

Review

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Review

Highly Pathogenic Avian Influenza (HPAI) H5 Clade 2.3.4.4b Virus Infection in Birds and Mammals

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Simple Summary: Avian influenza viruses are highly contagious respiratory viruses that severely impact bird populations, causing significant morbidity, mortality, and economic losses in the poultry industry worldwide. Particularly concerning are the Asian-origin H5N1 subtype highly pathogenic avian influenza viruses of clade 2.3.4.4b, which emerged in 2013 and have since spread across Asia, Europe, Africa, and America, leading to outbreaks in various poultry and animal species. The unique epidemiological and pathobiological characteristics specific to clade 2.3.4.4b viruses are discussed, emphasizing their distinct nature compared to other clades. Wild waterfowl, acting as natural reservoirs, frequently carry these viruses, posing threats not only to their populations but also to other wild bird species, including endangered ones. Furthermore, the adaptation of clade 2.3.4.4b viruses to mammalian species raises significant concerns about potential spillover events to humans. This review highlights the diverse outcomes of HPAI infections in different hosts, ranging from asymptomatic cases to fatal infection, influenced by host and virus-related factors. Understanding these complexities is vital for developing effective strategies to mitigate the impact of AIVs, safeguard poultry production, protect wildlife, and prevent potential public health crises.

Abstract: Avian influenza viruses (AIVs) are highly contagious respiratory viruses of birds, leading to significant morbidity and mortality globally and causing substantial economic losses to the poultry industry. Since their first isolation in 2013, the Asian-origin H5N1 highly pathogenic avian influenza viruses (HPAI) of clade 2.3.4.4b have undergone unprecedented evolution and reassortment of internal gene segments and have spread to Asia, Europe, Africa, and America, causing outbreaks in all the poultry categories. Novel epidemiological and pathobiological characteristics, distinct from other clades, are specific of clade 2.3.4.4b viruses. Wild waterfowl, the natural reservoir of AIVs, are frequently found infected with clade 2.3.4.4b viruses, which can also cause high morbidity and mortality in these birds. The sustained clade 2.3.4.4b virus circulation in waterfowl has also led to virus infection in other wild bird species, with implications for the conservation of endangered species. Furthermore, clade 2.3.4.4b viruses have been isolated in various wild and domestic mammals worldwide, and critical mutations related to virus adaptation to mammalian species have been identified, raising concerns about virus spillover to humans. The main clinical signs, and anatomopathological findings associated to clade 2.3.4.4b virus infection in birds and non-human mammals are hereby summarized.

Keywords: avian influenza; pathobiology; wild birds; poultry; wild mammals; domestic pets; virus spillover; public health

1. Introduction

Avian influenza viruses (AIVs) belong to the species *Alphainfluenzavirus influenzae* (previously influenza A virus or FLUAV), genus *Alphainfluenzavirus*, family Orthomyxoviridae, [1,2]. AIVs are highly contagious respiratory viruses of birds, responsible of high morbidity and mortality worldwide. These are single-stranded, negative-sense, segmented and enveloped RNA viruses which include eight gene segments coding for 11+ structural and non-structural/regulatory proteins [3–5]. The polymerase complex, composed of the polymerase basic protein 1 (PB1), polymerase basic

protein 2 (PB2), and the polymerase acidic protein (PA), altogether with the nucleoprotein (NP), the matrix protein 1 (M1), the nonstructural protein 1 (NS1), and the nuclear export protein (NEP) are found inside the lipid envelope, while the membrane ion channel (M2), the hemagglutinin (HA), and the neuraminidase (NA) are embedded within the envelope. The molecular properties of HA and NA proteins determine FLUAV classification into subtypes [6]. A total of 19 HA (H1-H19) and 11 NA (N1-N11) has been recognized, with H17N10 and H18N11 “flu-like” subtypes exclusively isolated in bats [7–11].

Wild waterfowl (Anseriformes, Charadriiformes) are considered the natural reservoir of 17 of 19 HA (H1-16 and H19) and 9 NA (N1-9) AIV subtype combinations and they carry AIVs mostly subclinically [12]. Spillover events of AIVs from wild birds to domestic poultry typically results in asymptomatic or mild to moderate respiratory disease, diarrhea and drop in egg production. On occasion, the circulation of LPAI H5 and H7 virus subtypes in poultry lead to virus evolution and increased disease severity with up to 100% fatality rates. Therefore, based on the disease severity in chickens, AIVs are further classified as either low or highly pathogenic (LPAI or HPAI). Cases associated with increased pathogenicity of AIVs are restricted to the H5 and H7 subtypes, which are reportable diseases to the World Organization for Animal Health (WOAH) [13–15]. Eurasian H5 HPAIVs are a serious concern for the WOAH, since these viruses are zoonotic, potentially pandemic, and manifest increased host range and tissue tropism.

Eurasian H5s are the immediate descendants of the A/goose/Guangdong/1/1996(Gs/GD)-like H5N1 HPAI virus that was first detected in sick farmed geese in the Guangdong province, China, in 1996 [16,17]. After becoming endemic in domestic poultry in Asia, HPAI viruses from this phylogenetic lineage have found a way back into the natural host (migratory aquatic birds) resulting in significant geographic spread worldwide to Asia, Africa, Europe, and America, and causing significant losses to the poultry industry [18,19]. Since their first emergence, the HA gene of H5 Gs/GD HPAIV has genetically diversified into an overabundance of clades and subclades. Among these, clade 2.3.4.4b has undergone explosive expansion in wild birds and domestic poultry and almost entirely replaced other circulating clades in just a short period of time [19–21]. Strains from clade 2.3.4.4b have been reported to cause severe systemic infections and high mortality rates among waterfowl as well as mass mortality events in sea birds, marine mammals and others [22]. Recently, livestock and dairy herds have tested positive in Minnesota, Texas, Kansas and Michigan [23]. While sustained mammalian transmission has not been documented yet, the persistent recirculation of this clade in wild and domestic mammalian populations is concerning for potential virus adaptation and species-jump.

Barriers to interspecies adaptation include changes within the viral polymerase complex to achieve successful replication within mammalian cells, and improved receptor binding activities of the HA and NA proteins [24]. The conformation of the HA protein is of particular importance, as it mediates binding to the host cell receptor through a receptor binding site (RBS), and the initiation of the virus cycle [25]. AIVs typically prefer α 2,3- linked sialylated glycans present more abundantly in respiratory and gastrointestinal tracts of avian species, while mammalian FLUAVs preferably bind to α 2,6-linked sialylated glycans predominant in mammalian tissues [26]. Upon endocytic uptake, the acidification of the endosome triggers a conformational change in the HA that mediates fusion between the viral envelope and the endosomal membrane. This allows the virus genome to be released into the cytoplasm [27]. Since HA is synthesized as an inactive precursor (HA0), this must be cleaved at a specific site, the cleavage site (CS), into HA1 and HA2 to become functional and induce membrane fusion and release of virus genome into the cytoplasm [27–29]. The amino acid motifs at the CS determine the spectrum of enzymes that can cleave and activate the HA [30]. Most LPAIVs contains a single arginine residue (monobasic) at the HA CS which is cleaved extracellularly exclusively by trypsin or trypsin-like proteases, only secreted in lung epithelium [31] and intestinal tract [32], therefore mediating localized infections and hence milder disease [33]. However, when the CS contains multiple basic amino acids (polybasic CS), it can be cleaved by a family of serine proteases [34], which are ubiquitously expressed and allow virus replication in different cell types,

causing an often fatal systemic disease [35,36]. Therefore, the CS sequence has been used as a reference for classifying AIVs into pathotypes by the WHO [37].

The HA protein also contains the major antigenic determinants targeted by the humoral host's immune response which undergo heavy selection pressure [38–43]. When non-synonymous nucleotide substitutions accumulate in a process called *antigenic drift*, the resulting virus can escape neutralization by host antibodies [44]. More pronounced genetic variations can also result in changes in viral properties such as receptor binding, replication, and transmission [45]. Furthermore, AIV's segmented genome enables *antigenic shift*, a phenomenon that occurs between two or more viruses that infect the same cell and consists in the reassortment of the gene segments and the emergence of an entirely novel virus. In poultry, reassortment events between different AIVs have contributed to the emergence of several novel emerging zoonotic subtypes with internal gene cassettes from other circulating subtypes [46]. This has also paved the way to the emergence of pandemic influenza in humans [47,48]. Therefore, given the significant implications for veterinary and public health, recognizing the early signs and clinicopathological manifestations of HPAI H5 Gs/Gd 2.3.4.4b clade viruses is crucial to promptly act interventions to mitigate its spread across susceptible species. The review hereby presented aims at summarizing the main clinical, pathological, and histopathological findings of clade 2.3.4.4b virus infections in main avian and mammalian hosts. To accomplish this, two databases, namely PubMed (<https://pubmed.ncbi.nlm.nih.gov>) and Scopus (<https://www.scopus.com/>), were queried from the 1st of February 2023 to March 30th 2024. The literature search was performed using the following keywords: 'highly pathogenic avian influenza or 'clade 2.3.4.4' and 'wild birds' or 'ducks' or 'poultry' or 'chicken' or 'turkeys'. Additional pertinent information was extracted from public databases, notably the Global Initiative on Sharing All Influenza Data (<https://gisaid.org>), Center for Disease Control and Prevention (<https://www.cdc.gov>), World Organization for Animal Health (<https://www.woah.org/en/disease/avian-influenza/#ui-id-2>), U.S. Department of Agriculture, Food and Drug Administration, Animal Plant Health and Inspection Services (USDA-APHIS) (<https://www.aphis.usda.gov>).

