Phenotypical characterization of clade 2.3.4.4b highly pathogenic avian influenza H5 genotypes in ducklings and pigs

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I. Introduction

Highly Pathogenic Avian Influenza virus (HPAIV) clade 2.3.4.4b of the subtype H5 has become a worldwide panzootic (One Health High-Level Expert Panel 2023). Originated from Asia, the particular goose/Guangdong (gs/GD) lineage spread to Europe, Africa and subsequently to the Americas (Bevins et al. 2022; Caliendo et al. 2022a) reaching even the Antarctic region at the end of 2023 (Bennison et al. 2023; Wille et al. 2023; WOAH 2024b). In Europe HPAIV H5 has become enzootic in wild birds partially with mass mortality in a variety of wild bird species (EFSA Scientific Report 2023a; EFSA Scientific Report 2023b). This creates a constant thread to poultry and concomitant deadly infections in wildlife: Beside marine mammal species such as seals (Leguia et al. 2023; Ulloa et al. 2023) also land-predators such as foxes (Bordes et al. 2023), minks (Agüero et al. 2023), martens, tanuki and bears (Alkie et al. 2023) are victims of spillover infections. Also farmed fur animals in Spain and Finland, cats kept as pet animals in Poland and lately dairy cows in the USA were affected by HPAIV (Rabalski et al. 2023; Domańska-Blicharz et al. 2023; WOAH 2024b). This observation raises concerns about the zoonotic potential of clade 2.3.4.4b HPAIV H5 strains and their adaptation to mammalian species (Günther et al. 2023; Bruno et al. 2023a; Abdelwhab et al. 2023). In addition, the steadily increased incidence of HPAIV H5 since 2020 resulted in a variety of reassorted HPAIV H5 genotypes with different gene composition including the NA segment (King et al. 2022a; Pohlmann et al. 2023). Phylogenetic analyses show differences in distribution and frequency patterns of genotypes (Pohlmann and Harder 2023) but influence of reassortment events on pathobiological properties and epidemiological consequences remain uncertain.

The current work aims to link available genetic information and knowledge on spatiotemporal distribution of genotypes to a biological phenotype. This includes the question whether a HPAIV H5N1 clade 2.3.4.4b genotype predominant in 2021 had gained properties to infect pigs. Secondly, the virulence in mallard ducks as surrogate species for epidemiology of HPAIV in wild birds, has been assessed. The obtained data reveal, that dominant HPAIV H5N1 genotypes are highly virulent for ducks but unable to establish a productive infection in pigs. These findings indicate that the current HPAIV panzootic in birds is best described by a 'transmission-virulence trade-off' model but fortunately infection chains did not favor mammalian adaptation.

II. Literature Overview

2.1 Avian Influenza

Avian Influenza is a viral disease caused by Avian Influenza viruses (AIV) that affects numerous bird species. Recognized as disease entity in 1878 in Italy (Perroncito 1878), the disease was later known as "fowl plague" (Beaudette 1925). In most susceptible poultry species, i.e. chickens and turkeys, AIV of virulent subtypes H5 or H7 categorized as highly pathogenic AIV (HPAIV) induce a peracute to acute systemic disease with mortality reaching rates of 100% (Modrow et al. 2021; Swayne and Suarez 2000; Webster et al. 1992b; American Association of Avian Pathologists 2013).

The primary reservoir of AIV are wild waterfowl of the order *Anseriformes* (particularly ducks) and *Charadriiformes* (gulls, shorebirds) (John Wiley & Sons, Inc 2008). In these wild birds the majority of AIV-strains do not induce disease and therefore have been undetected for a long period of time (Stallknecht and Brown 2008; Olsen et al. 2006; Boon et al. 2007). With respect to their virulence, those strains do induce only mild disease in chickens are called low pathogenic AIV (LPAIV). The first described LPAIV from free-living birds was derived from a common tern (*Sterna hirundo*) in 1961 (Becker 1966), followed by the discovery of antibody-positive wild birds in 1972. Since then, continuous reports of infected wild birds of various species provided evidence of susceptibility of numerous bird species to AIV-infection. Altogether AIV infection has been reported in up to 100 species of 12 avian orders and is therefore considered a widely distributed virus infection within the class *Aves* (Alexander 2000; Kaleta et al. 2005; John Wiley & Sons, Inc 2008). However, for some species, AIV infection is not relevant. For example, pigeons are generally susceptible to AIV-infection but natural infections are very rare with only single documented cases associated to HPAIV outbreaks (Cardona et al. 2009a).

Even though restricted primarily to the avian host, selected AIV subtypes may have an extended host range and bear the risk to adapt to mammalian species, giving rise to a potentially new zoonotic epidemic like pandemic H1N1 in 2009 (WHO 2009; CDC 2009; Pensaert et al. 1981). Considering the high transmissibility and the clinical impact, HPAIV is a listed disease for the world organization of animal health (WOAH) and category A disease according the EU animal health law ((EU) 2016/429).

2.2 Avian Influenza Virus

Avian Influenza viruses belong to the family of Orthomyxoviridae, genus Alphainfluenzavirus. The enveloped virions are pleomorphic with an average diameter of 80-120 nm and encompass a single-stranded, negatively orientated RNA genome. Separated in eight segments (ICTV 2011), the genome codes for 9 structural (PB2, PB1, PA, HA, NP, NA, M1, M2, NS2) and up to 5 nonstructural proteins (PB2-S1, PB1-F2, PB1-N40, PA-x, NS1). These are not associated to the virion (John Wiley & Sons, Inc 2008; Alexander and Brown 2009). Each segment contains a single-stranded RNA molecule of negative polarity that is encapsidated by the nucleocapsid (NC).

2.2.1 Discrimination of Avian Influenza viruses

2.2.1.1 Subtypes

Avian Influenza viruses can be differentiated in subtypes according to their two surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA). Originally based on serological differentiation, the subtyping today relies on phylogenetic grouping (Spackman et al. 2003a). To date 16 HA and 9 NA subtypes are described in avian species, with subtype H5, H6, H7 and H9 being the most relevant for poultry (*Animal Influenza Second Edition* 2017; Modrow et al. 2021). Subtypes H17 and H18 so far have only been detected in bat species (Tong et al. 2012; Tong et al. 2013)

2.2.1.2 Pathotypes

The majority of AIV strains can be categorized as low pathogenic (LPAIV), inducing no or only a moderate disease with no or low mortality rate. The infection with LPAIV is accompanied by mild respiratory or gastrointestinal symptoms. In contrast H5 and H7 subtypes can mutate in Galliformes poultry to highly pathogenic viruses (HPAIV), inducing severe disease in particular in chicken and turkeys. Classical discrimination is done by determination of the intravenous pathogenicity index (IVPI) in six to eight weeks old chickens (WOAH 2024b): After intravenous inoculation, clinical signs are scored daily during a ten days observation time, according to the criteria healthy [0], mildly ill [1], severely ill [2] or dead [3]. HPAIV is defined as strains with an IVPI of 1.2 or higher, while strains with an IVPI of less than 1.2 are considered LPAIV. Molecular mechanism of virulence is based on a stretch of amino acids within the precursor of the hemagglutinin (HA_0), serving as recognition site for cellular proteases (see chapter 2.3.2).

2.2.1.3 Clades

Phylogenetic analysis of viral genes and the deduced proteins allow further subtyping of the individual AIV genes. For HPAIV H5 of the A/goose/Guandong/96 (gs/GD) lineage a standardized nomenclature has been established in 2008 by a WHO working group assigning all descendants to the (gs/GD) lineage (WHO, OIE, FAO H, N, and Evolution Working Group 2008): Based on the established criteria HPAIV H5 of the gs/GD lineage are today categorized in 10 clades (0-9), divided further in 2nd, 3rd and 4th order of subclades. According to these standards, a clade is characterized by presence of a unique common node within the phylogenetic tree with of at least four virus isolates of different origin. The Kimura-2 model is used to differentiate clades; an average distance of >1.5% clearly distinguishes distances between two clades, a distance of at least <1.5% clearly distinguishes distances within clades. The bootstrap value should be at least 60% at the clade-defining node based on 1000 bootstrap replicates in the neighbor-joining model. Furthermore, the clades can be differentiated on the basis of their first associated prototype and its regional and sometimes spatio-temporal occurrence (WHO, OIE, FAO H, N, and Evolution Working Group 2008). For more detailed information see chapter 2.7.2.1.

2.2.1.4 Genotypes

Like for H5, the other AIV genes undergo constant diversification in their different reservoirs and can be distinguished by phylogenetic analysis. Due to reassortment of individual gene segments between different AIV strains, new AIV strains with a mixture of genes from two parental AIV viruses emerge (see chapter 2.5). Accordingly, applying information of full genome sequences, differences of the gene constellation of isolates can be recognized and applied to categorize strains into different genotypes. A most obvious difference between genotypes would be the introduction of a different NA subtype into a H5N1 virus, like emerged H5N3 or H5N8 genotypes. However, these analyses reveal a constant flexibility also of the internal genes.

In the U.S. for example, an influenza data pipeline called GenoFLU was generated to automate genotyping assignment. The tool is fed with individual gene segment sequences of a virus isolate, which are compared to predefined segment identification sequences of predefined genotypes. If the new sequence fed in differs by more than 2% from the nearest predefined identification sequence, this indicates a new reassortment. Based on this deviation, phylogenetic trees are created for each segment and the genotypes are assigned on this basis. Such deviating sequences may also serve as new identification sequences for the database in order to be able to assign new genotypes in the future (GenoFlu 2023).

At European level (European Reference Laboratory-EURL), the assignment of virus isolates to genotypes involves storing entire genomes of newly sequenced virus isolates in the GISAID EpiFlu™ database. The sequences of each of the 8 gene segments are then aligned and compared with the most closely related sequences by BLAST in the GISAID EpiFlu™ database. Maximum likelihood trees (with 1000 bootstrap values) are then created from all gene segments, the topology of which forms the basis for genotyping by allowing conclusions to be drawn about monophyletic patterns within the trees. The patterns need to fulfil the following criteria: firstly, each HPAI H5Nx cluster must have a bootstrap value of >90 and secondly, they must have a higher commonality with low pathogenic influenza viruses (which are likely to be the donor of the gene segment) than those in other H5Nx clusters. These gene segment donors were identified in advance by the BLAST search, a corresponding reference sequence was selected for each segment and each cluster and is thus the origin of each new cluster. Each cluster is labelled with an identification number (ID) and associated color code. The combination of the 8 different IDs of each gene segment ultimately defines the new genotype. The resulting numerical code is converted into a pseudonym: there is first a letter abbreviation for the lineage (e.g. EA, Eurasian), then the year of discovery in numbers and finally letters (A, B, C), which indicate the wave the genotype originates from (1 letter -2020-2021; 2 letters -2021-2022) (Fusaro et al. 2024b).

At national level (Germany) (Pohlmann 2023b), naming of a new genotype includes the locality of discovery (according to ISO 3166 alpha-2), the date of first discovery (YY-MM) and the NA subtype.

However, this nomenclature and specifications for defining a genotype has to be standardized on the European and international level. For further information see chapter 2.7.2.

2.3 Viral proteins

2.3.1 Polymerase complex (PB2, PB1, PA)

The RdRP complex consists of the proteins Polymerase basic protein 1 (PB1), Polymerase basic protein 2 (PB2) and Polymerase acidic protein (PA). With its different proteins it has the function of a viral RNA-dependent RNA polymerase (RdRP). The PB2 protein, encoded on segment 1, has a primer function by binding 5´cap structures of host cell mRNA to initiate viral RNA transcription. The PB1 protein, encoded on segment 2, in the center of the viral ribonucleoprotein (vRNP), is responsible for chain extension by binding 5´and 3´ends of viral RNA (vRNA) and complementary RNA (cRNA). The PB1 segment codes for a second protein termed PB1-F2, which is translated from an alternative open reading frame (ORF) (Chen et al. 2001b). This protein is associated with both pro-apoptotic and polymerase-enhancing functions (McAuley et al. 2010b; Kamal et al. 2017). The function of the PA protein, encoded on segment 3, includes genome replication but its function has not yet been fully elucidated (Eisfeld et al. 2015; Vigeveno et al. 2020; Hohensee et al. 2024; Günl et al. 2023).

2.3.2 Hemagglutinin (HA)

Encoded on segment 4, the HA of Influenza A is a glycoprotein, embedded as trimer in the viral envelope membrane. It initiates attachment of the virion to α2.3-or α2.6-glycosidated N-acetyl neuraminic acid as receptor on the cell surface of host cells and subsequent mediates fusion of the viral and endosomal membrane (Modrow et al. 2021). For diagnostic purposes this property has been used to detect viral presence by agglutination of erythrocytes, exploited as hemagglutination assay (HA). Synthesized as a precursor protein called HA_0, the protein has to be activated by cleavage into the HA_1, and the HA_2 subunits by intracellular or extracellular host proteases (Rott 1992) (Suzuki et al. 2002; Wiley et al. 1987). Ubiquitously occurring intracellular enzymes such as Furin-or Subtilisin like proteases preferentially process polybasic HA-cleavage sites thus enable activation of HA_0 and productive replication in various tissues including the brain of the infected individuum. By now this association of virulence to a polybasic cleavage site is restricted to HPAIV H5 and H7 HA-subtypes. In contrast HA_0 of LPAIV have a mono basic cleavage site which can be processed only by extracellularly trypsin-like serin-proteases that are restricted to the respiratory and gastrointestinal tract. In consequence, productive LPAIV infection remains localized (Bruin et al. 2022; Modrow et al. 2021; Garten et al. 2015).

2.3.3 Nucleoprotein (NP)

The structural nucleoprotein (NP) is encoded on segment 5 and is forming the nucleocapsid, that encapsulates the viral genomic RNA, localized within the virion. It is associated to the vRNP, that facilitates viral transcription and replication (Portela et al. 2002; Beaton and Krug 1986).

2.3.4 Neuraminidase (NA)

The Neuraminidase, coded on segment 6, is the second viral envelope protein embedded as tetramer in the viral membrane. It has the enzymatic function of Sialidase and cleaves sialic acids (SA), specifically α-2.3 and α-2.6-linked sialic acids, that are attached to newly formed virions. This function is considered to mediate the release of newly budded virions from the plasma membrane of infected cells and also prevents reattachment of the virus to already infected cells (Wagner et al. 2002; Air and Laver 1989). Historically, based on the serological differentiation, NA subtypes (1-9) are integral part of the nomenclature of an AIV-isolate defined by a different combination of HA and NA proteins (Hinshaw et al. 1981; McAuley et al. 2019; Harris et al. 2006).

2.3.5 Matrix proteins

Segment 7 encodes for two structural proteins, the M1 and M2 proteins (Modrow et al. 2021). The matrix protein M1 is associated with the inside of the viral membrane. It has interactions with the cytosolic sections of HA-, NA-, M2-proteins and with the nucleocapsids and mediates packaging of the nucleocapsids into the newly generated virus particles (Modrow et al. 2021). The M2 protein is located as tetrameric homopolymer within the viral envelope membrane and forms an ion channel through the viral membrane. This ion channel is responsible for acidification of the virus interior during the early stage of infection, and induces a relaxation of the interaction between the M1 proteins and the nucleocapsids with subsequent translocation of the M1 proteins into the cell nucleus. In the further course of infection, the M2 protein is incorporated into Golgi vesicles. The resulting pH shift is considered to be necessary to prevent the premature HA0 /$HA_{1/2}$ structural change (Modrow et al. 2021).

2.3.6 Non-Structural proteins

In addition to structural proteins associated to the virion, IVA also code for so-called non-structural proteins, such as the NS1 and NS2/NEP which are only expressed in the infected host cells. Further recently discovered non-structural proteins, are PA-X, PB1-F2, M42, being non-essential but having crucial roles in adaption of virulence and pathogenicity (Hao et al. 2020).

Segment 8 codes for two proteins (Lamb and Choppin 1979), the NS1 and NS2-protein. NS1 is transported after translation into the cell nucleus and can downregulate antiviral defense mechanisms of the cells (Lin et al. 2007) such as the interferon pathway by influencing two independent routes: On the one hand, NS1 is able to bind dsRNA that is synthetized during viral replication cycle and in consequence inhibits pre-transcription of sensors necessary for IFN activation such as Retinoic-acid inducible gene I (RIG-I) or Protein kinase R (PKR). On the other hand, NS1 directly interacts with cellular factors that are induced by IFN to affect cellular gene expression.

A second non-structural protein, NS2/ NEP (nuclear export protein), mediates transport of newly formed viral nucleocapsid segments from the cell nucleus into the cytoplasm and prevents re-entry of vRNP

into the nucleus by interaction with the M1 protein (Seo et al. 2002; Krug 2014; Garcia-Sastre 2001; Eisfeld et al. 2015; Hao et al. 2020).

2.4 Replication cycle

The initial step of viral replication of IVA is receptor-specific attachment of the virion to the cell by binding of the HA-protein to the sialic acids (SA) receptor on the host cell. Those are distributed differently in different species: Avian species are known to express mostly α-2.3-linked SA whereas in mammal species, especially in humans, the α-2.6-linked SA dominate. Specificity of the HA to a certain SA-receptor is therefore considered as key determinant for species adaptation, whereas AIV has affinity to α-2.3-linked SA and mammalian adapted IVA bind preferentially to α-2.6-linked SA receptors. In poultry, SA receptors are mainly located in the respiratory tract; in chickens in particular, α-2.3 SA are located in the upper respiratory tract (nasal cavity, trachea) and in the lungs. In some species like turkeys and also swines, both receptor types can be found. Turkeys provide SA receptors in the upper and lower respiratory tract with α-2.6 SA particularly common in the lungs. Swines on the other side, do contain both receptor types in respiratory organs. In the trachea and bronchus there is higher extent of α-2.6 SA receptors in comparison to α-2.3 SA, whereas in the bronchiole and alveoli of the lungs there are similar high amounts of both receptor types (Graaf and Fouchier 2014) as well as in the small intestine and colon (Nelli et al. 2010). Both receptor types are found in equal proportions in the lungs and in the gut, with higher α-2.3 SA affinity in the lamina propria (Zhao and Pu 2022). As ferrets have similar distributions of α-2.6 and 2.3-SA´s in the respiratory tract to humans, such as α-2.6 SA in the trachea and bronchus, α-2.3-SA in the Lamina propria and submucosal areas, and both receptor types inside the lung´s alveoli, they serve as widely used animal model for Influenza virus infections (Graaf and Fouchier 2014). It is assumed that species that possess both receptor types play a decisive role in the development of new host adaptations.

After viral attachment, receptor-mediated endocytosis of the virion is triggered, resulting in intracellular localization of the virus within an endosome that will subsequently undergo acidification. The lowered pH is inducing a conformational transformation of the HA protein with exposure of the fusogenic region within the amino-terminal end of the HA_2 fragment as well as opening of the M2 ion channel in the viral membrane. The fusion peptide subsequently inserts itself into the endosome membrane and mediates fusion of the viral with the endosomal membrane. In the following process called uncoating, the vRNP is released from the virus particle into the cytoplasm of the host cell. By accessing the importin-α and importin β-nuclear import pathway, the vRNP are transported from the cytoplasm into the cell nucleus. After translocation of the vRNP into the nucleus through nuclear pores, replication of the influenza A genome is initiated and starts with viral Polymerase complex dependent synthesis of viral RNA by first transcription of complimentary RNA (cRNA) followed by transcription into viral RNA (vRNA). Initiation of transcription involves a PB2 protein-dependent cellular mechanism called “cap-snatching”

where the viral PB2 binds 5' caps of host RNAs, followed by PB1, PB2 and PA mediated elongation of the viral RNA. The resulting viral mRNA is then exported for translation from the cell nucleus into the cytoplasmatic ribosomes by the viral NS2/NEP proteins thereby influencing cellular mechanisms to favor viral transport. Translation of the mRNA is divided: Viral proteins associated to viral envelope (HA, NA and M2) are translated at ribosomes associated along the rough endoplasmic reticulum (ER) and transported to and integrated into the cell membrane via the Golgi apparatus network. Before transport the M2 protein regulates acidification of the Golgi-Vesicles to prevent early induction of the HA-complexes. Within the Golgi apparatus HA_0 with a polybasic cleavage site will be cleaved into its active form by Furin and subsequently transported to the cell membrane by complex formation and glycosylation. The assembly of subunits is thought to be key factor for transfer to the plasma membrane (Copeland et al. 1988).

In the second pathway, proteins of the viral RdRp NP, M1 and the non-structural proteins are translated in cytosolic ribosomes. After replication vRNPs are transported to the cell periphery by cellular proteases like Rab11 GTPase (Eisfeld et al. 2011) and are probably attached there by the M1 protein. The new IAVs assemble in the cell periphery. At the site of budding from the cell membrane self-assembly takes place, thereby inducing a change of the shape of the host membrane into buds. Subsequent release from the host cell depends on the sialidase activity of the NA. The sialidase removes local SA residues on the host cell membrane and thereby prevents the HA and further released viral particles from re-attaching to the SA receptors on the cell surface (see chapter 2.3.4).

2.5 Continuous evolution of Influenza viruses

A key feature of influenza viruses is their continuous variation in conjunction with adaptability (Shao et al. 2017; Kim et al. 2018). Like most RNA viruses IVA have endogenously high mutation rate, expected to be 9.01×10^{-5} substitutions/site (Visher et al. 2016; Kawasaki et al. 2023), caused by the inaccurate viral RdRp (Steinhauer et al. 1992) and generating incorrect complementary bases during the replication process. These created point mutations, particularly those surround regions of the HA and NA sensitive to binding of neutralizing antibodies, affect the efficacy of established immunity within a population and thereby allowing mutated viruses to persist longer in a population (Caton et al. 1982). These continuous accumulations of mutations affecting antigenic sites is referred to as *antigenic drift* (Modrow et al. 2021; Kim et al. 2018; Both et al. 1983), a mechanism that can give rise to virus variants that escape host-specific antibody response and affect vaccine efficacy (Kim et al. 2018).

Besides, Influenza viruses are able to exchange individual genome segments with other Influenza strains, a process known as reassortment. When segments important for protective immune response are affected, like the HA-or NA-protein, such reassorted variants are associated to a sudden change of antigenic properties, a mechanism referred to as *antigenic shift* (Kim et al. 2018; Webster et al. 1982). Reassortment can occur when virus strains with different HA and NA proteins are replicating simultaneously in the same organism (Mostafa et al. 2018; Kim et al. 2018). If the infection with both

virus strains takes place in the same cell, a mixture of new reassortants can arise. Compared to the parental strains these reassortants might acquire new antigenic properties and possibly have different replication kinetics that might alter virulence. (Steel and Lowen 2014).

Newly released virus particles should contain the correct combination of all 8 virus segments. As this is mostly not the case, only around 10% of the newly created viruses are infectious (Modrow et al. 2021)

2.6 Clinic of an Influenza A infection

The clinical outcome of an AIV infection varies beside the pathotype (HP/ LP) of the specific virus strain also between host species that are affected (Alexander 2000; Swayne 2007; Pantin-Jackwood et al. 2009) and varies between subclinical, mild or severe disease with varying mortality rates (Pantin-Jackwood et al. 2009; Swayne 2007).

Differences in pathogenesis of LPAI and HPAI viruses are the result of varying replication sites. Replication of LPAIV is localized in epithelial cells, within the nasal cavity the trachea and air sacs leading to rhinitis and tracheitis as common clinical findings. LPAIV do also replicate in gastrointestinal epithelial tissues. Sometimes it can also replicate in the renal tubules and pancreas leading to tubular and interstitial necrosis as well as necrosis of pancreatic cells (Swayne 2007; Swayne 2009). Viral replication of HPAIV mostly also initiates from epithelial cells in the nasal cavity but can disseminate into the submucosal tissue and induce viremia with systemic spread and replication in almost all organs including the brain. Tropism to endothelial cells leads to altered permeability of blood vessels and by this to edema, haemorrhages and necrosis (Swayne 2007).

2.6.1 Manifestation in gallineous species

Infection with LPAIV might not be associated with detectable signs of disease. Often LPAIV infection is associated with a high morbidity but low mortality rate (Pantin-Jackwood et al. 2009). Mild clinical signs are dominated by respiratory distress or clinical signs associated to the gastrointestinal tract (Pantin-Jackwood et al. 2009). Signs might include coughing, sneezing and excessive ocular discharge as well as decreased activity, fatique, loss of appetite and diarrhea (Pantin-Jackwood et al. 2009). However, under field conditions clinical signs can be severe and associated to general weakness, signs like ruffled feathers, depression, deceased activity, egg production and weight loss (John Wiley & Sons, Inc 2008). Infections with LPAIV provide a classic breeding ground for secondary bacterial infections. These can exacerbate clinical signs and increase findings of severe pneumonia, air sac inflammation and peritonitis.

