetic analysis showed that these viruses were most closely related to HPAI GsGd viruses circulating in northwestern Europe in spring 2021. Our analysis of wild bird migration suggested that these viruses may have been carried across the Atlantic via Iceland, Greenland/Arctic or pelagic routes. The here documented incursion of HPAI GsGd viruses into North America raises concern for further virus spread across the Americas by wild bird migration.

Introduction

The A/Goose/Guangdong/1/96 (GsGd) lineage of highly pathogenic avian influenza (HPAI) H5 virus first emerged in poultry in Southeast Asia more than 25 years ago. During the first decade of circulation of this lineage, the hemagglutinin (H) genes diversified into multiple genetic clades. GsGd viruses of clade 2.3.4.4 started to dominate outbreaks globally from 2014 onwards with clade 2.3.4.4b currently emerging as a particularly fit virus. The GsGd lineage, and particularly clade 2.3.4.4b, is expanding both its geographical spread and its host range (1-4). Therefore, this lineage of HPAI H5 virus is an increasing threat to the health of poultry, wildlife, and humans worldwide, as well as a growing economic problem for the global poultry sector (3,5).

In recent years, HPAI GsGd H5 outbreaks have frequently occurred in Europe (3-8). For the first time in 2005, the virus spread from Asia to Russia, western Europe, Africa and the Middle East, causing high mortality in wild birds and poultry (8). This spread was a result of unprecedented long-distance transport of HPAI virus, in which wild migratory ducks, geese and swans were implicated. The last wild bird case for clade 2.2 and derivatives in Europe was detected in Germany in January 2009 (6). Another European incursion of a clade 2.3.2.1 virus occurred in 2009 but was not detected further west than Romania and Bulgaria, after being detected in Qinghai in China and Tyva in Russia (7). In 2014/2015 a new intercontinental outbreak of HPAI H5 virus occurred, and since then HPAI H5 viruses have repeatedly caused large outbreaks in wild birds and poultry in Europe (5,6). In addition, there are also growing concerns about the zoonotic risks, and in December 2021, the European Centre for Disease Prevention and Control raised the risk level for virus transmission to occupationally exposed people from ‘low’ to ‘low/moderate’ (5,9).

In December 2021, there was a die-off of domestic birds on an exhibition farm in St. John’s, a city on the Avalon Peninsula of the island of Newfoundland, on the Atlantic coast of Canada. The cause was diagnosed as HPAI H5N1. This was the first report of HPAI H5 in the Americas since June 2015, when the virus spread with wild birds across the Bering Strait to the Pacific coasts of Canada and the USA via the Pacific Flyway, one of the main avian migration routes (10). Genetic analysis as reported in the OIE report showed that the hemagglutinin (H) gene corresponded to Eurasian HPAI viruses circulating in spring 2021 (11). This implied that the virus had been carried across the Atlantic, a route that has been recorded before for LPAI viruses but not for any HPAI virus (12,13). Therefore, the goal of this study was 1) to investigate in detail whether the HPAI cases in Newfoundland were linked to the recent (2020/2021) or currently ongoing (2021/2022) HPAI outbreaks in Europe, and 2) to indicate the most likely scenario by which the virus crossed the Atlantic with migratory birds.

Results

Epidemiological description of exhibition farm outbreak

The index farm where highly pathogenic avian influenza (HPAI) H5N1 virus in captive birds occurred was an exhibition farm in St. John’s, Province of Newfoundland and Labrador, Canada. The farm housed 409 birds of different species, namely chickens, guineafowl, peafowl, emus, domestic ducks, domestic geese, and domestic turkeys. On 9th December 2021, the farm owner first noticed mortality. On 13th December, the farm owner reported the

increased mortality to a local veterinarian. Autopsies on four chickens showed swollen heads and cutaneous haemorrhages. Oropharyngeal and cloacal swabs from these chickens tested positive for H5 avian influenza virus at the Atlantic Veterinary College, University of Prince Edward Island, and the Canadian Food Inspection Agency (CFIA) was notified. On 16th December, by which time 306 birds (mostly chickens, turkeys and guineafowl) had died, staff of the CFIA collected tissue samples from dead chickens, as well as oropharyngeal and cloacal swabs and sera from different species of captive birds still present (Table 1), after which all remaining captive birds were culled. All oropharyngeal and cloacal swabs tested positive for HPAI virus of the subtype H5N1 by real-time RT-PCR, and all sera tested positive for influenza nucleoprotein antibodies by ELISA. On 20th December, the CFIA confirmed the diagnosis of HPAI of the subtype H5N1.

Bird type	**Number on site**	**Number of oropharyngeal swabs**	**Number of cloacal swabs**	**Number of sera**
Turkey (*Meleagris gallopavo*)	4	4	4	4
Silkie chicken (*Gallus gallus*)	8	8	8	0
Emus (*Dromaius novaehollandiae*)	2	2	0	0
Peafowl (*Pavo cristatus*)	5	5	5	0
Domestic goose (*Anser anser*)	14	0	14	5
Domestic duck (*Anas platyrhynchos domesticus*)	20	0	20	7
Guineafowl (*Numida meleagris*)	0	0	0	0
Chicken-other (*Gallus gallus*)	4	4	4	1
Total	57	23	55	17

Table 1. List of samples for virological and serological analysis collected by CFIA on 17 December 2021 from different species of captive birds still present at the farm.

Wild birds were frequently observed co-mingling with the captive population. Captive birds except emus were allowed to move freely in and out of the open pens in which they were housed. At an on-site pond, domestic ducks were reported to mingle with free-living mallards (scientific names of wild birds in Table 2), and a snowy egret had been observed around 2nd to 6th December. Other wild birds reported on the farm were common starlings, feral pigeons, and unspecified gulls.

Retrospectively, HPAI H5N1 virus also was identified in tissues of a great black-backed gull found at a nearby pond in St. John's. The gull had been found ill on 26th November 2021 and taken to a local wildlife rehabilitation centre, where it died the following day.

Common name	**Scientific name**
Atlantic puffin	*Fratercula arctica*
Black-legged kittiwake	*Rissa tridactyla*
Barnacle goose	*Branta leucopsis*
Black-headed gull	*Chroicocephalus ridibundus*
Brunnich's guillemot	*Uria lomvia*
Common eider	*Somateria mollissima*
Common starling	*Sturnus vulgaris*
Eurasian teal	*Anas crecca*
Eurasian wigeon	*Mareca penelope*
Feral pigeon	*Columba livia domestica*
Great black-backed gull	*Larus marinus*
Great skua	*Stercorarius skua*
Greater white-fronted goose	*Anser albifrons*
Greylag goose	*Anser anser*
Lesser black-backed gull	*Larus fuscus*
Light-bellied brent goose	*Branta bernicla*
Mallard	*Anas platyrhynchos*
Northern fulmar	*Fulmarus glacialis*
Northern pintail	*Anas acuta*
Pink-footed goose	*Anser brachyrhynchus*
Purple sandpiper	*Calidris maritima*
Red knot	*Calidris canutus*
Snow goose	*Anser caerulescens*
Snowy egret	*Egretta thula*
Tufted duck	*Aythya fuligula*
Whooper swan	*Cygnus cygnus*

Table 2. Common and scientific species names of the birds mentioned in the text.

Phylogenetic analysis

Phylogenetic analyses were performed to compare the genome sequences of the Newfoundland viruses from the exhibition farm birds and a great black-backed gull found nearby to other influenza viruses in the database. Based on BLAST analysis all eight gene segments of the virus had a Eurasian origin, and the virus was identified as a clade 2.3.4.4b H5N1 virus. Based on maximum likelihood and time-resolved trees inferred by use of whole genome sequences, the Newfoundland viruses had a shared common ancestor with European viruses circulating in early 2021 (Figure 1, Figure 2). The dates for the most recent common ancestor (MRCA) of all gene segments ranged from December 2019 to April 2021 (Table 3). There was no evidence that the Newfoundland viruses were genetically closely related to other current or recent viruses circulating in Europe. In contrast to currently circulating European viruses, the sequences of the Newfoundland viruses had no evidence of reassortment with other avian influenza viruses after ancestral emergence (Figure 3). The virus from the great black-backed gull was highly similar to those from the exhibition farm, except for a small number of nucleotide differences in the neuraminidase (N) gene segment.

Analysis of avian migration and potential routes for HPAI H5 virus to be carried across the Atlantic with migrating birds

There are several pathways for HPAI H5N1 virus to be carried across the Atlantic with migrating birds, based on the multitude of migration routes of wild birds and their overlapping ranges at breeding, stop-over, and wintering sites. Ring-recovery data confirm the regular movements of wild birds from Europe to Iceland and other North Atlantic islands, and from there to North America, and also provide evidence for direct movements of for example seabirds and gulls (Supplementary Table 1). Ringed individuals with a European origin have been found on Newfoundland for 10 of the 24 species in Supplementary Table 1: barnacle goose (1 ringed individual), Eurasian wigeon (5), Eurasian teal (1), great skua (13), European herring gull (1), black-headed gull (1), black-legged kittiwake (102), purple sandpiper (1), Brunnich's guillemot (15), and Atlantic puffin (50). Given that the most likely ancestor of the virus detected in Newfoundland was circulating in Northwest Europe between the beginning of the 2020/2021 outbreak in Europe in October 2020 and April 2021 (see above), likely routes include spring migration of bird species moving to Icelandic, Greenlandic or Canadian High Arctic breeding grounds, or migration directly across the Atlantic Ocean (Figure 4).

Gene segment	Node date	Lower bound	Higher bound
PB2	13 February 2021	6 March 2017	24 April 2021
PB1	4 January 2021	30 August 2020	17 April 2021
PA	22 February 2021	12 September 2020	24 April 2021
NS	18 January 2021	5 April 2020	2 June 2021
NP	31 August 2020	25 August 2018	23 April 2021
NA	5 April 2021	27 February 2021	24 April 2021
HA	20 August 2021	17 June 2021	19 October 2021
MP	8 August 2021	18 April 2021	29 November 2021

Table 3. Dates for the most recent common ancestor (MRCA) of all gene segments.

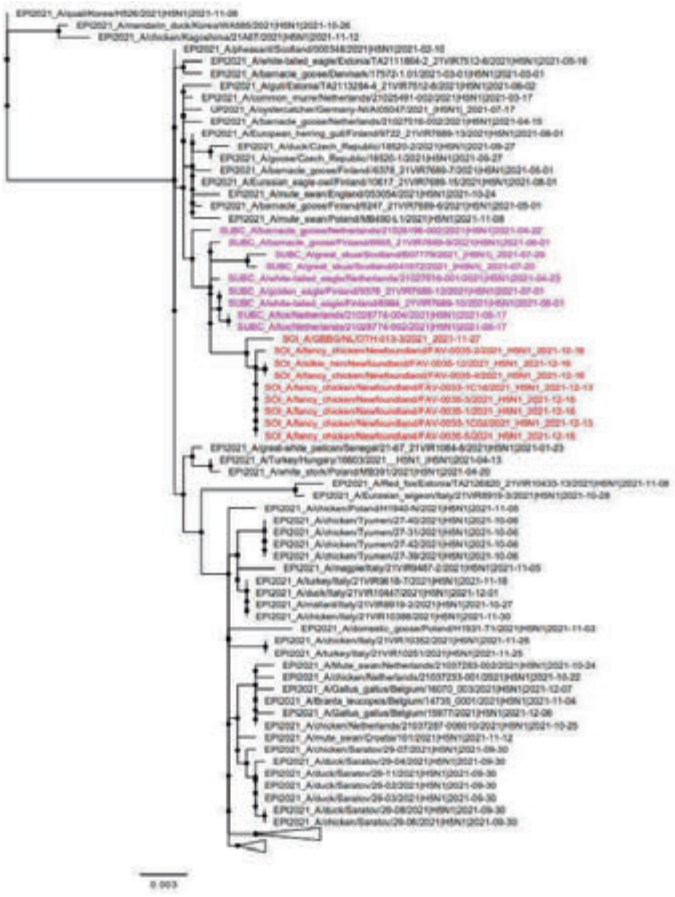
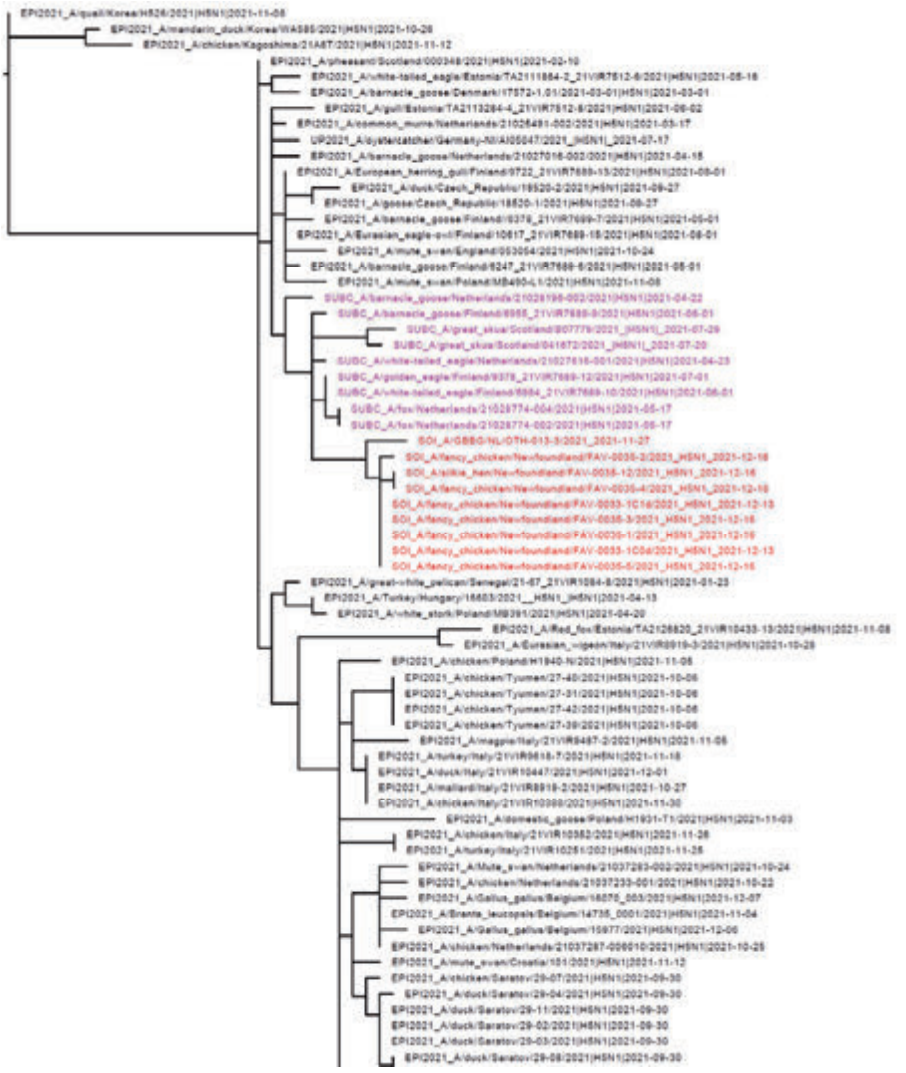


Figure 1. Maximum likelihood phylogenetic tree of the H5 HA gene. Relationships among the European 2021 H5 2.3.4.4b HPAI strains (magenta) and the Newfoundland wild bird and outbreak strains (red) are shown. The tree is rooted by the outgroup and nodes placed in descending order. Clades are collapsed for clarity.