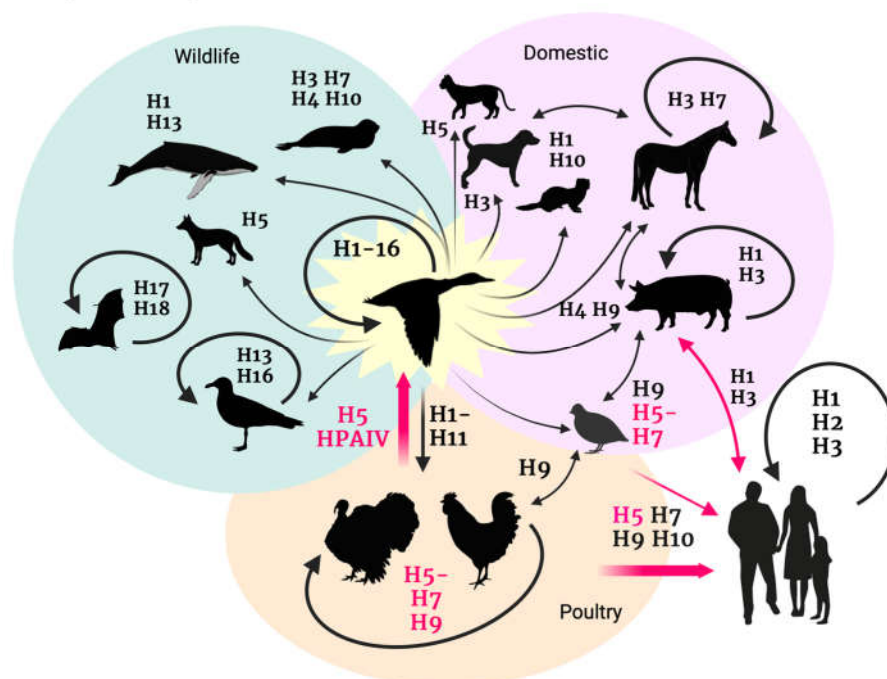


Figure 1. Avian influenza transmission flow from the natural reservoir (aquatic birds) to poultry, humans, and other animal species. Figure generated with BioRender.com.

2. H5 Genetic Evolution and Epidemiology

Due to the persistent circulation of Gs/Gd H5Nx HPAI viruses in poultry in Asia, viruses have readapted to waterfowl species and found their way back to their natural reservoir, the wild waterfowl. Through waterfowl migrations, four waves of intercontinental transmission of Gs/Gd lineage H5Nx virus from Asia to other continents have been identified since 2003 onward [21]. This has led these viruses to undergo reassortment of the internal gene segments with other influenza viruses and genetic evolution of the hemagglutinin H5 gene. The genetic diversity of the H5 Gs/Gd hemagglutinin has resulted in the identification of ten distinct clades (0 to 9) and multiple subclades, as outlined by a unified nomenclature [49,50]. Viruses from different clades have exhibited long-distance dissemination, and HPAI H5Nx viruses within the 2.3.4.4 clade have recently gathered significant attention due to their unprecedented global spread and their ability to infect a wide range of species, including not only domestic and wild birds, but also humans and other mammals [19,51]. Initially isolated from a Chinese live poultry market in 2010 [52], a subtype H5N8 virus of clade 2.3.4.4 caused multiple outbreaks in poultry and wild birds in South Korea [53]. Since its emergence, the HA gene of clade 2.3.4.4 has then undergone reassortment events with other avian influenza viruses from different regions acquiring various NA subtypes, including N1, N2, N3, N4, N5, N6 and N8, and has evolved into several subgroups [54]. The first intercontinental spread of the H5 clade 2.3.4.4c to North America dates to 2014-2015, with H5N8 viruses introduced most likely via migratory birds through the Bering Strait to breeding grounds in Alaska [55]. After reassorting with LPAI gene segments from North American wild birds [56], a H5N2 reassortant virus of clade 2.3.4.4c became predominant and affected over 50 million birds in the U.S. in spring 2015 [19,21,57]. After sporadic detections in wild birds, the H5 clade 2.3.4.4c viruses in North America were lastly isolated in late 2016 [58]. During 2014/2015, HPAIVs clade 2.3.4.4 were simultaneously introduced into Europe and East Asia, and have been circulating altogether with other clades, notably 2.3.2.1, following fall bird migration [19,21,55].

Since their first detection in domestic ducks in Eastern China (2013) and South Korea (2014) [53,59], the H5Nx clade 2.3.4.4b viruses were isolated from wild birds in Qinghai lake, China, in 2016, and spread on a large scale to Europe, Africa, Middle East, and Asia via migratory birds [19,60,61]. In Europe, 1,207 HPAI H5Nx poultry outbreaks and 1,590 wild bird mortality events were reported during the 2016/2017 virus epidemic, mostly due to H5N8 of clade 2.3.4.4b [62,63]. Sporadic outbreaks of HPAI H5N6 or H5N8 viruses then continued from 2018 to early 2020 in East Europe, until the emergence of a novel H5N1 virus genotype of clade 2.3.4.4b in October 2020 in The Netherlands [64–67]. Since fall 2020, a surge in HPAI H5Nx cases due to 2.3.4.4b viruses has been observed across the Eurasian continent, with an unprecedented number of H5N1 outbreaks in poultry and cases in wild birds that peaked during the 2021-2022 epidemic season [68]. This led to the largest HPAI epidemic ever, with more than 2,000 outbreaks in 37 European countries and over 40 million birds culled during 2021-2022 [69]. At least 19 different H5N1 clade 2.3.4.4b genotypes were characterized in Europe, originating from multiple inter- and intra-subtype reassortment events [69,70].

The transatlantic spread to North America of H5N1 clade 2.3.4.4b HPAI viruses occurred in late 2021. Notably, a die-off of domestic birds on the Atlantic coast of Canada was attributed to an HPAI H5N1 virus, which was also isolated from a great black-backed gull (*Larus marinus*). Strain characterization confirmed its placement within the 2.3.4.4b phylogenetic clade, signifying a new virus introduction into North America via migratory wild birds from Eurasia [71,72], possibly via Iceland [73]. A further intercontinental virus spread to North America then occurred via the Pacific and the North Atlantic-linked migratory flyways later in 2022 [74–76]. After the H5N1 virus spread westward and southward, extensive genetic reassortment with North American LPAIVs has brought to 10 different gene constellations composed of six major and 11 minor genotypes until April 2022 [77]. As of August 2023, the circulation of HPAI H5Nx viruses of clade 2.3.4.4b in the U.S. has resulted in 58 million domestic birds affected [78] and the detection of the virus in 7,152 wild birds [79]. The H5 HPAIVs of clade 2.3.4.4b introduction to Central and South America occurred in late 2022, through migratory birds [80]. Die-offs of wild aquatic birds and wild mammals, and cases in poultry have therefore been reported in countries along the Pacific Americas flyway, such as Peru and Chile

[81–84]. H5 HPAI cases have also been reported in countries on the east side of the Andes Mountains, with large outbreaks in poultry, wild birds and wild mammals in Brazil, Argentina, and Uruguay, occurring during 2023 [18,70,85,86]. First detections of HPAIVs of clade 2.3.4.4b in wild aquatic birds in the Antarctic and sub-Antarctic regions of Falkland Islands, South Georgia and South Sandwich Islands, were reported from September to December 2023, indicating the HPAI spread to new bird population groups [87,88].

Sequence data submitted to the GISAID Epi-Flu Database (<https://gisaid.org/about-us/acknowledgements/epiflu/>) demonstrates the global dominance of HPAI H5Nx viruses of clade 2.3.4.4b that have emerged over time (Figure 2). With respect to the other 2.3.4.4 virus clades, H5N6 subtype of 2.3.4.4d-h clades has been predominantly isolated in China since 2014 and the geographic distribution of these isolations has been limited to East Asia [89–91]. Clade 2.3.4.4a H5N6 virus has only been detected in Asia, where it circulates endemically since 2013, and distant geographical spread to other regions has not been reported yet [89].

