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Outbreaks of highly pathogenic avian influenza (HPAI) epidemics in Baltic Great Cormorant *Phalacrocorax carbo* colonies in 2021 and 2022

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ABSTRACT

Capsule: Over 1,700 Great Cormorants died from H5N1 highly pathogenic avian influenza (HPAI) at 11 breeding colonies in the Baltic Sea region, and one in the North Sea, in summer 2021 and 2022.

Aim: To determine the nature and impacts of the 2021/2022 HPAI outbreaks on Great Cormorants in the Baltic Sea region.

Methods: We collected data on HPAI outbreaks in Baltic Great Cormorant colonies through questionnaires, national virological and serological surveys, and avian influenza surveillance databases to better understand the 2021/2022 outbreak.

Results: The HPAI H5 clade 2.3.4.4b (goose/Guangdong lineage) caused mortality in five Great Cormorant breeding colonies in Denmark, three in Germany, two in Sweden and single outbreaks in Estonia and Latvia (all ground-nesting colonies). HPAI H5 monitoring of Great Cormorants in Germany since 2006 showed an increasing prevalence from 2022. Great Cormorant H5N1 viruses belonged to a genotype circulating in wild ducks since 2021. After 2021/2022 morbidity and no subsequent outbreaks, breeding colony sizes returned to above-normal levels in 2023.

Conclusion: After H5N1 HPAI outbreaks in 11 exclusively ground-nesting colonies of Great Cormorants in the Baltic Sea (and one in the North Sea) in 2022, breeding numbers recovered in 2023. Lack of subsequent mortality suggested immunity, but serological investigations are needed to determine the prevalence of protective antibodies in this and other coastal birds.

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Wild birds are recognised as the key reservoir for the circulation of low-pathogenic avian influenza A viruses (LPAIV), primarily transmitted by ingestion of material and water contaminated with virus-positive faeces. Such viruses typically do not cause clinically overt disease, due to adaptation, despite often highly productive virus replication (Olsen *et al.* 2006, Kuiken 2013). Although often asymptomatic, these infections can be transmitted to domestic birds and become highly pathogenic, particularly in gallinaceous poultry through a specific

mutation in the haemagglutinin gene, observed exclusively in the H5 and H7 subtypes (Xie *et al.* 2023).

The goose/Guangdong (gs/GD) H5-lineage is a descendent of an HPAI H5 virus first identified in 1996 in Guangdong province, China (Xu *et al.* 1999). Continued evolution, reassortment and adaptation in gallinaceous and anseriform poultry species have established these viruses in East Asian poultry, causing widespread morbidity, mortality, and substantial economic losses (Lycett *et al.* 2019). Spill-over

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infections of gs/GD HPAI viruses into wild bird populations have subsequently led to mass mortality events among wild birds (Wille & Barr 2022), and their global spread since 2005 (e.g. Lycett *et al.* 2016, Caliendo *et al.* 2022, Gamarra-Toledo *et al.* 2023a). Currently, descendants of this virus are spreading at an unprecedented rate, with high rates of reassortment generating a large number of genotypes. At least 10 different clades of the H5 subtype have evolved, each consisting of numerous subclades (Xie *et al.* 2023). The numbers of wild bird species affected by (*i.e.* testing positive for) gs/GD HPAI are increasing each year, but it was not until the summer of 2021 that it was reported from a ground-nesting, colonial-breeding, fish-eating species of seabird in north-western Europe (Adlhoj *et al.* 2022).

Globally, highly pathogenic H5N1 was reported from five dead or dying wild Great Cormorants (*Phalacrocorax carbo* hereafter 'Cormorants') in a Ukrainian ground-nesting colony in 2006 (Chirniy *et al.* 2008). In Africa, the Cape Cormorant *P. capensis* ($N=104$ fatalities), Crowned Cormorant *Microcarbo coronatus* ($N=2$) and White-breasted Cormorant *P. lucidus* ($N=16$) have been affected by highly pathogenic H5N8 since 2017 (Peyrot *et al.* 2022, Roberts *et al.* 2023), and highly pathogenic H5N1 since 2021 (Abolnik *et al.* 2023, Molini *et al.* 2023). During the 2021/22 southern hemisphere breeding season (October–January) mortality was observed among White-breasted Cormorants, Bank Cormorants *P. neglectus* and large numbers of Cape Cormorants. At least 15,000 and 900 Cape Cormorants died at two major ground-nesting South African colonies, before the virus spread to a Namibian ground-nesting colony, where another 6,500 birds succumbed (Abolnik *et al.* 2023, Molini *et al.* 2023).

In late 2021, H5N1 reached North America (Ruiz-Saenz *et al.* 2023), causing significant mortality among Double-crested Cormorants *P. auratus* ($N=2,779$). In October/November 2022, HPAI H5N1 reached South America for the first time (MINSA 2022, PAHO/WHO 2022, Ariyama *et al.* 2023), affecting waterbirds and marine mammals, including pelicans *Pelecanus* spp., cormorants, Sanderlings *Calidris alba*, sea lions (Otariidae) and dolphins (Delphinidae) (Leguia *et al.* 2023). Among the seabirds, fatalities were recorded from Guanay Cormorants *Leucocarbo bougainvilliorum* ($N=28,921$), Neotropical Cormorants *Nannopterum brasilianus* ($N=54$) and Red-legged Cormorants *Poikilocarbo gaimardi* ($N=5$, Harvey *et al.* 2023, Gamarra-Toledo *et al.* 2023b).

While this history demonstrates that cormorants are highly susceptible to HPAI, relatively few Cormorants

were testing positive for HPAI virus in Europe in the epizootic year 2016–2017. No virus introduction appeared to take place at any breeding colony of European Cormorants, despite their co-occurrence with other wild bird species that had been severely affected. Only one case of H5N6 was reported in a Cormorant in southern Denmark, in March 2018 (Liang *et al.* 2021).

This situation changed dramatically in the summers of 2021 and 2022. In the Baltic Sea region, Cormorants nest in trees and low bushes, safe from ground predators in mainland colonies, but they do also nest on the ground on islands lacking trees and predatory mammals, especially in Denmark, Sweden, Finland and Estonia. There are few ground-nesting colonies along the German Baltic coast. Because ground-nesting Cormorants are situated much closer together than those nesting in colonies in trees and shrubs, we paid particular attention to virus prevalence in relation to nesting site types, and in this paper we describe the progress and extent of a major outbreak among Cormorants in the Baltic region in 2021 and 2022.