EPI2021_A/quail/Korea/H826/2021|H5N1|2021-11-08
EPI2021_A/mandarin_duck/Korea/WA585/2021|H5N1|2021-10-26
EPI2021_A/chicken/Kagoshima/21A8T/2021|H5N1|2021-11-12
EPI2021_A/pheasant/Scotland/000348/2021|H5N1|2021-02-10
EPI2021_A/white-tailed_eagle/Estonia/TA2111884-2_21VIR7612-6/2021|H5N1|2021-08-16
EPI2021_A/barnacle_goose/Denmark/17572-1.01/2021-03-01|H5N1|2021-03-01
EPI2021_A/gull/Estonia/TA2113284-4_21VIR7612-8/2021|H5N1|2021-08-02
EPI2021_A/common_murre/Netherlands/21025491-002/2021|H5N1|2021-03-17
UP2021_A/oystercatcher/Germany-NI/AI05047/2021_|H5N1|_2021-07-17
EPI2021_A/barnacle_goose/Netherlands/21027016-002/2021|H5N1|2021-04-15
EPI2021_A/European_herring_gull/Finland/9722_21VIR7689-15/2021|H5N1|2021-08-01
EPI2021_A/duck/Czech_Republic/18520-2/2021|H5N1|2021-09-27
EPI2021_A/goose/Czech_Republic/18520-1/2021|H5N1|2021-09-27
EPI2021_A/barnacle_goose/Finland/9378_21VIR7689-7/2021|H5N1|2021-05-01
EPI2021_A/Eurasian_eagle-owl/Finland/10617_21VIR7689-15/2021|H5N1|2021-08-01
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SUBC_A/great_skua/Scotland/807779/2021_|H5N1|_2021-07-29
SUBC_A/great_skua/Scotland/041672/2021_|H5N1|_2021-07-29
SUBC_A/white-tailed_eagle/Netherlands/21027616-001/2021|H5N1|2021-04-23
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SUBC_A/white-tailed_eagle/Finland/9884_21VIR7689-10/2021|H5N1|2021-06-01
SUBC_A/fox/Netherlands/21028774-004/2021|H5N1|2021-05-17
SUBC_A/fox/Netherlands/21028774-002/2021|H5N1|2021-05-17
SOI_A/GBBG/NL/OTH-013-5/2021_|2021-11-27
SOI_A/fancy_chicken/Newfoundland/FAV-0035-3/2021_H5N1_2021-12-16
SOI_A/silkie_hen/Newfoundland/FAV-0035-12/2021_H5N1_2021-12-16
SOI_A/fancy_chicken/Newfoundland/FAV-0035-4/2021_H5N1_2021-12-16
SOI_A/fancy_chicken/Newfoundland/FAV-0033-1C1a/2021_H5N1_2021-12-13
SOI_A/fancy_chicken/Newfoundland/FAV-0035-3/2021_H5N1_2021-12-16
SOI_A/fancy_chicken/Newfoundland/FAV-0035-1/2021_H5N1_2021-12-16
SOI_A/fancy_chicken/Newfoundland/FAV-0033-1C5a/2021_H5N1_2021-12-13
SOI_A/fancy_chicken/Newfoundland/FAV-0035-5/2021_H5N1_2021-12-16
EPI2021_A/great-white_pelican/Senegal/21-67_21VIR1084-8/2021|H5N1|2021-01-23
EPI2021_A/Turkey/Hungary/18683/2021__H5N1_|H5N1|2021-04-13
EPI2021_A/white_stork/Poland/MB391/2021|H5N1|2021-04-20
EPI2021_A/Red_fox/Estonia/TA2126820_21VIR10433-13/2021|H5N1|2021-11-08
EPI2021_A/Eurasian_nigeon/Italy/21VIR8919-3/2021|H5N1|2021-10-28
EPI2021_A/chicken/Poland/H1940-N/2021|H5N1|2021-11-05
EPI2021_A/chicken/Tyumen/27-40/2021|H5N1|2021-10-06
EPI2021_A/chicken/Tyumen/27-31/2021|H5N1|2021-10-06
EPI2021_A/chicken/Tyumen/27-42/2021|H5N1|2021-10-06
EPI2021_A/chicken/Tyumen/27-39/2021|H5N1|2021-10-06
EPI2021_A/magpie/Italy/21VIR9487-2/2021|H5N1|2021-11-08
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EPI2021_A/turkey/Italy/21VIR10251/2021|H5N1|2021-11-25
EPI2021_A/Mute_swan/Netherlands/21037283-002/2021|H5N1|2021-10-24
EPI2021_A/chicken/Netherlands/21037233-001/2021|H5N1|2021-10-22
EPI2021_A/Gallus_gallus/Belgium/18075_003/2021|H5N1|2021-12-07
EPI2021_A/Branta_leucopsis/Belgium/14735_0001/2021|H5N1|2021-11-04
EPI2021_A/Gallus_gallus/Belgium/15977/2021|H5N1|2021-12-06
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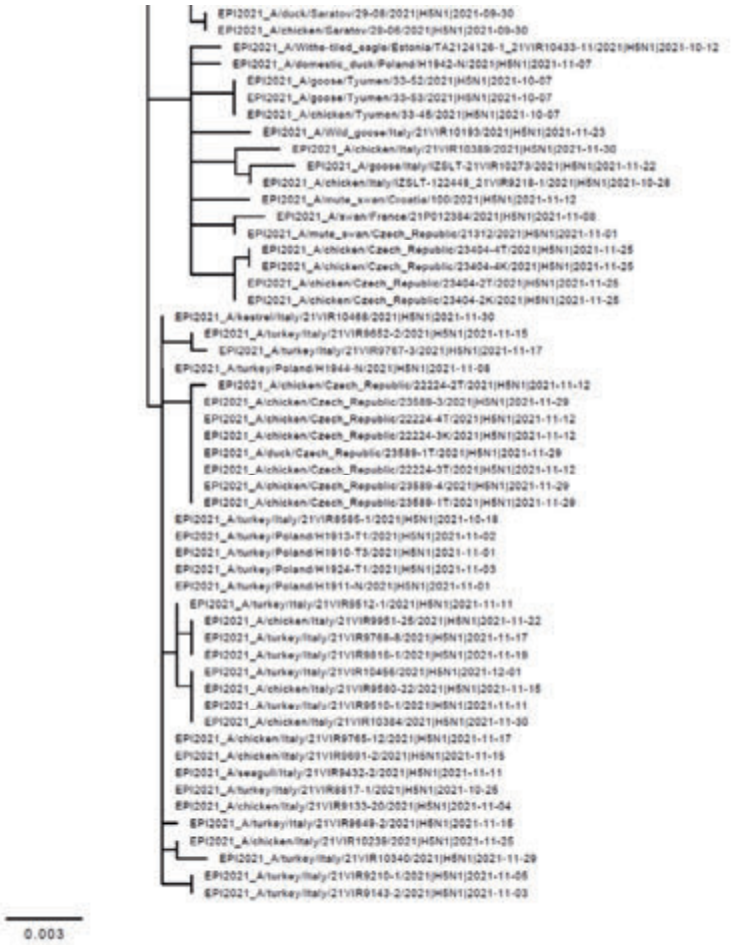