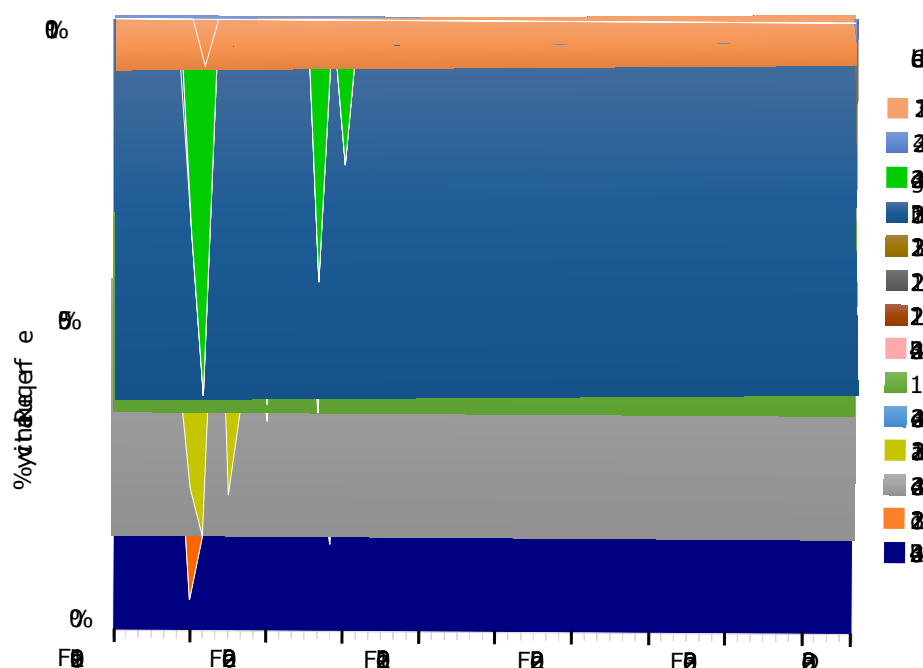


Figure 2. Relative frequency (%) over time of HPAI H5 Gs/Gd virus clades global isolations from February 2019 to December 2023. Based on data from GISAID's EpiFlu Database and reproduced on Microsoft Excel [92].

The H5 clade 2.3.4.4b viruses have exhibited novel epidemiological and pathobiological characteristics, distinct to other clades. An elevated pathogenicity has been observed in several duck species [93,94], considered as the AIV natural hosts, accompanied by asymptomatic infections in certain dabbling ducks [95–97]. Frequent die-offs have been observed, particularly in colony-breeding seabirds [98–102], which have been attributed to a shift in the HPAI virus epidemiology among waterbirds, resulting in endemic circulation of these strains [103,104]. Due to its sustained circulation in the free-ranging avifauna, H5N1 HPAI is therefore considered as a new threat to wild bird conservation [105]. Globally threatened species, as defined by the IUCN Red List version 2022-2 [106], have been already decimated due to the infection, such as the above-mentioned seabirds [83], cranes (the hooded cranes, *Grus monacha*; the white-naped crane, *Grus vipio*, and red-crowned crane, *Grus japonensis*), Dalmatian pelicans (*Pelicanus crispus*), Bald eagles (*Haliaeetus leucocephalus*) and California condors (*Gymnogyps californianus*) [107–110]. With respect to California condors, given the impact of HPAIV H5N1-related mortalities on this critically endangered vulture, the emergency use of a vaccine to prevent additional deaths of these birds has been authorized by government

authorities [111]. Detection of an HPAI H5N1 virus of unknown clade in dead wild bird carcasses in the Galápagos islands has recently raised concerns for the conservation of endemic species such as the Galápagos lava gull (*Leucophaeus fuliginosus*) and the Galápagos penguin (*Spheniscus mendiculusi*) [112].

In the context of poultry, variable and even relatively lower mortality rates have been documented in experimental or natural infections with clade 2.3.4.4b viruses [113,114], with transmissibility rates varying within infected flocks [115,116]. Notably, certain HPAI H5N1 clade 2.3.4.4b virus genotypes, especially circulating in wild birds, have been associated with increased frequency of infections in non-human mammals [68,99,117], raising concerns for public health due to the identification molecular markers of virus adaptation to mammalian hosts [118,119].

Given the significant implications for veterinary and public health, recognizing the early signs and clinicopathological manifestations of HPAI H5 Gs/Gd 2.3.4.4b clade viruses is crucial to promptly act interventions to mitigate its spread across susceptible species. The review hereby presented aims at summarizing the main clinical, pathological, and histopathological findings of HPAI H5Nx clade 2.3.4.4b virus infections in main avian and mammalian hosts. To accomplish this, two databases, namely PubMed (<https://pubmed.ncbi.nlm.nih.gov>) and Scopus (<https://www.scopus.com/>), were queried from the 1st of February 2023 to the 9th of August 2023 to access articles published from the 1990s onwards. The literature search was performed using the following keywords: ‘highly pathogenic avian influenza’ or ‘clade 2.3.4.4’ and ‘wild birds’ or ‘ducks’ or ‘poultry’ or ‘chicken’ or ‘turkey’ or ‘game bird’ or ‘mammal’. Throughout the document, when HPAIV subtypes are mentioned without any specific H5 genetic clade designation, it refers to the H5 hemagglutinin clade 2.3.4.4b.

3. Wild Aquatic Birds

3.1. Anseriformes Order

Within the Anseriformes order (ducks, geese, and swans), HPAI H5Nx viruses of clade 2.3.4.4b have been linked to a diverse range of infection outcome and a variety of clinical and pathological manifestations, depending on the viral strain, the species considered, and the age of the infected birds. Experimental challenges of waterbird species with H5 clade 2.3.4.4b viruses are synthesized in Table 1. Data on domesticated Pekin ducks, which are equivalents to mallards [120], included in this paragraph are reported in Table 2 under the domestic poultry section.

Table 1. Experimental infections of highly pathogenic avian influenza (HPAI) H5Nx viruses of clade 2.3.4.4b in wild waterbirds included in this review.

Species (age)	Age	Virus	Route of infection (dose)	Morbidity* (%)	Mortality (%)	S or A	DT	Transmission	Ref.
Mallard	2 wk	A/Tufted-duck/Denmark/11470/LWPL/2016 (H5N8)	IC (10 ³ EID ₅₀ /mL)	0/5 (0)	0/5 (0)	n.a.	n.a.	No	[94]
			IC (10 ⁵ EID ₅₀ /mL)	5/5 (100)	4/5 (80)	S	5.8	Yes	
			IC (10 ⁷ EID ₅₀ /mL)	5/5 (100)	4/5(80)	S	3.2	Yes	
	2 wk	A/American Wigeon/South Carolina/22-000345-001/2022 (H5N1)	IC (10 ³ EID ₅₀ /mL)	5/5 (100)	1/5 (20) [†]	S	n.a.	Yes	[121]
			IC (10 ⁵ EID ₅₀ /mL)	5/5 (100)	1/5 (20) [†]	S	n.a.	Yes	
			IC (10 ⁷ EID ₅₀ /mL)	5/5 (100)	4/5 (100) [†]	S	n.a.	Yes	

	6 wk	A/herring gull/Poland/MB082B/2016 (H5N8)	IO and IN (10 ⁷ EID ₅₀ /mL)	12/12 (100)	7/12 (58)	S	3-14	n.a.	[96]
	8 wk	A/mute swan/Shimane/ 3211A002/2017 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	8/8 (100)	0/8 (0)	A	n.a.	n.a.	[122]
	>9 mo	A/EurasianWigeon/NL/4/2016 (H5N8)	IC (10 ⁷ TCID ₅₀ /mL)	7/7 (100)	0/7 (0)	A	n.a.	n.a.	[123]
Tufted duck	>9 mo		IC (10 ⁷ TCID ₅₀ /mL)	7/7 (100)	1/7 (14)	S	4	n.a.	
Eurasian wigeon	7 wk	A/duck/Neth/16014829- 001005/2016 (H5N8)	IO and IT (10 ⁶ EID ₅₀ /mL)	9/10 (90)	2/10 (20)	A	7-8	n.a.	[124]
		A/duck/Neth/17017236- 001005/2017 (H5N6)	IO and IT (10 ⁶ EID ₅₀ /mL)	10/10 (100)	9/10 (90)	S	3-6	n.a.	
Common teal	(n.d.)	A/mute swan/Shimane/ 3211A002/2017 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	8/8 (100)	0/8 (0)	A	n.a.	n.a.	[125]
Herring gull	8 wk	A/herring gull/Poland/MB082B/2016 (H5N8)	IO and IN (10 ⁷ EID ₅₀ /mL)	12/12 (100)	11/12 (90)	S	1-6	Yes	[126]

S, Symptomatic; A, asymptomatic infection; MDT, mean death time (days); IC, intratracheal route; IN, intranasal route; IO, intraocular route; IT, intratracheal route; IV, intravenous route. n.a., not applicable. n.d., not defined. *Number of individuals showing neurological signs that were euthanized. *Infected individuals based on RT-PCR or virus isolation or hemagglutination inhibition assay.