Methods

Case study data collection

In early 2023, we sought to obtain an overview of known cases of HPAI outbreaks in Baltic Cormorant colonies and in European colonies elsewhere in 2022 and earlier. Members of the Wetlands International Cormorant Research Group, and other ornithologists known to work in Cormorant monitoring and research, were asked to supply information on (1) colonies experiencing unusual mortality patterns and their extent, (2) the number of breeding pairs (hereafter 'BP'), (3) outbreak start date and duration, (4) whether HPAI was confirmed by laboratory analysis (yes/no), and (5) the type of colony (specifically ground-nesting versus arboreal/bush/shrub-nesting).

Factors affecting risk of HPAI outbreaks

We compiled data on the number of BP, HPAI outbreaks and whether birds nested on the ground or in trees for each of the 120 colonies in Denmark (all colonies) and Germany (all colonies in Schleswig-Holstein and Mecklenburg-Western Pomerania) in 2022. We assessed whether infections were spatially clustered by fitting a generalized linear mixed-effects model (GLMM) with a binomial error structure, using

the sdmTMB package (v0.1.0, Anderson *et al.* 2022; all statistical analyses were performed in R v.4.2.0, R Core Team 2022), taking colony infection status (0 = not infected, 1 = infected with HPAI) as the dependent variable, and the intercept as the sole predictor. We tested for spatial covariation between colonies by fitting the model with and without a spatial random field, that we constructed using geographic coordinates in the equidistant Mercator projection and the packages INLA (v22.12.16, Lindgren & Rue 2015) and sf (1.0-14, Pebesma & Bivand 2023). The significance of the spatial random field was evaluated using a likelihood ratio test (LRT) comparing models fitted with and without the random effect (using two degrees of freedom for the Matérn correlation function; Zuur *et al.* 2017). Model fit was assessed via visual inspection of the distribution of residuals. We tested whether the probability of colony infection with HPAI was dependent on colony size (log-transformed BP), by fitting a similar spatially explicit binomial GLMM, again taking colony infection status as the dependent variable, and log-transformed colony size as a covariate, controlling for spatial covariation between colonies by fitting the same spatial random field as described above.

Finally, we tested whether breeding in trees or on the ground explained the infection status of a colony. Because there were no infected colonies in trees, colony infection status quasi-completely separated the predictor, which meant that it was not possible to fit a binomial model. Instead, we used Fisher's exact test to analyse the contingency table summarising infection status between ground- versus tree/bush/shrub-nesting colonies. We used the Haldane-Anscombe correction as implemented in the package pairwiseCI (v0.1-27, Schaarschmidt & Gerhard 2019) to derive an odds ratio with its associated asymptotic adjusted Woolf 95% confidence interval (Haldane 1940, Woolf 1955, Anscombe 1956, Lawson 2004).

Virological and serological sample collection and analysis

Samples were taken in 2022 from birds on the small island of Beuchel (on Rügen, Germany: 54°32' N, 13°17' E) following an outbreak of HPAI in Cormorants within a multi-species seabird colony dominated by intermixed Cormorants, Herring Gulls *Larus argentatus* and Mute Swans *Cygnus olor*. Thirty adult breeding Herring Gulls were caught with basket traps on 22 May 2022 and sampled by taking an oropharyngeal and cloacal swab, as well as a blood sample from the brachial vein. A total of 25 pre-fledged

juvenile Cormorants were sampled in the same way on 23 May 2022. Additionally, 12 pre-fledged juvenile Herring Gulls were sampled by taking oropharyngeal and cloacal swabs on 29 June 2022. From 30 April to mid-November 2022, sheep *Ovis aries* grazed the island to maintain short grass swards; they fed between and underneath the Cormorant nests and these 24 individuals were sampled on 16 October 2022. The numbers of Cormorant and gull BP, as well as numbers of their chicks, were monitored by regular counts throughout the whole breeding season.

Live bird samples were directly transferred to the Friedrich-Loeffler-Institut (FLI) for further processing. Serum was separated from blood cells after 10 min centrifugation at 500 x g and tested using ID.Vet nucleoprotein (NP)-ELISA following the manufacturer's guidelines. NP-ELISA positive sera were then further investigated in H5-specific ID.Vet ELISA. Results were interpreted by dividing the mean optical density values (OD, a measure of the density of the primary antibody that binds to the antigen in the ELISA reactions) of the test samples by the negative control mean (S/N ratio) as described in Chappell *et al.* (2014). Cut-offs were used as recommended by the manufacturer of the commercially available ELISA kits. Swab samples were processed as described earlier and screening, sub- and pathotyping done as previously reported in Pohlmann *et al.* (2023). From this, we derived cycle thresholds (Ct), a semi-quantitative value to categorise the concentration of viral genetic material in a sample following the testing by RT-qPCR as low, medium or high. Low Ct indicates high concentrations of viral genetic material, associated with high risk of infectivity and in this study, Ct values above 35 were considered negative.

Swab samples taken from carcasses were sent to the State Office for Agriculture, Food Safety and Fisheries Mecklenburg-Western Pomerania (LALLF) for initial diagnosis. Samples that tested positive for Influenza A matrix gene were sent to the National Reference Laboratory at the FLI for confirmation (Pohlmann *et al.* 2023). Swab samples from Cormorants found dead in Denmark were tested for the presence of avian influenza virus (AIV) by the Danish National Reference Laboratory essentially, as previously described (Liang *et al.* 2021), with the addition of routine testing also of brain swabs from February 2021 onwards.

Analysis of changes in HPAI prevalence over time

To assess changes in HPAI prevalence over time, we analysed data from the German National Database for

Animal Diseases (Tierseuchennachrichtensystem, TSN) and Database for Avian Influenza in Wild Birds (Aviäre Influenza Wildvogel-Datenbank, AI-DB) retrospectively. Exact binomial confidence intervals for the prevalence estimates were derived using the R scripts of Hollander *et al.* (2015). Data from the AIV passive surveillance from mid-2006 to 2022, and from the AIV active surveillance in live wild birds for the years 2007–2010 (the Danish AIV database (<https://ai.fvst.dk/>), and data from the spring 2006 (DVFA 2007)), were all included in the analysis.

