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Avian raptor surveillance – expert screening for highly pathogenic avian influenza viruses (clade 2.3.4.4b)

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Contents

I. Introduction

Only recently a comparably young pathogen group, high pathogenicity (HP) avian influenza viruses (AIV) of subtype H5 (HPAIV H5), has paved its way around the globe, sparing yet only Australia/Oceania and Antarctic regions. It emerged, somewhere in Southern China in the early-mid 1990s by spontaneous mutation in poultry from a precursor virus of low pathogenicity (LP). This so-called Goose/Guangdong (Gs/Gd) lineage of HPAIV hemagglutinin (HA) subtype H5 induced a highly fatal disease, also referred to as fowl plaque, in poultry or, following spillback transmission, in wild bird populations. Along with ongoing spread of the disease among avian hosts, heavily affected areas repeatedly have reported on infections of mammalian species, sporadically even humans, raising concerns about its zoonotic and pandemic potential. As yet, HPAI H5 viruses have not been brought under control.

More than 15 years ago, in winter 2005/2006, progeny of the Asian Gs/Gd lineage caused a first HPAIV H5 epizootic in Europe, ushering a new era of global awareness and invocations for preparedness on AIV. Over time, the genetics of the ancestral strain altered, resulting in different Gs/Gd-like HPAIV H5 subtypes and genotypes that have been phylogenetically classified into different clades. Meanwhile, viruses of clade 2.3.4.4b are riding the crest of the overall fifth wave of Gs/Gd-HPAI in Europe. The currently dominant HPAIV H5N1 subtype can be held responsible for two events that require special attention:

The prior epizootic circulation of clade 2.3.4.4b strains in Northern European countries became enzootic, in 2022, entailing disastrous economic consequences for poultry production and unpredictable impacts on several wild bird species potentially affecting biodiversity.

In winter of 2021/2022, a transatlantic spread of HPAIV H5N1 became evident and phylogenetic analyses revealed high similarity between strains emerging in North America and those circulating in Europe. However, the exact transmission routes for this event remained unclear for the time being.

Besides their role as reservoirs of LPAIV, and as vectors or key drivers in the wide geographic spread of HPAIV H5, individual wild birds also are simply victims of the disease. High infection pressure and mortality among primary avian hosts in the Anseriformes and Charadriiformes entail infections of secondary hosts at the end of the food chain: raptors and scavengers. Thus, their preying behavior on diseased, HPAIV infected birds or carcasses has long emphasized their (scientific) value as indicator species for HPAIV circulation. However, HPAIV H5 activity now overlaps with the breeding and hatching season of raptors in Europe, such as white-tailed sea eagles (*Haliaeetus albicilla*) and for the first time, novel age cohorts are exposed when HPAIV H5-positive prey is fed to nestlings. Ultimately, the almost global circulation of HPAIV H5 may pose a high risk to entire (sub)populations of avian apex-predators.

All the worse, HPAIV H5 is not the only emerging viral threat to wild bird populations - nor is it the only one with zoonotic potential and public health significance. Usutu virus (USUV) and West Nile virus (WNV), both of which recently became enzootic in (parts of) Europe, have been repeatedly detected in wild birds, including raptor species. Germany holds a special ecogeographical position due to the possibility of spatio-temporal co-circulation of enzootic orthomyxoviruses (HPAIV H5) and flaviviruses (USUV, WNV) in avian hosts.

This work addresses disease surveillance to (i) investigate the impact of HPAIV H5 on raptors and their breeding success, considering mass mortality events in prey species and in the context of species conservation. In addition to these more local observations, (ii) the suitability of these species as indicators for disease spread on a global scale is exemplified in the context of Iceland, as a stopover site during intercontinental spread of HPAIV H5 (clade 2.3.4.4b). (iii) A pilot study was conducted to identify overlapping hosts for HPAIV H5, USUV, and WNV in Germany that may provide an opportunity for even more advanced and broad surveillance approaches on zoonotic avian pathogens in future.

II. Review of Literature

1. Influenza A viruses

The various taxonomic classifications of viruses are constantly being re-evaluated by the International Committee on Taxonomy of Viruses (ICTV), including recent adjustments within the family *Orthomyxoviridae* [1]. It now includes nine genera (*Alphainfluenzavirus, Betavirus, Gammainfluenzavirus, Deltainfluenzavirus, Isavirus, Mykissvirus, Quaranjavirus, Sardinovirus* and *Thogotovirus*). The newly appointed group *Alphainfluenzavirus influenzae* represents the only species in the genus of *Alphaviruses* [2]. Influenza A viruses (IAV) are now classified as a taxonomic entity below species level, representing a highly relevant group of pathogens particularly for the world of birds, but also for mammals including humans [1]. Some special characteristics of IAV, including avian influenza viruses (AIV), are determined in the design of the overall genetic set-up, featuring an eightfold segmented single-stranded RNA genome of negative polarity, their (non-) structural proteins and two important features of replication, an error-prone RNA-dependent RNA-polymerase and the ability for reassortment, shaping the patterns on etiology and epidemiology.

1.1. Morphological characteristics and virus nomenclature

IAV are characterized by a negative-sensed single stranded RNA genome, that is organized in eight segments. Each segment encodes for at least one viral protein, including structural and non-structural ones [3, 4]. Each genomic segment is enwrapped by nucleoproteins (NP) and attached to a copy of the viral polymerase complex, formed by the polymerase acidic protein (PA), the polymerase basic protein 1 (PB1) and the polymerase basic protein 2 (PB2). All eight viral ribonucleoprotein complexes (vRNP) are engulfed by a proteinaceous shell formed by the viral matrix protein 1 (M1) which in turn is enveloped by a lipid bilayer membrane, that creates particles of around 80-120 nm in diameter, thus, forms a mainly spherical or occasionally filamentous, enveloped virion [5] (Figure 1). Besides the vRNPs also the nuclear export protein (or non-structural protein 2; NEP or NS2) and the M1, remain internal to the enveloped virion [6]. The matrix protein 2 (M2) forms as an ion channel within the viral envelope [6]. In contrary, the glycoproteins hemagglutinin (HA) and neuraminidase (NA) are anchored in spike-like fashion within the outer membrane [6]. Both, HA and NA, serve essential functions in viral replication and are utilized for further IAV subtyping and classification: Generally, the receptor binding sites of HA and NA are considered to remain highly conserved [7]. Still, even these sites are affected by high mutation rates due to the error-prone polymerase activity, lacking a proof-reading-function, during viral

replication [8]. Thus, until now in total 16 different HA and nine different NA were described among various avian hosts, whereas two novel HA (17,18) and NA (10,11) have been only detected in fruit bats from Middle and South America [9, 10]. Most recently, Fereidouni, Starick, Karamendin, et al. [11] described a further HA coding sequence, suggesting the existence of a novel HA 19 in an avian host. The final combination of HA and NA within an IAV is assigned to classify IAV **subtypes**, addressed as HxNy [5].

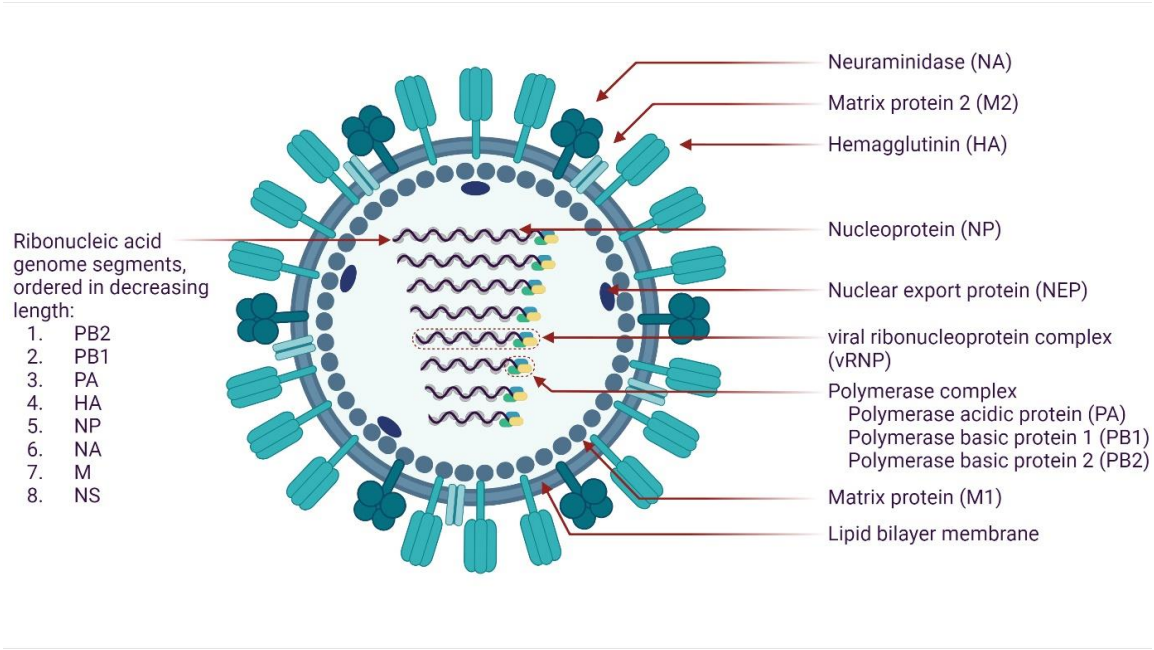

Figure 1 *Schematic structure of an influenza A virus particle. Created with BioRender.com. For permission rights see Appendix, legal permissions.*

Pathogenicity is applied as a second criteria for differentiation of IAV in avian hosts (AIV) which is substantial for the understanding of their epidemiology. Those IAV, that found their reservoir among aquatic birds or shorebird populations, are categorized as AIV of low pathogenicity (LPAIV) and usually do not cause any or merely mild clinical signs of an infection in these individuals [12]. For the initial process of an infection, IAV make use of enzymes "provided" by their hosts. Tissue specific proteases activate the HA pre-protein (HA_0), which is subsequently cleaved into disulfide-linked subunits HA_1 and HA_2 [13, 14]. The latter now presents the necessary formation to initiate the fusion process of viral and cellular membranes after the viral particle has attached to a host cell [15]. The amino acid sequence at the so-called cleavage site (CS), of the HA_0 decides which host proteases can split the pre-protein. A monobasic CS formation matches trypsin-like proteases, that exclusively occur on avian respiratory and

intestinal epithelia. As a result, the viral replication remains restricted to these tissues and causes only local infection of a generally mild clinical nature (LPAIV).

H5 and H7 subtypes have been observed to mutate into a highly pathogenic (HP) phenotype [16], in most cases associated with LPAIV transmission to and virus replication in Galliform poultry species [17]. A spontaneous mutation of the HA-segment might lead to a polybasic CS that renders it cleavable for furin-like proteases. Those are ubiquitous host-proteases, and, thus, virus replication is enabled in most organs, leading to systemic, clinically often fatal infections [18]. The exact mechanisms, how polybasic CSs are acquired remain yet unclear. Three main hypotheses were recently reviewed by de Bruin, Funk, Spronken, et al. [19]: (i) single nucleotide substitutions, (ii) backtracking or stuttering movements of the polymerase-complex that entail duplications within the nucleic acid sequence or (iii) non-homologous recombination events between viral or host RNA species and the original HA RNA (only described for HA H7). Polybasic sequences at the virus' CS lead to its classification as HPAIV according to the World Organisation for Animal Health (WOAH; formerly Office International des Epizooties [OIE]) regulations [20].
Furthermore, the decision on clustering into the HP group can be made by an *in vivo* assay based on the intra-venous pathogenicity index (IVPI). Values > 1.2, based on observations in chickens after standardized inoculation characterize the virus isolate as HP [20].

Given the numerous possibilities to classify IAV, a standardized nomenclature has been established to catalogue influenza viruses, referring to isolates and sequences [21]:

- Group of influenza virus (e.g. A, B, C, D; according to prior taxonomical classification as genera)
- Host species (omitted, when of human origin)
- Location of the origin
- Isolate number or laboratory-depending internal sample-identification
- Year of isolation/detection
- Pathotype in case of H5/H7 subtypes (optional)
- Subtype composition of HA and NA (optional)

For example, an HPAIV H5 isolate retrieved from a tufted duck (*Aythya fuligula*) in Germany during the 2016 HPAI epizootic was named A/tufted duck/Germany-SH/AR8444/2016 (HP H5N8; [22]).

1.2. Essential strategies supporting genetic variety and emerging variants of IAV

The above described genomic and structural characteristics serve as basis for the overall genetic flexibility of IAV. The continuous emergence of new strains can be explained with two main mechanisms:

Genetic drift occurs coincidentally as an effect of the error-prone RNA-dependent RNA-polymerase lacking a proof-reading function [8]. Hence, it is estimated that approximately every $1x\ 10^{-4}$ base is substituted by a mismatching base per replication cycle, resulting in a point mutation [23]. Although those changes might lead to less functional or non-functional protein structures, regarding antigenicity they may provide an evolutionary advantage, especially when affecting the HA segment or within the polymerase. The HA amino-acid structure acts as main target antigen for the host's immune response and changes in the corresponding epitopes might aid to elude host-specific immunity [23]. The process of genetic drift can be characterized as rather slowly, but steady [24]. However, compared to other viruses it is still faster and, thus, is of immense importance [23]: Over time, single coding point-mutations affecting the HA and NA proteins may lead to an enhanced immune escape. Kayali, Kandeil, El-Shesheny, et al. [25] emphasized the potential challenges due to the enzootic presence of HPAIV in Egypt: Since 2008, vaccination has become the main, and eventually only, measure in the pathogen control program, that was applied to all types of poultry holdings for multiple years. Over time, novel HA gene variations evolved in parallel as a result of genetic drift. However, these no longer matched the antigenic response induced by the previously used vaccine strains. As a result, vaccination contributed indirectly to the emergence and positive selection of escape mutants, which continued to cause outbreaks in the following years [25].

Another less frequent, but possibly more effective phenomenon is represented by the ability of IAV to exchange entire genome segments, referred to as reassortment, and leading to so-called *genetic shift* [5]. This event requires the simultaneous infection of a single cell with two IAV of different genetic set-ups (genotypes). When the genome segments are assembled during viral replication, the segments of the two "parental" virions may mix and be randomly redistributed to form new sets of eight segments comprised in a new reassorted virion [5]. If HA or NA segments are involved, that process may reveal novel subtypes with advanced immune escape propensity in naïve host populations or even provide options to broaden the host spectrum, when IAV of different host species become mixed [5]. Although the circumstances necessary for this to occur are highly subject to chance, high infection pressure and the flexibility of IAV in host selection provide such opportunities not infrequently [5]. Unlike genetic drift, genetic shift may immediately provide a clear evolutionary advantage and is closely monitored for IAV between so far known source and sink regions [22, 26-28].

2. Avian influenza

2.1.Transmission routes and disease patterns among different host groups

2.1.1. Avian hosts

Reservoir species

Wild waterfowl and shorebirds are the natural reservoir for LPAIV and until now a wide range of wild bird species has been identified as hosts for various subtypes [29-31]. The large taxonomic orders of Anseriformes and Charadriiformes are the most represented host groups in Europe [32], species that are also highly abundant in other continents [33]. Among them, various different behavioral patterns can be found in terms of migration (e.g. residential or long-distance migration), breeding (e.g. colony or non-colony breeders) and habitat preferences (e.g. pelagic, coastal, inland).

The main mode of transmission of LPAIV is via feco-oral exposure [23]. Therefore, many individuals congregating in large flocks at migration stop-over sites or sharing the same feeding, moulting or breeding grounds provide excellent circumstances for virus transmission. In particular, dabbling ducks, such as mallards (*Anas platyrhynchos*), have a high level of exposure, as they filter the surface of water, which may be contaminated with virus-containing feces, in search of food [31].

Outside the host, the tenacity of AIV is depending on various environmental conditions. In water, the virus remains infective for prolonged periods of days to months in cold temperature and neutral pH ranges, depending also on the water salinity [34, 35]. Moreover, Keeler, Dalton, Cressler, et al. [36] and Ramey, Reeves, Lagassé, et al. [37] suggested strain-specific differences in the perseverance of AIV in surface water.

LPAIV-affected waterfowl individuals often show no clinical signs of an infection [30] or only mild to unspecific indications (e.g. diarrhea) [12].

Poultry species

Poultry species (Anseriformes and Galliformes) are highly susceptible to AIV. Virus transmission from wild birds to poultry ("spill over"), accelerates its replication within a flock, given the high density of genetically similar host individuals. LPAIV infections in poultry can either remain asymptomatic or become associated with mild to moderate depressions and temporally limited phases of decline in laying performance or weight gain [17]. However, as described previously (see 1.1) AIV of subtype H5 or H7

could potentially switch from low pathogenicity to high pathogenicity phenotypes by *de novo* mutation in gallinaceous species. Outbreaks of HPAI in poultry are commonly known as fowl plague. In chicken and turkey flocks, HPAIV infection causes acute death, with flock mortality rates of up to 100% within few days [38]. Other potential (clinical) signs comprise depression, respiratory problems and sudden declines in food and water consumption [17]. Domestic Anseriform species, similar to wild waterfowl with HPAIV infections, may not exhibit a clear clinical picture, but can display a spectrum from unspecific to severe neurological signs [17].

AIV is introduced into poultry flocks either through direct contact with wild birds on e.g. free-range farms or backyard holdings, or indirectly through inadequate biosecurity measures in large commercial holdings, or via bridging hosts (refer to below). Within a poultry flock (HP)AIV spreads through secretions and excretions.

Due to the moderate to high tenacity of AIV, feces-contaminated equipment or clothing applied within animal care, could lead to the transfer of the pathogen between herds or holdings (secondary spread). Likewise, the movements of infected individuals (animal trade), their feces or possibly products (e.g. eggs), as well as improper disposal of carcasses or slurry, might contribute to the spread of the pathogen [39]. Those circumstances can also allow HPAIV to return to the environment and re-infect wild birds ("spill back"), as seen in the emergence of the Asian HPAIV Goose/Guangdong (Gs/Gd) lineage (refer to paragraphs 3.1 and 3.2).

HPAIV infections in poultry and in wild birds are classified as a notifiable animal disease, mainly due to their high morbidity and mortality rates after acute disease onset, resulting in significant economic impact. Until very recently, within the European Union outbreaks of HPAI in poultry have been combated solely by immediate culling of affected flocks to prevent further spread of the disease. In view of changing patterns in the temporal and spatial occurrence of viruses almost worldwide (see section 3.3), the possibility of vaccination campaigns has recently been revived as a supportive tool for disease control also in Europe. However, this option would call for further surveillance approaches, active as well as passive, to avoid undetected virus circulation within vaccinated flocks and identification of field strains to guarantee the appropriate choice of vaccine strains over time [40].

Vector and victim species

Following spill back events, infections with HPAIV H5 strains in wild waterfowl may sometimes occur without clinical signs, as described after experimental infection of mallards [41, 42]. At the same time, many epizootics were characterized by die-offs of those groups of host species [43, 44] pointing to strain-specific viral pathogenicity. In addition to general fatigue, infected birds can show aggravated

respiration and neurological manifestation, such as ataxia or head tilting and, often, HPAI is associated with acute deaths [17].

As Anseriformes and Charadriiformes do not necessarily show clinical signs of an infection, it could be inferred they remain mobile, even while shedding the virus. Thus, they can become vector species and carry the virus over particular geographical ranges. Global Consortium for H5N8 and Related Influenza Viruses (2016) [45] demonstrated the spread of the Asian Gs/Gd-lineage via migratory wild birds following the re-introduction of HPAIV from poultry to wild bird populations.

Bridging species

Besides long-distance migratory wild birds as potential vectors contributing even to the supra-regional and even transcontinental spread of (HP)AIV, for regional scenarios another group of hosts must be considered: Bridge hosts. As various definitions suggest, they are competent virus hosts but not necessarily highly susceptible [46, 47]. Their behavior allows them to become (mechanical) vectors for virus transmission, as stated by Le Gall-Ladevèze, Guinat, Fievet, et al. [46] and Caron, Cappelle, Cumming, et al. [47]. For instance, poultry flocks in inland areas without adjacent open water source are usually an unappealing environment for AIV reservoir species, such as waterfowl. Therefore, the introduction of (HP)AIV might be geographically and habitat-specifically rather unlikely. Yet, bird species that depend on an aquatic habitat and feed near human populations in an opportunistic fashion could fill this gap, leading to a potential virus introduction. Recent observations have demonstrated that the roles of the reservoir, victim and bridging host are not always sharply separated and can overlap. A new HPAIV H5N1 genotype, called genotype BB, dominates the current HPAI outbreaks in Europe after reassorting with a gull-adapted LPAIV H13 [44]. Black-headed gulls (*Chroicocephalus ridibundus*) are the most affected wild bird species; however, the same genotype has also been found in poultry and fur farms [48]. Gull species might have been involved in the initial reassortment of the virus, as a reservoir. Still, they also suffer severely from the infection and are true victims. In addition, black-headed gulls breed in both coastal and inland areas and can be considered as synanthropic species [49], posing as a bridge host by foraging in the vicinity of settlements or agricultural infrastructure.

Indicator species

Indicator species represent target species that are instrumentalized for effective sampling strategies, to assess the occurrence of (HP)AIV circulation in a certain region or population. This term is not consistently used among research groups, but mainly refers to the identification of key wild bird species, which might enable a more efficient and economic surveillance of pathogen circulation within targeted

sampling approaches: Different criteria can be set for this purpose; often the species abundance in relation to the confirmation of a (former) infection with a certain pathogen is addressed [50-54].

A general suitability as indicator species relies either on the opportunity to frequent virus exposure as a primary host species (e.g. reservoir species) or result in pathogen contact when feeding on infected primary hosts or virus-positive carcasses, and, thus, becoming secondary hosts. In the current literature, especially scavenging and hunting bird species are designated as (bio)indicators for passive HPAI surveillance due to their position at the top of the food chain [51, 55-57]. There is barely any knowledge on natural infections with LPAIV in raptor species, although after experimental infection seroconversion has been observed [58, 59]. In contrast, infections with HPAIV H5 strains typically result in acute fatal disease of raptors or lead to protracted severe neurological signs, that may necessitate euthanasia of the individual if retrieved and submitted to rescue centers [56, 57, 59-61].

This work uses the term “raptors” to refer to birds belonging to the taxonomical orders Accipitriformes, Falconiformes and Strigiformes, whereas the term “birds of prey” usually excludes the owl families Tytonidae and Strigidae.

2.1.2. Mammalian spill over and dead-end infections

In the beginning of the 1980s, first findings of AIV in harbor seals (*Phoca vitulina*) of the New England coast, United States of America, signaled a potential, previously undetected inter-species transmission potential of AIV [62]. Recently increasing case numbers of currently circulating HPAIV H5 strains have been found in wild terrestrial and aquatic carnivores in different parts of the world [63-74], but also in livestock, e.g. pigs [75], fur animals and pets, such as cats [76-83]. These individuals were either found dead or afflicted with severe neurological signs, including tremors, convulsions, ataxia and opisthotonos [66, 71, 72, 74]. It remains to be determined whether the majority of these infections inevitably leads to death of the wild or domestic carnivore or whether a substantial proportion undergoes mild or even asymptomatic infection signaled by seroconversion [84]. The most likely reason of these cases is alimentary contact with infected avian prey [68], and often these cases were restricted to single reports per species and transmission event [85], as such most likely excluding carnivore-to-carnivore transmission. Therefore, mammals still represent dead end hosts. In contrary, one recent observation described a massive die-off among South American sea lions (*Otaria flavescens*) in Peru and Chile. It is still unclear, if this represents a localized accumulation of single alimentary infections rather than an intra-species transmission between mammalian hosts; sea birds of the same region had been massively affected by HPAIV at the same time [71, 72].

Recent outbreaks in Poland showed that this risk of infection is not limited to wild mammals, but also threatens domestic carnivores. Here, outbreaks in domestic cats (*Felis catus*) could most likely be traced back to feeding HPAIV-positive food (e.g., fresh chicken meat) [81].

Reports from fur animal farms in Spain in October 2022 [86] and Finland in July 2023 [83] are of particular interest. Both reports describe outbreaks caused by the HPAIV H5 BB-genotype and suggested that the virus may have initially entered affected farms through infected wild birds (gull species; [83, 86]). Numerous individuals (American minks [*Neovison vison*], foxes [*Vulpes sp.*] and racoon dogs [*Nyctereutes procyonoides*]) developed clinical signs (lethargy, ataxia, tremors or diarrhea) and died [83, 86]. The swift spread in the affected farms cautiously suggested the possibility of HPAI H5 virus transmission between mammalian hosts although another common source, e.g. contaminated feed, could not be excluded either.

Infections with HPAIV H5 in mammals have been carefully recorded, because more effective adaptations of the virus to mammals and thus even to humans can occur. Investigations on the viruses of Spanish and Finnish fur animals revealed mutations within the PB2 segment, indicating a potential adjustment to mammalian hosts already reported in other infections in carnivorous species [69, 85, 87]. Given that mustelids such as ferrets (*Mustela putorius furo*) serve as model animals for influenza-related respiratory diseases in humans, any potential adaptations to these species are of the utmost public health concern [88].

2.1.3. Human spill over and human-avian-interfaces

The most recent clade 2.3.4.4b strains seem to remain highly adapted to avian hosts up until now. Still, there have been reports on human infections with HPAIV H5 strains, indicating spill-over from avian sources. Several AIV subtypes, including Gs/Gd HPAIV H5 of different clades, have been described to sporadically infect humans in Eurasia, Africa and recently in South America [89-91]. Clinically, disease in infected humans varies from asymptomatically infections to severe pulmonal dysfunctions exacerbating towards the acute respiratory distress syndrome and death. Noteworthy, elder HPAIV H5 strains that circulated until 2014 were responsible for a greater number of human cases associated with more severe clinical patterns and outcome, compared to strains of the 2.3.4.4b lineage. This is exemplified by more than 850 confirmed cases registered between 2003 and 2019 worldwide [92] associated with decreasing case fatality rates (CFR) over time from 60% (2003-2009), to 54% (2010-2014) and 30% (2015-2019). Within the last three years, the current 2.3.4.4b strains could be held responsible for a

total of 17 confirmed cases and 3 deaths (CFR 18%), representing a low zoonotic potential given the unprecedented vast number of animal cases during that period.

Retrospective epidemiological investigations of human cases generally revealed close contact of affected patients to infected birds. The perhaps most obvious possibility for direct or indirect contact with birds is represented within the poultry sector, along the chain of food production: Workers in farms (industrial, large-scaled farms or private backyard-farms), slaughterhouse workers or consumers when handling raw eggs and meat can be exposed. Numerous sporadic cases have been described, all pointing to spill over events originating from heavy exposure to infected poultry [89-91, 93]. Although circulation of HPAIV-contaminated poultry products in the food chain must be avoided by all means, such events have already been reported [94]. Contaminated feed for carnivorous pets and zoo animals likewise has given rise to clustered cases of HPAI in cats and large felids [81, 82].

Traditional hunting for waterfowl species seemed to pose a particular risk for transmission from hunted species to humans [95]. For falconry, i.e. hunting via a bird of prey species, different findings regarding the transmission of (HP)AIV from prey to predator species have been made by Khan, Shuaib, Rhman, et al. [96] and Kohls, Hafez, Harder, et al. [97], however no such transmission route is known so far for involved humans. Other studies describe the possible virus transmission from game birds to domestic chickens, pointing out the risk of an HPAIV H5 infection of e.g. backyard poultry via hunted waterfowl [98].

Another interface with avian species in captivity can be found in private holdings (e.g., exotic avian pets, hobby breeders) or zoological gardens (e.g., care takers, veterinarians). Hereby, the variation of species seems unlimited and covers representatives of dozens of different avian orders, many of them proven susceptible for AIV. The risk of HPAIV H5 incursions into zoological gardens or wild bird rescue centers has been well documented [63, 99, 100], including requirements for high biosafety measures when handling wild birds.

The latter is highly recommended for any interaction with wild birds, including free-ranging individuals, that are kept/handled for scientific purposes, such as bird ringing activities for potentially endangered species. Hereby, free-ranging birds are caught, marked/ringed and released back into nature to later on apply gained knowledge for e.g. species conservation efforts [101-103].

2.2. Diagnostic tools for disease control and their challenges

The demands for the diagnoses of an animal disease as HPAI are rapidity and precision to achieve fast and detailed information on the occurrence of (HP)AIV, their characterization in terms of subtype and

pathogenicity or induced host response (humoral immunity). Depending on the diagnosis, legally prescribed measures will be initiated for cases that require notification, along with additional surveillance or control measures.

Virus isolation represents the reference method for initial characterization of a novel AIV. For routine testing, meanwhile, a less time and resource intensive approach became established: the *detection of viral RNA* via reverse transcription polymerase chain reaction (RT-PCR), often in form of real-time RT-PCR (RT-qPCR). Hereby RNA extraction from clinical or field samples is followed by generic screening targeting fragments of the M1 or NP genes as highly conserved regions for IAV in general [104-106]. Subsequent specific tests allow the identification of H5 or H7, mainly targeting at the HA_2 region [107, 108]. Furthermore, combined (multiplexed or arrayed) RT-qPCR assays enable the simultaneous detection and identification of AIV, different subtypes (incl. NA), pathotypes and possible differential diagnoses such as Newcastle Disease [109]. Given the rapid mutation rate, especially for the HA segment [23], these methods are continuously adapted, to avoid cross-reactivity signals between subtypes or even false negative test results. In addition, reassortment events might include segments with so far unknown or LPAIV-associated segments, that requires a comprehensive validation of these assays. Still they remain limited for strains of different (avian) host species, temporal and spatial occurrence [109, 110].

Oligonucleotides used for diagnostic purposes, such as primers and probes for RT-qPCR, are selected based on available sequences to ensure the correct detection of chosen target regions. An approach by Hoffmann, Stech, Guan, et al. [104] focused on the conserved end regions of all IAV-segments and, thus, enabled the amplification of all eight segments with a universal primer set.

Whereas previously mainly partial genome sequences were generated via Sanger-sequencing, during the last years whole genome sequencing (WGS) via next generation sequencing (NGS) became common for AIV [111]. Hereby, especially nanopore platforms provide possibilities of high-throughput analyses in combination with rapid protocols [111].
In addition to in-depth characterization of the virus, the WGS aids to identify adaptations to different hosts, trends towards an increased zoonotic potential [20] and enables phylogenetic and phylogeographic analyses [28, 112].

Various AIV strains lead to various clinical manifestations. Therefore, not only the characterization of the virus itself, but also the *host immune response* is of particular interest. Again, generic screenings, targeting mainly antibodies against the NP, reveal prior exposure to IAV in general [20]. Hereby enzyme-linked immunosorbent assays (ELISA) applying monoclonal antibodies in competitive or blocking set-ups, allow for a species-independent identification of antibodies. Neutralizing antibodies mainly target

the HA surface protein [113]. There are commercial ELISA kits available for subtyping of HA antibodies, another common method is the hemagglutination inhibition test (HI). Known to be highly specific the HI requires careful choice of utilized antigens as mismatches can lead to possible test insensitivity [20].

The preferences of AIV for replication on mucosal surfaces within the respiratory or gastrointestinal system, point out the suitability for oropharyngeal/tracheal and cloacal swabs as reliable samples, taken from single individuals or pooled [105, 114]. In combination with the neurotropism of HPAIV, from deceased birds especially lung and brain samples should be considered for diagnostic screenings [61]. The latter is important in particular for the detection of viral RNA in carnivore hosts, since tracheal or rectal swabs might reveal low viral load or negative findings only [63, 66, 85], although, this might vary between clade 2.3.4.4b strains [84].

Furthermore, environmental samples (surface water, lake sediment or feces samples) can be applied for screenings on AIV. Although, often lower genome detection rates and fewer chances for virus isolation compared to direct avian sampling were reported, these methods delivered insights into the diversity of subtypes circulating among different wild bird species [115-117]. Nonetheless active surveillance based on environmental sampling approaches could not clearly reflect ongoing epizootics, as described by Ahrens, Selinka, Wylezich, et al. [115], and therefore passive surveillance on samples from deceased animals remained the current preference.

Collected swab and tissue samples are recommended to be instantly stored in a suitable medium, kept cool and dark. The storage conditions have to be chosen already with regard to the planned analyses [20], but also require compliance for biosafety standards and transport condition (e.g. virus cultivation medium vs. inactivating, but genome stabilizing buffers).

3. The HPAIV H5 Goose/Guangdong lineage

HPAIV H5 viruses currently affect wild bird populations and cause losses in poultry flocks almost worldwide, except for Antarctica and Australia/Oceania [118]. Those strains share a common HA H5 progenitor, that evolved in China at the end of the twentieth century.

3.1. Origin

In 1996, an isolate was retrieved from a goose with clinical signs in the Guangdong province in China in the context of an HPAI outbreak among domestic geese that revealed a morbidity rate of around 40% [119]. One year later a child succumbed to its influenza pneumonia in the bordering Special Administrative Region Hongkong, as causative agent an avian influenza virus was detected [120].

Although lacking a direct connection between both events, later on phylogenetic investigations revealed the human infection in 1997 caused by a genetically highly similar H5 virus, as detected one year before among the HPAI outbreak in the goose flock [119]. This finding marked the first confirmation of an HPAIV infecting humans and most likely representing the cause of severe illness and death [120]. Within the following months five from a total of 17 people that became infected with HPAIV H5 died [120]. The reports of human cases shortly after the first occurrence in poultry species (geese and chicken) and the high mortality rate in humans fueled discussions on the zoonotic potential of HPAIV H5 strains, suitable intermediate hosts and inter-species barriers [119-121]. In consequence, the global motivation for the surveillance of avian influenza viruses was strengthened, pursuing the idea to prevent spill-over events of HPAIV H5.

Genetic investigations on HPAIV H5 causing further outbreaks in Chinese chicken farms in 1997 showed, that despite the HA segment, the other seven genome segments did not match the Gs/Gd-strain, but genomes of further AIV circulating in that region [119]. Thus, genetic shift early exemplified the future challenges in handling this animal disease. It showed, that the first step of all control measures, the culling of the (potentially) affected poultry flocks [121], could not remain the only solution and highlighted the importance of sampling wild birds for proper monitoring.

Until the early years of the new millennium, findings of Gs/Gd-like HPAIV H5 occurred exclusively among poultry species or wild birds found dead in a closer surrounding to affected farm in Southeast Asia. Thus, wild birds were supposed to represent victims and dead-end hosts of spill-over-events [122, 123]. The developments in spring 2005 were all the more worrying when die-offs among wild birds were observed of different taxonomic orders (Anseriformes, Charadriiformes and Suliformes) caused by an HPAIV H5 around 2000 kilometers apart in a Western Chinese region at Qinghai Lake. Phylogenetic analyses still suggest an introduction from a poultry holding in Southeast Asia, but remarkably here a further adapted Gs/Gd-like HPAIV H5 was transmitted between wild birds, confirming aquatic waterfowl for the first time as a potential host and vector [123].