Dabbling ducks (*Anatidae*, *Anatinae* subfamily) have conventionally been regarded as more resistant than gallinaceous birds to overt HPAI clinical disease while maintaining high susceptibility to infection [127,128]. Notably, a sustained viral circulation in these species has been detected by surveillance efforts [67,117]. Among these, the mallard (*Anas platyrhynchos*) stands out as the most abundant and widely distributed duck species, serving as a crucial reservoir for LPAIVs [129]. Mallards’ higher resistance to HPAIV disease may be partially explained by the differences in cell signaling pathways compared to gallinaceous bird. Mallard possess RIG-I signaling pathway which receptor-mediated IFN-β signaling in infected cells and the rapid induction of ISGs that critically limit HPAI virus spread and viremia [130,131]. However, experimental infections of mallards with HPAI viruses of clade 2.3.4.4b have exhibited an array of clinical signs, contingent upon the specific strain and the dose used for challenging the birds, and the age of the animals. Little to no clinical signs and low to absent mortality rate was found in SPF mallards infected with a 2017-H5N6 clade 2.3.4.4b virus isolated in Japan, namely A/mute swan/Shimane/3211A002/2017 [122]. However, when considering experimental infections with HPAI H5Nx viruses of clade 2.3.4.4, an increased pathogenicity has been assessed for ducks infected with clade 2.3.4.4b viruses circulating in Europe during 2016-2017, in comparison to viruses of clade 2.3.4.4c [124,132]. This finding has been associated to differences in the translated amino acids in the viral genome, including internal genes, which confers novel pathobiological characteristics to the virus [133,134]. In a recent study, the synergic effect of HA, NP, NS and, to a lesser extent, NA proteins contributed to the increased receptor binding affinity, sialidase activity, interferon antagonism, and replication that conferred heightened virulence in H5N8 HPAIV of clade 2.3.4.4b-infected mallards, in comparison to H5N8 HPAIV of clade 2.3.4.4c [135].

A spectrum of clinical signs can be observed during experimental infections with 2016-H5N8 clade 2.3.4.4b HPAIVs in mallards or Pekin ducks. These signs range from mild nonspecific indications of HPAI infection (ruffled feathers, drooped wings, lethargy, and inappetence) to neurological signs, with a mortality rate ranging from 0 up to 80% depending on the strain used [94,96,124,136]. A notable weight loss has been associated to intratracheal experimental inoculation of mallards with 10⁷TCID₅₀/mL of A /Eurasian Wigeon/NL/4/2016 (H5N8) or 10⁷EID₅₀/mL of A/Tufted-duck/Denmark/11470/LWPL/2016 (H5N8), compared to sham-inoculated control groups

[94,123], with a dose-dependent effect [94]. Post-mortem examination (PME) of mallards experimentally infected with 2016-H5N8 HPAIVs of clade 2.3.4.4b revealed microscopic lesions similar to those described with other Gs/GD lineage H5N1 HPAI viruses, such as congestion, petechial hemorrhages and/or ecchymoses, and necrotizing lesions in the brain and visceral organs [94,96,123,132]. Microscopic lesions associated with the infection differed according to the day of sampling [96]. At 4 dpi, multifocal mild to moderate lymphohistiocytic infiltrations and, rarely, mild parenchymal necrosis, were observed in the myocardium, liver, and brain, where also a multifocal gliosis was noticed. Lastly, the immunohistochemistry (IHC) results indicated the predilection of H5N8 for nervous tissue, myocardium, respiratory epithelium, and hepatic and pancreatic cells [96]. Virus shedding was predominantly linked to the respiratory route [93,123,132,136], consistent with previous experimental infections of ducks with other HPAIV clades [61,137–140], but in contrast to LPAIVs preferential replication in the gastrointestinal tract of ducks [141,142]. However, heightened cloacal shedding was observed during experimental infection of SPF Pekin ducks with intranasal and intratracheal inoculums of 10^7 EID₅₀/mL of clade 2.3.4.4b viruses circulating in Europe during 2016–2017, namely A/duck/Neth/16014829-001005/2016 and A/duck/Neth/17017236-001005/2017, in comparison to an 2014-H5N8 HPAIV [124]. The phenomenon of “increased enterotropism” within recently circulating clade 2.3.4.4b viruses has been supported by immunohistochemical analysis in naturally infected individuals [143,144]. Virus antigen was detected in both the digestive and respiratory tracts of 2016-H5N8 and 2020-H5N8 HPAIVs infected wild ducks, as well as in the brain, liver, heart, and pancreas, in conjunction with necrosis and inflammation [144]. In the context of histopathological examination of naturally infected mallards with different subtypes of clade 2.3.4.4b viruses, the H5N1 HPAIV infection was linked to severe pancreatic necrosis [145], while H5N8-infected ducks, on the contrary, rarely exhibited mild pancreatic necrosis [146].

Despite frequent isolation of the virus in hunted, deceased, or moribund mallards during the 2021–2023 HPAI epidemics in Europe, the incidence of cases identified through passive surveillance (i.e., testing deceased or critically ill birds) has been lower in comparison to other waterbird species such as geese, swans, or terns. Historically, naïve geese and swans have been proven to be more susceptible to Gs/GD H5N1 HPAIV infection and disease than mallards [99,117,147–149]. Regardless, pre-exposure to homologous or heterologous AIVs may also modulate the disease outcome. This has been previously demonstrated for mallards and wood ducks (*Aix sponsa*) challenged with other Gs/Gd strains isolated during 2005 and belonging to clade 2.2 [150,151]. Pre-exposure to homo- or heterosubtypic LPAIVs or HPAIVs resulted protective against experimental HPAI virus challenge with 2.3.4.4b viruses, with a negligible infectious virus shedding from either the pharynx or cloaca insufficient to sustain a chain of virus transmission [93,96,123]. Following a less-virulent HPAIV challenge in wild ducks, a long-term immunity (> one year) has been assessed [123]. Absent or mild microscopic lesions (mild lymphoplasmatic infiltrates in the liver, heart, muscle, and proventricular mucosa) were observed in birds pre-exposed to LPAIVs or HPAIVs [93,96,123]. Considering the protective effect of an extant immunity against HPAI infection, apparently healthy but actively-infected mallards could potentially disseminate the virus and contribute to local or long-scale environmental contamination [96].

Although HPAI H5N8-infected satellite-tracked mallards were still capable of migration, suggesting a potential wide dispersion of the virus [152], also other species of dabbling ducks are regarded as efficient long-distance carriers of HPAI viruses along migratory routes. The initial evidence of this was presented with the isolation of H5N8 HPAI viruses of clade 2.3.4.4c in fecal samples collected from healthy Eurasian wigeons (*Mareca penelope*) during the years 2014 and 2015 in the Netherlands [153,154], as well as in Russia [155]. Subsequently, during the period of active AIV surveillance in Italy from 2020 to 2021, Eurasian wigeons sampled and examined displayed no apparent clinical signs despite ongoing infections with HPAI H5Nx viruses of clade 2.3.4.4b. These infected wigeons exhibited active viral shedding through both the oropharyngeal and cloacal routes [97]. However, when subjected to controlled experimental infections with clade 2.3.4.4b viruses, namely A/duck/Neth/16014829-001005/2016 (H5N8) or A/duck/Neth/17017236-001005/2017 (H5N6) inoculated intranasally and intratracheally at a dose of 10^6 EID₅₀/mL, listlessness, ruffled feathers,

reduced appetite, diarrhea, nasal discharge, and ultimately, neurological signs were observed and a notable mortality rate of 20% for H5N8-challenged individuals and 90% for H5N6-challenged individuals was recorded [124]. This was in line with the heightened pathogenicity of 2016-2017 HPAI viruses of clade 2.3.4.4b in ducks, in comparison with milder clade 2.3.4.4c infections [124,156]. In Europe, during the HPAI epidemic season of 2016-2017, it was estimated that up to 5% of the wintering population of Eurasian wigeons in the Netherlands might have succumbed to H5N8 virus circulation among wild birds [98]. Regarding HPAI virus shedding in Eurasian wigeons, an increased cloacal shedding was assessed for clade 2.3.4.4b viruses in experimentally inoculated individuals, in comparison to clade 2.3.4.4c virus inoculation [124]. Interestingly, IHC results of naturally infected individuals with HPAI H5N8 viruses circulating in Europe during 2016 confirmed virus replication in the intestinal tract [144]. However, in the case of individuals infected with the 2020-H5N8 HPAI virus strain, an intermediate level of virus attachment to intestinal epithelia was observed, suggesting incompatibility with virus replication at this site. Instead, these infected individuals displayed a high level of neurotropism, along with multifocal encephalitis characterized by areas of gliosis, neuronal degeneration, and necrosis [144].

Among dabbling ducks, Eurasian teals (*Anas crecca*) have also been proposed as potential long-distance vectors of HPAI infections due to asymptomatic disease [97,156,157]. Experimental infection of common teals with HPAI H5 2.3.4.4b viruses isolated in Japan during 2017, namely A/mute swan/Shimane/3211A002/2017 (H5N8), resulted in prolonged tracheal shedding and non-detectable clinical signs [125].