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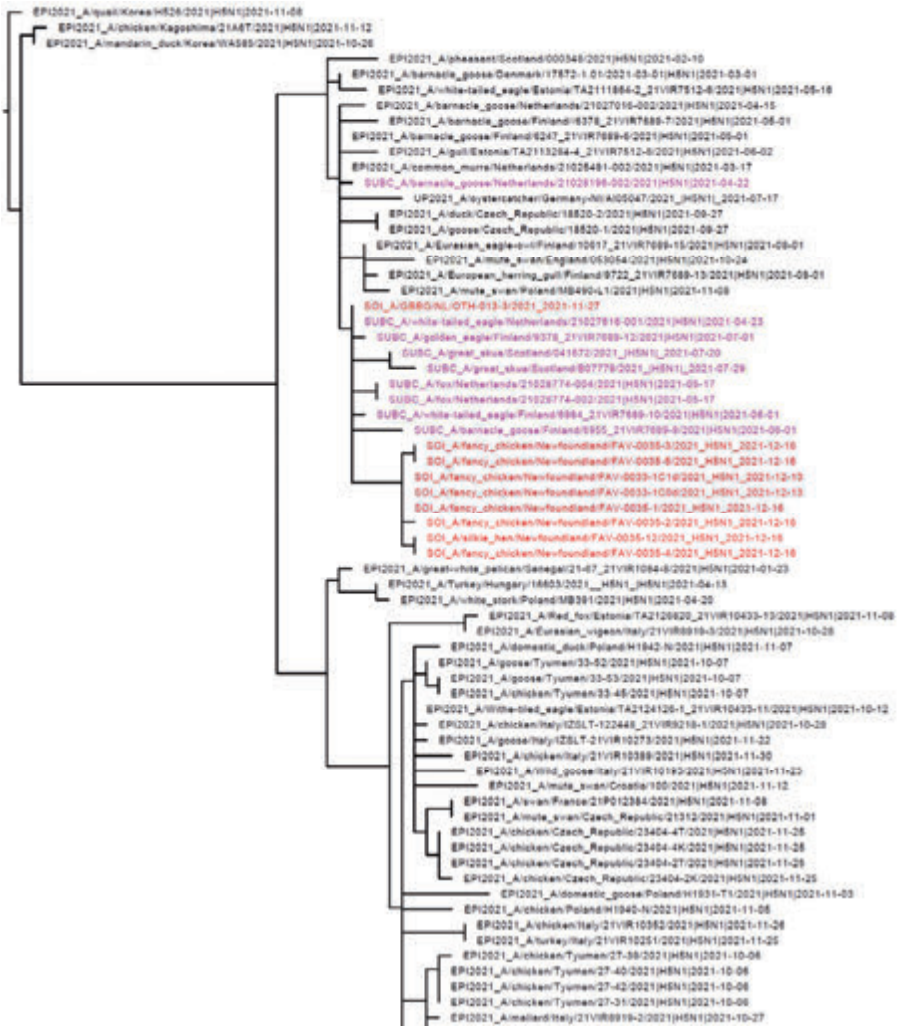
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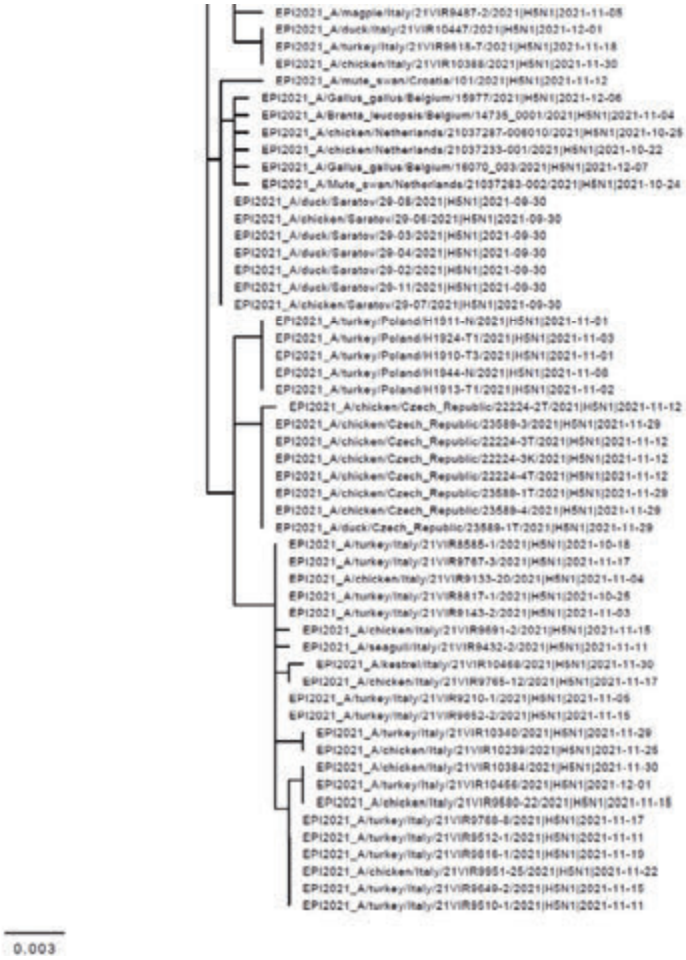
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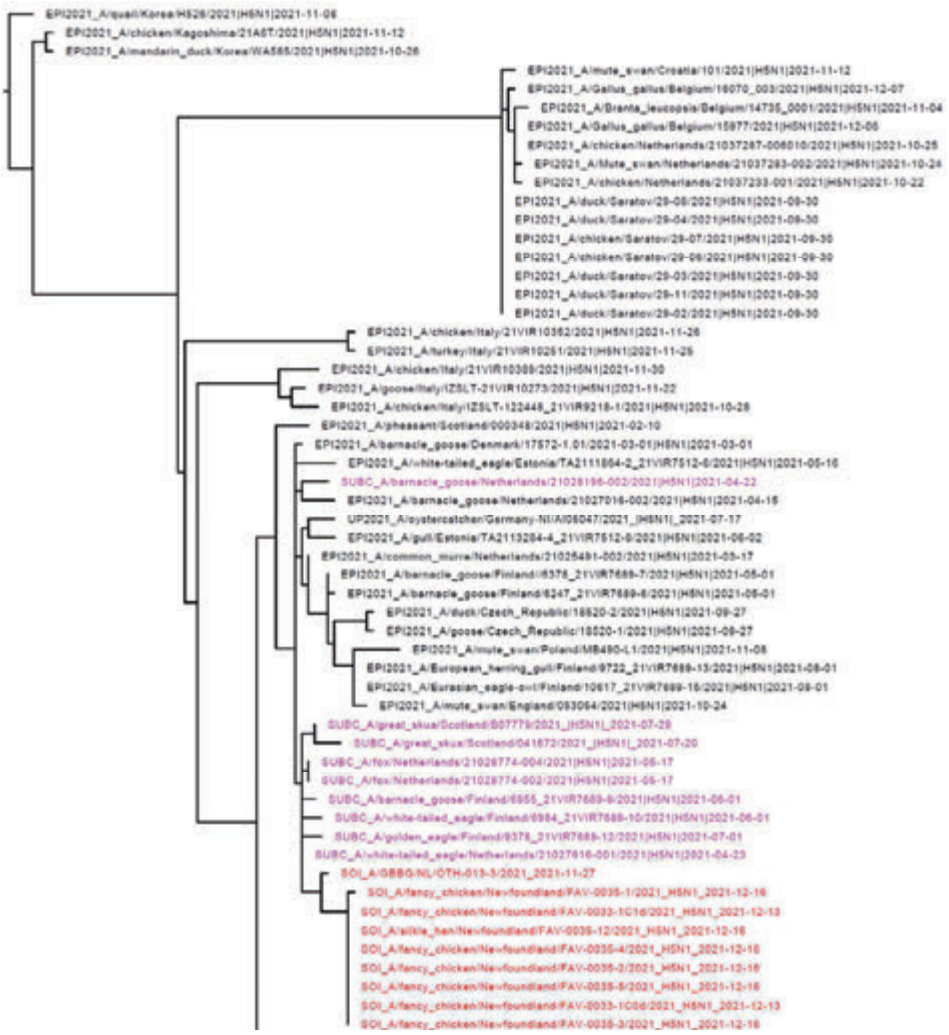
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EPI2021_A/gull/Estonia/TA2113264-4_21VIR7512-9/2021|H5N1|2021-06-02
EPI2021_A/common_murre/Netherlands/21025491-002/2021|H5N1|2021-05-17
EPI2021_A/barnacle_goose/Finland/6378_21VIR7689-7/2021|H5N1|2021-06-01
EPI2021_A/barnacle_goose/Finland/6247_21VIR7689-6/2021|H5N1|2021-06-01
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EPI2021_A/goose/Czech_Republic/18620-1/2021|H5N1|2021-09-27
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SUBC_A/fox/Netherlands/21028774-004/2021|H5N1|2021-05-17
SUBC_A/fox/Netherlands/21028774-002/2021|H5N1|2021-05-17
SUBC_A/barnacle_goose/Finland/6955_21VIR7689-9/2021|H5N1|2021-06-01
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SUBC_A/golden_eagle/Finland/9378_21VIR7689-12/2021|H5N1|2021-07-01
SUBC_A/white-tailed_eagle/Netherlands/21027616-001/2021|H5N1|2021-04-23
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EPI2021_A/turkey/Italy/21VIR9649-2/2021|H5N1|2021-11-16
EPI2021_A/seagull/Italy/21VIR9432-2/2021|H5N1|2021-11-11
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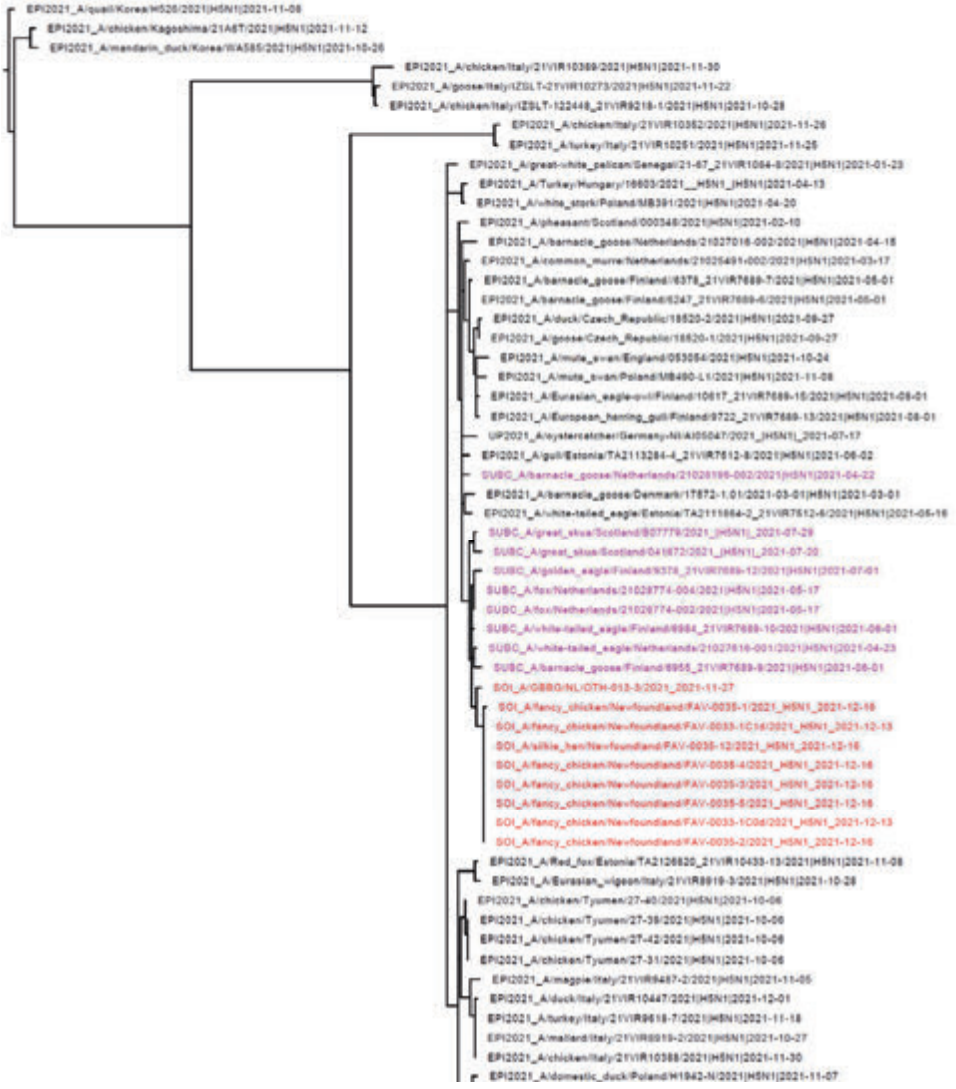
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EPI2021_A/turkey/Italy/21VIR9618-7/2021|H5N1|2021-11-18
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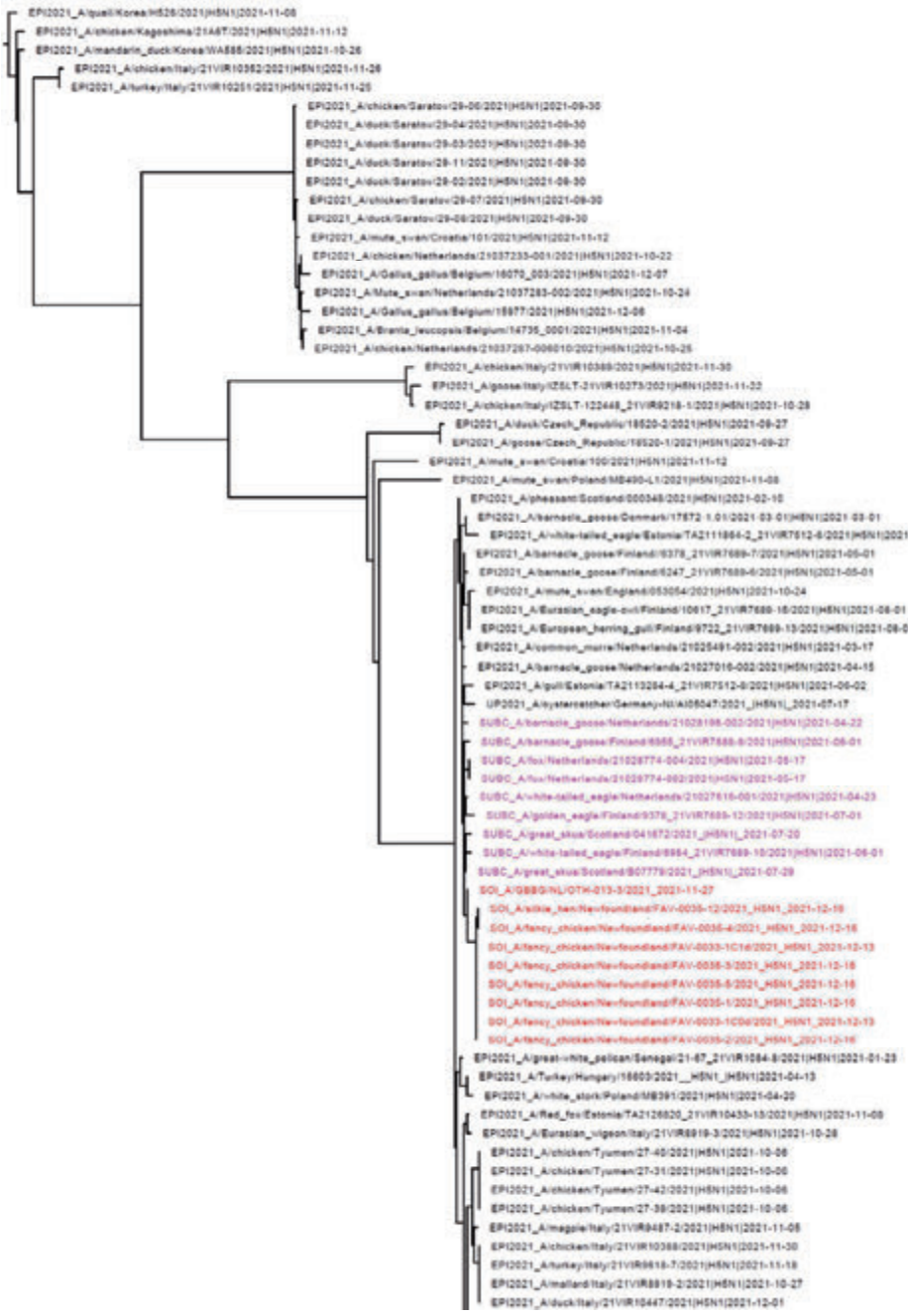
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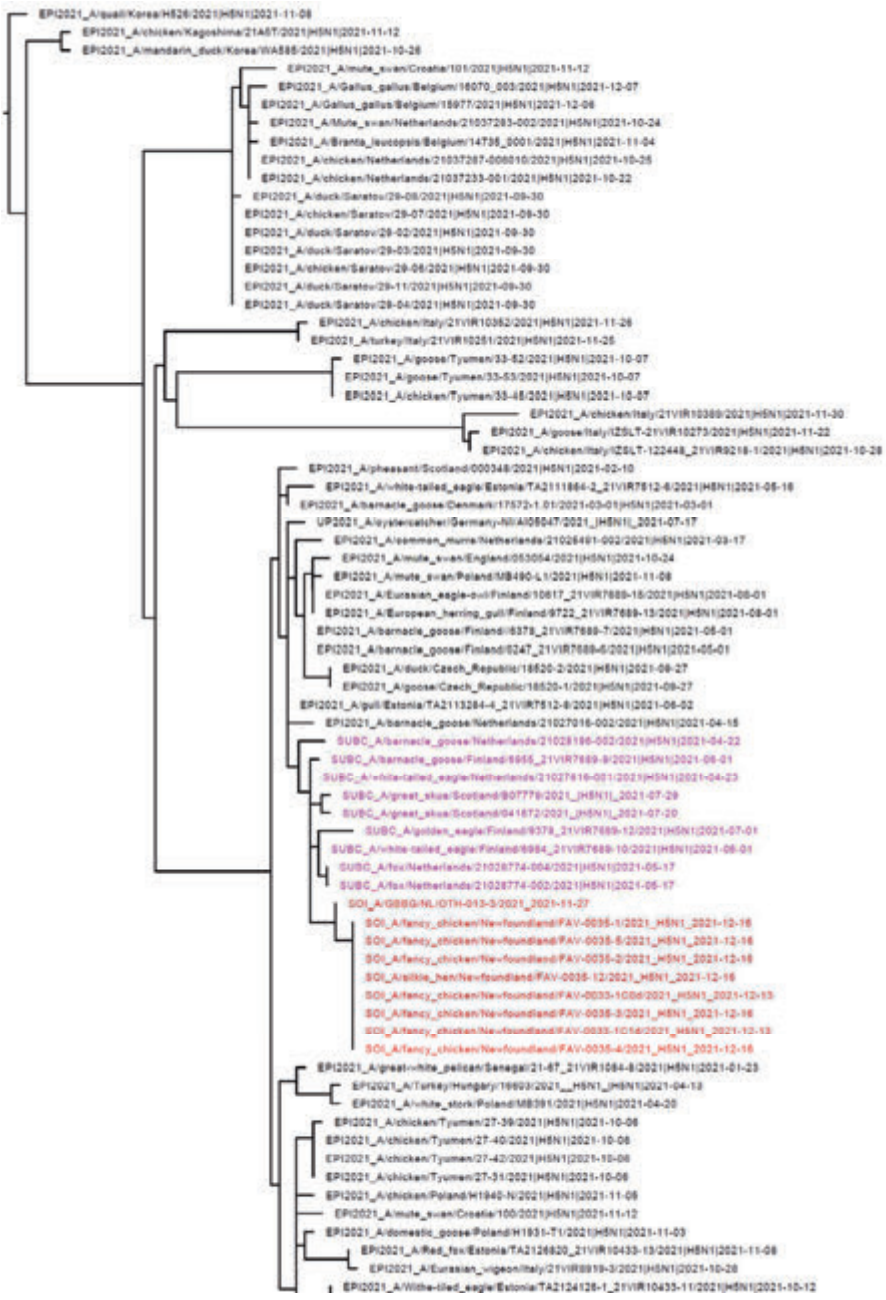
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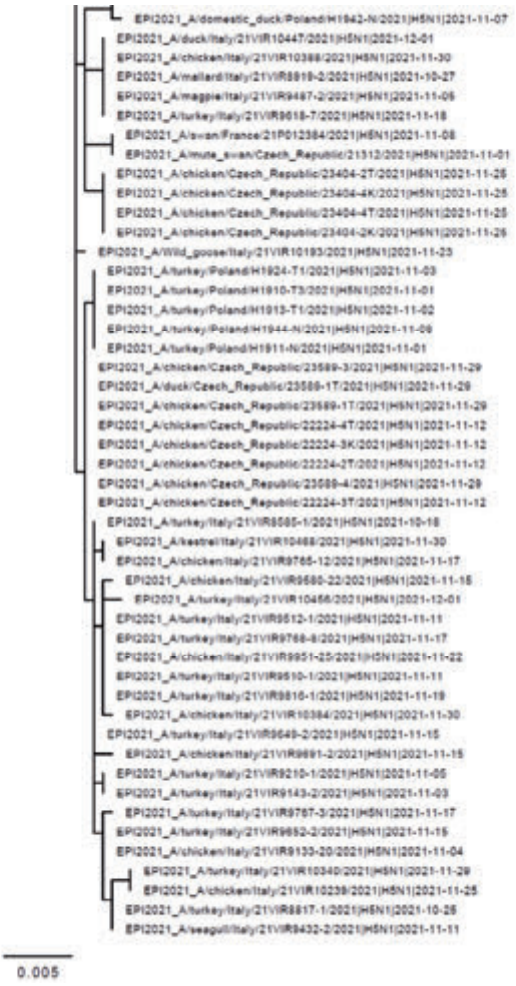
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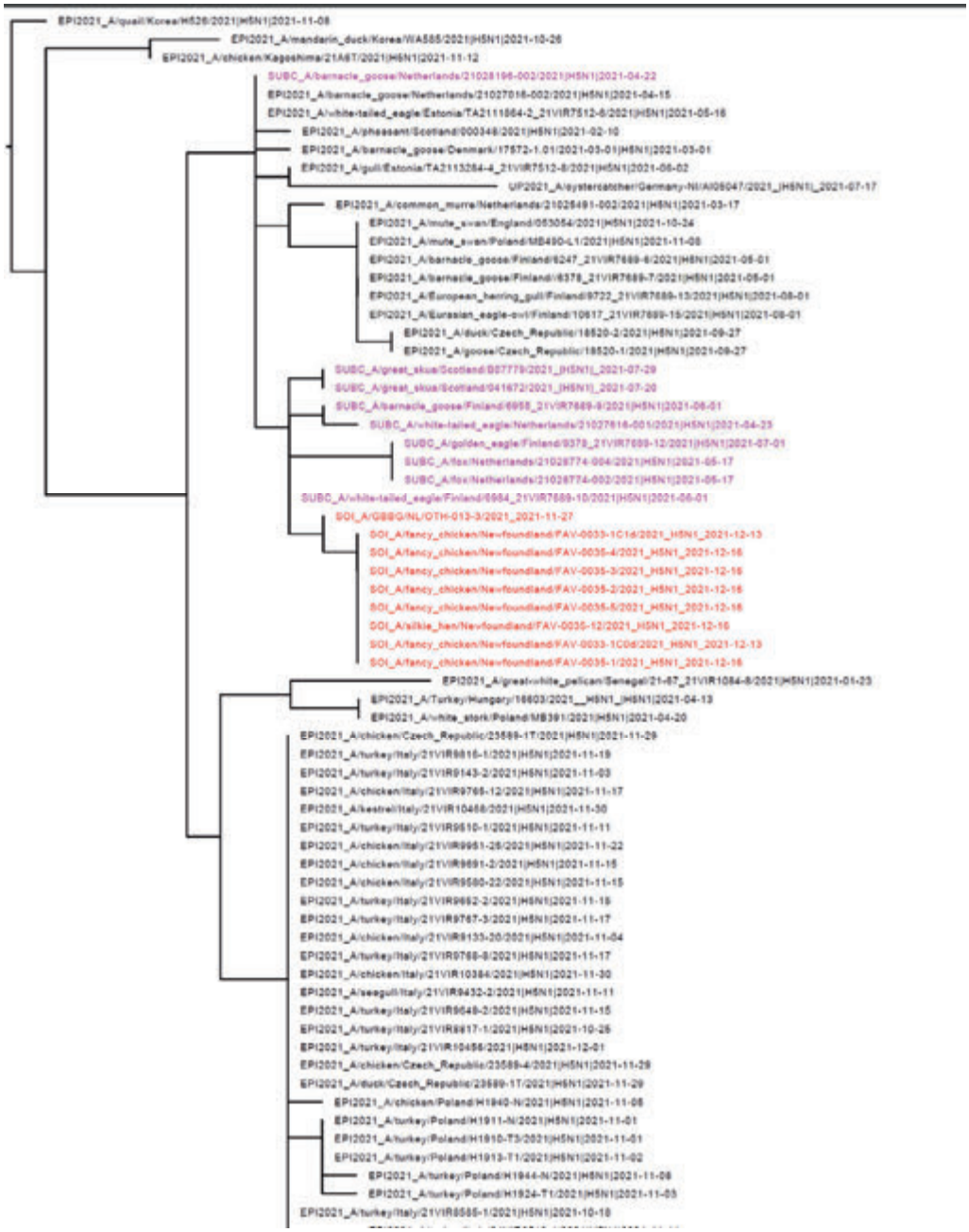
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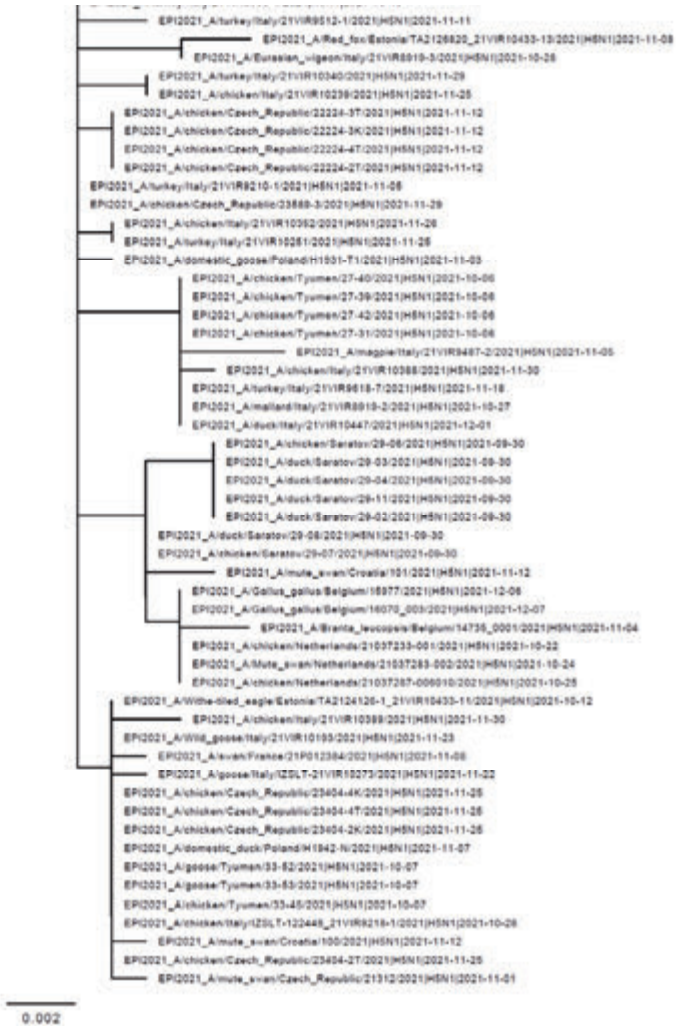
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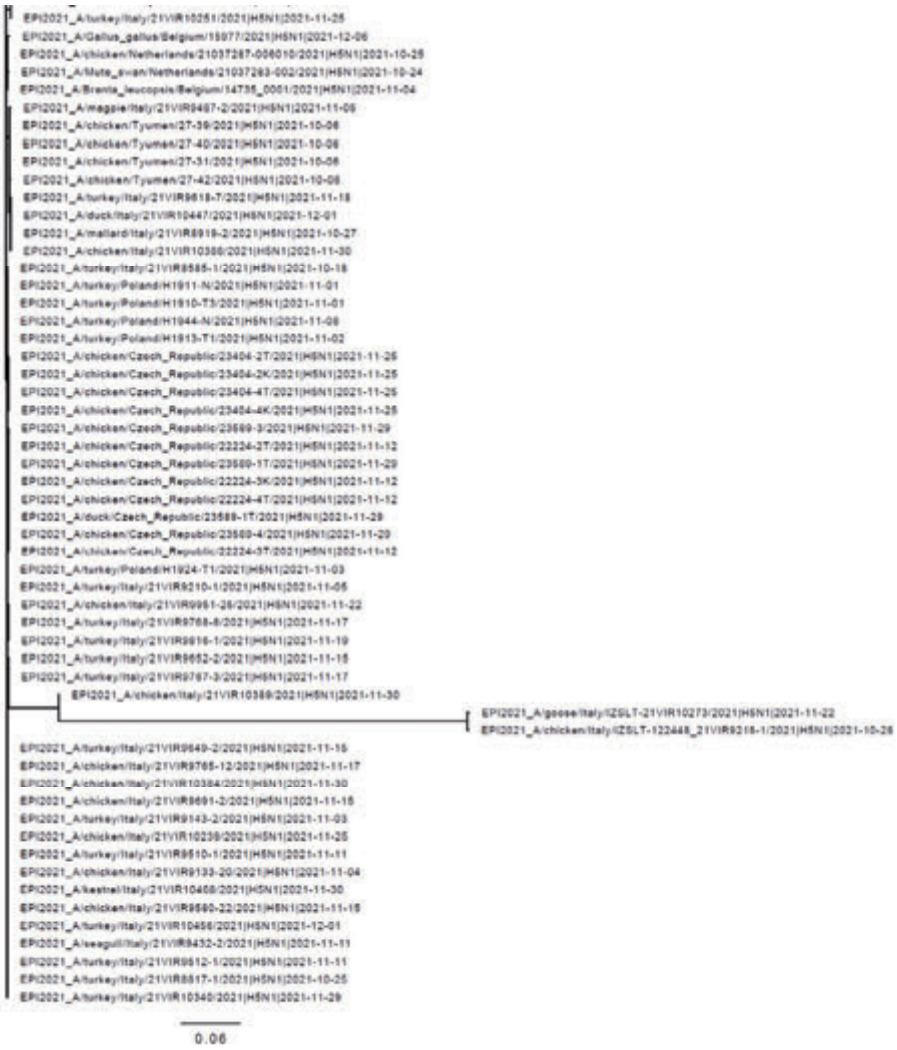
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EPI2021_A/chicken/Czech_Republic/23404-4T/2021|H5N1|2021-11-26
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EPI2021_A/turkey/Italy/21VIR9768-8/2021|H5N1|2021-11-17
EPI2021_A/turkey/Italy/21VIR9916-1/2021|H5N1|2021-11-19
EPI2021_A/turkey/Italy/21VIR9652-2/2021|H5N1|2021-11-15
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EPI2021_A/chicken/Italy/21VIR10389/2021|H5N1|2021-11-30
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EPI2021_A/chicken/Italy/21VIR9691-2/2021|H5N1|2021-11-16
EPI2021_A/turkey/Italy/21VIR9143-2/2021|H5N1|2021-11-03
EPI2021_A/chicken/Italy/21VIR10239/2021|H5N1|2021-11-25
EPI2021_A/turkey/Italy/21VIR9510-1/2021|H5N1|2021-11-11
EPI2021_A/chicken/Italy/21VIR9133-20/2021|H5N1|2021-11-04
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EPI2021_A/chicken/Italy/21VIR9690-22/2021|H5N1|2021-11-16
EPI2021_A/turkey/Italy/21VIR10456/2021|H5N1|2021-12-01
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EPI2021_A/turkey/Italy/21VIR8817-1/2021|H5N1|2021-10-25
EPI2021_A/turkey/Italy/21VIR10340/2021|H5N1|2021-11-29
0.06