This observation marked a next milestone in the history of the spread of Gs/Gd-lineage HPAIV H5: If migratory wild birds might have to be considered as vectors of HPAI, this disease harbored the potential of going global [123]. In fact, these early descendants of the Gs/Gd HPAIV H5 were capable of spreading fast and viruses of this clade, termed 2.2, were the first HPAIV H5 of Asian origin that paved their way onwards to Europe [124].

3.2. Brief historical background of Gs/Gd-like descendants arriving in Europe

Following spring 2005, outbreak events were reported along Russia and its southern neighboring countries Kazakhstan and Mongolia, in poultry farms and wild bird species [125]. Not even one year after the outbreaks at Qinghai Lake (April 2005), HPAIV H5 of the Gs/Gd-lineage was deteted in Central Europe (February 2006) and can be held responsible for the first epidemic in Germany [126-128]. Strains of the same clade (2.2) re-emerged in a second wave in 2007, but have not been reported ever since in this country [127, 128].

Further HPAIV H5, all of which have the H5 Gs/Gd-ancestor in common, evolved, reassorted and subsequently were classified into the clade-nomenclature system by the WHO OIE FAO H5N1 Evolution Working Group [129]. Despite a single case in 2009 in a wild bird, Germany was spared from further incursions until 2014, when a new clade, 2.3.4.4, of HPAIV occurred at the Northern coast of Germany as its first detection in Europe (November 2014-January 2015) [125, 130]. It was described initially in a poultry holding, but was found as well among wild birds and affecting a zoological holding in Germany [100, 130, 131]. Again, these viruses showed close similarity with Asian HPAIV H5 strains, that split into two closely related clusters. King, Harder, Conraths, et al. [132] summarized the phylogenetic coherences, leading to co-existing "*sister lineages clustering within clade 2.3.4.4* [that were] (…) *subsequently split into group A, termed Donglim-like, aka 2.3.4.4a and group B, termed Gochang-like, aka 2.3.4.4b*" [132-134].

Although the scale of phylogenetic investigations was limited, one of the main hypotheses describes the introduction of HPAIV H5 2.3.4.4a-like strains via infected, but migration-competent wild birds from Asia to Europe [130]. This was strengthened by the contemporary occurrence of those viruses in North America [135]. Lee, Torchetti, Winker, et al. [136] investigated the possibility of HPAIV H5 clade 2.3.4.4a spread along waterfowl migration paths between their summer and winter grounds within Asia and between Asia and North America, and highlighted the concomitant findings of reassortment events with LPAIV [136-138]. Likewise, the Global Consortium for H5N8 and Related Influenza Viruses proofed the major involvement of long-distance bird migration for the spread of this avian disease globally [45]. Notably, the American HPAIV H5 epizootic of 2014/2015 remained limited to the North American continent [139].

The genetically distinct sister-lineage, clade 2.3.4.4b, reached Europe in 2016 [22, 130, 133, 134]. Besides numerous affected countries, Germany reported cases from November 2016 until March 2017 among wild birds, zoo birds and poultry holdings [140, 141]. In contrast, a second influx of clade 2.3.4.4b caused an epidemic in 2017/2018 mainly among wild birds (December 2017-May 2018). A comparably mild, third incursion occurred in in Germany in the first quarter of 2020 [112].

Gs/Gd-like descendants and reassortants dominate the HPAIV activity since 2016. The fifth wave of these strains reached Germany in October 2020 and persisted with single cases until August 2021 [69, 142]. The fifth epizootic in autumn/winter 2020/2021 led to two main consequences for the European and global HPAI situation:

3.3.HPAIV H5 (clade 2.3.4.4b) became enzootic in Northern Europe, 2021-2022

At the European level, HPAIV was still sporadically detected during summer 2021 [143]. The incidences increased again during autumn 2021, marking a starting point of the enzootic status of the virus gained in European countries. When in autumn 2021 cases in wild birds increased again, the questions arose, whether this was caused by flaring of over-summering HPAI H5 viruses among residential bird species or by new incursions via migratory bird species. Whereas on one hand reassortment of genotypes outside Europe in Africa and Russia was confirmed, a monophyletic sublineage (called B1) continued to circulate in wild birds in Northern European countries [144] and completed the first year of a HPAIV H5 enzootic during summer 2022 in Northern Europe [145, 146] (Figure 2).

This event was characterized by a presumably high virus prevalence in (certain) wild bird populations that resulted in high losses among wild and captive birds. Especially, partially endangered, seabird colonies were hit severely, involving mass mortality in several breeding colonies along shorelines of the Baltic and the North Sea [147, 148].

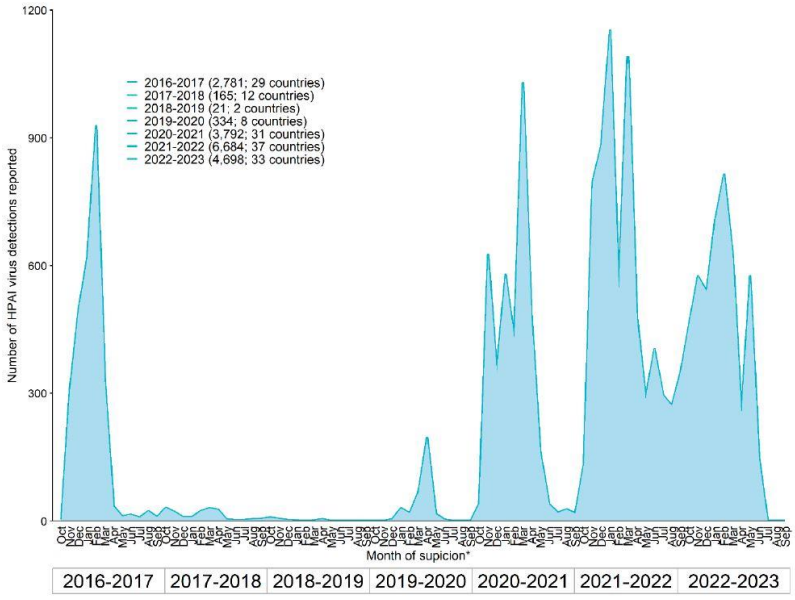

Figure 2 *Illustration of European reports on highly pathogenic avian influenza (HPAI) virus over time (1 October 2016 to 23 June 2023) as published in the "Avian influenza overview April – June 2023" by the European Food Safety Authority (EFSA) [48]. For permission rights see Appendix, legal permissions.*

3.4.HPAIV H5 of clade 2.3.4.4b became panzootic

Around the change of the year 2021/2022 HPAIV H5 viruses were confirmed to be the cause of high mortality rates among captive birds, mainly poultry and a deceased gull, in Newfoundland, Canada [149]. As to current knowledge, this was only the second introduction of HPAIV H5 viruses into North America. In 2015, Gs/Gd viruses had been introduced by migrating water birds via the Bering strait and spread along the Pacific coastline [150].

In December 2021, HPAI affected poultry farms were found along the Atlantic Ocean coast at the Eastern shore of North America (St. John's, Newfoundland and Labrador, Canada) [149]. Subsequent phylogenetic analyses revealed their affiliation with clade 2.3.4.4b, and a close similarity to European viruses, that circulated within the 2020/2021 epizootic in Europe [149]. In consequence, the HPAIV H5 progenitor of the North American strains was most likely circulating in Europe. Caliendo, Lewis, Pohlmann, et al. [149] questioned three main options, how wild birds could have been involved to enable the virus' transatlantic jump: incursions from European wild birds i) via Iceland, ii) via High Arctic or Greenlandic regions or iii) direct crossing of the Atlantic Ocean via the pelagic route – all possibilities remained unconfirmed at that time (Figure 3).

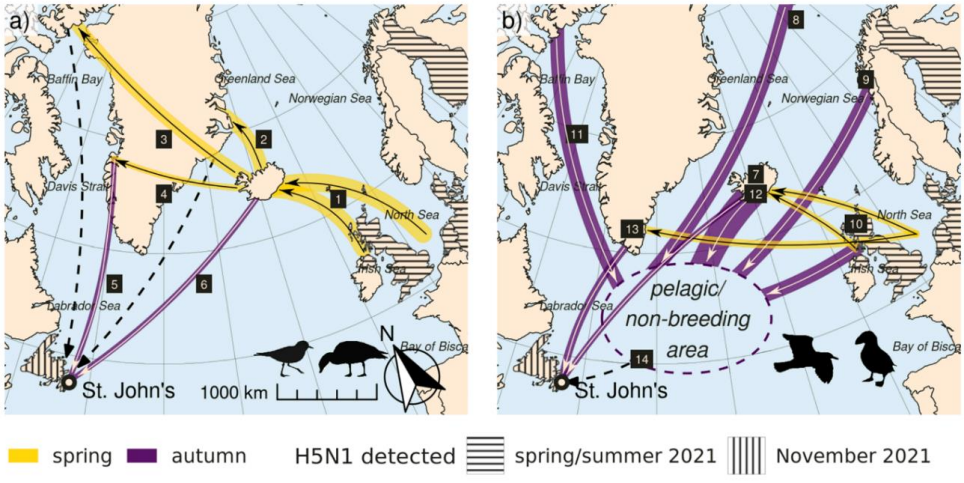

Figure 3 Map illustrating bird migration routes that were possibly involved in the transatlantic spread of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 to North America in winter 2021/2022 as originally published by Caliendo, Lewis, Pohlmann, et al. [149]. For permission rights see Appendix, legal permissions.

3.5.Outlook: HPAIV H5 (clade 2.3.4.4b) as a global threat

After the virus incursion during winter 2021/2022 and its maintenance in spring and summer, residential bird populations in Northern American countries mixed with avian individuals returning from over-wintering grounds in Middle and South America. In consequence, virus spread towards South America was enabled *vice versa* during fall migration, in 2022, followed by outbreaks in several Latin American countries [32, 71, 151, 152] (Figure 4). With respect to its established enzootic status in Europe, Asia and Africa, entailing economic losses in poultry farms, and endangering wild bird species conservation, Gs/Gd HPAI viruses have so far only spared Australia/Oceania [153] and the Antarctic region [154]. Populations of highly endangered species, such as Sandwich tern (*Thalasseus sandvicensis*), bald eagle (*Haliaeetus leucocephalus*) or Californian condors (*Gymnogyps californianus*) have already suffered badly from HPAIV H5. In particular, the Antarctic region is at extraordinary high risk of pathogen incursion. As described for other AIV subtypes (H6N8), migrating avian species, such as brown skuas (*Stercorarius antarcticus*) might bridge South American and Antarctic regions as vectors and, thus, might even enable the introduction of current HPAIV H5N1 strains into resident Antarctic bird populations [155]. The close geographical distance and recent outbreaks in the southern regions of South America are depicted in Figure 5.

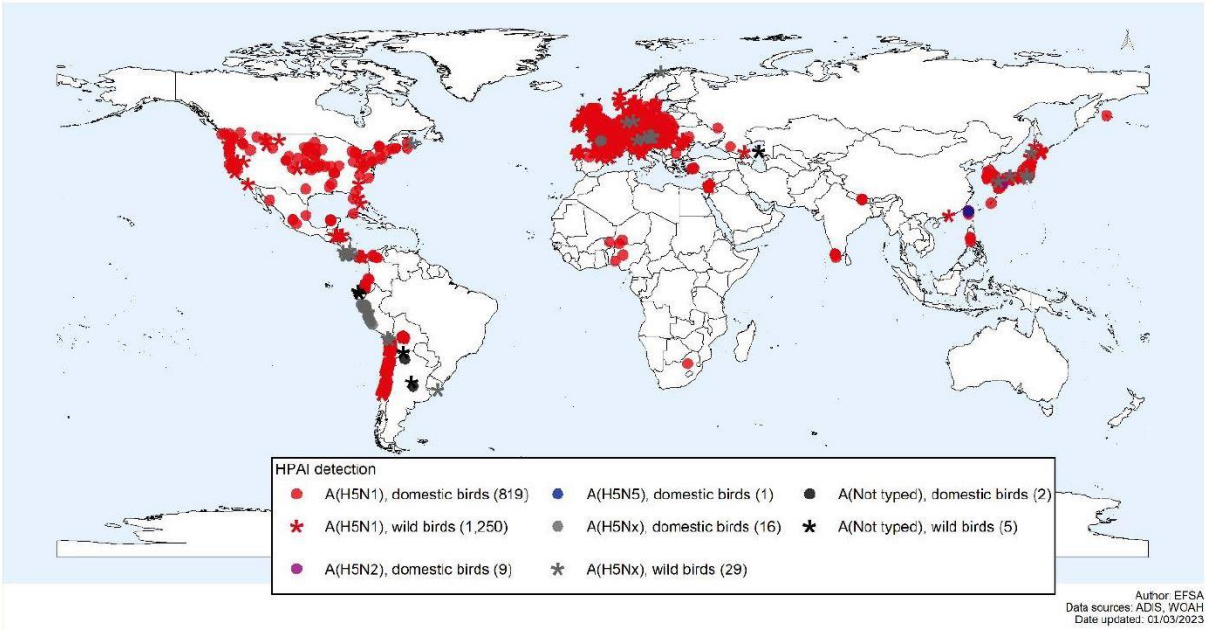

Figure 4 *Illustration of the global spread of highly pathogenic avian influenza viruses from December 2022 to March 2023 as published in the "Avian influenza overview December 2022 – March 2023" by the European Food Safety Authority (EFSA) [32]. For permission rights see Appendix, legal permissions.*

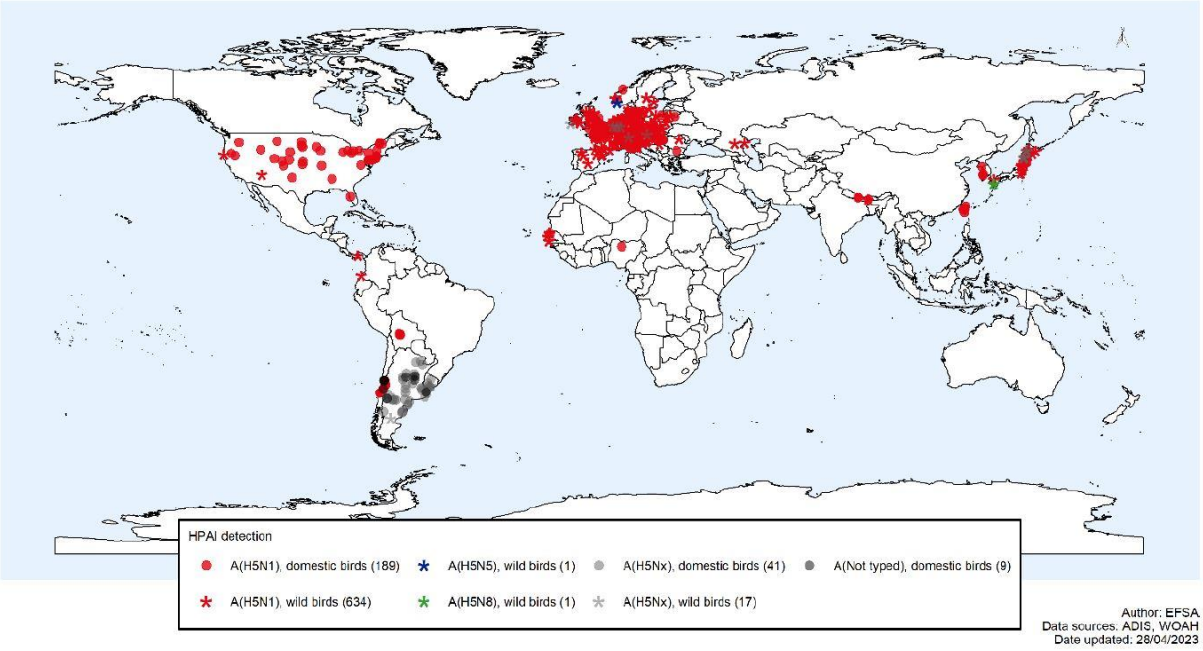

Figure 5 *Illustration of the global spread of highly pathogenic avian influenza viruses from March 2023 to April 2023 as published in the "Avian influenza overview March – April 2023" by the European Food Safety Authority (EFSA) [44]. For permission rights see Appendix, legal permissions.*

4. Other viral pathogens with zoonotic potential in European bird populations

Despite a current omni-presence of HPAIV in wild birds and concerns of interspecies transmission to mammals, including humans, it should not be neglected that there are further viruses circulating in wild bird populations. For European wild bird populations, the most relevant pathogens belong to the group of arthropod-borne (arbo) viruses: West Nile virus (WNV) and Usutu virus (USUV), likewise with certain zoonotic potential. Both viruses belong to the genus of *Flaviviruses* within the family of *Flaviviridae*. As many representatives of this family, both are maintained within an enzootic cycle between an avian host and (mainly ornithophilic) mosquitoes. Further characteristics are described in Table 1.

Both arbo-viruses depend on the activity of their vector species, which explains their peak occurrence in the summer months. This is in contrast to HPAIV H5, which used to occur mainly during the winter months but has since exhibited a trend of continuing into year-round presence. The enzootic trend of HPAI H5 viruses places Central European countries like Germany at a special position, with the possible simultaneous presence of orthomyxoviruses and flaviviruses in wild birds. So far, nationwide surveillance approaches focused on either HPAIV H5 *or* USUV and WNV, as summarized in various reports including the announcement of their first detections in German bird populations and holdings: HPAIV H5 clade 2.2. in 2006 [127], respectively clade 2.3.4.4 in 2014 [45]; USUV (Europe 3 lineage) in 2011 [156] and WNV (lineage 2) in 2018 [157].

Table 1 *Brief comparison of highly pathogenic avian influenza virus (HPAIV) of subtype H5 of the Goose/Guangdong (Gs/Gd)-lineage, Usutu virus (USUV) and West Nile virus (WNV) with regard to their classification, structure, geographical origin, occurrence in Germany, transmission routes and host species, including clinical appearance in humans.*

	HPAIV H5 (Gs/Gd-lineage)	USUV	WNV	References
Virus classification (family, genus)	Orthomyxoviridae, Alphavirus	Flaviviridae, Flavivirus		[1, 2, 158, 159]
Genome structure	Negative-sensed single stranded RNA, Segmented RNA	Positive-sensed single-stranded RNA		
Genome size (base pairs, bp)	Approximately 13.5 kbp	Approximately 11 kbp		[159, 160]
Virion characteristics	Enveloped spherical (100-120 nm) or filamentous (length of >300 nm)	Enveloped Spherical (50 nm)		[159, 160]
Originating from	Asia	Africa		[119, 161, 162]
Affected host groups (based on RNA confirmation)	Wild birds, captive birds, including poultry species, mammals			Examplatory: [76, 132, 163-169]
Main reservoir	for LPAIV: Wild aquatic waterfowl and shorebird species	Mainly Passeriformes		[23, 170, 171]
Transmission route	Feco-oral and direct contact	Endemic cycle between mosquito (mainly *Culex sp.*) and avian host, possibly direct contact		Examplatory: [23, 165]
Possible symptoms in humans	Mild respiratory symptoms, severe flu symptoms possible (current HPAIV H5N1 strains, at this time)	fever, asthenia, myalgia, headache	Flu-like clinical picture including fever, in rare cases development of West Nile Neuroinvasive Disease (WNND)	Examplatory: [48, 91, 172, 173]

III. Study Objectives

Objective I: **Exploring the occurrence and impact of HPAIV H5 (clade 2.3.4.4b) in avian raptor species in Northern Europe** *Publications I, II, III*

Since 2006, the occurrence of Gs/Gd HPAIV H5 has been associated with the migration of aquatic wild birds and manifested in occasional epizootics during winter months. This pattern changed when current HPAIV H5 strains of clade 2.3.4.4b became enzootic in wild bird populations of northern European countries. Here, the resulting threats of a year-round presence of HPAI for hunting and scavenging bird species was examined. (i) There is an increased risk of exposure due to potentially increased availability of HPAIV-infected prey and (ii) there is an overlap of viral activity with the breeding season, exposing novel age groups (nestlings) to the pathogen, with yet unknown future effects on species conservation.

Objective II: **Tracking the panzootic spread of HPAIV H5 activity (clade 2.3.4.4b) by utilizing raptor samples for whole-genome sequencing (WGS)** *Publications III, IV, V*

In winter 2021/2022, a transatlantic spread of HPAIV H5 from Europe to North America has been described, however, the exact routes of transmission initially remained unclear. By examining samples of white-tailed sea eagles (*Haliaeetus albicilla*) from Iceland, we retrospectively confirmed that Iceland was a stopover for the now panzootic HPAIV H5 (clade 2.3.4.4b). This demonstrates how surveillance of raptor species, used here as an indicator of virus spread, could serve as an early warning system to increase awareness and preparedness for viruses with particular zoonotic potential and high economic impact.

Objective III: **Outlook on the suitability of raptor species as indicators for further emerging viral pathogens with zoonotic potential** *Publication VI*

Zoonotic arbo-viruses such as West Nile virus or Usutu virus have recently emerged as another important group of avian pathogens in European wild bird populations. In combination with the enzootic HPAIV H5 in Northern European countries, Germany is now facing the challenge of a possible co-circulation of these pathogens. Identifying temporal, geographic and, potentially, species-specific overlaps in their occurrence, might foster harmonized and more efficient surveillance of key wild bird species for avian diseases with zoonotic potential.

IV. Results

The publications included in the results section of this thesis are listed according to their respective study objectives. Their reference sections and the numbering of tables and figures are not repeated at the end of this thesis and remain presented in the respective publication style. All publications are labelled with their respective Digital Object Identifier (DOI) so that supplementary material can be retrieved even if it is not included in the results section of this work.

Publication I: „Red knots in Europe - a dead end host species or a new niche for highly pathogenic avian influenza?“

Publication I

Red knots in Europe - a dead end host species or a new niche for highly pathogenic avian influenza?

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MICROBIOLOGY SOCIETY
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Red knots in Europe: a dead end host species or a new niche for highly pathogenic avian influenza?

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Abstract

The 2020/2021 epidemic in Europe of highly pathogenic avian influenza virus (HPAIV) of subtype H5 surpassed all previously recorded European outbreaks in size, genotype constellations and reassortment frequency and continued into 2022 and 2023. The causative 2.3.4.4b viral lineage proved to be highly proficient with respect to reassortment with cocirculating low pathogenic avian influenza viruses and seems to establish an endemic status in northern Europe. A specific HPAIV reassortant of the subtype H5N3 was detected almost exclusively in red knots (*Calidris canutus islandica*) in December 2020. It caused systemic and rapidly fatal disease leading to a singular and self-limiting mass mortality affecting about 3500 birds in the German Wadden Sea, roughly 1% of the entire flyway population of *islandica* red knots. Phylogenetic analyses revealed that the H5N3 reassortant very likely had formed in red knots and remained confined to this species. While mechanisms of virus circulation in potential reservoir species, dynamics of spill-over and reassortment events and the roles of environmental virus sources remain to be identified, the year-round infection pressure poses severe threats to endangered avian species and prompts adaptation of habitat and species conservation practices.

DATA AND MATERIALS AVAILABILITY

All sequencing data has been deposited in the GISAID EpiFlu sequence database and can be found under the accession numbers listed in Table S1 (available in the online version of this article).

BACKGROUND

Between mid- and end-December 2020, embedded in the most devastating and protracted highly pathogenic avian influenza (HPAI) clade 2.3.3.4b H5 epidemic until then in Germany, Europe and beyond, thousands of red knots (*Calidris canutus*) were found dead or dying along the shores or birds falling literally dead from the sky in the German Wadden Sea.

Red knots, a bird species belonging to the snipe family (*Scolopacidae*), are considered one of the very few species of *Charadrii* with evidence of high rates of previous avian influenza virus (AIV) infection [1]. Two subspecies of red knot occur in Europe: the *canutus* subspecies breeding in Siberia with a population of app. 250000 individuals (2010–2014) and the *islandica* subspecies breeding in Greenland and Canada with a population of 310000–360000 individuals (2013–2017 [2]). The *canutus* subspecies only visits the Wadden Sea areas of the southern North Sea en route to Western Africa during its autumn (mainly July–August

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Keywords: H5N3; highly pathogenic avian influenza (HPAI); mortality; reassortant; red knot (*Calidris canutus islandica*).
Abbreviations: AIV, avian influenza virus; Ct value, cycle threshold value; gs/GD, goose/Guangdong; HA, haemagglutinin; HACS, hemagglutinin cleavage site; HP, highly pathogenic; HPAI, highly pathogenic avian influenza; HPAIV, highly pathogenic avian influenza virus; LPAIV, low pathogenic avian influenza virus; MCC, maximum clade credibility; ML, maximum likelihood; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, non-structural; Oph-cl, oropharyngeal-cloacal; PA, polymerase acidic; PB, polymerase basic; RT-qPCR, reverse transcription real-time (quantitative) PCR.
Eight supplementary figures and one supplementary table are available with the online version of this article.

and October) and spring migration (May), while a proportion of the *islandica* subspecies may spend also the entire winter season from November to February in the Wadden Sea [3–6]. The Wadden Sea in central Europe is one of the most important staging sites for waders such as red knot worldwide, and according to a regular monitoring scheme, about 75% of the flyway population of red knots uses this area, i.e. a maximum of ca. 350000 individuals (both subspecies together [5]). Red knots use high tide roosts in large flocks in various places along the entire Wadden Sea coast [6]. While the trend of the *canutus* population in the Wadden Sea is stable (but in Schleswig-Holstein continuous declining since the end of the 1990s), the long-term trend 1987/1988–2019/2020 of the *islandica* population in the Wadden Sea remains negative, mainly due to the decline in the Schleswig-Holstein part, and the short-term trend 2010/2011–2019/2020 is stable [5]. In the European Red List of Birds, the red knot is classified in the category 'near threatened' [7]. In the Wadden Sea area, red knots are exclusively molluscivore and known to primarily feed on Baltic tellins (*Limecola balthica* [8]).

Wild birds of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* [gulls and allies (*Lari*), waders (*Charadrii*) and auks (*Alcae*)] are considered the main reservoir of AIV. In Europe, the majority of low pathogenic avian influenza viruses (LPAIV) have been found in water birds, particularly dabbling ducks [9]. Prevalences of LPAIV grossly vary in shorebirds in different regions globally, while in Europe, AIV have only sporadically been found in waders, and LPAIV infections appear to be more widespread in *Charadrii* birds in other regions of the world [10–12]. A particular AIV hotspot in waders has been reported in North American Delaware Bay, one of the largest wintering and stop-over sites of shorebirds globally [13].

Prior to the emergence of HPAI Goose/Guangdong/1996 (Gs/Gd) lineage, isolation of HPAI viruses from *Charadriiformes* was reported only once in 1961, when an HPAIV [A/tern/South Africa/61 (H5N3)] caused the death of more than 1000 common terns (*Sterna hirundo*) in South Africa [14].

After the emergence and continuous evolution of Gs/Gd HPAI H5 viruses, birds of the order *Charadriiformes* sporadically were found infected. Clade 2.3.4.4b of the Gs/Gd lineage was specifically successful in spreading to Europe and Africa since 2016. The lineage caused a major epidemic in white-winged terns (*Chlidonias leucopterus*) along the shores of Lake Victoria, Africa, in 2017 [15].

The 2020/2021 HPAI H5 epidemic in Europe surpassed all previously recorded European outbreaks in size, genotype constellations and reassortment frequency, with records of over 3500 cases of lethally affected wild birds reported with laboratory-confirmed HPAIV H5 infection from 28 countries [16]. Many 10 000 of wild birds likely succumbed to infection without being virologically diagnosed. The 2.3.4.4b lineage proved to be highly promiscuous with respect to reassortment with cocirculating LPAIV. During the winter/spring season of 2020/2021 and continuing in 2022, several subtypes including H5N8, H5N1, H5N5, H5N4 and H5N3 and more than 30 genotypes have been identified in Europe [17, 18]. While most sub- and many genotypes affected a range of both poultry and wild bird species, a unique HPAI H5N3 reassortant virus caused unusually high mortality rates only within a specific migratory wading bird niche, almost exclusively affecting red knots.

Here, we describe the brief but disastrous epidemic of a HPAIV clade 2.3.4.4b H5N3 reassortant in December 2020.

METHODS

Dead birds at the SchleswigHolstein Wadden Sea National Park were collected and documented by rangers. While the birds were safely disposed by veterinary authorities, a batch of 513 individual red knot carcasses was collected in December 2020 and kept frozen until August 2021 when they were dissected and assessed macroscopically under Biosafety Level 3 at the Friedrich-Loeffler-Institut, Isle of Riems in Germany.

Organ samples and combined oropharyngeal-cloacal swabs for virological analyses were collected during post-mortem examination of ten randomly selected red knot carcasses representing 2% of the total of 513 dissected red knots. RNA-extraction from homogenized tissue material and swab sample medium was carried out as described by Koethe *et al.* [19]. Initially all RNA samples were tested for avian influenza virus genome applying generic, reverse transcription real-time PCR (RT-qPCR) targeting the influenza A virus nucleoprotein (NP), including an internal control assay [20, 21]. In a subsequent step for each individual carcass, one RNA-sample with the lowest cycle threshold value (Ct value) in NP-qPCR was applied for further sub- and pathotype analyses using further previously described RT-qPCRs (RITA) [22]. The limit of detection of the subtyping PCRs is similar to that of the generic RT-qPCRs used and ranges between 25 and 250 per reaction [22].

To pinpoint the virus target tissue and cell tropism, immunohistochemistry was performed for virus antigen detection using a primary antibody against the matrix 1 protein of influenza A virus (ATCC clone HB-64) as specified, and the distribution of viral antigen was recorded in an ordinal scoring scale (score 0–4) as described by Blaurock *et al.* [23].

Viral full-genome sequences were generated from three red knots collected in Germany-Schleswig-Holstein and one red knot from Germany, Lower Saxony. Full-genome sequencing of AIV-positive samples was executed by a previously described nanopore-based real-time sequencing method with prior full genome amplification [24]. For this, RNA extraction with the Qiagen Mini Viral Kit (Qiagen, Germany) and subsequent genome amplification with universal AIV-End-RT-PCR using Superscript III

One-Step and Platinum Taq (Thermo Fisher Scientific, USA) with one primer pair (Pan-IVA-1F: TCCCAGTCACGACGTCGTAG CGAAAGCAGG; Pan-IVA-1R: GGAAACAGCTATGACCATGAGTAGAAACAAGG), binding to the conserved ends of the AIV genome segments, was conducted. After purification of the PCR products with AMPure XP Magnetic Beads (Beckman-Coulter, USA), full-genome sequencing on a MinION platform (Oxford Nanopore Technologies, ONT, UK) using Rapid Barcoding Kit (SQK-RBK004, ONT) for transposon-based library preparation and multiplexing was performed. Sequencing was directed according to the manufacturer's instructions with a R9.4.1 flow cell on Mk1C device with MinKNOW Software Core (v4.3.11). Live basecalling of the raw data with Guppy (v5.0.13, ONT) was followed by a demultiplexing, quality check and trimming step to remove low quality, primer and short (<50 bp) sequences. After sequencing, full-genome consensus sequences were generated in a map-to-reference approach utilizing MiniMap2 [25]. Reference genomes are a curated collection of all haemagglutinin (HA) and neuraminidase (NA) subtypes alongside an assortment of internal gene sequences chosen to cover all potentially circulating viral strains. Polishing of the final genome sequences was done manually after consensus production according to the highest quality (60%) in Geneious Prime (v2021.0.1, Biomatters, New Zealand). For phylogenetic analysis, sequences from EpiFlu were retrieved where search was restricted to clade 2.3.4.4b H5N3 sequences or Eurasian non-GS/GD collected June 2019–May 2021. Respective accession numbers and data source acknowledgement can be found in Table S1 (available in the online Supplementary Material).

Segment specific multiple alignments were generated using MAFFT (v7.450) [26], and subsequent maximum likelihood (ML) trees were calculated with RAxML (v8.2.11) [27] utilizing model GTR GAMMA with rapid bootstrapping and search for the best scoring ML tree supported with 1000 bootstrap replicates. Time-scaled trees of concatenated genomes of the same H5N3 genotype were calculated with the BEAST (v1.10.4) software package [28] using a GTR GAMMA substitution model, an uncorrelated relaxed clock with a lognormal distribution and coalescent constant population tree models. Chain lengths were set to 10 million iterations and convergence checked via Tracer (v1.7.1). Time-scaled summary maximum clade credibility trees (MCC) with 10% post-burn-in posterior were created using TreeAnnotator (v1.10.4) and visualized with FigTree (V1.4.4). The robustness of the MCC trees was evaluated using 95% highest posterior density confidence intervals at each node and posterior confidence values as branch support.

RESULTS

Mass die-off of red knots

In Germany, more than 16 000 deceased or moribund waders and waterfowl had been identified in the Wadden Sea area of Schleswig-Holstein (predominantly in the district of Nordfriesland) between 25 October 2020 and end of March 2021 (Fig. 1). Among all avian species, the highest number of HPAI cases was found in Barnacle geese (*Branta leucopsis*, 46%), red knots (21%) and Eurasian wigeons (*Mareca penelope*, 10%) (Figs 1 and 2). While other species were found dead in high numbers throughout longer periods, 3329 red knots were found dead mainly within only a short period between 14 and 23 December 2020 (Fig. 2).

The mass die-off in red knots occurred at a high tide roost on the peninsula of Nordstrand in the federal state of Schleswig-Holstein, Germany (54° 29′ 15″ N; 8° 49′ 04″ E; Fig. 1). Although both subspecies of the red knot cannot be distinguished securely morphologically, the virus outbreak described in this study affected exclusively individuals of the *islandica* subspecies as it took place in late December and therefore affected wintering birds. Most of the birds were already dead on the beach, while a few others were still able to fly and suddenly dropped dead from the sky to the ground. Other individuals were apathic and showed clear signs of neurological disorder with no escape reactions. In addition to red knots, only a common buzzard in the same region and time was detected with the same virus sub- and genotype, HPAIV H5N3. All other dead water birds were mainly HPAIV H5N8 positive at that time.

Necropsy

A batch of 513 individual red knot carcasses was dissected and assessed macroscopically. Of these, around 80% were adults and 20% were juveniles in their first calendar year. The sex distribution was uniform among adults and juveniles, respectively (overall 49% of females and 51% of males were found). The macroscopic inspection of the 513 individuals showed a good to moderate body condition (according to Camphuysen and van Franeker (2007))[29]. A common finding was a nearly empty gut and a varying chest muscle thickness. In none of the individuals, the gut showed macroscopic lesions, whereas there were 10 (2%) individuals showing lesions of the liver. Furthermore, a total of 334 individuals (65%) showed internal bleeding in the lung, and another total of 278 individuals (54%) had kidneys that were coloured whitish or were covered with white dots.