Turning attention to diving ducks (Anatidae, Aythyinae, Oxyurinae, and Merginae subfamilies), these inhabit freshwater, brackish, and coastal wetlands, often sharing ecological niches with dabbling ducks. Diving ducks are susceptible to AIV infection, yet limited reports have been published due to research and surveillance activities primarily focusing on dabbling ducks [158–164]. Following HPAI H5N8 of 2.3.4.4b clade natural infection, a significant number of tufted ducks (*Aythya fuligula*) were found deceased in Germany, displaying macroscopic changes such as severe hepatic necrosis, multifocal petechiae, and varying degrees of lung hyperemia and oedema [165]. IHC examination revealed the presence of AIV nucleoprotein (NP) antigen associated with necrotic lesions in the liver, heart, brain, spleen, pancreas, and thymus [165]. However, a different infection outcome is suspected to be influenced by the bird's immunological status. Similar to mallards, tufted ducks previously challenged with a less-virulent HPAIV (2014-H5N8 virus of clade 2.3.4.4c) before exposure to a more virulent 2016-HPAI H5N8 virus of clade 2.3.4.4b remained asymptomatic, avoiding body weight loss and mortality, in contrast to immunologically naïve control individuals directly challenged with the 2016-H5N8 [123]. Depending on the clade virus considered, HPAIV infection can however manifest different levels of pathogenicity. For instance, experimentally challenged common pochards (*Aythya ferina*) with an H5N8 HPAI clade 2.3.4.4c isolate from Europe (A/chicken/ Netherlands/emc-3/2014) became infected without evidence of disease, primarily shedding the virus through the pharynx [156]. These findings contrast with previous observations from experimental inoculation with an HPAI H5N1 virus of clade 2.2.1, which resulted in labored breathing, increased recumbency, neurologic signs, and mortality within 4 dpi [166]. Adults and juvenile ruddy ducks (*Oxyura jamaicensis*) and lesser scaups (*Aythya affinis*) were susceptible to experimental infection with HPAI H5N2 and H5N8 viruses of clade 2.3.4.4c obtained during 2014 [167]. Age-associated differences in clinical outcomes were observed, with higher disease susceptibility noted in juvenile ruddy ducks. Absence of clinical disease in adult ruddy ducks and lesser scaups suggested their potential reservoir role. However, low virus shedding, and a short duration of shedding indicated the inefficient role of these diving ducks in maintaining and disseminating the virus [167].

Geese and swans (Anatidae, Anserinae subfamily) are highly susceptible to HPAI clade 2.3.4.4b infection, with wild populations experiencing severe impact [69,168–170]. IHC results from naturally infected graylag goose (*Anser anser*) revealed 2016-HPAI H5N8 virus of clade 2.3.4.4b virus replication not only within the respiratory tract but also in the intestinal epithelium of deceased individuals [143]. Grossly, the primary pathological changes attributed to HPAIV H5Nx natural

infection encompassed multifocal necrosis in the liver and pancreas, pin-point hemorrhages in the brain, sub-pericardial hemorrhages, and multifocal lung hemorrhages [144]. Notably, gross findings in H5N8-infected black swans (*Cygnus atratus*) were scarcely undetectable, with occasional mild pancreatic or splenic necrosis [146]. Hydropericardium was identified in a mute swan (*Cygnus olor*) that underwent necropsy and tested positive for HPAI H5N1 clade 2.3.4.4b virus through rRT-PCR detection [145]. As reported by Floyd, *et al.* [171], in cases of HPAI H5N8 clade 2.3.4.4b virus infection in wild mute swans, gross findings were associated with liver and epicardial petechiae in one instance, and air sac opacity in another, contingent on the individual examined. Microscopic examination of tissues from one bird unveiled multifocal, necrotizing, nonsuppurative myocarditis, hepatitis, splenitis, nephritis, and encephalitis, accompanied by the intralesional presence of influenza A virus antigen, as observed in IHC analysis [171].

3.2. Charadriiformes Order

Gulls and other members of the Laridae family, within the Charadriiformes order, order have exhibited a marked susceptibility to HPAI H5Nx viruses of clade 2.3.4.4b infection [99]. Instances of mass mortality among adult seabirds have been documented across Europe, America, and Africa [81,99,102,172–174]. This underscores the rapid dissemination of infection attributed to their colony-breeding behaviors and scavenging tendencies. In the context of experimental infection, 8-week-old herring gulls (*Larus argentatus*) challenged with an HPAI H5N8 virus of clade 2.3.4.4b at 10⁷EID₅₀/mL intraocular and intranasal inoculation (A/herring gull/Poland/MB082B/2016 (H5N8)) displayed a swift and severe onset of clinical signs as early as 24 hours post-infection [126]. These signs encompassed heightened body temperature, depression, recumbency, reduced appetite, opisthotonos, torticollis, head tremors, paralysis, conjunctivitis, dyspnea, and nystagmus, alongside episodes of diarrhea. Within the 2-7 dpi period, 11 out of 12 directly infected gulls succumbed or were humanely euthanized due to the high fatality rate associated with the infection [126]. Similar to ducks, a greater survival rate was noted when birds had prior exposure to LPAI H5N1 virus infection. However, differently from ducks, pre-exposed exhibited abundant viral shedding from both the oral cavity and cloaca. PME subsequent to H5N8 experimental infection encompassed necrotizing inflammation of tissues, internal organ congestion (primarily intestines and lungs), and hemorrhages in subcutaneous tissues across the head, cerebral hemispheres, proventriculus, bursa of Fabricius, kidneys, liver, and spleen [126].

Natural HPAI H5N1 clade 2.3.4.4b infection in several species of terns, such as sandwich terns (*Thalasseus sandwicensis*), swift terns (*Thalasseus bergii*), common terns (*Sterna hirundo*), resulted in instances of die-offs [173,175,176]. A report from the Netherlands reported that afflicted sandwich terns exhibited marked debilitation, rendering them unable to take flight and predominantly lethargic; some were observed with wings outstretched. In later stages of the illness, certain individuals presented with opisthotonos and, on occasion, flipped over backwards. Among the four adult birds subjected to necropsy and subsequently confirmed by PCR analysis, IHC results revealed viral antigen expression in the pancreas (N = 3), duodenum (N = 4), or lung and nasal tissue (N = 1), colocalized with necrosis and inflammation [175].

In July 2021, in Scotland, notable die-offs of great skuas (*Stercorarius skua*) were observed, with instances escalating to the extent that mass mortality events were recorded among various breeding populations [177]. Birds submitted for PME were generally in good physical condition, occasionally displaying vent soiling or an empty proventriculus and gizzard. Histologically, the frequently encountered changes associated with HPAI H5N1 infection encompassed severe pancreatic necrosis, along with mild to moderate meningoencephalitis. In rare instances, findings included myocardial necrosis, moderate adrenal necrosis, mild hepatic necrosis, and proventricular necrosis. During the summer 2022, an additional decrease in the number of breeding colonies of great skuas was observed in the Shetland islands of the UK, coinciding with an unusually high occurrence of deceased individuals attributed to HPAI H5N1 clade 2.3.4.4b infection [178]. While PME or histopathological analyses were not conducted, a comprehensive description of clinical manifestations was provided. Affected individuals exhibited distinct neurological symptoms, including walking in circular

patterns, stumbling over their own feet, rolling movements, and a drooping head. As their condition deteriorated and they succumbed, these birds demonstrated severe hyperextension and spasticity in the head and neck region [178].

Within the Charadriiformes order, HPAIVs of clade 2.3.4.4b have been also detected in various species of wild shorebirds [54,179]. These hold a central role in the ecology of LPAIVs in North America [180], though they are less frequently sampled during HPAI surveillance efforts in Europe [100,101]. To date, experimental infections involving HPAI H5 viruses of clade 2.3.4.4b have yet to be published for these birds. However, a study conducted by Wille, *et al.* [181] reported the presence of antibodies against this H5 clade in red-necked stints (*Calidris ruficollis*) sampled during the 2016-2017 austral summer, thereby indicating prior virus exposure in this long-distance migratory wader.

3.3. Other Aquatic Wild Birds

The incursions of HPAI H5Nx viruses of clade 2.3.4.4b have also impacted African wild birds, particularly those in South and West Africa, owing to the presence of wetlands that host substantial numbers of potentially infected migratory birds from Eurasia during the winter months [182]. In January 2019, notable mortalities were documented among African penguins (*Spheniscus demersus*), an endangered penguin species, in Namibia due to HPAI H5N8 infection [183]. Prior to demise, afflicted individuals exhibited symptoms such as emaciation, torticollis, twitching, incoordination, corneal opacity, and lethargic or comatose behavior. Upon post-mortem examination, the carcasses appeared externally normal, though a grayish-greenish soiling of the peri-cloacal region was observed in two cases. Necropsy findings unveiled fibrinous strands within the coelomic cavity, widespread visceral congestion, and segmental hemorrhages along the gut in all instances. Instances of mass mortalities among African penguins due to H5N8 HPAIV infection had been previously documented in the Western and Eastern Cape Provinces of South Africa, alongside other migratory aquatic species, including Cape gannets (*Sula capensis*) and Cape cormorants (*Phalacrocorax capensis*) [176], in absence of further clinic-pathological examination. Additionally, Cape cormorants, an endangered bird species endemic to the southwestern African coasts, recently experienced HPAI H5N1 clade 2.3.4.4b outbreaks resulting in a significant decline in a Namibian colony in 2022 [184]. Lastly great white pelicans (*Pelecanus onocrotalus*) infected with HPAIV H5N1 displayed paralysis, dyspnea, diarrhea, and subcutaneous hemorrhages [185].