Figure 2. Maximum likelihood phylogenetic tree of the H5 gene segments. Relationships among the European 2021 H5 2.3.4.4b HPAI strains (magenta) and the Newfoundland wild bird and outbreak strains (red) are shown. The tree is rooted by the outgroup and nodes placed in descending order; order: HA, NA, PA, PB1, PB2, NP, MP, NS.

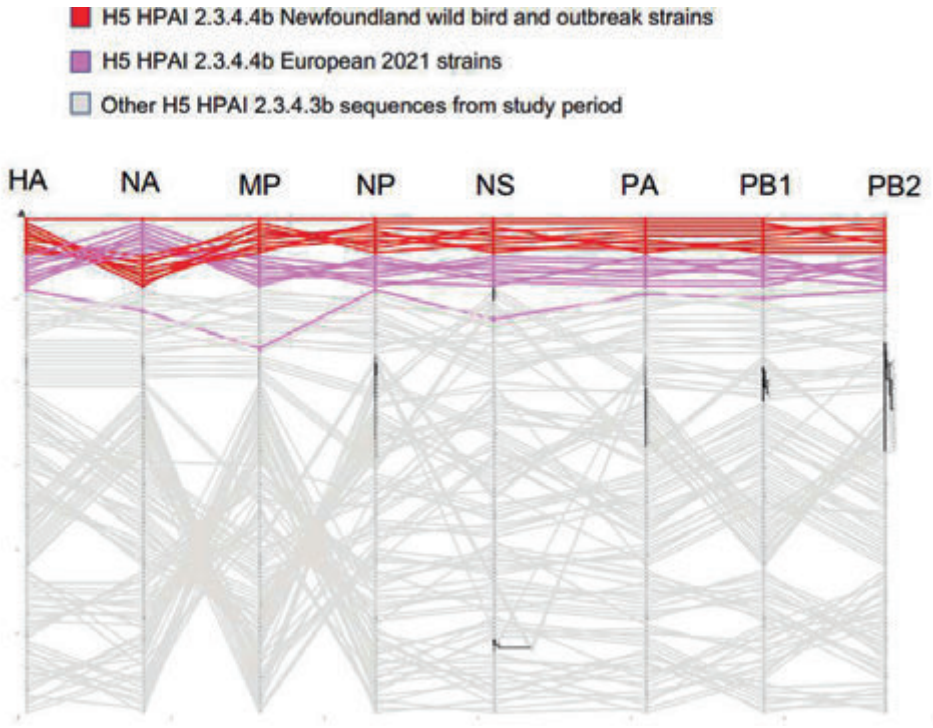

Figure 3. Phylogenetic incongruence analyses. Maximum likelihood trees for the H and N gene segments and internal gene segments from equivalent strains were connected across the trees. Tips and connecting lines are coloured according to the legend.

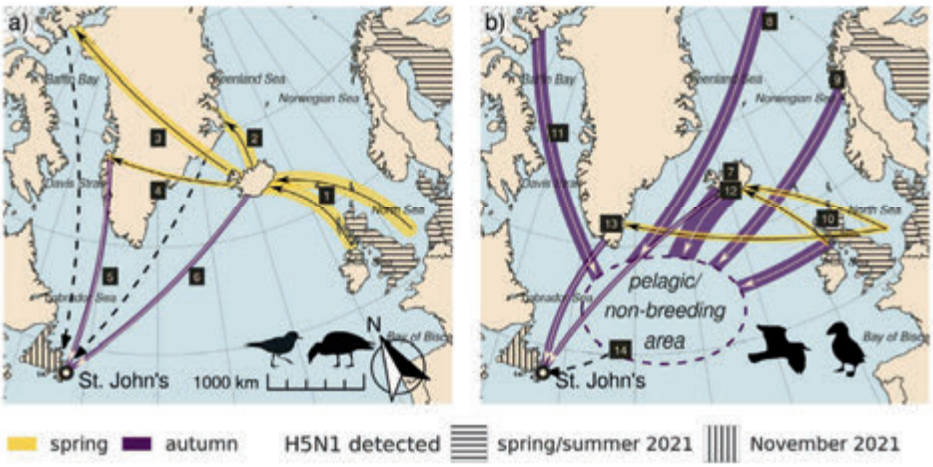

Figure 4. Maps of transatlantic migration. Putative virus transmission pathways between Europe and Newfoundland via migratory waterfowl/shorebirds (a) and pelagic seabirds (b). Many Icelandic waterfowl and shorebirds (panel a) winter in Northwest Europe and return to Iceland to breed in spring (1), including whooper swans, greylag geese, pink-footed geese, Eurasian wigeons, Eurasian teals, northern pintails, common ringed plovers and purple sandpipers. Some bird populations use Iceland as a stopover site, and continue to breeding grounds in East Greenland (2; barnacle geese and pink-footed geese), the East Canadian Arctic (3; light-bellied brent geese, red knots, ruddy turnstones) and West Greenland (4; greater white-fronted geese). Migratory birds from Europe share these breeding areas with species that winter in North America, including Canada geese and snow geese from East Greenland and the East Canadian Arctic (5), and some Iceland-breeding species of duck, including small numbers of Eurasian wigeons, Eurasian teals, and tufted ducks (6). Several seabird species (panel b), such as gulls, skuas, fulmars and auks, have large breeding ranges in the Arctic. After the breeding season many species become fully pelagic and can roam large parts of the northern Atlantic. The mid-Atlantic ridge outside Newfoundland is an important non-breeding area for seabirds, and is frequented by auks from Iceland (7), Svalbard (8) and Norway (9), including large numbers of Atlantic puffins and Brünnich guillemots, and by black-legged kittiwakes and northern fulmars originating from Iceland, Norway and the United Kingdom (7-8, 10). There these birds are joined by seabirds from Canadian and Greenlandic waters (11). Direct migratory links to Newfoundland occurs through greater and lesser-black backed gulls as well as black-headed gulls from Iceland and Greenland (12, 13), and gulls also link the pelagic and the coastal zone around Newfoundland (14). Thickness of the lines highlights the relative approximate population sizes. Dashed lines show where small numbers of individuals, or vagrants, provide a potential pathway. For more details on species and population numbers see Table 2.

Figure 4 was prepared using the software R (version 4.0.5, https://www.r-project.org/) and the following packages: - ggplot2 (version 3.3.5, https://cran.r-project.org/web/packages/ggplot2/index.html), - sf (version 1.0.5, https://cran.r-project.org/web/packages/sf/index.html).

The first possible route via Iceland seems to be the strongest link between Newfoundland and Europe (14-17), because it is a meeting point of breeding bird populations which winter in Europe as well as along the East coast of North America. Numerous species, totaling almost two million individual birds, migrate annually from northwestern Europe to breeding grounds in Iceland and beyond. Several populations breed on Iceland, including swans (whooper swan) (Supplementary Table 1), geese (greylag goose, pink-footed goose), ducks (Eurasian wigeon, Eurasian teal, Northern pintail), gulls (great- and lesser black-backed gull, black-headed gull, black-legged kittiwake) and shorebirds (common ringed plover, purple sandpiper, Supplementary Table 1). In addition, several species (e.g. barnacle geese and pink-footed geese) migrating to breeding grounds further away (Greenland and/or Canadian High Arctic) make spring and autumn stopovers in Iceland, (18,19). This creates potential for the virus to have been spread northwards to Iceland (or further northward) in spring, where it could have circulated among breeding birds, or transmitted during autumn migration by species returning from the Arctic. Several Iceland-breeding species of ducks (Eurasian wigeon, Eurasian teal, tufted duck), gulls (lesser black-backed gull, black-legged kittiwake, black-headed gull) and alcids (Brunnich's guillemot, Atlantic puffin) winter along the Atlantic coast of North America in variable numbers (Supplementary Table 1). If the virus was transmitted to one of these populations during their stay on Iceland, it could have been spread to Newfoundland during the subsequent autumn migration. Importantly, Iceland-breeding Eurasian wigeons or Eurasian teals could be responsible for both the journey to Iceland from European wintering grounds, as well as the journey from Iceland to Newfoundland, where these species are frequently encountered as vagrants (Supplementary Table 1) (20, 21).

The second possible route is via species that migrate from northwestern Europe to the Canadian High Arctic and/or Northwest Greenland. These include shorebirds (e.g. ruddy turnstone, red knot) and some geese (light-bellied brent goose and greater white-fronted goose). If the virus circulated in these breeding populations and then moved to other coastal marine bird populations bordering Baffin Bay, which include huge numbers of colonial seabirds and marine waterfowl (22, 23), the virus could have followed a coastal or even pelagic route south with the large autumn migration of Arctic marine birds (various sea ducks, auks and larids) (24,25) to emerge in Newfoundland. Alternatively, shorebirds and waterfowl may have played a role: several European-wintering populations have overlapping breeding grounds with populations wintering along the east coast of North America. Regarding geese, greater white-fronted geese share breeding grounds in western Greenland with Canada geese (26,27), which migrate south along the Canadian Atlantic coast. Also, brent geese have overlapping breeding grounds with snow geese (18). In addition, individual barnacle geese and pink-footed geese breeding in Greenland could also have travelled south to Newfoundland carrying the virus, as these birds are regular vagrants to the North American Atlantic coast (28). While geese occur only in small numbers on Newfoundland, two barnacle geese and four pink-footed geese, probably originating from Greenland breeding grounds, were observed in the autumn of 2021. St. John's is the first major population center on a coastal route south from Baffin Bay/Davis Strait and along the Labrador Shelf, so emergence in eastern Newfoundland is consistent with this route.