Virologic and immunohistochemical results

As shown in Table 1, influenza A virus genome was detected in every tested individual bird in at least eight out of nine tissue samples collected. Ct values ranged from 12.8 to 38.5. All individuals were confirmed to harbour HPAIV H5N3 RNA. The most prominent viral loads were detected in brain samples. Consistently, viral loads were also found in all ten oropharyngeal/cloacal swab samples, assuming virus shedding, although no virus isolation was conducted. In accordance with virus genome detection data, the brain was consistently affected (10 out of 10 birds). Further, the air sacs (8/10), the heart (7/10) and the kidneys (6/10)

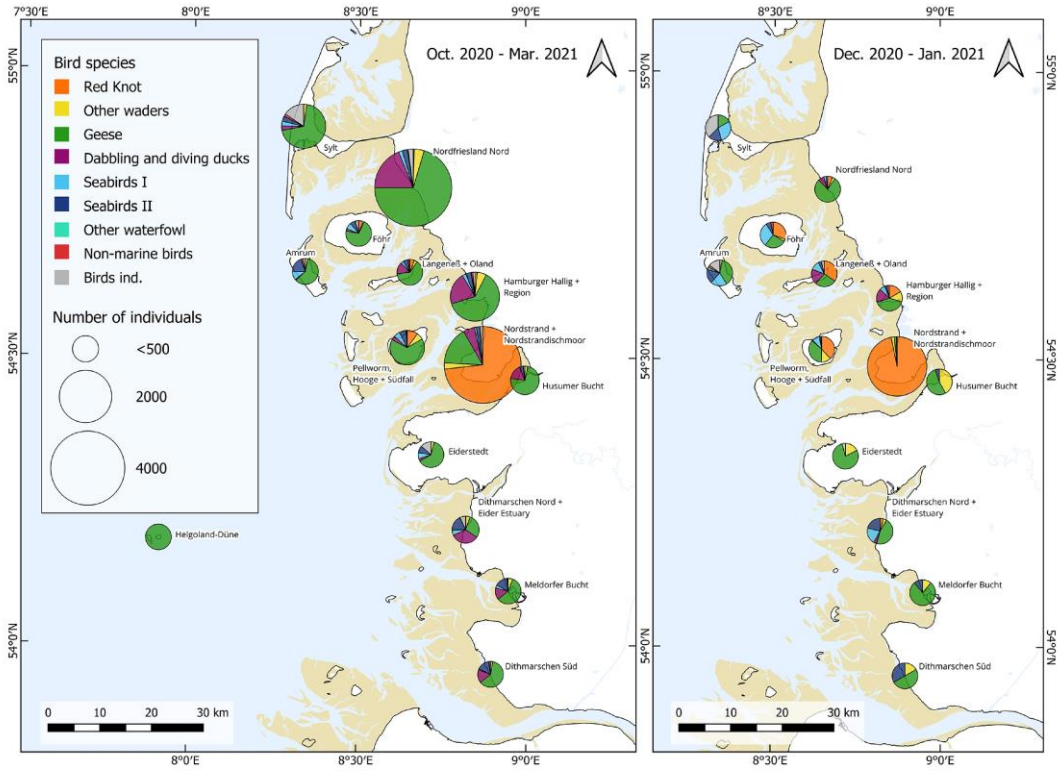

Fig. 1. Location and species composition of collected dead birds in the period October 2020 to end of March 2021 (left) and during the mass die-off in the period of 15 December 2020 to 15 January 2021(right) in the northern German Wadden Sea. Note: red knots are marked in orange. Seabirds I: seaducks, mergansers, grebes, tubenoses, gannets, cormorants, auks. Seabirds II: gulls, skuas, terns.

regularly exhibited virus antigen. Individual animals (1 or 2/10) showed virus protein labelling in the liver, lung, pancreas, large intestine and skeletal muscle. No antigen was found in the proventriculus, gizzard and small intestine. The identified target cells comprised predominantly tissue-specific epithelial cells (parenchyma); however, some red knots presented with focal endotheliotropism or infection of smooth muscle cells. Details on target cells and antigen distribution are shown in Table 2, while representative tissue slides are shown in Fig. 3.

Phylogenetic analysis

The goal of our phylogenetic analysis was to reconstruct the evolutionary relationship of HPAI H5 viruses and to identify common ancestors and the patterns of divergence over time. The search of clade 2.3.4.4b H5N3 sequences in the phylogenetic databases yielded a total of 13 genome sequences comprising HPAIV from 'red knots' (*n*=5), 'knot waders' (*n*=2), 'Eurasian curlew (*Numenius arquata*)' (*n*=1), 'common buzzard (*Buteo buteo*)' (*n*=2), 'peregrine falcon (*Falco peregrinus*)' (*n*=2) and 'common kestrel (*Falco tinnunculus*)' (*n*=1). Samples were collected in Denmark (*n*=1, 'common kestrel'), France (*n*=2, 'red knot', 'curlew'), Ireland (*n*=2, 'knot wader'), Northern Ireland (*n*=2, 'peregrine falcon'), The Netherlands (*n*=2, 'common buzzard') and Germany (*n*=4, 'red knots'). Exact sample details can be found in Table S1.

All respective H5N3 viruses belong to the H5 clade 2.3.4.4b while carrying a polybasic hemagglutinin cleavage site (HACS; PLREKRRKRGLF), thus fulfilling the legal criteria for high pathogenicity. Both the HA and matrix protein are highly similar to the previously described lineage of HPAI H5N8 viruses circulating in Central Europe from October 2020 onwards [30]. All further six segments point towards reassortment with Eurasian avian lineage LPAIV genes as indicated by phylogenetic analyses of each the eight segments from a broad set of viruses covering geographic locations of Eurasia affected by HPAI (Supplementary Material; for the NA-gene, only NA3 genes were considered). The results are summarized in Fig. 4. Closest relatives of the polymerase segments (polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA)) were found in LPAI from wild birds, poultry, environmental and mixed samples across Europe from fall 2020 to early 2021 (PB2 and PB1 in A/

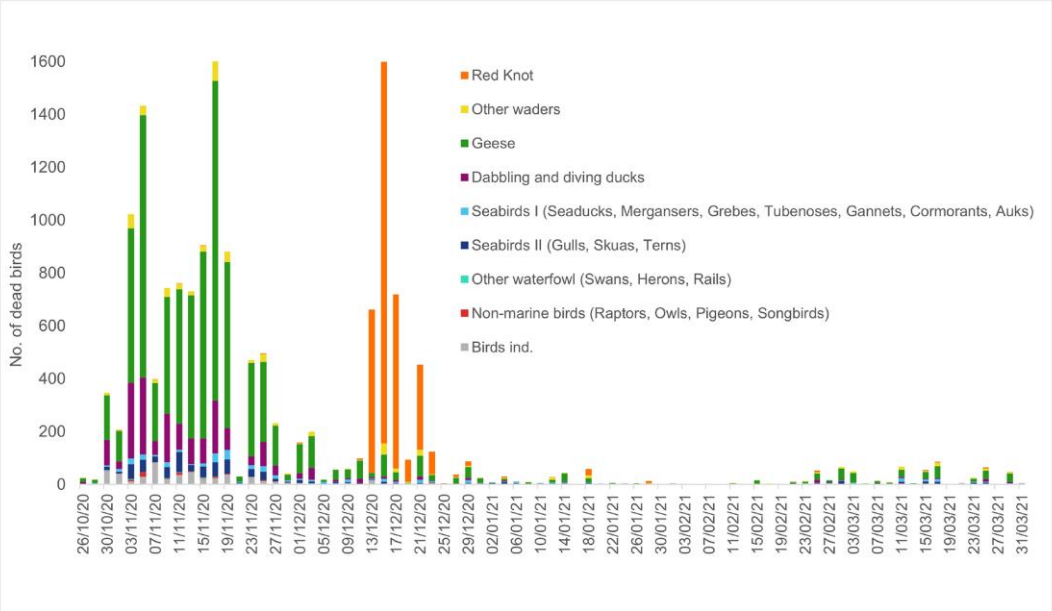

Fig. 2. Number of dead birds allocated to different bird groups recorded in the Schleswig-Holstein Wadden Sea area within the period October 2020 to end of March 2021.

turkey/Germany-BB/AI00868/2021, A/mallard/France/20P017917/2020, A/turkey/England/018179/2021; PA in A/environment/England/030642/2020). The NP segment was previously detected in a LPAIV H5N8 identified in Germany in September 2020 (A/guinea fowl/Germany-NW/AI01184/2020). The NA, NP and non-structural (NS) segments are most closely related to LPAIV genes detected in Europe over the past few years (A/mallard/France/20P017917/2020 (H5N3), A/turkey/England/018179/2021 (H5N3); NS in A/mallard/Denmark/12946-11/2020 (H7N5) A/Anas_platyrhynchos/Belgium/10413_0003/2020 (H5N2)). The H5N3 reassortant was one of several reassortment events resulting in different HPAIV subtypes [17].

Segment-wise phylogenetic analyses of a broad set of sequences representative of different host species and geographical locations affected by HPAI H5 viruses indicate, in addition to high similarities over the entire genome range between 99.85 and 99.55% identity, a singular reassorting event generating the common ancestor of all HPAI H5N3 viruses from Germany analysed here. This timeframe fits to the result of the Bayesian phylogenetic inference analyses where the ancestral virus was calculated to have emerged in early November 2020.

DISCUSSION

The current study describes the first outbreak of a new reassortment of an HPAI virus subtype H5N3 of clade 2.3.4.4b causing a mass mortality in December 2020 in a group of *Charadrii* bird species in the German Wadden Sea that was never affected before in such high numbers. Mass die-offs of various bird species in the Wadden Sea have been reported before and were mainly attributed to food shortages [31, 32] and the effects of severe weather [31, 33]. Large botulism outbreaks occurred in the Elbe estuary in the 1980s and mid-1990s [34]. In 2019, *Vibrio cholerae* was identified as a cause of high chick mortality here [35]. In winter 2016/17, a first major avian influenza outbreak in wild birds (mainly wigeons) was detected in the Dutch Wadden Sea [36]. Otherwise, monitoring of dead birds in the Wadden Sea in the past years has revealed a low degree (incidences<5%) of avian influenza infection [37].

The flyway population of red knots of the subspecies *islandica* is estimated 310 000–360 000 individuals [2] and shows a long-term declining trend which is currently recovering slowly since 2015 [5]. The mortality described in this study affected around 3500 red knots (which must be regarded as a minimum number given that likely not all dead individuals were found and accessible to counting) in the federal state of Schleswig-Holstein in Germany alone. Hence, the mass mortality event impacted around 1.0–1.1% of the whole flyway population. The exact numbers of red knots spending the winter in the Wadden Sea of the federal

Table 1. (a) Distribution of viral genomic load based on NP2-RT-qPCR resp. H5-RT-qPCR in organ samples and/or swab samples of red knots found dead in Germany. (b) Sub- and pathotyping of influenza A virus RNA detected in brain samples of ten red knot carcasses collected in Germany

(a) Red knot carcasses										
Tissue sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Brain	+	+	+++	+++	+++	+++	+++	+	+++	+++
Heart	(+)	+	+++	+++	+++	++	++	+	+	+
Kidney	(+)	+	+++	+++	++	++	++	+	+++	(+)
Liver	N.A.	(+)	++	(+)	++	(+)	(+)	(+)	+	(+)
Lung	(+)	+	++	++	++	++	++	++	++	+
Duodenum	(+)	+	+	+	+	+	+	(+)	(+)	(+)
Laying guts	(+)	++	+++	++	++	N.E.	++	N.E.	+++	+
Feather follicle	(+)	(+)	+	–	(+)	N.E.	(+)	–	–	–
Oph-cl swab	(+)	(+)	+	+	+	+	(+)	+	+	(+)
(b) Brains of Red knot carcasses										
RT-qPCR	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
H5	++	++	+++	+++	+++	+++	++	++	+++	+++
N3	++	++	+++	+++	+++	+++	++	++	+++	+++
HP-2.3.4.4b	++	++	++	+++	+++	+++	++	++	+++	+++

Influenza A virus generic RT-qPCR results of red knots no. 1–10 shown as Ct values: –, ≥40 (negative); (+), ≥30–40; +, ≥25–30; ++, ≥20–25; +++, ≤20. Red print indicates samples chosen for HP H5 subtype-confirmation. Liver sample of red knot no. 1 was not analysable. Feather sample of red knot no. 6 was not examined (N.E.). Sex: #6, #8 male, all other birds female.
RT-qPCR results for further sub- and pathotyping from chosen IAV-positive samples. Ct values are shown as described in Table 1a.

state of Schleswig-Holstein are unknown, but are estimated several ten thousand individuals (K. Günther, pers. comm.). Thus, the affected birds likely comprise a significant proportion of the population of wintering red knots in the Wadden Sea.

More than 500 red knots were subsequently dissected. No distinct pathological findings were present and birds seemed to be in good condition which may indicate a peracute course of the fatal disease which also was described earlier in an experimental infection with a HPAI H5 virus in red knots [38]. A randomly selected number of samples taken for virological examinations yielded exclusively positive results indicating systemic HPAIV H5N3 infection. Accordingly, it can be assumed that all the red knots died of that infection. Furthermore, contemporary reports of mortalities of red knots being positive for HPAIV H5N3 in the Netherlands, France and the UK indicate a supraregional epidemic although significantly smaller numbers of red knots were impacted.

Red knots are known to use high tide roosts together with other *Charadriiformes* [6]. At the same time and location other species, in particular barnacle geese, but in smaller numbers, also dunlins and curlews were found dying from HPAIV infection, but they tested positive for other H5 subtypes, mostly HPAIV H5N8. The HPAIV H5N3 reassortant was almost exclusively found in the red knots with the exception of buzzards, falcons and a kestrel that are assumed to have scavenged on the infected red knots. A single reassortment pathway involving gene segments from different HPAI H5 and LPAI viruses generated the common ancestor of all analysed HPAI H5N3 viruses from Germany. It was calculated that the ancestral virus emerged in early November 2020 and subsequently spread supra-regionally to other parts of northern and western Europe, but obviously on a smaller scale. After this event, this specific reassortant has not reappeared.

Red knots form dense social groups, both on their high tide roosts and within their foraging sites [39]. This might have facilitated intra-specific infection and restricted spread to other species. Other wader species such as oystercatchers (*Haematopus ostralegus*) that were largely spared from the epidemic are known to seek out (and defend) their own foraging sites on the intertidal mudflats separate from red knots [40–42].

Former studies have shown that red knots in the Wadden Sea are highly mobile and may switch between foraging sites and high tide roosts, respectively, in short periods of time [43, 44]. In addition, virological investigations confirmed viral loads in oropharyngeal and cloacal samples suggesting virus shedding and transmission. Therefore, the question arises, why the virus did not spread to other places of the Wadden Sea and particularly to other high tide roosts in the vicinity to a greater extent. High

Table 2. Virus antigen detection in tissues from ten red knot carcasses collected in Germany.

		Virus target tissues and cells in red knots									
Tissue	**Target cells**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Brain	Neurons, glial cells, ependymal cell, choroid plexus epithelium	++	++	+++	+++	++	++	++	++	++	++
Heart	Cardiomyocytes	–	+	++	+++	++	++	+++	+	–	–
Air sac	Epithelium, luminal debris	–	+	+++	+	+	+	++	+	+	–
Kidney	Tubular epithelium	–	–	++	+++	+++	+	+	–	+++	–
Liver	Hepatocytes	–	–	+	–	+	–	–	–	–	–
Lung	Bronchial epithelium	–	–	–	–	++	–	–	–	++	–
Pancreas	Acinar epithelium	–	–	+	+	–	–	–	–	–	–
Proventriculus/gizzard	–	–	–	–	–	–	–	–	–	–	–
Small intestine (duodenum)	–	–	–	–	–	–	–	–	–	–	–
Large intestine, caecum)	Smooth muscle	–	–	+	–	–	+	–	–	–	–
Skeletal muscle	Myocyte	–	–	–	+	–	–	–	–	–	–
Skin	Feather follicle	–	–	+	–	–	–	–	+	–	–
Blood vessel, any tissue	Endothelium	–	–	+	–	–	–	+	–	–	–
Blood vessel, any tissue	Vessel, smooth muscle	–	–	–	+	–	–	–	–	–	–

Immunohistochemistry against influenza matrix 1 protein. –, negative; +, focal; ++, multifocal; +++, coalescent; ++++, diffuse

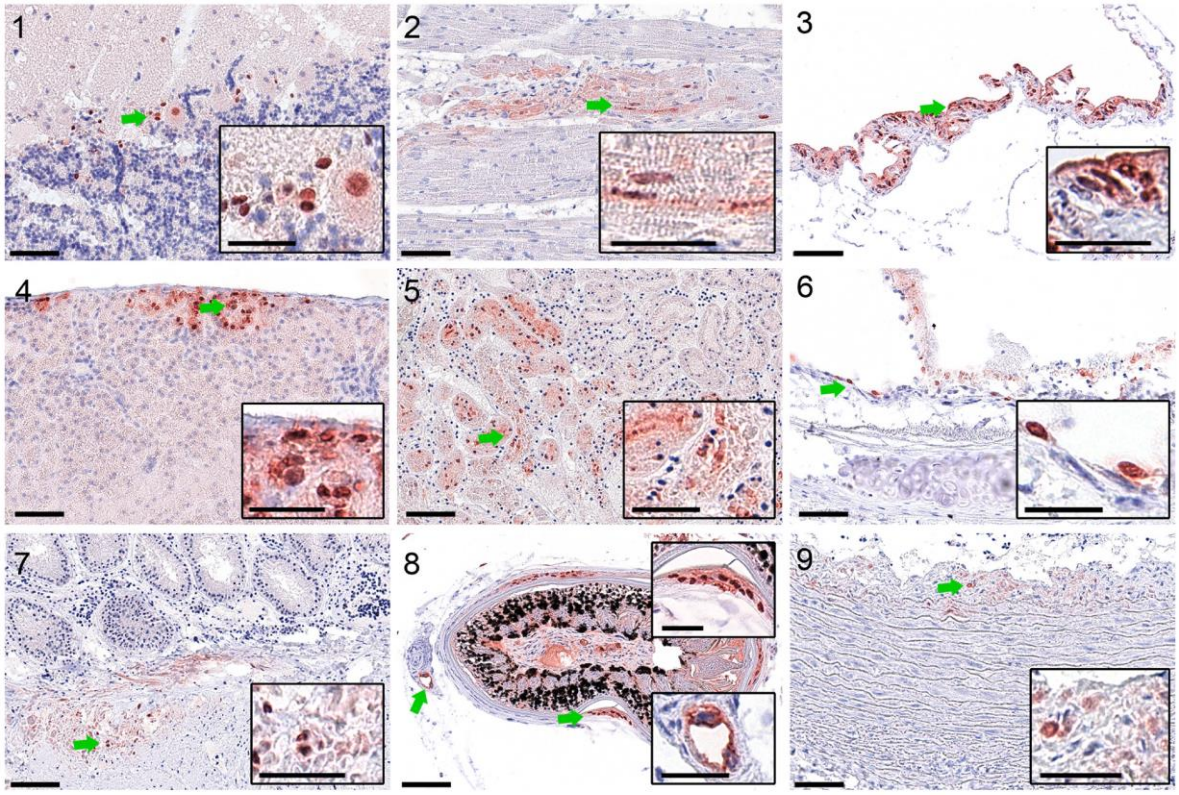

Fig. 3. Influenza virus target cell tropism in red knots. Shown is the influenza A matrix 1 protein (bright red staining) in neurons and glial cells, brain (1); cardiomyocytes, heart (2); epithelium, air sacs (3); hepatocytes, liver (4); tubular epithelium, kidney (5); bronchial epithelium, lung (6); smooth muscle cells, caecum (7); feather follicle epithelium and vascular endothelium, skin (8); smooth muscle cells, artery (9). Green arrows indicate the region of interest depicted in the inlay. Scale bars indicate 50 µm (original) or 25 µm (inlays).

virulence of the HPAIV H5N3 reassortant might provide an explanation: a peracute onset of disease with prominent neurological disorders as judged by the massive viral infection in brain tissues and reported clinical signs could have immobilized the birds rapidly whereby avoiding significant spread to other places in the Wadden Sea. Prominent loads of HPAIV H5N3-specific viral RNA were also found in heart muscle samples of dead red knots. Myocardial infection also would be in support of a hypothesis of rapid deaths as witnessed 'birds falling dead from the sky'.

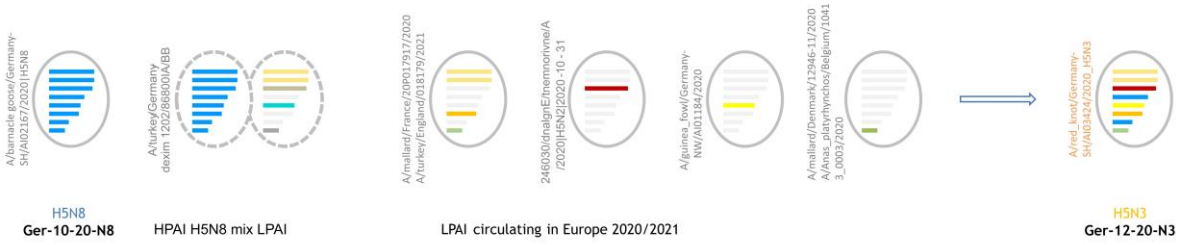

Fig. 4. Visual representation of likely reassortment pathways of HPAIV clade 2.3.4.4b and Eurasian avian lineage LPAI viruses resulting in HPAI H5N3 viruses found in red knots in Europe. Dashed lines indicate mixed samples. Virus names of the closest relatives are given. The underlying trees can be found in Supplementary material 1–8. HPAI H5N3 of the red knots are composed of genes (in colour) of different viruses (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, NS).

Former unrelated mass mortality events associated with starvation have shown that juvenile birds were impacted significantly more strongly than adults [33]. Although the age composition of live red knots in the Wadden Sea during the year of the study is not known, the low proportion of juveniles among the birds found dead suggests that both age classes died in similar proportions. At least a high proportion of adults was concerned which is a crucial finding in terms of the impact on the overall population, as shorebirds are relatively long-lived and mortality of experienced adult birds is known to impact population dynamics significantly [45].

While the subtype HPAIV H5N3 seems to have completely vanished with the red knot fatalities, yet another subtype, HPAIV H5N1, emerged as the predominant virus on the Wadden Sea coast of Lower Saxony and Schleswig-Holstein since 2021, subsequently dominating all other infections in Germany, Europe and beyond. Since then, more than 4600 cases of HPAIV H5N1 infection in wild birds have been reported from 34 countries of the European Union, with Germany reporting the most cases in wild birds (EURL Avian Flu Data Portal izsvenezie.it). For the first time ever, the H5N1 subtype was still circulating during the summer months, with multiple introductions into breeding colonies of waterbird and seabird species and subsequent mass mortalities [46]. During these outbreaks, no cases of red knots were reported from Germany.

Red knot subspecies *Calidris canutus rufa* and *Calidris canutus roselaari* have been investigated serologically in the USA (Delaware Bay and Alaska) with high antibody abundance against the LPAIV subtypes H3, H4, H10 and H11 [1, 47]. Serological investigations of red knots have also been carried out in the East Atlantic Flyway with positive findings, while virological investigations yielded mostly negative results [12]. It is unknown whether the affected red knots may have been pre-exposed to AIV before they were infected with HPAIV H5N3. Since colony-breeding seabirds or wintering populations of red knots seem to be highly susceptible to lethal HPAIV H5 infection, they are more likely to be the victim of a spill-over event from a yet unknown source of infection than a potential future HPAIV reservoir species. However, more information is needed on the potential for and magnitude of survival of *Charadrii* species, including *Scolopacidae* and *Sternidae*.

CONCLUSION AND OUTLOOK

Since 2020, the avifauna of the Wadden Sea has been affected by HPAI H5 clade 2.3.4.4b on a larger scale. Although the incidence of HPAIV H5N3 infections with fatal outcomes in probably around 1% of the entire flyway population of *islandica* red knots was a singular and self-limiting event, similar reassortments in *Charadrii* species cannot be excluded in the future.

A combination of emergence of new reassortants, prolonged infection transmissions and introduction into breeding colonies of shorebirds will likely have the potential to severely impact the bird populations breeding, wintering and resting in the Wadden Sea World Heritage Site. In fact, such mass mortalities due to yet another gs/GD HPAIV reassortant of subtype H5N1 have been described in summer of 2022 [46, 48]. In order to assess the resilience and level of threat of rare species, like the red knot, serological studies are needed to provide evidence on natural immunity and to estimate survival rates.

More frequent opportunities for possible spill-over to scavenging mammals due to amassed presence of avian carcasses harbouring high viral loads increase the risk for adaptation of HPAIV H5 clade 2.3.4.4.b viruses to mammals including humans. Hunted mammals like foxes, raccoon dogs and stray cats shall be serologically investigated for influenza H5-specific antibodies to explore the extent of spill-over events in the region.

Observations, bird census, collection of deceased birds and mammals and their investigations for influenza viruses are crucial in understanding the evolution of influenza viruses and depend on strong collaboration of ornithologists, conservationists, state veterinarians, virologists and decision-makers. Persons (rangers, bird ringers) in contact with potentially infected birds should be vaccinated against human influenza strains to prevent reassortments. Precautionary measures avoiding human exposure during carcass removal are indispensable.

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Conflicts of interest
The authors declare that they have no competing interests.

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Publication II: „Mass mortality among colony-breeding seabirds in the German Wadden Sea in 2022 due to distinct genotypes of HPAIV H5N1 clade 2.3.4.4b"

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Mass mortality among colony-breeding seabirds in the German Wadden Sea in 2022 due to distinct genotypes of HPAIV H5N1 clade 2.3.4.4b

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Mass mortality among colony-breeding seabirds in the German Wadden Sea in 2022 due to distinct genotypes of HPAIV H5N1 clade 2.3.4.4b

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Abstract

Mass mortality was observed among colony-breeding seabirds in the German Wadden Sea area of the North Sea during the summer months of 2022. Several species' colonies were affected, most notably sandwich terns (*Thalasseus sandvicensis*), common terns (*Sterna hirundo*) and Germany's only northern gannet (*Morus bassanus*) colony on the island of Heligoland. Mortality in some tern colonies reached 40%, while other colonies were almost spared. In all cases, infections with the high-pathogenicity avian influenza virus (HPAIV) subtype H5N1 of clade 2.3.4.4b were identified to have caused the epidemic. Phylogenetic analysis of whole-genome sequences revealed that the outbreaks were dominated by two genotypes, Ger-10–21 N1.2 and Ger-10–21 N1.5, previously identified in Germany. Spatiotemporal analyses of phylogenetic data suggested that these viruses could have entered the continental North Sea coastal region via the British Isles. A close linkage of viruses from tern colonies in the German Wadden Sea was evident with further connections to breeding colonies in Belgium and the Netherlands, and further spread to Denmark and Poland. Several of the affected species are endangered, such that negative effects of epizootic HPAIV infections on populations are feared, with uncertain long-term consequences.

INTRODUCTION

Infections by high-pathogenicity avian influenza viruses (HPAIVs) of clade 2.3.4.4b of the H5 goose/Guangdong (gs/GD) lineage have emerged repeatedly in Germany since 2016 [1]. Regional and temporal accumulations of HPAIV-infected wild birds were detected at the German coasts of the Baltic and North Sea, with the worst affected species varying between seasons [1]. In 2016/17, for example, the majority of cases were observed in diving duck species, such as tufted ducks (*Aythya fuligula*) and common pochards (*Aythya ferina*), whereas geese species such as barnacle geese (*Branta leucopsis),* at the North Sea coast, and to a lesser degree also graylag geese (*Anser anser*), have dominated the epizootic since 2020 [2]. The 2020/21 and 2021/22 HPAI winter periods exceeded all previously recorded HPAI epizootics in Germany in terms of the number of wild bird cases recorded, the genetic diversity of the viruses and the duration of virus activity [3]. Moreover, a geographical shift of wild bird cases towards the Wadden Sea coast was observed [4].

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Keywords: seabirds; highly pathogenic avian influenza; mass mortality; H5N1; transmission; species conservation.
Abbreviations: BA, Baysian factor; HA, hemagglutinin protein; HPAI, highly pathogenic avian influenza; HPAIV, highly pathogenic avian influenza virus; MCC, maximum clade credibility; NA, neuraminidase protein; Q, quartal of a year.
Sequence accession numbers are listed in Table S1. Data associated with spatiotemporal spread are accessible at https://zenodo.org/ under DOI: 10.5281/zenodo.6901960.
All supporting data, code and protocols have been provided within the article or through supplementary data files. Three supplementary figures and two supplementary tables are available with the online version of this article.

This trend culminated in HPAI-associated mass mortality of a large flock of wintering red knots (*Calidris canutus islandica*) from the Greenland/Canada population in December 2020 (Globig *et al*., in preparation). In addition, an increased incidence was observed in raptor and gull species [5].

From May 2022, a new and unprecedented situation was observed in Europe when several colony-breeding water- and seabird species, such as the sandwich tern (*Thalasseus sandvicensis*), the common tern (*Sterna hirundo*), the northern gannet (*Morus bassanus*), the black-headed gull (*Chroicocephalus ridibundus*), and – mainly at the Baltic Sea coast – the cormorant (*Phalacrocorax carbo*) started showing mass mortality induced by HPAIVs [6]. Other European colony breeding species affected by HPAI in the breeding season 2022 are the Eurasian spoonbill (*Platalea leucorodia*) in the Netherlands and, in other parts of northwest Europe, common guillemots (*Uria aalge*). This development turned out to be embedded in a larger scale expansion of HPAIVs of the gs/GD lineage of Southeast Asian origin into the Northern Atlantic and onward to North America [7, 8]. In early May 2022, AI was detected in a sandwich tern colony on the German Baltic coast (Langenwerder). In late May 2022, high mortality was observed in breeding colonies of sandwich terns in Calais, France, and in the Dutch islands of Zeeland and Texel, and HPAIV of the H5N1 subtype was detected. At Shetland and the Orkney Islands, UK, outbreaks of HPAIV infections at northern gannet and great skua (*Stercorarius skua*) breeding spots were observed in 2021 and from April 2022 onwards. In early June 2022, HPAIVs of subtype H5N1 were detected for the first time in colony-breeding seabirds at the German Wadden Sea coast (sandwich terns, common terns).

Here we assemble data documenting the impact of HPAIV H5N1 on colony-breeding seabirds in Germany and analyse phylogenetic and phylogeographical aspects of virus incursion and circulation within and between the breeding colonies.

METHODS

Ornithological data collection

Since 1991, the three countries bordering the Wadden Sea coastline of the North Sea, Denmark, the Netherlands and Germany, have committed themselves to jointly monitor birds in the Wadden Sea in a co-ordinated scheme that has its roots in the survey for oiled birds (e.g. [9]). The surveillance programme is coordinated by the Common Wadden Sea Secretariat. The methods of recording dead birds differ depending on the species and have been detailed by Hälterlein *et al.* [10]. Many breeding colonies in the German Federal States of Schleswig-Holstein and Lower Saxony have been recorded in recent years using drones and aerial photographs. Breeding pairs of gannets on Heligoland are recorded according to Südbeck *et al.* [11] and bird flu monitoring was carried out at Heligoland daily on the main island and the dune. Carcasses in the gannet colony were collected on three dates during the outbreak. Drift line checks take place at regular intervals at designated coastline sections. All dead animals are recorded and documented by various institutions such as the national park administration or nature conservation associations [12].

Three German Federal States share the Wadden Sea coastline of Germany: Schleswig-Holstein, Hamburg and Lower Saxony (Fig. 1). In both Schleswig-Holstein and Lower Saxony, no colonies were entered during the breeding phase to avoid disturbing the birds. It was feared that the birds could adopt other habitats in the wake of the disturbance and that this could contribute to further spread of HPAI viruses. At Minsener Oog, Lower Saxony, however, a transect of ~1.3 km was established near the colony and the dead birds were recorded during the breeding season on a daily basis. The number of dead birds over time was used to monitor the progression of infection within the colony. The colonies on the island of Norderoog were checked after the end of the breeding season on 23/24 July. All carcasses were registered with GPS co-ordinates. Time of death and, in the case of chicks, age, were estimated. Affected parts of the colony that were not recorded (~40–50% of the area) were extrapolated. At the common tern colony at the Banter See, Lower Saxony, the colony area and the lake were searched twice a day and all dead terns were collected. On the island of Neuwerk, Hamburg, the carcasses of birds were collected and disposed of but not recorded, such that an assessment of the number of dead birds is currently not available. Future breeding pair counts may, however, allow us to retrospectively estimate the impact of HPAI 2022 on sandwich terns at this island, as well as at other locations, since even for the other locations mortality estimates are currently based on the sightings of dead birds, and may be underestimated.

General uncertainty of exact mortality rates exists because (i) some dead birds will not have been found, such that the number of dead birds will be underestimated to an unknown extent; (ii) retrospective calculations on the basis of next season's counts may suffer from unknown levels of (additional HPAI-induced) mortality in the wintering areas; (iii) the number of breeding pairs is not always easy to estimate, due to variable numbers of replacement and second clutches, with perhaps more pronounced difficulties in the disrupted breeding season of 2022; (iv) many populations contain prospectors and/or non-breeders that may have been found dead, but do not appear in estimates of the number of breeding pairs; and (v) the breeding site fidelity of some species is not very pronounced and relocations frequently take place.

Molecular virus detection and phylogenetic analyses

Clinical material, mainly oropharyngeal and cloacal swabs, was collected from wild bird carcasses obtained from affected colonies or washed ashore. RNA was extracted with the Qiagen Mini Viral kit (Qiagen, Germany). We used real-time RT-PCR (RT-qPCR) to test for the presence of AIV-specific RNA, as well as for sub- and pathotyping [13] .

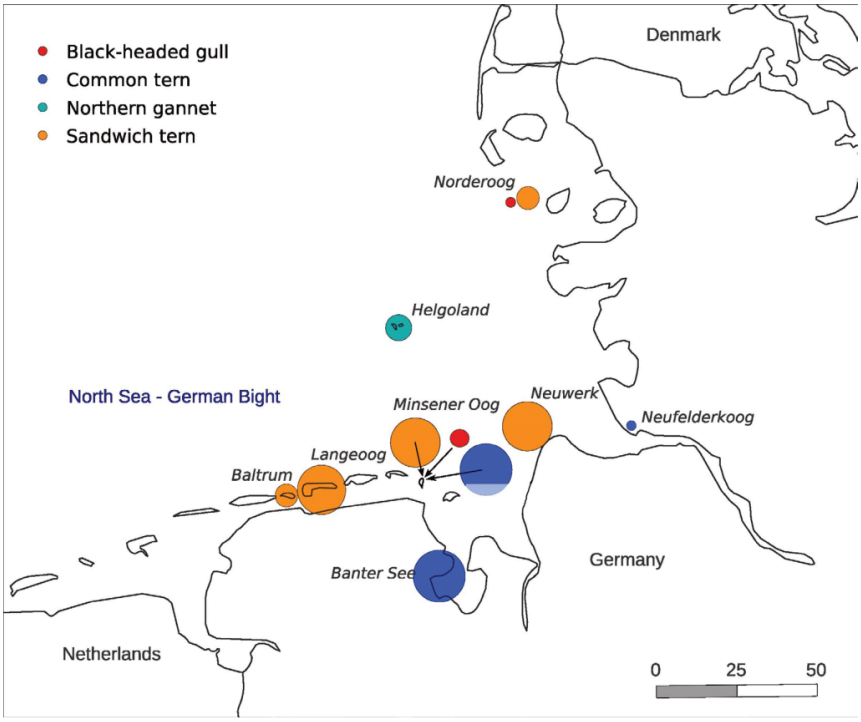

Fig. 1. Map of affected seabird breeding colonies along the North Sea coast of Germany. The colour of the dots represents species, as shown in the legend, and the size of the dots depicts the level of mortality, as detailed in Table 1. Scale, km.