Phylogenetic Analysis

Whole genome sequencing of samples taken from the two confirmed German HPAI outbreaks in Cormorant colonies (Figure 1: #7 Heuwiese, #8 Beuchel) was performed using a nanopore-based amplification method (King *et al.* 2022). Whole genome sequences were generated from samples taken at three of the confirmed Danish HPAI outbreaks in 2022, using Nextera libraries on an Illumina MiSeq system as previously described (Liang *et al.* 2021), except that the MiSeq Reagent Kit v2 was used with 2×150 cycles.

For phylogenetic analyses, a sequence dataset was compiled from public databases where the search was restricted to clade 2.3.4.4b H5N1 sequences collected from wild birds during the breeding seasons in 2021 and 2022 (April–July) in the Baltic Sea coastal countries (Denmark, Estonia, Finland, Germany, Latvia, Lithuania, Poland, Russia, Sweden). A total of 512 sequences from 64 viruses (25 isolates from summer 2021 and 39 isolates from summer 2022) were compiled. Multiple alignments of concatenated coding sequences of whole genomes were generated using MAFFT (v7.450) (Katoh & Standley 2013), and subsequent maximum likelihood (ML) trees were calculated with RAXML (v8.2.11) (Stamatakis 2014) utilising model GTR GAMMA with 1,000 iterations rapid bootstrapping and a search for the best-scoring ML tree. Genotypes were assigned and designated as described by Pohlmann *et al.* (2022). Data sources or availability and acknowledgment of data providers are given in Supplemental material Table S1.

Results

Case study data

Respondents reported one outbreak of unusual mass mortality of Cormorants in the 2021 breeding season and 10 in 2022 in the Baltic Sea area, with one also suspected in 2022 on the Danish North Sea coast

(Figure 1). Of these, four were subsequently confirmed (i.e. laboratory confirmed as HPAI H5N1) and seven suspected (i.e. an unusually high mortality of Cormorants in colonies but not confirmed by testing). The cases reported were as follows:

Case 1: The first Baltic case of elevated mortality among Cormorants and other seabirds was reported in May 2021 from **Kuralaid** island in Pärnu county, **Estonia** (58°26'N, 23°40'E, Figure 1: #1). At the time, the island hosted a Cormorant colony of 1,367 BP nesting on the ground or in low bushes. A visit on 25 May 2021 found 71 dead Cormorants and many birds sick with typical neurological symptoms (i.e. uncoordinated movements). Other dead animals recorded were 13 Herring Gulls, two Oystercatchers *Haematopus ostralegus*, two Mute Swans, a Greater Black-backed Gull *Larus marinus* and four Grey Seals *Halichoerus grypus*. On 2 June 2021, a visiting veterinarian took samples from six dead birds (including three Cormorants) on the island, all of which tested positive for HPAI at the National Centre for Laboratory Research and Risk Assessment, the national reference laboratory for the avian influenza virus (the former Veterinary and Food Laboratory).

As the island was not revisited during the 2021 breeding season, we lack information on breeding success at this colony in that year. Many Cormorant skeletons were found on the island during a visit in 2022. In 2021, higher than normal mortality was observed in neighbouring regions among the Cormorants and gulls; all birds collected and sampled tested positive for HPAI H5N1. In mid-May 2021, two dead young White-tailed Eagles *Haliaeetus albicilla* tested HPAI H5N1-positive from Matsalu, Pärnu county (58°43'N 23°42'E). A dead Cormorant found in Pärnu county in August 2021 also tested HPAI H5N1 positive. In 2022, however, the monitoring of 203 islands harbouring seabird colonies throughout Estonia revealed no unusual avian mortality.

Case 2: In late April 2022, the Danish Nature Agency received a report of 30 dead Cormorants in **Hårbølle Harbor** on Møn, **Denmark** (54°53'N, 12°08'E), 1.3 km southeast of the large ground-nesting Cormorant colony of **Malurtholm** (Figure 1: #2). Three dead Cormorants collected on 2 May 2022 were submitted for analysis. All three, and another individual collected on 18 April 2022 a few kilometres away, tested positive for HPAI H5N1. The Malurtholm Cormorant colony was visited during the regular monitoring activities on 4 June 2022, when 403 dead adult Cormorants were recorded, which were estimated to have died three to four weeks earlier (with no signs of recent mortality), as well as 424 active nests and 200 living chicks. There were no observations of