4. Game Fowls

Minor gallinaceous species have been recognized as susceptible to HPAIV infection, although have been rarely investigated in comparison to domestic poultry or wild aquatic birds [186]. Furthermore, game birds have been considered as potential bridge host for AIV incursion in poultry farms [186]. In the context of HPAI H5 clade 2.3.4.4b, the literature offers scant reports. Wild ring-necked pheasants (*Phasianus colchicus*) have been found HPAIV of clade 2.3.4.4b-infected in several European countries, demonstrating systemic virus tropism resembling that in chickens with high morbidity and mortality [54,187]. Notably, in Finland, mass mortality events of pheasants released in hunting grounds led to the spillover of an HPAI H5N1 virus of clade 2.3.4.4b that severely affected wild carnivores [188]. Intranasal and intraocular experimental infections of pheasants with different doses (10^5 or 10^7 EID₅₀/mL) of A/pheasant/Denmark/12106-3/2018, an H5N8 virus of clade 2.3.4.4b, showed evident clinical signs (drooped wings, huddling, ruffled feathers and lethargy, associated to neurological signs in some individuals), up to 100% mortality and efficient transmission to naïve pheasant or chicken contact birds, confirming the role of pheasants as bridging host for the infection of commercial poultry [189]. Similarly to experimental infection, naturally infected pheasants with 2017-HPAI viruses of clade 2.3.4.4b displayed severe clinical signs with neurological disorders [190,191]. PME findings aligned with prior observations in other gallinaceous species, characterized by necrotic and hemorrhagic lesions in visceral organs [186,190,191]. However, a novel and consistent discovery was the identification of diphtheroid plaques in the oropharyngeal mucosa, linked to viral-induced necrotizing stomatitis of the epithelium [190]. IHC results demonstrated viral antigen consistently detected in parenchymal and endothelial cells within the encephalon, heart, and nasal

mucosa, and less frequently in the kidney, pancreas, and liver. PCR results indicated a higher virus shedding via the oropharynx [190].

Evidence from necropsied naturally HPAIV H5N1 clade 2.3.4.4b-infected pheasants, peafowl (*Pavo cristatus*), and guinea fowls (*Numida meleagris*) in the UK has primarily revealed pancreatic and splenic necrosis, accompanied by hydropericardium and epicardial petechiae [145].

5. Pigeons and Doves

Pigeons and doves are susceptible to HPAI infection, however do not usually exhibit clinical signs and are considered ineffective vectors of the virus due to low viral shedding [192]. Nevertheless, varying outcomes of infection can be observed depending on the viral clade used for experimental infection [90,193]. Natural infections of H5N8 HPAIVs of clade 2.3.4.4b in wild columbids in South Africa during 2017 resulted in atypical die-offs, possibly exacerbated by concurrent avian orthoavulavirus infection [176]. A noteworthy instance of natural HPAI H5N1 clade 2.3.4.4b infection was documented in a wood pigeon (*Columba palumbus*) that was found dead in a German wildlife sanctuary [194]. Gross findings included mild enlargement of the spleen and few greyish discolorations in the pancreas. mild splenic enlargement and a few greyish discolorations in the pancreas. Histologically, acute lymphohistiocytic meningoencephalitis was observed, accompanied by mild perivascular cuffing and neuronal necrosis in the grey matter of the cerebral hemispheres and brain stem. Additionally, the pancreas displayed severe multifocal to coalescing necrotizing pancreatitis. IHC results unveiled a pronounced neurotropism of the virus [194].

6. Scavengers and Raptors

Due to their feeding ecology, scavengers (such as corvids, nocturnal and diurnal raptors) and birds of prey (falcons, hawks, eagles, owls) have the potential to come into contact with AIV-infected prey or carcasses [195]. Consequently, HPAI epizootics involving clade 2.3.4.4b viruses have had severe impacts on these bird populations [69,98,196,197]. In the context of the Accipitriformes order, natural HPAI H5N1 virus of clade 2.3.4.4b infection in an African fish eagle (*Ichthyophaga vocifer*) resulted in gross findings of carcass dehydration and oedematous brain [198]. In 2017, die offs in white-tailed eagles (*Haliaeetus albicilla*) were attributed to H5N8 clade 2.3.4.4b infection [196]. Infected birds found alive exhibited neurological symptoms including torticollis, opisthotonus, limber neck, ataxia, and circling movements. PME revealed scant or absent gross lesions typically associated with HPAI. However, histopathological and immunohistological investigations unveiled oligo- to multifocally necrotizing lesions in the cerebrum, cerebellum, and brain stem. Immunohistological and virological analyses confirmed the presence of influenza A virus NP antigen and/or H5N8-specific RNA in organ samples [196]. PME of HPAI H5N8-naturally infected common buzzards (*Buteo buteo*) found in the Netherlands did not reveal any external lesions, with birds in a moderate to good state of nutrition, highlighting the acute nature of their deaths [199]. Among the 11 birds examined, only two displayed gross abnormalities, including well-delimited, multifocal hemorrhages in the heart and brain of one individual, and a swollen liver with a rounded margin in another. Multifocal encephalitis with foci of gliosis, neuronal degeneration, and necrosis was reported in the majority of the birds, indicating the neurotropic nature of the viral strain. Furthermore, the highest amount of virus based on PCR results was detected in the myocardium, indicating the cardio-tropism of the strain, with focal and extensive myocardial necrosis observed. Interestingly, necrotic lesions were found in the proventriculus of two common buzzards, with viral antigen present in the epithelial cells [143].

Among corvids (Passeriformes order), virus histochemistry revealed the presence of HPAI H5N8 virus antigen in both respiratory and digestive tract of naturally deceased Eurasian magpies (*Pica pica*) [143], which are often found around poultry houses and have been regarded as bridge hosts for avian influenza transmission between domestic and wild birds [195].

7. Domestic Poultry

HPAIV infection in chickens and turkeys are typically associated with high morbidity and mortality. Clinical signs manifest as systemic virus replication and damage to visceral organs, as well as cardiovascular and nervous systems [200]. However, a fulminating disease with sudden death is also frequently observed. HPAI-infected chickens have an early and increased production of proinflammatory molecules and succumb to cytokine storm [201–203]. Gross lesions can vary based on factors such as the virus strain, the duration between infection and death, and the age and species/breed of the poultry affected [13]. Regarding HPAIV H5Nx of clade 2.3.4.4b, both experimental and natural infections have been documented in major poultry species. An overview of the experimental challenges involving HPAIV viruses of clade 2.3.4.4b hereby gathered is reported in Table 2.

Table 2. Selected studies reporting experimental infections with highly pathogenic avian influenza (HPAI) H5Nx clade 2.3.4.4b strains in domesticated poultry species.

Species	Age	Virus	Route of infection (dose)	Morbidity* (%)	Mortality (%)	S or A	MDT	Transmission	Ref
Pekin duck	1 wk	A/tufted duck/ Germany/AR8444-L01987/2016 (H5N8)	IM ¹ (10 ⁶ TCID ₅₀ /bird)	10/10 (100)	10/10 (100)	S	2	Yes	[132]
	5-6 wk	A/wigeon/Wales/052833/2016 (H5N8)	IN and IO (10 ⁷ EID ₅₀ /mL)	8/8 (100)	0/8 (0)	A	n.a.	Yes	[136]
			IO and IN (10 ⁵ EID ₅₀ /mL)	5/5 (100)	1/5 (20)	S	n.a.	Yes	
			IO and IN (10 ³ EID ₅₀ /mL)	5/5 (100)	2/5 (40)	S	n.a.	Yes	
	7 wk	A/duck/Neth/16014829- 001005/2016 (H5N8)	IN and IT (10 ⁷ EID ₅₀ /mL)	10/10 (100)	0/10 (0)	A	n.a.	n.a.	[124]
		A/duck/Neth/17017236- 001005/2017 (H5N6)	IN and IT (10 ⁷ EID ₅₀ /mL)	10/10 (100)	6/10 (60)	S	≥3	n.a.	
Domestic duck	14 mo	A/tufted duck/Germany/AR8444L01987/20 16 (H5N8)	IN (10 ⁶ EID ₅₀ /mL)	10/10 (100)	10/10 (100)	S	1-8	Yes	[93]
	adult	A/tufted duck/ Germany/AR8444-L01987/2016 (H5N8)	IO and IN (10 ⁶ TCID ₅₀ /bird)	10/10 (100)	2/10 (20)	A	4-5	Yes	[132]
	2 wk	A/mandarin duck/ Korea/H242/2020 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	5/5 (100)	0/5 (0)	A	n.a.	Yes	[204]
		A/duck/Korea/HD1/2017 (H5N6)	IN (10 ⁷ EID ₅₀ /mL)	5/5 (100)	0/5 (0)	A	n.a.	Yes	
Muscovy duck	1 wk	A/tufted duck/ Germany/AR8444-L01987/2016 (H5N8)	IM ¹ (10 ⁶ TCID ₅₀ /bird)	10/10 (100)	10/10 (100)	S	≥2	n.a.	[132]