Three wild bird species involved in the Iceland and/or Greenland/High Canadian Arctic routes deserve particular attention. Eurasian wigeon have been prominently involved in

outbreaks in Eurasia, and are considered prime candidates for carrying HPAI virus over long distances (29). Also, during the first stages of an outbreak they are one of the first species to be detected HPAI virus positive, often without clinical signs. Barnacle geese and greylag geese, which congregate in Iceland, were in the top three most abundant species detected H5-positive in Europe in late winter and early spring 2021 (5). Given that both greylag and barnacle geese have populations breeding on Iceland/Greenland and wintering in Europe (particularly the UK), these two species are high on the list of probable vectors that transported the virus to Iceland/Greenland and finally to Newfoundland. The high involvement of infected geese in the HPAI dynamics, which was not seen before October 2020, together with the unusually high levels of HPAI H5 virus presence in wild birds in Northwest Europe in spring 2021, might also explain why HPAI H5 virus spread to Newfoundland this winter (2021/2022), and not in the previous winters (2020/2021, 2016/2017, 2014/2015, 2005/2006). It is, however, striking that no cases of HPAI H5 virus have been recorded on Iceland in 2021.

A third possible, pelagic, route is directly across the Atlantic Ocean. Such a route could have started with coastal and pelagic seabirds in Northwest Europe, where the virus may have remained undetected for much of the summer of 2021. A subsequent migration of seabirds to Newfoundland waters in the autumn of 2021 could have brought the virus to North America. The large populations of black-legged kittiwakes and northern fulmars that breed in the U.K. have long been known to frequent Newfoundland waters (30), and these movements have been corroborated by recent telemetry studies (31). Further, recent telemetry information reveals that millions of pelagic seabirds breeding all across the Atlantic congregate over the Mid-Atlantic Ridge in the central North Atlantic at all times of year (32), making a pelagic transmission route a possibility. From the pelagic wintering grounds off Newfoundland, a species that uses both pelagic and coastal habitats, possibly a gull, may have brought the virus to shore in St. John's. Trans-Atlantic transmission via seabirds has been suggested for LPAI viruses, including detection of mosaic Eurasian-North American viruses in gulls and alcids (12, 33-35).

For the time period and geographical frame considered, HPAI-H5-positive species included ducks (Eurasian wigeon, mallard, common eider), geese (barnacle, greylag, brent, pink-footed and greater white-fronted goose), swans (whooper), gulls (black-headed, herring, lesser black-backed, great black-backed), and shorebirds (red knot, ruddy turnstone) (Supplementary Table 2). Of these 15 species, ringed individuals with a European origin have been recorded on Newfoundland for barnacle goose (1 ringed individual), Eurasian wigeon (5), great skua (13), and black-headed gull (1) (Supplementary Table 1). Ringed individuals with a European origin have been found on Newfoundland for 5 species which were found to be HPAI-H5-positive between October 2020 and April 2021, such as Barnacle Goose (1), Eurasian Wigeon (5), Great Skua (13), Black-headed Gull (1). These species might be considered to be possible carriers of HPAI H5 virus from Europe in late winter 2020/2021 or early spring 2021 partly or all the way to Newfoundland. However, given the incompleteness of sampling and the possibility of wild birds carrying HPAI virus subclinically, the involvement of other wild bird species in transatlantic virus transport cannot be ruled out.

Having reached the Avalon Peninsula of Newfoundland via one of above routes, the virus may have spread further within the abundant local populations of ducks and gulls wintering

in the city of St. John's. The peridomestic populations of some of these species may be candidates for incursion of the virus into the farm in St John's.

Conclusions

In conclusion, the HPAI H5N1 viruses that were detected in Newfoundland in November and December 2021 originated from Northwest Europe and belonged to HPAI clade 2.3.4.4b. Most likely, these viruses emerged in Northwest Europe in winter 2020/2021, dispersed from Europe in late winter or early spring 2021, and arrived in Newfoundland in autumn 2021. The viruses may have been carried across the Atlantic by migratory birds using different routes, including Icelandic, Greenland/Arctic, or pelagic routes. The unusually high presence of the viruses in European wild bird populations in late winter and spring 2021, as well as the greater involvement of barnacle and greylag geese in the epidemiology of HPAI in Europe since October 2020, may explain why spread to Newfoundland happened this winter (2021/2022), and not in the previous winters.

The incursion of these HPAI viruses, which appear to be well-adapted to certain wild birds, raised concern at its first detection about the potential of HPAI virus to become established and spread in the Americas via wild birds (OFFLU reference). The concern was that if these viruses become established in the Atlantic Flyway, they could rapidly spread west to Mississippi, Central and Pacific Flyways. The implication of this scenario would be high wild bird mortality, risk for incursion into poultry holdings and those of other captive birds, as well as zoonotic risk. In fact, as of 4 April 2022, this virus has now been detected in wild birds in three flyways in North America and disease in commercial and/or backyard poultry flocks has been detected in 3 Canadian provinces and 24 US states (https://www.usgs.gov/centers/nwhc/science/distribution-highly-pathogenic-avian-influenza-north-america-20212022). Large scale mortality events have not been reported in wild birds, with most positive wild birds detected via testing of hunter-collected birds or testing of individual dead birds (https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-wild-birds).

To prevent and mitigate the risk of viral spread, it will be vital to further increase surveillance of wild birds in North America and South America, as well as at migration stop-over stations in Iceland and Greenland. This should include virus detection with whole genome sequencing to enable molecular epidemiology. Collecting wild bird mortality reports can give an idea of the impact of the outbreak on local wild bird populations, and active surveillance is critical to identify vector species. The overlap of migratory movements of wild waterbirds along the Atlantic coast of North America with densely populated poultry areas may increase the risk of viral incursion into poultry farms, emphasizing the need for appropriate biosecurity measures and spatial planning of the poultry sector. The spread of HPAI H5 viruses from Europe to North America stresses the importance of close international cooperation and data exchange to better understand the global epidemiology of avian influenza, e.g. by swiftly identifying where these viruses emerged from and mitigating endemic disease in poultry to avoid these production systems potentially acting as future sources for emerging variants (35) , and is a call to re-assess the poultry sector in a way that embraces the One Health perspective: to sustainably balance and optimize the health of people, animals and ecosystems (https://www.who.int/groups/one-health-high-level-expert-panel).

Materials and methods

Phylogenetic analysis

Methods for phylogenetic analysis were the same as Sagulenko 2018 and Poen 2019 (36,37).

Full genome sequences were obtained from nine clinical or postmortem samples of captive birds at the exhibition farm, and from one postmortem sample of a great black-backed gull from a nearby city pond.

We searched for H5NX whole genome sequences in GISAID from Europe, Asia and Africa where samples were collected from 01-01-2021 through 27-12-2021. To these existing data we added eight unpublished sequences from Newfoundland, and three additional unpublished sequences from European wild birds collected in the timeframe to the GISAID database.

We aligned the sequences using MAFFT v7.407 and trimmed to the starting ATG and ending STOP codon. Maximum-likelihood trees were inferred using IQ-TREE 2.1.3. and 1000 replicates for the Shimodaira–Hasegawa approximate likelihood ratio test. We used TreeTime, a Python-based framework for phylodynamic analysis using an approximate Maximum Likelihood approach to estimate ancestral states, and reroot trees to maximize temporal signals.

Analysis of avian migration

We evaluated the possible routes along which wild birds can migrate from Europe to North America, based on knowledge on existing migration routes as well as the retrieval of identification (bird) rings. We compared the information with the data of HPAI H5 virus-positive birds from Northwest European countries (i.e., UK, Ireland, Norway, Finland, Denmark, Germany, Netherlands, Belgium, France) that are the starting points, or are situated along these migratory routes. For the analysis, we prioritized the most abundant bird species, that also most frequently tested H5-positive during the 2020/2021 outbreak in Europe, as detected during active and passive surveillance (38).

We focused on bird species susceptible to avian influenza (waterfowl, gulls, shorebirds and seabirds) which either bred or made a migratory stopover on Iceland, this being the most likely connection between Europe and Newfoundland. We identified wintering grounds, staging sites and breeding grounds based on literature, using mostly the database of Birds of the World (39, 40). We estimated the population sizes breeding in Iceland or passing through Iceland during migration using Fox & Leafloor (2018), Icelandic Institute of Natural History (2021) and van Roomen (2018) (40-42).

We provided the number of individuals observed in Newfoundland from Ebird data (19). We extracted all observations from complete lists done between September – December, 2011 to 2021 on Newfoundland. For rare species (with less than 10 records annually) we also included sightings from incomplete lists. For every year and species, we calculated the maximum number of observed individuals per location, and added these to calculate the total number of individuals observed in Newfoundland for every year. We then calculated the average number of individuals observed annually between 2011 – 2021, and the number observed in 2021. We identified the most likely origin of birds encountered in Newfoundland using the database Birds of the World, (39-43).

Ring-recovery data were obtained from the EURING Migration mapping Tool MMT, an online tool under development, that provides information on movements of ringed birds between pre-set areas within Europe and to other areas of the world, based on the EURING databank. These data were augmented with published (individuals recovered up to 2002, Lyngs 2003), (12-16) and unpublished data (to 2011) of birds ringed in Greenland supplied by Copenhagen Bird Ringing Centre. All records of individual birds moving between Northwest Europe (Norway & Sweden, Germany & Denmark, Belgium & Netherlands, Great Britain & Ireland) and Iceland and Faroe Islands, or Svalbard and other North Atlantic islands or Greenland, and individual birds moving between these areas and Canada or USA were selected. Prior to selection, unlikely records (finding date before ringing date, finding or ringing location not accurate etc.) were removed. For species not considered in the Migration Mapping Tool, only records of birds moving between Northwest Europe and Greenland, Canada or USA were available.

To further evaluate which wild bird species might have been involved in transatlantic transport of HPAI H5 virus, we compared above bird migration patterns with reports of HPAI-H5-positive wild birds in Europe. We limited our evaluation to the period of six months up to April 2021, the latest MRCA date of the Newfoundland virus gene segments (see above), and to the coastal countries in Northwest Europe, which act as the main wintering areas for wild birds that migrate across the Atlantic.

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Chapter 3.3

Highly Pathogenic Avian Influenza Virus (H5N8) Outbreak In a Wild Bird Rescue Center, The Netherlands, consequences and recommendations

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Abstract: Since the emergence of the Goose/Guangdong H5 lineage in 1996 and spillover of highly pathogenic avian influenza (HPAI) from poultry to wild birds, outbreaks have become increasingly frequent in wild birds. The latest outbreak in the Netherlands occurred in the fall-winter of 2020-2021 and was linked to incursions of HPAI H5N8 virus. During the larger national outbreak, wild birds in rehabilitation center "Vogelklas Karel Schot (VKS)" in Rotterdam presented with clinical signs compatible with HPAI, including head shaking, torticollis and abnormal gait. During an epidemiological investigation at VKS, water samples from the pools in the enclosures and oropharyngeal and cloacal swabs from 128 birds of different species were analyzed for the presence of H5N8 virus. Forty-five birds and the pool water tested positive for the virus. The outbreak at VKS was likely introduced by one or more infected geese (*Anser anser, Anser anser domesticus, Branta leucopsis*), after which the virus spread via pool water and with the relocation of infected birds within the center. In principle, such outbreaks are preventable. We here report recent updates about HPAI to provide guidance to help avoid future incursions of HPAI into wildlife rescue centers.

Introduction

Wild birds, and waterfowl in particular, have co-evolved with low pathogenic avian influenza viruses, with the virus remaining largely nonvirulent for its host (1). However, since the emergence of the Goose/Guangdong H5 lineage in 1996 and repeated spillover of viruses of this lineage from poultry to wild birds, highly pathogenic avian influenza (HPAI) outbreaks are becoming increasingly frequent in wild birds (2, 6, 12-15). From the spring of 2020, HPAI H5N8 has been reported in poultry separately in the Republic of Iraq (May 2020), in the Russian Federation (July 2020), and in Kazakhstan (September 2020) (5,13). In the Netherlands, H5 HPAI virus was detected on 16 October 2020 in nine Eurasian wigeons (*Mareca penelope*). Since 23 October 2020 a national housing order was imposed by the Dutch Minister of Agriculture, Nature and Food Quality for captive birds, including those in commercial and hobby farms, zoos, and wildlife rehabilitation centres.

Wild bird rescue centers recover sick and injured wild birds and may then rehabilitate the birds and release them back to the wild. Rescuing wild birds during HPAI outbreaks can be detrimental, because of the high infection risk to the whole bird collection, and because there are no accepted guidelines for the quarantine and care of wild birds suspected of HPAI virus infection.

In this study, we report the case of the 2020 HPAI H5N8 outbreak at Vogelklas Karel Schot (VKS) in Rotterdam. VKS is one of the largest bird rescue centers in the Netherlands and receives a yearly average of 12,000 wild birds, including more than 50% water birds, for rehabilitation. This study examined the potential events that exposed the birds in the center to the infection, and reports the results of diagnostic work during the investigation of the outbreak. The study also aims to bring more awareness of the challenges that wild bird rescue centers experience during HPAI outbreaks. Furthermore, we provide guidelines to advise rescue centers toward prevention and management of HPAI outbreaks in birds under their care.

Materials and Methods

VKS bird center enclosures

At the start of the outbreak, 135 wild birds were housed at VKS in eight main enclosure units (Figure 1). Birds were categorized (Table 1) as whether they were tested (Groups 1 and 2) or not tested, and by testing date (Group 1 on 6 November; Group 2 on 23 November).

Birds in the quarantine area had access to one indoor freshwater pool, and birds in enclosure 2 had access to two roofed, outdoor pools (Figure 1). The two outdoor pools were separated from each other by concrete walls and had independent water circulation. Anseriformes, gulls and the other water birds were housed in enclosures 2 and 8. The raptors were kept in individual indoor and outdoor enclosures (enclosures 3, 6, and 7). The Passeriformes and Columbiformes were housed in roofed aviaries (enclosures 1, 4 and 5). The same VKS care takers oversaw different enclosures.

Newly admitted birds were housed in individual cages in the quarantine area. However, due to insufficient quarantine space and high number of birds under care, not every bird was routinely quarantined individually. The quarantine area also provided access to a communal indoor water pool used for rehabilitation during the quarantine period. This pool was

accessed by different birds at the same time. At the end of the quarantine period, water birds were translocated to enclosure 2. The premises were disinfected weekly with Virkon®S (Biosecurity b.v., Someren, 5711LV, the Nederlands) at 1% solution as per manufacturer's instructions.

Virological and serological investigation

Oropharyngeal and cloacal swabs were collected using sterile cotton swabs and each placed in 1 ml virus transport medium (18). From each of the three water pools, 1 ml of water was collected in a sterile 2 ml tube. Samples were analyzed for the presence of avian influenza virus (H5) RNA using matrix- and H5-specific real-time reverse-transcription PCR (RRT-PCR) assays, followed by haemagglutinin (HA) and neuraminidase (NA) gene sequencing as previously described.20 Samples were characterized as HPAI H5 virus by detection of a multi-basic cleavage site upon Sanger sequencing of the HA gene. Full length HA and NA sequences and full genome sequences for a subset of virus isolates were obtained by Sanger sequencing. All sequences were deposited in a public database (http://www.gisaid.com).

Nucleoprotein (NP)-specific antibodies against avian influenza virus were detected in sera by using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (Idexx A Ab Test ; Idexx Laboratories BV, Hoofddorp, 2132LR, the Netherlands). Samples were tested according to the manufacturer's instructions. A sample was considered NP positive when the signal-to-noise ratio (i.e., ratio of the mean optical density [ODx] of the sample/ODx of the negative control) was 0.5 or lower.

Between 26-28 October, 2020, a barnacle goose (Branta leucopsis), a domestic goose (Anser anser domesticus) and a greylag goose (Anser anser) were admitted to the rehabilitation center. The barnacle goose and domestic goose were housed in individual cages in the quarantine area. These two geese were lethargic and the domestic goose showed body tremors. The greylag goose showed no obvious neurological signs, was not lethargic and presented with corneal opacity. The greylag goose was placed in the indoor pool area for a short observation (about 30 minutes) and was then housed in an individual cage in the quarantine area while condition was deteriorating. The indoor water pool was also used by three juvenile mute swans (Cygnus olor), two juvenile Egyptian geese (Alopochen aegyptiaca) and ten juvenile mallards (Anas platyrhynchos) at the same time as the greylag goose. Between 28-30 October, the three geese (barnacle goose, domestic goose and graylag goose) became unresponsive and died within 48 hours of admission. The carcasses were disposed without further testing or examination. The reconstruction of events suggests that this likely represented the first introduction of the virus to the center. However, it is difficult to prove retrospectively which goose or geese were infected.