In contrast, commonly symptoms resulting from HPAIV infection in gallineous poultry are characterized by severe systemic infection in all parenchyma. The most frequent occuring symptoms are onset of a sudden and severe decrease of food and water intake, general depression and huddling, reduced vocalization, swollen sinuses, wattles and comb, dyspnea, coughing and sneezing such as

mucosal production from the beak and also gastrointestinal disorders like altered faeces and diarrhea (WOAH 2024b). In more prolonged subacute courses of disease, central nervous symptoms become manifest mostly after 1.5–4 days (Perkins, L E. L. and Swayne, D. 2001) such as torticollis, nystagmus, head shaking, visual disturbances, disorientation, balance disorders and paralysis (Alexander et al. 1986; Pantin-Jackwood et al. 2009). Due to viral endotheliotropism, petechia in the head appendages and feet are prognostic sings, caused by severe necrosis and inflammation with water retention or blood congestion (Pantin-Jackwood et al. 2009; Swayne 2007; Breithaupt). Beside acute to subacute disease with death within 1.5 – 5.5 days (Swayne 2007), peracute courses of HPAI are frequently observed, with sudden death of the birds due to multi-organ failure without showing symptoms of disease. Mean time of onset of disease (MTO) after experimental infection varies from 1.6 to 5 days depending on virus strain (Alexander et al. 1986). The estimated time between HPAIV introduction into poultry flocks and appearance of clinical signs is around 4 days (Elbers et al. 2005; Stegeman et al. 2004) but 14 days can elapse before mortality increases noticeably (Bos et al. 2007).

The clinical signs of other fowl species such as quail, guinea fowl, and pheasants are similar to those of chicken, e.g., HPAIV infection is associated with high morbidity and mortality (Bertran et al. 2014; Perkins, L E. L. and Swayne, D. 2001) beginning with nonspecific symptoms such as lethargy, inappetence, splayed feathers and usually progressing to neurological symptoms such as gait abnormalities, head movements, disorientation. In most cases, the neurological signs, as well as multi-organ failure in the animals, ultimately result in the death of the animals. Patho-morphologically, pancreatic necrosis is also common in these species, along with swelling of the spleen and kidneys with urate collections. Lymphatic tissue and the digestive tract are also commonly affected in these species, which can often be readily seen by mucosal hemorrhage, as well as bagging and hemorrhage of Peyer's plaques. The clinic usually depends on the HPAIV strain.

2.6.2 Manifestation in waterfowl

Waterfowl are generally susceptible to AIV infection, but are more resistant to disease than gallinaceous birds and may be even clinically unrecognized carrier of HPAIV. However, there are HPAIV strains that induce disease in those species (Pantin-Jackwood et al. 2013b). In particular in the panzootic with currently circulating HPAIV H5N1 clade 2.3.4.4b viruses waterfowl and Sea birds are clinical affected and cases of mass mortality have been reported (EFSA Scientific Report 2023a; EFSA Scientific Report 2023c; EFSA Scientific Report 2023d). However, clinical signs in wild waterfowl are scarcely described, as mostly, recovered wild birds are already dead. In domestic ducks, clinical signs are better defined: Disease is associated with general depression and weight loss. Often diarrhea, polyuria and/or respiratory distress is observed, sometimes also conjunctivitis. More specific but not pathognomic are disorders of the central nervous system including ataxia and convulsions (Cardona et al. 2009b; Songserm et al. 2006b; James et al. 2023). Besides, differences in clinical outcome between duck species have to be considered: While Muscovy ducks (*Cairina moschata*) generally being more sensitive to

disease, Pekin ducks (*Anas platyrynchos var. domesticus*) are more resistant (Pantin-Jackwood et al. 2013b; Spackman 2009; Cagle et al. 2012). For example clinical severity after experimentally infection with HPAIV H5N8 circulating in Germany in 2014 induced severe disease and mortality in Muscovy ducklings with developing progressive central nervous disorder whereas mortality in Pekin ducklings was negligible (Grund et al. 2018). Further there were also different clinical outcomes in duck species after experimental infection with HPAIV H5N1, with severe respiratory distress and neurological disorder only in tufted ducks (*Aythya fuligula*) and pochards (*Aythya ferina*) accumulating within 3-4 days post infection (pi), such as loss of balance and tremors associated with viral encephalitis, with signs being far more severe in tufted ducks (Keawcharoen et al. 2008). Tufted ducks in particular appear to be susceptible to severe disease progression associated with HPAIV. There is evidence of high mortality rates after natural and experimental infection with HPAIV H5N1 of clade 2.2 and 2.3.2 (Bröjer et al. 2015; Bröjer et al. 2009). Beginning as early as day 2 pi, neurological deficits such as ataxia, uncoordinated head movements and postures as well as tremors were observed with increasing severity. The clinical signs were accompanied by reduced food intake with weight loss (Bröjer et al. 2009).

In addition to ducks, other waterfowl species such as geese are also affected by HPAIV but clinical disease is less well described and resembles the subacute course. Animals become apathic and may develop central nervous signs including disorientation, torticollis and conjunctivitis. High mortality rates are particularly observed in barnacle goose (*Branta leucopsis*) and graylag goose (*Anser anser*). Investigations into natural infection with AIV revealed a high affinity for neurotropism in geese (EFSA Scientific Report 2021; Caliendo et al. 2022d). There are also reports of mild diarrhea (Cardona et al. 2009b; Eurosurveillance Editorial Office 2007; Perkins, L E. L. and Swayne, D. 2002).

Differences of HPAIV manifestation between Galliformes and Anseriformes birds are considered to be linked to the innate immunity of the host. Central role in the innate immune system are pattern recognition receptors (PRRs) that ensure quick identification of so called pathogen associated molecular patterns (PAMPs) of viral pathogens. PRRs are important to control Influenza infections in birds and can be divided into Retinoic acid-inducible gene-I-like receptors (RLRs), Toll-like-receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Located in cytosol and cell surfaces (Park et al. 2019), PRRs induce antiviral responses by upregulation of antiviral effectors such as chemokines, cytokines (Evseev, D. and Magor, K. E. 2019) Interferons (IFN), Interleukins (IL) as well as Growth-factors and Tumor necrose factors. Recent findings demonstrate that different avian species are equipped with divergent PRR expression, that might explain differences in host response upon AIV-infection (Campbell and Magor 2020; Evseev, D. and Magor, K. E. 2019; Adams et al. 2009; Cornelissen et al. 2012; Cornelissen et al. 2013; Mishra et al. 2017). Thus it is considered that a rapid onset and robust immune system with a fast endowment of viral recognition receptors such as RIG-1 in ducks can limit infections early on (Campbell and Magor 2020).

In the same line, studies point to increased cytokine production, particularly of Il-6, observed in chickens after HPAIV and LPAIV infection compared to Peking ducks (*Anas platyrynchos*) (Burggraaf et al. 2014). Similar findings with regard to rapid but controlled and time-limited IFN-response after inoculation with HPAIV H5N1 in Pekin ducks were reported (Saito et al. 2018). In consequence increased cytokine production and thus proinflammatory response is considered to contribute to the more severe pathogenesis of HPAIV in chickens.

Next to differences of virulence and susceptibility of the host species, age has an influence on pathogenesis of AIV infection. This becomes most apparent in Anseriformes with strains where adult birds might be subclinical infected with HPAIV but young birds show severe signs of disease. For example experimental infection with HPAIV H5N8 clade 2.3.4.4b virus did induce moderate mortality in adult (33.3%), but 100% mortality in 1-week-old pekin ducks within two days (Grund et al. 2018). Similar studies compared infection of pekin ducklings at different ages with 4 circulating HPAIV H5N1 clade 2.3.4.4 strains, and demonstrated that younger individuals were significantly more susceptible to disease, particularly with respect to severe neurological disease and death (Pantin-Jackwood et al. 2007). Likewise infection of 8-or 12-week-old pekin ducks with an clade 2.2 HPAIV H5N1 strain, induced 100% mortality within 4 days only in the group with the younger animals, while the older animals showed only mild clinical signs (Löndt et al. 2010). These experimental results are in agreement with observations of natural infection in the field, for example higher HPAIV prevalence in juvenile than in adult birds when studying free-living animals in Alaska between 2006 and 2007 (Ip et al. 2008). The same was true for studies, where a population of mute swans was sampled and at the same time only the youngest group showed no seroconversion after previous contact with AIV (Pybus et al. 2012). This could be due to the gradually maturing immune system and higher neutralizing antibody titers of older individuals. This phenomenon was also investigated in 1-to 8-week old Pekin ducklings leading to the result that genes associated with cell damage tend to be upregulated more in even younger Pekin ducklings after inoculation with HPAIV H5N6 virus of clade 2.3.4.4. (Jang and Heui Seo 2020).

2.6.3 Manifestation in other species (Birds of prey, pet birds)

During monitoring programs of current panzootic, the highest rate of HPAIV H5 infected wild birds were waterfowl with around 5,100 individuals as of mid-march 2024 (Fusaro et al. 2024a). However, in raptor species, detection rates were also high (around 1200 individuals), especially buzzards (Caliendo et al. 2022c; EFSA Scientific Report 2023b; Fusaro et al. 2024a) and colony-breeding seabird species (around 3,700 individuals). This is in agreement with reports from recent years on documented sick or dead birds, highlighting that birds of prey are highly susceptible to disease with fatal outcome (Caliendo et al. 2022b; Krone et al. 2018; Shearn-Bochsler et al. 2019). Investigated cases of buzzards demonstrate systemic viral spread including neurotropism with manifestation of a severe encephalitis (Caliendo et al. 2022c; van den Brand et al. 2015). Observations on live birds describe none pathognomonic clinical signs like anorexia or apathy as well as slight to severe weakness (Lecu et al. 2009; Munster et al. 2007;

Gaidet et al. 2007; Caron et al. 2017) but also neurological signs like torticollis, head shaking, flying disability and lack of coordination to abnormal gait to sudden death (Caliendo et al. 2022b). Clinical signs associated to experimental infection of American kestrels (*Falco spaverius*) were initial decreased food intake and subsequently dose-independent severe clinical disease with neurologic manifestation and death within 6-7 days post inoculation (Hall et al. 2009; Uno et al. 2020; Fujimoto et al. 2022). Furthermore, there have been reports of an HPAIV H5N8 clade 2.3.4.4b infected white-tailed sea eagle, that succumbed to death (van den Brand et al. 2015; Krone et al. 2018). Surprisingly, experimental infection of white-tailed sea eagles (*Haliaeetus albicilla*) with HPAIV H5N6 clade 2.3.4.4e did not induce clinical disease (Fujimoto et al. 2022). However, swab samples taken during the trial demonstrated viral excretion. Investigation of organs at the end of the observation time detected viral RNA in the lungs, heart, liver, proventriculus, spleen, colon and kidney of a bird necropsied on day 3 pi and in heart, liver, proventriculus and spleen of a bird necropsied on day 8 pi. No viral RNA could be detected in the brains of both individuals. These findings are proving susceptibility to infection with this clade but apparently resistance to disease. As large proportions of birds of prey are carnivores (Caliendo et al. 2022c; EFSA Scientific Report 2023a; EFSA Scientific Report 2023b) the source of infection is suspected to be by feeding on infected prey or their carcasses (Nadjafzadeh et al. 2013), which was reproduced experimentally as well (Bertran et al. 2012).

Regularly HPAIV is also reported from exotic species kept in zoological gardens, wild bird rescue centers or farms raising game birds. This includes prominent species like ostriches, especially in countries with widespread ostrich farming like South Africa (Abolnik et al. 2016), but also quail, pheasant and a variety of exotic birds that are kept as pet birds (Alexander 2000). Described clinical signs are decreased food intake up to inappetence, poor growing and slow weight gain in ostrich chicks, infertility, lethargy and also neurological disorder with death within 6-7 days (Caliendo et al. 2022b; EFSA Scientific Report 2023c; United States Department of Agriculture (USDA) Foreign Agricultural Service 2021).

2.6.4 Manifestation in mammalian hosts

Avian influenza viruses are generally restricted to birds. This is in part due to the specificity of receptor affinity of AIV to mainly α-2.3-SA, while mammalian IVA have a higher affinity to α-2.6-SA receptors (see chapter 2.4). However, host species that express a dual receptor configuration on epithelial cells of the upper airways like pigs and turkeys (Ito and Couceiro 1998) (Modrow et al. 2021) are susceptible to both mammalian adapted IVA and AIV and are classically considered intermediate hosts (Scholtissek 1994; Scholtissek 1990). Reassortment of influenza viruses of different species within the swine, avian viruses in particular can acquire the ability to adapt to the mammalian host. When entering the human population, these new emerging IVA can give rise to a new human Influenza pandemic.

IAV crossing species barriers is well established for the Spanish flu, which arose around 100 years ago and is associated to the death of around 40 million individuals worldwide (Crosby 2003; Taubenberger

2006). Phylogenetic and structure analyses support the notion that an avian Influenza virus was the origin of the virus responsible for the Spanish flu and is considered the ancestor of all subsequent human and swine H1N1 influenza viruses (Taubenberger 2006). Three further human influenza A pandemics during the last 50 years ("Asian" influenza in 1957, "Hong Kong" influenza in 1968, "Russian" flu in 1977) probably originated from animal and avian influenza viruses as well (Taubenberger 2006; Mostafa et al. 2018).

The general importance of pigs as a reservoir for emerging human IVA was highlighted in 2009, when an H1N1 virus originating from pigs led to a human influenza pandemic (Fraser et al. 2009; Dawood et al. 2009; Patel et al. 2010).

Beside this classical model with the swine as intermediate host, repeated cases of direct infections of humans from avian sources have been documented (WHO 2024a; CDC 2023b). In these cases, close contact with the animals, their excretions or products was assumed to play a major role in transmission (Webby and Webster 2003; Cardona et al. 2009a; Webster 2004). Live-markets or the slaughter process of backyard chickens were identified as a common source of infection (Bruno et al. 2023a) with infection via the nasal route or conjunctiva tissues (CDC 2023b; CDC 2023a). Symptoms of human infection with AIV regularly vary between no or mild, like conjunctivitis, upper respiratory disease or flu-like symptoms and atypical gastrointestinal disease (Glisson 2013) but can also cause severe disease (e.g. Pneumonia), encephalitis and death caused by multi-organ failure (CDC 2023a; CDC 2024c). Clinical disease in humans infected with AIV is not necessarily correlated to virus pathogenicity in birds (CDC 2024c). With nearly 900 cases between 1997-2024 and a case fatality rate of almost 50%, HPAIV H5N1 virus of the gs/GD lineage is of particular concern (WHO 2024b; CDC 2024c; CDC 2024b). However, only very rare cases have been linked to human to human-transmission so far (Buxton Bridges et al. 2000; CDC 2024a; Katz et al. 1999; Ungchusak Kumnuan et al. 2005; Wang et al. 2008).

Since 2014, several human infections with HPAI H5N6 and H5N8 viruses emerged, reporting asymptomatic, mild or severe disease (CDC 2024c; CDC 2024b). Besides, cases of HPAIV H7 virus infections have been reported in Canada (2004; H7N3), in the USA and the Netherlands (2003; H7N7). Of great concern were human infections of H7N9 between 2013 and 2017 in China (CDC 2024c). The virus has been initially circulating in eastern China, causing only sporadic but fatal human infections (Gao et al. 2013) while being mostly asymptomatic in poultry which enabled rapid silent spread (Pantin-Jackwood et al. 2014). In the following years, there were repeated waves of outbreaks, but the number of people affected did not increase significantly until 2016, where the H7N9 virus evolved into highly pathogenic, leading to 1560 human infections in total (Su et al. 2017; Li and Chen 2021; Shi et al. 2017). From September 2017 vaccination of poultry provided effective virus control in poultry and went along with a drastic reduction of human cases (Shi et al. 2018). Nevertheless, routine surveillance is still detecting circulating virus in live poultry markets, slaughterhouses and poultry farms until today (Hou et al. 2024).

Beside AIV H5 and H7, infections with other (low pathogenic) subtypes like H6, H9 and H10 have been detected (Wei et al. 2013; Butt et al. 2005; Song and Qin 2020; WHO 2024d; CDC 2024c).

Natural infections of non-human mammals with AIV of the H5N1 subtype were reported repeatedly since 2003 (Plaza et al. 2024a). Up to 2019, natural HPAIV H5-infections occurred in up to 10 countries worldwide, mostly in carnivorous species such as tigers (*Panthera tigris*) (Thanawongnuwech et al. 2005; Keawcharoen et al. 2004; He et al. 2015), domestic cats (*Felis catus*) (Songserm et al. 2006a; Leschnik et al. 2007; Amonsin et al. 2007), dogs (*Canis lupus familiaris*) (Songserm et al. 2006a) and raccoons (*Nyctereutes procyonoides*) (Plaza et al. 2024a; Reperant et al. 2009; Root and Shriner 2020; Horimoto et al. 2011).

With continuing spread of clade 2.3.4.4b HPAIV H5 and high prevalence in wild bird populations in Asia, Europe, Africa, the Americas and even the Antarctic, spillover infections to mammalian species increased. From 2020 to present, there is evidence of natural HPAIV infection in over 48 mammal species worldwide, most of which can be classified as terrestrial or at least semi-aquatic (Plaza et al. 2024a) and usually associated with severe neurological signs and high mortality rates. Most affected species are carnivores and include foxes (*Vulpes vulpes*) (Bordes et al. 2023; Rijks et al. 2021), lynxes (*Lynx lynx*) (Tammiranta et al. 2023), minks (*Neovison vison*) (Agüero et al. 2023; Kupferschmidt 2023), Tanuki (*Nyctereutes procyonoides viverrinus*) (Hiono et al. 2023) or mountain lions (*Puma concolor*) and lately also cats (Rabalski et al. 2023; Briand et al. 2023). The route of infection is considered via ingestion of infected animals or carcasses. However, cases of herbivores and omnivores such as otters (*Lutra lutra*) (Plaza et al. 2024a; Tammiranta et al. 2023) have been reported.

Beside terrestrial mammals, H5-infection of purely marine mammals are also observed in the ongoing panzootic, such as dolphins (*Tursiops truncatus*) (Murawski et al. 2023; Kayano et al. 2024; EFSA Scientific Report 2022b) or Harbor-(*Phoca vitulina*) and gray seals (*Halichoerus grypus*) at North Atlantic coasts (Puryear et al. 2023), Netherlands and Germany (Mirolo et al. 2023). Also Harbor porpoises (*Phocoena phocoena*) were affected in Sweden (Thorsson et al. 2023). A special event was reported affecting American sea lions (*Otaria flavescens*). Animals were found dead or stranded and mortality included over 5000 dead individuals at the coasts of Peru, around 4500 in Chile and similar numbers in Argentina (Gamarra-Toledo et al. 2023; Ulloa et al. 2023; Plaza et al. 2024b). Analyzed sequences from August 2023 in Argentina point to several virus introductions into the American sea lion population with highest similarities to HPAIV H5N1 viruses from pinniped species from other South American countries. This would indicate a transmission between see mammals highlighting the importance of pinniped-migration for this epidemiological event (Rimondi et al. 2024) and a transmission between mammals.

Since March 2024, there have been reports of HPAIV H5N1-infected ruminants in the USA. The animals initially attracted attention due to an acute drop in milk production, with high virus loads being detected in the milk secretions (United States Department of Agriculture (USDA) Foreign Agricultural

Service 2024a). The routes of entry are still unclear-there are indications of HPAIV-infected wild birds in the vicinity of the stables, but in addition to indirect contacts such as feed or drinking water as a point of entry, direct virus transmission between cows via the milking equipment and transport of infected cows to farms in different states is also currently being discussed (Burrough et al. 2024). Persons who had been in direct contact with infected cattle were found to be infected with HPAIV H5N1, which was accompanied by conjunctivitis. At present, there are no indications of similar infection events in ruminants outside the USA. However, work is currently underway to establish a sensitive diagnostic test schedule at least in Germany (Friedrich-Loeffler-Institut 2024).

The repeated host range expansion of AIV into non-avian hosts increases the risk of an adaptation to mammalian hosts and ultimately to the human population. Already the intense circulation of AIV have repeatedly led to mutations that facilitate species spillover. Mutations in the HA-gene are of special interest, as they might, like the Q226L mutation located in the HA receptor binding domain, have an impact on receptor binding preferences of AIV i.e. α-2.3-SA to α-2.6-SA (Schrauwen and Fouchier 2014; Imai et al. 2012b; Zhang et al. 2012; Chutinimitkul et al. 2010; Imai et al. 2012a; Maines et al. 2011). Additionally, amino acid exchanges at position K182 and R192 in the HA´s of clade 2 H5N1 viruses were found to increase interaction with human type receptors (Yamada et al. 2006). Besides the HA, additional segments have been unraveled to be associated with adaptation to mammalian hosts: Important alterations associated with cross-species adaptation of AIV have been identified in the viral polymerase complex (RdRp), the NP protein, the envelope protein NA and the non-structural protein NS1.

Well established mutations of the RdRp are mutations in the PB2 protein, namely amino acid E627K (Hatta et al. 2001; Hatta et al. 2007; Bortz et al. 2011; Fornek et al. 2009; Kim et al. 2010), D701N (Gabriel et al. 2005; Gao et al. 2009; Le et al. 2009; Li et al. 2005b) and T271A (Bussey et al. 2010) that correlate with an increase of the polymerase activity in mammalian hosts whereas 590S/591R (Mehle and Doudna 2009) and 158G (Zhou et al. 2011; Ilyushina et al. 2010) are found in both mammalian as well as in avian hosts (Cauldwell et al. 2013; Cauldwell et al. 2014; Mostafa et al. 2018). Further mutations linked to increased polymerase activity are 253N/591K, 253N/291K and 526R/627K. In particular, the substitution of a glutamic acid with lysine at position E627K in PB2 has been shown to be an important mutation, responsible for shifting temperature optimum of the RpDP. For example, an AIV strain with a 627K mutation replicates also at 33°C, a temperature present in the human upper respiratory tract, compared to 41°C present in the avian gastrointestinal tract (Massin et al. 2001; Cauldwell et al. 2014; Mostafa et al. 2018). Besides, the commonly detected mutation within PB2 (701N) has been recognized to increase the replication rate and transmissibility of the virus in mice and ferrets (Gabriel et al. 2005; Zhou et al. 2013; Mostafa et al. 2018; Cauldwell et al. 2014). Also in the PB1 protein mutations are described associated with adaptation to mammalian hosts: Site N375S (Taubenberger et al. 2005) is considered as “host-range signature” amino acid and consists of asparagine in avian strains, while human strains carry serine at this position. In addition, G375S substitution has

been observed in IVA originated from swine after spillover infection to humans (Kawaoka et al. 1989). However, the exact mechanisms how these mutations might influence host switch are still unclear (Taubenberger et al. 2005; Naffakh et al. 2008; Cauldwell et al. 2014). A mutation in PB1-F2, encoded on an alternative ORF, namely N66S has been shown to increase pathogenicity of AIV in mice (Zamarin et al. 2006; Conenello et al. 2007) and facilitate secondary bacterial infections (Krumbholz et al. 2011; McAuley et al. 2007; McAuley et al. 2010a).

Likewise, the NP protein is thought to play an important role in affecting host range by shifting adaptation of AIV to mammalian organisms. Mutation of N319K for example has been shown to enhance avian viral replication in mammalian cells (Gabriel et al. 2005). In addition, mutations at 100I/V, 283P and 313Y of the NP have been described that enable avian influenza viruses to overcome the human intracellular restriction factor MxA in order to establish themselves in human populations (Götz et al. 2016).