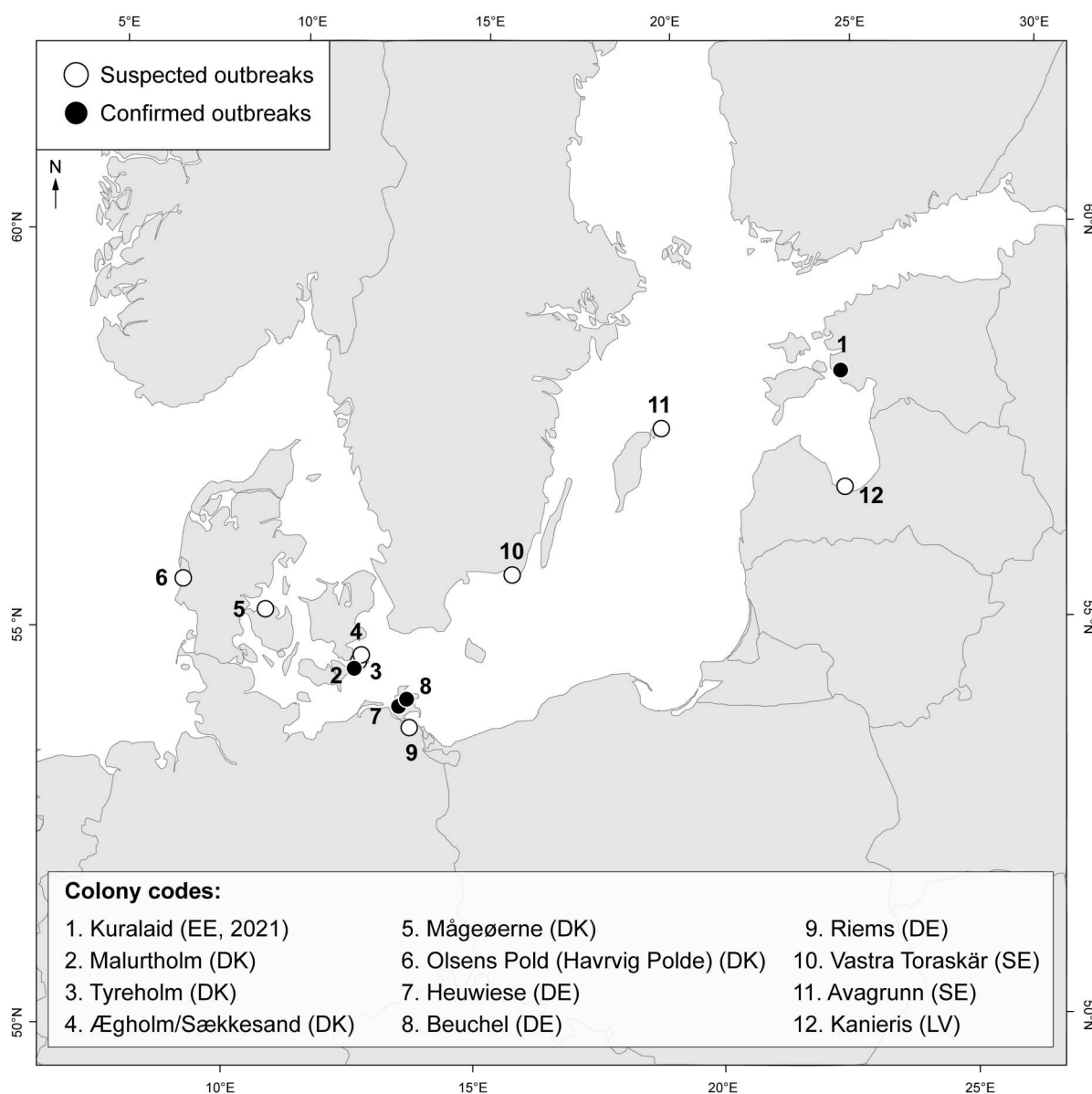


Figure 1. Location of HPAI outbreaks at Cormorant colonies in the Baltic Sea area during the breeding seasons in 2021 and 2022, including the single colony on the Danish North Sea coast (numbered 6, see text for full details).

dead or sick chicks. However, the number of active nests was much lower than during the previous year, when a total of 1,163 nests were counted.

Case 3: Reduced numbers of nests (517 compared to 1,055 the year before) and > 210 dead Cormorants (some long dead) were recorded in and around the ground-nesting colony on **Tyreholm**, in northern Møn, **Denmark** (55°01'N, 12°14'E) (**Figure 1**: #3), during the visit on 19 May 2022. Another 273 (mostly) long-dead adults were found during a follow-up visit on 28 June 2022. However, the colony had not collapsed, as up to 200 juvenile Cormorants (maximum 25 days old) were healthy and no recent fatalities of young birds were observed. Juvenile birds can show a higher viral

prevalence than adults (Webster *et al.* 1992, Kleijn *et al.* 2010, Høye *et al.* 2011, Van Dijk *et al.* 2014), but, compared with younger and middle-aged breeders, it might be expected that the immune system of older breeders could be less efficient in protecting against the fatal effects of exposure to HPAI (Lee *et al.* 2023). However, among 120 ringed Cormorants recovered among the breeders that died at the two neighbouring Danish colonies of Tyreholm and Malurtholm (above) during the outbreak, there was no sign of over representation of younger or older individuals (see Supplemental material Figures. S1-S3).

Case 4: On **Ægholm/Sækkesand**, north of Møn, **Denmark**, ((**Figure 1**: #4), two ground-nesting

colonies in close proximity at 55°04'N, 12°13'E and 55°04'N, 12°15'E) had no active nests recorded during the counts of Cormorants and other breeding birds on 31 May 2022, compared to 530 nests in the previous year. Ten dead adults were found close to nests on Ægholm, 16 on Sækkesand and several more along the coast. Dead Cormorants on Ægholm and Sækkesand were partially eaten, probably by White-tailed Eagles.

Case 5: Away from Møn, dead Cormorants were found in larger than usual numbers in the ground-nesting colony on **Mågeøerne**, north Funen, **Denmark** (Figure 1: #5; 55°34'N, 10°06'E). During the count on 5 May 2022, 15 dead adult Cormorants were found, all of which had been dead for several weeks.

Case 6: In the ground-nesting colony at **Olsens Pold** (**Havrvig Polde**) in Ringkøbing Fjord on the North Sea coast of **Denmark** (Figure 1: #6; 55°56'N, 08°10'E), 23 dead adult Cormorants were visible on drone images captured on 9 June 2022.

The surveys on the islands around Lolland/Great Belt (Nakskov Fjord, Vensholm, Rågø Sand, Vresen) and at Denmark's largest Cormorant colony in Stavns Fjord did not reveal unusual numbers of dead Cormorants in 2021 or 2022.

Along the **German** Baltic coast, outbreaks appeared at the end of April and beginning of May 2022 in two colonies on small islands in the lagoons around the island of Rügen:

Case 7: Mortality of Cormorants was observed on the island of **Heuwiese** (Figure 1: #7; 54°26' N, 13°07' E), an uninhabited bird sanctuary in the western lagoon area of Rügen. The island hosts a large ground-nesting colony of Cormorants, a colony of Herring Gulls and a colony of breeding Mute Swans. On 6 May 2022 a count of 343 Cormorant nests was made on the island, with 20–25 nests were still under construction. Around that time the carcasses of 20 Cormorants (19 adults and one second-year bird) and four Herring Gulls (one adult and three immatures, all scavenged by White-tailed Eagles) were found. Several live Cormorants showed neurological symptoms and H5N1 was confirmed on 12 May 2022 for four out of five of the tested Cormorants that were immediately collected as freshly dead. On 18 May 2022 another 12 dead Cormorants and two dead Herring Gulls were collected, by which time breeding activities had ceased and the colony had been abandoned.