Full-genome sequencing of selected HPAIV-positive samples was executed by a previously described nanopore-based real-time sequencing method with prior full genome amplification [14]. Samples were selected on the basis of their viral load, the species origin and the location.

HPAIV genome amplification was conducted by universal AIV-End-RT-PCR using Superscript III One-Step and Platinum *Taq* (Thermo Fisher Scientific, USA) using a single primer pair (Pan-IVA-1F: TCCCAGTCACGACGTCGTAGCGAAAGCAGG; Pan-IVA-1R: GGAAACAGCTATGACCATGAGTAGAAACAAGG), which binds to the conserved ends of the viral genome segments.

After purification of the PCR products with AMPure XP Magnetic Beads (Beckman-Coulter, USA), full-genome sequencing on a MinION platform (Oxford Nanopore Technologies, ONT, UK) using the Rapid Barcoding kit (SQK-RBK004, ONT) for transposon-based library preparation and multiplexing was performed. Sequencing was directed according to the manufacturer's instructions with a R9.4.1 flow cell on Mk1C device with MinKNOW Software Core (v4.3.11).

Live basecalling of the raw data with Guppy (v5.0.13, ONT) was followed by a demultiplexing, quality check and trimming step to remove low-quality, primer and short (<50 nt) sequences.

After sequencing, full-genome consensus sequences were generated using a map-to-reference approach utilizing MiniMap2 [15]. Reference genomes are a curated collection of all haemagglutinin (HA) and neuraminidase (NA) subtypes alongside an assortment of internal gene sequences chosen to cover all potentially circulating viral strains.

Polishing of the final genome sequences was done manually after consensus production according to the highest quality (60%) in Geneious Prime (v2021.0.1, Biomatters, New Zealand).

Segment-specific and concatenated whole-genome multiple alignments were generated using MAFFT (v7.450) [16] and subsequent maximum-likelihood (ML) trees were calculated with RAxML (v8.2.11) [17] utilizing model GTR GAMMA with rapid bootstrapping and searching for the best scoring ML tree supported with 1000 bootstrap replicates or alternatively with FastTree (v2.1.11) [18]. Time-scaled trees of concatenated sequences of the different genotypes were calculated with the BEAST (v1.10.4) software package [19] using a GTR GAMMA substitution model, an uncorrelated relaxed clock with a lognormal distribution and coalescent constant population tree models.

Table 1. Mortality of seabirds in breeding colonies and along the drift line of the German Wadden Sea coast, June–August 2022

Species	Location*	Colony size†	Carcasses		Adult mortality‡	Carcasses§ (drift line)
			Adult	Juvenile		
Sandwich tern	Minsener Oog	4765	2967	2807	31.1%	
	Baltrum	649	14	NA	1.1%	
	Langeoog	165	112	38	33.9%	1904
	Neuwerk	>660	>850	NA	>60%	
	Norderoog	6442	650	2900	5,0%	
Common tern	Banter See	690	510	1350	37.0%	
	Minsener Oog	190	176	74	46.3%	105
	Neufelderkoog	1070	7	94	0,3%	
Black-headed gull	Minsener Oog	2852	121	151	2.1%	350
	Norderoog	1670	29	101	0,9%	
Northern gannet	Heligoland	1485	259	689	8.7%	697

*Depicted in Fig. 1.
†No. of breeding pairs.
‡Calculated by dividing the no. of adult carcasses by twice the no. of breeding pairs.
§Total no. counted along the German North Sea shore line.
NA, not available.

Spatiotemporal analysis was modelled on discrete sampling locations (countries) using a symmetric model, applying Bayesian stochastic search variable selection (BSSVS) procedures. Bayes factors (BFs) were calculated and the potential geographical pattern of dissemination visualized considering BFs >3 and posterior probabilities >0.7 as significant. For inferring the detailed spread of cases, their latitude and longitude location coordinates were derived via adaptive optical means. A continuous relaxed random walk model with lognormal distribution was applied. Chain lengths were set to 20–50 million iterations, depending on the data set, and their convergence checked via Tracer (v1.7.1). Time-scaled summary maximum-clade credibility (MCC) trees with 10% post-burn-in posterior were created using TreeAnnotator (v1.10.4) and visualized with FigTree (V1.4.4). The robustness of the MCC trees was evaluated using 95% highest posterior density (HPD) confidence intervals at each node and posterior confidence values as branch support. Spatiotemporal spread was inferred on MCC trees using SPREAD (v1.0.7) [20] and visualized with QGIS (V3.24.3, QGIS.org). Associated data and underlying source data are available in the Zenodo repository https://zenodo.org/ under DOI 10.5281/zenodo.6901960.

RESULTS

High adult mortality at breeding colonies of seabirds along the German Wadden Sea coast was found during summer 2022

Breeding colonies of terns in Germany are mainly scattered across small flat sandy islands along the Wadden Sea coast (Fig. 1). The largest sandwich tern colonies in Germany at Minsener Oog, Lower Saxony, and Noorderoog, Schleswig-Holstein, were affected differently by HPAI (Table 1). On Minsener Oog, adult mortality amounted to 31.1%, while on Norderoog it remained at 5.0%.

In the beginning of June, HPAIV was only detected sporadically in dead adult and young birds on Norderoog. In late June the mortality of adult sandwich terns increased exponentially after the late settlement of birds that had presumably already made unsuccessful breeding attempts in other colonies during this breeding season (among these a high proportion of birds ringed in the Netherlands), and most of the juveniles died from the infection. The largest common tern colony in Germany at Neufelderkoog, Schleswig-Holstein, is very elongated, with large distances between the nests, and was almost unaffected by HPAI (mortality rate 0.3%). This very low impact also applies to Noorderoog, where common terns breed in the vicinity of the sandwich tern colony. The colonies at the Banter See and on Minsener Oog, both Lower Saxony, in contrast, showed the highest mortality rates of >37% of adult birds.

In the black-headed gull (*Chroicocephalus ridibundus*) colony on Minsener Oog, Lower Saxony, a mortality rate of 2.1% was recorded, similar to what was seen at Norderoog island.

For the northern gannets on Heligoland, Schleswig-Holstein, a mortality rate of 8.7% was calculated, based strictly on dead birds counted within the colony. Since more than 400 individuals were additionally recorded dead during drift line inspections, the actual mortality is likely higher, up to 22.5% if all of the washed up birds were Heligoland breeders.

Mortality was due to HPAIV H5N1 infection

All samples (*n*=63) examined from species listed in Table 1 tested positive for HPAIV of the H5N1 subtype. Viral loads varied considerably but were in general high for fresh carcasses retrieved from breeding colonies (cycle thresholds 14–21). Considerably lower virus loads were detected in carcasses found along the coastal drift lines, especially when in a state of enhanced decomposition. This mainly affected findings of northern gannets (cycle thresholds >34).

A total of 111 full-genome sequences were obtained from German samples, of which 23 originated from colony breeders (4 gannets, 13 terns, 2 cormorants, 4 gulls, (Supplementary Material), whereas the other sequences originated from other wild birds, predators and poultry holdings. For each case, the RT-qPCR-based sub- (H5N1) and patho- (HP) typing was confirmed. All haemagglutinin sequences clustered with the gs/GD clade 2.3.4.4b. Further genetic analyses were used to map the relationship of HPAIV H5N1 from colony-breeding seabirds and to trace trajectories of introduction and spread within and among the breeding colonies. This showed that the outbreak in Germany since May 2022 was dominated by two known genotypes that have been found to circulate in Germany since October 2021: Ger-10–21 N1.2 and the reassortant Ger-10–21 N1.5 [7]. These two genotypes also prevailed in previous outbreak events in wild birds as well as in poultry from early 2022 and comprise ~75% of all sequences obtained across various regions of Germany.

Detection of three genetic clusters of HPAIV H5N1 cycling within and between breeding colonies around the North Sea

The German sequence data were supplemented with publicly available sequences, resulting in curated data sets for each reassortant of 191 sequences for analysing tern and cormorant colonies (genotype Ger-10–21 N1.5) and 109 sequences for analysing gannet colonies (genotype Ger-10–21 N1.2). Data sources and acknowledgments are listed in Table S1 (available in the online version of this article). Bayesian stochastic search variable selection (BSSVS) with calculation of BFs is summarized in Table S2.

The only German breeding colony of northern gannets is located on the North Sea island of Heligoland. Although this colony was affected by HPAIV (see above), no samples from that location yielded valid sequences that could be included in the analyses. Instead, four northern gannet samples collected during drift line inspections in Germany in the summer of 2022 confirmed positive for HPAI H5N1 viruses and yielded sequence information identifying them as belonging to the Ger-10–21 N1 genotype. A single gannet sample collected in February 2022, however, clustered with the HPAIV H5N1 genotype Ger-10–21 N5. Viruses of this genotype were detected in the German North Sea and Baltic Sea coastal regions in Q4 2021 in other wild birds (https://doi.org/10.5281/zenodo.6838094) and travelled westward in Q1 2022. These viruses were consistently detected in samples from Germany until 1 March 2022.

Phylogenetic analysis via maximum likelihood (Figs S1 and S3) shows that these viruses are closely related to contemporary viruses collected in the UK and are related to viruses from the Netherlands. An incursion of these viruses into the continental North Sea coastal region via the British Isles could be confirmed by spatiotemporal analysis via time-scaled MCC phylogeny of subsets of clustered sequences and inferring their spread (cluster 1, Figs 2a, S2a and 3a). The potential incursion via the British Isles could, however, not be corroborated by ornithological findings: Although ringing data of gannets washed up on the North Sea island of Sylt revealed that they hatched in British breeding colonies, this merely reflects that they were likely ringed as chicks in the UK, but they could still have been regular breeding birds of the Heligoland colony for years. At the Heligoland breeding site, gannets from very different hatching colonies can be found.

The tern cases (common and sandwich terns) are caused by a different reassorted virus: Ger-10–21 N1.5 (Fig. S1). This genotype has been present in Germany since at least October 2021. The tern cases in Germany can be assigned to two different clusters within this genotype (Figs 2b, c, S2b, c and S3b): cluster 2 could mainly be traced back to infections of geese in Q1 2022 in the Netherlands. These viruses moved further eastward, affecting wild birds and colony-breeding great cormorants in Mecklenburg-Vorpommern on the Island of Rügen. Subsequently, white-tailed eagles (*Haliaeetus albicilla*) on the island of Rügen and sandwich tern colonies were infected by this virus, causing widespread fatality (Figs 2b, S2b and S3b). Spatiotemporal analysis revealed the further spread of this genotype from Germany to Poland and to the Danish North Sea coast.

Cluster 3 reflects a close linkage of tern colonies in the German Wadden Sea. The virus was detected in common terns at the North Sea coast in Wilhelmshaven and in Sandwich terns on the island of Minsener Oog in Lower Saxony, part of the Wadden Sea, in June 2022. Similar viruses caused infection in sandwich terns in the Netherlands and Belgium. The virus was subsequently also detected further north in Germany in sandwich terns found dead on Hallig Hooge nearby the Island of Norderoog in Schleswig-Holstein (Figs 2c and S2c).

DISCUSSION

The mass mortality among seabirds in the area of the North Sea – triggered by HPAIV H5N1 in the summer of 2022 – is unprecedented. During the breeding season, seabird colonies have high population densities, and the close physical contact of the birds to one another is likely to increase HPAIV transmission rates greatly. According to observations made in a number of tern

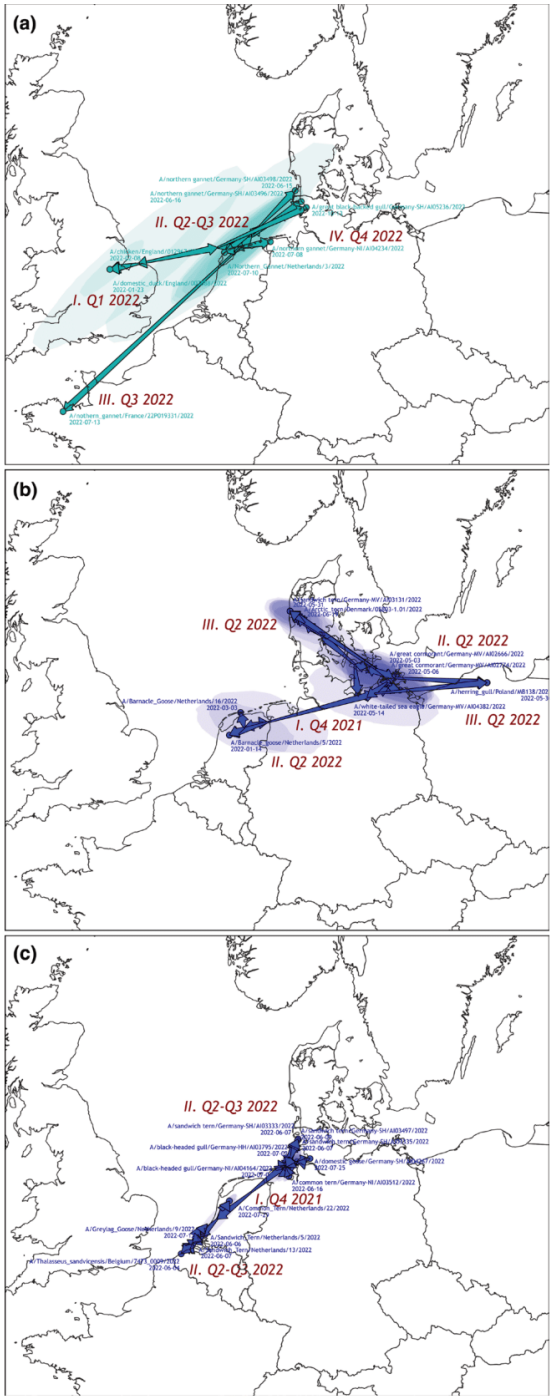

Fig. 2. Cluster areas (polygons) with their directed spread (arrows) of HPAIV genomes (points) inferred by spatio-temporal phylogeography with noted key steps of virus dissemination. (a) Cluster 1 viruses from northern gannets Q1–Q2 2022. (b) Cluster 2 viruses from common and sandwich terns Q1–Q2 2022. (c) Cluster 3 viruses from common and sandwich terns Q2–Q3 2022.

colonies in Germany, the spread of HPAIV within a colony seemed to require some time to develop from an easily overlooked latent phase with few individual infections and deaths to a fulminant epizootic. As populations shrink due to rising mortality, dispersal of affected birds or the regular end of breeding activities, infection chains are disrupted. However, the risk of epizootic infections did not appear to be distributed evenly, as significant differences in mortality rates were observed between different HPAIV-affected colonies of the same species. Differences in the time of HPAIV incursion into the colony could be one explanation. Additionally, however, it needs to be considered that the presented mortality rates are based on rough calculations made using numbers of dead birds found and the estimated number of breeding pairs per population, whereas the exact population size of most colonies is unknown and probabilities of finding dead birds may differ among populations as well.

The current summer cases among colony breeding birds may have particularly grave consequences for the continuation of at least some of the heavily hit colonies. In recent years, an increase of cormorant populations in the Wadden Sea has been observed. The population of sandwich terns was stable, while the populations of black-headed gulls, common terns and common eiders decreased [9]. All of the aforementioned species require advanced ages for sexual development (e.g. gannets >5 years), and the average breeding success per year is quite low, e.g. in common terns 0.12–1.57 fledglings/brood [21], and increases with age [22], such that the loss of older, more experienced breeders is especially detrimental [23]. As such, it can be assumed that the loss of a large number of adult birds will have a negative long-term impact on population development. The complete impact of the HPAI epizootic in 2022 on long-term population patterns will not be known until breeding pair counts in the future. If colonies are similarly affected by HPAI in upcoming years, it may be damaging to population trends in the Wadden Sea in the event of an enzootic entrenchment of HPAIV.

There are currently no preventive measures in place to protect seabird colonies from future HPAIV reincursion events, and the intervention options are few. Most importantly, the early collection and safe disposal of all carcasses at the beginning of an outbreak found in breeding areas by appropriately trained personnel aids in the reduction of virus loads in the environment [24]. Not only do the carcasses themselves remain infectious (especially muscles and feathers, depending on temperature, 3–4 weeks at 20 °C [25]), but carcasses floating in water will flush out infectious viruses. Virus-contaminated surface water has been shown to act as a highly potent transmission medium [13]. AI virus persistence in surface water is strongest at a low temperature (<17 °C), a neutral-to-basic pH (7.0 to 8.5), low salinity (<0.5 ppt) and a low dissolved ammonia concentration (<0.5 mg l^{-1}). Small shallow fresh water pools in the vicinity of breeding colonies in Lower Saxony are often visited by terns for bathing and grooming and might have contributed to virus spread via contaminated surface water. However, even in sea water with salinity >3 p.p.t. it was estimated to take 3 days to achieve a 1 log reduction of virus titre [26].

From an epizootiological perspective, seabird colonies amplified HPAIV H5N1 in the North Sea and other North Atlantic regions in the summer of 2022. This undoubtedly aided in maintaining year-round virus replication, establishing enzootic HPAIV circulation in Northern Europe, and increasing virus presence in the environment. The consequences of persistently high HPAIV infection pressure are severe for both avian wildlife and poultry rearing, particularly in free-range poultry operations. In addition, the presence of HPAIV H5N1 in wild bird populations increases the risk of infection in mammalian species such as foxes, mustelids and seals [27–30]. Overall, the risk of emerging HPAIV variants with zoonotic propensity increases.

Controlling HPAI in poultry globally is fundamental to lowering the risk of virus spillover from poultry to wild birds [31], while also lowering the risk of human exposure. Strict biosecurity measures for poultry holdings, combined with a test-and-cull strategy, have been shown to be effective in areas where outbreaks occur infrequently. Risks of spill-out events of HPAI virus from infected poultry premises to natural habitats of wild birds, e.g. during culling operations or removal of manure and waste, must be minimized. The same strict hygiene principles should also be employed when entering wild bird habitats, visiting wild bird breeding colonies or handling wild birds for other purposes (e.g. ringing). However, vaccination of poultry may be required as an additional layer of protection when incursion pressure remains consistently high. HPAI vaccination options in wild birds have yet to be explored. Baited vaccination of foxes and other carnivores against rabies or wild boar populations against European swine fever has a long history of success in Europe [32]. Discussions must be initiated to determine whether similar vaccine options can be envisioned for use in some of the much more diverse, mobile, volatile and endangered wild bird populations. Access will be shaped by foraging habits, with gulls being much less fastidious compared to terns, which strictly feed on small live fish.

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Author Dr Lina Stacker was not available to confirm co-authorship, but the corresponding author Dr Harder affirms that author Dr Stacker contributed to the paper and vouches for author Dr Stacker's co-authorship status.

Author contributions

A.P., T.H. and M.B. conceived the study; O.S., F.P., B.H., V.H., E.B. and S.B. provided samples and ornithological population data; J.K., L.S., A.G., Ch.H., A.G., Y.L. and C.H. carried out the experiments and generated sequences; AP and JK analysed sequence data; A.P., T.H., O.S. and S.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript, and agreed with the final version.

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Publication III: „Pathogen-prey-predator relations of avian raptors during epizootics of highly pathogenic avian influenza virus HPAIV H5N1, clade 2.3.4.4b, in Germany"

Publication III

Pathogen-prey-predator relations of avian raptors during epizootics of highly pathogenic avian influenza virus HPAIV H5N1, clade 2.3.4.4b, in Germany

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Pathogen-prey-predator relations of avian raptors during epizootics of highly pathogenic avian influenza virus HPAIV H5N1 (clade 2.3.4.4b) in Germany

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Article impact statement

Adapted surveillance measures were developed to assess risks for the conservation of avian raptors due to the panzootic spread of HPAIV.

Abstract

Transition of highly pathogenic clade 2.3.4.4b H5 avian influenza virus (HPAIV) from epizootic to enzootic status in Northern European countries was associated with severe losses and even mass mortalities among various wild bird species. Both avian and mammalian raptors hunting infected debilitated birds or scavenging on virus-contaminated avian carcasses contracted HPAIV infection. This precarious pathogen-prey-predator relation further worsened when in 2021 and 2022 outbreaks in Germany overlapped with the hatching season of avian raptor species. Retro- and prospective surveillance revealed avian raptors as important indicators of HPAIV and its genetic diversity on the one hand. On the other hand, their role as victims of HPAIV is stipulated. The first case of an HPAIV H5N1-related death of a white-tailed sea eagle (*Haliaeetus albicilla;* WTSE) hatch in Germany, 2021, followed by several such cases in 2022, and a low overall seropositivity rate of 5.0-7.9% among WTSE nestlings, raised fears of a serious negative impact on reproduction rates of WTSEs and other birds of prey when HPAIV becomes enzootic in an ecosystem. However, comparably stable breeding success of WTSE in the study area in 2022 and a potentially evolving natural immunity raises hope for a less severe long-term impact.

Keywords

Highly pathogenic avian influenza virus H5N1, white-tailed sea eagle, *Haliaeetus albicilla*, nestling, breeding success, wild bird surveillance, maternal immunity

Introduction

The current panzootic of highly pathogenic (HP) goose/Guangdong (gs/GD) clade 2.3.4.4b avian influenza virus (AIV) causes immense damage in poultry holdings and severe die-offs in wild birds worldwide (Caliendo, Lewis, et al., 2022). Along with an enormous extension in geographic range, the recent gs/GD HPAIV H5 lineage has gained an enzootic status in European wild bird populations (A. Pohlmann et al., 2022) causing immense clinical impact and high mortality in several endangered wild bird species.

The natural history of influenza A viruses (IAV) of low pathogenicity (LPAI) identifies wild water birds of the Anseriformes and Charadriiformes as reservoir hosts. Extended co-evolution ensured efficient virus replication and spread while not impacting the clinical status of the avian hosts (Globig et al., 2013; Globig et al., 2009; Yoon, Webby, & Webster, 2014). However, species of these orders are equally susceptible, and clinically highly vulnerable, to HPAIV which arise sporadically by spontaneous mutation in galliform poultry infected with LPAI precursor viruses of subtypes H5 or H7 (Pantin-Jackwood & Swayne, 2009). During the 1990s, such HPAIV (i.e. the gs/GD lineage) arose in Chinese poultry populations and reached migratory wild bird populations by spill-over infections in Far East Asia since the early 2000 years. In fact, migratory waterfowl has been identified as long-distance vectors of gs/GD HPAIV H5 (Global Consortium for H5N8 and Related Influenza Viruses (2016), 2016). Along with HPAIV dissemination in wild water birds, avian raptors of the orders Accipitriformes, Falconiformes and Strigiformes are increasingly affected (EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), EURL (European Reference Laboratory for Avian Influenza), Adlhoch, Fusaro, Gonzales, Kuiken, Marangon, Niqueux, Staubach, Terregino, Aznar, Chuzhakina, et al., 2022; EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), et al., 2022a, 2022b; EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), EURL (European Reference Laboratory for Avian Influenza), Adlhoch, Fusaro, Gonzales, Kuiken, Marangon, et al., 2023). Due to the high public attention that many of these species receive, their feeding behaviour on diseased and weakened prey or infected carcasses and their apparently high susceptibility, they were marked out as indicator species for (passive) HPAIV disease surveillance (Caliendo, Leijten, van de Bildt, Fouchier, Rijks, & Kuiken, 2022; El Zowalaty et al., 2022; Günther et al., 2022; Krone et al., 2018; Nemeth et al., 2023; Redig & Goyal, 2012; van den Brand et al., 2015).

The recently established year-round presence of gs/GD HPAIV H5 in European wild bird populations poses major threats to avian raptors: (i) Increased infection pressure due to multiple opportunities of ingesting HPAIV H5 infected prey (Banyard et al., 2022; Anne Pohlmann et al., 2023; Rijks et al., 2022) and (ii) a temporal overlap of virus presence with the hatching season of raptor chicks. Mortality among nestlings of white-tailed sea eagles (*Haliaeetus albicilla*, WTSE) in Estonia in 2021 (Estonian University of Life Sciences, 19.05.2021) and bald eagles (*Haliaeetus leucocephalus*) in North America in 2022 due to alimentary HPAIV H5 infections (Nemeth et al., 2023) have been reported already.

Within a nationwide retrospective and regional prospective surveillance for HPAIV H5 infections in raptor species in Germany we: *(i)* report on (HP)AIV infection rates in raptors since 2016, (ii) screened archived samples of avian predators collected across Germany since 2010, and (iii) prospectively sampled raptor nestlings during ringing activities in Mecklenburg-Western Pomerania (MWP), Germany. This region, severely affected by gs/GD HPAI in 2021-22, holds the highest density of WTSE breeding pairs in Germany and harbours important stop-over sites for migratory water birds (Herrmann, Krone, Stjernberg, & Helander, 2023; Krone et al., 2018).

Material and Methods

Sample and data sets

All samples obtained in a prospective or retrospective surveillance approach were collected in Germany including individuals from the taxonomic orders of Accipitriformes, Strigiformes and Falconiformes. For reasons of endangered species protection listing precise breeding locations was omitted throughout this manuscript.

Retrospective surveillance on HPAIV in raptor species

The avian influenza database represents a governmental, non-public database on all virological data regarding AIV infections in wild birds. Data were selected with respect to the orders Accipitriformes, Falconiformes and Strigiformes, subsequently considered as raptors, and on HPAI-specific results on March 6, 2023, for the years 2016 to 2022, covering the activity of HPAIV clade 2.3.4.4b strains.

The database survey was compiled by raptor samples archived by the Leibnitz Institute for Zoo and Wildlife Research, Berlin. These organ samples (mainly brain, lung or liver) had not been examined for HPAIV previously as they were collected in frame of unrelated research projects. The carcasses were collected across the whole geographic range of Germany. All samples were examined at the Friedrich-Loeffler-Institut, Isle of Riems, Germany. Additionally, WTSE sera retrieved as part of different research projects in the federal states of Brandenburg, MWP and Thuringia were included.

Prospective surveillance in raptor nestlings and rehabilitated raptor species

The majority of individuals was sampled as nestlings of ten different species, when handled within their first weeks of age during scientific bird ringing activities in spring 2021 (April to July) and 2022 (May and June) in MWP, Germany (see 2.1.3 for permissions). The ringing of birds allows for an unambiguous and unmistakable individual identification of an animal (and thus a sample) from that point on. Some birds were sampled in a wild bird rescue centre in Greifswald, MWP (June, July and October 2021 and May and June 2022), covering five different species. The sample-identification comprises serial numbers indicating the affiliation to a nest/location, while letters represent the sampled individuals per sampling nest/location (e.g., two individuals at location (nest) #6 are named #6A and #6B). All birds were physically examined for general behaviour and clinical signs of infection, e.g. laboured breathing or neurological disease manifestation. A complete sample set included two separate swabs (oropharyngeal and cloacal) and a venous blood sample, taken from the wing vein. In some cases, only a subset of samples was taken, either to reduce the time of handling, due to situation-dependent field-work aspects, or according to the bird's size or clinical condition.

Ethical statement

Organ samples from raptor carcases were collected during post-mortem examinations in the context of different research projects with ecotoxicological objectives and, therefore, no additional permits were required for our retrospective analyses. The serum samples collected in prior studies were approved by the authority of the Federal State of MWP, Germany (LALLF reference number 7221.3-3.2-004/19) and by the authority of the Federal State of Brandenburg (LAVG reference number 2347-A-10-1-2019).

The prospective sampling of avian raptors in MWP, Germany, was approved by the authority of the Federal State of MWP, Germany (LALLF reference number 7221.3-2-003/21, approved 24 March 2021).

Molecular analyses

Swabs were stored in virus cultivation medium (Sigma-Virocult®). Archived swabs and organ samples were kept at -70 °C until final analyses. RNA extraction from swabs and supernatants of homogenated organ samples was performed using the Macherey-Nagel NucleoMag® VET-Kit on a KingFisher Flex Purification System (Thermo Fisher Scientific), following the manufactures' instructions. A heterologous internal control RNA was added during the RNA extraction process (B. Hoffmann, Depner, Schirrmeier, & Beer, 2006), to assure successful extraction process. RNA was screened by real-time reverse transcription polymerase chain reaction (RT-qPCR) for presence of IAV-specific generic targets in matrix (M) or nucleoprotein (NP) genes (Fereidouni et al., 2012; E. Hoffmann, Stech, Guan, Webster, & Perez, 2001). Positive samples were further sub- and pathotyped by RT-qPCR protocols as described previously (Hassan et al., 2022).

Serological analyses

Samples of coagulated blood were transported cooled and dark until separation from serum and blood cruor by ten minutes of centrifugation (3500 rpm). Serum was stored at -20 °C after heat inactivation for 30 minutes at 56°C. Sera were screened using competitive enzyme-linked immunosorbent assays for IAV-specific antibodies. In a first step, all samples were applied to the ID Screen® Influenza A Antibody Competition Multi-species assay, detecting generic antibodies against the NP. In case of positive findings, those samples were screened by using the ID Screen® Influenza H5 Antibody Competition assay to detect antibodies against the HA of subtype H5. The cut-off values for sample to negative (S/N) ratios were used as recommended by the manufacturer: S/N%≤45% positive, 45<S/N%<50 indeterminate and S/N%≥50 negative for antibodies against NP, respectively S/N%≤50% positive, 50<S/N%<60 indeterminate, S/N%≥60 for H5. Due to limited sample volumes, a single test per sample and step was performed. A single sample, for which sufficient volume was available, was additionally analysed in a hemagglutinin inhibition (HI) test against a set of reference antigens supplied by the European Reference Laboratory Padova, Italy (H5N1, Eurasian AIV: A/ck/Scotland/1/59; H5N3, Eurasian AIV: A/Teal/England/7394-2805/06; H5N8, HPAIV gs/GD: A/tk/Italy/7898/14; Newcastle disease virus Clone 30).

Sequencing and genetic analyses

HPAIV-positive samples were considered for sequencing when revealing distinct viral loads of Cq (quantification cycle)-values below 30. The sequencing workflow described by King, Harder, Beer, and Pohlmann (2020) was followed. Retrospective sequences from other studies and from databases were included for comparison and genotype assignment. Genotype differentiation and derivation of reference sequence were done with a combined phylogenetic and similarity-based method. Genotypes were assigned, and new genotypes were differentiated if they are clustering separately with robust bootstrapping values (>80) or if differences greater than 2% were observed when comparing nucleotides at segment level. The first complete genome sequence of a newly detected genotype was used as a reference sequence, and additional references for a genotype were derived as needed. Genotype names are filed to include locality (three digits), date of first discovery (month-year), and NA subtype. When multiple genotypes of one subtype were assigned within the same locality and date, the names were numbered consecutively. Detailed methodology and overview of reference sequences are available as technical note under https://doi.org/10.5281/zenodo.8233814.

Breeding success rate and breeding pair numbers of white-tailed eagles in MWP, Germany

We utilized data on the breeding success rate and the number of overall breeding pairs for WTSEs in MWP, from 2002 to 2022. These data sets were compiled by the "Working Group for Conservation of

Large Birds MWP" and provided by the Agency for Environment, Nature Conservation, and Geology MWP.

Statistical analyses

For the calculation of the 95% confidence intervals (95%CI) (Clopper & Pearson, 1934) and the Fisher-test (Fisher, 1936) we applied R version R4.2.2 (R Core Team, 2021). The 95%CI is provided for the detection rate of (HP)AIV RNA positive species or groups of species. We utilized the Fisher-test to verify, if there is a significant difference between the findings on NP-specific antibodies in WTSE nestlings and all other sampled raptor nestlings (significant value is considered as $p<0.05$).

Results

Retrospective sample screening confirms large to medium-sized raptors highly affected by HPAIV H5

In a nationwide retrospective surveillance organ samples from 232 birds of ten different species collected between 2010-2022 were analysed retrospectively: Ospreys (n=2; *Pandion haliaetus*), Northern goshawks (n=1; *Accipiter gentilis*), common buzzards (n=46; *Buteo buteo*), red kites (n=16; *Milvus milvus*), barn owls (n=23; *Tyto alba*), common kestrels (n=28; *Falco tinnunculus*), tawny owls (n=28; *Strix aluco*) and peregrine falcons (n=3; *Falco peregrinus*), WTSE (n=82) and Eurasian eagle owls (n=3; *Bubo bubo*). Different age cohorts, from nestlings to adult individuals, were represented (Figure 1).

The general German wild bird surveillance revealed yearly HPAIV H5 detection rates between 0.0% (95%CI 0.0-2.3) and 7.6% (95%CI 5.2-10.6) in raptors for the years 2016 to 2022 (Figure 2). The highest detection rate of HPAIV-positive raptors is found in WTSEs (13.3 %; 95%CI 8.79-19.00), buzzard sp./common buzzards (6.55%; 95%CI 4.43-9.27 and 4.75%; 95%CI 3.69-5.99, respectively), Northern goshawks (4.6%; 95%CI 2.23-8.31) and peregrine falcons (3.89%; 95%CI 1.27-8.81) – followed by other raptor species mentioned in the supplementary material Table S1. Two of the HPAIV H5-positive WTSE samples from 2021 were identified as nestlings from a single breeding location in Schleswig-Holstein (SH), Germany.

Prospective surveillance in WTSE and their nestlings revealed increased HPAIV H5N1 infection rate since 2021

A prospective surveillance of nestlings started in early 2021 and was carried out in the context of scientific bird ringing. 252 individual birds of eleven different species were sampled (Figure 1). The majority of samples was obtained between April to July 2021 (n=118) and May to June 2022 (n=124) from nestlings on their nests in natural habitats in the German Federal State of Mecklenburg-Western Pomerania (MWP), Germany. Additionally, ten birds (2021: n=7, 2022: n=3) were sampled in a wild bird rescue centre, of which seven birds were considered as adults and three as fledglings/juveniles. The majority of the nestlings showed no clinical signs. However, for few birds (n=13) healed injuries (red kite, n=1 and lesser-spotted eagle [*Clanga pomarina*], n=1; both adult), poor nutritional status (red kite, n=1, nestling and WTSE, n=1, adult) and increased agitation associated with capture/handling (WTSE, n=2, nestlings) were noted. Two WTSE nestlings appeared mildly (n=1) or markedly depressed (#84A; n=1), and five nestlings showed mild serous rhinorrhoea. During fieldwork, nine WTSE nestlings were found dead, either on the nest or in close proximity under the eyrie in varying states of decay. One of them had been sampled alive approximately two weeks prior to death (#84A). In October 2021, a

juvenile WTSE (#72A) was found with neurological disorders (e.g. ataxia) and unable to fly. It was sampled by a veterinarian, including a blood sample for confirmative diagnosis of an expected lead intoxication. The bird died on the following day. A detailed overview on all samples is provided in Tables S2-S4.