Table 1. Mutational changes within AIV towards mammalian adaption

Gene	Mutation	Function	Reference
HA	Q226L	• Exchange of receptor binding preference from avian to human receptors	(Schrauwen and Fouchier 2014; Chutinimitkul et al. 2010; Imai et al. 2012a; Maines et al. 2011; Zhang et al. 2012);
	182, 192	• Increase of interaction with human type receptors	(Yamada et al. 2006)
PB2	E627K	• Determination of temperature optimum of the RpDP • Increased polymerase activity and avian viral replication in mammals or mammalian cells • Contribution to increased pathogenicity in mammals	(Hatta et al. 2001; Hatta et al. 2007; Bortz et al. 2011; Fornek et al. 2009; Kim et al. 2010)
	D701N	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Gabriel et al. 2008; Gao et al. 2009; Le et al. 2009; Li et al. 2005b)
	T271A	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Bussey et al. 2010)
	590S591/R	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Mehle and Doudna 2009)
	158G	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Zhou et al. 2011; Ilyushina et al. 2010)
	253N/591K/291K	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Mok et al. 2011; Mostafa et al. 2018)
	526R/627K	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Mostafa et al. 2018)
PB1	N375S	• Host range signature	(Taubenberger et al. 2005)
	G375S	• Adaption to mammals	(Naffakh et al. 2008)
PB1-F2	N66S	• Increase of pathogenicity in mice • Facilitate secondary bacterial infections	(Zamarin et al. 2006; Conenello et al. 2007; Krumbholz et al. 2011; McAuley et al. 2007; McAuley et al. 2010b)
NP	N319K	• Increased polymerase activity and avian viral replication in mammals or mammalian cells	(Gabriel et al. 2005; Gabriel et al. 2008)
	100I/V, 283P, 313Y	• Overcome human intracellular restriction factor mxA	(Götz et al. 2016)

2.7 Epidemiology

2.7.1 Transmission

AIV infection routes may vary, depending on the viral strain, affected species and bird density, as well as environmental conditions such as temperature and presence of suitable vectors (Alexander 2007). The virus is excreted via body fluids, e.g. fecal material and mucous of the beak cavity containing the secretions of the sinus (Webster et al. 1978). Consequently, the fecal-oral route is considered of uttermost importance for transmission of influenza viruses (Kida et al. 1980; Webster et al. 1992b). Furthermore, water contaminated by mucous fluids of the beak cave has an important role for AIV transmission. This assumption is supported by observations in experimentally infected ducks viral excretion via the oral route was similar to the fecal route (Hénaux et al. 2011) and may even exceed the fecal excretion in many species (Germeraad et al. 2019). Experimental studies on HPAIV H5N8 inoculated mule ducks (*Cairina moschata*×*Anas platyrhynchos*) also proved for excretion of high viral titers in both orally as well as cloacally derived swabs as early as 2 dpi, although clinical symptoms only occurred in animals from 4 dpi. The virus could even be detected via the conjunctiva and the feather pulp at such an early stage. In this setting, virus could be detected very early in environmental samples such as aerosol, dust, but also infectious virus in drinking water samples from 1-14 dpi and in pool water samples from 2-5 dpi, depending on the viral strain tested (Filaire et al. 2024). The importance of virus contaminated water was further highlighted by findings, that 10^2 tissue culture infectious units ($TCID_{50}$) per liter in a pool were sufficient to start infection with subsequent high infectious virus loads in bell drinkers (Ahrens et al. 2022). Furthermore, both HPAIV as well as LPAIV could be detected in bell as well as in nipple drinkers in naturally infected poultry flocks (Sieverding and Hafez 2023). In general, it has been proven, that beside transmission of Influenza directly from animal to animal, indirect transmission by vectors play an important role in viral spread (Modrow et al. 2021). On the surface of such contaminated objects, virus may remain infectious over a prolonged period of time with high variations depending on the temperature. For example, in water it has been demonstrated that at 4°C infectious virus could be recovered after 32 days of storage (Webster et al. 1978) with considerable variations between different virus strains (18.0-176.2 days) (Brown et al. 2009). Increasing water temperatures were found to have a negative effect on viral persistence: for example at 17°C virus could be recovered between 30-74 days (Brown et al. 2009), while no infectious virus was recovered after 7 days at 22°C (Webster et al. 1978) or 3-11 days at 28°C (Brown et al. 2009). Likewise, lower pH (generally under 6.6) and higher salinity (generally over 25,000 ppm) (Brown et al. 2009) have a negative effect on viral infectivity. As virus might be present on all surfaces, transmission might occur by contaminated eggs (Li et al. 2006), but thereby infection remains strictly horizontal (Alexander 2007; Spackman 2023).

Beside inanimate vectors AIV can be spread by infected birds, such as LPAIV circulating in his natural reservoir species, Anseriformes wild birds. Thereby the virus can be transmitted over long distances

especially during migration (Stallknecht et al. 2016). This has also been demonstrated for HPAIV and intercontinental spread of HPAIV has been linked to wild birds. Such an observation is supported by phylogenetic analyses, demonstrating that HPAI H5N1 viruses detected in captive birds in Canada, wild birds in the United States and cases in poultry holdings in North America in December 2021 (WOAH and OIE 2021) were closely related to European viruses from spring 2021, with all eight viral gene segments having Eurasian origin (Caliendo et al. 2022a; Bevins et al. 2022). Further on, data from ringed wild birds confirm migration routes from Europe to North America, with stopover in Iceland and Greenland (Caliendo et al. 2022a), countries serving as breeding grounds for bird populations (Hall et al. 2014). In addition, passive surveillance efforts from 2022 revealed HPAIV H5N1 infected wild birds in Iceland. Interestingly, the detected virus had segments originated from both European and north American clusters, confirming HPAIV in this region and highlighting Iceland a possible stepping stone for intercontinental virus distribution (Günther et al. 2022).

2.7.2 Emergence and evolution of the goose/Guandong (gs/GD) lineage virus

Since its first documented occurrence in 1878, Avian Influenza, or known as “fowl plague” (Beaudette 1925) was a constant threat to poultry. From 1950 onwards, up to 39 de novo emergence events on AI have been identified, with conversion of LPAIV H5 or H7 to highly pathogenic pathotype in poultry. However, all these outbreaks remained confined and stringent restrictions helped to eradicate all of these viruses (Dhingra et al. 2018). This situation changed with the emergence of a new strain that was first recognized 1996 in infected commercial geese from the Guandong Province, China (Xu et al. 1999) and today known as goose/Guandong (gs/GD) lineage. Between 1997 and 2003, long-term surveillance detected multiple reassortants of gs/GD derived viruses in poultry in China (Zhang et al. 2009; Liu et al. 2003; Li et al. 2005a) and repeatedly in healthy appearing ducks (Chen et al. 2004). Subsequently, viruses of the (gs/GD) lineage re-emerged in China from 2003 with outbreaks in poultry (Li et al. 2010; Guan et al. 2002; Webster et al. 2002) as well as in wild birds (Chen et al. 2005).

After virus detection in migratory birds in 2003/2004 in Asian countries (Chen et al. 2005) and initial reports of high wild bird mortality in Northwest China (CDC 2009; Lvov et al. 2010; Zhou et al. 2016; Chen et al. 2006; Chen et al. 2005), intercontinental dissemination of HPAI H5N1 viruses of the gs/GD lineage began. Followed by reports of HPAI viruses, designated to the gs/GD lineage from East and Southeastern Asia, the virus could spread to Russia and Africa in the following years (WOAH 2024b; Olsen et al. 2006). The initial introduction of HPAIV H5 gs/GD lineage into Europe was in autumn of 2005, starting from the eastern towards western countries, including Greece, Italy and Germany (Olsen et al. 2006; Weber et al. 2007; Globig et al. 2009). Since the first introduction (Starick et al. 2008) Germany has experienced repeated entries of HPAIV H5 gs/GD lineage descendant viruses over the years with varying dimensions (Harder et al. 2015; King et al. 2020b; Pohlmann et al. 2017; Starick et al. 2008).

2.7.2.1 Clades and subclades up to 2.2.2

During this journey, viruses of the (gs/GD) lineage underwent a constant diversification, leading to classification of 10 (0-9) genetic clades and subclades (WHO, OIE, FAO H, N, and Evolution Working Group 2008; WHO, OIE, FAO H5N1 Working Group 2012). Thereby, especially clade 2 viruses stand out with a high degree of diversity which is reflected by the separation into five second order subclades (Neumann et al. 2010). From a European point of view, respective clade 2.2 viruses caused outbreaks between 2006-2009 in several countries (Cattoli et al. 2009), including Germany. A splitting of separate cases in 2006/2007 in Germany into subclades 2.2.1 and 2.2.2 also took place (King et al. 2021; WHO, OIE, FAO H, N, and Evolution Working Group 2008; WHO, OIE, FAO H5N1 Working Group 2012).

2.7.2.2 First occurrence of Clade 2.3.4.4 in China

From today´s perspective, clade 2.3 is of special interest with its diversification in Africa and Asia (2.3.2.1), in Asia, Europe and North America (2.3.4) and separated clade 2.3.4.4 viruses of 2014 in Asia. This particular subclade subsequently was dispersed to Africa, Europe and North America and included new H5N2, H5N5, H5N6 and H5N8 reassortants (WHO, OIE, FAO H, N, and Evolution Working Group 2008).

Ancestral strains of clade 2.3.4.4 HPAIV H5N8, that later account for outbreaks in Europe, were first isolated around 2010 in China (Zhao et al. 2013; Li et al. 2010; Gu et al. 2011; Zhao et al. 2012). Recurrence of clade 2.3.4.4 H5N8 was reported from January 2014, during surveillance programs on live poultry markets in China (Ma et al. 2018), in Japan and in three different premises of commercially kept ducks in the Donglim Reservoir, South Korea (Kim et al. 2016; Yoon et al. 2015). Viruses from diseased ducks of the Korean outbreaks were designated as Gochang I, Buan II and Donglim III and identified to belong to two different clusters: clade 2.3.4.4a (Buan/ Donglim-like) and 2.3.4.4b (Gochang-like) (Lee et al. 2014; Kang et al. 2015; Kim et al. 2014).

2.7.2.3 Introduction of Clade 2.3.4.4 in Europe

In September 2014, shortly after outbreaks in Asia, a clade 2.3.4.4a (Buan/Donglim-like) virus was isolated in an Eurasian wigeon (*Anas penelope*) in Russia (Lee et al. 2017; Marchenko et al. 2015). This was followed by the first European outbreak caused by clade 2.3.4.4a viruses recognized in Germany in autumn of 2014. A farm of fattening turkeys in the North-East of Mecklenburg-Western Pomerania was struck (Conraths et al. 2016; WOAH 2016) with subsequent detection of H5N8 in an Eurasion teal (*Anas crecca)* bird on the island of Ruegen in late November 2014. Cases in wild birds and individual outbreaks in poultry built up in January 2015 in Mecklenburg-Western Pomerania, in summer in Lower Saxony and in December in Bavaria (TSIS-TierSeuchenInformationsSystem 2024b). These clade 2.3.4.4a viruses circulated in Europe until the end of 2015 (Globig et al. 2017). Phylogenetic analyses of newly emerged H5N8 reassortants indicated that new European and the Russian 2.3.4.4a viruses were derived from precursors circulating in East Asia between 2009-2012 (Kim et al. 2014; Gu et al. 2013).

After a period without detections of HPAIV, clade 2.3.3.4b (Gochang) H5N8 reassortants emerged in autumn 2016: After being detected in Russia during summer 2016, first cases were reported in wild birds in Hungary and Poland (EFSA Scientific Report 2017; Pohlmann et al. 2017; King et al. 2020b; FAO 2016). While no cases of HPAIV H5 were registered during spring and summer of 2016 in Germany, the new clade 2.3.4.4b HPAIV H5N8 virus emerged in November, becoming evident by mortality in tufted ducks (*Aythya fuligula*) (Pohlmann et al. 2017). First detected at Lake Plön in the North (Schleswig-Holstein), shortly after there was HPAIV H5N8 clade 2.3.4.4b identification in wild birds in the south at Lake Constance in Baden-Württemberg (Globig et al. 2017). Spread of HPAIV H5N8 into wild birds, mainly wild water birds like *Anseriformes, Charadriiformes* and *Ciconiiformes* went on across many German federal states during December 2016 and was also occasionally introduced into poultry premises such as turkey fattening farms in Lower Saxony (TSIS-TierSeuchenInformationsSystem 2024a). By September 2017 more than 1,100 cases in wild birds as well as around 100 outbreaks of HPAI H5 in captive birds had been confirmed, for Germany coining the most severe HPAI epidemic so far (Globig et al. 2017). Throughout Europe, the H5N8 subtype caused up to 2,600 outbreaks in poultry and captive birds but was especially associated with a high detection rate in wild birds (Napp et al. 2018; EFSA Scientific Report 2017). Experimental studies on Pekin and Muscovy ducks demonstrated that clade 2.3.4.4b induced mortality was higher than that of the previous clade 2.3.4.4a H5N8 variant from 2014 (Grund et al. 2018).

Further H5 clade 2.3.4.4b reassortants emerged between 2018-2020 in European countries, but remained locally and timely restricted (Xie et al. 2023). In Germany, circulation of clade 2.3.4.4b H5N8 continued in 2018 and simultaneously new H5N6 (Poen et al. 2019) and H5N5 reassortants emerged (Pohlmann et al. 2019), although these variants did not reach the detection rate from previous years (EFSA Scientific Report 2018). No avian influenza outbreaks in wild birds were notified in Germany in 2019 (EFSA Scientific Report 2019).

In January 2020, an H5N8 clade 2.3.4.4b was detected in a wild bird in Eastern Germany, close to the Polish border (Spiegel 2020; Friedrich-Loeffler-Institut 07.01.2021). Phylogenetic analyses found the initial ancestor of this H5N8 virus in Egyptian poultry, possibly introduced by wild birds through the Black sea-Mediterranean flyway, following circulation patterns as in previous seasons (Xie et al. 2023; Napp et al. 2018).

With regard to the German viruses of the H5N8 subtype, these could be further differentiated into two frequently detected genotypes (DE 20-10 N8 and DE 21-02 N8). With a sharp increase of H5N8 cases in wild birds and outbreaks in poultry across Europe, a new H5N1 subtype emerged in eastern European countries from mid-2020. Two separate lineages of this virus formed, one in European countries, displacing the prevailing H5N8 subtype and spreading to North America by the east atlantic flyways (Günther et al. 2022; Gass et al. 2023), while the other was introduced via southern European countries and the Mediterranean flyways to Africa (Xie et al. 2023). This clade 2.3.4.4b H5N1 successor was

genetically highly flexible with appearance of multiple H5Nx subtypes, from which genotype DE 21-02 N1, first detected in February 2021, initially emerged (Pohlmann et al. 2022; King et al. 2022a). Clade 2.3.4.4b H5N1 genotypes have since then been responsible for panzootic virus spread between 2021-2023 (Xie et al. 2023). In addition to that finding, the virus emergence shifted its formerly seasonal activity to a now year-round, enzootic occurrence (Pohlmann et al. 2022; EFSA Scientific Report 2023c).

Since September 2021, there have been new cases of HPAIV H5N1 in wild birds and poultry farms in Europe, leading to hundreds of infected wild birds, including up to 4,000 red knots (*Calidris canutus*) (Pohlmann et al. 2023), cranes (*Grus grus*) and barnacle geese (*Branta leucopsis*) (Friedrich-Loeffler-Institut 13.09.2021). From January 2022, up to 1600 HPAIV H5 outbreaks in poultry and numerous infected wild birds have been reported in Europe, exceeding the previous year´s dimensions, with France, Hungary, Italy, Poland and Germany in particular being affected (Friedrich-Loeffler-Institut 10.01.2022). Unusual cases of HPAIV H5N1 infection-related mass mortality in colony breeders (terns (*Sterna hirundo*), gulls (*Larus ridibundus*), Northern gannets (*Morus bassanus*)) in coastal areas of northern Europe were reported in May 2022 (Pohlmann et al. 2023). In addition to the unprecedented epidemic in Europe, individual African and Asian countries also reported isolated outbreaks of HPAIV H5N1 until early summer 2022. While the numbers in these countries remained low, however, there have been an increasing number of cases of HPAIV H5N1 in Canada and the USA (Friedrich-Loeffler-Institut 08.07.2022). From October 2022, HPAIV H5N1 outbreaks in domestic poultry were again reported in several European countries. For the first time, the virus was also detected in countries in South America, including Colombia and Argentina (Pan American Health Organization 2023) and also in Greenland. Since December 2022, infections of mammals with clade 2.3.4.4b HPAIV H5N1 have been reported in several countries worldwide, including the Netherlands (Vreman et al. 2023; Bordes et al. 2023), France, Spain (Agüero et al. 2023) and Canada (Alkie et al. 2023). The monthly case numbers of infected wild birds have remained at a consistently high level across Europe since the end of 2022. As in previous years, the infections primarily affected coastal birds. However, since the end of November 2023, there have been higher numbers of infected wild birds throughout Europe and also in the Americas (Friedrich-Loeffler-Institut 07.12.2023). The first cases of HPAIV H5 of clade 2.3.4.4b detected in Antarctica in October 2023 were able to spread to several species such as skuas and terns in the following months (Friedrich-Loeffler-Institut 07.12.2023; EFSA Scientific Report 2023c).

The number of outbreaks in domestic poultry and wild birds in Europe has been at a high level again since December 2023. From February 2024, the number of reported cases in both wild and domestic birds has dropped again (Friedrich-Loeffler-Institut 14.03.2024), but sporadic cases have continued to occur, such as reports in March 2024 of infected ruminants in the USA (United States Department of Agriculture (USDA) Foreign Agricultural Service 2024) (see chapter 2.6.4).

2.7.2.4 A Plethora of Clade 2.3.4.4b H5Nx genotypes arises

By week 52 of 2021 until the beginning of 2022, a massive extension of up to 16 differing HPAIV H5Nx clade 2.3.4.4b genotypes could be identified by sequencing of isolates of varying spatio-temporal distribution (Fig. 1) (Pohlmann 2023b).

The H5N1 genotype DE 21-10 N1.2, first detected in October of 2021 dominated the German HPAIV epidemic in 2021 with up to 120 case reports, followed by DE 21-10 N1.5 with 177 identified cases throughout the following year 2022. In addition to these two dominant genotypes, further genotypes such as DE 21-12 N1.2 – N1.5 were detected in December of 2021, in January 2022 (DE 22-01 N1.1) and later in April (DE 22-04 N1.1) (Pohlmann 2023b; Pohlmann 2022).

Infections with genotypes DE 21-10 N1.2 and prevailing DE 21-10 N1.5 remained active during the summer and were responsible for outbreaks in colony-breeding seabirds (Pohlmann et al. 2023; Pohlmann et al. 2022; Falchieri et al. 2022; Friedrich-Loeffler-Institut 08.07.2022). Complemented by new genotypes DE 22-09 N1.1, DE 22-11 N1.1 and DE 22-12 N1.1 over the course of the year, all previously known genotypes have been repressed by February 2023 at latest with emergence of a new genotype (DE 23-02 N1.1) (Ahrens et al. 2024). This new predominant genotype could be isolated from up to 50 cases in wild and domestic birds over summer of 2023, but was no longer detectable from September 2023 on. Instead, four other H5N1 genotypes, (DE 23-11 N1.1 -N1.4), appeared in wild and domestic poultry, each with a different spatial distribution, (Friedrich-Loeffler-Institut 12.01.2024; Pohlmann 2023b). Also during 2023 HPAIV H5 remained endemic, mostly affecting shorebirds and gulls as wild bird species (Ahrens et al. 2024).

New genotypes, such as DE 24-01 N1.1 and DE 24-02 N1.1-N1.2 have been appearing since the beginning of 2024, simultaneously circulating with their predecessors. The aforementioned genotypes are repeatedly detected in current virus isolates of 2024, although none of them can be crystallized as predominant in Germany (Pohlmann 2023b).

Present, the occurrence of wild and domestic animal populations affected by HPAIV infections is continuing, even if the sample density is discontinuous.

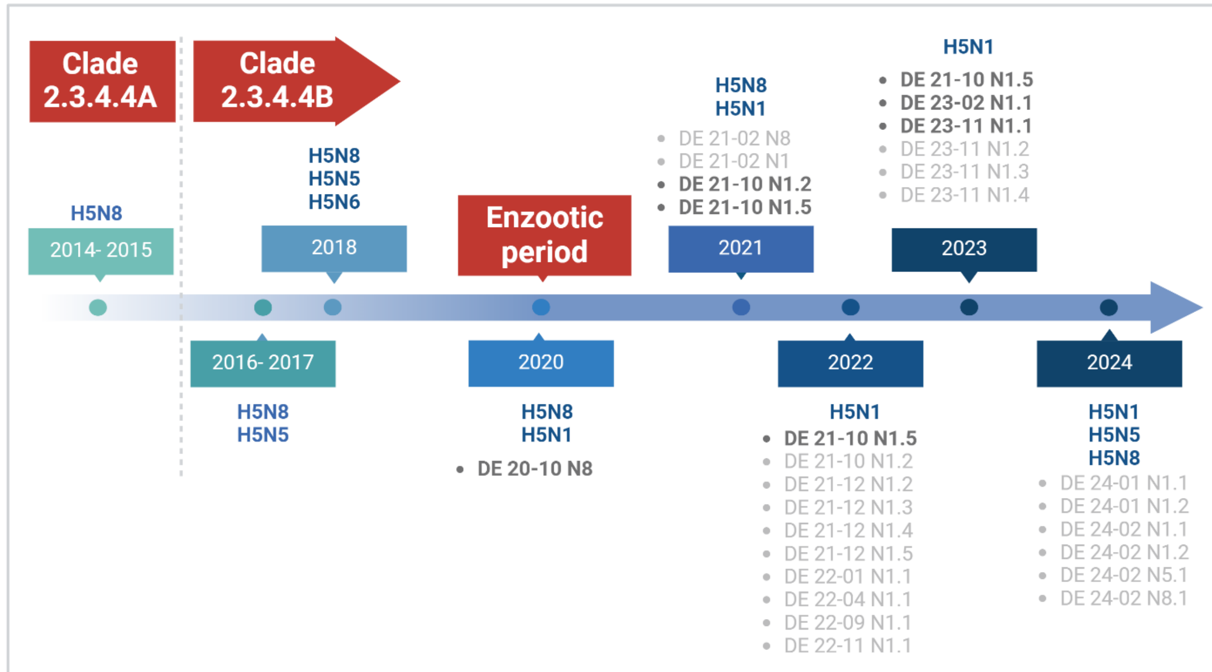

Figure 1. **Temporal development of HPAIV H5 clade 2.3.4.4b in Germany between 2014-2024.** Modified from (Pohlmann 2024b; Pohlmann and Harder 2023; *Created in BioRender, Piesche, R. (2025) https://BioRender.com/a79a003*)

III. Study objectives

Avian Influenza, in particular the highly pathogenic H5 subtype has established a worldwide panzootic that imperil not only wild bird species and poultry but also encompass the risk to extend host range and become the next human pandemic. Continuous evolution of HPAIV H5 viruses from the gs/GD lineage, driven by genetic shift and drift bear the risk to alter pathobiology within the avian hosts and may contribute to an extension of the host range. The extended epidemiological dispersion of clade 2.3.4.4b HPAIV H5 with a plethora of newly emerged sub-and genotypes in 2021/2022 indicated a possible transformation of AIV. To gain insight into possible altered biological properties of HPAIV H5 genotypes, the present project is assessing the virulence and host range of the relevant genotypes from Germany, subdivided into two main objectives:

3.1 Characterization of HPAIV H5N1 clade 2.3.4.4b genotypes in juvenile ducks as indicator for evolutionary host adaptation

According to the "avirulence hypothesis", it is postulated that microorganisms that are less harmful, are more successful to establish endemicity and are more prosperous on the evolutionary scale. This work addresses the question whether decreased virulence in ducklings is associated with dominance of phylogenetically different clade 2.3.4.4b H5Nx genotypes.

In addition, the connection between virulence and the genetic backbone may provide clues to virulence factors that promote or reduce the pathogenicity of individual genotypes. In the course of this work, virus isolates of relevant current HPAIV H5N1 genotypes were prepared and characterized. Virulence was tested in a newly established intra muscular pathogenicity index (IMPI) duckling model, applying the inoculum to one-week old Pekin ducklings (*Anas platyrhynchos*) intra muscularly. Pekin ducks were chosen as infection model as they are the domesticated form of mallards, and are considered as important aquatic wild bird species for the epidemiology of HPAIV. Further experiments in Pekin ducklings were addressing the shedding profile and pathological manifestation after occulo-nasal inoculation for representative HPAIV H5N1 isolates with high and moderate duckling virulence. This work establishes the IMPI-duckling model to elaborate differences between HPAIV in waterfowl and highlights the importance of phenotypical characterization of different genotypes for a better understanding of the epidemiology of the current HPAIV H5 panzootic.

3.2 Elaboration of the zoonotic potential of a predominant HPAIV H5N1 clade 2.3.4.4b subtype in food-producing animals

Infections of numerous mammalian species have highlighted the panzootic potential of currently circulating HPAIV H5N1 strains. Of particular concern would be susceptibility of pigs to clade 2.3.4.4b HPAIV H5N1: pigs harbor a variety of mammalian adapted influenza A viruses (IVA) and it is well established that they can serve as a "mixing-vessel", i.e. facilitating the emergence of reassortants between AIV and IAV. In an animal experimental study, the question was addressed whether pigs are

susceptible to genotype DE 21-10 N1.5 of HPAIV H5N1 clade 2.3.4.4b, that dominated epidemiology in Germany in 2022. Outcome of infection should be tested after occulo-nasal or oral route inoculation by assessing clinical course, excretion profile, immune response and tissue tropism.

IV. Results

The reference section of each manuscript follows the style of the respective journal and is therefore not listed at the end of the document.

