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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

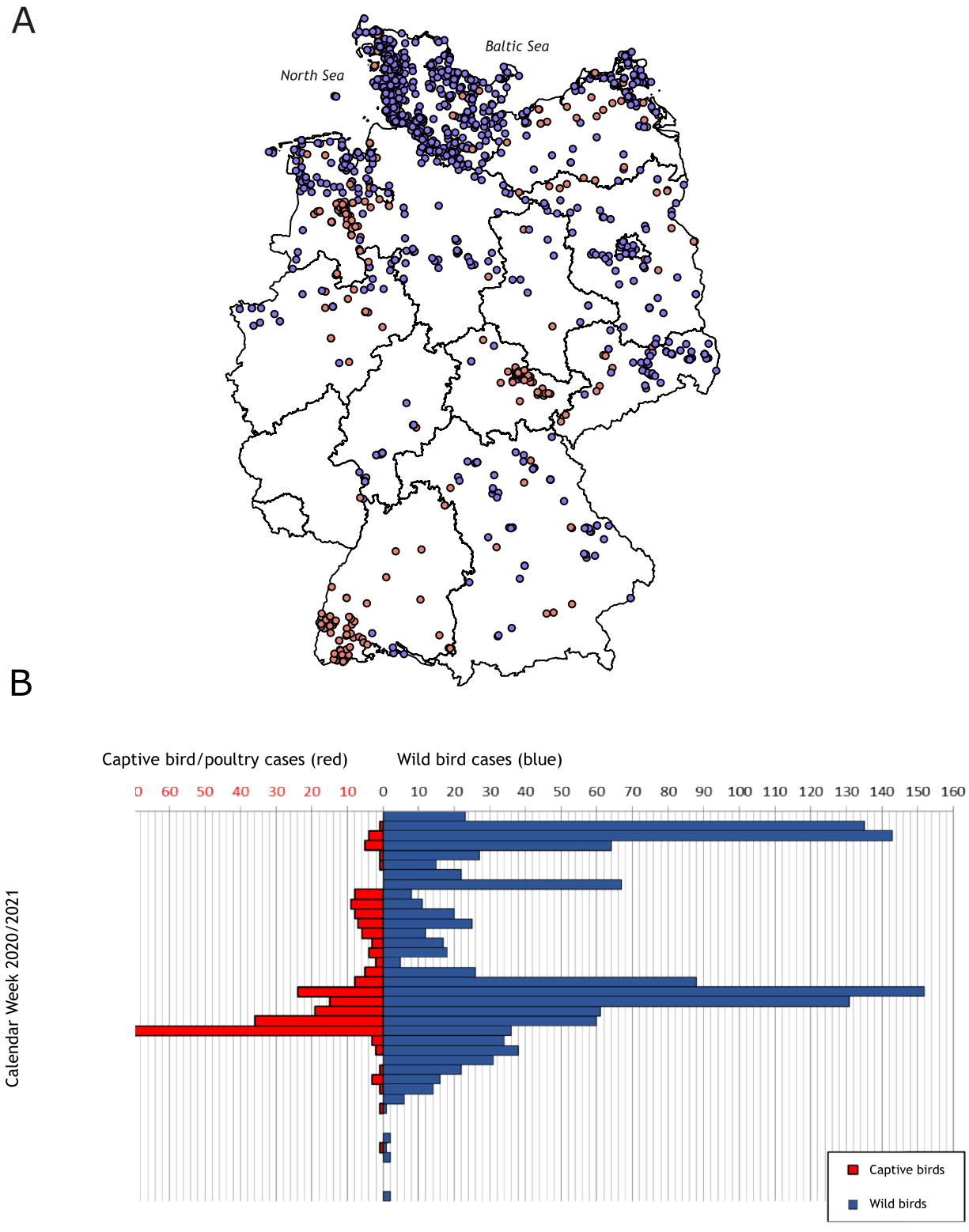

Figure 1. HPAIV case counts and geographic distribution according to data collected by the German animal disease notification system (Tierseuchennachrichten—TSN). (A) Geographic map of reported cases in Germany, 26 October–1 November 2020 (week 44) to 26 July–1 August 2021 (week 30). (B) Dynamics of 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).