As compared to the nationwide surveillance, detection rates for the prospective-regional sampling approach in MWP, Germany, in 2021 to 2022 for HPAIV H5-positive individuals ranged from 3.3% (95%CI 1.4-6.4; n=241) in nestlings to 9.1% (95%CI 0.2-41.3; n=11) in non-nestlings. This is based on the examination of a total of 252 oropharyngeal and 230 cloacal swab samples from 252 individual birds. All samples collected from ospreys (n=4), Northern goshawks (n=10), common buzzards (n=3), red kites (n=59), the lesser-spotted eagle (n=1), black kites (n=5; *Milvus migrans*), sparrow hawks (n=10; *Accipiter nisus*), common kestrels (n=11), tawny owls (n=6) and peregrine falcons (n=3) remained negative. In contrast, nine out of 140 WTSEs were confirmed positive for HPAIV H5N1 (clade 2.3.4.4b). Of these, eight samples were obtained from nestlings, sampled in spring 2022 (Figure 2). Two of these were sampled when nestlings were alive (#77A and 84A), whereas nestlings #77B, 79A, 79B, 84B, 140A and 141A were found dead (Figure 3). HPAIV H5N1 was detected not only in swabs but also in organ samples of these six carcasses. Animal #84A was found dead two weeks after ringing, but its carcass was excluded from the necropsies, due to advanced decay. In addition, a juvenile WTSE (#72A) showing neurological disorders before death tested positive in swabs and organ samples (Figure 3). Highest viral genome loads were found in brain, heart, lung and liver samples (Figure 3).

Passive surveillance in avian raptor species in Germany partially mirrors regional diversity of gs/GD HPAIV H5 genotypes

Further virological characterization work and genome-wide sequence analyses on samples in the period between calendar week (CW) 44 in 2020 to CW 48 in 2022 comprised a total of 33 HPAIV H5 genotypes in wild and captive birds, as well as in poultry (Anne Pohlmann, 2023). Eight genotypes (Ger-04-21-N1, Ger-10-20-N5, Ger-10-20-N8, Ger-10-21-N1.2, Ger-10-21-N1.5, Ger-12-21-N1.3 and Ger-12-21-N1.4) were also found in raptors (Figure 2). Genotype Ger-11-21-N1.4 was detected in a buzzard and remained the only finding of that genotype in Germany (highlighted in grey, Figure 4).

Serological evidence of increased AIV, but not H5-specific, exposure rates in WTSE nestlings

In total, 71 (2021) and 114 (2022) serum samples from nestlings of seven different raptor species and seven (2021) and three (2022) serum samples taken from non-nestlings of five different raptor species were prospectively screened for AIV-reactive antibodies (Figure 5-A). Nestlings positive for nucleoprotein (NP)-reactive antibodies were found exclusively for WTSEs (8 out of 116; 6.9%) of which in only one case antibodies against the H5 subtype could be confirmed unambiguously (#103A). In hemagglutinin inhibition (HI) testing this serum revealed the highest titre against a gs/GD-lineage among several H5 antigens of different origins and therefore is highly likely to be clade 2.3.4.4-specific (Table S5). In an adult red kite and WTSE, and in a juvenile WTSE (#72A), NP-antibodies were also detected. H5-specific antibodies could be confirmed for both adult birds, whereas for the juvenile bird (#72A) the result remained indeterminate (Figure 5-A).

Furthermore, 161 serum samples from WTSEs, taken during prior investigations in 2006-2011, 2013-2019 and 2021 were retrospectively examined. Those WTSE sera retrieved within prior studies are juxtaposed with serological findings in WTSEs from our prospective surveillance (Figure 5-A). Significantly fewer birds tested seropositive for NP-specific antibodies before 2021, and evidence for H5-specific antibodies was confirmed only in 2021 and 2022 (Figure 5-B).

No evidence for declining breeding success rate of WTSE in MWP, Germany, despite concurrent enzootic HPAIV H5N1 circulation

As shown in the regions screened in Germany, the amount of WTSE breeding pairs in 2022 was situated in the upper range compared to previous counts over the last two decades (Figure 6-A). The breeding success rate in 2022 when HPAIV H5N1 was highly prevalent in two regions (Isle of Rügen and Isle of Usedom) averaged that of the preceding years (Figure 6-B). The breeding success rate indicates the proportion of those breeding pairs of which at least a single nestling fledged compared to all pairs that had started breeding in the respective year.

Discussion

Raptor populations are frequently threatened by a number of anthropogenic factors. These include encroachment of their habitats (Newton, 1979), increased toxicological burdens (Badry et al., 2020; Nadjafzadeh, Hofer, & Krone, 2013) and collision with man-made structures, such as wind energy plants (Heuck et al., 2019), among others. In many countries, including Germany, a high level of conservation effort is required to compensate for these negative anthropogenic factors and has succeeded to stabilize, or even promote growth of avian raptor populations. New infectious diseases associated with high mortality, such as HPAI, might challenge these recent achievements. Avian raptors are especially exposed to pathogens and opportunistic microbiota, when they prey on infected, weakened animals and some species are even scavenging on carcasses. However, hunters and facultative scavengers should have evolved increased resistance to infectious threats from their prey (Zepeda Mendoza et al., 2018; Zou et al., 2021), and even provide beneficial functions by removing potentially infectious carcasses (and their pathogens) from the ecosystem (Plaza, Blanco, & Lambertucci, 2020). Nevertheless, from an evolutionary perspective, HPAIV H5 (gs/GD) is a very recent pathogen in wild birds and yet, no such resistance mechanisms could have been positively selected in avian raptors, including specialized scavengers like vultures (Ducatez et al., 2007). Conceivably, HPAIV H5 infection in immunologically naïve avian raptor species has been shown to induce severe and often fatal disease. These findings have prompted investigations of using avian raptors as indicator species to monitor geographical expansion of HPAIV activity in general and incursion events of HPAIV H5 into new regions, as recently described for the transatlantic spread of HPAIV H5, clade 2.3.4.4b, via Iceland (Günther et al., 2022; Lee et al., 2019).

Our retrospective analysis clearly confirmed a high infection risk for raptors at the end of the food chain, in particular for large to medium-sized avian raptor species, during the epizootic years 2016/17 and since 2020. Thus, the informative status of avian raptors with respect to virological investigations regarding HPAIV is obvious. This became apparent also when analysing the HPAIV H5 genotypes of raptor-born viruses: The HPAI epizootic 2020-2021 in Germany was caused by numerous different subtypes and genotypes of HPAIV H5 (King et al., 2022). Almost a quarter of all different genotypes was also found in various raptor hosts. Their frequency in raptors is proportional to their occurrence in other avian hosts. An exception is genotype Ger-11-21-N1.4 (A/buzzard/Germany-SH/AI07099/2021-like) which has been found exclusively in a single unspecified buzzard. This suggests the origin of Ger-11-21-N1.4 in another primary, avian host, that remained unspecified and undetected, e.g. due to a very localized and restricted occurrence of this virus strain. WTSEs seemed to be particularly informative targets within (passive) surveillance approaches, showing that over time approximately every eighth individual WTSE tested was confirmed positive for HPAIV H5 (Table S1). Indeed, we have been able to provide data to confirm the role of raptor species as suitable indicators for a general HPAI-surveillance and to highlight their importance to reflect even temporal and geographical patterns of genetic variants.

During the epizootic 2016/17, juvenile and immature WTSEs have been affected more frequently than (sub)adult ones; nestlings were not affected at all since the virus was only recorded outside the hatching season (Krone et al., 2018). HPAIV H5N1 in a WTSE hatch (n=2) has first been found in the Northern German federal state SH in May 2021. To our current knowledge this is the first detection of HPAIV H5 in nestlings of WTSEs in Germany and matched with a report from Estonia over the same time period (Estonian University of Life Sciences, 19.05.2021). Virological testing during the second year of prospective sampling in MWP, Germany, yielded similar observations of sporadically infected WTSE nestlings (Figure 3) in 2022, when eight nestlings of five locations have been confirmed positive for HPAIV H5, clade 2.3.4.4b.
At the time of sampling, nestlings #77A and #84A did not show severe neurological signs, as described for HPAIV-infected birds before, only a markedly, but unspecific, depression (#84A). In both occasions, another HPAIV-positive nestling was found dead in or in close proximity to the nest. HPAIV RNA was detected in all organ samples taken from these deceased nestlings and confirmed systemic infections in accordance to prior findings during the 2020-21 epizootic by Caliendo, Leijten, et al. (2022).

In contrast to the virological HPAIV testing, few studies have focused on serum antibody analysis. Previous studies failed to detect AIV-reactive antibodies in raptor nestlings during similar sampling approaches in Northern Europe (Gunnarsson et al., 2010; Lee et al., 2019). Here, we analysed raptor sera on a larger scale and detected antibodies against IVA NP (n=8; in 2017, 2021 and 2022) and H5 (n=1; in 2022) exclusively in WTSE nestlings. The fact that we were able to also confirm one H5-seropositive case (#103A; Figure 5-A,) in two independent assays (refer to methods), suggests the reliability of the commercial kits utilized but not validated for raptors due to the lack of reference sera.

NP-antibody detection rates in WTSE nestlings of 5.0% (2021) and 7.9% (2022) appear low given the massive HPAIV H5 outbreak scenarios and the presumed high likelihood of parental female WTSE for exposure during the last two years. Still the single WTSE nestling #103A sampled in 2022 remained the only evidence in a nestling of antibodies against H5 of clade 2.3.4.4 (Figure 5A, Table S5). Due to the highly variable age at which the animals are ringed (and thus sampled), we cannot rule out the possibility that samples were taken, at least in some cases, at a time when maternal antibodies had already declined below detection levels and an active specific immune response had not been generated by the nestling.
Thus, the data may present a vast underestimation of the true seroprevalence in adult female WTSEs. No literature on the stability of maternal antibody levels in WTSEs after hatching exists, but studies among other avian species suggest a rapid decline of maternal antibody levels (van Dijk, Mateman, & Klaassen, 2014; Velarde, Calvin, Ojkic, Barker, & Nagy, 2010), depending on the initial level of yolk-derived antibodies and the respective test sensitivity. Testing younger nestlings closer to hatch might have provided more conclusive results but there is a minimum age of more than five weeks that needs to be considered when sampling is to be combined with ringing.
The case of the WTSE nestling #103A exemplifies the problems of interpretation in a seropostive case: It has been sampled between six to seven weeks after hatching and tested positive for H5-antibodies. Assuming the time period of decreasing maternal antibodies as a matter of a few weeks, not days or months after hatching, the sero-response of nestling #103A cannot be clearly associated with either maternal antibodies or seroconversion after direct contact with HPAIV H5. The latter, unconfirmable, assumption would raise hope that even WTSE nestlings, under certain circumstances, may overcome HPAIV H5 infection.
Sampling of adult birds would have allowed direct measurements of seroprevalence rates; however, adult raptors are accessible for blood sampling on exceptional occasions only. Sampling of large-sized adult raptors is mainly limited to wild bird rescue centers, when birds are admitted for care and such sample set would be skewed by the dominance of samples from non-healthy birds. One out of eight blood sampled non-nestling raptors, other than WTSE, revealed NP- and H5-reactive antibodies (Table S4). The seroconversion of this adult red kite is interpreted as an indication that some raptor species can overcome an AIV H5 infection. Also, a single adult WTSE out of eleven blood sampled adults (9.1%) revealed H5-antibodies, simultaneously the only adult WTSE tested in 2022.

Overall, sampling of nestlings is an elegant option to gain insights into wild avian raptor populations. Although only few trained experts are concerned, direct contact with, as shown here, nestlings shedding HPAIV H5 asymptomatically cannot be excluded. Zoonotic transmission routes via injuries caused by the bird´s beak or claws contaminated with feces or carrion remains can be envisaged when ringing or sampling avian raptors. Strict hygiene measures are required and even cessation of bird ringing activities in confirmed HPAI hotspot regions should be considered to securely prevent spill-over events to humans, but also to avoid bird ringers (unknowingly) becoming vectors between (breeding) locations via contaminated equipment, clothes and shoes. Avian rehabilitation centers and clinics face a similarly high risk of being confronted with HPAIV-H5-positive wild birds. Caliendo, Mensink, et al. (2022) pointed out the importance of increased awareness for those institutions, including continuous education of employees, for adequate quarantine measures to prevent inadvertent spread. Routine virological screening of admitted raptors is highly recommended as exemplified here by the case of WTSE #72A shown here initially misdiagnosed for lead intoxication, one of the most common causes of death in (sub)adult WTSE, but instead being HPAIV H5-infected.

Promoting and maintaining a stable reproduction ratio over many years supported by a high number of breeding pairs is essential for a stable population of long-lived k-strategists such as WTSEs. Reducing this rate by removing adult breeding-competent individuals or by impacting hatching and upbringing of chicks may ultimately lead to population instability. The enzootic status of HPAIV H5 in Germany combines these risks for WTSE as shown here for juvenile to (sub)adult WTSE deaths and five affected hatches in MWP in 2022. Contrary, however, to the expectations and previous reports from bald eagle populations in North America (Nemeth et al., 2023), the overall breeding success rate for this region remained unaffected (Figure 6), even if further cases might have remained undetected. It remains to be determined whether cross-immunity of the parental female birds and, therefore, maternal antibodies in their nestlings might have contributed. Our serological data gave some evidence in that direction.
Furthermore, there is a staggered start of breeding of WTSE pairs within the same region, preventing that all clutches hatch within a very narrow period of time. This would reduce the influence of time-sensitive risk factors to the overall population, mainly related to weather conditions but probably extrapolatable also to the prevalence of pathogens in prey. However, such effect may vary in (coastal) regions where WTSEs have stronger ties to water areas with seabird colonies. The latter have been hit severely by HPAIV H5, culminating even in mass mortalities (Anne Pohlmann et al., 2023). Removal of possibly HPAIV-infected carcasses by human activities has been shown to have positive effects on such colonies (Knief et al., 2023). Yet, this kind of carcass removal can never be as efficacious (and timely) as the scanning activities of birds of prey. In addition, the hunting behaviour e.g. of peregrine falcons cannot be manipulated to be distracted from infected and weakened live prey. Thus, it cannot be excluded that detrimental impacts will develop nevertheless over time in case the enzootic HPAI H5-status in the regional wild bird populations is to continue. With the current continuation of HPAIV H5 circulation in Europe black-headed gulls (*Chroicocephalus ridibundus*) became the dominantly affected species; while no increase in WTSE cases are reported, peregrine falcons and Eurasian eagle owls are found HPAIV H5-infected at increasing rates. In this context, particular adaptations of new emerging genetic variants of gs/GD HPAIV such as the gull-adapted reassortant genotype BB to certain prey species may lead to shifting risks for different raptor species (EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), EURL (European Reference Laboratory for Avian Influenza), Adlhoch, Fusaro, Gonzales, Kuiken, Mirinaviciute, et al., 2023).

Conclusions

Overall, our results on HPAIV H5 found in raptor species, particularly WTSE, common buzzards, Northern goshawks, peregrine falcons and Eurasian eagle owls, during passive surveillance confirm their suitability as important indicators for the occurrence of the pathogen, including detection of temporally and geographically restricted variants. Prospective screening of avian raptor nestlings revealed the

412 presence of maternal antibodies or seroconversion in WTSE chicks. The still low AIV-seropositivity rate
413 of nestlings in the examined WTSE population indicates a particular risk for naïve nestlings to alimentary
414 HPAIV infections on the nest and after fledging. This became evident by multiple findings of systemic
415 fatal infections in WTSE nestlings in MWP in 2021 and 2022. As yet, no direct detrimental influence on
416 breeding success rates of WTSE was evident in the region. The combination of scientific bird ringing and
417 sampling for disease surveillance seems highly appropriate in terms of coordinated species protection
418 but requires heightened awareness and strict hygiene measures to avoid inadvertent pathogen
419 carryover and human exposure.
420

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430 Supportive information

431 Additional supporting information may be found in the online version of the article at the publisher's
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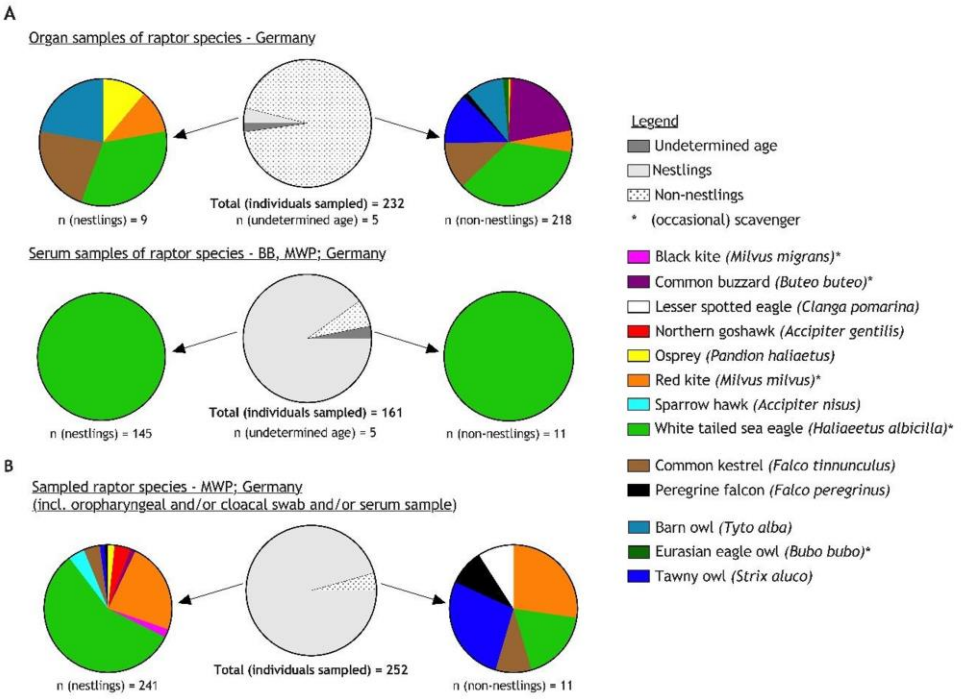
579 # Figures



580

581 ***Figure 1*** *Overview of individual avian raptors sampled within the retrospective (A) or prospective (B) surveillance approach in*
582 *this study, including additional information on age cohorts and scavenging behavior.*

583

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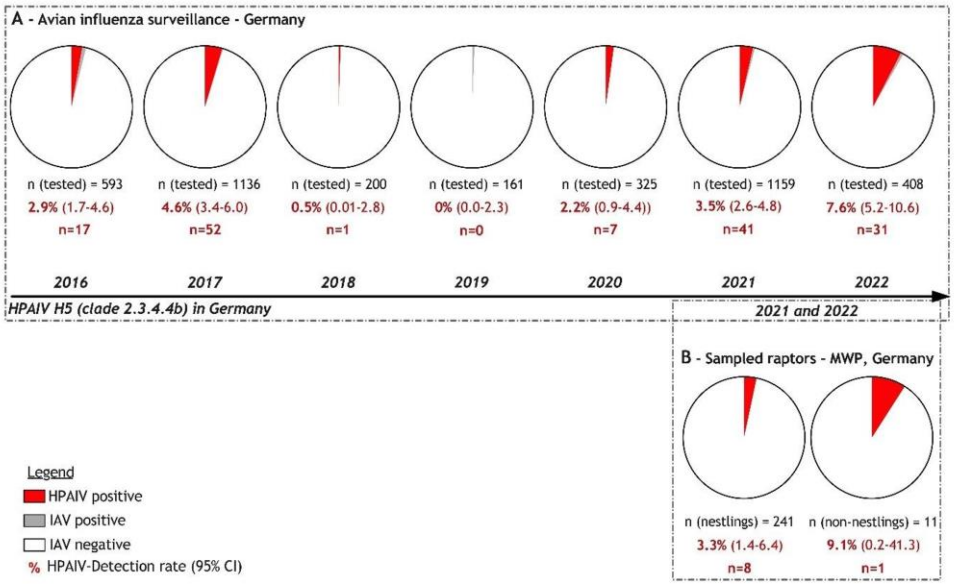

585

586 ***Figure 2*** *Numbers of raptors screened for influenza A viruses (IAV) and testing positive for gs/GD highly pathogenic avian*
587 *influenza viruses (HPAIV). The proportion of HPAIV-positive birds is given for the retrospective surveillance across the whole of*
588 *Germany between 2016-2022 (A) and nestlings and non-nestlings (B), sampled within the prospective-regional sampling*
589 *approach in Mecklenburg-Western Pomerania (MWP), a Federal State in the Northeast of Germany (2021 and 2022).*

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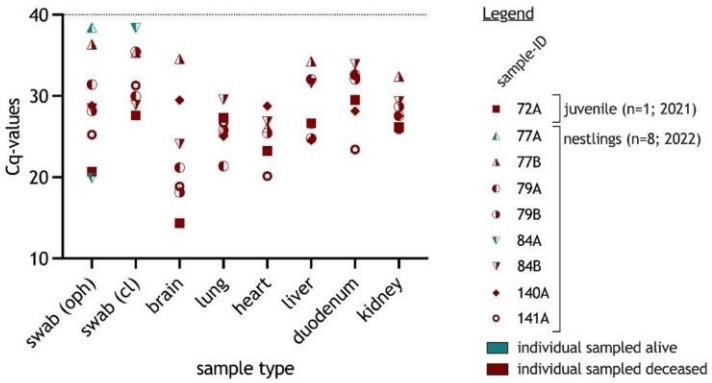

591

592 ***Figure 3*** *Distribution of relative viral load (low Cq values indicate high viral loads) in HPAIV H5N1-positive white-tailed sea eagles,*
593 *screened by RT-qPCR. The results are presented per sample matrix (oropharyngeal or cloacal swab or organ) and individual bird.*
594 *Individuals are assigned numbers and letters, where numbers indicate a specific nest and letters the different nestling therein.*
595 *Samples taken from individuals alive are shown in blue, samples taken from dead nestlings are shown in red.*

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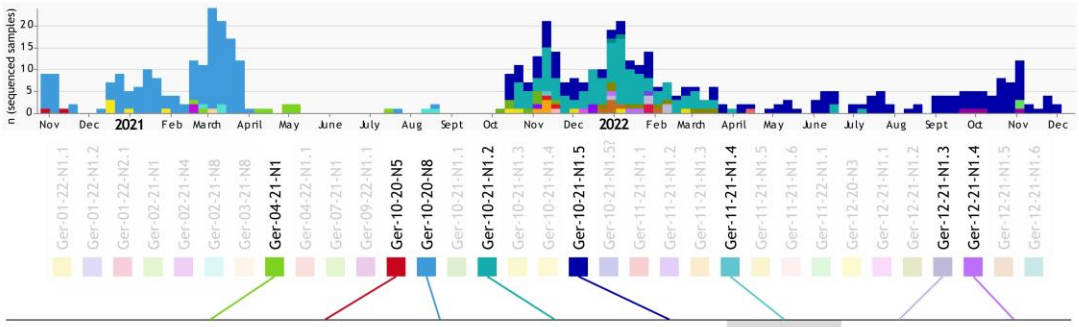


n (total sequences)	3	2	162	122	168	1	5	3
n (raptor host)	1	1	3	11	9	1	2	1
Affected raptor species	Buzzard sp.	Common buzzard	Common kestrel, Eurasian eagle owl, peregrine falcon	Buzzard sp., Eurasian eagle owl, Northern goshawk, peregrine falcon, undefined raptor, white-tailed sea eagle	Eurasian eagle owl, undefined raptor, white-tailed sea eagle	Buzzard sp.	Buzzard sp., long-eared owl	Common kestrel
n (other host)	2	1	159	111	159	-	3	2
Other affected hosts species	*Anseriformes*	poultry	*Anseriformes*, *Charadriiformes*, poultry, *Rheiformes*; mammalia (seals)	*Anseriformes*, *Charadriiformes*, *Ciconiiformes*, *Gruiformes*, *Passeriformes (Corvidae)*, poultry, *Suliformes*	*Anseriformes*, *Charadriiformes*, *Pelecaniformes*, poultry, *Suliformes*; mammalia (coati)	-	*Columbiformes*, *Charadriiformes*	*Charadriiformes*
Expansion pattern	regional (NI)	regional (MWP)	all over Germany	all over Germany	all over Germany	single case (SH)	SH, NW, BB	regional (SH)

Figure 4 *HPAIV H5 genotype variation in Germany. Between calendar week 44 in 2020 and 48 in 2022, 33 distinct genotypes were found in total (unique colour codes, upper panel). Of these, eight were found in avian raptors(highlighted), and additional information on their temporal occurrence and distribution, raptor host species (groups) and other affected hosts is summarized. Ger-11-21-N1.4 is shaded in grey emphasizing that it is the only genotype found exclusively in a raptor host. Abbreviations of the German federal states: BB - Brandenburg; MWP – Mecklenburg-Western Pomerania; NI - Lower Saxony; NW – North Rhine-Westphalia; SH - Schleswig-Holstein.*

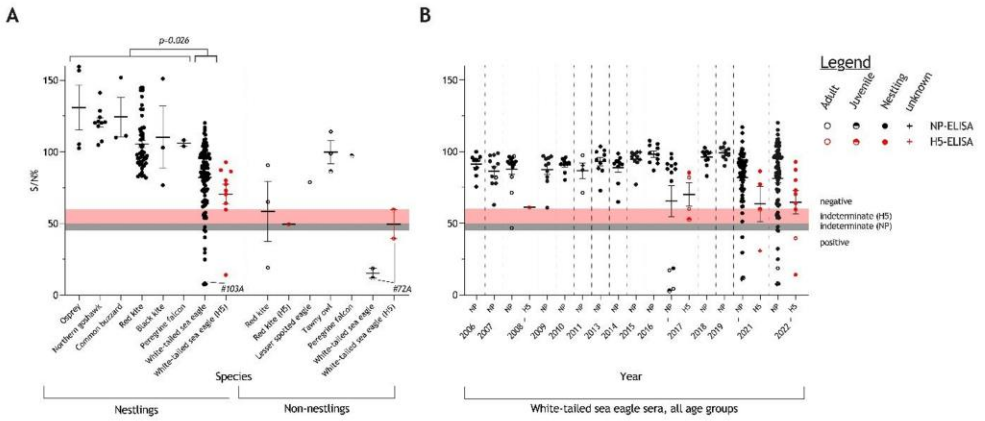
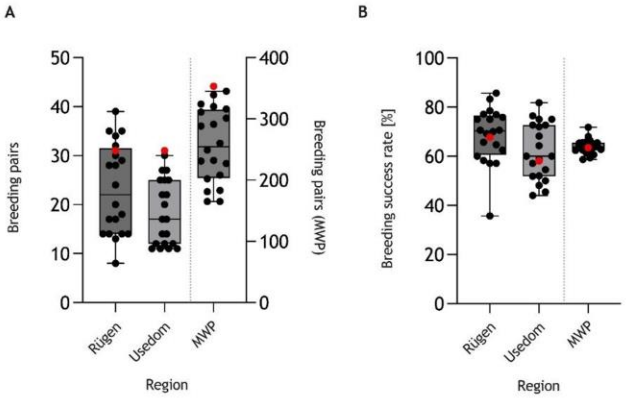


Figure 5 *Serological results of samples retrieved from avian raptors. The results are shown in percent inhibition values measured by competition ELISA to detect antibodies against the nucleoprotein (NP, black symbols, grey indeterminate range 45-50%; positive <45%). NP-positive sera were further tested against the hemagglutinin H5 (H5, red symbols, pinkish indeterminate range 50-60%; positive <50%). A) Serological status of individuals sampled within the prospective-regional surveillance approach in Mecklenburg-Western Pomerania (MWP), Germany in 2021 and 2022. The data are grouped per species and age, indicating mean and standard error of the mean. Data points for certain individuals are highlighted by specific numbers; numbers correspond to listings in Supplementary Tables 1-3. P-value < 0.05 is confirming significant differences comparing NP-positive findings in white-tailed sea eagle (WTSE) nestlings (n(positive)=8; n(non-positive)=108) and nestlings of all other raptor species (n(positive)=0; n(non-positive)=69) via the Fisher-test. B) Serological status of WTSE analyzed retrospectively (2001-2019) or obtained within a prospective targeted surveillance approach in MWP, Germany (2021-2). The data are stratified by year of sample origin and by age cohort indicating mean and standard error of the mean.*



617

618 ***Figure 6*** *Number of breeding pairs (A) and breeding success rate (B) of white-tailed sea eagles in two selected regions of the*
619 *federal German state of Mecklenburg-Western Pomerania (MWP; Isle of Rügen and Isle of Usedom) and in total MWP, 2002-*
620 *2022. Red dots indicate the data for 2022 when HPAIV H5N1 of clade 2.3.4.4b was enzootically prevalent in those regions.*

Publication IV: „Highly pathogenic avian influenza virus incursions of subtype H5N8, H5N5, H5N1, H5N4, and H5N3 in Germany during 2020-21"

Publication IV

Highly pathogenic avian influenza virus incursions of subtype H5N8, H5N5, H5N1, H5N4, and H5N3 in Germany during 2020-21

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Rapid Communication

Highly pathogenic avian influenza virus incursions of subtype H5N8, H5N5, H5N1, H5N4, and H5N3 in Germany during 2020-21

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Abstract

From October 2020 to July 2021, five different subtypes (H5N8, H5N5, H5N1, H5N4, and H5N3) and seven genotypes of highly pathogenic avian influenza viruses (HPAIV) belonging to clade 2.3.4.4b were detected in a broad array of avian hosts in Germany. Initial incursion by wild birds with an unprecedented involvement of charadriiforme species at the Wadden Sea coast only carrying subtype H5N3, lateral spread between poultry with detection of novel reassortants and mixed infections in poultry holdings, suspected spillback of HPAIV from poultry to wild birds, and detection of HPAIV-infected wild birds during the following summer in 2021 were hallmarks of this epizootic. Local reassortment events with low pathogenic AIV strains were detected by phylogenetic analyses, with a dominating HP H5N8 and later HP H5N1 strain responsible for most cases. In addition, the first-ever described HPAIV strain of subtype H5N4 could be genetically characterized.

Key words: HPAIV; H5N8; H5N5; H5N1; H5N4; H5N3; reassortment; third-generation sequencing; MinION; nanopore sequencing.

1. Introduction

Since the first incursion into Europe of highly pathogenic avian influenza virus (HPAIV) subtype H5N8 of clade 2.3.4.4b in 2016, Germany has seen a whole series of novel incursions with distinct reassortants (King et al. 2021). Viruses of clade 2.3.4.4b, derived from the goose/Guangdong (gs/GD) lineage first detected in China, 1996, have demonstrated an unprecedented tendency for reassortment, resulting in a promiscuous array of sub- and genotypes (Xu et al. 1999). Following the extensive and diverse epizootic in 2016–18 that took a major toll on wild bird populations and the poultry production sector (Globig et al. 2017; Pohlmann et al. 2018), a novel incursion of another clade 2.3.4.4b HPAI H5N8 variant in February of 2020 resulted in only a minor outbreak in small holdings and captive birds (Swieton et al. 2020; King et al. 2020b). The detection of further clade 2.3.4.4b HPAI H5Nx viruses in October 2020 in Germany entailed the largest recorded HPAI epizootic in the country to date (EFSA 2021) and, on the other hand, marks the beginning of a new epidemic with a wide variety of reassortants, countless cases of infections in wild birds, and the introduction of HPAIV H5Nx into poultry farms with additional secondary outbreaks.

This study aims to portray a comprehensive (phylo-) genetic analysis of all detected sub- and genotypes from the 2020–21 HPAIV season in Germany.

2. Material and methods

Full-genome sequencing of AIV-positive samples was executed by a previously described nanopore-based amplification method (King et al. 2020a). In short, RNA extraction with the Qiagen Mini Viral Kit (Qiagen, Germany) and subsequent AIV-End-RT-PCR with Superscript III One-Step and Platinum Taq (ThermoFisher Scientific, USA) for universal whole genome amplification was conducted with one primer pair (Pan-IVA-1F: TCCCAGTCACGACGTCGTAGCGAAAGCAGG; Pan-IVA-1R: GGAAACAGCTATGACCATGAGTAGAAACAAGG). After purification of the PCR products with AMPure XP Magnetic Beads (Beckman-Coulter, USA), full-genome sequencing utilized the Mk1C MinION platform (Oxford Nanopore Technologies, ONT, UK) in combination with the Rapid Barcoding Kit (SQK-RBK004, ONT) for sample multiplexing. Sequencing was directed according to the manufacturer's instructions with a R9.4.1 flow cell. Live base-calling of the raw data with Guppy (v.4.0.11 and v.4.3.4, ONT)

was followed by a demultiplexing, quality check, and trimming step to remove low quality, primer, and short (<50bp) sequences. After sequencing, full-genome consensus sequences were achieved in a map-to-reference approach utilizing MiniMap2 (Li and Birol 2018). Reference genomes are a curated collection of all HA and NA subtypes alongside an assortment of internal gene sequences chosen to cover all potentially circulating viral strains. Polishing of the final genome sequences was done manually after consensus production according to the highest quality (60 per cent) in Geneious Prime (Biomatters, New Zealand). Data included in this study have been deposited in the EpiFlu™ GISAID database (www.gisaid.org/). Respective accession numbers can be found in Supplementary Table S1.

Segment-specific and concatenated whole-genome maximum likelihood trees were generated with RAxML (Stamatakis 2014) utilizing the model GTR GAMMA with rapid bootstrapping and search for the best-scoring maximum likelihood tree together with 1,000 bootstrap replicates. For phylogenetic incongruence analysis trees were imported and tips of identical strains aligned using Dendroscope (V3.8.1). Time-scaled trees of the HA sequences of all genotypes were calculated with BEAST (V1.10.4) software package (Suchard et al. 2018) using a GTR GAMMA substitution model, an uncorrelated relaxed clock with a lognormal distribution, and coalescent constant population tree models. Chain lengths were set to 50 million iterations and convergence checked via Tracer (V1.7.1). Time-scaled summary maximum clade credibility trees (MCC) with 10 per cent for the post-burn-in posterior were created using TreeAnnotator (V1.10.4) and visualized with FigTree (V1.4.4). The MCC trees show estimates of the time and their 95 per cent highest posterior density (HPD) confidence intervals at each node.

Geographical distribution was visualized with QGIS (V3.16, QGIS.org). Geographical geojson vector maps were obtained from http://opendatalab.de/projects/geojson-utilities/ with open data provided by the German Federal Agency for Cartography and Geodesy (https://gdz.bkg.bund.de/).

3. Results

3.1 Evidence of the largest HPAI epizootic among wild birds and poultry in Germany

Since the first HPAIV H5 report on 26 October 2020, the majority of cases in wild birds and outbreaks in captive birds (wild or domestic bird species held in captive enclosures such as zoos) were recorded from November 2020 to March 2021. Overall, more than 1,300 wild bird cases and over 250 outbreaks in poultry holdings (mainly turkey and layer chicken holdings) were confirmed as HPAI H5 positive during the season (data as of 18 October 2021). Two monthly maxima in November 2020 and March 2021 were observed in the wild bird population, affecting mainly species of the Anser genus, while domestic bird cases peaked in March/April 2021. The geographical distribution of wild bird cases shows a focus on the coastal sites of Germany, particularly the Wadden Sea of the North Sea coastline (Fig. 1). Remitting notifications of single cases continued until July 2021, with 'final' cases in a Eurasian oystercatcher (*Haematopus ostralegus*, H5N1) and mute swans (*Cygnus olor*, H5N8). Since October 2021, cases of H5N1 have been accumulating, especially yet again in Eurasian wigeon (*Mareca penelope*) and Barnacle geese (*Branta leucopsis*).