During 28-30 October 2020, the three juvenile mute swans were transferred from the indoor to the outdoor pools in enclosure 2. On 31 October 2020, one of the transferred mute swans, the two Egyptian geese and the ten mallards, residents of the indoor pool in the quarantine area, presented with head-shaking, torticollis, and abnormal gait. Due to compatibility of these clinical signs with HPAI virus infection, VKS contacted the Netherlands Food and Consumer Product Safety Authority (NVWA) to report the case and to discuss control measures. NVWA started an investigation at the center and, together with VKS management, implemented control measures including : testing for HPAIV; immediate disinfection of the premises with Virkon®S; depopulation of the enclosures connected to the indoor and

outdoor pools (quarantine area and enclosure 2) and closure of these enclosures; limitation of personnel in contact with the birds to staff rather than volunteers; and restrictions on new admissions (new admissions were coded low-risk and high-risk based on the likelihood of HPAIV infection , and low-risk admissions were diverted to a satellite rescue center).

On 6 November 2020, oropharyngeal and cloacal swabs were collected from 68 birds (Group 1) to gain more information about the epidemiology of the outbreak (Table 1). The samples were tested for the presence of HPAI H5 virus by PCR. At the same time, water samples were collected from the three pools. All the birds in Group 1 were euthanized by intravenous injection of T-61® (MSD Animal Health, Boxmeer, 5831AN, the Netherlands).

Results

Forty-five birds tested positive for HPAI H5 virus (Table 2). The number of positive birds was similar between adults and juvenile for the same species, when both age groups were present. Overall, oropharyngeal swabs tested positive more frequently (number positive / number tested) for virus than cloacal swabs (number positive / number tested). The water samples from the three pools were positive for virus (Indoor Pool, Ct = 27.0; Outdoor Pool 1, Ct = 26.2; Outdoor Pool 2, Ct = 27.4). Sequencing and molecular characterization of the virus detected in the birds and in the water revealed that HPAI virus subtype H5N8 had caused the outbreak (http://www.gisaid.com, isolates ID: EPI_ISL_1575129, EPI_ISL_1575130, EPI_ISL_1575131, EPI_ISL_1575132, EPI_ISL_1575133, EPI_ISL_1575134, EPI_ISL_1575135, EPI_ISL_1575136).

Based on examination for clinical signs consistent with HPAI, no new cases of HPAI virus infections were reported at VKS for more than two weeks after 6 November and, on 23 November 2020, a diagnostic investigation was performed on the surviving low-risk birds at the center to assess their health status and to plan a possible release into the wild. Blood samples, oropharyngeal and cloacal swabs were collected from 56 remaining birds at VKS (Table 1, Group 2). These birds were housed in separate enclosures from birds in Group 1 (temporary quarantine area and enclosures 1,4 and 5), did not show clinical signs consistent with HPAI, and had not had contact with Group 1. Group 2 consisted of 15 carrion crows (Corvus corone), 13 Eurasian magpies (Pica pica), 10 western jackdaw [Coloeus (Corvus) monedula], 7 Eurasian collared doves (Streptopelia decaocto), and 6 feral pigeons (Columba livia domestica) that were all at the center during the outbreak and housed in enclosures 1,4 and 5; and of 1 northern goshawk (Accipiter gentilis), 1 common kestrel (Falco tinnunculus), 1 domestic goose, and 2 mute swans that were admitted at the center after 6 November 2020 and housed in a temporary quarantine area. All birds in Group 2 tested negative for both HPAI H5 virus and antibodies against avian influenza virus NP, were relocated to a different center, and released after rehabilitation.

			Number of birds		
Order	Family	Species	Group 1 #	Group 2*	Not tested
Anseriformes	Swans	Mute swan *Cygnus olor*	23	2	
		Black swan *Cygnus atratus*	1		
		Barnacle goose *Branta leucopsis*			1
	Geese	Greylag goose *Anser anser*	3		1
		Egyptian goose *Alopochen aegyptiaca*	2		
		White domestic goose *Anser anser domesticus*		1	1
	Ducks	Domestic duck *Anas platyrhynchos domesticus*	3		
		Mallard *Anas platyrhynchos*	2		8
		Indian runner duck *Anas platyrhynchos domesticus*	4		
		Call duck *Anas platyrhynchos domesticus*	1		
Podicipediformes	Grebes	Great crested grebe *Podiceps cristatus*	2		
Gruiformes	Coots	Eurasian coot *Fulica atra*	3		
Charadriiformes	Gulls	Common gull *Larus canus*	2		
		Black-headed gull *Chroicocephalus ridibundus*	1		
		Lesser black-backed gull *Larus fuscus*	8		
		Herring gull *Larus argentatus*	9		
Accipitriformes	Accipitridae	Common buzzard *Buteo buteo*	2		
		Northern goshawk *Accipiter gentilis*	1	1	
Falconiformes	Falcons	Common kestrel *Falco tinnunculus*		1	
Strigiformes	Owls	Barn owl *Tyto alba*	1		
Passeriformes	Corvidae	Carrion crow *Corvus corone*		15	
		Eurasian magpie *Pica pica*		13	
		Western jackdaw *Coloeus (Corvus) monedula*		10	
Columbiformes	Columbidae	Eurasian collared dove *Streptopelia decaocto*		7	
		Feral pigeon *Columba livia domestica*		6	
Total			68	56	11

#Tested on 6 November 2020, *Tested on 23 November 2020

TABLE 1 Birds housed at Vogelklas Karel Schot, the Netherlands, and dates of testing for avian influenza virus, during an outbreak of highly pathogenic avian influenza virus (H5N8) in October-November 2020.

				Number positive for HPAI H5 virus by PCR					
Order	Family	Bird species	Number sampled	Ad	Juv	Ad + juv	OP swab	CL swab	Pooled OP + CL swabs
Anseriformes	Swans	Mute swan	23 (11 ad, 12 juv)	9*	8*	17	10	9	7
		Black swan	1 (1 ad)	1*	na	1	1	1	na
	Geese	Greylag goose	3 (3 ad)	3*	na	3	2	2	1
		Egyptian goose	2 (2 ad)	2*	0	2	2	1	na
	Ducks	Domestic duck	3 (3 ad)	3*	na	3	3	3	na
		Mallard	2 (1 ad, 1 juv)	1*	1*	2	2	1	na
		Indian runner duck	4 (4 ad)	4	na	4	4	4	na
		Call duck	1 (1 ad)	1	na	1	1	1	na
Podicipediformes	Grebes	Great crested grebe	2 (2 juv)	na	0	0	0	0	na
Gruiformes	Coots	Eurasian coot	3 (2 ad, 1 juv)	2	1*	3	3	2	na
Charadriiformes	Gulls	Common gull	2 (2 ad)	2	na	2	2	0	na
		Black-headed gull	1 (1 juv)	na	0	0	0	0	na
		Lesser black-backed gull	8 (4 ad, 4 juv)	0	1	1	1	1	Na
		Herring gull	9 (6 ad, 3 juv)	3	2	5	5	1	Na
Accipitriformes	Accipitridae	Common buzzard	2 (2 ad)	1*	na	1	1	0	Na
		Northern goshawk	1 (1 ad)	0	na	0	0	0	Na
Strigiformes	Owls	Barn owl	1 (1 ad)	0	na	0	0	0	Na
Total			68 (44 ad, 24 juv)	32	13	45	37	26	8

ad, adult (second-year bird and older); juv, juvenile (first-year bird); OP, oropharyngeal; CL, cloacal; na, not applicable.

TABLE 2 Group 1: Results of PCR for presence of highly pathogenic avian influenza virus H5 in birds sampled on 6 November 2020 during an outbreak of highly pathogenic avian influenza (H5N8) at Vogelklas Karel Schot, the Netherlands.

*showed neurological signs (i.e., head shaking, torticollis, abnormal gait)

FIGURE 1. Plan of wild bird rescue centre Vogelklas Karel Schot, the Netherlands, showing where different groups of birds were housed the eight main enclosures during an outbreak of highly pathogenic avian influenza (H5N8) in October-November 2020.

Legend: Enclosure 1 Passeriformes and Columbiformes, 2 Anseriformes, gulls and the other water birds, 3 Raptors, 4 Passeriformes and Columbiformes, 5 Passeriformes and Columbiformes, 6 Raptors, 7 Raptors, 8 Anseriformes, gulls and the other water birds, IP indoor pool, OP1 outdoor pool 1, OP2 outdoor pool 2, K kitchen and food preparation area

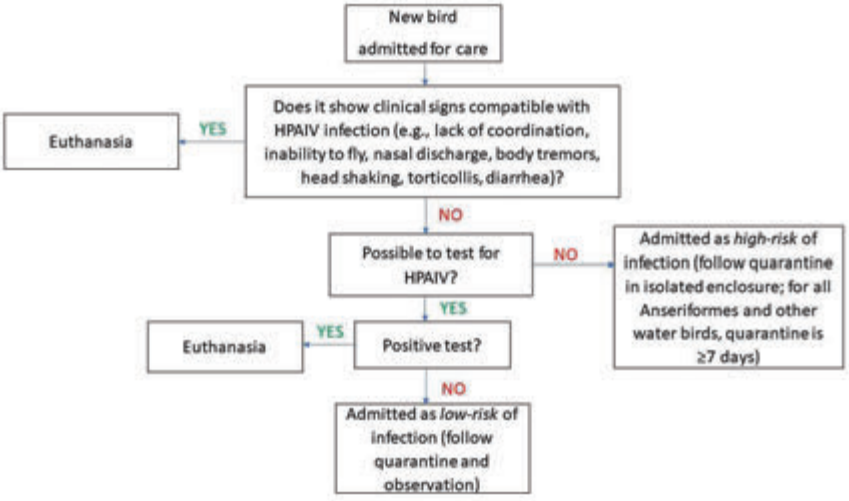

FIGURE 2. Recommended guidelines to control HPAIV infection in rescue centers at the admission of new birds, particularly in high-risk circumstances (e.g., during a national HPAI outbreak).

Discussion

This study describes a HPAI H5N8 outbreak at the VKS bird center, likely introduced by one or more infected geese. The infected geese were admitted during the early stage of a larger national HPAI outbreak in the Netherlands. The virus likely spread from the infected geese to other birds in the quarantine area via water in the shared pool, and then was transferred with the relocation of these birds to the outdoor pools. Multiple waterfowl and other water bird species housed together in the indoor and outdoor enclosures were infected. Swans are particularly sensitive to disease from HPAI virus infection, and were among the first species to show signs of infections at VKS.9 Raptors, also susceptible to disease from HPAIV infection, were housed separately and did not have direct contact with the other birds of Group 1. It is possible that care providers were the vector, possibly via contaminated clothes or shoes, from infected birds to the raptors of Group 1.

Columbidae and Corvidae at VKS were housed in aviaries, separate from the water birds. Despite being cared for by the same caretakers, they were not infected during the outbreak. The birds of Group 2 were not infected and could be spared from culling, so that their rehabilitation continued.

Reviewing the events leading to the VKS outbreak, we are of the opinion that, in principle, such outbreaks are preventable. Nevertheless, HPAI being an emerging disease in wild birds, it is possible that rescue center employees may not always have access to the most recent literature on HPAI. We here report recent updates about HPAI to provide guidance to help avoid future incursions of HPAI into wildlife rescue centers.

Suggested guidance

Birds admitted for care. Wild birds belonging to the group of Anseriformes (ducks, geese, swans) are more likely to act as a vector of HPAIV (2,6,11-15). The European Food Safety Authority (EFSA) maintains an updated list of the wild bird species found dead and most often found positive for HPAIV (4). However, during the latest HPAI outbreak, non-listed species like barnacle goose also played a significant role in the infection (5). More practically, all wild, admitted Anseriformes can be infected with the virus and should be considered as a possible source of infection for the other resident wild birds. Figure 2 shows our recommended guidelines to control the risk of introducing HPAIV in rescue centers at the admission of new birds. Other water bird species can also harbor the virus. For example, studies have shown that gulls can be infected with HPAI viruses (2,8). Infected raptors often become terminally ill, and may be admitted at a terminal stage of disease during a HPAI outbreak.18 Resident raptors are highly susceptible to infection and should be prevented from contact with suspected or confirmed infected birds. There are several reports of naturally and experimentally infected Passerines, but their role in the dynamic of the disease is not yet completely understood, (16) and they are likely less important as potential sources of HPAI virus.

Seasons of autumn and winter are the main risk periods for HPAI outbreaks, and it is especially in these seasons that extra care should be taken when accepting wild birds from the higher risk groups (4,5).

Clinical signs. Experimental infections have shown that some Anseriformes (e.g., mallards and Eurasian wigeons, Mareca penelope) can be infected with HPAI virus without showing clinical signs (10,19). However, these species can excrete substantial amounts of infectious virus, posing risk of infection to other birds. There are no pathognomonic signs of HPAI virus infection, but some wild birds can present lack of coordination, inability to fly, nasal discharge and diarrhea. When the virus spreads to the brain, the neurological signs are more prominent and can also manifest as repetitive body tremors, head shaking, torticollis and sudden death (10).

At admission to VKS the domestic goose showed lethargy and body tremors, the barnacle goose and greylag goose showed more unspecific signs, including lethargy. Wildlife rehabilitators should be educated to recognize these clinical signs, and know the correct procedures to prevent the spread of infections.

Quarantine. Quarantine should be a standard procedure in wild bird centers and adequate quarantine facilities should be always available. Experimental infections showed that infected birds usually have a mean virus excretion time of seven days after inoculation (1,19). When applied to the concept of quarantine, this suggests that more than seven days are required in order to be effective. To reduce the risk of contamination, quarantine units should have dedicated cleaning and animal care tools, and infection protocol for personnel, such as separate enclosure handling, or protective equipment and disinfection. Many different disinfectants (i.e., diluted sodium hypochlorite) are effective to inactivate the virus and can be used to regularly clean tools and cages. The United States Environmental Protection Agency (EPA) maintains an online list of registered disinfectants (3). Personal protective equipment (e.g., disposable gloves, googles, gowns) is highly encouraged when handling recently acquired birds, for both prevention of virus spread to other birds, and prevention of becoming infected with a potentially zoonotic HPAI virus. HPAI virus can also contaminate and disperse via infected food and water.1 A stricter quarantine, longer in time and without interaction between the birds under care, of the infected geese could have prevented the spill over of the infection from the quarantine unit to the other birds at the VKS center.

Separation of birds, such as relocation of resident birds to an indoor facility, preventing access of feral birds, and housing the birds divided as per different risk groups, are also necessary to reduce the risk of HPAI virus incursion in the wildlife center during a HPAI outbreak in the geographical region. Consider euthanizing birds that show early signs of HPAI at admission, and immediately remove the carcasses of dead birds, as virus is still present in carcasses (21).

Testing. A qualified veterinarian should inspect the birds at admission and, during HPAI outbreak in the geographical region, should contact appropriate authorities for official testing of suspect cases. Real time PCR (RT-PCR) is the preferred test for HPAI virus detection for active and passive bird surveillance (7,17,20). Experimental and field studies have shown that HPAI viruses are mainly excreted via the oropharynx, and less frequently via the cloaca (1,10,17,19). In the case of limited resources, pool samples by first swabbing the oropharynges and then the cloaca with the same swab. HPAI outbreaks like the one at VKS

rescue center, could be prevented by testing suspected infected birds at admittance of care. HPAI is a notifiable disease. Restrictive measures, including temporary site closure and the culling of resident birds, may be necessary to control and eradicate an outbreak.