Case 8: On 30 April 2022, larger numbers of dead Cormorants were found on the small island of **Beuchel**, located in the Neuendorfer Wiek bay on the island of Rügen (Figure 1: #8; 54°32' N, 13°18' E). On 3 May 2022, the carcasses of 270 full-grown Cormorants (225 adults and two second-year birds,

plus 43 individuals not possible to age that were scavenged by White-tailed Eagles) and seven nestlings, as well as one dead adult Herring Gull, were removed from the island. Fifteen Cormorants were tested for HPAI and all were confirmed positive for HPAI H5N1 on 6 May 2022. During the following weeks, more Cormorants and a few Herring Gulls were found dead or sick. These records included, on 7 May 2022, two freshly dead adult Cormorants, three adults still alive with neurological symptoms and 45 carcasses scavenged by White-tailed Eagles. On 14 May 2022 there were 18 dead Cormorants (six adults, one second-year, seven pulli; four adults scavenged by White-tailed Eagles). On 22 May 2022 there were 11 dead Cormorants (three adults, six pulli; two adults scavenged by White-tailed Eagles).

Beuchel supports breeding colonies of Cormorants (two-thirds of which are ground-nesting, the remainder nesting in dead small bushes), Herring Gulls and Mute Swans, all of which come into direct contact with each other. On the island, Herring Gulls feed on the eggs and chicks of other seabirds, and they also regularly take fish dropped by breeding Cormorants that may also be contaminated with faeces.

In contrast to the colony on Heuwiese, the Beuchel colony did not collapse in 2022 as breeding continued in some nests and about 100 chicks fledged from the first broods by the end of May. Two ringed chicks from that year were later recovered well away from the colony, which indicates that some young Cormorants had survived to disperse. In mid-May 2022, about 150 pairs started a second breeding attempt. Among them was a ringed adult bird, likely a breeding bird from the Heuwiese colony, where it had been observed in 2013, 2015, 2017 and 2021, indicating that this second breeding attempt included birds from Heuwiese. Of these second broods, some 150 juveniles fledged.

Although Herring Gulls had direct contact with the Cormorant colony, only a few adult birds died in 2022, although many pairs stopped breeding. Herring Gull chicks died at least partly because of HPAI infection (based on laboratory tests) and ultimately breeding success was extremely low, with 424 BP fledging about 20 young. The sheep on the island showed no signs of disease.

Case 9: Another Cormorant colony on the southern coast of the Greifswald Lagoon, on the small island of **Großer Werder Riems** (Figure 1: #9; 54°10' N, 13°21' E), suffered increased mortality in early May 2022, when 30 dead Cormorants and three dead Herring Gulls were reported, but later there was no evidence that breeding numbers had been seriously affected. On this island,

Cormorants nest densely in small bushes and on the ground.

At other monitored Cormorant colonies on Rügen (Nonnensee/Bergen in central Rügen and Mellnitz-Üselitzer Wiek at the southern coast of Rügen) there were no reports of unusual mortality. In contrast to Heuwiese and Beuchel, these other colonies are not ground-nesting, with nests being constructed in tall trees. At Niederhof, a tree-nesting colony 32 km south of Beuchel and 20 km south-southwest of Heuwiese, a single Cormorant with neurological symptoms was found during a count on 3 May 2022, but, despite frequent visits and checks, no further dead or sick birds were recorded.

In **Sweden**, mass mortality of Cormorants was reported from two ground-nesting colonies in 2022, these being on **Torraskär** (Karlskrona archipelago) and **Avagrunn** (north-east Gotland):

Case 10: On **Vastra Torraskär** (Figure 1: #10; 56°07' N, 15°40' E), in a large colony with approximately 1,500 ground-nesting BP of Cormorants, several hundred dead birds were found during a visit at the beginning of August 2022, but the carcasses were not tested because they were considered to be too old to establish the cause of death.

Case 11: On **Avagrunn** (Figure 1: #11; 57°56' N, 19° 17' E), about 50 dead Cormorants were found during a colony visit in June 2022, but they were not tested for HPAI. There is evidence that H5N1 was circulating on Gotland in summer 2022, with two dead Cormorants collected in July in the northern part of the island, and one in the western part, all testing positive for H5N1 (C. Bröjer, National Veterinary Institute, SVA, pers. comm.). However, these birds were not collected from breeding colonies.

Case 12: At **Kanieres Lake** (Figure 1: #12; 57°00' N, 23° 29' E), the biggest Cormorant colony in **Latvia**, some signs of unusual mortality were reported in summer 2022, in the late breeding or post-breeding period, but details were not available. Cormorants in this colony breed in trees and form dispersed satellite colonies around the lake shore, which are difficult to access.

In contrast to the widespread and numerous deaths of Cormorants at the above-mentioned colonies during the summer of 2022, no increased mortality

was reported at any of the affected colonies in 2021, or elsewhere in the Baltic region during the 2023 breeding season.

Factors affecting the risk of HPAI outbreaks

There was spatial covariation between Cormorant colonies in their risk of being infected (LRT $P = 3.6 \times 10^{-22}$), which means that infected colonies were clustered spatially (Figure 1). The risk of a colony being infected with HPAI did not increase with its size, but we observed a trend in the expected direction, with larger colonies being more likely to become HPAI-infected, although the result was not statistically significant (β [95% CI] = 1.67 [−0.326–3.67], $P = 0.10$). Nesting on the ground increased the likelihood of HPAI-infection significantly (odds ratio = 33.2, 95% CI = 1.8–596.8, $P = 4.5 \times 10^{-4}$). Notably, not a single tree-breeding colony was affected by HPAI in Denmark or Germany (Table 1).

Virological and serological sample collection and analysis

On 22 and 23 May 2022, a total of 27 adult Herring Gulls and 25 near-fledged juvenile Cormorants were caught, ringed and tested for HPAI virologically and serologically (Table 2). Herring Gulls showed antibodies against Influenza A, and six tested positive for Influenza H5-subtype antibodies in the ELISA (Figure 2), while no HPAI H5 virus excretion was evident at the time of sampling. All swab samples taken from the juvenile Cormorants were negative in the RT-qPCR (Table 2). Only two juvenile Cormorants showed weak antibody reaction in NP ELISA (Figure 2), which were H5 negative. All 24 sheep sera sampled in mid-October on Beuchel were serologically negative for influenza A virus antibodies

Table 2. Summary of virological and serological results for the birds and sheep tested on Beuchel, Germany.