4.1 Publication I

Dominant HPAIV H5N1 genotypes of Germany 2021/2022 are linked to high virulence in Pekin ducklings

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Dominant HPAIV H5N1 genotypes of Germany 2021/2022 are linked to high virulence in Pekin ducklings

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Highly pathogenic avian influenza viruses (HPAIV) of H5 clade 2.3.4.4b pose an ongoing threat worldwide. It remains unclear whether this panzootic situation would favor low virulent phenotypes expected by the 'avirulence hypothesis' of viral evolution. Assessing virulence in Pekin ducklings in an intramuscular infection model revealed that the two genotypes that dominated the epidemiological situation in Germany during the period 2021 and 2022 (EU-RL:CH and EU-RL:AB) were of high virulence. In contrast, rare genotypes were of intermediate virulence. The genetic constellation of these reassortants pointed to an important role of the viral polymerase complex (RdRP), particularly the PB1 genome segment, in shaping virulence in ducklings. Occulo-nasal infection of ducklings confirmed the phenotypes for two representative viruses and indicated a more efficient replication for the high virulence strain. These observations would be in line with the 'virulence-transmission trade-off' model for describing HPAIV epidemiology in wild birds in Germany.

Highly pathogenic avian influenza virus (HPAIV) causes severe disease and mortality in both wild and domestic poultry species on a global scale and therefore poses a significant threat to conservation efforts and the global agricultural industry[1]. Lately, HPAIV H5 of the so-called Goose/Guangdong lineage (gs/GD) lineage have spread to almost all continents[2,3], most recently even to the Antarctic region. Mainly detected in avian species, the virus is emerging increasingly also in marine and terrestrial mammals, epitomizing the risk of avian-to-mammalian spillover infection and subsequent adaptation[4,5]. As a member of the genus of alpha-influenza viruses within the family of Orthomyxoviridae, HPAIV is an enveloped virus and contains a single-stranded negative-sense RNA genome. The genome is divided in eight segments coding for a total of up to 17 proteins. Each virion comes with a RNA-dependent RNA polymerase (RdRP)[6], composed by the association of three polymerase proteins: polymerase basic 2 (PB2), polymerase basic 1 (PB1), and polymerase acidic (PA), with viral genomic RNA encapsidated by the nucleoprotein (NP). Two glycoproteins embedded in viral membrane, the hemagglutinin (HA) and the neuraminidase (NA)[7] mediate viral attachment to the cellular α-2.3/ 2.6-N-acetyl neuraminic acid receptors with subsequent cell entry and sialidase activity to facilitate release of the new virion from infected cell[8–10]. Today, 16 HA subtypes and 11 NA subtypes have been identified in avian species with wild Anseriformes considered as their natural reservoir. Recently, an H19 subtype has also been suggested in birds[11]. Within the reservoir species, co-infections with different subtypes are considered the sources for versatile reassortment events that are associated with emergence of new genotypes with gene constellations differing from their parental viruses. The majority of avian influenza viruses (AIV), however, induce only a local infection in the respiratory- and gastro-enteric tract and are of low pathogenicity (LPAIV). A molecular marker for low- pathogenic (LP) phenotypes is a monobasic proteolytic cleavage site within the HA-precursor protein (HA_0). In contrast to all other 14 HA subtypes, in H5 and H7, the proteolytic site can mutate to a polybasic proteolytic cleavage site that enables systemic viral replication, thereby causing severe clinical disease, classified as notifiable highly pathogenic avian influenza (HPAI) in galliform birds[10]. Mutations from LPAIV to HPAIV are mostly associated with galliform infections, although few exceptions have occurred. Such de novo emergence events of HPAI from LPAIV precursor have been documented 39 times since the 1950s[12], causing outbreaks of different magnitudes but mainly restricted to poultry. The majority of these highly virulent viruses were not able to prevail over a prolonged period of time within a population, due also to consequent restriction measures, such as depopulation, applied to eradicate these viruses. In this respect HPAIV H5 of the gs/GD lineage in 1996 is a notable exception as its successors persist and emerge until today[12–14]. Based on the HA gene, 10 clades (0-9) can be distinguished within the gs/GD lineage[15], which in turn have established themselves in different regions such as Asia, America, Africa and Europe. Today, the ongoing panzootic is driven by

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clade 2.3.4.4b viruses. Precursor viruses of clade 2.3.4.4 split, before 2013, into two separated clusters, A (Buan/ Donglim- like) and B (Gochang-like), which had been introduced to Europe in 2014 and 2016 respectively[16,17]. Reassortants of the introduced HPAI H5N8 clade 2.3.4.4b viruses were subsequently the origin of today's HP H5Nx genotypes that established endemicity in wild birds in Europe[18,19] and disseminated to the Americas[14] and Africa. Since 2020, swarms of reassortants were observed with up to five different NA subtypes: Initially, H5N8 and H5N5 strains[16], sharing high identities with Asian sequences were encountered which were replaced by H5N1 genotypes, that subsequently dominated the epizootic in Germany[19,20] and Europe. Between October 2021 and beginning of 2022 sixteen different H5N1 genotypes have been discovered in Germany from outbreaks in poultry and cases in wild birds. Within this swarm, certain genotypes seem to dominate the epizootic. For example, genotype DE 21-10 N1.2 (EU-RL:CH) was the most frequently detected one at the end of 2021[21], but in 2022 was superseded by genotype DE 21-10 N1.5 (EU-RL:AB). For an overview of frequency of different genotypes in Germany and Europe see the associated dashboard[22]. While a lot of efforts have been undertaken to characterize the plethora of emerging genotypes, information on the phenotypic characteristics that might shape the HPAIV H5 pandemic are limited. In particular it is not addressed so far whether dominance of certain genotypes is associated with adaptation and concomitant decrease of virulence as postulated according to the "avirulence hypothesis" originally put forward by Theobald Smith[23]. According to this model, high virulence is in opposition to an expansion of a given pathogen population, as a fast "immobilization" of the host shortens the infection cascade and restricts further spread. On the opposite, in case of subclinical infections the mobile host can be a source of spread of the pathogen for a longer period of time, i.e. lower virulence in the host would help a virus to spread and eventually persist in a population. However, when replication of a pathogen can be assumed to cause some inevitable damage to the host, an increase in the number of pathogens increases both transmission and virulence. This consideration was taken into account when the "virulence-transmission trade-off" hypothesis was introduced[24]. This model, like the "avirulence hypothesis" assumes, that from an evolutionary point of view, it is not beneficial for the pathogen to kill its host before being passed to another host. However, within this model evolution will lead to intermediate virulence, but, depending circumstances, initial virulence may also increase. To study virulence of different HPAIV genotypes in a relevant reservoir species we selected the Pekin duck (*Anas platyrhynchos var. domesticus*). Pekin ducks are the domesticated form of the mallard duck (*Anas platyrhynchos*)[25], the most abundant wild duck species in the Northern hemisphere, and a well-known natural host of gs/GD HPAIV. Mallards exhibit complex migratory patterns, and frequently switch between partly resident and partly migratory behavior, making them an important species for both dispersal and local maintenance of AIVs[13,26,27]. In general, Anseriformes are highly susceptible to both LPAIV and HPAIV[28], but clinical disease due to HPAIV infection is often mild compared to commercial galliform poultry species[27,29–31]. In recent years, clinical outcome of infections with clade 2.3.4.4 HPAIVs in ducks have been shown to vary depending on the virus strain, from mild courses of disease induced by viruses of 2014, to severe lethal courses including neurological disorders after 2016[29,32,33]. It remains unclear whether and to what extent virulence in mallard ducks, a globally prevailing wild water bird species, might influence the dominance of certain genotypes during an epizootic. Results obtained during active surveillance efforts, detecting HPAIV shedding in hunted mallards, i.e. birds capable to fly[34] supported the hypothesis that virulence of circulating HPAIV might have decreased and that lower virulence for mallards might go along with dominance of such pathotypes in wild birds in general. This hypothesis was further promoted by experimental infection studies and field investigations[35–38] demonstrating subclinical infection of HPAIV H5 infection in Pekin and mallard ducks. In general, such observations might be in line with the "avirulence hypothesis". To investigate whether such "adaptation" took place during the epizootic 2021/22, we pathotyped in ducklings HPAIV H5N1 genotypes that co-circulated at the end of 2021 (week 52) and compared that to subsequent detection rates of HPAIV H5N1 genotypes in Germany. Pathotyping followed the previously published approach of inoculating HPAIV parenterally into week-old ducklings[32]. However, due to tiny size of veins at this age, a less error- prone parenteral inoculation route, i.e. intramuscular (i.m.) was chosen. This system, referred to as the "intramuscular pathogenicity index" (IMPI) testing has been shown to provide a finer distinction of pathogenicity in semiquantitative terms compared to the more categorical yes/no results that are usually obtained with chickens and the intravenous inoculation system (IVPI). Based on the IMPI results our studies point out that higher assertiveness of individual genotypes in the field went hand in hand with a significant higher virulence in Pekin ducklings. These data on altered pathogenicity in the Pekin duckling model shed light on the driving forces of the current panzootic, with dominance of the most virulent variants thereby superseding HPAIV strains of lower virulence.

Methods

Viruses

Details of the viruses used in this study are given in Table 1. Basis for selecting the viruses was the database featured in the public dashboard "HPAIV genotypes in Germany" [22], which holds sequence data from the National Reference Laboratory for Avian Influenza in Germany. Retrospectively, these data indicate dominance of certain genotypes. For the phenotypic characterization we focused on genotypes present in the 52nd calendar week of December 2021, a week when 7 different genotypes were detected in Germany. It was possible to obtain isolates of 6 genotypes of week 52 (#3, #4, #6, #7, #8, #9), while isolation of a seventh genotype detectable in week 52, namely DE 21-12 N1.6, failed. In addition, derived from the two sites genotype #8 was exclusively detected, genotype #5 and two further isolates of genotype #4 (#4:1 and #4:2) were included. The three isolates of genotype #4 take its massive and long-term presence throughout 2022 into consideration and represent isolates from three timepoints throughout 2022, i.e. February (#4:1), June (#4:2), and July (#4:3). Viruses were isolated from swab samples of deceased birds sent to the national reference laboratory for avian influenza at the Friedrich-Loeffler-Institute (FLI). For virus isolation, samples were first enriched in cell culture (Immortalized chicken hepatocytes ([LMH cell line, ATCC (CRL-2117), 1997]) by infecting them with 500 µl of 1:10 prediluted and filtrated (Millex® Syringe- driven Filter Unit −0.22 µm) (SigmaAldrich) viruses and then incubating them for at least 96 h at 37 ₹C and 5% CO_2 concentration, in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific), supplemented with 0.86 g/L NaHCO3 (Carl Roth) and 10% Fetal Calf Serum (FCS). At the end of the period, the cells were visually checked for a cytopathic effect (CPE) such as formation of virus- associated circular plaques or general cell necrosis. Cell culture supernatants were examined for viral hemagglutination capacity using a hemagglutination assay (HA)[39]. HA assay positive supernatants were then added in a quantity of 200 µl for propagation in the allantoic cavity of 10-day-old embryonated eggs from chicken specific pathogens free (SPF). Inoculated eggs were then incubated at 37.5 °C for at least 96 h and death of the eggs was monitored every 12 h by candling. Amnio-allantoic fluids (AAF) were then harvested from eggs harboring dead embryos and centrifuged for 10 min at 1500 revolution per minute (rpm) before storage of the supernatants at -70 °C until further use.

Each isolate used for animal experiments was characterized by full genome sequencing and subsequent genotyping as described in ref.[40].

Virus titration

Immortalized chicken hepatocytes [LMH cell line, ATCC (CRL-2117), 1997] were cultivated at 37 °C and 5% CO_2 concentration, in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific), supplemented with 0,86 g/L $NaHCO_3$ (Carl Roth) and 10% Fetal Calf Serum (FCS). For virus titration, LMH cells were incubated in 96 well plates without FCS but supplemented with TPCK Trypsin (2 µg/ml). For determination of tissue culture infectious dose 50 ($TCID_{50}$), four serial tenfold dilution rows were prepared of each virus stock and each dilution row was

added in triplicates on 96 well plates with 50 µl per well. Development of cytopathic effects (CPE) was visually scored by microscopic investigation. After 72 h of incubation formation of CPE had fully developed in form of virus- associated circular plaques or general cell necrosis. $TCID_{50}$ was then calculated according to the Reed & Muench formula[41].

Animal experiments

All animal experiments were in accordance with relevant guidelines and regulations of the state office for agriculture, food safety and fishery in Western pomerania (LALLF) and have been have been approved by an independent ethics commission and granted permission by the state office for agriculture, food safety and fishery in Western pomerania (LALLF) under the registration number LALLF 7221.3-2-009/19. Pathotyping of HPAIV was done by intramuscular (i.m.) inoculation of one-week old Pekin ducklings (*Anas platyrhynchos var. domesticus*) as described[32]. Shortly, one day old ducklings were obtained from a commercial hatchery. The parental flocks are routinely screened serologically to exclude AIV infections at a prevalence of 5% (90% confidence). In addition, 10 ducklings from the same batch were euthanized for testing and all yielded seronegative results as well as negative PCRs from swab samples. After an acclimatization phase of one week, all animals were healthy and randomly separated by allocating individual picked birds alternating to nine different groups with ten birds each. Each bird was inoculated with 0.1 ml of a 1/10 diluted AAF from infected eggs into the caudal femoral muscles. The group size, virus quantity and dilution scheme were based on IVPI standards[39]. Birds of each group were kept on the floor of separated rooms and were allowed to move freely within an enclosure of 6.46 m^2. They had access to food, drinking water ad libitum with an additional water tub for bathing and dry places with infrared lamps to warm up.

Subsequently, birds were clinically monitored for 10 days which included daily veterinary check- ups and supervision by animal care staff supported by a 24 h daily video surveillance program, accessible to all persons involved in the experiment. The scoring scheme provided categorization of animals as healthy [0], sick [1] severely sick [2] or dead [3], a wording adopted from the IVPI definition format. "Sick" reflects a general body condition, where animals appear weak, less enthusiastic to follow the cage mates. At this stage animals respond to external impulses and are willing to eat and drink. "Severely sick" encompasses a body condition where vital sings as described above are missing and/or sings of central nervous disorders are evident, and corresponds to the defined human endpoints of disease. Animals reaching these endpoints in the context of the experiment were euthanized by stunning by means of a blunt blow to the head and subsequent bleeding by opening the carotid arteries on both sides, with final dislocation of the cervical vertebrae and severing of the spinal cord at the neck (Annex IV of Directive 2010/63/EU; No. 2 c). These animals were scored as dead from the following day on. Based on such scoring, a clinical intramuscular pathogenicity index (IMPI) was calculated according to the standard protocol for the intravenous pathogenicity index (IVPI) of avian influenza viruses[39].

For occulo-nasal (o.n.) infection, one-week old Pekin ducklings, raised as described for the IMPI, per groups of ten ducklings, were inoculated with 0.1 ml of the same virus stock from respective genotypes, again 1/10 diluted as for the IMPI (Supplementary Table 1). Per animal 1 drop of virus suspension was conjunctivelly applied while the rest of inoculum was applied through the nares onto the choanal epithelia. From one day post infection (dpi) on, two additional ducklings were housed together with the ten inoculated animals to serve as sentinels for virus transmission. The animals were kept under the same conditions as the within the IMPI trials, i.e. they had unrestricted access to bathing water and ad libitum feeding and drinking supply. Clinical supervision by veterinarians included daily health checks as well as supervision by trained animal care staff for the whole duration of the experiment. Clinical score values per animal for an observation time of 10 days were used to calculate a group clinical score according to the previous IMPI, similar to calculation of the IVPI used in chickens. In addition, choanal and cloacal swab samples were taken daily for the first seven dpi, added into tubes with 1 ml cell culture medium supplemented with Enrofloxacin (Baytril®; 20 µg/ml) and stored at −70 °C until further analysis. Due to the extended survival of ducklings during the IMPI, the monitoring period of the o.n. experiment was prolonged for up to 21 days after inoculation.

A postmortem examination with tissue sampling was performed on acutely diseased animals for o.n. inoculated animals or from surviving animals on the last day of the experiment i.e. on10 dpi for the IMPI groups and 21 dpi for the o.n. experiment. Samples of brain, lung, duodenum/pancreas, kidney, liver and heart were taken, added into tubes containing 1 ml of cell culture medium with Enrofloxacin (Baytril®; 20 µg/ml) and stored at −70 °C until further analysis. Blood was collected from all remaining ducklings at the final day of the experiment.

Pathology

Histological evaluation was performed on three ducks each, that were either moribund and euthanized, i.e. o.n. inoculated animals infected with

Table 1 | Characteristics of German HPAIV-H5N1 clade 2.3.4.4b genotypes investigated in this study

Genotype			Accession number[a]	Virus isolate	$TCID_{50}$/dose[b]	IMPI
code	Germany-RL[c]	EU-RL[d]				
#1	DE 20-10 N8		accession 5146288	*A/chicken/Germany-NW/AI 3705/2021 (H5N8)*	1.40×10^6	2.52
#2	DE 02-21 N8		accession 4804850	*A/seal/Germany-SH/AI 5373/2021 (H5N8)*	2.86×10^3	2.69
#3	DE 21-10 N1.2	CH	accession 18006762	*A/European herring gull/Germany-MV/AI 1411/2022 (H5N1)*	5.02×10^7	2.92
#4:1	DE 21-10 N1.5	AB	accession 17693298	*A/European herring gull/Germany-SH/AI 1196/2022 (H5N1)*	9.90×10^6	3.00
#4:2	DE 21-10 N1.5	AB	accession 17693301	*A/brent goose/Germany-SH/AI 2407/2022 (H5N1)*	5.48×10^7	2.95
#4:3	DE 21-10 N1.5	AB	accession 16096050	*A/chicken/Germany-NI/AI 4286/2022 (H5N1)*	2.86×10^7	2.99
#5	DE 21-11 N1.1		accession 17693297	*A/European herring gull/Germany-SH/AI 7088/2021 (H5N1)*	2.40×10^7	1.65
#6	DE 21-12 N1.2		accession 18006937	*A/chicken/Germany-MV/AI 1026/2022 (H5N1)*	2.00×10^6	1.52
#7	DE 21-12 N1.3		accession 10261376	*A/pigeon/Germany-NW/AI 951/2022 (H5N1)*	7.47×10^6	2.96
#8	DE 21-12 N1.4		accession 5098132	*A/red knot/Germany-SH/AI 616/2022 (H5N1)*	8.28×10^5	1.22
# 9	DE 21-12 N1.5		accession 18006938	*A/black-headed gull/Germany-HH/AI 1073/2022 (H5N1)*	2.40×10^7	1.26

[a]Accession number according to the gisaid.org EpiFlu™ platform.
[b]TCID50/dose per 0.1 ml inoculum.
[c]genotype designation by the national reference laboratory for avian influenza in Germany (RL); code for the country and federal state code is given according to ISO 3166.
[d]genotype designation by the EU reference Laboratory (RL) for avian influenza (provided where available).

genotype #4:3 (3 dpi) or genotype #8 (5-6 dpi), respectively (Fig. 3; Supplementary Figures 2–4). In addition, for genotype #8 organ samples were taken from animals that survived until the end of the observation time, i.e. 21 dpi for o.n. inoculated and 10 dpi for i.m. inoculated animals. Samples from nasal conchae, lung, heart, kidney, brain, and spinal cord (thoracal, lumbar) were fixed in 10% neutral buffered formalin. Tissues were paraffin-embedded and 2–4 µm-thick sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was performed for viral antigen detection using a primary antibody against the M protein of IAV (ATCC clone HB-64) as described in ref. 42. Slides were scanned using a Hamamatsu S60 scanner and analyzed using NDPview.2 plus software (Version 2.8.24, Hamamatsu Photonics, K.K. Japan). HE stained sections of all tissues were evaluated and described. Following IHC, the distribution of IAV matrix protein was recorded on an ordinal scoring scale with scores 0 = no antigen, 1 = oligofocal, affected cells/tissue < 5% or up to 3 foci per tissue; 2 = multifocal, 6%–40% affected; 3 = coalescing, 41%–80% affected; 4 = diffuse, > 80% affected. The target cells were identified based on the morphology. Evaluation and interpretation were performed by a board-certified pathologist (DiplECVP) in a blinded fashion.

Real- time RT-qPCR

For further processing swab samples of the o.n. inoculated birds (Supplementary Table 2) were thawed and vortexed in a Thermomixer (Thermomixer comfort® Eppendorf) at room temperature for 5 min. Organ samples were directly homogenized in tubes containing a steel bead by a tissue lyser (TissueLyser II Qiagen, Hilden, Germany) while oscillating for 3 minutes at 30 Hz. RNA was extracted from 100 µl of centrifuged supernatant from swab- and organ samples by the Macherey-Nagel NucleoMag® VET-Kit according to the Manufacturer´s instructions using the Biosprint 96 extraction robot (Qiagen, Hilden, Germany) and eluted in 100 µL elution buffer.

Subsequently, preparations were tested by real-time RT-PCR (RT-qPCR) for the presence of influenza A virus specific matrix gene RNA (M1.4) using the AgPath ID One-Step RT-PCR Kit (Ambion-Applied Biosystems) as described in ref. 43. PCR tests were carried out in a CFX96™Real-Time-System C1000™ thermal cycler (BioRad, Munich, Germany). Estimation of virus genome equivalents per milliliter (VE/mL) in each sample is based on correlation of the individual Cq values to an intra-assay calibration curve of a defined HPAIV H5N1 virus stock. The value is derived from standard titration experiments of infectivity of a virus suspension in cell culture medium. The $\log_{10}$ dilutions are tested in parallel by RT-qPCR. The resulting correlation curve of $TCID_{50}$ and Cq values are used to derive the VE value.

Serology

Blood samples were collected from all ducklings reaching the respective end of the experiment alive. Blood was drawn into a tube containing heparin, plasma separated by centrifugation and then subjected to heat inactivation at 56 °C for 30 minutes. Plasma samples of the ducklings were screened by ELISA for Influenza A-specific antibodies using two different commercial test kits as indicated for the specific sera (IDEXX AI MultiS- Screen Ab Test or ID screen® Influenza A Antibody Competition Multi- species ELISA). The cut-off for the IDEXX ELISA used was <0.5 S/N (Signal to noise ratio) for positive sera, while sera with a value of >0.5 were assessed as negative. Positive results were set for the ID- vet ELISA at a cut-off of <45% S/N (%) inhibition, while results >50% S/N (%) inhibition were considered negative. The range in between was to be considered questionable. Subsequently, hemagglutination inhibition (HI) assays were performed on ELISA- positive samples using homologous and heterologous antigens AIV, respectively, according to standard procedures[39]. By tilting the plates, the agglutination of the plate could be assessed, whereby the HI titre of a sample corresponds to the highest serum dilution, which causes a complete inhibition of 4 HAU (Hemagglutination Units) of the antigen[39]. For HI assays, duck samples were pretreated with KJO_4-solution to remove non-specific inhibitors.

Statistics

Statistical analyses on significance were performed as indicated where appropriate. Differences in IMPI clinical scores between all groups was statistically evaluated using unpaired two-tailed Mann- Whitney- U tests. The data on oropharyngeal and cloacal excretion of the two occulo-nasally infected groups #4:3 and #8 were statistically evaluated using Kruskal-Wallis One-Way- ANOVA on ranks, Tukeys tests and following unpaired two-tailed Mann-Whitney-U tests when appropriate. *P*- values as well as associated effect sizes are given in the text or in the supplemental material (Supplementary Figure 1; Supplementary Table 6; Supplementary Table 6.1). Statistical analysis was applied with help of the statistical analysis tool GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA, USA) as well as SigmaPlot™ version 11 (SigmaPlot Software, Grafiti LCC, Palo Alto, CA, USA).

Sequencing

Full-genome sequencing for genotype confirmation of all isolates from passaged viruses on LMH cells as well as AIV-positive organ samples was done as described[40]: after initial RNA extraction via the Qiagen Mini Viral Kit (Qiagen, Germany) and subsequent AIV-End-RT-PCR applying Superscript III One-Step, sequencing was executed by a nanopore-based amplification method. 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.

Genotyping

The designation of genotypes was applied on a national level, based on Germany as country of virus isolates' origin, the year and month of the first detection of a particular genotype within a typed sample, and finally the NA subtype as described in refs. 19,40,44. Matching nomenclature proposed at the European level applied by the European Reference Laboratory (EU-RL) was used where appropriate. Consecutive hash numbers enable an easier identification of the genotypes used here (Table 1).