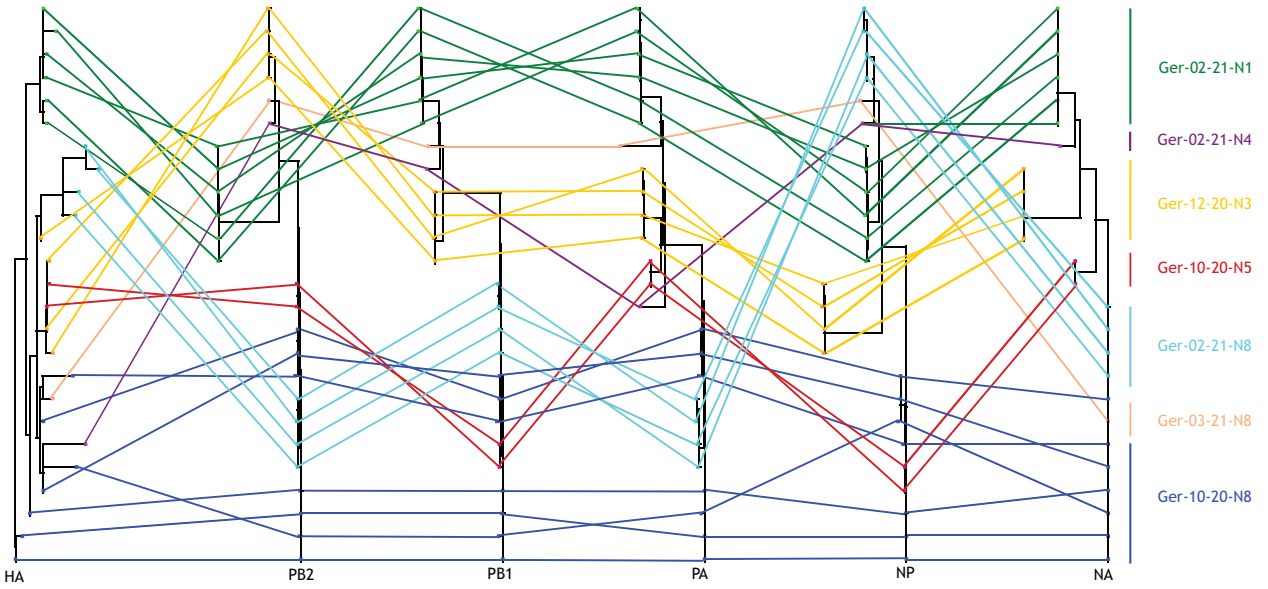

Figure 2. Phylogenetic incongruence analysis. Maximum likelihood trees of the HA, PB2, PB1, PA, NP, and NA segments from representative strains of all detected sub- and genotypes from October 2020 to July 2021 in Germany, calculated utilizing RAxML with model GTR GAMMA (fast bootstrapping) and 1000 bootstrap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