	Cormorant	Herring Gull	Sheep
Date of investigation	23 May 2022	22 May 2022	16 October 2022
Age	Unfledged juv.	Adult	Adult
NA* / Number of samples investigated	0 / 25	0 / 30	Not done
Number of NP-antibody positives / Number of samples investigated	2 / 25	27 / 27	0 / 24
Number of H5-antibody positives / Number of samples investigated	6 / 27	0 / 2	Not done

*NA = detected nucleic acid for Influenza A matrix protein presence in oropharyngeal and/or cloacal swabs

Table 1. Relative frequency of HPAI infection at tree-nesting versus ground-nesting Cormorant colonies in Germany and Denmark.

	Infected	Non-infected	Total
Tree-nesting	0	78	78
Ground-nesting	7	35	42
Total	7	113	120

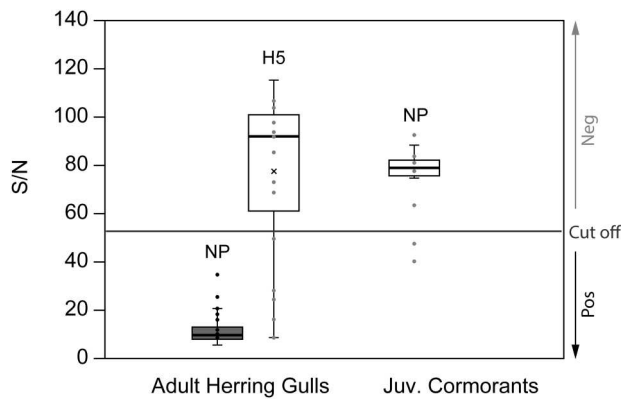


Figure 2. S/N values in blocking NP and H5 IDVet ELISA for adult Herring Gulls and juvenile Cormorants sampled in May 2022 on Beuchel island, Rügen, Germany. The S/N ratios are derived by dividing the mean test optical density (OD) values (a measure of the density of the primary antibody that binds to the antigen in the ELISA reactions) of the test samples by the negative control mean as described in Chappell *et al.* (2014). The NP ELISA enables rapid serological diagnosis, suited for influenza A antibody screening. All NP tested Herring Gulls showed influenza A antibodies (grey shaded box and whisker), very likely having had a recent influenza A infection, since all S/N ratio values fell below the threshold cut-off for this run of tests. Subsequent testing with H5 ELISA surprisingly found most of these tested H5-negative (central box and whisker plot), but confirmed the presence of H5-specific antibodies in five of these individuals. Two of the near-fledged Cormorants showed weak antibody reactions to NP ELISA but were also H5 negative (right box and whisker plot).

(note that although this ELISA is a multi-species test, it has not been validated with sheep sera).

Analysis of changes in HPAI prevalence over time

Since 2006, in Germany a total of 3,764 avian influenza tests have been performed on Cormorants, all of which

were registered in the German Wild Bird Avian Influenza Monitoring Database. In the HPAI outbreak year of 2006, HPAI (H5N1) was only detected in three out of 793 sampled Cormorants (prevalence = 0.38% [95% CI 0.07–1.10]. HPAI H5 (N8, N5)) was detected in Cormorants in the outbreak years of 2016 and 2017 (prevalence = 1.27% [95% CI 0.03–6.85] and 2.96% [95% CI 1.43–5.37], respectively), although the majority of birds tested negative. Since 2021, HPAI (H5N8; H5N1) has been recorded regularly in Cormorants, with the highest prevalence rate hitherto seen in 2022 (12%, 95% CI 7.40–19.12; Figure 3; Supplemental material Table S2).

Since 2006, a total of 226 Cormorants have been tested for avian influenza in Denmark. Most of these were found dead, with the exception of 82 live birds tested in 2009 without clinical signs of avian influenza infection (Supplemental material Table S3). Of these 226 birds, avian influenza was detected in four Cormorants (but not of the H5 or H7 subtypes), HPAI H5N6 virus was detected in one Cormorant in March 2018 and HPAI H5N1 virus in four Cormorants in April–May 2022.

Phylogenetic Analysis

The analysis of the H5N1 genomes from the summers of 2021 and 2022 revealed genetic clusters that confirmed the emergence of specific genotypes during this period (Supplemental material Figure S4). Three distinct genotypes could be identified. Firstly, in the summer of 2021 the genotype DE-21-02-N1, euC B1, (A/barnacle goose/Germany-NI/AI01605/2021-like; A/Eurasian Wigeon/Netherlands/1/2020-like) was present in the region studied. In Estonia, three analysed genomes from a White-tailed Eagle, a Hen Harrier *Circus cyaneus* and a

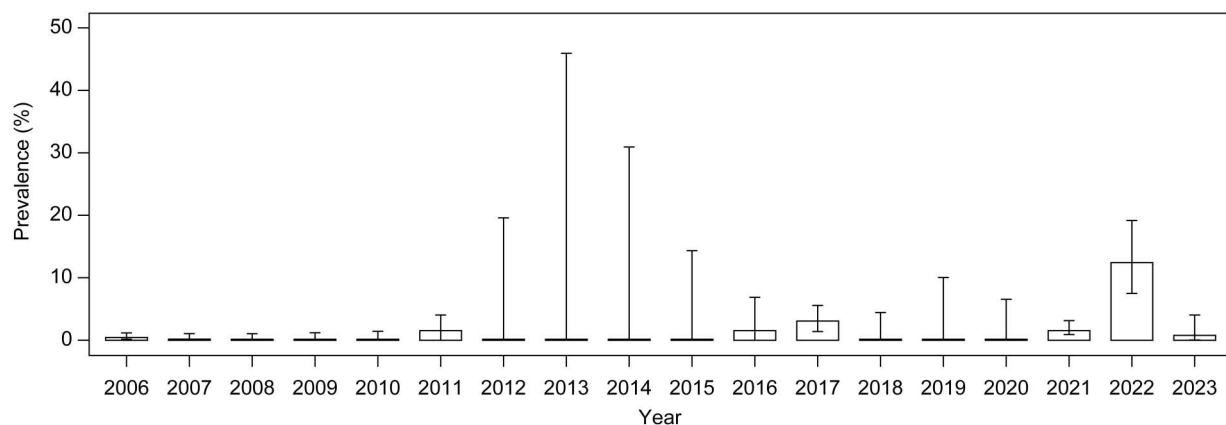


Figure 3. Prevalence detection (in percent) and 95% confidence intervals for all investigated Cormorant samples collected in Germany between 2006 and the end of 2023. Each bar represents data gathered between 1 January and 31 December of the year identified. For more detail, including sample sizes, see Table S1.