Phylogenetic analysis

Based on the classification of different genotypes, closely related sequences were selected by blasting the non-redundant nucleotide database at the NCBI (National Center for Biotechnology Information; NCBI; Bethesda, MD, USA) and the EpiFlu™ platform of the Global Initiative on Sharing All Influenza Dara (GISAID) (https://blast.ncbi.nlm.nih.gov/; https://platform.epicov.org/), entering accession numbers or FASTA sequences of genotypes used in our trials as well as applying search pattern filters on the Influenza Type A, H1-10, N 1-10, animal hosts, location between Europe, Asia and America as well as on clade 2.3.4.4 viruses. Additional filters were applied on required segments such as PB2 and PB1. For further information on retrieved sequences please see Supplementary Table 5. Sequences got downloaded and further analyzed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms software version 10.2.4 (Kumar, Stecher, Li, Knyaz, and Tamura 2018)[45]. The alignment was fed with additional, related sequences[19,46]. Sequences were finally aligned using the MUSCLE algorithm contained in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms software version 10.2.4 (Kumar, Stecher, Li, Knyaz, and Tamura 2018) applying the following parameters: maximum 16 iterations, cluster method UPGMA. Phylogenetic analyses were run on nucleotide sequences using the Phylogeny tool in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms software version 10.2.4 (Kumar, Stecher, Li, Knyaz, and Tamura 2018). More precisely, Maximum likelihood (ML) trees were computed utilizing a General Time Reversible Model (GTR) + gamma (G) with rapid

bootstrapping and search for the best scoring ML tree supported with 1000 bootstrap replicates. Correspondingly protein sequences were translated and phylogenetic trees based on the ML method with 1000 bootstrap values were created again using the MEGA X Phylogeny tool. The resulting phylogenetic nucleotide and translated protein trees were color- coded according to their genotype- distribution using a vector graphics editor (CorelDRAW 2017) and representative virus isolates referencing certain genotypes were marked by a dot.

Results

HPAIV H5N1 genotypes of clade 2.3.4.4b differ in virulence for ducklings

Phylogenetic analyses on current genotypes in combination with the epidemiological information reveal, that despite the diversity of simultaneously circulating genotypes, the current epizootic as a whole was dominated by single, individual genotype (Fig. 1A)[44]. For the phenotypical characterization of co-circulating HPAIV H5N1 clade 2.3.4.4b genotypes we focused on the last week of December 2021 (week 52), a time period when seven different genotypes of HPAIV H5N1 were present in Germany and compared the virulence to frequency of detection in subsequent year 2022. Representative virus isolates of six out of these seven genotypes present in December could be obtained from samples obtained in 2021-2022 by cultivation on LMH-cells. No isolate could be obtained from one genotype, namely DE 21-12 N1.6. Conformity with original samples was demonstrated by full- genome sequencing, verifying their distinct genotypes (Fig. 1B, genotypes #3 - 9). For comparison, one H5N1 genotype first detected in November (#5) as well as two older H5N8 clade 2.3.4.4b viruses representing two further distinct genotypes (#1, #2) were included in the pathotyping approach. Both H5N8 genotypes circulated in Germany until April 2021, but have since than been replaced by H5N1[20]. Genotype #5 as well as two more viruses of genotype #4 (#4:1 and #4:2) were included as they are derived from the same sites as genotype #8 (January 2022), representing viruses from November 2021 (#5).

In seven-day old pekin ducklings, both H5N8 viruses of genotypes #1 and #2 induced 100% mortality within three to six dpi, resulting in an IMPI score of 2.52 and 2.69, respectively (Fig. 1B; graphical representation and data for individual animals are listed in Supplementary Figure 1; Supplementary Table 10). However, their pathogenicity was exceeded by genotype #3 (IMPI 2.92), a genotype emerged in October of 2021 and dominating the German HPAIV epidemic with a total of 60 (43.8%) of the sequenced HPAIV H5N1 viruses up to the end of 2021 (Supplementary Table 1). In frequency of detection genotype #4 was the second highest disseminated genotype, detected in 43 (31.4%) of HPAIV H5N1 viruses sequenced in 2021 (Fig. 1A; Supplementary Table 1). Compared to both precursor H5N8 viruses, IMPI scores of three genotype #4 virus isolates were significantly higher ($p < 0.005$, $r_U > 0.6$) with scores of 3.00 (#4:1), 2.95 (#4:2) and 2.99 (#4:3), respectively, see Supplementary Figure 1; Supplementary Table 6 for statistical analysis) and inducing mortality within 2 dpi. These later three HPAI H5N1 2.3.4.4b viruses of the same genotype #4 have been recovered in February 2022 (#4:1), April 2022 (#4:2) and July 2022 (#4:3) and were included to evaluate consistency of IMPI scores (Table 1). The results indicate, that the virulence of this genotype was stable and even slightly higher than genotype #3: Whereas for genotype #3, 7 of the 10 inoculated ducklings were alive on one dpi, two genotype #4 isolates induced death in all (#4:1) and nine (#4:3) ducklings within one day, resulting in significant higher IMPI scores (#4:1 $p = 0.0031$, $r_U = 0.7$ and #4:3 $p = 0.0174$, $r_U = 0.6$). With regards to frequency of detection the order of the genotypes changed in the following year 2022, with 133 of 222 sequenced viruses belonging to genotype #4 (59.9%), in contrast to only 60 (27.0%) of the sequenced viruses from 2022 belonging to genotype #3. Nevertheless, during 2021 and 2022 genotypes #3 and #4, highly virulent for ducklings by IMPI, together made up 75 and almost 90% of all sequenced HPAIV in Germany.

Of almost equally high duck virulence was an isolate representing genotype #7, inducing 100% mortality within 24 h (IMPI of 2.96). This particular virus was recovered from a wood pigeon (*Columba palumbus*) that was euthanized during an outbreak in a wild bird sanctuary in January 2022[47]. However, this genotype remained a minor population detected generally, with one detection up to the end of 2021 and four in 2022 in poultry and wild birds (see Supplementary Table 1), constituting 1.8% of sequenced viruses in the period 2021 and 2022.

The remaining three genotypes that co-circulated in December 2021 (#6, #8 and #9) remained rare detections, representing 10 (2.8%), 3 (0.8%) and 6 (1.7%) of the total number of viruses sequenced in Germany for the period up to the end of 2022. Obtained virus isolates from 2022 from genotypes #6, #8 and #9 were of moderate duck virulence with significant lower IMPI scores compared to dominate genotype #3 (#8 $p = <0.0001$, $r_U = 1$; #9 $p = <0.0001$, $r_U = 1$) and genotype #4 ($p = <0.005$, $r_U = 1$) (Supplementary Figure 1; Supplementary Table 6): besides birds that succumbed to infection two to six days after inoculation, single birds showed only moderate or no clinical signs at all up to the end of the observation period, resulting in IMPI scores between 1.22 and 1.52. For example, in the group with the lowest IMPI, i.e. the genotype #8 virus, only four out of nine birds died or had to be euthanized because of severe signs of disease dominated by disorders of the central nervous system (CNS), while five birds had recovered from slight apathy at day 7 to 9 after infection (dpi) (Fig. 2; Supplementary Fig. 1).

Outcome of infection with moderate HPAIV H5N1 genotypes

All ducklings that survived until the end of the observation period of 10 days had seroconverted by ELISA (Supplementary Table 4) proving they all had become infected. In addition, organ samples taken at the end of the experiment from ducklings of the genotype #8-infected group- showing the lowest IMPI value - tested virus positive: Viral RNA was detected in the brain of all 5 ducklings on 10 dpi (9.1×10^3– 2.8×10^5 VE/mL). However, attempts to isolate infectious virus from late brain samples of genotype #8-infected ducklings failed. To a lesser extent, residual viral RNA was detected in the heart (4 of 5), the lungs (3 of 5) and the duodenum (2 of 5) of genotype #8 infected ducklings. Likewise, ducklings infected with genotypes #6 or #9, survived the ten-day observation period ($n = 6$ of each group), showing no (#9: $n = 4$) or only mild signs of depression (#6: $n = 4$) but harboring viral RNA in the brain (#9: $n = 4$; #6: $n = 6$) (Supplementary Table 3). The detailed histological evaluation of 3 ducks, euthanized 10 days after i.m. inoculation with genotype #8, revealed that virus antigen was still detectable multifocally in neurons and oligo focally in glia cells of the CNS in 2 out of 3 animals. Although clinical signs were lacking at 10 dpi, all ducks showed a moderate to severe, subacute, necrotizing meningoencephalitis predominantly in the cerebrum and brainstem, but hardly in the cerebellum (Fig. 3). No virus antigen was detected in the remaining examined tissues, however, 2 out of 3 ducks exhibited mild, chronic interstitial pneumonia, partially with cellular debris in the air sac and one animal showed minimal, chronic myocarditis. No lesions were recorded in the kidneys (data not shown). For details on virus antigen detection, see supplementary table (Supplementary Table 9).

These results revealed striking differences in duckling pathogenicity between different genotypes of HPAIV H5N1 of clade 2.3.4.4b, indicating that epidemiologically dominant genotypes were more virulent for ducks. Interestingly, ducklings surviving infection up to day 10 dpi with genotypes with moderate virulence, although clinically inconspicuous, showed meningoencephalitis and virus antigen-positive neurons and glial cells in the brain, confirming a general neurotropism of the different HPAIV H5 genotypes.

Genotype-related distinct virulence for ducklings is also verified by the occulo-nasal inoculation route

To investigate the influence of the inoculation route on duckling virulence, one isolate of high (genotype #4:3; IMPI: 2.99) and moderate duckling virulence (genotype #8; IMPI: 1.22) were tested in ducklings applying occulo-nasally inoculation route, mimicking a more natural route of infection compared to parenteral inoculation. Like after the i.m. inoculation, the virulence of the two genotypes differed dramatically in ducklings:

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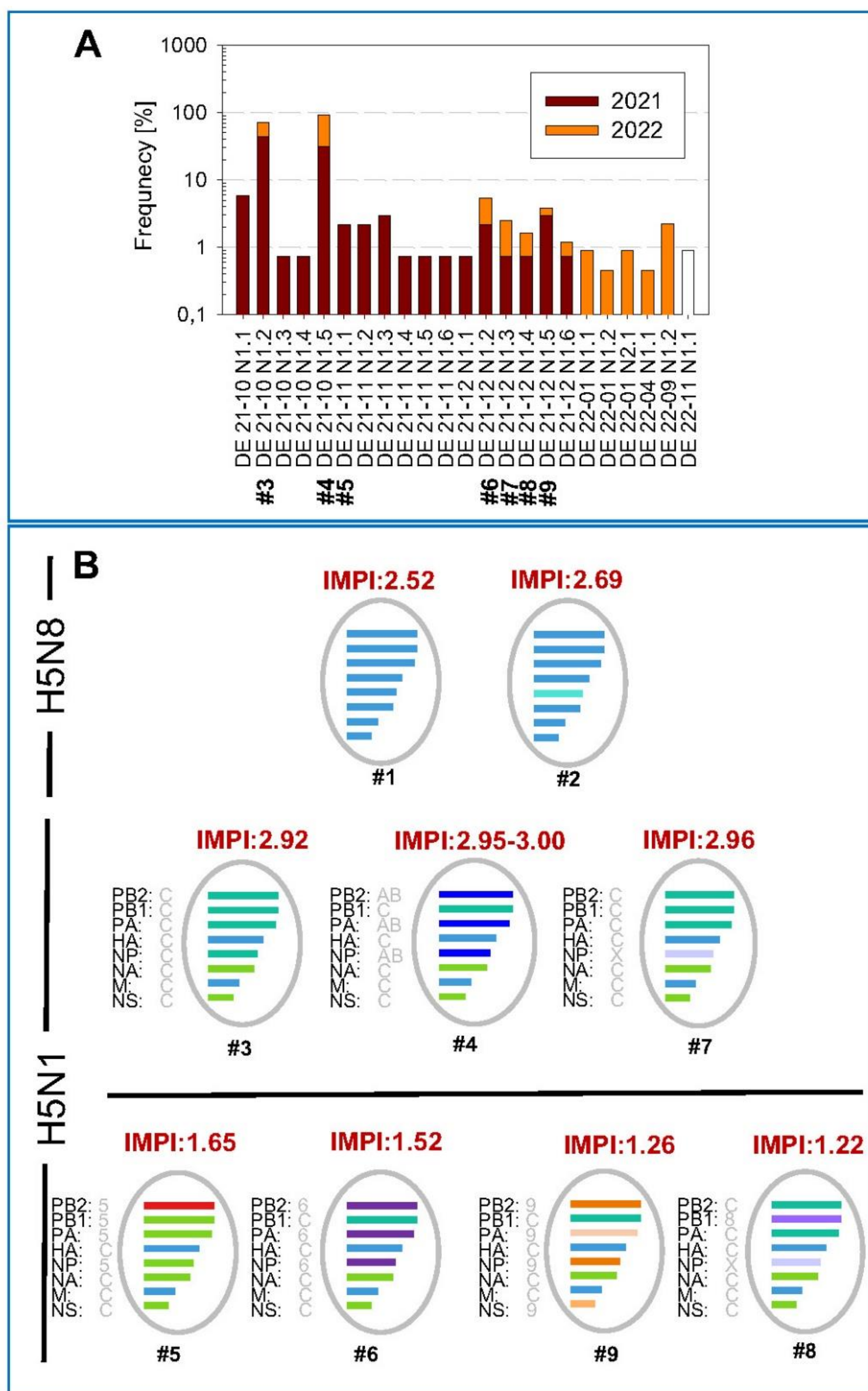

Fig. 1 | Geno- and phenotype of co-circulating HPAIV H5 in Germany during 2021-2022. HPAIV H5N1 clade 2.3.4.4b genotypes co-circulating in December 2021 (week 52) in Germany were characterized with respect to their duckling virulence by IMPI and compared to 2 HPAIV H5N8 genotypes, the subtype dominating HP epidemics until April 2021. **A** Detection frequency of genotypes prevalent in Germany during 2021 and 2022 are given and tested viruses are indicated (#). **B** Depiction of genetic composition of the tested genotypes, based on analyses described previously[21]. The genotypes are shown with their IMPI score, that is given above the virus cartoons. The horizontal black line separates genotypes with high (upper panel) from those with intermediate virulence (below). The origin/relation of the segments to other genotypes is given by letters besides the segment names with designation based on the EU-RL nomenclature).

https://doi.org/10.1038/s44298-024-00062-0 Article

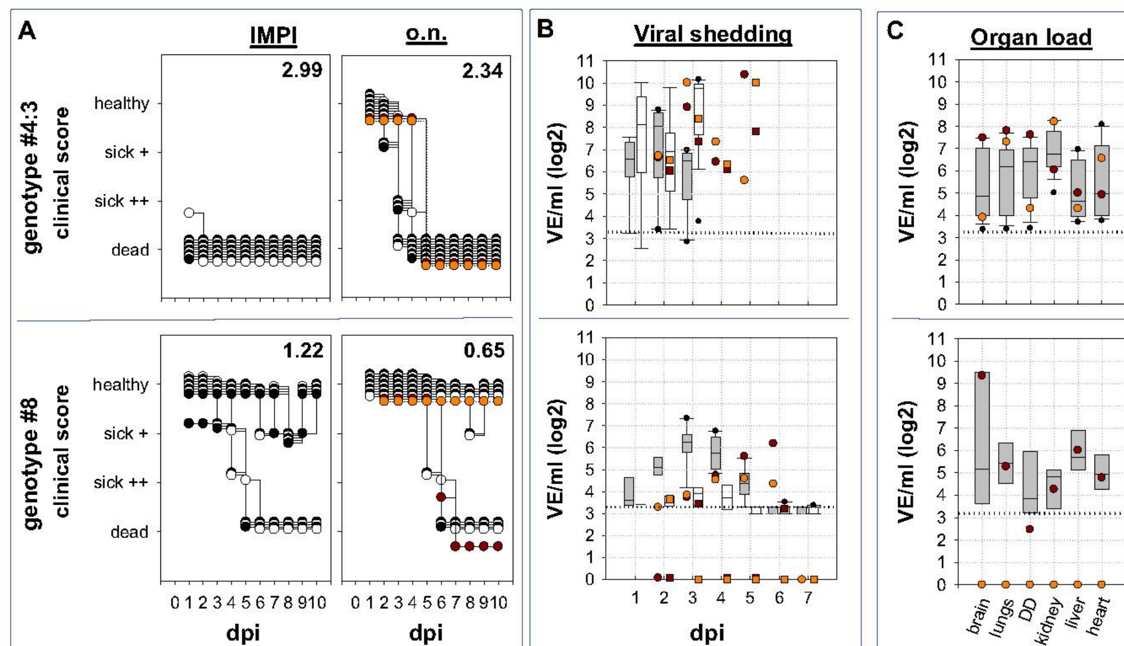

Fig. 2 | Clinical course and viral shedding after o.n. HPAIV H5N1 infection with genotypes of high and moderate duckling virulence. Depicted are individual daily clinical scores after i.m (IMPI) and o.n. inoculation with genotypes #4:3 and #8 respectively. Two different sentinel animals in each of the o.n. inoculated groups are individually marked by orange and red dots, respectively. **A** Clinical disease was observed up to 10 dpi and to better compare both types of infection clinical scores (numbering in the top right corner of each graph) were determined after ten days. In the stacked format of the graph white and black dots are used only to contrast different individual inoculated ducks. **B** Viral shedding was tested by pharyngeal - (grey boxes) and cloacal swabs (transparent boxes) by RT-qPCR and results are standardized to virus equivalents (VE/ml) as described in material and methods. Viral shedding from two sentinel animals per group is given individually by orange and red dots for pharyngeal shedding as well as squares for cloacal shedding. Please see Supplementary Table 6 for further information on statistical analysis. The dotted horizontal line represents the cut-off. **C** Likewise, organ samples from o.n. inoculated ducklings of both groups were investigated, taken either at the time of euthanasia or at the end of the observation time. Again, sentinel animals are indicated by an orange and a red dot.

Whereas genotype #4 induced mortality in all ten inoculated and both sentinel ducks, six out of ten ducklings inoculated with genotype #8 and one sentinel animal survived infection and showed no clinical signs after day 8 p.i. All seven surviving ducklings from genotype #8 group were subsequently observed for 21 dpi and remained clinically unremarkable and seroconverted (Supplementary Table 4; 4.1). However, compared to the i.m. inoculation, the course of disease after o.n. inoculation was delayed resulting in lower clinical scores (CS) for both genotypes: CS of 2.34 vs. IMPI of 2.99 ($p < 0.0001$, rU=1) for genotype #4 and CS of 0.65 vs. IMPI of 1.22 ($p = 0.0383$, rU=0.6) for genotype #8 (Fig. 2A; Supplementary Table 6). Concerning viral replication, the higher virulence of genotype #4 virus was associated with a faster increase of viral shedding in o.n. inoculated ducklings (Fig. 2B). Starting at 1 dpi already 9 out of 10 pharyngeal (1.75×10^3 to 3.46×10^7 VE/mL).The highest viral load was detected in pharyngeal swabs from both sentinal animals with 1.02×10^{10} and 2.38×10^{10} VE/mL on 2 and 5 dpi respectively. Pharyngeal viral shedding remained on that high level up to the death of the animals. In comparison, in the group of genotype #8 inoculated ducklings, 8 out of 10 pharyngeal swabs were virus positive on 1 dpi, but at a considerable lower level (5.13×10^3 to 2.87×10^5 VE/mL) than in the genotype #4 group. During the following days, pharyngeal viral shedding increased, with 10 out of 10 swab samples testing positive on 3 and 4 dpi, with the peak of viral shedding observed on day 4 (5.49×10^3–2.15×10^7 VE/mL) remaining up to 28 times lower (Supplementary Table 2) compared to genotype #4 inoculated ducklings. Remarkably, cloacal shedding remained at very low levels in this group: within the first 7 dpi, only single cloacal swabs tested positive at low loads with significant differences in shedding between groups on 3 dpi ($p = 0.004$, $r_U = 0.94$). Nevertheless, both sentinel animals of the genotype #8 group tested positive in pharyngeal swab samples from day 3 or 6 dpi, demonstrating successful virus transmission. Blood samples taken on the last day of the observation period, i.e., 21 dpi, confirmed seroconversion in all six surving inoculated and one sentinel animal of the genotype #8 group by ELISA (Supplementary Table 4) and also by HI. Testing with different antigens, sera showed preferential binding to homologous antigen with HI-titers between 6-8 [$\log_2$], compared to HI-titers between 4 and 6 [$\log_2$] to heterologous antigens from previous years (2014 and 2016) (Supplementary Table 4.1).By occulo-nasal inoculation mimicking a more natural route of infection the dualism of highly and moderately virulent genotypes of HPAIV H5N1 clade 2.3.4.4b was confirmed.

Clinical manifestations after occulo-nasal inoculation reveal dominance of neurological signs

Diseased animals inoculated with highly virulent genotype #4 had to be euthanized on day 3 because of progredient CNS disorders. Virological examination of these birds revealed presence of influenza A virus RNA in almost all tested organs (Supplementary Table 2), with the highest viral load detected in the kidneys, with virus loads ranging from 2.09×10^3 VE/mL to 1.86×10^6 VE/mL. Histopathological analysis of organs of three acutely deceased birds revealed an acute, necrotizing meningoencephalomyelitis in all birds with intralesional virus antigen detection particularly in the forebrain (score 4) and brain stem (score 1-3), and to a lesser extent in the cerebellum (score 0-3) (Fig. 3) and spinal cord with dorsal root ganglia (score 1-3) (Supplementary Figure 2). Target cells comprised neurons, glia cells, ventricle and central canal epithelium. Furthermore, a severe, acute necrotizing rhinitis, sinusitis, air sacculitis and pneumonia was recorded, with intralesional virus antigen (score 2-3) detected in the olfactory and

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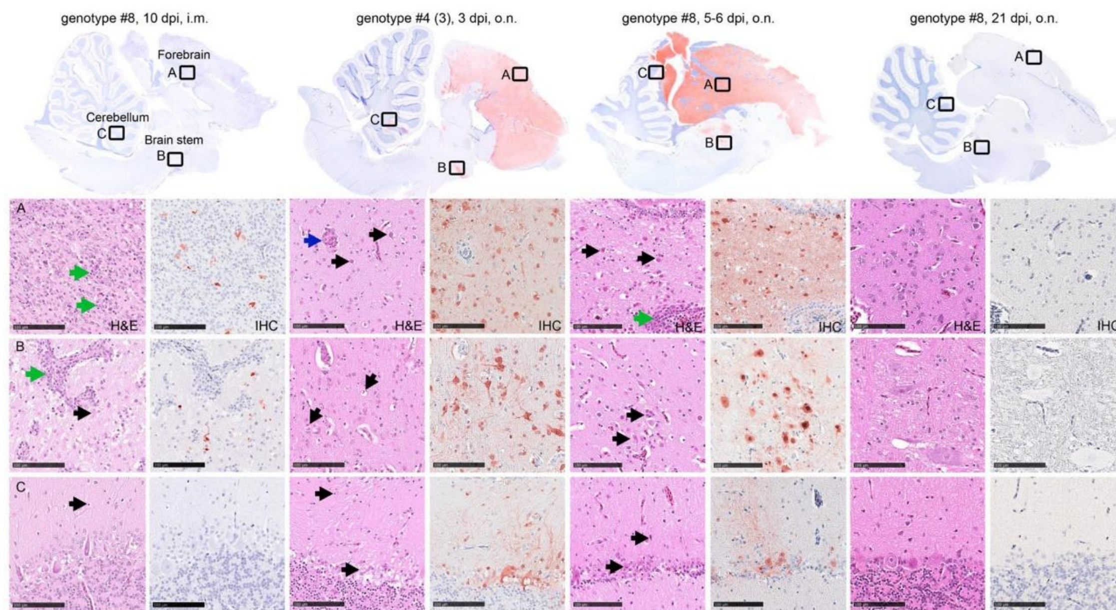

Fig. 3 | Pathological findings and Influenza A virus antigen detection in the brain. Shown are HE staining and antigen detection in different brain areals of ducks inoculated with genotype #8 (10 dpi, i.m.), genotype #4:3 (3 dpi, o.n.) or genotype #8 (5-6 dpi, o.n.; 21 dpi, o.n.). Necrotizing meningoencephalitis, with intralesional virus antigen detection, was present following i.m. inoculation with genotype 8 and in moribund/deceased birds after o.n. inoculation with genotype #8 or genotype #4 (3). The (**A**) cerebrum and (**B**) brain stem, were most affected, the (**C**) cerebellum was significantly less affected. Blue arrows indicate activation of blood vessels, black arrow point to necrotic neurons and glial cells, green arrows show immune cell infiltrates, perivascular and disseminated in the neuropil. Dpi days post infectio-n,i.m. intramuscularly, o.n. occulo-nasally, hematoxylin-eosin stain, H&E, immunohistochemistry IHC. Bar 100 μm.

respiratory epithelium of the conchae, in the respiratory sinus and air sac epithelium as well as in the pulmonary bronchial, parabronchial and capillary epithelium (Supplementary Figure 3; 3.1).