Vaccination. European Union legislation (Council Directive 2005/94/EC) forbids vaccination in poultry, and although vaccination protects against the clinical signs of the disease, birds may still become infected and spread the virus. Vaccination of captive wild birds is allowed under certain circumstances in zoos and closed avian collections (7). Wild bird rescue centers may also qualify for preventative vaccination. Many commercial vaccines licensed for poultry are based on low pathogen avian influenza viruses and may not provide a sufficient protection against HPAIVs. Vaccines against H5-viruses could provide a stronger protection from disease, however species-specific information on post-vaccination antibodies production is not always available. The genetic heterogenicity of at the time circulating HPAI H5 viruses might also have an impact on the vaccine performances (13). Finally, active surveillance to monitor HPAIVs in wild birds is partially based on serological tests (17). Vaccinated, rehabilitated birds that are returned to the wild may represent a confounding factor for the information gathered during surveillance.

Education. HPAI viruses can pose a threat for humans, poultry industry and wild birds. Although recent strains of HPAI viruses in circulation hold a low zoonotic potential, human infections have been reported (5). To control infection outbreaks, the public should be informed about the epidemiology of the disease and the infection risk when encountering a sick wild bird. Wild bird rehabilitation centers are well positioned to be an important connection between the scientific community and the public.

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Chapter 4: Summarizing discussion

General discussion

In the past highly pathogenic avian influenza viruses were mainly confined to poultry. However, in 1996 this paradigm shifted with the emergence and circulation of the Goose/ Guangdong (GS/GD) lineage of H5 HPAI viruses, and the subsequent introduction of HPAIVs into wild bird populations. This thesis presents new evidence on the pathogenesis and epidemiology of recently circulating GS/GD H5 HPAI viruses in wild birds, highlighting the key factors that likely contributed to the adaptation of HPAIV to these new hosts.

Long-term persistence of HPAIV in wild waterfowl

The dynamics of avian influenza virus infection in bird populations have changed dramatically in recent years, resulting in major mortality of wild and domestic birds. The 2014/2015 emergence of the GS/GD HPAI H5N8 virus (clade 2.3.4.4) and its derivatives, which spread from southeast Asia to Europe and North America, caused outbreaks in poultry holdings of seven countries with a loss of over 50 million head of poultry (1-4).

This virus spread via infected migratory wild birds from breeding grounds along migration routes to wintering sites in North America and Europe (4). Epidemiological analysis and experimental infection studies showed that wild ducks (including Eurasian wigeons and mallards) can be infected with 2014 H5N8 virus without clinical or pathological evidence of disease (5-12). Two years later, the 2016-2017 H5N8 outbreak also spread intercontinentally along the wild bird migratory pathways, and caused a large and widespread epidemic in Europe. In the Netherlands alone, more than 13,600 wild birds were reported dead (13). During these outbreaks, HPAIV H5N8 was isolated from apparently clinically healthy free-living wild ducks with some birds also presenting HPAI H5 virus-specific antibodies (3,8,13). These findings were a first indication that long-term persistence of HPAIV in wild waterfowl was possible. **Chapter 2.1** describes an experimental study that provided an explanation for the field dynamics of the infection survival rate during the 2014/2015 and 2016/2017 H5N8 outbreaks in wild birds. By serially infecting wild ducks (mallards and tufted ducks) with two HPAIVs (i.e., the less virulent 2014 H5N8 and the more virulent 2016 H5N8), this study showed that ducks infected with a less virulent HPAIV developed immunity that was protective against a subsequent infection with a more virulent HPAIV 9 months later. Mallards and 2014-H5N8-inoculated tufted ducks survived the infection with 2016 H5N8. The 2014-H5N8-infected mallards showed detectable clinical signs of disease (i.e., weight loss), and one of the 2014-H5N8-infected tufted ducks showed clinical signs of disease, consisting in mild neurological signs. Post inoculation with the more virulent 2016 H5N8, control mallards showed weight loss and control tufted ducks manifested increased mortality. Pharyngeal viral excretion was higher than cloacal excretion, in accordance with previous experimental studies in these and other wild bird species (5-8). Infectious virus was excreted up to five days post infection, and likely transferred from the pharynx to drinking water. This is in agreement with the idea that water can be an important source of infection of HPAIV for birds (14,15). Following 2014 H5N8 infection, the proportion of birds with detectable influenza-specific antibody declined from 100% in tufted ducks and 78% in mallards after 1 month, to 33% in tufted ducks and 29% in mallards after 9 months. After inoculation with 2014 H5N8, presence of NP-serum antibodies did not correlate well with

evidence of previous infection, nor as a predictor of reinfection outcome. This result demonstrated that the value of AIV NP-antibodies serology in wild birds is limited in time, because of the short window of detection of AIV serum antibodies. Control mallards excreted a relatively high quantity of infectious virus and thus may be suitable vectors of HPAI H5 viruses. However, after reinfection with HPAIV H5N8, mallards did not excrete any infectious virus. This result may have an important consequence in the field because it excludes a significant role for previously infected mallards (and possibly other duck species) in spreading infectious HPAIVs.

Adaptation of HPAIV to wild birds

Chapter 2.2 describes how the wide dissemination of the 2016 H5N8 virus among wild birds in Europe resulted at least partly from a change in tissue tropism. From the respiratory tract, similar to older HPAIV viruses, the virus also adapted to the intestinal tract, as in LPAIVs. The increased enterotropism of 2016 H5N8 implies a more efficient faecal-oral transmission and an increased chance to persist long term in the wild waterbird reservoir. This study concluded that 2016 H5N8 had tropism for both digestive tract and respiratory tract of at least four wild bird species (Eurasian wigeon, tufted duck, black-headed gull, Eurasian magpie) based on virus antigen detection in naturally infected birds. This means that 2016 H5N8 mirrors the enterotropism of LPAIV, without having lost the respirotropism of older viruses of the GS/GD lineage. The implication of increased tropism for the digestive tract is that relatively more virus is excreted from the cloaca and contaminates the water bodies on which wild waterbirds reside.

In October 2020, new HPAIVs were isolated in wild birds in Europe (16,17). The 2020/2021 HPAI epizootic was particularly relevant because it registered record numbers of HPAI-related infections and mortalities of wild birds (16,17). In the Netherlands, mortality of wild birds was substantially higher than the average mortality estimates in previous years (**chapter 3.1**). Geese species, such as the barnacle goose, accounted for the highest number of mortalities and infections. This was a different scenario compared to the 2016/2017 HPAI outbreak, when duck species, such as Eurasian wigeon and tufted duck experienced the highest number of mortalities. In 2020/2021, 32 wild bird species were found HPAI virus-infected, indicating that the virus host range locally expanded to previously unreported species. The abundant circulation of HPAI virus in new host species indicates that the virus had well adapted to wild birds. Temporally the virus persisted in local bird populations for over one year, confirming the fact that it can be spread and maintained long-term in wild bird populations. A consequence of the unusual persistence of the virus over summer was that naïve, newly hatched birds were exposed and died as a consequence of infection (16). From the spring of 2021, the virus was also detected in several mammalian species, a validation that the 2020/2021 H5 virus has an increased zoonotic potential (16,17). During the outbreak, HPAI virus reassorted with locally circulating low pathogenic avian influenza viruses, so that six virus subtypes were identified (H5N1, H5N2, H5N3, H5N4, H5N5, H5N8). The most frequently detected subtypes were HPAI H5N8 virus during the first part of the outbreak, and HPAI H5N1 virus from the second part the outbreak. The higher prevalence of HPAI H5N1 virus, a common trend across Europe (16), may be an indication that this subtype had a greater fitness in wild birds.

Chapter 2.3 investigated whether 2020 H5N8 virus followed the trend of its predecessor, 2016 H5N8 virus, of increased enterotropic potential in certain bird species. The study was based on the hypothesis that enterotropism is likely to be maintained in bird species like mallard and Eurasian wigeon that are hypothesized as being vectors for long-distance viral dispersion. Furthermore, enterotropism could be also evident in new host species, like graylag goose and barnacle goose, with the advantage of virus perpetuation in these globally abundant host populations and their movements. The findings of this study partially supported the hypothesis that 2020 H5N8 virus had higher tropism for the intestinal tract of wild birds than 2016 H5N8 virus. The 2020 H5N8 virus had slightly higher level of attachment to the intestinal epithelium of Eurasian wigeon and graylag goose. In mallards the level of attachment for the intestinal tract of 2020 H5N8 virus was unchangingly high compared to 2106 H5N8 virus. The tufted duck is a less likely long-distance vector for HPAIV, and 2020 H5N8 virus had a low level of attachment to the intestinal epithelium of this species. Cloacal swabs were consistently positive for viral RNA in naturally infected Eurasian wigeons and barnacle geese, and more reliable in detecting the infection in barnacle geese. In addition, the jejunum of the five infected Eurasian wigeons was consistently positive for virus RNA by RT-PCR. These findings contrasted with previous studies, where cloacal excretion of HPAI virus infected birds was usually uncommon and, if present, lower than pharyngeal excretion and supported the possibility of HPAI virus replication in the intestinal tract of wild birds (5-7).

Eurasian wigeons and geese have similar biological traits that may be relevant for the epidemiology of HPAI. Eurasian wigeons have already been described as long-distant vector; HPAI virus infection in barnacle and graylag geese is instead a more recent and less studied event. Although migratory goose species were not previously found to disperse LPAIVs (18), this concept should be revisited for HPAI viruses given the novel involvement of barnacle geese and graylag geese in recent HPAI epizootics. Another common trait between Eurasian wigeons and geese is their feeding biology. Apart from dabbling, Eurasian wigeons use a diverse feeding strategy that includes foraging by grazing on land (19,20). During migration, wigeons make use of a great variety of wetland habitats, including wet farm fields, and often feed on grass, leaves, stems and roots. The use of water-rich land for resting and feeding is also common feature in geese, as these birds regularly congregate and feed on improved agricultural pastures (21,22). During these events, a high density of birds makes use of limited surface space. The close contact, and the fact that birds contaminate the grass that they eat with potentially infected feces, may increase chances of AIV infection via the fecal-oral (or fecal-oral) route in flocks of Eurasian wigeons as well as geese.

Respirotropism was retained for 2020 H5N8 virus, based on patterns of virus attachment and virus antigen expression observed in this study. The combination of these two mechanisms (i.e., respirotropism and enterotropism) could give HPAI viruses an advantage over LPAIVs that are mainly excreted via the orofecal route.

This study also described the virology and pathology of HPAI virus infection in barnacle geese, a new host species for the virus. Infection with HPAI H5 viruses in barnacle geese was characterized by a high level of neurotropism and presented with brain lesions associated with virus antigen expression. Most likely, this also represented the cause of death for the birds. Brain lesions were also compatible with neurological signs (i.e., incoordination, body tremors, torticollis) shown by many birds, and described by field operators during the

2020/2021 HPAI surveillance (16,17). The high neurotropic potential of HPAI viruses has been reported in domestic species and it is more often reported also in wild birds (23-25). Pronounced neurotropism is a maintained trait for HPAI H5N8 clade 2.3.4.4b viruses (11). The mortality of waterbirds from the neurotropism of 2020 HPAIV H5N8 could result in reaching yet more new host species. Infected waterbirds with neurological signs are more visible and trigger hunt response of their predators; they are easier prey to catch and more likely to be eaten (26-29).

For the first time since the HPAI H5N1 epidemic in 2005/2006, during the 2020/2021 HPAI H5 epidemic wild mammalian carnivores were reported HPAI virus infected; European foxes, gray seals, and harbor seals were found dead, probably from contact or ingestion of infected wild birds (16,17). HPAI in wild birds has a relatively limited history, and it is possible that virulence levels have not yet been optimized for transmissibility by natural selection (28-29); and the continuously evolving dynamics of HPAI in wild birds may bring new directions for virulence and tropism of HPAI virus in wild birds.

Raptors are at high risk of infection during outbreaks (25). Chapter **2.4** showed that common buzzards may function as bio-sentinel for the presence of HPAI virus in wildlife and determined the most suitable samples to collect during surveillance. This study aimed to determine the virus distribution and associated pathological changes in the tissues of Common buzzards that died with HPAI H5 virus infection during the 2020–2021 epizootic. Eleven freshly dead, HPAI H5 virus-positive Common buzzards were necropsied. Based on RT-PCR, all birds were systemically infected with HPAI H5N8 virus, as viral RNA was detected in cloacal and pharyngeal swabs and in all selected tissues of the birds. Based on histology and immunohistochemistry, the most common virus-associated pathological changes were necrotizing encephalitis and necrotizing myocarditis. The proventriculus of two birds showed virus-associated necrosis, indicating tropism of this virus for the digestive tract. This study advised to collect at least a miniset of samples including brain, heart, liver, and spleen, as these tissues were positive both by RT-PCR and for virus-antigen-associated lesions.

During the larger 2020/2021 national HPAIV outbreak, wild birds in rehabilitation center Vogelklas Karel Schot (VKS) in Rotterdam presented with clinical signs compatible with HPAI, including head shaking, torticollis and abnormal gait (**chapter 3.3**). During an epidemiological investigation at VKS, water samples from the pools in the enclosures and oropharyngeal and cloacal swabs from 128 birds of different species were analyzed for the presence of H5N8 virus. Forty-five birds and the pool water tested positive for the virus. The outbreak at VKS was likely introduced by one or more infected geese, after which the virus spread via pool water and with the relocation of infected birds within the center.

New routes of viral incursion

Infected wild birds were able to spread the HPAIV from Europe to North America (**chapter 3.2**). In December 2021, there was a HPAI H5N1-die-off of domestic birds on an exhibition farm in St. John's, a city on the Avalon Peninsula of the island of Newfoundland, on the Atlantic coast of Canada. This was the first report of HPAI H5 in the Americas since June 2015, when the virus spread with wild birds across the Bering Strait to the Pacific coasts of Canada and the USA via the Pacific Flyway, one of the main avian migration routes (30,31). Genetic analysis showed that the H gene corresponded to Eurasian HPAI viruses circulating

in 2021 (32). This implied that the virus had been carried across the Atlantic, a route that had not been recorded before for any HPAI virus.

The HPAI H5N1 viruses that were detected in Newfoundland in November and December 2021 originated from Northwest Europe and belonged to HPAI clade 2.3.4.4b. Most likely, these viruses emerged in Northwest Europe in winter 2020/2021, dispersed from Europe in late winter or early spring 2021, and arrived in Newfoundland in autumn 2021. The viruses may have been carried across the Atlantic by migratory birds using different routes, including Icelandic, Greenland/Arctic, or pelagic routes. The unusually high presence of the viruses in European wild bird populations in late winter and spring 2021, as well as the greater involvement of barnacle and greylag geese in the epidemiology of HPAI in Europe since October 2020, may explain why spread to Newfoundland happened in 2021/2022, but not in the previous years.

Incursions of recent HPAI viruses, which appear to be well-adapted to wild birds, raises concern about the potential of HPAI virus to become established and spread intercontinentally via wild birds. The implication of this scenario would be high wild bird mortality, higher risk for incursion into poultry holdings and those of other captive birds, as well as zoonotic risk.

Conclusion and future perspectives

AIV is a genetically variable, multi-host pathogen with a high replication rate, and can rapidly adapt to new ecological niches. AIV strains with the highest fitness have been recently maintained for more than one season in wild waterfowl. GS/GD H5 HPAIVs now appear to be able to spread long-distance along interconnected migratory flyways, independently of their circulation in poultry. For this reason in the last decade the risk of HPAIV incursions from wild birds into poultry holdings was substantially higher. This constitutes a novel source of exposure for poultry holdings and requires novel biosecurity measures.

Multidisciplinary studies, such as epidemiological, ornithological, and phylogenetic analyses, are necessary to understand the evolving dynamics of HPAI in wild migratory birds. Despite the long-term surveillance performed in North America and Eurasia, the understanding of the epidemiology of HPAI viruses in relation to the ecology of their host species is still limited. The potential role of wild birds in the intercontinental spread of HPAIV demands an increase of our knowledge about connections between migratory waterfowl populations that is necessary to improve and innovate surveillance and management of AIV in wild birds as well as in poultry. Knowledge on the level of risk that migratory waterfowl pose for long-distance spread of AIV along interconnected migratory flyways, including the potential for certain HPAIV to be maintained indeterminately in wild waterfowl populations, is vital to shape effective and more targeted temporal and geographical surveillance strategies and risk analyses.