Herring Gull belonged to this genotype. The gull was collected close to the affected Cormorant colony at Kuralaid. In the summer of 2022 the genotype DE-21-10-N1.2, euC B2 (A/white-tailed eagle/Germany-MV/AI05975/2021-like) and genotype DE-21-10-N1.5, euAB, (A/Eurasian wigeon/Germany-SH/AI05952/2021-like; A/duck/Saratov/29-02/2021-like genotype) were recovered and identified; the latter is characterized by reassorted PB2, PA and NP segments. The genomes obtained from case #7 at Heuwiese (A/great cormorant/Germany-MV/AI02774/2022) and case #8 at Beuchel (A/great cormorant/Germany-MV/AI02666/2022) were very similar (99.85% or a difference of 20 nucleotides), belonging to genotype DE-21-10-N1.5_euAB and clustered together with sequences from other colonial breeding waterbirds (Common Guillemot *Uria aalge*, Black-headed Gull *Chroicocephalus ridibundus*, Sandwich Tern *Thalasseus sandvicensis* and Arctic Tern *Sterna paradisaea*) and avian raptors (Peregrine Falcon *Falco peregrinus*, White-tailed Eagle and Tawny Owl *Strix aluco* from Germany, Poland and Denmark; Figure S4).

Sequences from Cormorants in Denmark belong to two different genotypes: A_great_cormorant_Denmark_04557-1.01_2022 was assigned to genotype DE-21-10-N1.5_euAB, whereas A_great_cormorant_Denmark_03254-1.02_2022 and _great_cormorant_Denmark_04559-1.02_2022 are genotype DE-21-10-N1.2_euC B2, which clusters with avian raptors (Red Kite *Milvus milvus*, Common Buzzard *Buteo buteo* and Hen Harrier) from Denmark and Estonia, and colony-breeding Northern Gannets *Morus bassanus* from Sweden and Germany (Figure S4).

Discussion

Highly pathogenic avian influenza viruses of the H5 goose/Guangdong (gs/GD) lineage emerged in Europe in autumns and winters of 2006, 2014, 2016 and 2020. Until 2017 each of the virus lineages typically disappeared in the following year. Since 2021, however, all detected viruses have belonged to clade 2.3.4.4b and have persisted throughout the summer, causing outbreaks among wild birds and poultry on an ever-increasing scale (e.g. Pohlmann *et al.* 2023). A new development, suggesting the enzootic status of the virus, was witnessed in summer 2022, when the HPAI H5 virus entered northern European populations of colony-nesting seabirds on a large scale, among them the Cormorant. The outbreaks described here occurred exclusively in ground-nesting colonies, where the virus caused high mortality among adults and chicks, although the latter may have died indirectly through loss of parental care, lack of food or predation by gulls and raptors.

Away from the Baltic Sea, we received year-specific information about Cormorant colonies from Austria (R. Parz-Gollner), Belgium (J.-Y. Paquet), France (L. Marion), Greece (S. Kazantzidis), Italy (S. Volpony), the Netherlands (M. v. Eerden), Latvia (K. Millers) Norway (S.-H. Lorentsen) and the United Kingdom (S. Newson). Although there were HPAI outbreaks in various seabird colonies in these countries in the same year (e.g. Dalmatian Pelican *Pelecanus crispus* colonies in Greece, Alexandrou *et al.* (2022), Gannet *Morus bassanus* colonies in the United Kingdom and Germany (Lane *et al.* 2023), Sandwich Tern colonies in France, Belgium, the Netherlands, Germany and the United Kingdom (Knief *et al.* 2024)), none of the respondents reported Cormorant colonies being affected. However, routine passive surveillance for HPAI in some countries has detected HPAI H5N1 virus-positive Cormorants (two in Belgium, three in the United Kingdom), providing evidence for the presence of the virus in these areas, but for some reason not causing outbreaks in breeding colonies.

The close proximity of nests among ground-nesting Cormorants on islands covered by low vegetation is different from the situation where they nest in trees, or, as in the United Kingdom, on cliffs. In the case of arboreal or cliff-nesting colonies, the distance between nests is larger, both horizontally and vertically, than for ground-nesters. The nearest-neighbour distances between nest centres of Danish ground-nesting Cormorants rarely exceed 1.5 m at Tyreholm, Ægholm and Sækkesand, which ensures constant close physical contact with other birds, regurgitated pellets and faeces, the latter of which can carry high virus loads if birds are infected. In arboreal breeding colonies the excrement falls to the ground, and Cormorants do not come into regular contact with this material.

The proximity hypothesis is supported by the fact that in the spring of 2022, outbreaks of HPAI H5 occurred in colonies of several different species of densely colonial ground-nesting birds in north-western Europe, which were not previously known to be subject to HPAI mortality. These included Sandwich Tern (Knief *et al.* 2024) and Northern Gannet colonies (Lane *et al.* 2023), which both also breed at high densities. On Helgoland, as elsewhere, those Northern Gannets breeding on the cliffs were less affected than those breeding on the flat surfaces on top of the rocks, again supporting the proximity hypothesis (Ballstaedt *et al.* 2023, Lane *et al.* 2023).

The transmission of HPAI virus between neighbouring ground-nesting colonies is well explained by the known movement patterns of

Cormorants. Ring recoveries confirm the exchange of birds, e.g. between different colonies on the island of Rügen, including Heuwiese, Beuchel and others (Behringer *et al.* 2022). Furthermore, there is evidence that breeding birds visit multiple colonies during the breeding season. In 2019, 10 adult Cormorants resting on Beuchel were equipped with GPS loggers, and these turned out to be nesting in three different colonies: Beuchel, Heuwiese and Nonnensee (central Rügen; S. Garthe, *in litt.*).

Our genetic analysis showed a high similarity between the H5N1 virus genomes from the two H5N1-positive Cormorant colonies near the island of Rügen and those from other colonial breeding species in Germany, Poland and Denmark, also suggesting that proximity of breeding increases the risk of virus spread. Phylogenetic analysis confirmed that different virus genotypes were present in our study region in summer 2022, with two different genotypes in Cormorants. This was consistent with a previous study on mass mortality in colonial breeders, which suggested different routes of virus entry and spread between colonies of terns and Cormorants along the Baltic and North Sea coasts, and of Northern Gannets along the North Sea coast that year (Pohlmann *et al.* 2023). The different genotypes found in Danish Cormorants underline these different incursion routes into Denmark in summer 2022.