A total of 176 full-genome sequences and further 10 partial genome sequences were attained for analyses. This selection covers 42 wild and 134 domestic bird whole-genome sequences, and aims to portray a comprehensive subset of the epizootic with respect to time, location, and species affected. A detailed overview of the sequenced samples can be found in Supplementary Table S1.

3.2 Genotyping reveals co-circulation of up to five sub- and seven genotypes of clade 2.3.4.4b HPAI H5 viruses

For genotyping, segment-based phylogenetic analysis was done on all German sequences, comparing publicly available international sequences from the same time span and integrating similar sequences from databases representing possible ancestors (Supplementary Figs S1 and S2). Phylogenetic incongruence analysis of the German viruses was generated to give an outline of the different reassortants (Fig. 2).

Five HPAIV H5 subtypes encompassing seven genotypes were identified throughout the epizootic in Germany. Here, genotypes are labelled according to their first detection date in Germany and their NA subtype (Ger-Month-Year-Nx) in line with previous publications (Pohlmann et al. 2018; King et al. 2020b). All sequences analysed revealed H5 viruses expressing an identical polybasic haemagglutinin cleavage site (PLERRKKRG) confirming high pathogenicity grading.

Initially and concurrently, both HPAI H5N8 and H5N5 strains were detected in deceased wild birds found along the North Sea Coast of Germany. The earliest identified H5N8 genotype (termed **Ger-10-20-N8)** arose to be the dominating strain, responsible for >90 per cent of all sequenced cases. This H5N8 genotype shared a high identity for all segments with previously described strain A/chicken/Iraq/1/2020 (H5N8, EPI_ISL_623074) collected in May 2020 in Iraq and frequently found since summer 2020, first in Central Asia and subsequently in autumn 2020 in Europe and Southeast Asia (Xu et al. 2017).

Ger-10-20-N8 formed the genetic backbone for most genotypes detected in Germany. For example, an H5N5 subtype reassortant (**Ger-10-20-N5**) shared six segments with Ger-10-20-N8. Its N5 NA segment clustered with an H5N5 strain identified in the Russian Federation in 2020, while the PA segment showed closest relations to low pathogenic avian influenza viruses (LPAIV) of subtype H3N1 also detected in the Russian Federation in 2018 (Fig. 3).

In addition to Ger-10-20-N8, two further HPAI H5N8 genotypes were discovered in poultry only. Termed **Ger-02-21-N8** and **Ger-03-21-N8**, these novel reassortants were identified in February and March 2021, respectively. While Ger-02-21-N8 only differed from the original Ger-10-20-N8 strain by a novel NP segment that clustered with LPAIV from Europe, Ger-03-21-N8 showed a completely different genetic constellation. Ger-03-21-N8 differed in every segment excluding the HA, NA, and MP genes. The new segments could all be traced back to previously sequenced LPAIV from Germany and Europe. The NS segment of Ger-03-21-N8 was identified prior to the epizootic in September 2020 in a LPAI H5N8 strain from Germany (A/guinea fowl/Germany-NW/AI01184/2020, EPI_ISL_661312). While Ger-03-21-N8 was initially discovered in a poultry holding in North Rhine-Westphalia, the PB1, PA, and NP segments belonging to Ger-03-21-N8 were likewise discovered in a poultry holding in Brandenburg (January 2021, A/turkey/Germany-BB/AI00868/2021, Fig. 3—mixH5N8) where a

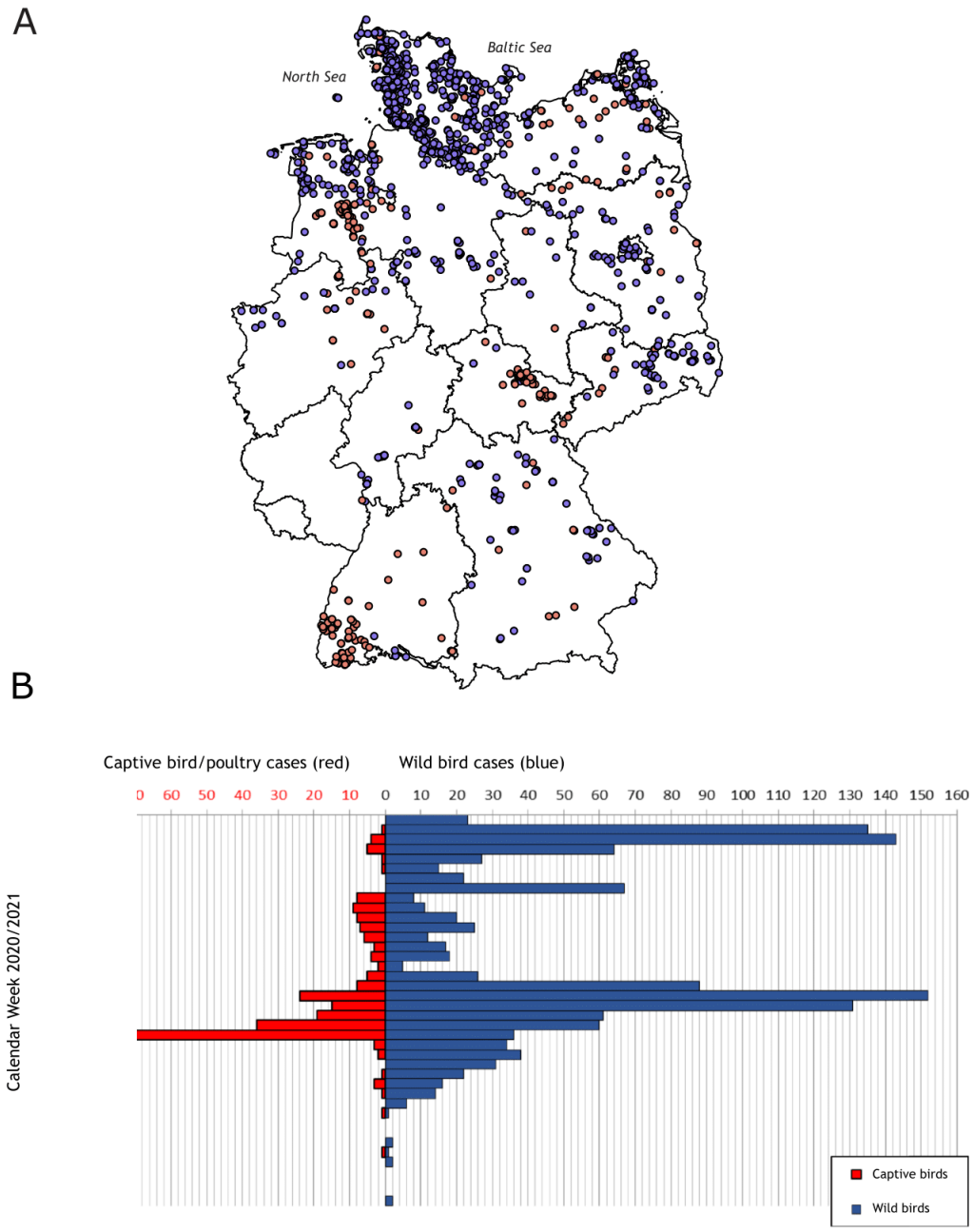

Figure 1. HPAIV case counts and geographic distribution according to data collected by the German animal disease notification system (Tierseuchennachrichten—TSN). (A) Geographic map of reported cases in Germany, 26 October–1 November 2020 (week 44) to 26 July–1 August 2021 (week 30). (B) Dynamics of case counts according to wild bird and poultry reports in Germany, 26 October–1 November 2020 (week 44) to 26 July–1 August 2021 (week 30). Wild bird cases and outbreaks in captive birds (poultry including zoos) are distinguished.

mixed infection of Ger-10-20-N8 alongside the respective segments was discovered. Here, the genotype Ger-10-20-N8 was identified as a full-genome alongside the novel PB1, PA, and NP segments related to the respective genes from Ger-03-21-N8 and an additional novel PB2 segment, also most closely related to European LPAIV (Fig. 3).

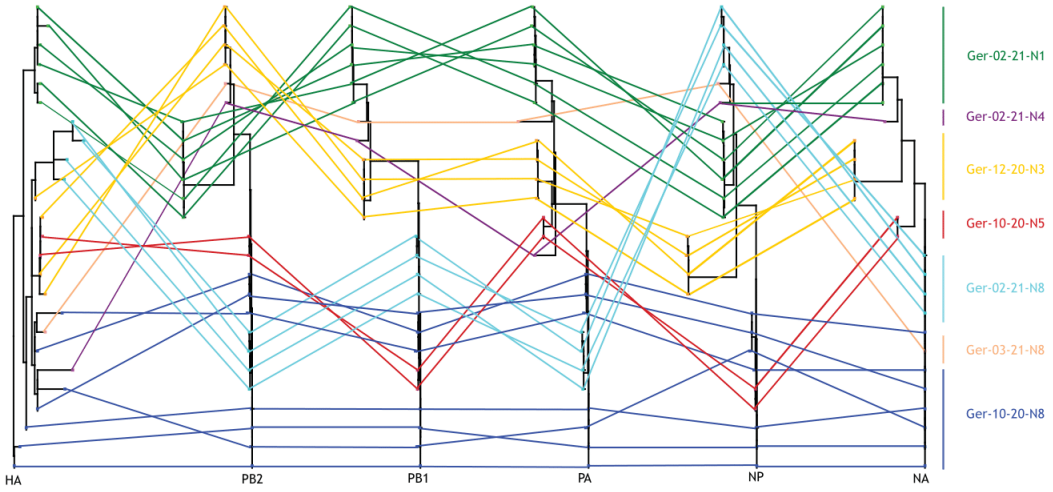

Figure 2. Phylogenetic incongruence analysis. Maximum likelihood trees of the HA, PB2, PB1, PA, NP, and NA segments from representative strains of all detected sub- and genotypes from October 2020 to July 2021 in Germany, calculated utilizing RAxML with model GTR GAMMA (fast bootstrapping) and 1000 bootstrap replicates. Strains were connected across trees and tips and genotypes are designated and coloured consistently.

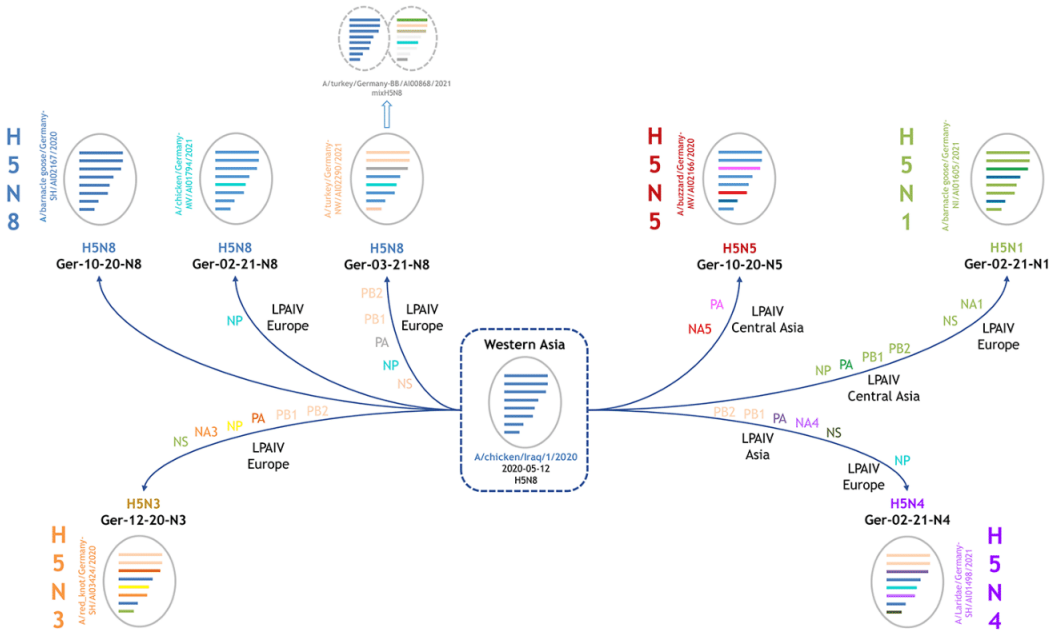

Figure 3. Schematic reassortment analyses of the detected sub- and genotypes in Germany, October 2020 to July 2021. Putative precursor segments are labelled according to geographic origin and coloured consistently dependent on relations throughout all reassortants.

European LPAIV also played an important role in the genetic constellation of the detected HPAIV H5N3 (**Ger-12-20-N3**) subtype. Here, only the HA and MP segments of the genetic backbone were retained. All other segments showed the closest relations to European LPAIV, including the PB2 and PB1 segments found in the previously described Ger-03-21-N8 genotype (2021AI02290), and the NP segment reverting to the German LPAIV H5N8 sample (2020AI01184).

In addition to the HPAIV H5N3 strain, a completely novel H5N4 subtype was identified in Germany (**Ger-02-21-N4**). To date, this was the first detection of an HPAIV of this subtype worldwide. Once again carrying the backbone HA and MP genes,

all other segments shared their closest relations to further Eurasian viruses. The N4 segment itself was similar to a H7N4 strain from Bangladesh, 2019. Only a few cases of this subtype were reported in the wild bird population, affecting gull and duck species.

Although several European countries identified a HPAI H5N1 strain early on in the European epizootic, Germany detected its first HPAI H5N1 case, not before February 2021 (**Ger-02-21-N1**). Once again, the common core backbone was represented by the HA and MP segments. In addition, the PB2, PB1, PA, and NP segments shared the closest relation to LPAIV found in Central Asia, while the NA1 and NS genes showed evidence of their origin in European LPAIV. These findings are in line with the previously described genetic constellation of the H5N1 subtype in other European countries. This reassortant affected both the wild bird and poultry populations and was responsible for many cases reported during the spring/summer/autumn months of 2021 (April–July). The 'final' German cases reported in July 2021 (H5N8, Ger-10-20-N8; H5N1, Ger-02-21-N1) attest an extended circulation period within the wild bird population.

3.3 Time-scaled phylogenetic analysis of Ger-10-20-N8 shows multiple incursions into Germany

A detailed time-scaled MCC phylogeny of all German and publicly available international HA segments (Supplementary Fig. S2) indicates several independent incursions and simultaneous co-circulation of various strains in Europe. The dominant Ger-10-20-N8 genotype likewise shows multiple incursions into the German wild bird population. Comparison of Ger-10-20-N8 to international sequences indicate similarities to viruses collected eastward, northward, or northwest ward related to the main outbreak region in Germany, the North Sea coastal region. This is in line with the findings that viruses of the same genotype were present in Europe throughout the season (EFSA 2020, 2021).

3.4 Time-, location-, and species-related restriction of HPAIV H5 sub- and genotypes in Germany

During the 2020/21 avian influenza season, 14 federal states in Germany reported HPAIV infections. From the get-go, the geographic distribution showed an accumulation of wild bird cases in the federal state of Schleswig-Holstein, especially accumulating at the Wadden Sea coastline (Fig. 1A). While the majority of HPAI wild bird reports in 2020 and throughout most of 2021 derived from Northern Germany (Bremen, Hamburg, Mecklenburg-Vorpommern, Lower Saxony north of Hannover, and Schleswig-Holstein), cases from Central (Berlin, Brandenburg, Hesse, North Rhine-Westphalia, Lower Saxony south of Hannover, Saxony, Saxony-Anhalt, and Thuringia), and Southern Germany (Bavaria, Baden-Württemberg, and Rhineland-Palatinate) were mainly detected from February to April 2021 (Supplementary Figure S3).

While keeping in mind that more poultry outbreak samples were sequenced than wild bird cases, subtypes H5N5 and H5N1 alongside genotype Ger-10-20-N8 (H5N8) showed no host specificity, affecting wild birds and poultry (Galliformes) alike. Affected wild bird species mainly belonged to the Anseriformes order. Ger-02-21-N8 and Ger-03-21-N8 were only detected in poultry holdings, but carried segments related to LPAIV found in wild birds. On the contrary, subtype Ger-12-20-N3 (H5N3) affected nearly only wild birds belonging to the Charadriiformes species along the Wadden Sea coast, where a mass mortality event within the respective bird population was recorded. Only few additional H5N3 cases were recorded in predatory bird species hunting on Charadriiformes in the Wadden Sea. Subtype H5N4 was only detected in wild birds, affecting gull and duck species. Precise enumeration of the affected bird

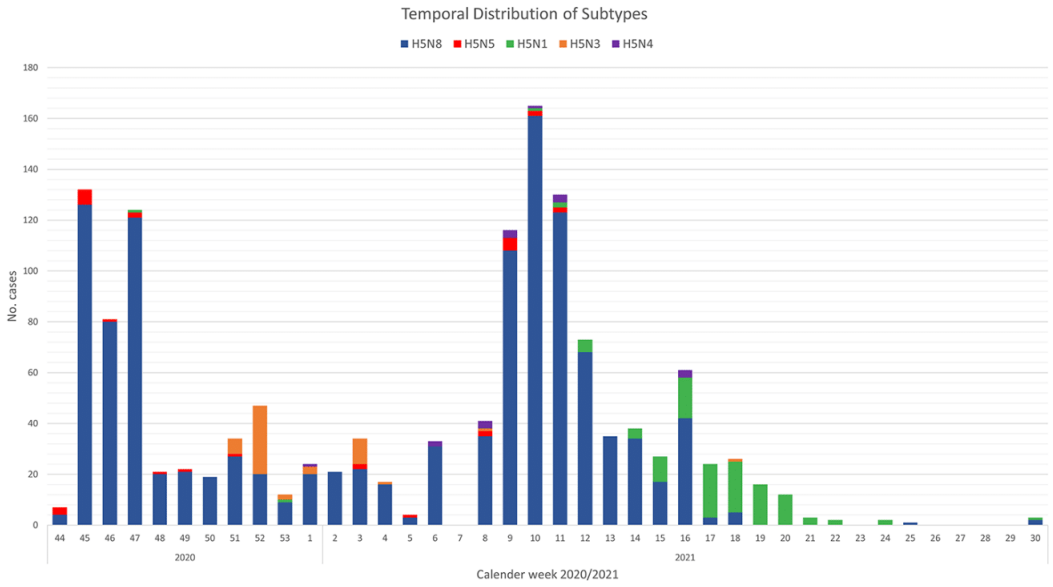

Figure 4. Temporal distribution of the subtypes detected in wild birds in Germany, 26 October–1 November 2020 (week 44) to 26 July–1 August 2021 (week 30), based on data collected by the German National Reference Laboratory for AI (NRL AI).

species conferring to the subtype can be found in Supplementary Table S2.

With regard to the temporal subtype distribution, H5N8 cases dominated the winter season of 2020/21. Alongside the leading Ger-10-20-N8 genotype, spring 2021 saw a large influx of novel H5N1 cases, continuing into the summer months. Only few H5N5 and H5N4 cases were recorded, with H5N5 more present in the beginning of the epizootic and H5N4 detected later in spring 2021. H5N3 cases showed an extensive peak during December 2020, with nearly all cases confined to this month (Fig. 4).

4. Discussion

Yet again, clade 2.3.4.4b HPAIV have been responsible for a major epizootic in Germany and Europe. The 2020–21 outbreak has outbid all previously recorded clade 2.3.4.4b outbreaks in Germany in regards to both case count and genetic diversity. In addition, the long circulation period with detections until summer 2021 in the wild bird population adds to the mix of worrying characteristics. Here, the possibility of endemic HPAIV circulation in Europe has become a genuine threat and must be very carefully observed. Genotype Ger-10-20-N8 was found from October 2020 to July 2021 in Germany, and H5N8 viruses of this genotype were simultaneously dominating HPAIV outbreaks in Europe. In addition, H5N1 genotype Ger-02-21-N1 became established in early 2021 and has been in circulation until at least the middle of December 2021 in Germany. Other European countries have reported continued detection of HPAIV throughout summer and into autumn 2021, including for example Belgium (July 2021), The Netherlands, Poland, and Finland (August 2021), and France (September 2021) (FAO 2021).

After detection of a clade 2.3.4.4b HPAIV H5N8 in Russian poultry workers in December 2020 (Pyankova et al. 2021), and detection of the same reassortant (Ger-10-20-N8) in other mammalian species in a wildlife rehabilitation centre in the UK (Floyd et al. 2021) and dead seals in Germany (Postel et al. 2022), concerns were raised regarding the zoonotic potential of the circulating strains. However, no HPAIV H5N8 sequences analysed from avian hosts showed any mutations that concur with enhanced zoonotic potential. Nevertheless, the identified case in the Russian Federation alongside previously detected human cases of HPAIV clade 2.3.4.4b H5N6 (WHO 2018) underlines the potential of clade 2.3.4.4b viruses to adapt and mutate according to the host species.

Of all detected sub- and genotypes, the HA and MP segments are highly similar throughout, and are thus able to be designated as the conserved 'core genome'. As all other segments, including the NA segment, appear exchangeable in combination with this specific core genome, the likelihood of novel reassortment events leading to new, potentially zoonotic or endemic phenotypes is high and needs careful analysis. In addition, the remarkable number of identified genotypes highlights the need for precise and rapid full-genome sequencing. Although standard RT-qPCR testing is indispensable for diagnostic work, whole-genome evaluation including variant analysis for potential host shift mutations is of utmost importance, particularly concerning the reported mammalian cases. Additionally, the identification of mixed infections and reassortants of one subtype is only possible with the help of full-genome sequencing.

As Eurasian LPAIV play an important role as a source of reassorted segments, the lack of whole-genome sequencing of LPAIV circulating in Europe hampers the analyses, and enhanced active surveillance programs for LPAIV are strongly recommended. As many of the novel reassortants were only identified in Europe, for example, the H5N4 and H5N3 subtypes, this suggests active local reassortment with circulating European LPAIV. Here, intensified passive and active surveillance of LPAIV strains would greatly aid in the assessment of potential (zoonotic) novel AIV, possibly prior to their emergence.

5. Summary

The role of migratory birds and their pathways in the spread and transmission of HPAIV has been thoroughly investigated (Lycett et al. 2020). The 2020–21 avian influenza season in Germany surpassed all previously recorded HPAIV outbreaks in size and genetic variation. The introduced clade 2.3.4.4b HPAI H5Nx viruses affected both the wild bird population while causing vast economic losses in the poultry sector, and showed an unprecedented tendency for reassortment, even when compared to the major 2016–18 epizootic in Europe. In addition, the possible zoonotic potential and endemic threat emphasize the potential danger of the continuously reassorting clade 2.3.4.4b viruses. Intensified active and continued passive surveillance in combination with full-genome sequencing could aid in early detection and contribute to risk assessment of new HPAIV reassortants and variants.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

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Data availability

All sequencing data included in this study have been deposited in the EpiFlu™ GISAID database (www.gisaid.org), accession numbers can be found in Supplementary Table S1.

Conflict of interest: The authors have no conflicts of interest to declare.

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Publication V: „Iceland as Stepping Stone for Spread of Highly Pathogenic Avian Influenza Virus between Europe and North America"

Publication V

Iceland as Stepping Stone for Spread of Highly Pathogenic Avian Influenza Virus between Europe and North America

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Iceland as Stepping Stone for Spread of Highly Pathogenic Avian Influenza Virus between Europe and North America

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Highly pathogenic avian influenza viruses (HPAIVs) of hemagglutinin type H5 and clade 2.3.4.4b have widely spread within the northern hemisphere since 2020 and threaten wild bird populations, as well as poultry production. We present phylogeographic evidence that Iceland has been used as a stepping stone for HPAIV translocation from northern Europe to North America by infected but mobile wild birds. At least 2 independent incursions of HPAIV H5N1 clade 2.3.4.4b assigned to 2 hemagglutinin clusters, B1 and B2, are documented for summer–autumn 2021 and spring 2022. Spread of HPAIV H5N1 to and among colony-breeding pelagic avian species in Iceland is ongoing. Potentially devastating effects (i.e., local losses >25%) on these species caused by extended HPAIV circulation in space and time are being observed at several affected breeding sites throughout the North Atlantic.

Potentially zoonotic highly pathogenic avian influenza (HPAI) viruses (HPAIVs) of subtype hemagglutinin (HA) 5 (H5) emerged from a domestic geese flock in southern China in the mid-1990s. Since then, descendants of this so-called goose/Guangdong (gs/GD) lineage have continued to circulate, evolved into various clades, and formed a plethora of subgenotypes and genotypes that threaten poultry production worldwide (*1*,*2*). Because of repeated incursions from poultry into migratory aquatic wild bird populations in Asia, these viruses have spread, since 2005, in several waves westward and southward across Eurasia, into Africa and eastward, through the Bering strait, into North America. Infected but mobile migratory birds aided in linking geographically widely separated areas along overlapping flyways; palearctic breeding areas were serving as an additional link between Eurasia and America during 2014 (*3*,*4*).

Because Europe was facing the most severe HPAIV epizootics in the influenza winter seasons of 2020–21 and 2021–22 in terms of case numbers and genetic diversity of characterized viruses (*5*,*6*), concerns about spread to North America, this time by westward virus spread, were renewed. By December 2021, HPAI H5N1 detection in wild birds in Canada was reported, followed by numerous additional wild bird cases and incursions into poultry holdings along the eastern coastline of the United States (*7*,*8*). Phylogenetic analyses of the viruses in North America confirmed a close relationship to HPAIV H5N1 genotypes from Europe (*7*–*9*). Although the outcomes of the transatlantic HPAIV transfer are evident, the steps taken by the virus to cross the Atlantic are not. We present data supporting HPAIV transfer from Europe to North America by bird migration through Iceland.

Incursion of HPAIV H5N1 into Iceland

Although low pathogenicity avian influenza virus (AIV) strains have been detected in sea birds around Iceland (*10*,*11*), outbreaks of HPAIV were not reported from Iceland until spring 2022. However, retrospective screening of wild bird samples from

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Iceland showed that an HPAI case was in a juvenile white-tailed sea eagle (*Haliaeetus albicilla*) found dead in the southern Westfjords, Iceland, during October 2021 (*12*). This bird had been equipped with a satellite transmitter (global positioning system/global system for mobile communications) as a nestling on July 24, 2021. After fledging on August 11, 2021, the eagle stayed in the nesting area of its parents and moved within a range of 1.6 km^2 (95% minimum complex polygon) for ≈2 months. The juvenile eagle died at the shore of the region in Iceland on October 8, 2021, and was kept frozen until necropsy in the spring of 2022.

Postmortem examination showed a female weighing 5,540 g that had extensive subcutaneous and body cavity fat tissue indicating a good nutritional condition. Gross pathologic alterations (fibrinous pericarditis, swollen hyperemic liver, spleen, and kidneys) were indicative of a severe infectious disease, which led to an acute death of the young eagle. We analyzed organ samples for AIV by using quantitative reverse transcription PCR as described (*13*). HPAIV of subtype H5N1 was found at high viral loads in all tissue samples examined, including the brain (cycle threshold 16.2).

Despite an appeal from the veterinary authorities in Iceland to the general public to report finding of sick or dead wild birds, only 17 birds came to be sampled and AIV was tested in the first 9 months of 2021, and all samples were AIV negative. In the beginning of 2022, the veterinary authorities in Iceland enhanced passive surveillance through reports from the public of sick or dead wild birds. In mid-April, a common raven (*Corvus corax*) and a pink-footed goose (*Anser brachyrhynchus*) tested HPAIV H5N1 positive. In addition, in the same period, a northern gannet (*Morus bassanus*) tested positive for H5N1, but HPAI could not be confirmed. The raven was found on a farm in southern Iceland where 6 days later a backyard chicken flock on the same farm showed abruptly increased mortality rate, and chicken carcasses tested HPAIV H5N1 positive. Consequently, public awareness and reporting of dead wild birds increased markedly after a press release on these first findings.

From April 2022 onward, including the already identified wild birds, HPAIV H5N1 was detected in 21 wild birds from 10 species: northern gannets (n = 7), European herring gull (*Larus argentatus*) (n = 2), great black-backed gull (*Larus marinus*) (n = 2), great skua (*Stercorarius skua*) (n = 2), greylag goose (*Anser anser*) (n = 2), pink-footed goose (n = 2), barnacle goose (*Branta leucopsis*) (n = 1), black-headed gull (*Chroicocephalus ridibundus*) (n = 1), common raven (n = 1), and lesser black-backed gull (*Larus fuscus*) (n = 1). Because in 1 sample from a northern gannet, neuraminidase 1 could not be confirmed, the bird was reported as positive for HPAIV H5Nx (last updated on June 21, 2022).

Phylogeographic Identification of ≥2 Virus Introduction Events

We performed direct MinION (Oxford Nanopore Technologies, https://nanoporetech.com) full-genome sequencing as described (*5*) for 3 samples from Iceland (2022AI02104: white-tailed eagle, brain tissues; 2022AI02564 and 2022AI02565: backyard chickens, oropharyngeal swab specimens) that were immediately available for analysis and showed high viral loads. Presence of HPAIV H5N1 of clade 2.3.4.4b was confirmed. Phylogenetic and phylogeographic analyses of the genomes (Appendix, https://wwwnc.cdc.gov/EID/article/28/12/22-1086-App1.pdf) and associated data (*14*) showed close relationships to HPAIV H5N1 viruses from Europe and North America, grouping in 2 different HA clusters (B1 and B2) recently defined in clade 2.3.4.4b viruses from Europe (Figure 1) (*6*,*15*–*18*).

Those findings point to ≥2 independent incursions into Iceland. The sequence from Iceland isolated during 2021 clusters in the B1 HA cluster between sequences from countries in northern Europe (the Netherlands, Ireland) and sequences from Canada and eastern coastal states of the United States (Figure 2). Analyses of concatenated genome sequences showed no evidence of reassortment with other AIV strains currently or recently circulating in Europe. Time-scaled phylogenetic analyses and inferred phylogeography (Figures 1, 2) demonstrate the circulation of similar viruses of the B1 HA cluster in northern Europe from the winter of 2020 to spring and summer of 2021 (*6*), and point toward viral spread from locations on the British Isles to Iceland and from there onwards to Canada and eastern coast of the United States.

White-tailed sea eagles are known to be a resident bird species in Iceland, and introduction of virus with this species is highly unlikely. Instead, the white-tailed sea eagle infection is likely caused by feeding of the eagle on infected, therefore weakened, prey or scavenging on carcasses, as described for raptor species (*19*). Some of the contemplable prey species of the taxonomic orders of *Anseriformes* or *Charadriiformes*, including geese, gulls and waders, are known to migrate from the British Isles and the North Sea region and are confirmed to have been infected in spring and early summer of 2021 in their overwintering areas (*20*–*22*). Iceland is situated along overlapping flyways that connect the Eastern and Western Hemispheres,

and it has been suggested that Iceland connects virus movements between mainland Europe and North America (*7–11*,*23*).

In addition, HPAIV H5N1 genomes from 2 chickens dying in a backyard farm on Iceland during April 2022 were sequenced and could be traced back to a second, independent incursion featuring viruses of HA cluster B2. Inferred phylogeographic analysis showed that viruses collected in northern Asia were a possible source of this second introduction into central Europe and further spread throughout the continent (*6*). The Iceland chicken sequences cluster between viruses of HA cluster B2 collected from the British Islands and Ireland during the winter of 2021/2022 (Figures 1, 2). Viruses of this HA cluster (B2) have not been detected in North America to date.

Epidemiologic, Conservational, and Public Health Concerns of Expanded HPAIV Circulation

Our data provide evidence for 2 translocation events of HPAIV H5N1 clade 2.3.4.4b viruses from central Europe through the British Isles into Iceland observed during October 2021 with a most recent ancestor in summer 2021 (most recent common ancestor 2021.5). Onward transmission to Newfoundland and possibly additional regions in the North Atlantic raises several concerns.

Large breeding colonies of pelagic bird species, such as puffins, northern gannets, and kittiwakes are located along the coasts of the North Atlantic. Confirmed HPAIV H5N1 infection in 9/12 gannet carcasses and daily public reporting of sick and dead gannets in the Reykjanes Peninsula, Iceland, since beginning of April 2022 underline that these colonies are now in danger of HPAIV H5 outbreaks of larger scale, which might affect the continuity of these local populations. Concerns extend to local populations of species with narrowly circumscribed breeding/resting ranges in the North Atlantic region such as great skua, long-tailed skua, red knots, pink-footed geese, and barnacle geese, as well as birds of prey exposed during opportunistic scavenging (e.g., white-tailed sea eagles and great skuas) and active hunting of weakened, infected prey (e.g., gyrfalcons [*Falco rusticolus*]). Therefore, enhanced passive surveillance should focus on such spots and scavenging and colony-breeding species.

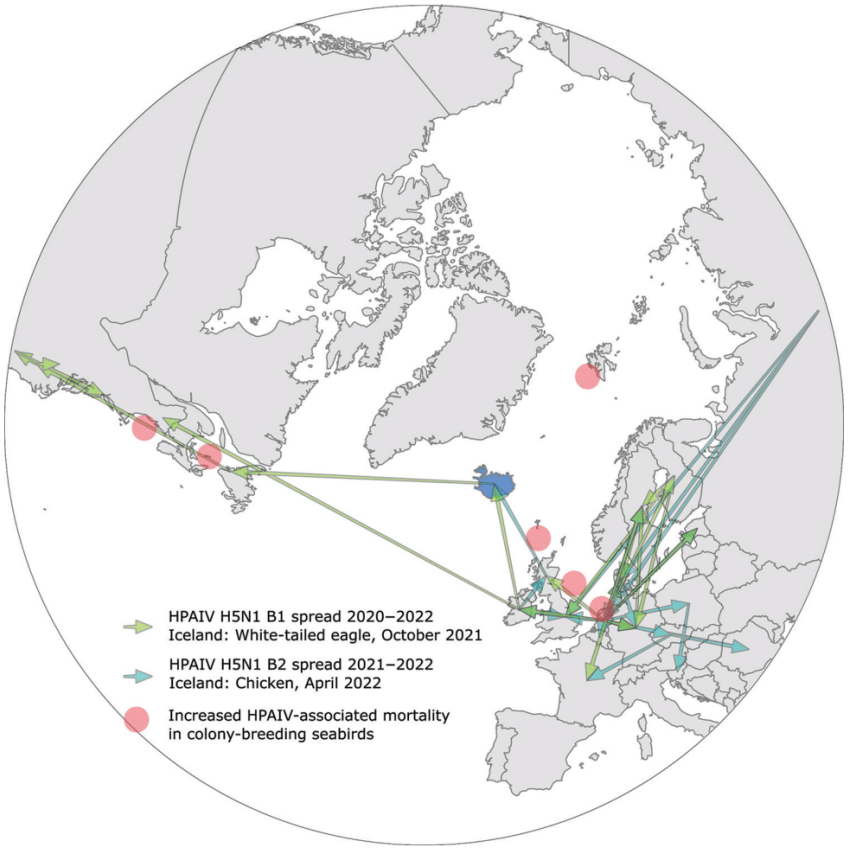

Figure 1. Polar map view of the palearctic and nearctic realm, and inferred spread of hemagglutinin clusters B1 and B2 of highly pathogenic avian influenza viruses (HPAIVs), subtype, clade 2.3.4.4b and their incursion routes to Iceland (blue) during 2021 (green arrows) and 2022 (turquoise arrows). Red dots indicate geographic locations where current (summer 2022) HPAIV-associated mass deaths in pelagic or colony-breeding seabirds have been reported. Data from were obtained from various sources (*15–18*).