A deeper understanding of the evolving HPAI dynamics is also relevant for implementing transformative changes towards a more sustainable poultry sector. In particular, an up-to-date understanding of the route and risks of AIV incursion from wild birds into poultry holdings, including identification of high priority bridge species and reservoir species and their roles in the dynamics of avian influenza introduction and spread, will provide new directions for

strengthening the biosecurity measures of poultry holdings. More targeted, early detection systems will facilitate faster detection methods and reduction of risk of viral incursion in poultry holdings. In addition, scientific-based interaction with the poultry industry and the involvement of the end-consumers may provide more sustainable approaches towards the prevention and control of the current and future HPAI emergencies.

Chapter 5: References

Chapter 1

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Chapter 6: Summary/ Nederlandse Sammenvatting

Summary

Highly pathogenic avian influenza viruses of the Goose/ Guangdong (GS/GD) lineage are an emerging threat to wild birds. HPAI viruses have originally evolved in poultry but are now making use of wild waterfowl as a new environmental niche. Since first emergence in 1996, GS/GD HPAI H5 viruses have successfully adapted and circulated widely in several wild bird species. Wild waterfowl now constitute an important vector for HPAI viruses and their global spread. Wild Anseriformes offer HPAI viruses the great evolutionary advantage to travel via their migratory routes and the opportunity to change their genetical pool by reassorting with circulating LPAI viruses. The adaptation of HPAI virus to wild birds provides an additional route of virus incursion into poultry holdings, and expands the geographic range over which HPAI virus poses a threat to human and animal health.

In **Chapter 2**, wild ducks were serially infected with HPAI viruses. Under experimental conditions, previous infection protected tufted ducks 100% from clinical signs including body weight loss and mortality, compared to sham-inoculated tufted ducks that were clinically affected. This could explain why susceptible species that are infected in repeated HPAI virus outbreaks have disparate outcomes: some remain apparently healthy because they have been infected in previous years, while others die because they are immunologically naïve. Upon re-infection, virus excretion was minimal. Only a minority of infected ducks still presented serum antibodies at 9-month post-infection, indicating a short window of detection of AIV serum antibodies.

The wide dissemination of the HPAI virus among wild birds in Europe in 2016 may have resulted at least partly from a change in tissue tropism. From the respiratory tract, as in older HPAI viruses, the virus also adapted to the intestinal tract of certain wild bird species. The increased enterotropism of 2016 HPAI H5N8 virus implied a more efficient faecal-oral transmission and an increased chance to persist long term in the wild waterbird reservoir. The 2020 HPAI H5N8 virus also had slightly higher level of attachment to the intestinal epithelium of wild birds. Barnacle geese were a key host during the 2020/2021 outbreak in Europe and experienced high rates of infection and mortalities. Barnacle geese have increased chances of AIV infection via the fecal-oral (or faecal-oral) route, by aggregating in close contact and ingesting grass contaminated with potentially infected feces. Raptors such as common buzzards were also at high risk of HPAI virus infection by ingesting infected preys during the outbreak. Common buzzards may function as bio-sentinel for the presence of HPAI virus in wildlife.

Chapter 3 showed that during the 2020/2021 HPAI outbreak in the Netherlands, mortality of wild birds was substantially higher than the average mortality estimates in previous years. Interestingly, 32 wild bird species were found HPAI virus-infected, indicating that the virus host range locally expanded to previously unreported species. The abundant circulation of HPAI virus in new host species indicates that the virus had adapted well to wild birds. Temporally the virus persisted in local bird populations for over one year, confirming the fact that it can be spread and maintained long-term in wild bird populations. A consequence of the unusual persistence of the virus over summer was that naïve, newly hatched birds were

exposed and died because of the infection. From the spring of 2021, the virus was also detected in several mammalian species, a validation of the fact that the 2020/2021 H5 virus has an increased zoonotic potential. During the outbreak, infected wild birds were able to spread HPAI viruses from Europe to Canada. The HPAI H5N1 viruses that were detected Canada in December 2021 originated from Northwest Europe and belonged to HPAI clade 2.3.4.4b. Most likely, these viruses emerged in Northwest Europe in winter 2020/2021, dispersed from Europe in late winter or early spring 2021, and arrived in Canada in autumn 2021. The viruses may have been carried across the Atlantic by migratory birds using different routes, including Icelandic, Greenland/Arctic, or pelagic routes. The unusually high presence of the viruses in European wild bird populations in late winter and spring 2021, as well as the greater involvement of geese in the epidemiology of HPAI in Europe since October 2020, may be an explanation for this new event. The unusually high presence of HPAI virus in wild birds was also a cause of the higher risk of viral incursion and related outbreak in a wild bird rehabilitation centre in Rotterdam.

In conclusion, the adaptation of HPAI virus to wild birds provides an additional route of virus incursion into poultry holdings and expands the geographic range over which HPAI virus poses a threat to human and animal health. The work in this thesis provides new evidence for more targeted decision making for HPAI control.

Nederlandse Sammenvatting

Hoogpathogene aviaire-influenzavirussen van de Goose/Guangdong (GS/GD)-lijn vormen een opkomende bedreiging voor wilde vogels. HPAI-virussen zijn oorspronkelijk geëvolueerd in pluimvee, maar maken nu gebruik van wilde watervogels als een nieuwe ecologische niche. Sinds de eerste opkomst in 1996 hebben GS/GD HPAI H5-virussen zich met succes aangepast en verspreid onder verschillende wilde vogelsoorten. Wilde watervogels vormen nu een belangrijke vector voor HPAI-virussen en hun wereldwijde verspreiding. Wilde Anseriformes bieden HPAI-virussen het grote evolutionaire voordeel om via hun trekroutes te reizen en de mogelijkheid om hun genetische pool te veranderen door opnieuw te sorteren met circulerende LPAI-virussen. De aanpassing van het HPAI-virus aan wilde vogels biedt een extra route voor het binnendringen van virussen in pluimveebedrijven en vergroot het geografische bereik waarbinnen het HPAI-virus een bedreiging vormt voor de gezondheid van mens en dier.

In **Hoofdstuk 2** werden wilde eenden serieel geïnfecteerd met HPAI-virussen. Onder experimentele omstandigheden beschermde eerdere infectie tufteenden 100% tegen klinische symptomen, waaronder verlies van lichaamsgewicht en sterfte, in vergelijking met schijn-geïnoculeerde tufteenden die klinisch waren aangetast. Dit zou kunnen verklaren waarom vatbare soorten die zijn geïnfecteerd bij herhaalde HPAI-virus-uitbraken, uiteenlopende resultaten hebben: sommige blijven ogenschijnlijk gezond omdat ze in voorgaande jaren zijn geïnfecteerd, terwijl andere sterven omdat ze immunologisch naïef zijn. Bij herinfectie was de virusuitscheiding minimaal. Slechts een minderheid van de geïnfecteerde eenden vertoonde 9 maanden na infectie nog steeds serumantilichamen, wat wijst op een korte detectieperiode van AIV-serumantilichamen.

De brede verspreiding van de HPAI-virus onder wilde vogels in Europa in 2016 kan op zijn minst gedeeltelijk het gevolg zijn geweest van een verandering in weefseltropisme. Vanuit de luchtwegen, zoals bij oudere HPAI-virussen, heeft het virus zich ook aangepast aan het

darmkanaal van bepaalde wilde vogelsoorten. Het verhoogde enterotropisme van het 2016 HPAI H5N8-virus impliceerde een efficiëntere fecaal-orale transmissie en een verhoogde kans om op lange termijn in het reservoir van wilde watervogels te blijven. Het 2020 HPAI H5N8-virus had ook een iets hogere hechting aan het darmepitheel van wilde vogels. Brandganzen waren een belangrijke gastheer tijdens de uitbraak van 2020/2021 in Europa en kenden hoge besmettings- en sterftecijfers. Brandganzen hebben een grotere kans op AIV-infectie via de fecaal-orale (of fecaal-orale) route, door samen te klonteren in nauw contact en door gras op te nemen dat besmet is met mogelijk geïnfecteerde uitwerpselen. Roofvogels zoals gewone buizerds liepen ook een hoog risico op HPAI-virus-infectie door geïnfecteerde prooien op te nemen tijdens de uitbraak. Buizerds kunnen fungeren als bio-schildwacht voor de aanwezigheid van HPAI-virus in dieren in het wild.

Hoofdstuk 3 liet zien dat tijdens de HPAI-uitbraak van 2020/2021 in Nederland de sterfte van wilde vogels aanzienlijk hoger was dan de gemiddelde sterfteschattingen in voorgaande jaren. Interessant is dat 32 wilde vogelsoorten werden gevonden met HPAI-virus geïnfecteerd, wat aangeeft dat het virusgastheerbereik plaatselijk is uitgebreid tot voorheen niet-gemelde soorten. De overvloedige circulatie van HPAI-virus in nieuwe gastheersoorten geeft aan dat het virus zich goed had aangepast aan wilde vogels. Tijdelijk bleef het virus in lokale vogelpopulaties meer dan een jaar bestaan, wat bevestigt dat het virus zich op lange termijn kan verspreiden en in stand kan houden in wilde vogelpopulaties. Een gevolg van de ongebruikelijke persistentie van het virus in de zomer was dat naïeve, pas uitgekomen vogels werden blootgesteld en stierven als gevolg van de infectie. Vanaf het voorjaar van 2021 werd het virus ook gedetecteerd bij verschillende zoogdiersoorten, een bevestiging van het feit dat het H5-virus 2020/2021 een verhoogd zoönotisch potentieel heeft. Tijdens de uitbraak konden besmette wilde vogels HPAI-virussen van Europa naar Canada verspreiden. De HPAI H5N1-virussen die in december 2021 in Canada werden gedetecteerd, zijn afkomstig uit Noordwest-Europa en behoorden tot HPAI-clade 2.3.4.4b. Hoogstwaarschijnlijk zijn deze virussen in de winter van 2020/2021 in Noordwest-Europa opgedoken, in de late winter of het vroege voorjaar van 2021 vanuit Europa verspreid en in de herfst van 2021 in Canada aangekomen. De virussen zijn mogelijk via verschillende routes over de Atlantische Oceaan vervoerd door trekvogels, inclusief IJslandse, Groenland/Arctische of pelagische routes. De ongebruikelijk hoge aanwezigheid van de virussen in Europese populaties wilde vogels in de late winter en het voorjaar van 2021, evenals de grotere betrokkenheid van ganzen bij de epidemiologie van HPAI in Europa sinds oktober 2020, kunnen een verklaring zijn voor deze nieuwe gebeurtenis. De ongebruikelijk hoge aanwezigheid van HPAI-virus bij wilde vogels was ook een oorzaak van het hogere risico op virale inval en gerelateerde uitbraak in een opvangcentrum voor wilde vogels in Rotterdam.

Concluderend kan worden gesteld dat de aanpassing van het HPAI-virus aan wilde vogels een extra route biedt voor het binnendringen van virussen in pluimveebedrijven en het geografische bereik vergroot waarbinnen het HPAI-virus een bedreiging vormt voor de gezondheid van mens en dier. Het werk in dit proefschrift levert nieuw bewijs voor meer gerichte besluitvorming voor HPAI-controle.

Chapter 7: About the author

Curriculum vitae

Valentina Caliendo obtained her degree in Veterinary Medicine from the University of Napoli, Italy. Her passion for veterinary medicine and curiosity for diverse cultures motivated her to travel across many countries to expand her skill, and work with different taxa. For several years, she provided veterinary care for a large raptor collection in the Middle East. In 2017, her interest in avian medicine and medical research led her to pursue a doctorate at Erasmus Medical Center, the Netherlands, to explore the dynamics of avian influenza in wild birds, and that resulted in this thesis. While pursuing her doctorate, Valentina successfully qualified as diplomate of the American College of Zoological Medicine.

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Courses

Courses	
2018	Course on Virology
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2018	Course on Laboratory Animal Science
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Presentations

2018-2022	Lab meetings, Department of Viroscience, Erasmus MC
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Teaching

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List of publications

V. Caliendo, L. Leijten, M. W. G. van de Bildt, M. J. Poen, R. A. M. Fouchier and T. Kuiken. 2022. Long-term protective effect of serial infections with H5N8 highly pathogenic avian influenza virus in wild ducks. Journal of Virology 2022, 96(18): e01233-22.

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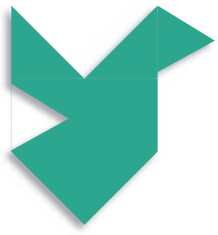
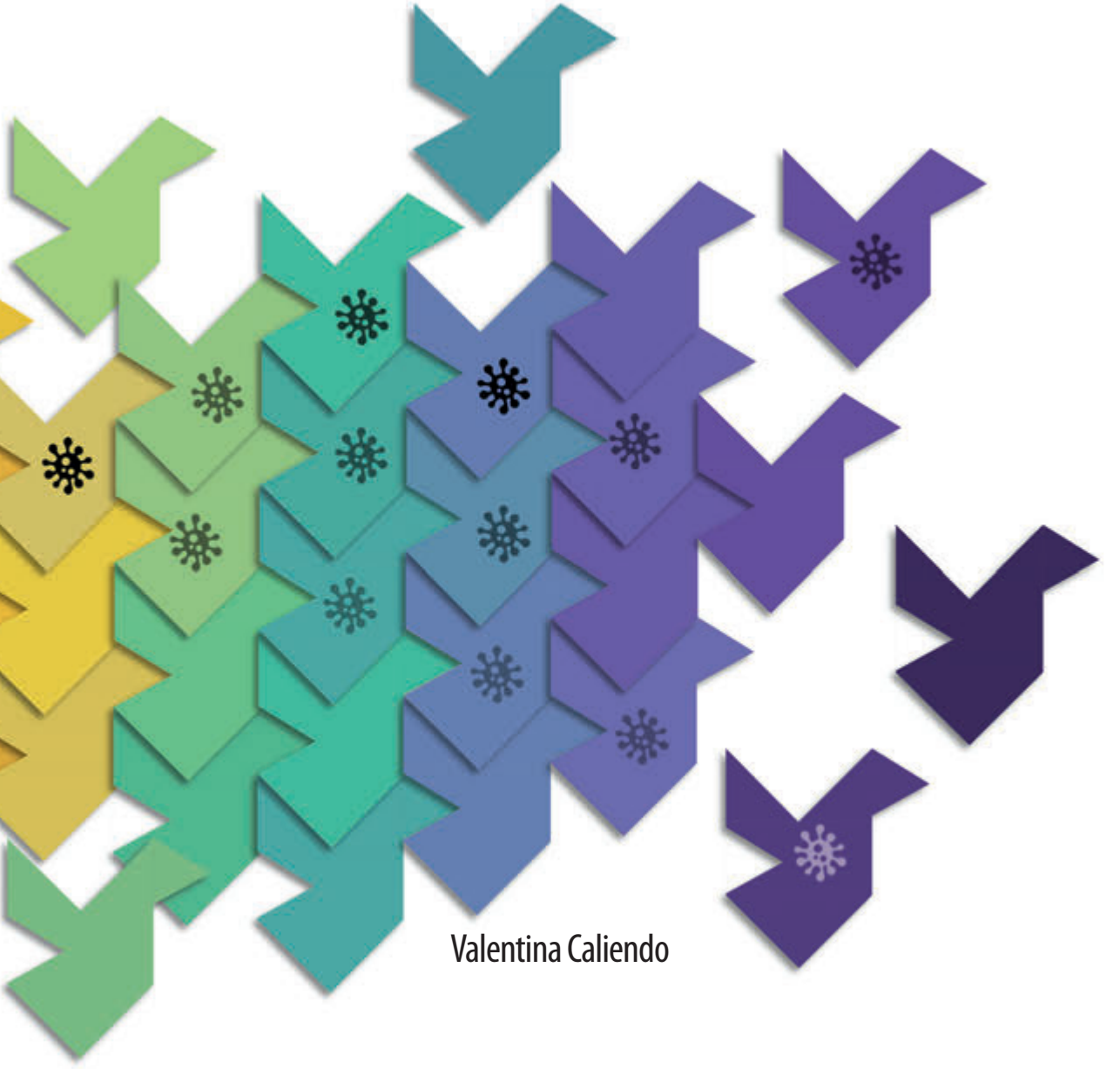


Pathogenesis and epidemiology of the Goose/Guangdong lineage of highly pathogenic avian influenza in wild birds



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