The investigation on Beuchel found that, while a large proportion of the Cormorants breeding in April died at the end of the month due to infection with the HPAI H5 virus, there were hardly any deaths among the adult ground-nesting Herring Gulls breeding in close proximity to the Cormorants. Serological tests showed that all tested Herring Gulls had influenza A antibodies and some H5-specific antibodies, suggesting that they had been exposed to AIV and some also to an H5 virus. Whether the birds were in the process of producing H5-specific antibodies or whether the nucleoprotein antibodies cross-reacted with the circulating HPAI H5 virus strain remains an open question, but it appears that by some mechanism they were either protected from overt clinical disease and survived, or had not been sufficiently exposed to the virus to start an infection. Herring Gulls (and other larger gull species) are scavengers that feed on dead birds, Cormorant pellets etc., and should therefore come into frequent contact with the virus. Despite this, there were few reported outbreaks causing mortality, these being on Trischen (Germany) and in the Netherlands, and one Great Black-backed Gull *Larus marinus* in Denmark. Why these species showed lower susceptibility than, for example, Black-

headed Gulls (which are not exposed in the same way yet died in large numbers from HPAI across Europe in 2023) remains an enigma.

Evidence from several colonies suggested dead Cormorants were increasingly scavenged by White-tailed Eagles as the outbreaks continued. In 2021, two young White-tailed Eagles in Estonia died of HPAI at the same time and near the area of mass mortality among Cormorants and gulls in Pärnu county (Kotkaklubi 2021). In Sweden, a White-tailed Eagle infected with HPAI was found on the northern island of Gotland, a region where HPAI infections in Cormorants were confirmed during the same summer (Swedish Veterinary Institute, SVA). In 2022, eight nestlings from five White-tailed Eagle nests in Mecklenburg-Western Pomerania were infected with the HPAI virus. Three of these nests were in western Rügen, close to the affected Cormorant colonies on Heuwiese and Beuchel. All except two of the eagle nestlings had died before the nests were accessed for ringing, and one of the survivors was also later found dead. All dead eagle nestlings tested positive for HPAI, but, remarkably, only nestlings and recently fledged eagles were affected. There were no records of dead juvenile, immature or adult eagles that tested positive for HPAI, and for every dead White-tailed Eagle the cause of mortality was routinely investigated by the Institute for Zoo and Wildlife Research Berlin, which includes testing for HPAI (Piro *et al.* 2023, Günther *et al.* 2023). These results suggest that White-tailed Eagles are highly susceptible to HPAI H5N1 during the nestling period, once protection by maternal antibodies has ceased, and that the birds later gain immunity through exposure to influenza viruses. Despite the loss of eagle nestlings due to HPAI infection, there were no apparent effects at the population level, even on the island of Rügen, where the reproductive output of White-tailed Eagles was not lower than in previous years (Günther *et al.* 2023).

On Beuchel, some Cormorant chicks survived the outbreak and were virologically and serologically tested prior to fledging. Active virus circulation was ruled out, and antibodies were found in only two juvenile Cormorants, suggesting either that the maternal antibodies had disappeared or that the parents and the chicks had not come into contact with the virus.

Serological tests for H5 antibodies in sheep sera collected from animals grazing in the infected ground-nesting Cormorant colony on Beuchel were all negative, despite repeated testing at various dilutions. Although the ELISA test kit we used has not been validated with ruminant sera, the result suggests that

the sheep were not infected, which is supported by the observation that the animals never showed clinical symptoms despite being in direct contact with the faeces and carcasses of infected Cormorants.

The high mortality of Cormorants in the affected colonies did not have a noticeable effect on the population level. In 2023, BP numbers in the south-western Baltic Sea (Denmark, Schleswig-Holstein, Mecklenburg-Western Pomerania combined) were slightly higher than in the previous year (46,600 BP in 2023 versus 44,400 BP in 2022, our unpublished data). There was no detectable effect either in the affected colonies; both Heuwiese and Beuchel hosted approximately the same number of BP in 2022 and 2023.

Conclusions

It appears that arboreal nesting reduces the risk of transmission of the H5N1 virus among Cormorants, because all affected colonies were ground-nesting, where birds are frequently in close proximity to each other and their faecal material. This hypothesis is supported by single observations of symptomatic birds at tree-nesting colonies where outbreaks did not subsequently occur. Despite local losses of breeding Cormorants in 2022 at some Baltic colonies, there was no impact at the population level. The number of dead birds (about 1,700 recorded in total, though the real number of fatalities was likely higher) was still relatively trivial out of a Baltic Sea breeding population of 200,000–220,000 BP, with an unknown (but certainly high) number of non-breeders (Herrmann *et al.* 2021). The geographical clustering of outbreaks suggests connectivity between nearby colonies, confirmed by observations of marked birds visiting other colonies around their breeding site during the breeding season.

Three different genotypes were found in or around different colonies in three different countries, which generally corresponds with the circulation of those genotypes in wild birds in the respective regions, and is in accordance with the emergence of a plethora of genotypes from 2021 onwards. Data from Influenza A investigations in wild birds since 2006 show that Cormorants have generally been susceptible to H5, but the numbers of infected birds were far lower before 2021. Since 2021, when the virus became prevalent during the breeding season, the numbers of positively-testing Cormorants increased. This indicates a low transmission risk of the virus during the non-breeding season, but a high transmission risk in summer, especially in ground breeding colonies, where bird densities are high. The presence of H5 antibodies in Herring Gulls showing no disease symptoms hints at

this species as a potential vector. With mounting fears regarding the viral spill-over from birds to mammals, the lack of detection of antibodies in blood samples taken from ruminants grazing between Cormorant nests suggests they are difficult to infect, despite the fact that the sheep were clearly exposed to the virus in and around the colony. The reason for the lack of outbreaks in Cormorant colonies in 2023 requires serological investigations to determine the prevalence of protective antibodies in this population, and to gain a better understanding of how such disease outbreaks affect the survival and population growth rates of different species of coastal birds.

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Disclosure statement

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

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