Likewise, organ samples from four ducklings that died in the moderately virulent genotype #8 group between day 5 and 6 p.i., had particularly high viral loads in the brain (2.53×10^5 to 4.77×10^8 VE/mL) and infectious virus was recovered from the euthanized birds. Also a sentinel animal to this group, that was euthanized on day 6 p.i., while suffering from central nervous disorder, had a particular high viral load in the brain (2.21×10^9 VE/mL) (Supplementary Table 2). Lower viral loads were also detected in the lungs, gastrointestinal tract, kidneys, heart, and especially liver, indicating systemic viral spread. Histological examination of three ducklings that succumbed during acute infection, identified a moderate, acute to subacute, necrotizing meningoencephalitis with oligofocal to diffuse antigen detection (score 1–4) showing the same distribution pattern and target cells as described after 3 dpi for genotype #4, except for the spinal cord, that appeared unaffected. Oligofocally, individual ducks tested positive for virus matrix protein in the submucosal glands of the beak, the sinus and air sac as well as in the myocardium associated with necrosis (Supplementary Figure 3; 4). Although lacking virus antigen, the regenerative lesions in the olfactory and sinus epithelium (Supplementary Figure 3) and mild interstitial pneumonia (not shown) indicate past infection. No findings were recorded for the kidneys at 5-6 dpi.

Remarkably, at the end of the experiment on day 21 p.i. residual amounts of viral RNA were still detected by RT-qPCR in the brain of four out of six inoculated surviving animals; but not in other organs tested (Supplementary Table 2). The evaluation of the H&E and IHC slides did not yield any pathologic findings or virus antigen in the remaining tissues (Supplementary Figures 2–4). Similar to the IMPI group of the same genotype, at this late stage, no infectious virus was recoverable. Histological examination did not find any specific lesions in the brain (Fig. 3), but identified an irregular architecture of the olfactory mucosa and squamous metaplasia in the respiratory epithelium of the conchae in 2 out of 3 ducks, lacking antigen labelling (Supplementary Figure 3). Again, this could be an indication of a past infection with regeneration of the affected mucosal areas.

Genotype-related molecular determinants distinguishing highly from moderately virulent genotypes in ducklings

Molecular comparison of the various HPAIV H5N1 genotypes, as depicted in Fig. 1B, reveals that only 3 genes of genotype #3, the virus that dominated the HPAIV H5N1 epidemic in December 2021, are present in all other viruses. Besides the HA gene only NA- and M-gene of all investigated HPAIV H5N1 viruses are belonging to the same phylogenetic branch. However, the number of reassorted genes varied considerably for the different genotypes studied: For the other two HPAIV H5N1 genotypes with IMPI score of >2, either a single gene (genotype #7: NP) or three genes coding for proteins that are part of the polymerase complex (genotype #4: PB2, PA and NP) differ compared to genotype #3. Likewise, in the group of viruses with moderate duckling pathogenicity (IMPI < 2), an exchange of up to 4 segments could be observed. In addition to genes encoding for proteins of the polymerase complex (PB2, PB1, PA and NP), the NS gene (genotype #9) is derived from genetically divergent branches as genotype #3. The single exception within the group of viruses with moderate duckling virulence, is genotype #8: Compared to highly virulent genotype #7, only the PB1 gene is exchanged. Even though genotypes #7 and #8 share a related NP-gene that differs from genotype #3, this exchange alone obviously did not affect the IMPI. This points to PB1 as an important determinant for the decrease of duckling virulence of genotype #8. Phylogeny of the PB1 gene clearly distinguishes genotype #8 from the other HPAIV H5N1 viruses studied (Fig. 4A). This specific branch of genotype #8 (Fig. 4B) encloses HPAIV H5N3 from the same species than the studied H5N1 genotype #8 virus, i.e. red knots, circulating in 2020 and 2021 in Germany and France[48]. Besides, closely related PB1 genes were present in a variety of subtypes of low pathogenicity like H1, H3, H4 or H10 that were detected in samples from

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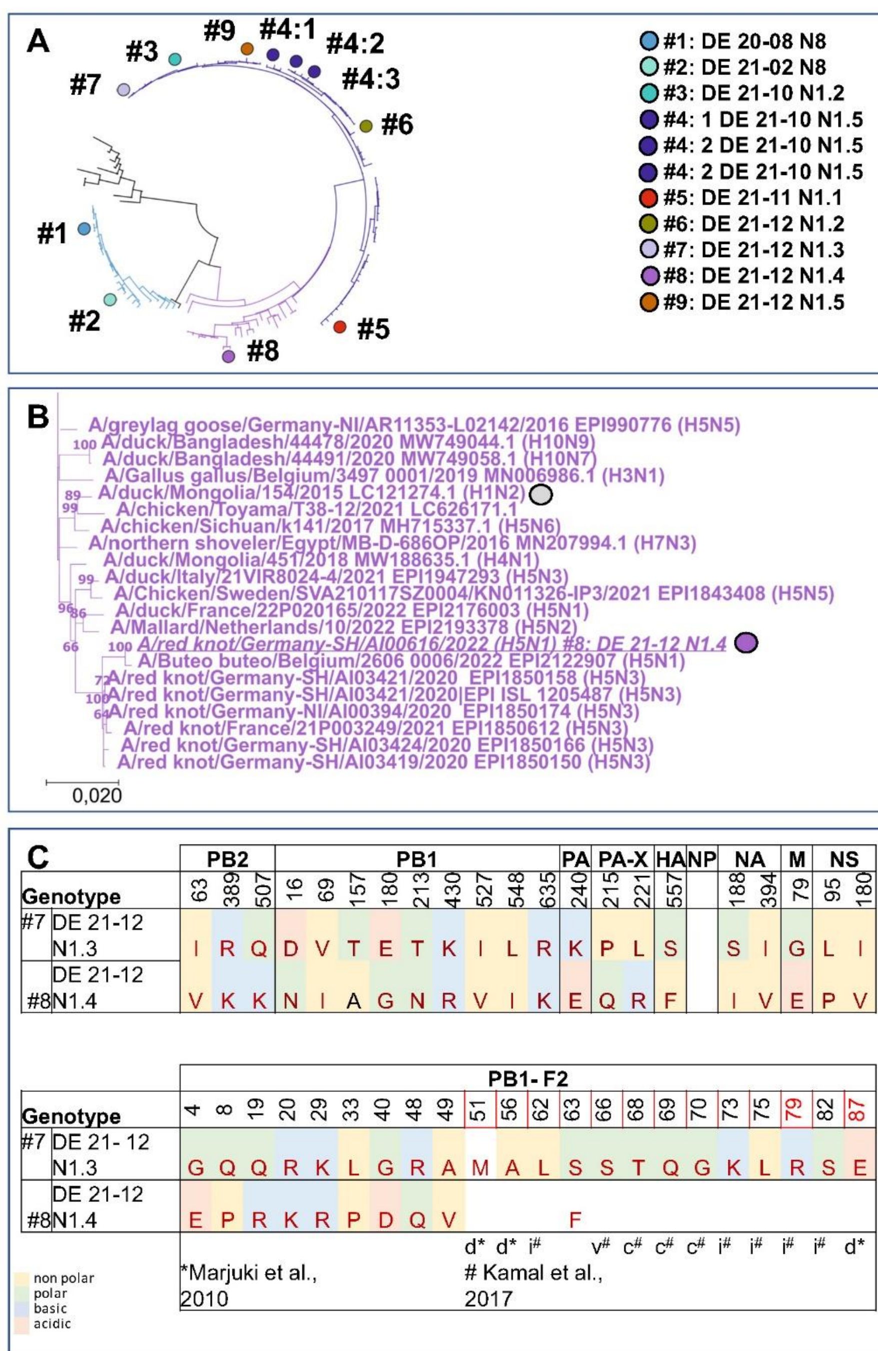

Fig. 4 | Analysis of PB1. Schematic representation of the phylogenetic relationship based on the PB1 gene of the genotypes tested. The unique color scheme has been used to distinguish genotypes in Germany since 2020, adapted from a custom palette out of the dashboard "HPAIV genotypes in Germany"[22] (**A**). **B** Shown is a detailed image of the genotype #8 group, presenting related viruses included in the phylogenetic analysis with genotype #8 and the oldest virus with a related PB1 gene indicated by dots (**B**). **C** Differences of deduced amino acids between highly duckling virulent genotype #7 and moderate duckling virulent genotype #8 are given for PB1 and PB1-F2. Sites recognized as being part of a inflammatory motif (i), cytotoxic motif (c) or correlated to virulence in mice but not in ducks (v) are indicated as reviewed[79] Kamal et al. In addition, sites associated with reduces pathogenicity in ducks (d) as described in ref. 85, are indicated but did not show differences.

wild birds, with the earliest detection in 2015 (A/duck/Mongolia/154/2015 (H1N2), grey dot). At protein level, genotype #8 virus accumulated altogether nine mutations in PB1 compared to genotype #7 (Fig. 4C). These mutations within the PB1 stretch all over the protein, but did not include the catalytic residues 446 or 447 (Fig. 4C). However, at position 180, located in the nuclear localization motif[49], glutamic acid in the highly virulent genotype #7 was replaced by a Glycine (E180G) in the moderately virulent genotype #8 (Fig. 4C). Besides, amino acid (aa) substitutions in PB1-F2, a protein translated from an alternative open reading frame (ORF) of segment 2 were present. Altogether, deduced PB1-F2 proteins of genotype #7 and #8 differed in 10 of the 90 aa (11,1%), which is remarkable compared to only 9 different aa in the PB1 ORF (aa 1,2%; 755aa). In contrast, proteins of the other five gene segments had no (NP) or only single point mutations compared to genotype #7 (Supplementary Table 7). This genetic comparison of natural occurring reassortants highlights that multiple genes may affect duckling virulence. Natural phylogenetic analysis in particular of highly and moderate duckling virulent genotype #7 and #8 respectively, indicate that point mutations within PB1 and PB1-F2 may attribute to this phenomenon of shift of duckling virulence.

Discussion

Numerous different genotypes of HPAIV H5 of clade 2.3.4.4b were circulating in avian species simultaneously in Germany over the last years. When analyzing virulence of an array of genotypes that were detected in the country within week 52 during winter 2021[22,44], genotype-specific differences in duckling virulence ranging from moderate to highly virulent became evident. In the succeeding months of 2022, two of these genotypes came to dominate the epidemic in Germany in 2022, i.e. genotype #3, and its successor, genotype #4. Both were highly virulent for ducklings with scores of 2.92 and 2.95-3.00 (out of a maximum of 3), respectively. In contrast, less virulent genotypes, such as #5, #6, #8 and #9 with markedly lower IMPI scores ranging from 1.65 down to 1.22 (Fig. 1B; Supplementary Figure 1), faded out in the epidemic of the following months of 2022. In light of these results it is intriguing to argue that genotypes of higher duck virulence may gain epidemiological advantages in wild birds over those with reduced virulence. In this respect our results are not consistent with the 'avirulence hypothesis'. Alternatively, the "virulence-transmission trade-off" model of virulence evolution of pathogens seems more appropriate in describing the epidemiological situation of HPAIV in Germany in the period 2021/2022. In the latter model, virulence and transmission can be positively correlated at least as long as transmission of a certain viral lineage to other hosts is advantageous compared to other lineages and not limited by the number of susceptible individuals in the given host populations[50,51], i.e. here the huge multispecies metapopulations of wild aquatic birds and poultry. Such view would be in line with the concept that virulence in a certain host can be higher if a pathogen is a generalist and can easily jump between host species[52]. In this respect, the growing number of susceptible species reported in the course of the recent global spread of the HPAIV 2.3.4.4b virus (now including even species from the Antarctic ecosystem) add to the huge reservoir of a metapopulation of avian hosts accessible to these viruses. In that context, virulence associated with a high case-fatality rate in a host species might not be considered as a limiting factor, but rather as a concomitant of an increased production and release of virus progeny[51]. In fact, a comparison of LPAIV and HPAIV H5N2 viruses in chicken[53] provided evidence that despite mortality due to HPAIV between day three to day 10 post infection, the reproduction number R_0 of the HPAIV infection was higher than that of the corresponding LPAIV, leading to early infection of the HPAIV sentinel animals, i.e. between day three and four post infection. Even though the authors acknowledge limitations of their studies with respect to extrapolation to the field, our observations strengthen the view, that all factors promoting transmissibility are key drivers for the current evolution of HPAIV H5. The results of the o.n. infection study that we also performed here, comparing genotype #4 with genotype #8, i.e. a highly prevalent virus with high duckling virulence versus a virus with low prevalence and intermediate duckling virulence, support the latter notion, indicating superior virus shedding of the more virulent genotype also after inoculation along more natural infection routes (Fig. 2). However, it is important to note that individual host-specific factors may also contribute: Several ducks infected with the high virulence genotype #4 proved to be "super-shedders", while in two animals the viral load in throat swabs was picayune, not exceeding a maximum titre of 2×10^3 VE/mL. In contrast, the two infected sentinel animals reached a titre of 10^{10} VE/mL, a 10^7-fold difference in virus release (Fig. 2). Beside the quantity of excreted virus, the route of shedding should be considered: While in the group inoculated with high virulence genotype #4 high amounts of virus were present in pharyngeal as well as in cloacal swabs, shedding by the enteral route was almost negligible for the moderate virulence genotype #8. Recently, surface water was demonstrated to be an important medium for HPAIV transmission[54,55]. In conjunction with the notion that shedding of evolutionary successful LPAIV is dominated by the enteral route[28,56], this finding would clearly emphasize the importance of the enteral route of shedding for the transmission dynamic of HPAIV H5 in wild water birds. The putative relevance of an indirect transmission mode may be another factor in favor of the virulence-transmission trade-off model: If the pathogen is spread by a fomite i.e., by surface water, transmission does not depend on direct interaction of infectious and susceptible individuals. Thus, infection chains can be sustained even though the host was swiftly killed by the pathogen. Dominance of genotypes with high virulence for ducklings would then indicate that, as a net effect, transmission efficacy and high virulence are not negatively correlated for certain clade 2.3.4.4b variants during the enzootic situation in Germany and ultimately contribute to their wide dispersal. However, these assumptions have to be taken with caution, since several limitations of the study have to be considered:

(i) Pekin ducks, as a surrogate of mallards, have been tested here as a single avian species at a very young age: It cannot be excluded that results would differ with other species or other age cohorts. Yet, mallards, being the closest relative in the wild of domestic Pekin ducks, are an abundant species with a global distribution that is deemed to play an important epidemiological role in the field.

(ii) All isolates were derived from diseased or dead hosts, introducing a possible bias towards more virulent genotypes: As such, it seems all the more astonishing that even then, a substantial number of strains have been identified that expressed only moderate pathogenicity in ducklings even in this harsh infection model. In general, HPAIV monitoring in the EU is focused for almost two decades on passive monitoring systems. Few HPAIV detections have been made by active monitoring investigations. Yet, it cannot be excluded that other genotypes exist which predominantly cause clinically mild or even asymptomatic courses of infection that are missed by passive surveillance.

(iii) An inoculation model, the IMPI, was chosen that is not regularly used for HPAIV pathotyping: Routine IVPI pathogenicity testing in chickens most often creates only categorical (yes/no) results (HP or not; dead/alive) that are not useful to pinpoint gradual differences in pathogenicity between strains. In previous experiments[32] we showed that i.m. inoculation into week-old ducklings, in contrast to IVPI in chickens or occulo nasal application in adult ducks, provided a readout system that allowed a finer distinction of pathogenicity in semiquantitative terms. In this respect age has to be considered. It is well known, that younger ducks are more susceptible to disease[57–59]. Thus, choosing one-week old ducklings represents a sensitive way to distinguish virulence in particular of strains with low or moderate duck virulence like clade 2.3.4.4c or 2.2 viruses[60]. For other strains with high duck virulence, expressing IMPI scores close to the endpoint of 3, like genotypes #1-#4, an infection model using older birds might be more suitable to distinguish differences in virulence[61]. In any case, our results emphasize, that the IMPI is a model system that allows to differentiate duck virulence.

(iv) Intramuscular inoculation is an artificial route and may have skewed the course and outcome of infection: Intramuscular infection is a well approved infection mode for testing vaccine efficacy according the

European pharmacopoeia, for example for Newcastle disease. Like the IVPI, it is a parenteral application, thereby circumventing primary local replication at the entry site. A faster systemic spread could explain the differences in onset of a clinical manifestation that was indeed present when testing genotypes #4 and #8 in parallel trials where the same virus has been inoculated occulo-nasally and i.m. However, principal differences in virulence between genotypes were evident regardless of the inoculation mode (parallel testing of o.n. and i.m. routes for genotypes #4 and #8 (Fig. 2)) but the IMPI model provided differentiation of virulence in a pronounced way.

(v) The inoculation dose was not normalized between the groups and differed between 2.86×10^3 (genotype #2; IMPI 2.69) and 5.48×10^7 $TCID_{50}$ (genotype # 4:2; IMPI 2.95) (Table 1): Like in the diagnostic IVPI, we followed the rule that a virus stock should have an HA-titer of >16. Under this rule we observed that the viruses with the lowest (#2) and the highest (#4) viral titers induced almost the same IMPI despite a gap in infectivity of 4 log_{10} steps. This would strengthen the notion deduced from the official (legal) IVPI testing that the titre of infectivity, i.e. the inoculum dose is not a key determinant for pathogenicity provided it exceeds a certain threshold. Indeed, this assessment would be in line with observations from titration experiments in ducks with a clade 2.3.4.4b HPAIV H5N1[29,61]. In this example (Spackman et al.[29]), onset and severity of clinical signs after occulo- nasal infection were indistinguishable for groups inoculated with a dose of 10^4 or 10^6 $TCID_{50}$. However, in birds inoculated with 6.3×10^1 $TCID_{50}$ per bird, onset of clinical disease started two days later; this would clearly influence a clinical score as applied here. In conclusion, this would support the notion that the inoculum has to exceed a certain threshold, beyond which a clinical disease index is no longer correlated to the amount of infectivity in the inoculum. Yet, for further standardization of similar experiments, a minimum amount of 10^5 $TCID_{50}$ should be recommended.

Investigating pathogenesis for high and moderate virulence genotypes #4 and #8, respectively, after occulo-nasal application, verified differences of virulence between the two genotypes, and demonstrated a slower progression to disease after o.n. compared to i.m. virus application: Whereas after i.m. application of genotype #4, nine of ten ducks were dead by 1 dpi, clinical signs started in the o.n. group at 2 dpi, but became apparent only in three of ten ducklings. Likewise, for genotype #8, incubation time was prolonged from 3 dpi to 5 dpi for the i.m. and o.n. inoculated groups, respectively, before individual birds showed signs of disease. However, regardless of the genotype, it became evident, that all diseased birds suffered from a systemic infection, as demonstrated by virus detection in various organs. Abundant viral replication was detected for both genotypes in the brain, with a dominance of viral antigen during the acute phase in the forebrain. Most interestingly, viral antigen was present in histopathological examinations of two out of three i.m. inoculated ducklings infected with moderate duckling virulence genotype #8, at 10 dpi, i.e. at a time when none of the animals showed any clinical signs of disease. Antigen detection was accompanied by severe, subacute, necrotizing meningoencephalitis in both antigen-positive birds. However, the third/remaining duckling presented moderate inflammation, lacking intralesional virus antigen, indicating a potential natural healing process. These observations would argue, that also in animals showing only moderate clinical signs of disease after genotype #8 infection, virus invasion into the CNS was sufficiently controlled. The conclusion of limited brain infection of the moderately virulent genotype #8 could explain the puzzling observation in o.n. inoculated ducklings euthanized at the end of the experiment at 21 dpi, where residual amounts of viral RNA were detected in the brain without histological lesions (Fig. 3). The latter indicates that for some animals, brain invasion was prevented or at least significantly limited after occulo-nasal infection. The rapid onset of infection control across the blood-brain barrier would support the notion that it is related to innate immunity, and independent of the specific immune response[62,63]. In our view, neuronal infection with a virus of moderate virulence for ducklings could provide a valuable system to study these virus-host interactions.

For the majority of genotypes with reduced duckling virulence, several genome segments differed in comparison to dominant high virulence genotype #3. In three of the four genotypes with decreased duckling virulence, i.e. genotypes #5, #6 and #9, at least two of three segments coding for the viral RNA-dependent RNA polymerase (RdRP)[64,65] were changed, i.e Polymerase basic protein 2 (PB2), Polymerase basic protein 1 (PB1), and Polymerase acidic protein (PA) respectively. In addition, the nucleoprotein (NP) was different compared to genotype #3. NP, forming a helical scaffold that packages and protects the viral genome[66], is associated to viral RNA (vRNA) and together with the RdRP is the integral part of the viral ribonucleoprotein (vRNP) complex. These observed changes of segments coding for the vRNP complex indicates a contribution to decreased duckling virulence, a notion that is supported by experimental data for other strains[67–71]. However, genotype #4, the genotype that subsequently dominated HPAIV H5N1 epidemiology in 2022 (see Fig. 1B), also differed with respect to RdRP, i.e. PB2, PA and NP, compared to genotype #3 (see Fig. 1A). Considering the complex interactions of RdRP in conjunction with its interplay with NP (for review, see[72,73]), it is conceivable that a new composition in reassorted genotypes affects viral transcription and replication, and thus virulence, in a pleiotropic manner. In this context, differences in virulence would not only be associated with a particular segment, but could also result from the context with other components of the replication machinery, i.e. the gene constellation. In addition, segment 8, which encodes non-structural protein 1 (NS1) and nuclear export protein (NEP), was different in genotype #9 compared to genotype #3 and may have contributed to the reduced virulence in ducklings, as shown in previous studies for other strains[74].

An exception from this observation is the moderate virulence genotype #8 in which only a single segment, i.e. PB1, differed from the high virulence genotype #7. Specific mutations within PB1 have been reported to facilitate an expanded host range[64] or to convey adaption to host innate immunity[75]. One striking difference was observed at position 180 (E180G), a site identified as one of two mutations within PB1 (E180D, M317V) that were shown to increase polymerase activity in chicken cells and plaque sizes in chicken and duck cells[71]. On the other side, a higher polymerase activity may not be directly correlated to virulence by producing higher virus loads as indicated by studies on HPAIV H5N8 viruses from outbreaks in 2014–15 to 2016–17, but rather influenced virulence through species-specific levels of induced IFN-ß[76]. This observation is in line with in vivo data, comparing innate immune responses in ducks and chicken with regard to virulence[77]. It remains to be investigated whether this phenomenon is linked to the observed differences in virulence of these specific genotypes.

Likewise, differences in PB1-F2 of moderately virulent genotype #8 are noteworthy. This protein, translated from an alternative open reading frame (ORF) of segment 2[78] is associated to both pro-apoptotic and polymerase enhancing functions[79,80]: In all tested HP AIV H5N1, but not in the H5N8 viruses (Supplementary Table 8), full length PB1-F2 ORF was present, a finding that is in line with the observations that the ORF is well-conserved in avian influenza virus isolates[81] and reported to be highly virus-isolate specific with respect to influence on virulence[80]. Studies in chicken indicate, that presence of PB1-F2 contributes to an optimized spread of the virus without increasing the virulence[82,83], while other studies describe a slightly prolonged survival time of chickens infected with PB1-F2 deletion mutants[84] and delayed onset of both clinical symptoms and systemic spread of virus in ducks[70]. Besides, aa substitutions in the N-terminal domain of PB1-F2, at positions T51M, V56A and E87G were accompanied with reduced virulence for mallard ducks and shortened time of virus shedding[85]. However, both genotypes, i.e. #7 an #8, that differed in segment 2, had an identical aa composition in these three sites, sharing two sites associated with lowered virulence and one aa site with increased virulence (underlined aa).

Combined with the observed differences in duckling virulence, our results would justify further work on the role of PB1 /PB1-F2 in species-specific virulence in Anseriformes.

Analysis of naturally occurring reassortant genotypes of clade 2.3.4.4b HPAIV H5 suggests that high virulence in ducklings was not a limiting factor for epidemiological dominance in Germany. From a swarm of seven concurrently circulating HPAIV H5N1, it appeared that viruses with high duckling virulence were the most successful in terms of geographical spread and duration of circulation[22], while genotypes with lower virulence remained in the minority during outbreaks and were detected in very limited geographical areas only. Therefore, assuming that the virulence-transmission trade-off model is more consistent with the emergence and dominance of high virulence strains, this would suggest that the size of the susceptible population is not currently a limiting factor for HPAIV evolution. Thus, these strains can be expected to retain high duckling virulence if this continues to promote improved transmissibility, as suggested for the strains studied here. Consequently, breaking this vicious circle would require a reduction in the susceptible population. In wild birds, this can only be achieved by gradually increasing population immunity mediated by survivors of previous infections. In poultry, however, this could be achieved by vaccination.