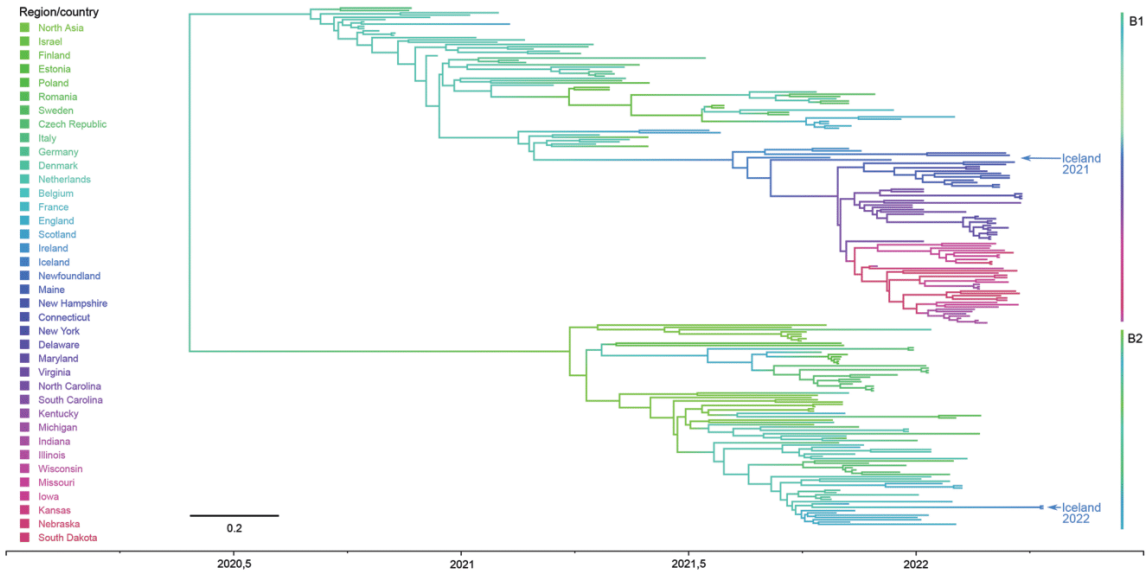

Figure 2. Phylogeographic tree of highly pathogenic avian influenza viruses. Taxa are colored according to their country of origin, and countries are arranged in geographic order from east to west. Arrows indicate viral genomes during 2021 and 2022 in Iceland and assigned to different hemagglutinin clusters B1 and B2. Method hints and basic data are presented in Hassan et al. (*13*). Scale bar indicates nucleotide substitutions per site.

The massively extended circulation in space and time of recent HPAIV H5N1 clade 2.3.4.4b viruses in migratory wild birds in the North Atlantic will further threaten endangered species. Grossly increased mortality rates for colonies of northern gannets and several tern species are being observed at several breeding sites throughout the North Atlantic (Figure 2). The most recent incursion of these viruses into wider palearctic areas of the Atlantic will inevitably lead to viral contamination of northern breeding habitats where ambient conditions prevail that are considered favorable for a prolonged retainment of viral infectivity outside avian hosts (*23*,*24*).

Increased alertness should now also extend to the Southern Hemisphere. In the 2 reported incursion events of gs/GD HPAI viruses into North America by migrating wild birds, during 2014 and 2021/2022, virus spread along the Pacific (2014) and the Atlantic coastline (2021) from north to south and further inland affecting wild birds and poultry in Canada, as well as in most of the United States (*7*–*9*). However, for unknown reasons, spread seems to be interrupted between North America and South America because no incursions had been reported during 2014/2015 or since 2021 from the Caribbean region and South America.

Similar observations have been made along the east side of the Pacific Ocean. Despite endemic presence of gs/GD HPAIV in several regions of Southeast Asia, and frequent incursions into migratory wild bird populations, cases have so far not been reported from Australia/Oceania (*4*). It is only at the most southern tip of Africa that gs/GD-like HPAIVs have reached and stayed within the Southern Hemisphere. However, this bridgehead of the virus might put geographically sequestrated subantarctic species, such as penguins and albatrosses, or the highly endangered avifauna of New Zealand at increased risk for exposure.

In conclusion, as shown by the rapid and devastating spread of HPAIV H5N1 through poultry holdings in North America after primary incursions from infected wild birds (*10*), the avian–human interface has expanded again. Infections in 1 human (*25*) and in several terrestrial scavenging carnivores, such as foxes, skunks, and raccoons (*12*), illustrate the increased risk for spillover transmissions.

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All sequence data including raw data are available in the INSDC (https://www.insdc.org) and GISAID database

via ENA (https://www.ebi.ac.uk) project accession no. PRJEB53596 and EPI (https://www.ncbi.nlm.nih.gov) isolate accession nos. 13245602, 13246267, and 13246657. Details of methods are described separately. All associated information is available on Zenodo (https://zenodo.org).

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Publication VI: „Continuous surveillance of potentially zoonotic avian pathogens detects contemporaneous occurrence of highly pathogenic avian influenza viruses (HPAIV H5) and flaviviruses (USUV, WNV) in several wild and captive birds"

Publication VI

Continuous surveillance of potentially zoonotic avian pathogens detects contemporaneous occurrence of highly pathogenic avian influenza viruses (HPAIV H5) and flaviviruses (USUV, WNV) in several wild and captive birds

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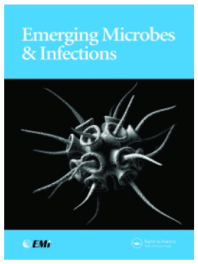

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Continuous surveillance of potentially zoonotic avian pathogens detects contemporaneous occurrence of highly pathogenic avian influenza viruses (HPAIV H5) and flaviviruses (USUV, WNV) in several wild and captive birds

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Continuous surveillance of potentially zoonotic avian pathogens detects contemporaneous occurrence of highly pathogenic avian influenza viruses (HPAIV H5) and flaviviruses (USUV, WNV) in several wild and captive birds

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ABSTRACT

Three avian viral pathogens circulate in Germany with particular importance for animal disease surveillance due to their zoonotic potential, their impact on wild bird populations and/or poultry farms: Highly pathogenic (HP) avian influenza virus (AIV) of subtype H5 (HPAIV H5), Usutu virus (USUV), and West Nile virus (WNV). Whereas HPAIV H5 has been mainly related to epizootic outbreaks in winter, the arthropod-borne viruses USUV and WNV have been detected more frequently during summer months corresponding to peak mosquito activity. Since 2021, tendencies of a potentially year-round, i.e. enzootic, status of HPAIV in Germany have raised concerns that *Orthomyxoviruses* (AIV) and *Flaviviruses* (USUV, WNV) may not only circulate in the same region, but also at the same time and in the same avian host range. In search of a host species group suitable for a combined surveillance approach for all mentioned pathogens, we retrospectively screened and summarized case reports, mainly provided by the respective German National Reference Laboratories (NRLs) from 2006 to 2021. Our dataset revealed an overlap of reported infections among nine avian genera. We identified raptors as a particularly affected host group, as the genera *Accipiter*, *Bubo*, *Buteo*, *Falco*, and *Strix* represented five of the nine genera, and highlighted their role in passive surveillance. This study may provide a basis for broader, pan-European studies that could deepen our understanding of reservoir and vector species, as HPAIV, USUV, and WNV are expected to further become established and/or spread in Europe in the future and thus improved surveillance measures are of high importance.

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KEYWORDS *Orthomyxoviridae*; *flaviviridae*; raptors; disease surveillance; host species; HPAIV H5; USUV; WNV

Introduction

In recent years, various epizootics have drawn attention to the increasing spread of animal pathogens, circulating between wildlife, livestock, and pet animals and being potentially spilled-over to humans. Three zoonotic viruses are especially relevant in wild bird populations that permanently reside, breed, and winter within or migrate through Germany: highly pathogenic (HP) avian influenza viruses (AIV) of subtype H5, and the flaviviruses Usutu virus (USUV) and West Nile virus (WNV). Until now, their spatio-temporal occurrence and host range in Germany has not been investigated in a combined retrospective study in order to identify any potential overlaps in the emergence and/or maintenance of these viruses.

The first cases of HPAIV H5N1 in Germany in winter 2006 were caused by incursions of the Asian H5 A/Goose/Guangdong/1/1996 (gs/GD) lineage. These viruses belonged genetically to clade 2.2 of the gs/GD lineage, whereas since 2014 clade 2.3.4.4 viruses are dominating [1]. Until 2021, temporal and spatial patterns of gs/GD HPAI virus emergence were correlated with the presence of migrating or resting wild waterfowl in Germany. The threat of incursion into poultry flocks has increased, and wild bird populations themselves have suffered severely during and amidst epizootic events [2–8]. Some waterfowl such as various dabbling duck species, which are a long-known reservoir for low pathogenic AIV, may not even show clinical signs after infection with HPAIV H5 [9,10]. The considerable contagiousness of HPAIV,

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the orofecal transmission route, and their substantial tenacity in the environment [11] in combination with the seasonal behaviour of birds, in particular mixed-species flocking at resting areas during migration or aggregating in winter, supports the interspecies distribution.

USUV and WNV belong to the group of arthropod-borne (ARBO) viruses, as they are mainly transmitted in an enzootic cycle between wild birds and ornithophilic mosquito species (especially *Culex sp.*) [12,13]. Thus, the virus activity is dependent on the susceptibility of the local bird species, but also on vector competence and availability associated to environmental conditions. The first evidence for USUV in Germany was detected in a *Culex pipiens pipiens* pool, trapped in summer 2010 in southwest Germany in the context of a mosquito-monitoring programme [14]. A regional outbreak in a local passerine bird population was described one year later, followed by regular detections across the country since 2018 [15,16] and a meanwhile enzootic status [17–19]. Several studies confirmed the vector competence of local arthropod species in Germany for USUV and WNV [12,13,20].

In contrast, WNV reached Germany not before 2018. In the following years, enzootic outbreaks in wild bird populations developed in the eastern part of the country based on various introduction events of WNV strains of Eastern European origin. Again, those outbreak events mostly correlated with main mosquito activity in summer months [12,21]. Although WNV was not confirmed all over Germany, it became enzootic in so called "hotspot areas" in Berlin and Central-Germany [16,18,20,22–26].

Recently, concerns have been raised that HPAIV H5 (clade 2.3.4.4b) might have established an enzootic status in Europe since 2021, as productive wild bird infections and outbreaks in poultry holdings are now occurring year-round including the summer months [27]. In this regard, spatio-temporal co-circulation of HPAIV H5, USUV, and WNV in German wild bird populations is to be anticipated in the near future.

We therefore evaluated this potentially year-round threat by conducting a data- and literature-based study to identify susceptible avian species for these pathogens in Germany. Our aim was to identify a possible overlap of species, genera, or avian groups that might be utilized as future indicators for the circulation of HPAIV H5, USUV, and WNV in Germany to support combined surveillance approaches of orthomyxoviruses and flaviviruses with zoonotic potential.

Material and methods

Reports and databases issued by the German Reference Laboratories (NRLs) for WNV and AIV, based at the Friedrich-Loeffler-Institut (FLI), Isle of Riems, were screened systematically to gain information on affected bird species. According to the first confirmation of each virus in Germany, the dataset included cases from 2006–2007, 2009, 2014–2017 and 2020–2021 (for infections with HPAIV H5), 2011–2021 (for infections with USUV) and 2018–2021 (for infections with WNV). The majority of these retrospectively examined cases were already part of monitoring programmes on the respective pathogens in Germany within the last years [1,3,5–8,15–19,22–25,28–33].

Information on affected hosts by at least one of the three viruses was collected, if the following criteria applied:

Sample origin and time

The sample had to be collected in Germany. The previous diagnostics of the reported cases were mainly based on swab samples or organ material for HPAIV H5 confirmation and organ material or blood clot for flavivirus detection (see original publications). We considered reported cases in wild birds and included reports on captive wild bird species, including zoological institutions and private facilities. Since it was not possible in all cases to ascertain the exact date of sampling or the time when a dead animal was found, we applied the date when the animal was found dead, the date when the animal was sampled or, if none of the former was available, the date when the sample arrived at the respective NRLs.

Animal disease

We screened the data for reports on HPAIV H5, USUV, and WNV infections. For AIV, we focused on infections with HP subtype H5, as these strains were the dominating ones in Europe since 2006 and are known to harbour zoonotic potential [1,34]. Our dataset covers different time periods related to the virus' first detection in Germany (HPAIV since 2006, USUV since 2011, and WNV since 2018) and therefore, comprises different genetic strains as investigated by prior independent studies that include sequences and corresponding metadata.

Infection status

We included individual birds that tested positive for viral RNA by RT-qPCRs as described by Michel et al. [16] and Hassan et al. [35], regardless of any clinical signs, pathological lesions, or death. A case within our dataset does not necessarily indicate the death or euthanasia of the affected animal, nor if the sample stands for a single individual or was one tested bird representing a group of birds at one sampling site. High detection rates are achieved in passive

surveillance approaches based on swab-sampled carcasses or organ material, thus, the majority of samples were most likely from deceased birds. However, for the analysed blood clot, the status of the individual remains unclear.

Species

We focused on avian host species and did not include reports in humans, mammalian, or arthropod-vector species. A report was excluded, if the taxonomy has not been indicated and the individual could not be classified at least on the taxonomic level of orders. Beside wild birds, we included cases in various captive birds, except domestic poultry species, in order to broaden the spectrum of contemplable host species. In this regard, the following species and species groups were considered poultry and were thus not included in the study: domestic fowl/chicken, domestic goose, domestic duck, quail, and turkey. Poultry is rarely known to perish following flavivirus infections. Therefore, poultry species are unlikely to be suitable targets for passive surveillance approaches, nor suitable reservoir hosts for all three avian pathogens mentioned above. Information on taxonomic identification was summarized with the most precise biological characterization (lowest taxonomic level) given: order, family, genus, and species. If it was not possible to assign the exact species name, the next higher, possible taxonomic level was applied.

Results

Identification of host species with overlapping occurrence for orthomyxo- and flaviviruses

In total, 4583 cases of avian individuals, infected by HPAIV H5, USUV, or WNV have been identified (Supplementary Table S1), whereby 73.1% refer to AIV ($n = 3351$), 22.7% to USUV ($n = 1042$), and 4.2% to WNV infections ($n = 190$; Figure 1(A)). Cases within our dataset were not usually screened for all three pathogens. For seven individuals, a co-infection with both flaviviruses was described. These seven cases were included twice in the list of detected infections, listed once for USUV and once for WNV, and marked with an asterisk in the column "co-infection" in Supplementary Table S1 [18,23]. Co-infections with flaviviruses occurred in 2018, 2019, and 2020 among captive and wild individuals of *Accipitriformes*, *Anseriformes*, *Charadriiformes*, *Passeriformes*, and *Strigiformes*. Thus, they represented 1.1% of all USUV and 3.7% of all WNV cases since both pathogens co-existed in Germany in 2018. No individual bird was confirmed to harbour a co-infection with one or both Flaviviruses together with HPAIV H5.

Regarding biological classification/taxonomy, in 3622 out of 4583 cases (79.0%), the avian species was indicated and 136 different avian species were covered in total. However, in 961 reports (21.0%) no precise species was indicated (Figure 1(B)). The less specific the biological classification (higher taxonomic level), the more cases were classified.

Figure 1(C) shows nine avian orders in which infections with all three pathogens were detected. HPAIV H5 infections were reported mainly in *Accipitriformes*, *Anseriformes*, *Charadriiformes*, *Falconiformes*, *Galliformes*, and *Pelecaniformes*. USUV infections prevailed in *Columbiformes*, *Passeriformes*, and *Strigiformes*. WNV infections represented the minority of cases in all bird orders. Highest numbers of WNV infections were identified in *Accipitriformes*, *Strigiformes*, and *Galliformes*, although in the latter order only a total number of seven cases was reported.

While for HPAIV H5 and USUV infections, cases were mainly reported in wild birds, WNV infections were identified in captive and free-ranging birds nearly equally distributed (Figure 1(D)). As indicated above, cases in poultry species have not been considered here.

Pronounced overlapping incidence for orthomyxoviruses and flaviviruses in raptors and scavengers

The comparison of the affected hosts revealed that all pathogens were detected at least once in three avian species (Figure 2): Northern goshawk (*Accipiter gentilis*), tawny owl (*Strix aluco*), and grey heron (*Ardea cinerea*). Therefore, those species represented an overlap in general predisposition. Reports for northern goshawks ($n = 74$) mainly referred to WNV infections (HPAIV H5 21.6%, USUV 4.1%, WNV 74.3%), whereas in tawny owls ($n = 7$) USUV and HPAIV H5 infections were described more often than WNV infections (HPAIV H5 $n = 3$, USUV $n = 3$, WNV $n = 1$). Grey herons ($n = 41$) were found only once positive for USUV or WNV, while HPAIV H5 infections were confirmed in the majority of the reports (HPAIV H5 95.1%, USUV 2.4%, WNV 2.4%).

The comparison on higher taxonomic levels gave a similar picture (Figure 2): In the genus *Accipiter spp.*, WNV infections dominated. In *Bubo spp.*, WNV infections were reported almost as frequently as HPAIV H5 infections. HPAIV H5 infections occurred mainly in the genera *Ardea spp.*, *Buteo spp.*, *Corvus spp.*, *Falco spp.*, *Larus spp.*, and *Mergus spp.* Only for the genus *Strix spp.* USUV infections were the most common reported infection.

A summary of cases in the avian host genera *Accipiter*, *Bubo*, *Buteo*, *Falco*, and *Strix* is displayed in Figure 2 under the term "raptor species".

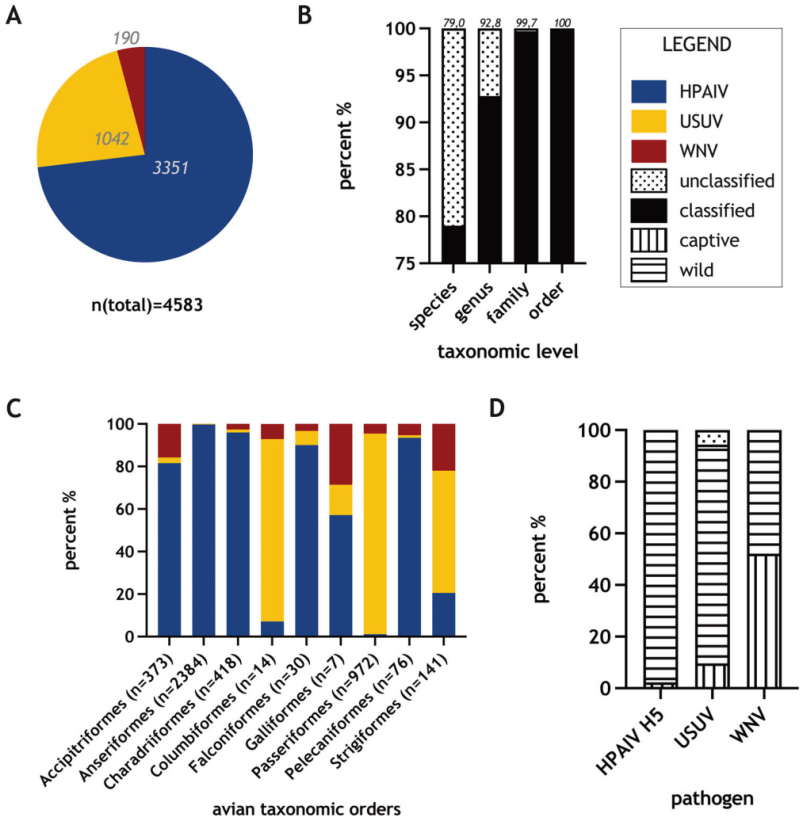

Figure 1. Overview of test results and avian taxa, which were tested positive by RT-qPCR for highly pathogenic avian influenza virus of subtype H5 (HPAIV H5), Usutu virus (USUV), and West Nile virus (WNV) in Germany from 2006 to 2021. (A) Number of positive RT-qPCR test for HPAIV H5, USUV, and WNV; (B) Eligibility to biological classification (species, genus, family, and order) displayed as percentage from the total number of samples; (C) Distribution of positive test results for HPAIV H5, USUV, and WNV in nine avian orders, reported for infections with all mentioned pathogens; (D) Origin of birds tested positive for HPAIV H5, USUV, and WNV, distinguishing between captive and wild.

Temporally overlapping co-circulation of HPAIV H5 and WNV in wild birds in Germany

For case reports of HPAIV H5 and WNV infections from 2016 to 2021, the date (sampling date or the date when the post-mortally tested individual was found) was associated. Where this information was not available, the arrival date of the sample at the NRLs at FLI, was considered instead. Figure 3 displays the temporal dynamics for both pathogens (see also Supplementary Table S2). For the USUV subset, this information was given only sporadically and therefore USUV data were not included here.

In general, until 2021 HPAIV H5 activity occurred mainly in autumn and late spring. However, HPAIV outbreaks in 2021 no longer revealed the seasonal pattern as in previous years and high numbers of wild bird cases were also reported during the summer.

WNV infections were reported from June to October except for one case in March. That case was confirmed in March 2021 for a WNV-positive tested Jandaya parakeet (*Aratinga jandaya*) kept in an aviary in a zoo in Berlin. It is very likely that this positive test was the result of a chronic infection, as viral genome detection was only possible in the kidney of the bird, but not in other organs.

Months with overlapping activities of WNV and HPAIV H5 were June, July, September, and October in the present study. Individuals, which were tested positive for WNV and also for USUV at the same time, were reported between the end of August and the beginning of September.

Discussion

Demands for disease monitoring in wild bird populations focusing on potentially zoonotic viruses increased recently, and in Germany, HPAIV, USUV, and WNV became the most relevant ones. Combining monitoring efforts might enable (i) broader

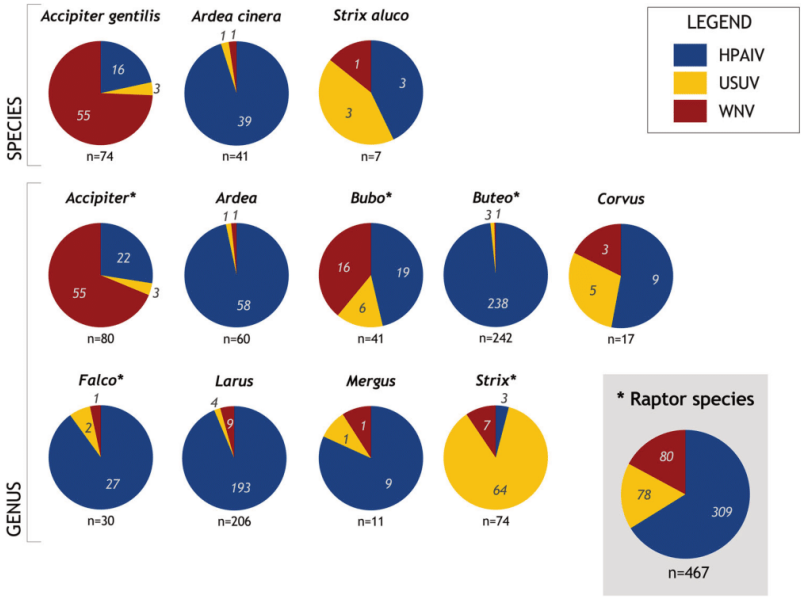

Figure 2. Distribution of pathogen detection in species, genera, and groups showing infections with three viruses (highly pathogenic avian influenza virus of subtype H5 (HPAIV H5), Usutu virus (USUV), and West Nile virus (WNV)) detected from 2006 to 2021. Genera belonging to the group of raptors are marked with an asterisk.

surveillance on virus activity in general, (ii) simplified and resource-sparing approaches by targeting suitable indicator species/groups, and to (iii) optimize risk assessments for spill-over events to humans.

Although excluded in this first attempt, the poultry sector represents a human-bird interface (e.g. farmers, veterinarians, consumers) and there is a risk of pathogen spill-back from poultry holdings into wild bird populations, particularly for HPAIV H5 (e.g. due to improper biosafety measures or in free-ranging flocks). For Arboviruses, the role of chicken, duck, and goose as reservoir or carrier is minor. These species have been discussed as indicator species for flavivirus circulation, but only on the basis of serological findings [36]. In this context, the absence of severe viremia, e.g. after WNV infections, hampers analyses on the genetic background of the respective virus strain, that could be obtained by RT-qPCR screening and sequencing within passive monitoring approaches in wild or zoo birds.

The majority of case reports were collected for infections with HPAIV H5, followed by USUV and comparably few cases of WNV (Figure 1(A)). This distribution might reflect the respective time period after the pathogens' first introduction into German wild bird populations: HPAIV H5 (clade 2.2) in 2006 [3] and clade 2.3.4.4 in 2014 [2], USUV (Europe 3) in 2011 [15], and WNV (lineage 2) in 2018 [25]. Moreover, the higher contagiousness and the direct transmission cycle of HPAIV H5 compared to the less effective, slower and primarily vector-dependent transmission of WNV and USUV might have led to this distribution.

About 79% of all reports could be assigned to the lowest taxonomic level of the precise avian species (Figure 1(B)). Multiple uses of generalizing terms such as buzzard, wild duck, thrush, or raptor prevented the identification of the exact avian species in 21% of the cases and only allowed the more general taxonomic classification up to the level of orders. Therefore, we emphasize the importance of accurate species identification during sampling to enable a precise data assessment. Animal photographs accompanied with samples and reports might help to determine the species retrospectively. Furthermore, e.g. (official) veterinarians in charge could be specifically trained for species identification or be provided with survey sheets. Molecular determination of host species, e.g. by utilizing DNA barcodes [37], might be done when a taxonomic classification is not possible based on morphology. Moreover, all data must be reported to the databases in a complete and detailed manner, and databases should be encouraged or forced to use appropriate species catalogues minimizing typing and reporting errors. Missing data on the exact species impedes the idea of picking certain species for monitoring attempts, not only in a combined approach. However, any knowledge about the host taxonomy might help to understand the spread of viral pathogens that is influenced not only by characteristics of the viral entity but also by the species-specific characteristics of the host. One

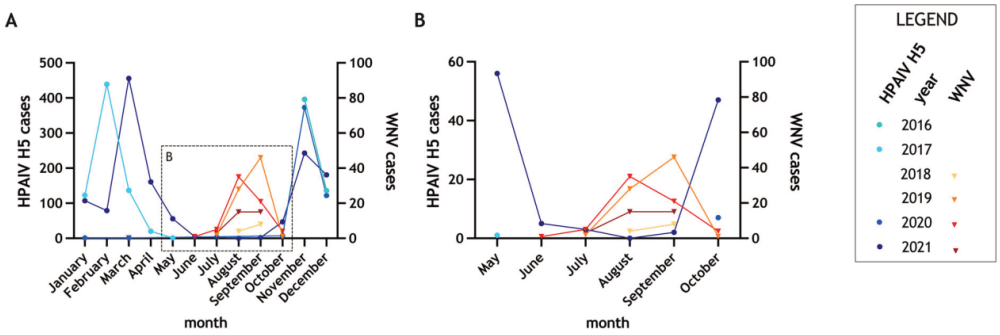

Figure 3. Time of highly pathogenic avian influenza of subtype H5 (HPAIV H5) and West Nile virus (WNV) infections reported in birds, stratified by month, displayed for the years 2016–2021. (A) Annual view, (B) Focus on summer and autumn months (May to October).

example might be the migrations of waterfowl and the association of this behaviour to the spread of HPAIV H5 [2].

Although we figured out avian groups affected by all three pathogens, none of the numerically abundant species/genera showed an evenly distributed pattern of the pathogens (Figure 1(C) and Figure 2). Choosing a single of those species/genera, e.g. representative for one pathogen, for monitoring, could harbour the risk of overlooking other pathogens.

Common to all but one overlapping genera (except *Mergus*) is the scavenging and/or hunting behaviour of the birds. Therefore, we regrouped subsets according to characteristics of host species, instead of only their taxonomic attribution: Datasets for five genera of birds of prey (*Accipiter*, *Buteo*, and *Falco*) and owls (*Bubo* and *Strix*) were summarized under the term raptors. Thus, a slightly more harmonized distribution pattern became apparent for infections with WNV, USUV, or HPAIV H5. The position as predator at the end of the food chain, feeding on carrion or hunting infected and therefore potentially weakened avian prey species results in a certain risk of increased exposure to pathogens and thus hunts towards their suitability as indicators.

Various studies describe raptor species as susceptible for orthomyxoviruses [8,38,39] or flaviviruses [22,40–42], mainly transmitted by the alimentary route. Medium-sized and larger raptors seem to be especially attractive to mosquitoes [43]. Some residential raptor species in Germany may be categorized as medium-sized and might attract public attention when found weakened or dead. Smaller birds such as most songbird species are more easily overlooked, except in the case of mass mortalities in a circumscribed region. Moreover, small-sized birds are often caught, especially when sick, and their carcasses are faster removed by raptors or by mammalian predators such as cats, foxes, martens, or racoons. In addition, the popularity of raptor species might support rescue attempts by citizens when they observe, for example, neurological signs of disease as described for infections with WNV or USUV but also with HPAIV (see Supplementary Table S3) and, thus, helps retrieving cases [8,22,39,41,42,44–55]. At the same time, the described circumstances could lead to a pre-selection of species of which samples will ultimately be tested within routine diagnostics. To that effect, it can only be speculated about the existence of further species (groups) possibly representing an overlap in general predisposition, but not being tested.

Although there was no indication for a single suitable species, our data suggest us to recommend to always test raptor species (or scavenging species) for all mentioned pathogens, especially in passive disease monitoring programmes. This synergizes with the relevance of raptors for ecotoxicological monitoring approaches (monitoring pesticides, rodenticides, and heavy metals). This comprises European programmes as ERBFacility, EuRapMon [56], and MEROS [57,58]. In order to optimize efficacious use of raptor samples, such monitoring programmes should be combined in future, if biosafety aspects are not violated.

After its introduction in 2018, infections with WNV lineage 2 were reported mainly during summer months – whereas HPAIV H5 outbreaks (clade 2.3.4.4b) were associated with epizootics in the winter semester (Figure 3). Since 2020 this situation has changed, as HPAIV-H5-cases of various species were reported in late spring and even during summer [27,59] culminating in HPAI-associated mass mortalities of colony breeding sea birds in Europe in summer of 2022 [60]. The tendency of HPAIV activity continuing in late spring and summer months was recognized in other European countries as well and exacerbated the concern of HPAIV H5 becoming enzootic in Europe [27]. The years 2020/21 therefore represented the first cycles of overlapping outbreak scenarios of HPAIV H5 and WNV in Germany, as WNV was detected from June to October 2020 and July to

September 2021. Mild winters and the ability of e.g. WNV-positive vectors to overwinter might also extend the time period of flavivirus occurrence in wild bird populations in the future [26].

The included co-infections with USUV and WNV in avian species were described as the first cases for Europe by Santos et al. [18] and later on by Ziegler et al. [23] (Supplementary Table S1). Given four years of co-existing WNV and USUV circulation, such co-infections seem to occur quite infrequently, possibly due to cross-protective immunity or interference effects amongst flaviviruses. However, no reports on interference between Flaviviruses and Orthomyxoviruses in birds (wild, captive, or poultry species) and co-infections with HPAIV H5 and WNV have been published. Such a scenario seems unlikely, as an infection with one of these pathogens ends lethally usually for the majority of avian hosts, except waterfowl species perhaps. Furthermore, once a pathogen is detected in a deceased bird, it is often considered as the causative agent of death and no further assays targeting other diseases are conducted. To investigate Flavivirus–Orthomyxovirus co-infections in birds, positive cases would have to be re-tested for the corresponding pathogen.

Surveillance measures at a national level could be improved if continued in the context of transboundary approaches, e.g. global climate change may affect bird migration routes or behaviour and, thus, potentially disease infection patterns.

Conclusion

This study on infections with HPAIV H5, USUV, and WNV among wild and captive birds in Germany succeeded in identifying a spatio-temporal overlap of affected host species or genera and pathogen occurrence. Although it could not be shown for a single bird species/genus, our data particularly highlight the role of raptors for combined passive surveillance of orthomyxoviruses and flaviviruses.

Due to the increasing and partially overlapping infection pressure of wild birds particularly by HPAIV H5 and WNV, orchestrated European-wide studies generating transnational datasets would allow a more comprehensive view on affected bird species across their habitats in the European geographic range. Such an approach might reveal further insights into reservoir and carrier species. These pathogen-targeting studies could be combined with existing ecotoxicological studies for synergistic effects aiming at important key wild bird species such as apex-predators. Given the zoonotic potential of both HPAIV H5 and WNV interdisciplinary collaboration among infectologists, environmental toxicologists, and ornithologists in a One Health frame is highly recommended. Parallel monitoring of vectors, humans, and susceptible animal hosts increases the likelihood, effectiveness, and timeliness of pathogen detection and the validity of pathogen distribution patterns offering various advantages for veterinary and human medicine.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics statement

No ethical approval was required as this study is based on analyses of available datasets provided by German National reference laboratories.

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V. Discussion

Between 2006 and 2021, epizootics of Gs/Gd-related HPAIV H5 occurred sporadically and mainly during the autumn and winter months in Germany and other European countries. Since 2016 clade 2.3.4.4b H5Nx viruses were dominant and also the beginning of the 2020/2021 epizootic in Europe followed previously known patterns of HPAI outbreaks with cases among (migratory) Anseriformes and Charadriiformes [174, 175]. Subsequently, a significant divergence from previous scenarios was seen: The emergence of an HPAIV H5N1 strain, that could lead to a persistent - and, from 2022, enzootic - presence of HPAI in various European wild bird populations.

Along with waterfowl, avian raptor species have been regularly reported in the context of HPAI outbreaks [51, 56, 57, 61, 176]. The rising numbers of cases in wild birds during epizootics in Germany (2006/2007, 2016/2017 and since 2020) suggested that also an increasing amount of hunting and scavenging birds is becoming exposed to the pathogen. The current shift in the HPAIV H5N1 outbreak dynamic in Northern Europe deserves careful surveillance for its impact on these species that frequently become secondary hosts.

Objective I: **Exploring the occurrence and impact of HPAIV H5 (clade 2.3.4.4b) in avian raptor species in Northern Europe**

The susceptibility of birds of prey to HPAIV H5 of different genetic subclades has been demonstrated by experimental studies in which the animals have been inoculated with the pathogen or have been fed with infected prey [53, 59, 60, 177]. Most of the inoculated individuals shed the virus rapidly after exposure and often died or had to be euthanized within a few days, due to severe neurological disease [53, 59, 177]. In consequence, raptor species, often top predators at the end of the food chain, owe a high risk of getting exposed to HPAIV H5 via (primary) prey species, and alimentary infection represents a highly relevant inter-species transmission route.
Here, secondary hosts are defined as raptors, but possibly further scavenging groups, such as Stercorariidae or Corvidae could be added. Essentially, the changing patterns of HPAIV H5 infections in primary hosts initially need to be understood, to follow up the impacts on secondary host species.