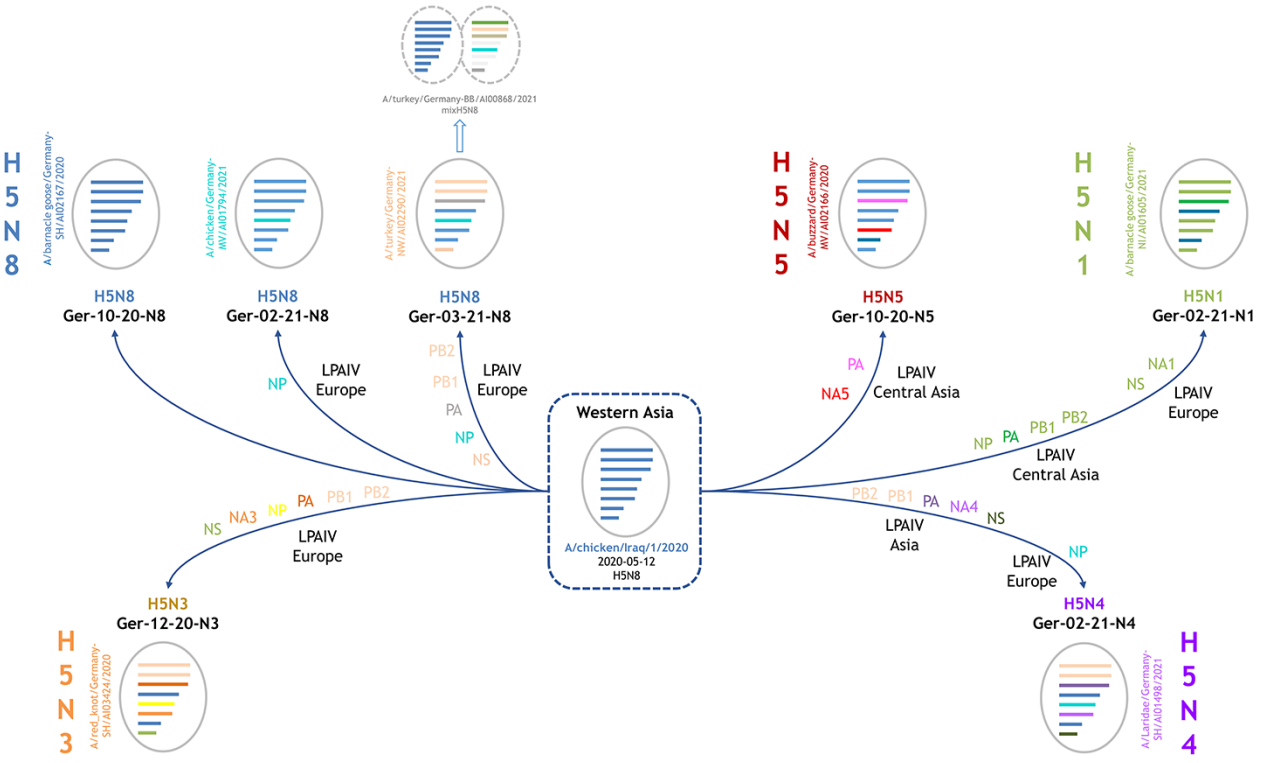

Figure 3. Schematic reassortment analyses of the detected sub- and genotypes in Germany, October 2020 to July 2021. Putative precursor segments are labelled according to geographic origin and coloured consistently dependent on relations throughout all reassortants.

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

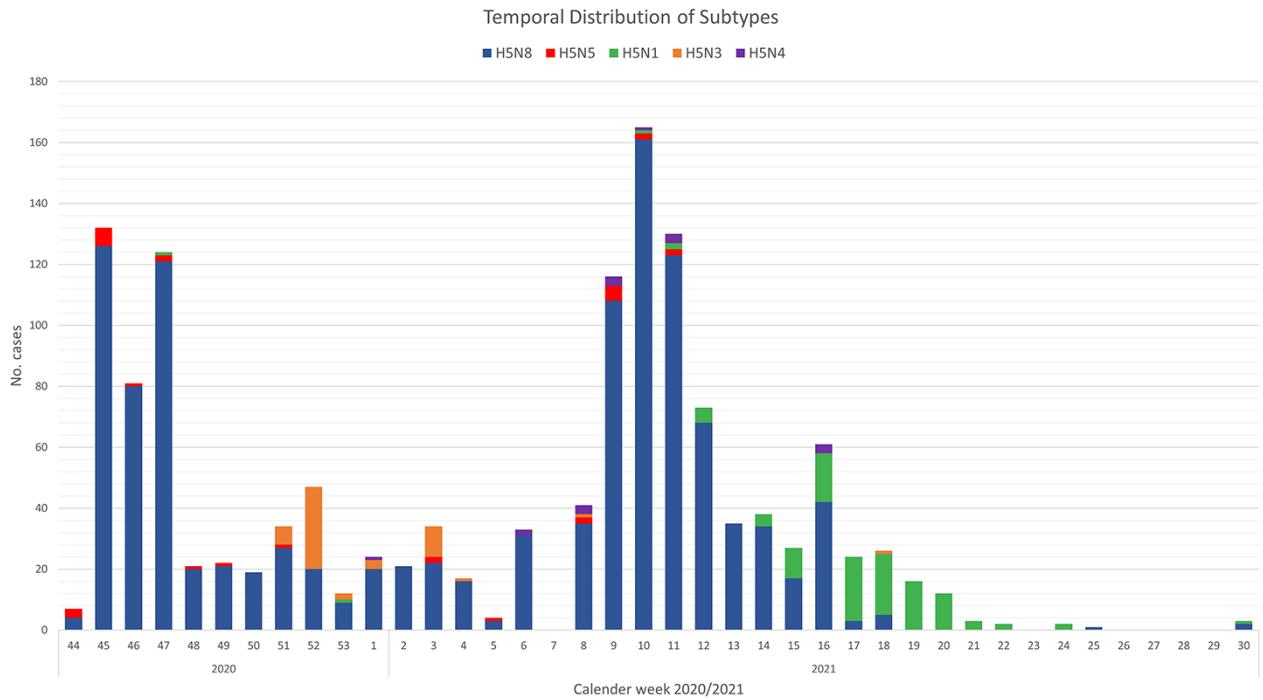

Figure 4. Temporal distribution of the subtypes detected in wild birds in Germany, 26 October–1 November 2020 (week 44) to 26 July–1 August 2021 (week 30), based on data collected by the German National Reference Laboratory for AI (NRL AI).

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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