Chicken – Ross 308	2 wk	A/Goose/Spain/ IA17CR02699/2017 (H5N8)	IN (10 ⁵ ELD ₅₀ /0.05mL)	15/15 (100)	1/15 (8)	S	6	n.a.	[205]
			IN (10 ⁵ ELD ₅₀ /0.05mL)	13/13 (100)	3/13 (25)	S	3.7	n.a.	
	4 wk	A/common- coot/Egypt/CA285/2016 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	10/10 (100)	10/10 (100)	S	8.1	Yes	[206]
		A/duck/Egypt/F446/2017 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	10/10 (100)	10/10 (100)	S	2.5	Yes	
		A/duck/Egypt/SS19/2017 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	10/10 (100)	10/10 (100)	S	4.2	Yes	
		A/American Wigeon/South Carolina/22-000345-001/2021 (H5N1)	IC (10 ^{3.6} EID ₅₀ /mL)	3/5 (60)	3/5 (60)	S	2.7	Yes	[207]
			IC (10 ^{5.6} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	2	Yes	
			IC (10 ^{7.6} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	1	Yes	
		A/Tufted duck/Denmark/11740- LWPL/2016 (H5N8)	IC (10 ^{1.5} EID ₅₀ /mL)	0/5 (0)	0/5 (0)	A	n.a.	No	
			IC (10 ^{2.8} EID ₅₀ /mL)	1/5 (20)	1/5 (20)	S	3	No	
			IC (10 ^{6.7} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	2	Yes	
Chicken – White Leghorn	5 wk	A/mandarin duck/ Korea/H242/2020 (H5N8)	IN (10 ⁷ EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	4.3	Yes	[204]
		A/duck/Korea/HD1/2017 (H5N6)	IN (10 ⁷ EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	2.2	Yes	
	6 wk	A/tufted duck/ Germany/AR8444-L01987/2016 (H5N8)	IV ² (n.d.)	10/10 (100)	10/10 (100)	S	2	Yes	[132]
		A/domestic duck/	IV ²	10/10 (100)	10/10 (100)	S	3	n.a.	[208]

Turkey – Commercial BBW		Siberia/49feather/2016 (H5N8)	(10 ⁷ EID ₅₀ /mL)						[116]
		A/tufted-duck/Denmark/11740/ 2016 (H5N8)	IC (10 ⁷ EID ₅₀ /mL)	13/13 (100)	13/13 (100)	S	2.1	No	
		A/turkey/Hungary/53433/2016 (H5N8)	IC (10 ⁷ EID ₅₀ /mL)	13/13 (100)	13/13 (100)	S	2.1	No	
	3 wk	A/American Wigeon/South Carolina/22-000345-001/2021 (H5N1)	IC (10 ^{3.6} EID ₅₀ /mL)	4/5 (80)	5/5 (100)	S	4.6	Yes	[207]
			IC (10 ^{5.6} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	3.4	Yes	
			IC (10 ^{7.6} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	2.6	Yes	
		A/Tufted duck/Denmark/11740-LWPL/2016 (H5N8)	IC (10 ^{1.5} EID ₅₀ /mL)	0/5 (0)	0/5 (0)	A	n.a.	No	
			IC (10 ^{2.8} EID ₅₀ /mL)	0/5 (0)	0/5 (0)	A	n.a.	No	
			IC (10 ^{6.7} EID ₅₀ /mL)	5/5 (100)	5/5 (100)	S	3	Yes	
	5-6 wk	A/wigeon/Wales/052833/2016 (H5N8)	Contact to infected ducks	12/12 (100)	12/12 (100)	S	2.96 to 8.54	n.a.	[136]

S, Symptomatic infection; A, asymptomatic infection; MDT, mean death time; IC, intrachoanal route; IN, intranasal route; IM, intramuscular route; IO, intraocular route; IT, intratracheal route; IV, intravenous route. n.a., not applicable. ¹Intramuscular pathogenicity index testing. ²Intravenous pathogenicity index testing. *Infected individuals based on RT-PCR or virus isolation or HI assay.

7.1. Chickens

Chickens (*Gallus gallus domesticus*) are highly susceptible to HPAI, with a severity of infection varies depending on the viral strain and host factors (species, breed, age at infection, and immune response) [25,209,210]. The inoculation of most HPAIVs in chickens causes evident clinical signs (e.g. apathy, nervous signs) and gross lesions (e.g. cutaneous oedema, cyanosis of the comb and wattles, hemorrhages in skin), and chickens usually die shortly after the infection [25,211].

With respect to 2016-HPAI viruses of clade 2.3.4.4b, SPF chickens intravenously infected with HPAI H5N8 strains, namely A/tufted_duck/Germany/AR8444L01987/2016 or A/domestic duck/Siberia/49feather/2016, showed systemic disease and rapid death within 2-3 dpi [132,208]. Classic lesions of HPAI infections were noticed in visceral organs, such as necrosis and necrotizing inflammation [132,208,212]. A pronounced neurotropism was noticed for A/domestic duck/Siberia/49feather/2016, with the highest titer observed in the brain ($6.75 \pm 0.07 \log_{10} \text{TCID}_{50}/\text{mL}$) [208]. Following natural route of infection though, HPAIVs H5Nx clade 2.3.4.4 have showed varying and relatively lower mortality rates in experimentally inoculated or contact-exposed chickens, in comparison to previous HPAIVs H5N1 of the Gs/GD lineage that caused almost 100% mortality [114,213,214]. In testing the pathogenicity and transmissibility of three H5N8 Egyptian isolates of clade 2.3.4.4b collected during 2016-2017, namely A/common-coot/Egypt/CA285/2016 (CA285), A/duck/Egypt/F446/2017 (F446) and A/duck/Egypt/SS19/2017 (SS19), in SPF chickens inoculated intranasally, a systemic viral replication and inflammatory and necrotizing changes in organs were noticed in all the infected groups [206]. Chickens challenged with the F446 strain exhibited higher transmissibility and pathogenicity (shorter mean death time, MDT), than CA285 and SS19, likely due to the presence of a more adapted reassorted polymerase complex. Higher viral titers in swabs and organs associated to a relatively delayed mortality (4-7 dpi MDT versus 3 dpi MDT) were noticed in comparison to an HPAIV H5N1 strain, namely A/chicken/Egypt/15S75/2015 (clade 2.2.1.2), used as a control [206]. Differently, a 100% mortality within 3 dpi was shown in SPF chickens inoculated with two HPAI H5N8 viruses of clade 2.3.4.4b isolated in Europe (A/turkey/Hungary/53433/2016 and A/tufted-duck/Denmark/11740/2016) [116]. Notably, a higher oropharyngeal viral shedding was observed compared to cloacal shedding, but no transmission to co-housed chickens occurred. Microscopic examination revealed necrosis of parenchymal cells, accompanied by lymphoplasmacytic or heterophilic inflammatory infiltrates, consistent with other groups infected with viruses belonging to 2.3.4.4a, 2.3.4.4c, 2.3.4.4d and 2.3.4.4e H5 clades. Remarkably, virus replication was prominently evident in villi enterocytes and capillaries, often leading to focal areas of necrosis with lymphoplasmacytic infiltrates, similar to the findings seen in HPAI H5 2.3.4.4a and 2.3.4.4e clade virus-challenged individuals [116].

During the 2020-2021 HPAI H5Nx of clade 2.3.4.4b virus incursions in Europe, PME of chickens succumbed to infections reported frequent cyanosis of the combs or reddening of the feet, facial edema, and pancreatic and splenic necrosis [146]. Notably, 10.3% (24 out of 230) outbreaks in broilers were associated with the absence of mortality and/or clinical signs [69]. This “*silent infection behavior*” was associated to minimal tracheal and/or cloacal viral shedding and, considering the potential delayed outbreak suspicion and diagnosis on farms due to scarce clinical signs, additional sampling strategies have been proposed for these poultry species [68,113]. The lower pathogenicity of a 2020-HPAI H5N8 virus of clade 2.3.4.4b (A/mandarin duck/Korea/H242/2020) in comparison to a 2016-HPAI H5N6 virus of the same clade (A/duck/Korea/HD1/2017) has been assessed in five-week-old SPF chickens experimentally challenged [204]. The results revealed that chickens inoculated with the 2020-HPAI H5N8 virus exhibited a longer survival period, with a MDT of 4.3 days, compared to the 2.2 days observed for the 2016-HPAI H5N6 virus. Notably, a higher viral shedding titer from both oral and cloacal routes was detected through virus isolation of HPAIV H5N8 from swabs, in comparison to the HPAI H5N6 strain. This finding suggests an increased risk of viral contamination on farms and delayed epidemic control [204].