The die-off in a subpopulation of red knots (*Calidris canutus islandica*, Publication I) in the Wadden Sea represented an exceptional match of a unique subtype (HPAIV H5N3) and a single host species, but otherwise exhibited typical characteristics of a seasonal HPAI outbreak in waterfowl or shorebird species as described in various European countries during previous epizootics with Gs/Gd clade 2.3.4.4b strains: the time of occurrence, the clinical picture and manifestation with fatal outcome. In contrast to other circulating subtypes at that time in Germany, barely any inter-species transmission of HPAIV H5N3 to

other primary hosts was detected, possibly because of the strict social demarcation behavior of red knots to other wader species and the high virulence leading to peracute deaths (Publication I).

Never before has been described such a great diversity of different reassortants/genotypes and such a high total number of reported HPAIV H5 cases during German or European epizootics. HPAIV H5N1 emerged as winner of the previous subtype competition and has since become established in different genotypes since October 2021 [178]. The following year, when HPAIV circulated enzootically in northern European countries, the transmission dynamics took on a new dimension. Breeding colonies of shorebirds and seabirds were affected by H5N1 for the first time during the spring and summer of 2022 [147], also in Germany (Publication II).

Both events (die-off in red knots in 2020/2021, Publication I, and mass mortality in breeding colonies in 2022, Publication II) were characterized by high mortality rates, e.g., >60% adult mortality among Sandwich terns [*Thalasseus sandvicensis*] (Publication II). Effective transmission possibly has been supported by the density of individuals within migrating flocks or breeding colonies and affected juveniles as well as adults. The loss of so many breeding pairs is expected to lead to negatively impact future breeding success and population stability, since experienced adults are meaningful for the reproductive performance of species with a comparably long lifespan (e.g., red knot > 25 years [179]) and late onset of sexual maturity. Knief, Bregnballe, Alfarwi, et al. [148] et al. expect a recovery time of far more than ten years for northwestern European Sandwich tern populations, even if the mass mortality event in spring 2022 remains unique. However, these scenarios appeared to be self-limited for their respective season, but only long-term observations will show the actual impact on the affected populations.

The HPAIV H5N3 remained highly host-specific in a primary host species known to be strictly tied to coastal habitats. As no reoccurrence of this subtype has been reported in this or any other species, red knots have most likely not become a new niche or reservoir group for this HPAIV H5 strain. In contrast, similar patterns in colony breeding seabirds reoccurred in 2023 and hit colonies of different bird species, notably black-headed gulls. This time, the majority of outbreaks was caused by again a novel H5N1 genotype (called BB), that emerged following reassortment with a gull-adapted LPAIV H13 strain (segments PA, NP and NS) [44]. Black-headed gulls represent potential bridging species, since their foraging behavior includes anthropologically influenced areas, such as parks or agricultural facilities. They have been already associated with the introduction of genotype BB into, e.g. fur farms [83] in 2023. Thus, gull species might remain a highly relevant group for current HPAI dynamics in wild bird populations and contribute to the wild bird-livestock-human interface.

During outbreaks of heavily affected shorebird populations (Publication I) and seabird colonies (Publication II), infected raptors (common buzzard [*Buteo buteo*] and white-tailed sea eagle) were found

in the same locations and were confirmed positive for the corresponding viruses. The high viral loads, described in the contemplable prey species (Publication I and II) elucidate the high risk of alimentary infection for raptors. Our investigations on Gs/Gd HPAI H5 viruses in raptor species in Germany (Publication III) confirmed these (occasional) scavenging species frequently affected, as are Northern goshawks (*Accipiter gentilis*) and peregrine falcons (*Falco peregrinus*), representing rather hunting birds of prey. Likewise, prior studies indicated the high risk of pathogen exposure associated with HPAIV H5 epizootics [51, 57, 61].

The overall detection rates of HPAIV (clade 2.3.4.4b) in raptor species in Germany (Publication III) matched the general occurrence of prior epizootics [132], respectively the ongoing enzootic [144], and display that alimentary transmission must have occurred frequently.

Considering the peaks of prior epizootics, until winter 2020/2021, reports on HPAIV H5 positive raptors invariably referred to fully fledged, independent individuals (mainly juvenile and subadult birds) [51, 57]. Now, our retrospectively analyzed samples (Publication III) enabled the confirmation of the first nestlings of white-tailed sea eagles that became victims to HPAIV during spring 2021. Further similar findings originate from North Germany in 2022. Deceased white-tailed sea eagle nestlings were found mainly in close proximity to affected seabird breeding colonies (Publications III and II) in 2022. Thus, in consequence of the shift from epizootic to enzootic HPAIV H5 presence in Germany, an entirely novel age cohort became affected, when parental birds fed infected prey to their nestlings. These observations fit with similar worrying observations from Europe [180] and North America [56], raising concerns on HPAIV becoming a threat to raptor species conservation. Whereas in the United States of America all age groups of bald eagles seem to suffer from infections with HPAIV H5, in Germany mainly white-tailed sea eagle nestlings tested positive, indicating adult individuals might be able to cope with the omnipresence of infected prey.

More detailed insights into the serological status of raptors, especially white-tailed sea eagles, are required for a better understanding of “pathogen-prey-predator relations” in highly affected regions (Publication III). Since catching healthy, adult raptors is a difficult task, raptor nestlings might substitute as a more accessible suitable target group. Seroconversion of female birds following pathogen contact, allows for the transfer of relevant antibodies to the yolk before egg laying. Maternal egg yolk antibodies (matAB) mean to protect the offspring during the first days to weeks after hatching, albeit the duration of this period is not precisely defined and certainly depends individually on the antibody titre of the female bird. Experiments revealed positively correlating antibody levels in the egg yolk with the serum AIV titre of the maternal bird and partially with its weight [181-183]. A correlation between matAb levels and the egg laying order was discussed controversially for different waterfowl species and might

indicate species-specific survival strategies [181, 182]. A high initial level of AIV-specific matAb supports a longer persistence in hatchlings and titres could be detected up to 28 days after hatching in quail chicks in captivity [183] and mainly two to three weeks after hatching in white ibis (*Eudocimus albus*) chicks in their natural environment [184]. However, no screening for AIV-specific matAb revealed positive findings in comparable attempts among raptor nestlings following prior European HPAIV-epizootics, 2006/2007 and 2016/2017 [52, 185].

Our field work to assess the infectiological and serological status in raptors likewise was conducted in collaboration with ornithologists performing scientific bird ringing focused on raptor nestlings. To current knowledge, our findings on seroconversion in nestlings of white-tailed sea eagles represent the first positive findings within such an approach in general, but can be summarized as an overall low AIV antibody-detection rate (2021: 5.0% and 2022: 7.9%, Publication III). One reason might be a conflict between the time point of sample collection and the persistence of matAb: Bird ringing of raptors usually is performed after a few weeks after hatching but before the offspring reach their capability to fly. Thus, blood sampling might take place at a time when the decay of matAb is already advanced or even completed leading to “false-negative” serological results and underestimating the seroprevalence in adult female birds. On the other hand, a seropositive nestling might already have experienced non-fatal H5 infection and has developed active immunity, in this case, the seroprevalence in female birds would be overestimated. Nonetheless, a first finding of a single white-tailed sea eagle nestling with HA H5-specific antibodies raises hope, that there might be long-persisting protective matAb, or even nestlings could overcome an HPAIV H5 exposure as well as seropositive parental birds.

Supported by the overall unaffected breeding success for white-tailed sea eagles from the same region in 2022 (Publication III), there seem to be no immediate risk for the white-tailed sea eagle population observed in our study. It remains open, if a cross-protective immune response in maternal birds after (HP)AIV H5 exposure during previous epizootics might have increased the chance of survival for their otherwise naïve nestlings, since no serological data to compare with do exist. Most likely, the naturally staggered start of the breeding and thus hatching season helps that not all nestlings are simultaneously exposed to pathogen hot-spots in the environment.

In May 2023, again similar observations were reported for further species, e.g., a Eurasian eagle owl (*Bubo bubo*) nestling tested positive for HPAIV H5N1 (clade 2.3.4.4b, genotype BB) in its nest, sitting right next to the prey remains of a black-headed gull (Figure 6). Possibly novel emerging “prey-predator-pathogen relations” (e.g., black-headed gulls, peregrine falcons and genotype BB HPAIV H5N1, Publication III) might have an even more severe outcome. Long-term effects on both primary and

secondary host populations cannot be ruled out and require further (serological) studies in avian prey and predator species.

Figure 6 Eurasian eagle owl (Bubo bubo) nestling with prey remains of a black-headed gull (Chroicocephalus ridibundus). The nestling has been sampled in the framework of scientific bird ringing in May 2023 and was tested positive for highly pathogenic avian influenza virus (AIV) of subtype H5N1 at the National Reference Laboratory for AIV (sample identification 2023AI05071), suggesting alimentary infection through virus positive prey. Photo by Andreas Buck, Arbeitsgemeinschaft Wanderfalkenschutz (AGW), 2023. For permission rights see Appendix, legal permissions.

At this time, the only possible prevention remains awareness for general and personal hygiene and concomitant responsibility when working or volunteering on wild birds to reduce the risk of an additional "anthropogenic" transmission (e.g. reuse of contaminated clothes or equipment when visiting e.g. colonies, nestling sites, backyard poultry farms). Furthermore, HPAIV ought to be(come) a highly relevant differential diagnoses, especially in wildlife rehabilitation centers (Publication III). Lowering the infection pressure, implies the instant removal and appropriate disposal of carcasses, but always competes with avian or mammalian predator or scavenging species that will efficiently screen their territories for food (Publications II and III). Strategies on suitable protection against HPAIV H5 clade 2.3.4.4.b strains in wild bird populations are just about to become enforced. Only recently a pilot campaign got admitted targeting endangered populations with particular high risk of an infection (California condor) and will represent the first in field study in terms of vaccinating scavenging wild bird species [186].

Potential hosts may develop mechanisms to co-exist with pathogens. Obligate scavengers, such as vulture species, may exhibit adaptations in digestion and immunity [187, 188]. However, the threat posed by infections with HPAIV H5 of the Gs/Gd lineage remains relatively recent. Adaptive evolutionary processes, in particular in the host, require time and are only to be expected in the distant future, if at all. Viral evolution might now be competing with the potentially growing immunity of wild bird host

populations. The high genetic flexibility of HPAIV H5 and its near-global occurrence could confer an advantage. Due to the large number of susceptible species of wild birds and the considerable genetic diversity in species with many individuals, it is to be expected that virus-host relationships will develop that could also allow HPAIV to co-exist with individual species. In order to determine whether there are viral or host adaptations that could result in reduced morbidity and mortality rates among raptors, long-term studies and consistent testing of sick or dead birds is warranted.

The susceptibility and vulnerability of raptors to HPAIV H5 so far has made them very valuable for passive surveillance, especially as a few positive birds say a lot since they are at the end of the food chain. Not only the general occurrence of HPAIV itself corresponded with cases in raptor species (Publication III), but also its circulation on a regional level could be matched with findings in raptor species (Publication III). Sequences of German HPAIV H5 cases even identified a single genotype that was found in a common buzzard only (Publication III). Thus, infections in raptor species can mirror local virus activity, as long as matching predator-prey relations are existent (preferred prey species susceptible for and affected by HPAI, prey size, infectious load in the prey or carcass).

These findings support the hypothesis of free-ranging raptors being invaluable indicators for HPAIV surveillance in their environment - at least for currently circulating HPAIV strains of the Gs/Gd lineage. This knowledge could be applied as well on a supra-regional level to understand the panzootic expansion of HPAI from Europe to North America.

Objective II: Tracking the panzootic spread of HPAIV H5 activity (clade 2.3.4.4b) by utilizing raptor samples for whole-genome sequencing (WGS)

In December 2021 cases of HPAIV H5 have been detected in North America, and subsequently their close relationship with recent European strains was confirmed [149]. This event marks only the second finding of HPAIV H5 strains of Eurasian/European origin in North America after clade 2.3.4.4 strains were detected for the first time in 2014 [135]. Back then an introduction by migratory bird species via the Bering Sea became evident [136]. In 2021, in contrast, a route crossing the Atlantic Ocean seemed more likely [149]. This was supported by the circumstance that European countries were facing the most severe and extraordinary epizootic scenario ever caused by HPAIV H5 Gs/Gd-strains, since late 2020. Initially, the exact route of crossing the Atlantic Ocean remained unclear and its understanding requires background information on the changing HPAIV H5 scenario in Northern European countries.

In Germany, a variety of subtypes and genotypes circulated mainly between autumn 2020 and spring 2021 (Publication IV). Unusually, this epizootic continued until summer 2021 and had resulted in an unprecedented range of affected individuals (>1300 reported wild bird cases and >250 poultry

holdings), species (42 wild bird species) and a total of five HPAIV subtypes (H5N1, H5N3, H5N4, H5N5 and H5N8), in form of seven genotypes (Publication IV). We investigated more than 170 cases by WGS for tracking changes in outbreak dynamics and follow-up incursion events. Genotypes are referred to with a three letter country code (Ger for Germany), their first months and year of occurrence and their NA designation (numbered consecutively in case of similar NA subtypes) – the same nomenclature was applied in Pohlmann, Starick, Grund, et al. [140], King, Schulze, Engelhardt, et al. [112] and Pohlmann, King, Fusaro, et al. [144]. In addition, where it is applicable, the corresponding nomenclature on the European level will be provided as "Ger-MM-YY-NA.X*//EU-nomenclature*" as compared by Pohlmann and Harder [189].

H5N1, H5N5 and H5N8 (Ger-10-20-N8*//A*) were detected in wild birds and domestic avian hosts, Ger-03-21-N8 and Ger-02-21-N8 occurred in poultry only (Publication IV). The latter has been later on additionally confirmed in harbor seals found dead summer 2021 [69]. An HPAIV H5N4 subtype was observed for the first time globally within this study, occurring in wild birds only. The same subtype has been reported at a similar time (early spring 2021) by a Swedish HPAI monitoring study exclusively in two peregrine falcons [190]. H5N3 has been highly associated with the die-off in red knots (Publication IV), whereas the other subtypes did not show signs of host specificity. All subtypes retained a conserved "core genome", comprising the HA and M genome segments, of a common West Asian ancestor and most of the genotypes were characterized by reassortments with different LPAIV of Asian or European origin (Publication IV). The complexity of this 2020/2021 epizootic highlighted the capability and tendency of current HPAI H5 clade 2.3.4.4b viruses for reassortment, possibly entailing more effective adaptations in wild birds or even pronounced zoonotic character over time (Publication IV).

Finally in October 2021, in Germany, five different HPAIV H5N1 genotypes (Ger-10-21-N1.1*//C*, -N1.2*//C*, -N1.3*//C*, N1.4 and -N1.5*//AB*) could be identified [178]. Pohlmann, King, Fusaro, et al. [144] suggested Ger-10-21-N1.1*//C* and -N1.3*//C* as evolving genotypes highly related to the previous Ger-10-21-N1*//C* and, grouped by a common monophyletic HA H5 designated "B1" at a European level. Ger-10-21-N1.2*//C*, -N1.4 and -N1.5*//AB* formed a second HA H5 cluster, B2. Thus, their occurrence was considered as independent incursion events prior to autumn 2021. Both clusters share a common HA H5 ancestor within clade 2.3.4.4b and have been found to circulate since autumn 2020 [144].

The overall high infection pressure and almost constant circulation of these Gs/Gd HPAIV H5 strains in European wild bird populations extended to regions, that serve as core wintering grounds for migratory avian species (e.g., British Isles). From here, wild bird movements possibly rendered the spread to North America [149]. Besides a conceivable direct spread of HPAIV via migratory non-breeding, pelagic species (mainly gulls [149]), North Atlantic islands have been discussed as intermediate stations including in

particular Iceland as part of the East Atlantic flyway from Northern Europe to Greenland, where the North American flyway is met [149, 191]. In Greenland and/or Iceland, the occurrence of LPAIV in gulls, auks, dabbling ducks and wild geese [191-194] has been investigated and partially suggested Greenlandic wild birds as "*mixing vessels for* [primarly LP] *AIV*" between North America and Europe [194]. However, neither in Iceland, nor in Greenland HPAIV H5 cases had been reported so far.

With publication V we succeeded to provide evidence that Iceland indeed had been "*a stepping stone for* [the] *spread of highly pathogenic avian influenza virus between Europe and North America*" (Publication V) around autumn 2021.

The first Icelandic sample set examined by us by WGS comprised organ samples of a single white-tailed sea eagle, that hereby was retrospectively confirmed positive for HPAIV H5N1, after the individual has been found dead in October 2021. The second set comprised samples from a backyard poultry holding, confirmed in April 2022 for HPAIV H5. Whereas initially the white-tailed sea eagle has been the very first and only detection of an HPAIV H5 in Iceland, half a year later the cases in domestic chicken in spring 2022 were accompanied by further HPAI reports in wild birds (Charadriiformes, Suliformes, Anseriformes and Passeriformes (Corvidae only); state June 2022, Publication V).

Phylogenetically, we grouped the sample sets into the two different HA H5 clusters, B1 and B2 [144]. This result clearly pointed to at least two independent incursions onto the island. The eagle-derived sequence clustered within B1, circulating in Europe during winter 2020 and summer 2021, and with early strains from North America. Neither the virus' transatlantic spread, nor the jump to Iceland presumably was caused by a white-tailed sea eagle, as those species are rather residential. Instead, "*infected, but mobile migratory wild birds*" presumably introduced the pathogen to Iceland (Publication V) and became prey for the eagle, without HPAI outbreaks having been noticed. Anseriformes and Charadriiformes species on their way from overwintering to oversummering areas may have been involved, as there were reports on HPAIV H5 affected populations in spring 2021, e.g. in the United Kingdom of Great Britain and Northern Ireland [195-197].

The ongoing severe HPAIV H5 activity in Europe, ought to have allowed a second introduction to Iceland the year after, in spring 2022. The sequences of the HPAIV from the backyard poultry belong to the second HA cluster (B2), evolving from a different Eurasian ancestor. Whereas in the beginning the HA H5 B2 cluster has not been reported from North America, recent investigations confirmed their presence since late 2022/early 2023. Those sequences were retrieved again from possibly scavenging species, an American crow (*Corvus brachyrhynchos*) and a red fox [198]. This proves separate, consecutive incursions of 2.3.4.4b HPAIV also to North America.

Although, on the North American continent, the introduced HPAIV rapidly started reassortments with local, American lineage LPAIV, no such reassortants have yet been detected in Europe or Asia indicating a one-directional spread in two consecutive years from Europe to North America [149, 198]. In addition, in February 2022 an HPAI H5 virus was detected in a bald eagle at the West coast of Canada with high genetic similarity to East Asian HPAIV segments of clade 2.3.4.4b, found in a white-tailed [sea] eagle in Japan the month before [199]. Therefore 2.3.4.4b HPAI viruses also entered the American continent via the Bering strait.

All of these reports demonstrate the complexity of how HPAIV are spreading worldwide. Hereby, WGS combined with phylogeographic analyses is a highly suitable methodology to provide conclusive information on the ancestry or adaptation of newly emerging strains. Surveillance focused on raptor and scavenging species provides an excellent opportunity to track the (supra)regional spread of HPAIV (Publications III and V) and, supported by WGS, its genetic composition (Publications III, IV, V). Although the Icelandic white-tailed sea eagle was clearly analyzed retrospectively, the identification of HPAI's presence in the area alerted the public and the authorities just before the second introduction event became visible in outbreaks among wild birds and poultry holdings (Publication V). The originally infected prey of the eagle clearly has been overlooked but not the large, conspicuous eagle. As such avian raptors tragically function (and can be exploited) as the “sharper eyes” of pathogen surveillance.

One more step before that, a deeper knowledge of regionally circulating LPAIV, likewise could serve as an additional early warning approach for emerging (HP)AIV (Publication IV). Noteworthy, the majority of described genotypes in Germany could be attributed to reassortment events with mainly local (European) LPAIV (segments; Publication IV). In order to obtain more whole-genome sequences, particularly active surveillance approaches would need to be further strengthened, e.g. in reservoir hosts. Although susceptible [59], enhanced sampling activities in raptor and scavenging bird species might not aid in this context, as barely reports on natural infections with LPAIV in free-ranging individuals of this group exist [55, 200].

Gs/Gd HPAIV H5 strains have evolved into a global problem and represent a complex threat on many levels, partially with high concerns on species conservation. Following its introduction to North America, HPAI H5 viruses of clade 2.3.4.4b came up against naïve bird populations and spread explosively. After about the first four months of HPAIV H5 circulation in North America (April 2022), already more than twice as many individuals had become infected compared to the 2014/15 outbreaks and likewise as in Europe broad genetic diversification by reassortment took place [201, 202]. By the end of 2022, the pathogen reached Central and South American countries [32] and meanwhile has spread via all three major American migration flyways (Pacific Americas Flyway, Mississippi Americas Flyway and Atlantic

Americas Flyway) reaching southern regions of South America [32]. Hence, the next continent that now faces the risk of HPAI introduction will be the Antarctica. Investigations on American LPAIV (H6N8) have proven wild bird movements between South America, the periantarctic islands and Antarctica itself [155]. The introduction of HPAIV into these southern climes could cause enormous damage, as little is known about the vulnerability of the species there. The Antarctic summer is also characterized by breeding bird colonies (chinstrap penguins [*Pygoscelis antarcticus*]), hunting species (brown skuas [*Stercorarius antarcticus*]) and scavengers (snow sheathbill [*Chionis albus*]) [155, 203], and the impact on various mammal species (seals) cannot be foreseen.

In order to consider the rapid global spread and increasing persistence of current HPAIV H5 strains, especially passive surveillance measures need to be introduced or maintained. Only sampling of free-living birds can provide the necessary insights into the current or expected occurrence of the disease. Hereby, the ability of raptors or scavengers to most efficiently locate carcasses and hunt weakened individuals before disease outbreaks are noticed by humans is of particular advantage. This idea of targeted sampling for HPAIV can be applied to diverse eligible regions, since almost every (terrestrial) ecosystem has its avian predators or scavengers [203, 204].

Objective III: Outlook on the suitability of raptor species as indicators for further emerging viral pathogens with zoonotic potential

Besides HPAIV H5, other viral pathogens in European wild bird populations are of particular interest, most importantly the two originally African flaviviruses, USUV and WNV. All three pathogens can be transmitted from avian hosts to humans as zoonosis, potentially causing severe disease. By 2020, German surveillance systems focused on either orthomyxoviruses (HPAIV) or flaviviruses (USUV, WNV), as exemplified by Globig, Staubach, Beer, et al. [127] and Michel, Sieg, Fischer, et al. [164]. Given the trends towards an enzootic circulation of HPAIV H5 (clade 2.3.4.4b; [144]) implying its occurrence during spring and summer, it was worth the comparison for possible overlaps in species-specific and temporal occurrence in Germany. In particular, the identification of common host species might help to flag key wild bird species for a broad and resource-sparing monitoring on avian zoonotic disease with public health interest.

Using four criteria, we analyzed more than 4,500 cases. The frequency of each virus reflected the time period of its respective discovery in Germany (HPAIV H5 of clade 2.2 since 2006 [127] and clade 2.3.4.4 since 2014 [45] > USUV since 2011 [156] > WNV since 2018 [157]). In addition, arbo-viruses tend to spread more slowly, as they require additional competent vectors in their transmission cycle.

Three overlapping host species (Northern goshawk, tawny owl [*Strix aluco*] and grey heron [*Ardea cinera*]), at least once positive with one of the pathogens, and five overlapping genera (*Ardea spp., Buteo spp., Corvus spp., Falco spp., Larus spp.* and *Mergus spp.*) have been identified. In none of the overlapping groups there was an even distribution of reports with all three pathogens (Publication VI). Surveillance based on only one of these species/genera might lead to too narrow a view of the pathogen most prevalent in that species group. Combining the findings of five out of nine genera within the non-taxonomic group of "raptors" revealed a slightly more equally distributed pattern of viral diseases (Publication VI). Raptor, and potentially scavenging, species seem to be not only indicators for HPAIV H5 surveillance, but could support as important key species for other avian pathogens. Again, their suitability as indicators relies on alimentary exposure via infected prey, that is as well described for Flaviviruses [205] in addition to the transmission via arthropod vectors.

Noteworthy, the clinical presentation of viral diseases in the identified target species may exhibit similarities and range from non-specific signs to predominantly neurological disorders (Publication VI). Not only, but especially in raptors and scavengers, infections with all pathogens should be considered as differential diagnosis in case of suspicion regardless of the study assignment/inquiry. To investigate the possibility of co-infections, simultaneous screening on all these pathogens would be beneficial. While co-infections with Flavivirus have been reported [206, 207], there are currently no reports of co-infections with HPAIV H5 and Flavivirus. Nonetheless, a detection of AIV-WNV co-infection has been documented in Italy in 2020 in a Yellow-legged gull (*Larus michahellis*) that tested positive for WNV RNA and LPAIV H13N6 [208]. It is currently unclear whether any interference exists between orthomyxoviruses and flaviviruses in the same host. In the future, wild birds, particularly reservoir species, may encounter co-infections more frequently.

Raptor species always have been attracting (public) attention. Hence, findings of dead or suffering medium-sized individuals from this species might be more often noticed or reported, since they occur in various habitat structures and possibly all around the year as residential species. Furthermore, avian predators already serve as target species within the field of ecotoxicology, on the assumption that they might as well accumulate residues of e.g., pesticides, rodenticides, heavy metals or pharmacological residues via prey [209-211]. Agreeing on joint sampling or sample sets could save resources and address animal welfare by limiting field sampling activities, while still making the best use of obtained samples. However, using the secondary host as an indicator species for sampling has the consequence of leaving the originally infected primary host, the prey species, unidentified. Thus, no information can be obtained regarding potential reservoir hosts or their behavior, which could contribute to the general understanding of disease transmission.

Throughout the entire data collection, a major problem was the inaccurate or even missing species designation in about one in five cases (Publication VI). When trying to identify key bird species for a pathogen or a combined surveillance approach, the lack of (precise) metadata impedes conceivable analyses on the host specificity of a virus or on certain host characteristics. Exact species determinations would help to gain insights into the virus epidemiology and could later be used e.g. for predictive models [212]. Consequently, not only for raptor species, but in general, it is important to know the exact species, perhaps even the sex and age of the examined individual when a sample is submitted for examination.

VI. Summary

Highly pathogenic avian influenza viruses (HPAIV) of subtype H5N1 have led to almost unprecedented mortality rates in various wild bird populations worldwide. The high infection rates, especially in Anseriformes and Charadriiformes, suggest an increasing risk for raptors and scavenging bird species as secondary hosts. This thesis addresses two main aspects in this context:

1. *HPAIV H5 infections in prey species cause increased infection rates in raptor species. Due to recent shifts in the activity and distribution patterns of the pathogen, a general expansion of this threat to novel age cohorts and entire populations of raptors is to be dreaded.*

Although an unprecedented number of HPAIV subtypes has been detected in wild birds during winter 2020/2021, our investigations revealed that only the unique subtype H5N3 could be held responsible for a die-off among red knots (*Calidris canutus islandica*) in the Wadden Sea. This clearly host-specific subtype was additionally detected in only one other species, a common buzzard (*Buteo buteo*). However, this subtype did not prevail in the further course of the ongoing epizootic. Instead, the H5N1 subtype has become progressively established in Northern Europe since 2021, reaching an enzootic status that, as our research has shown, also led to severe HPAI outbreaks in colony-breeding seabirds in the North Sea in spring 2022.

Previous seasonal epizootics in Europe during winter months, especially since the incursion of clade 2.3.4.4b, mainly affected juvenile to subadult raptors. The meanwhile enzootic circulation of HPAIV H5 also posed a threat to their nestlings in the rearing phase, as this work confirms for the first time for white-tailed sea eagles (*Haliaeetus albicilla*) in Germany, whose nestlings died of HPAIV H5 infections in coastal regions. While HPAIV H5 outbreaks in colony-breeding seabird species pose a serious threat to these populations, the white-tailed sea eagle population we studied does not appear to be at immediate threat.

2. *Due to their susceptibility and particular high risk at the top of the food chain, raptors are valuable indicator species in the context of passive disease surveillance.*

Targeted testing of raptor samples for HPAIV H5 could be used to assess the (supra)regional spread of these pathogens and their genetic spectrum. In particular, our investigations of samples from Iceland illustrated how only the retrospective examination of a deceased white-tailed sea eagle revealed a previously unknown HPAIV H5 incursion and, thus, simultaneously provided valuable evidence on the route of the transatlantic spread of clade 2.3.4.4b HPAIV from Europe to North America. In addition, raptor species provided an interface for further zoonotic wild bird pathogens, such as Usutu virus and West Nile virus.

Summary

The research presented in this thesis enabled initial assessments of the threat to biodiversity posed by HPAIV H5 in primary and secondary avian hosts in Germany, allowing comparison with data from global HPAI outbreak scenarios. The high genetic flexibility of these viruses will continue to emerge novel variants worldwide and, therefore, requires continuous molecular-epidemiological investigation, whereby the focus on raptors can be a particularly efficient way to support passive surveillance of HPAIV H5 and other zoonotic pathogens.

VII. Zusammenfassung

Hochpathogene aviäre Influenzaviren (HPAIV) des Subtyps H5N1 haben nahezu global zu noch nie dagewesenen Mortalitätsraten in verschiedenen Wildvogelpopulationen geführt. Die hohen Infektionsraten, insbesondere bei Gänsevögeln (Anseriformes) und Regenpfeiferartigen (Charadriiformes), lassen auf ein steigendes Risiko für Raubvögel und Aas-fressende Vogelarten als Sekundärwirte schließen. Die vorliegende Arbeit thematisiert in diesem Kontext zwei Hauptaspekte:

1. *HPAIV H5 Infektionen in Beutetierarten bedingen gesteigerte Infektionsraten in Raubvögeln. Aufgrund neuer Aktivitäts- und Verbreitungsmuster des Erregers ist eine allgemeine Ausweitung der Bedrohung auf neue Altersklassen und ganze Populationen von Raubvögeln zu befürchten.*

Obwohl im Winter 2020/2021 eine noch nie dagewesene Anzahl von HPAIV-Subtypen bei Wildvögeln nachgewiesen wurde, zeigten unsere Untersuchungen, dass einzig der Subtyp H5N3 für ein Massensterben von Knutts (*Calidris canutus islandica*) im Wattenmeer verantwortlich gemacht werden konnte. Dieser deutlich wirtsspezifische Subtyp wurde zusätzlich lediglich in einer anderen Spezies, einem Mäusebussard (*Buteo buteo*), nachgewiesen; dieser Subtyp setzte sich im weiteren Verlauf der Epizootie jedoch nicht durch. Stattdessen hat sich der H5N1-Subtyp seit 2021 in Nordeuropa etabliert und einen enzootischen Status erreicht, der, wie unsere Untersuchungen gezeigt haben, im Frühjahr 2022 auch zu schweren HPAI-Ausbrüchen bei koloniebrütenden Seevögeln in der Nordsee führte. Frühere saisonale Epizootien in Europa im Winterhalbjahr, insbesondere seit dem Auftreten der Klade 2.3.4.4b, betrafen hauptsächlich juvenile bis subadulte Raubvögel. Die nunmehr enzootische Zirkulation von HPAIV H5 stellt auch eine Bedrohung für deren Nestlinge in der Aufzuchtphase dar, wie diese Arbeit es erstmalig für an HPAIV H5-Infektionen verendeten Seeadler-Nestlingen (*Haliaeetus albicilla*) in Küstenregionen Deutschlands bestätigt. Während HPAIV H5-Ausbrüche bei koloniebrütenden Spezies die jeweiligen Populationen ernsthaft in ihrem Bestand gefährden, scheint die von uns untersuchte Seeadlerpopulation allerdings nicht unmittelbar in ihrer Existenz gefährdet zu sein.

2. *Aufgrund ihrer Empfänglichkeit und ihres besonders hohen Risikos an der Spitze der Nahrungskette sind Raubvögel wertvolle Indikatorarten im Rahmen der passiven Surveillance.*

Gezielte Untersuchungen von Raubvogel-Proben auf HPAIV H5 konnten genutzt werden, um die (über)regionale Ausbreitung dieser Erreger und deren genetisches Spektrum einzuschätzen. Insbesondere unsere Untersuchungen von Proben aus Island veranschaulichten, wie erst die retrospektive Untersuchung eines verendeten Seeadlers einen zuvor unbekannten HPAIV H5-Eintrag aufdeckte und damit gleichzeitig wertvolle Hinweise auf die Route der transatlantischen Verbreitung von HPAIV der Klade 2.3.4.4b von Europa nach Nordamerika lieferte. Zudem stellten Raubvögel eine

Schnittstelle für weitere zoonotische Wildvogel-Pathogene, wie das Usutu-Virus und das West-Nil-Virus dar.

Die hier versammelten Untersuchungen ermöglichten erste Einschätzungen zur Bedrohung der Biodiversität durch HPAIV H5 in Primär- und Sekundärwirten in Deutschland, welche den Vergleich mit Daten globaler HPAI Ausbruchsszenarien erlauben. Die hohe genetische Flexibilität dieser Viren wird auch weiterhin weltweit neue Varianten hervorbringen und erfordert daher eine kontinuierliche molekular-epidemiologische Aufarbeitung, wobei der Fokus auf Raubvögel ein besonders effizienter Weg sein kann die passive Surveillance dieser und anderer zoonotischer Pathogene zu unterstützen.

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IX. Appendix

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List of Abbreviations

AIV *avian influenza virus*
arbo *arthropod-borne*

bp *base pair*

CS *cleavage site*

ELISA *enzyme-linked immunosorbent assay*

Gs/Gd *Goose/Guangdong*

HA *hemagglutinin*
HI *hemagglutination inhibition test*
HP *high pathogenic*
HPAI *highly pathogenic avian influenza*
HPAIV H5 *highly pathogenic avian influenza virus subtype H5*

IAV *influenza A virus*
ICTV *International Committee on Taxonomy of Viruses*
IVPI *intra-venous pathogenicity index*

LP *low pathogenic*
LPAIV *low pathogenic avian influenza virus(es)*

M1 *matrix protein 1*
M2 *matrix protein 2*
matAB *maternal antibody*

NA *neuraminidase*
NEP *nuclear export protein*
NGS *next generation sequencing*
NP *nucleoprotein*
NS2 *non-structural protein 2*

OIE *Office International des Epizooties*

PA *polymerase acidic protein*
PB1 *polymerase basic protein 1*
PB2 *polymerase basic protein 2*

RNA *ribonucleic acid*
RT-PCR *reverse transcription polymerase chain reaction*
RT-qPCR *real-time RT-PCR*

USUV *Usutu virus*

vRNP *viral ribonucleoprotein complex*

WGS *whole genome sequencing*
WNND *West Nile Neuroinvasive Disease*
WNV *West Nile virus*
WOAH *World Organisation for Animal Health*

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