Data availability

Data is presented within the paper or in the supplementary files. All reference sequences are available on Zenodo under https://doi.org/10.5281/zenodo.8233814. Sequences in this study are available in the GISAID EpiFluTM database under accession numbers: A/chicken/Germany-NW/AI03705/2021 (accession 5146288), A/seal/Germany-SH/AI05373/2021 (accession 4804850), A/European herring gull/Germany-MV/AI01411/2022 (accession 18006762), A/chicken/Germany-NI/AI04286/2022 (accession 16096050), A/herring gull/Germany-SH/AI01196/2022 (accession 17693298), A/brent goose/Germany-SH/AI02407/2022 (accession 17693301), A/herring gull/Germany-SH/AI07088/2021 (accession 17693297), A/chicken/Germany-MV/AI01026/2022 (accession 18006937), A/pigeon/Germany-NW/AI00951/2022 (accession 10261376), A/red knot/Germany-SH/AI00616/2022 (accession 5098132), A/black-headed gull/Germany-HH/AI01073/2022 (accession 18006938).

Code availability

GraphPad Prism in software version 8.0.1 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. SigmaPlot™ in version 11 (SigmaPlot Software, Grafiti LCC, Palo Alto, CA, USA) was also used to double-check the statistical data. Hamamatsu S60 scanner (Hamamatsu Photonics, K.K. Japan) and software NDPview.2 plus in version 2.8.24 were used for histopathological analyses. Geneious Prime in version 2021.0.1, as well as the tool Minimap2 in version v2.24 as well as the phylogenetic analysis tool MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms software version 10.2.4 (Kumar, Stecher, Li, Knyaz, and Tamura 2018)[45] were used for sequence analyses. Further data on sequences etc. are provided within the supplemental material as well as available upon request.

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Author contributions

M.B., T.C.H. and C.G. devised the project and the main concept. R. P. and C. G. carried out the infection experiments. A. P., A K. A., R.P. and C.G. carried out sequence and phylogenetic analyses. A. B. carried out the pathological and histopathological examinations. R.P. and C.G. processed the experimental data, performed initial analysis and draft of the manuscript with input from T.C.H. Design of the figures was made by R.P. and C.G. with assistance of A.P. Statistical analyses was performed by R.P. and C.G. T.C.H and M.B. critically revised the article. All authors approved the final manuscript.

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4.2 Publication II

Low Susceptibility of Pigs against Experimental Infection with HPAI Virus H5N1 Clade 2.3.4.4b

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Low Susceptibility of Pigs against Experimental Infection with HPAI Virus H5N1 Clade 2.3.4.4b

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We found that nasal and alimentary experimental exposure of pigs to highly pathogenic avian influenza virus H5N1 clade 2.3.4.4b was associated with marginal viral replication, without inducing any clinical manifestation or pathological changes. Only 1 of 8 pigs seroconverted, pointing to high resistance of pigs to clade 2.3.4.4b infection.

Spread of highly pathogenic avian influenza (HPAI) virus H5N1 clade 2.3.4.4b of the goose/Guangdong (gs/GD) lineage, has exacerbated since early 2022 into a panzootic (*1*). Regional enzootic status in wild bird populations in Europe and North America, with lethal courses of HPAI virus infection in some species, produced large numbers of wild bird carcasses, easy prey for raptors and scavengers. Exposure of

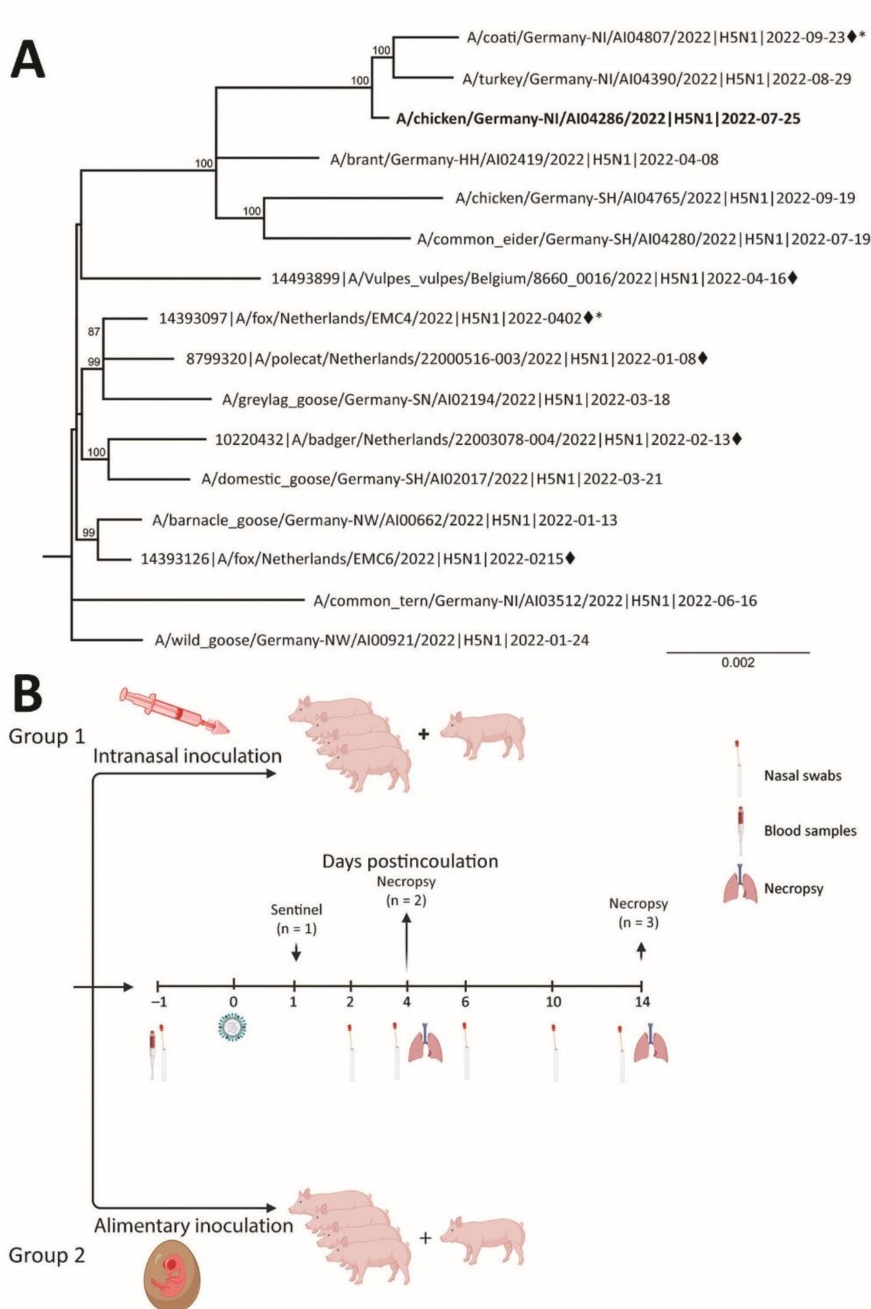

Figure. Phylogeny and experimental design for study of susceptibility of pigs against experimental infection with highly pathogenic avian influenza (HPAI) (H5N1) virus clade 2.3.4.4b. A) Maximum-likelihood phylogenetic tree (RAxML, https://cme.h-its.org/exelixis/web/software/raxml) based on 8 concatenated genome segments of selected recent HPAI H5N1 viruses from naturally infected avian hosts and from mammalian hosts (black diamonds) in Europe. Bold indicates study isolate A/chicken/Germany/AI04286/2022. Asterisks (*) indicate sequences with polymerase basic 2 E627K mutations. B) Scheme of the experimental setting of HPAI H5N1 virus infection of pigs. Four pigs, 4 months of age, were inoculated with 10^6 $TCID_{50}$ in 2 mL using mucosal atomization devices. Four pigs were each fed with 1 HPAI H5N1 virus–infected embryonated chicken egg, carrying ≈10^8–10^9 $TCID_{50}$/mL of allantoic fluid. Each pig was offered an egg, separately, in a trough and observed to complete consume it. Ten-day-old eggs were inoculated with 0.2 mL of clarified amnio-allantoic fluid of egg passage 1 and incubated for 3 days or until embryonic death was evident. Eggs were chilled until fed to pigs. Panel B created with BioRender.com and licensed by the company (agreement no. UC258UM8J3). $TCID_{50}$, 50% tissue culture infectious dose.

terrestrial carnivores and marine mammals resulted in sporadic infections, some of those terminating with fatal encephalitis (*2*). Frequent spill-over events, rather than consistent mammal-to-mammal transmission, were at the basis of these cases (Figure, panel A). However, recently reported HPAI outbreaks among sea lions along the Pacific coast of South America and an outbreak in a mink farm in Spain (*3*) may constitute first examples of avian-independent transmission chains and increase public health concerns about zoonotic transmissions of this virus. Still, the total of 11 human cases globally reported for the currently dominating H5N1 2.3.4.4b lineage did not point toward increased zoonotic propensity (*4*).

Possible adaption of avian influenza virus (AIV) to mammalian livestock hosts and subsequent

Table. Detection of seroconversion and of influenza A viral loads in tissues and nasal swabs of experimentally infected pigs exposed by intranasal or alimentary inoculation with HPAI virus H5N1 clade 2.3.4.4b virus*

			GEq, by qRT-PCR							
Infection route	Day postinoculation	Animal ID	Nasal swabs†	Conchae nasalis	Trachea	Lung	Ileocaecal tonsil	Colon	Brain	NP-ELISA seroconversion
Intranasal	4	1	30	–	–	–	–	–	–	–
	4	2	150	–	–	–	–	–	–	–
	14	3	30	10	–	–	–	–	–	–
	14	4	10	60	–	10	170	200	350	+
	14	Sentinel	–	–	–	–	–	–	–	–
Alimentary	4	1	–	–	5	–	–	–	–	–
	4	2	30	–	–	–	–	–	–	–
	14	3	20	–	–	–	–	–	–	–
	14	4	140	–	–	–	–	–	–	–
	14	Sentinel	–	–	–	10	–	–	–	–
Intranasal	4	1	300,000	10,000	860,000	5,400	NA	NA	NA	–
positive	4	2	83,000	35,000	56,000	1,500	NA	NA	NA	–
control‡	4	3	750,000	4,300	1,600,000	1,500	NA	NA	NA	–
	4	4	710,000	29,000	520,000	1,100	NA	NA	NA	–

*Shown are only tissues for which >1 animal has tested positive. GEq, genome copy equivalents per 0.1 mL calculated on the basis of qRT-PCR quantification of cycle values; HPAIV, highly pathogenic avian influenza; ID, identification; NA, not applicable; qRT-PCR, quantitative reverse transcription PCR; +, positive; –, negative.
†Nasal swabs positive at day 2 postinoculation for the intranasal group and day 4 postinoculation for the alimentary group.
‡Four pigs, infected with the same device and dose of a swine-adapted influenza A virus strain (subtype H1avN1, clade 1C2.1) of a former study are included for comparison (*11*). Pigs were sacrificed at day 4 post infection when no seroconversion was to be expected; gastrointestinal tissues and brain were not examined.

human exposure is of particular concern. In this respect, the role of pigs as a "mixing vessel" for HPAI viruses is largely unresolved. AIV can potentially be transmitted to pigs, and further reassortment with swine influenza A viruses (swIAVs) may contribute to the emergence of pandemic strains. Rare and subclinical infections of pigs with gs/GD HPAI virus have been confirmed serologically in Vietnam, Thailand, and France (*5*) and virologically in Indonesia (clades 2.1.1 and 2.1.3), Nigeria (clade 2.3.2.1c), China (clade 2.3.4), and Italy (clade 2.3.4.4.b) (*6*–*8*).

For our study, we purchased 4-month-old pigs (6 male, 4 female) from a conventional pig holding in Germany and exposed them nasally or by the alimentary route to high doses of the recent avian-derived HPAI virus H5N1 2.3.4.4b isolate A/chicken/Germany/AI04286/2022 (genotype Ger-10.21-N1.5). The egg-grown isolate was closely related to a mammal case but lacked any mammalian-adaptive mutations (Figure, panel A). We inoculated 2 groups of 4 pigs each intranasally or by feeding 1 infected embryonated chicken egg per animal. One sentinel pig per group was associated at day 1 postinoculation to assess the transmission by direct contact to those inoculated (Figure, panel B).

After exposure, HPAI virus H5N1 detection by real-time RT-PCR was limited to day 2 (intranasal group) and day 4 (oral group) postinoculation, with a range of 10–150 genome copy equivalents per 0.1 mL (Table). An avian-derived, swine-adapted H1avN1 strain intranasally inoculated into naïve pigs at the same dose and by the same device produced 3–4 log_{10} levels greater nasal excretion in comparison (Table) (*9*). It cannot be excluded that HPAI virus H5N1 detected in nasal swabs at day 2 postinoculation still represents residual inoculum. Correspondingly, except for 1 alimentary inoculated that showed tracheal viral loads at day 4 postinoculation close to the detection limit, samples from the remaining 3 pigs euthanized at day 4 postinoculation gave no indication of viral replication in respiratory or gut tissues, regardless of the inoculation route. Only in 1 intranasally inoculated animal, euthanized at day 14 postinoculation, was viral RNA detected at low levels in organ samples. In addition to samples from the respiratory and alimentary tracts, minute amounts of RNA were found also in a brain sample of that pig. The virus could not be isolated using chicken hepatoma cells and MDCK-II cells, and histologic and immunohistochemical investigations gave no evidence for inflammatory reactions or presence of viral antigen. Low viral loads in this pig impeded sequence analysis of eventual mutants. Nonetheless, this pig was the only animal that seroconverted at 14 dpi. In agreement with the virologic findings, none of the pigs showed any clinical signs or fever within 14 days of observation.

In conclusion, only 1 of 8 pigs inoculated intranasally with HPAI virus H5N1 underwent transient, low-level infection that resulted in the presence of viral RNA in several tissue specimens and seroconversion at 14 dpi. In naturally infected wild mammals, this virus was prominently detected in the brain (*2*).

Given the detection of viral RNA in the brain of 1 intranasally inoculated pig, it cannot be excluded that longer observation might have revealed continuing viral replication in the brain of this animal. Sialic acid α2,3-gal receptors dominate on porcine brain cells, which might have fostered replication of α2,3-adapted viruses, such as HPAI virus H5N1 (*10*).

Overall, we conclude that pigs are unlikely vehicles in transmitting this genotype of HPAI virus H5N1 clade 2.3.4.4b among pigs and across interfaces. However, considering the ongoing massive panzootic of this virus, a plethora of new genotypes of the circulating strain is emerging, with possibly higher permissiveness for pigs. Therefore, swine populations need to be part of HPAI virus surveillance programs, and periodic reassessment of prepandemic propensity of circulating HPAI virus H5N1 genotypes in the swine model is required.

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V. Discussion

After repeated incursion of HPAIV H5 of the goose/Guandong (gs/GD) lineage into Western Europe, including Germany, with temporal and spatial limited epidemics, clade 2.3.4.4b viruses established endemicity in Germany and lead to a worldwide panzootic. The concomitant continuous viral burden in wild birds went along with the emergence of a plethora of reassortants with detected 46 H5Nx genotypes between October 2020 and March 2024 (Pohlmann 2023b). This vast number of reassortants is a clear indication for the high prevalence of clade 2.3.4.4b HPAIV H5 in wild bird species as genetic reservoir for new gene constellations. It remains unclear, whether genetic composition of newly emerging genotypes influenced virulence thereby facilitating silent spread in wild bird species. Besides, regular deadly spillover infections to wild carnivores and farmed fur-bearing animals raises concern of adaptation of AIV to mammalian species.

The presented work addresses both questions: Is decreased virulence associated to viral fitness with regard to epidemiological dominance, and is a virus prevalent in dead mammalian species adapted to mammalian species i.e. able to replicate in pigs.

5.1 Reconciling dominance of phylogenetic genotypes to virulence in Pekin ducks (Study objective I)

The first study elaborated whether the dominance of certain genotypes (as measured by their detection frequency) is associated with a decrease in virulence in a suitable indicator species, as postulated by the 'avirulence hypothesis' originally proposed by Theobald Smith (Smith 1904).

As model system, Pekin ducklings (*Anas platyrynchos var. domesticus*) were chosen as they are the domesticated form of Mallards (*Anas platyrynchos*) (Qu et al. 2009) and are the most abundant wild duck species in the northern hemisphere (Anderson and Rhymer 1987; Kulikova et al. 2005; Kaminski and Elmberg 2014). Mallards, like Anseriformes in general, are highly susceptible to both LPAIV and HPAIV but may be resilient to clinical disease. Thus, Mallards are considered an important natural host to spread of gs/GD HPAIV (Olsen et al. 2006; Brown and Stallknecht 2008; Gobbo et al. 2021). Previous studies have shown that differences in virulence between HPAIV H5 genotypes of clade 2.3.4.4a and 2.3.4.4b could be demonstrated by experimentally inoculating mallard ducks: While the differences of clinical manifestations were rather marginal after infection of adult ducks due to clinical resilience against HPAIV, they were quite pronounced in juveniles (Grund et al. 2018). In consequence, one-week old ducklings were inoculated via the intramuscular route (IMPI) – in analogy to the well-established Intravenous Pathogenicity Index (IVPI), used to pathotype AIV. To enhance data reproducibility, the intramuscular route was selected as a less error-prone inoculation method due to the presence of small-sized veins in young ducklings. In addition, a one-week acclimatization period was implemented to reduce the risk of spontaneous death within the first week of life and thereby skew the clinical scoring. The results clearly demonstrate, that the IMPI in juvenile ducks instead of the IVPI in adult chickens is

able to differentiate virulence between HPAIV strains. Whereas highly susceptible chickens perish rapidly after inoculation with HPAIV and can therefore only give yes/no results for pathotyping, the clinical scoring in ducklings provided a graded evaluation of the virulence. This is in agreement with virulence studies carried out for viruses of clade 2.3.4.4c H5N8 2014 and clade 2.3.4.4b H5N8 2016 in chickens and ducks (Beerens et al. 2019). Likewise, differences in virulence between HPAIV H5 genotypes from different years and clades have also been shown recently in an ex vivo model using embryonated chicken and duck eggs (Bordes et al. 2024). However, both studies, compared strains that represented dominant genotypes at a particular time. In the recent approach, different HPAIV H5N1 genotypes circulating at the same time were compared. That included viruses that were the most abundant, as well as minor genotypes. In addition, preceding HPAIV H5N8 strains were included in this study. This approach enabled a retrospective correlation of virulence with respect to viral fitness on the epidemiological level.

Interestingly, all four genotypes that dominated for a certain time period were of particular high virulence in the ducklings with IMPI scores of 3.00-2.52 (LPAIV<1.2>HPAIV). In contrast, most genotypes that were found only sporadically demonstrated diminished virulence in ducklings with IMPI scores between 1.65-1.22. It is not possible to ascertain, whether this elevated virulence in ducklings is biased by the passive monitoring surveillance, i.e. detected viruses are derived from dead animals. This would possibly favour identification of viruses with high virulence and therefore could result in a significant bias in genotype detection. Nevertheless, a number of active monitoring activities have been conducted in which HPAIV has been identified (Gobbo et al. 2021; Pannwitz et al. 2009). To successfully identify genotypes with a milder virulence and thus gain a broader understanding of existing genotype developments over time, expanded active monitoring would be needed, for instance by follow-up studies of infected animals (Harder et al. 2023). However, identification of the genotypes with lower virulence by the current EU monitoring program indicates that also by passive monitoring a variety of phenotype can be detected.

In general, it has to be emphasized, that the results of the IMPI do not represent the clinical course of HPAIV infection in adult immune competent ducks. Multiple studies demonstrate that mortality in Pekin ducks, as a suitable infection model for infection of mallards, is low (Keawcharoen et al. 2008; van den Brand et al. 2018; Tanikawa et al. 2022). Animals can be subclinically infected with HPAIV of the gs/GD lineage and shed virus. Nevertheless, the results of the IMPI in conjunction with the prevalence data, indicate that HPAIV H5 of clade 2.3.4.4b with high virulence can persist and do not attenuate during the current endemic situation. This somehow contradicts the ‘avirulence hypothesis’ and argues that the 'virulence-transmission trade-off hypothesis' (Anderson and May 1982) is describing the current enzootic situation better. This model takes into consideration, that the death of the infected host shortens the period of time in which the pathogen can be transmitted. In this sense, variants have an evolutionary advantage, if virulence and transmission are positively correlated (Kun et al. 2023). A prerequisite for highly virulent viruses would be that they manage to spread before killing the initial host. In the

experiments it was evident, that after occulo-nasal infection, virus shedding started from 2 dpi before any onset of disease. In addition, viral load was higher in swab samples from ducks infected with the highly virulent genotype compared to a mildly virulent genotype. These findings are consistent with infection studies in mule ducks, which already excreted high virus titres at 1 dpi via the oropharyngeal, cloacal, conjunctival and feather route without showing any signs of infection (Filaire et al. 2024). The likelihood of virus transmission is increased in light of the rapid and high rate of excretion, particularly in ducks, even prior to the manifestation of disease symptoms. This phenomenon contributes to viral contamination of the residents environment (Filaire et al. 2024; James et al. 2023). A review of the situation in waterbirds in particular reveals that AIV not only persists in resident species, but that migratory and partially migratory species play a significant role in the continuous infection dynamics and transmission (Lisovski et al. 2018). This is evidenced by, next to others, the considerable numbers of animals congregating at stopover sites, which elevate the risk of virus transmission from and to non-migratory animals that they inevitably encounter. In addition to the sheer population density, migratory species are subject to an increased risk of infection due to the physical exertion of the journey and that they repeatedly encounter new virus strains on their route, against which their mostly strain-specific immunity is insufficient to fight, must be taken into account. Furthermore, bird migration is discontinuous for many species, like mallard ducks for example, with not all birds migrating at the same time but rather in phases that may be weeks or even months apart. This results in a phenomenon known as migratory replacement, in which the first animals, which may have already acquired immunity, are repeatedly replaced by later travellers without immunity. These individual migration patterns contribute to bird migration being a continuous source of new viruses to reassort with resident strains on the one hand and of repeatedly vulnerable hosts on the other (Lisovski et al. 2018). To further support the notion, that transmission is a key feature for dominance of certain clade 2.3.4.4b HPAIV H5 variants, and that interplay between migratory and resident wild bird species is crucial for virus evolution, further studies on transmission efficacy, determining the reproduction number R_0 would be needed.

While current HPAIV genotypes appear to circulate primarily in waterfowl and wild birds (James et al. 2023), circulation of HPAIV H5 of clade 2.3.4.4b in domestic poultry holdings has to be considered to contribute to the current endemicity. The increased prevalence of HPAIV in wild birds greatly promotes the risk of infection for highly susceptible domestic poultry species, making strict separation of domestic and wild fowl essential. While inter-species and inter-flock transmission of HPAIV was suspected in poultry outbreaks from 2020-2022, current outbreaks tend to be segregated. This may be due to lower virus shedding rates in poultry compared to waterfowl, or a suspected adaptation of the current strains to wild birds (James et al. 2023). These findings are supported by cases of silently infected commercial broilers with HPAIV H5N1, which also shed little virus but did not seroconvert (Gobbo et al. 2022). The combination of these circumstances is a cause for concern: although there are possible explanations for the absence of symptoms in broilers, such as the presence of resistance genes to clade 2.3.4.4b viruses in some breeds (Perlas et al. 2021), their basic susceptibility in the absence of immunity raises the

possibility of a new reservoir function for HPAIV in domestic poultry. Introduction of HPAIV into an enormous population of susceptible birds in a confined space, with various connections between holdings is setting selection criteria for highly transmissible variants (Gobbo et al. 2022). Thus, spillover events from infected poultry to wild birds would not only increase the overall virus burden, but also contribute to dominance of virulent clade 2.3.4.4b HPAIV H5 variants. Further, there is a risk of reassortment events with LPAIVs, generally harbored in domestic poultry. LPAIV, such as H6 or H9 for example serve as donors for viral segment exchanges and can lead to emergence of further HPAIV variants (McMenamy et al. 2024). A particular danger is posed by H6 viruses, which are now widespread in poultry worldwide and even of enzootic status in Asia (Everest et al. 2020; Li et al. 2019). In this context, biosecurity of poultry holdings is not only essential for protecting poultry flocks, but also as integral part to control clade 2.3.4.4b HPAIV H5 in general. In light of the worldwide growing demand for poultry products this might become a global challenge (OECD and FAO 2024). Already in the past, there has been an enormous increase of meat production which is indicative of the general poultry density. For example, within 12 years, from 2010 to 2022, poultry meat production in Asia increased by 36.3% (3.5×10^7 to 5.4×10^7 tons poultry meat/year), in China at least by 28.7% to 2.4×10^7 tons poultry meat/ per year and in Africa at least by 42.3% to 8.2×10^6 tons poultry meat/year. Likewise, a continuous growth in poultry meat production was observed in the Americas and Europe, too, (FAO 2024) (see Tab 2). Under this circumstances, it remains a challenge to minimize cross infection between the wild bird-poultry interface (Gilbert and Pfeiffer 2012).

Table 2. Development of poultry meat production in tons (t)

Area	2010	2022	Development (%)
Asia	3.5×10^7	5.4×10^7	36.3
China	1.7×10^7	2.4×10^7	28.7
Africa	4.7×10^6	8.2×10^6	42.3
Northern America	2.1×10^7	2.4×10^7	11.7
Southern America	1.7×10^7	2.3×10^7	24.5
Europe	1.6×10^7	2.2×10^7	27.5
sum	1.1×10^8	1.6×10^8	28.7
Source: modified from (FAO 2024)			

5.2 Evaluating of the susceptibility of pigs to a current HPAIV genotype in relation to the zoonotical risk (Study objective II)

While the role of poultry in the context of food supply and thus exposure risk for humans is already being assessed (Harder et al. 2016; Harder et al. 2009; Thomas and Swayne 2009), there is little information on other food-supplying animals. The role of the pig as such seems particularly interesting because it is itself susceptible to various types of influenza viruses and described as a reservoir host for

influenza A reassortants (Nelli et al. 2010; Hennig et al. 2022). The basic susceptibility of pigs to AIV has been proven several times in the field and experimentally in the past (Meseko et al. 2018; Hervé et al. 2021; Kaplan et al. 2017), but the result of an infection with current virus isolates of clade 2.3.4.4b is rather unclear. Furthermore, there is a risk that contagious viruses, reassorting in pigs, could be (re)transmitted into poultry and humans and by this gain properties to increase zoonotic adaptability and by this pandemic potential.

To investigate this question, adult pigs were infected with an isolate of a dominant genotype of subtype H5N1 clade 2.3.4.4b from 2022, that was found in most mammalia cases infected with HPAIV at that time (Pohlmann and Harder 2023; Pohlmann 2023b). The isolate was of the DE 21-10 N1.5 genotype, which was also subjected to the IMPI and occulo-nasal infection trial in ducklings, resulting in high IMPI scores. Inoculation routes were modelled on the most likely natural infection route both occulo-nasally or orally via ingestion of infectious material. While viral RNA could be detected in the brain of one animal and one animal also seroconverted, none of the animals showed any clinical signs of infection. Our analyses indicate that pigs did not support replication of the dominating HPAIV genotype. However, detection of viral genome in the brain of one intranasally inoculated pig in conjunction with seroconversion in only this one pig indicated viral spread. Whether this RNA resulted from the original inoculum or points to initial low viral replication remains unclear. Findings of viral RNA were limited to days 2 (intranasal inoculation group) and 4 (oral group) from swab samples with low detection levels, where residual inoculum detection cannot be excluded from the intranasal group findings. Currently, the risk of infection of domestic pigs with circulating HPAIV genotypes appears to be low. However, in the recent past, an increasing number of cases with severe disease symptoms and in particular central nervous symptoms have been reported (Bordes et al. 2023; Agüero et al. 2023), possibly due to the previously described expansion of avian host diversity of HPAIV carcasses inevitably accumulating more frequently. Death birds serving as food source for predators and scavengers, the oral route of infection seems most likely infection route (Brown et al. 2008; Reperant et al. 2008; Verma et al. 2023). Another aspect that might contribute to higher frequency of mammalian HPAIV H5 infections are adaptive mutations leading to altered receptor affinity of the host, and/or to a different temperature profile of the viral polymerase (Graaf and Fouchier 2014; Subbarao et al. 1993). Those adaptations have been linked to advanced replication capacities, opening up a broader host spectrum that includes mammals (Cauldwell et al. 2014; Maines et al. 2011; Mostafa et al. 2018). Likewise the number of reported human cases with HPAIV H5 infection increased to a 896 cases from 2003 to July 19th 2024, from which 463 ended with fatal outcome (52% lethality) (WHO 2024c). Fortunately the documented chains of infection pointed as source of infection to contact with infected poultry and could rule out human-to-human transmission (WHO 2024a; One Health High-Level Expert Panel 2023; Bruno et al. 2023b; Bruno et al. 2023a). For the human infections as well as cases of omnivorous and herbivorous mammal species, the infection route is not linked to alimentary inoculation. Instead, shared environments and close contact with infected birds (Gamarra-Toledo et al. 2023; Plaza et al. 2024a;

Zhou et al. 2009; Ramey et al. 2020), contaminated dust (Spekreijse et al. 2013), high virus loads on surfaces (Hauck et al. 2017) or in surface waters (Ahrens et al. 2022; Lebarbenchon et al. 2011; Zhang et al. 2014) are considered important sources of infection. In conjunction with the number of genotypes harboring mutations with zoonotic potential, the risk of adaptation of AIV to mammalian species should be considered as a constant threat. For this reason, susceptibility of pigs as an important intermediate host to emerging HPAIV H5 variants should be re-evaluated regularly and included in basic AIV surveillance. In addition, contact between poultry and kept pigs should be restricted, especially with regard to backyard holdings to reduce the risk of spillover infections.

5.3 Outlook on future perspectives characterizing the diversity of HPAIV H5 subtypes to assess risk potentials for target species, dead end hosts and humans

At least since 2021, clade 2.3.4.4b HPAIV H5Nx have been circulating intensively among wild birds and poultry with increasing global spread. The unprecedented reassortment activity of clade 2.3.4.4b viruses with LPAIV contributed to the constant emergence of new genotypes. The dominant genotypes apparently maintain duck virulence despite an overall enzootic character of the current epidemiology. Major factors contributing to the AI virus evolution are the reservoir in wild birds, the continuous introduction into domestic poultry, spill-back into wild birds and the potential for interspecies spread. In addition, climate change could represent a further challenge with regards to HPAIV ecology that should be considered: The phenomenon of global warming is likely to contribute to an increase in the risk of a global panzootic in the coming decades. This might occur as a result of a merging of individual scenarios: It is predicted that climate change will result in alterations to the migration patterns of migratory birds (Both and te Marvelde 2007; Gu et al. 2021; Gilbert et al. 2008). As a consequence, species that previously migrated to mild-temperated regions during the winter months will, in the future, remain in their summer habitats, thereby increasing the local population and virus density (Morin et al. 2018). In contrast, other species will lose their habitats due to climate-related destruction and will be forced to move to other, already colonized areas, again encountering avian species and their virus strains. It is also anticipated, that previously unexplored areas, such as the Arctic (van Hemert et al. 2014) and Antarctic, will be further colonized by new species in the future, thereby creating potential entry points for HPAIV. It is also probable that wild mammals, which are resident in principle, will have to travel longer distances in search of food and a safe habitat, which will increase the likelihood of contact with HPAIV. In light of current projections, a lack of geographical space (and wild bird habitat) is also likely to emerge in some countries, like in Asia (Murdiyarso 2000), if the global poultry industry continues to expand at the current pace and without accounting for shrinking ressources (Morin et al. 2018). This could create tighter interfaces with both wild birds and humans and might expand a perpetual and growing breeding ground for reassortment and the emergence of new HPAIV.

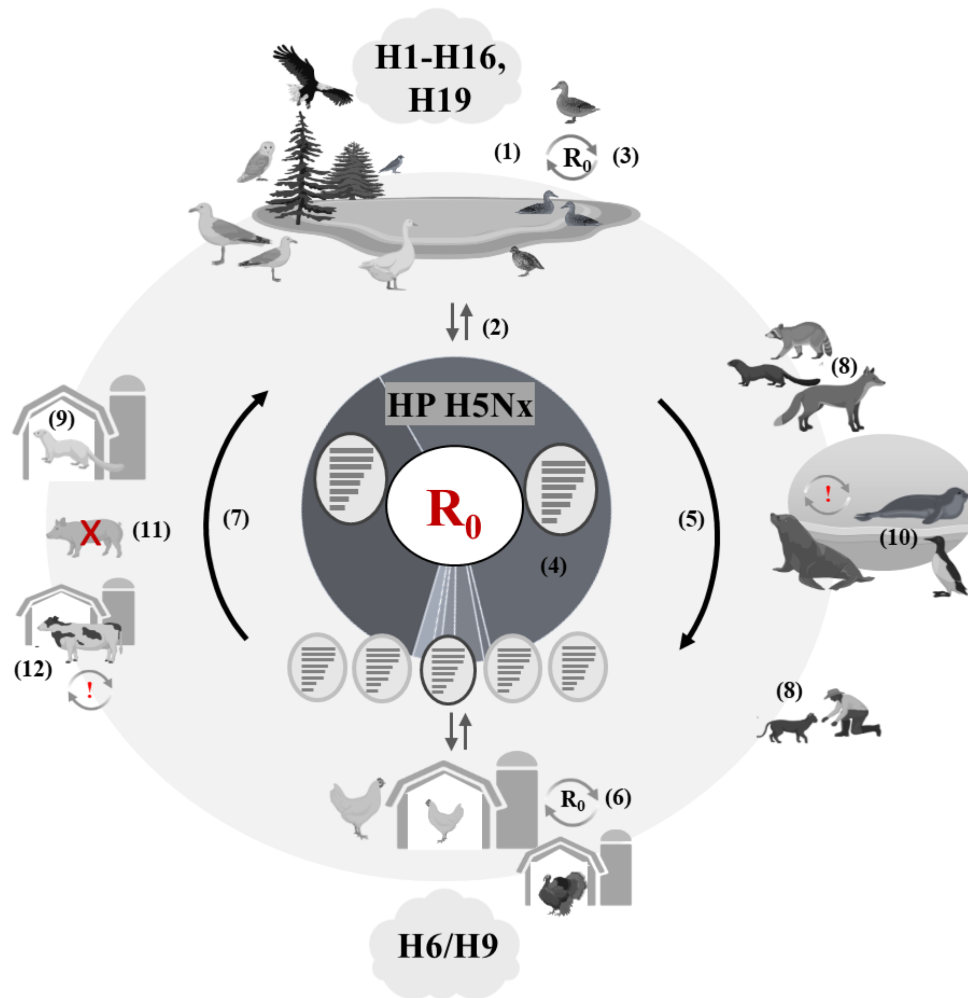

Figure 2. **Factors Driving Avian Influenza Virus Evolution**

Wild waterbirds are considered the natural reservoir for Avian influenza viruses (AIV) of all subtypes (1) with Mallards (*Anas platyrynchos*) considered as an important factor in the ecology of AIV. Co-infection with circulating HP AIV H5 of the gs/GD lineage can give rise to new reassortants (2). By modelling different scenarios, the key factor for ecological dominance of variants within an ecosystem of resident and migratory birds, was found to be the transmission rate, R_0 (3) (Lisovski et al. 2018). High virulence of HPAIV genotypes, as observed in epidemiologically dominant genotypes in this study (4) might thus be maintained within the complex multi-species ecosystem of wild birds. A prerequisite however, would be that the HP phenotype of new emerging variants favour transmission. Migratory patterns and the rapid excretion of the virus, especially in ducks, contribute to sustaining infection cycles and reassortment with resident strains.

High prevalence of HPAIV in wild birds increases the risk of virus incursions into domestic poultry (5). In poultry, resembling an avian host population of almost unlimited size, fast efficient virus spread is a key selection factor (6). Consecutive mutual spillover events between wild birds and poultry might contribute to conservation of virulent variants (7). In consequence, biosecurity is not only an important feature for protecting poultry, but should be considered as important factor to remove “save havens” for the virus and to lower overall HPAIV virus burden. Increased HPAIV H5 spillover into mammals since 2022 raises concern of possible adaptation (8). While sporadic cases in wild carnivore species are considered to be initiated by oral infection, outbreaks in farmed fur animals (9) and sea lions (10) along coasts of South America point to possible transmission within those species and could possibly accelerate adaptation. Concerns that new HPAV H5 variants are able to establish infection in pigs were not supported by infection experiments performed in this study nor by epidemiological investigations (11). However, the latest introduction of HPAIV into dairy farms in the U.S. with subsequent lateral spread (12) clearly indicates the risk of the ongoing HPAIV H5 panzootic and highlights the need for new research and surveillance strategies in order to understand influenza A virus ecology. Given the importance of efficient transmission, phenotypical virus characterization should include the determination of the basic reproduction number R_0. *(Created in BioRender, Piesche, R. (2025) htpps://BioRender.com/a79a003)*

Together, the aforementioned settings may have contributed locally at a different rate to undamped spread and now panzootic nature of HPAIV H5 clade 2.3.4.4b. In light of the current results of this thesis, indicating that mechanism of the "virulence-transmission trade-off model" are efficient drivers of the panzootic, further efforts have to be considered that facilitate reduction of the basic reproduction number R_0 to repel worldwide circulation and continuous adaptation. These activities should include combination of different strategies on a global scale:

I. In the case of wild birds, there is currently no reliable technology to prevent uncontrolled AIV infections. Consequently, the only viable option is to rely on elevated population immunity following natural infection. In addition, vaccination with new vector vaccines capable of limited replication and that can be applied via feed or water, might be an option for certain ecosystems. Besides, measures implemented to safeguard breeding grounds of migratory birds, thereby reducing the probability of avian influenza dissemination through disrupted ecosystems, might contribute to lower virus dissemination.

II. In the context of domestic poultry, application of vaccines could provide an additional option to lower susceptibility at the population level and reduce the spread of avian influenza within and in between farms. Reduced transmission would be an important factor also to limit virus mutation rate. Legislation in the EU has recently been amended to permit vaccination of poultry against HPAIV. However, potential vaccine candidates designed to provide reliable long-term protection are still in the testing phase.

III. Reliable vaccination will, however, only be successful in the future if it is combined with rigorous biosecurity measures. Worldwide efforts should be undertaken to establish common measures, that include strict hygiene management, rapid euthanasia and safe disposal of infected animals, tracing of contacts and contact routes. Genomic sequencing of detected AIV could help to track links of transmission and improve biosecurity concepts at the farm level but along the production chain, including farm supply and marketing of products. For highly intensified production sectors, a reduction of poultry flock size and/or population density might be necessary to consider, along with a critical review of personal consumption preactices of consumers.

IV. New strategies for expanded passive and active monitoring strategies for poultry and monitoring of farmed domestic mammals should be explored. This could include computer aided systems to evaluate the general health status of animals to obtain indications for an infection and allow early testing. Together with maintaining an efficient veterinary public service, this would further support measures that both, infected animals and their products exempt from transport and further use.

V. Additionally, monitoring programs have to be designed to assess the risk of human exposure. The general susceptibility of humans to HPAIV is a reason for increased vigilance. Considering the current prevalence of the HPAIV H5 among various species, including humans, the HPAIV

panzootic can only be controlled by a vigilant One Health approach. This approach relies on international cooperation and data sharing. Support to poultry sectors in developing countries to develop an appropriate infrastructure could be the basis to contain virus outbreaks before they spread further within a country or beyond borders.

In the future, phenotypical characterization of emerging HPAIV variants including determination of virulence and transmission dynamics in appropriate indicator species could serve as crucial information to forecast the imminence of actual circulating genotypes and assess the impact of applied measures.

VI. Summary

Current HPAI H5N1 clade 2.3.4.4b viruses in Europe have established enzootic status in wild bird populations with spillover infections to mammals. The prevalence of various genotypes of these viruses undulates with some genotypes becoming extinct and others dominant. This has raised speculation about a correlation between genotype and phenotypic virus characteristics. In particular, the question came into focus, whether a possible viral adaptation to mallards as an important migratory bird species with a large global population might have led to reduced virulence in mallards and thereby facilitate a more efficient “silent” spread. To address this question in this study, virulence was assessed in an in vivo model with juvenile mallard ducks (*Anas platyrhynchos*) involving infection via the thigh muscles to compare viruses via the intramuscular pathogenicity index (IMPI). The established IMPI model proved to differentiate between concurrently circulating HPAIV H5N1 genotypes, revealing that variants that dominated the epidemic were of high virulence (IMPI 2.92-3.00), inducing death of inoculated animals within 1-2 days. In contrast, minor genotypes were of intermediate virulence (IMPI 1.65-1.22) with 20-50% of the inoculated animals surviving infection. Comparable differences in clinical manifestation of representative strains with high and intermediate IMPI scores were also observed after occulo-nasal infection, inducing either death of all 10 inoculated animals within 4 days, or death of 4 out of 10 ducks in between day 5-6. Surviving ducklings in the group inoculated with the intermediate virulent variant were clinically healthy at the end of the 21day observation time and seroconverted. The analysis of genetic differences in these natural occurring genotypes point to the viral polymerase complex, in particular the PB1 segment as important for influencing virulence in ducklings. In conjunction with the epidemiological information of the genotypes tested, the results indicate that dominance of genotypes was not associated to attenuation but rather to sustained high virulence. These results are therefore best described by the 'transmission-virulence-trade-off' model: This would be in line with observed higher viral shedding in the group infected with highly virulent genotype. Further studies have to verify, whether this can be confirmed on the level of transmission.

Since an increasing frequency of avian-to-mammal spillover transmissions of clade 2.3.4.4b HPAIV is observed, a possible susceptibility of pigs was assessed as well. Pigs are an important livestock species and known as a mixing vessel between AIV and IVA from other species, in particular from humans. To this end, adult pigs were inoculated in this study by oral ingestion of a high dose of infectious material. This resembles the most likely current route of transmission between wild birds and predatory or scavenging mammals. Inoculated pigs did not reveal clinical signs and no shedding of virus was detected. An immunological response to infection was observed in only one animal which seroconverted weakly. Viral genome was detected in the brain of only one pig. In consequence, pigs are considered to be of low susceptibility for the 2.3.4.4b HPAIV genotype used, which was also the dominant one of 2022, proved during the IMPI trials in ducklings.

The results of the studies presented in this thesis highlight the importance of intensive and regular monitoring of new HPAIV reassortants. It became evident, that in vivo studies in relevant model species such as pigs and ducks are central to an assessment of the zoonotic pre-pandemic potential of H5N1 clade 2.3.4.4b viruses.

VII. Zusammenfassung

Die aktuellen HPAI H5N1-Viren der Klade 2.3.4.4b haben in Europa einen enzootischen Status in Wildvogelpopulationen erreicht und sind auf Säugetiere übergesprungen. Die Prävalenz der verschiedenen Genotypen dieser Viren schwankt, wobei einige Genotypen aussterben und andere dominant werden. Dies hat zu Spekulationen über einen Zusammenhang zwischen Genotyp und phänotypischen Viruseigenschaften geführt. Insbesondere rückte die Frage in den Mittelpunkt, ob eine mögliche Anpassung des Virus an die Stockente als wichtige Zugvogelart mit einer großen weltweiten Population zu einer geringeren Virulenz bei der Stockente geführt und dadurch eine effizientere „stille" Ausbreitung ermöglicht haben könnte. Um diese Frage in dieser Studie zu klären, wurde die Virulenz in einem In-vivo-Modell mit jungen Stockenten (*Anas platyrhynchos*) untersucht, bei dem die Infektion über die Oberschenkelmuskulatur erfolgte, um die Viren über den intramuskulären Pathogenitätsindex (IMPI) zu vergleichen. Das etablierte IMPI-Modell erwies sich als geeignet, um zwischen gleichzeitig zirkulierenden HPAIV H5N1-Genotypen zu unterscheiden. Es zeigte sich, dass die Varianten, die die Epidemie dominierten, von hoher Virulenz waren (IMPI 2,92-3,00) und den Tod der inokulierten Tiere innerhalb von 1-2 Tagen herbeiführten. Im Gegensatz dazu waren weniger häufigere Genotypen von mittlerer Virulenz (IMPI 1,65-1,22), bei denen 20-50% der inokulierten Tiere die Infektion überlebten. Vergleichbare Unterschiede in der klinischen Manifestation von repräsentativen Stämmen mit hohen und mittleren IMPI-Werten wurden auch nach einer okkulo-nasalen Infektion beobachtet, die entweder den Tod aller 10 inokulierten Tiere innerhalb von 4 Tagen oder den Tod von 4 von 10 Enten zwischen dem 5. und 6. Die überlebenden Entenküken in der Gruppe, die mit der intermediären virulenten Variante inokuliert wurde, waren am Ende der 21-tägigen Beobachtungszeit klinisch gesund und hatten serokonvertiert. Die Analyse der genetischen Unterschiede in diesen natürlich vorkommenden Genotypen deutet darauf hin, dass der virale Polymerasekomplex, insbesondere das PB1-Segment, einen wichtigen Einfluss auf die Virulenz bei Entenküken hat. In Verbindung mit den epidemiologischen Informationen der getesteten Genotypen deuten die Ergebnisse darauf hin, dass die Dominanz der Genotypen nicht mit einer Abschwächung, sondern vielmehr mit einer anhaltend hohen Virulenz verbunden war. Diese Ergebnisse lassen sich daher am besten durch das 'Transmission-Virulence-Trade-off'-Modell beschreiben: Dies stünde im Einklang mit der beobachteten höheren Virusausscheidung in der Gruppe, die mit dem hochvirulenten Genotyp infiziert war. In weiteren Studien muss überprüft werden, ob dies auf der Ebene der Übertragung bestätigt werden kann.

Da eine zunehmende Häufigkeit von Spillover-Übertragungen von HPAIV der Klade 2.3.4.4b von Vögeln auf Säugetiere beobachtet wird, wurde auch eine mögliche Anfälligkeit von Schweinen untersucht. Schweine sind eine wichtige Nutztierart und dafür bekannt, dass sie AIV und IVA von anderen Arten, insbesondere vom Menschen, übertragen können. Zu diesem Zweck wurden erwachsene Schweine in dieser Studie durch orale Aufnahme einer hohen Dosis infektiösen Materials inokuliert. Dies ähnelt dem derzeit wahrscheinlichsten Übertragungsweg zwischen Wildvögeln und räuberischen

oder aasfressenden Säugetieren. Die inokulierten Schweine zeigten keine klinischen Symptome, und es wurde keine Ausscheidung des Virus festgestellt. Eine immunologische Reaktion auf die Infektion wurde nur bei einem Tier beobachtet, das eine schwache Serokonversion zeigte. Virales Genom wurde im Gehirn von nur einem Schwein nachgewiesen. Folglich wird davon ausgegangen, dass Schweine eine geringe Empfänglichkeit für den verwendeten HPAIV-Genotyp 2.3.4.4b aufweisen, der auch der dominante Genotyp von 2022 war, der bei den IMPI-Versuchen an Entenküken nachgewiesen wurde.

Die Ergebnisse der in dieser Arbeit vorgestellten Studien unterstreichen die Bedeutung einer intensiven und regelmäßigen Überwachung neuer HPAIV-Reassortanten. Es wurde deutlich, dass In-vivo-Studien an relevanten Modellspezies wie Schweinen und Enten von zentraler Bedeutung für die Bewertung des zoonotischen, präpandemischen Potenzials von H5N1-Viren der Gruppe 2.3.4.4b sind.

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IX. Supplementary

9.1 List of abbreviations

AI	Avian Influenza
AIV	Avian Influenza Virus
BLAST	Basic local alignment search tool
CDC	Centers for Disease Control and Prevention
cRNA	complementary RNA
dpi	days post inoculation
EA	eurasian
EFSA	European Food Safety Authority
ER	Endoplasmatic Reticulum
EU	European Union
EURL	European Reference Laboratory
FAO	Food and Agriculture Organization
GISAID	Global initiative on sharing all influenza data
gs/GD	Goose/Guandong
HA	Hemagglutination assay, Hemagglutinin
HA_0	Hemagglutinin precursor
HP	highly pathogenic
HPAI	High pathogenic avian influenza
HPAIV	Highly pathogenic avian influenza virus
ID	identification number
IFN	Interferons, Interferon
IL	Interleukins
IMPI	Intramuscular pathogenicity index
IVA	Influenza Virus A
IVPI	Intravenous Pathogenicity Index
LP	low pathogenic
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic Avian Influenza Virus
M1	Matrix protein 1
M2	Matrix protein 2
mRNA	messenger RNA
MTO	Mean time of onset of signs
NA	Neuraminidase
NC	Nucleocapsid

NEP	Nuclear export protein
NLRs	Nucleotide-binding-oligomerization-domain-like Receptors
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
OECD	Organization for Economic Co-operation and Development
ORF	Open reading frame
PA	Polymerase acidic protein
PAMPs	Pathogen associated molecular patterns
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
pi	post inoculation
PKR	Protein kinase R
PRRs	Pattern recognition receptors
RdRP	RNA-dependant RNA polymerase
RIG-I	Retinoic acid-inducible gene I
RLRs	Retinoic acid-inducible gene I-like Receptors
RNA	Ribonucleic acid
SA	Sialic acids
TCID	Tissue culture infection dose
TLRs	Toll- like Receptors
TSIS	Tierseucheninformationssystem
vRNA	viral RNA
vRNP	viral Ribonucleoprotein
WHO	World Health Organization
WOAH	World Organization of animal health

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