A different pathogenicity of HPAIV infection can be observed with respect to the chicken breed/line considered [215–217]. SPF chickens and Ross 308 broilers were more resistant to HPAI

experimental inoculation with A/Goose/Spain/IA17CR02699/2017 (H5N8) of clade 2.3.4.4b virus in comparison to four Spanish local breeds (Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller, Euskal Oiloa) [205]. The more resistant chickens breeds displayed less frequency of severe clinical signs, lower mortality rates and lower number of animals shedding virus. This suggests that the genetic background may confer a higher natural resistance to different HPAIVs subtypes, in line with a previous report [218].

7.2. Turkeys

Turkeys (*Meleagris gallopavo*) are particularly susceptible to HPAI infection, often experiencing more severe impacts compared to other poultry species [12,219,220].

In relation to HPAIV strains of clade 2.3.4.4b circulating in Europe during the autumn 2016, SPF turkeys in contact with Pekin ducks infected with different doses of A/wigeon/Wales/052833/2016 (H5N8) (10^7 , 10^5 , 10^3 , 10^1 EID₅₀/mL given intraocularly and intranasally) all became infected and died [136]. The longest MDT (8.54 days) was observed in turkeys exposed to the lowest duck-dose group, and the onset of clinical signs, such as loss of balance, inability to seek food or water, tremors, and diarrhea, ranged between 6-14 hours prior to death. PME of dead or humanely euthanized turkeys revealed moderate splenomegaly and mottling. IHC results confirmed systemic infection and indicated the presence of AIV antigen in adrenal medulla ganglions, ependymal cells of the central nervous system (associated with pronounced lymphocytic meningitis and perivascular cuffing), thymus epithelia, and exocrine pancreas epithelia. Furthermore, IHC findings underscored the widespread distribution of the virus, including feather follicles, air sacs, thymus, liver, ovary/testes, nasal cavity, and gizzard [136]. PMEs in field cases of HPAI H5N8 and H5N1 virus infections in turkeys during the 2020-2021 outbreaks in the UK reported facial oedema in a small number of individuals, particularly around the masseter, submandibular, periocular, or conjunctiva areas. A substantial proportion of infected turkeys exhibited pancreatic necrosis, often appearing as multifocal to coalescing tan discoloration and, in some instances, forming target-like lesions. All H5N8-infected turkeys exhibited enlarged and rounded spleens, featuring multifocal white mottling. Kidney lesions were present in 33% of the examined turkeys, characterized by swollen parenchyma displaying areas of pallor and occasional petechiae within the renal subcapsular spaces or peri-renal fat. Pancreatic and splenic necrosis were also commonly observed in HPAI H5N1-infected individuals [145]. In an experimental setting comparing HPAI H5N1 of clade 2.3.4.4b infection in chickens and turkeys, the latter exhibited longer MDT (2.6-8.2 days *versus* 1-4 days for chickens), increased virus shedding and facilitated transmission to contact birds [207].

Following the introduction of the H5 clade 2.3.4.4b in North America in 2021 [71,72], Malmberg, *et al.* [221] investigated the pathological effects of HPAI H5N1 virus infection in wild turkeys found deceased nearby an infected backyard poultry farm. External gross lesions commonly associated with HPAIV infection in poultry, such as cyanosis, facial edema, petechiae, were not observed. Instead, the prevailing gross lesions included mild to marked, multifocal to coalescing necrosis of the ovary and oviduct, along with mottling of the spleen. Furthermore, moderate to marked, diffuse edema and congestion of the lungs were noted. Upon histopathological examination, all examined individuals displayed signs of acute multiorgan necrosis in various organs, including the lungs, liver, gonads, and lymphoid tissues of the spleen and gastrointestinal tract. Notably, no significant lesions were identified in the heart or trachea. A lower number of individuals showed necrotic or inflammatory lesions in the brain, pancreas, kidney, esophagus, and thymus [221].

7.3. Domestic Ducks and Geese

Consistent with field observations in wild waterfowl, experimental studies using 2016-2016 HPAI H5 viruses of clade 2.3.4.4b in domestic duck breeds resulted in high virus replication and a range of mortality rates [94,132,136].

Severe disease was reported in Egyptian ducks from a meat duck farm during an HPAI H5N8 outbreak in 2017 in Egypt [222]. Clinical signs included ruffled feathers and nervous system involvement such as abnormal head position, recumbency, and tremors, resulting in a 90% mortality

rate. Gross observations revealed multifocal areas of encephalomalacia in the cerebrum, petechial hemorrhages on the cerebellar surface, and cerebellar swelling. Furthermore, the lung, kidney, and spleen displayed congestion, while the pancreas exhibited multifocal areas of necrosis [222]. Previously, similar lesions in the brain were reported in HPAI H5N2-, H5N6- and H5N8 virus-infected domestic Pekin ducks [223] and H5N8-infected fattening ducks [224]. An increased neurovirulence of clade 2.3.4.4b virus infection [A/tufted_duck/Germany/AR8444L01987/2016 (H5N8)] in Pekin ducks inoculated by oculo-nasal route was reported in comparison to 2.3.4.4c virus infection [(A/turkey/Germany-MV/R2472/2014 (H5N8)] [132]. Ducks dying following 2016-HPAIV H5N8 of clade 2.3.4.4b experimental infection consistently exhibited moderate, multifocal, acute to subacute, necrotizing polioencephalitis with intralesional antigen-positive neuroglial cells and high viral titer levels in the brain, comparable to those of lung samples of the same animals. In contrast, no inflammatory changes and no antigen was detectable in the brains of 2014-H5N8 clade 2.3.4.4c challenged ducks [132]. The predominant neurotropism of HPAI virus in domestic ducks has been linked to a combination of early colonization of the central nervous system (CNS) in HPAIV H5N8-experimentally challenged individuals and of the prolonged survival after the onset of virus replication, allowing the virus and the host response to cause significant tissue damage to the brain tissue [225]. Genetic differences between HPAI-H5N8 viruses of clade 2.3.4.4c and 2.3.4.4b might influence the differences in duck pathogenesis, following continuous evolution through genetic reassortment [135,226]. Neurotropism of 2016-HPAI H5N8 virus strains in Pekin ducks has been linked to a combination of virulence factors expressed by the HA, NP, NS and, to a lesser extent, NA [227]. Also, species-related factors (e.g., breed, age, route of inoculation, challenge dose) can play a role in the severity of the disease developed following HPAIV infection in ducks. As an example, mallards and Pekin ducks are generally considered more resistant than Muscovy ducks to infection with previously circulating HPAI strains belonging to other H5 genetic clades [132,228–230]. However, H5 clade 2.3.4.4b virus infection exhibited high virulence even in mallards and Pekin ducks [94,132,227]. On the other hand, domestic ducks can display no clinical signs, prolonged viral shedding, and a 100% transmission rate to contact birds, as demonstrated in an experimental study involving A/mandarin duck/Korea/H242/2020 (H5N8) and A/duck/Korea/HD1/2017 (H5N6) intranasally inoculated at low to high doses (10^3 , 10^5 , and 10^7 EID₅₀/mL) [204]. These pathogenic features of HPAI virus in ducks have been linked to their epidemiological role as asymptomatic carrier in the field [204].

In the case of domestic geese, Zhu, *et al.* [231] documented an HPAI H5N8 outbreak in January 2021 on an egg-laying farm in Northeastern China. The overall mortality rate reached 50%, with affected individuals displaying cyanosis in the head, sarcoma, oral mucosa, tongue, and hemorrhagic conjunctiva and nostrils. PMEs of the coelomic cavity revealed lesions primarily localized in the digestive and respiratory tracts, including hemorrhagic trachea, larynx, liver, and glandular gastric papillae. Additionally, a diffuse hemorrhage was observed in the intestine and pancreas [231]. Natural cases of HPAI H5N1 infections in domestic geese in the UK were associated with pancreatic lesions [145].

8. Mammals

Since 2016, HPAI H5N1 viruses of clade 2.3.4.4b have been associated with global spillover events involving wild and domestic mammals and, in rare instances, humans exposed to infected birds [118,232]. Among wild mammal species, the infection with HPAI H5Nx viruses has been most frequently detected in predators or scavengers such as wild canids, felids, and marine mammals, that hunt infected wild birds, and/or feed on wild bird carcasses. With respect to outbreaks of H5Nx in farmed mammals, these were linked to virus introduction via wild birds through to direct or indirect contacts [233–235], or through exposure to infected poultry [236]. Notably, instances of mass mortality in pheasants released in hunting estates in southern Finland have been attributed to virus infections in wild mammals, underscoring the significance of robust surveillance efforts in wildlife [188]. While conclusive evidence of mammal-to-mammal transmission remains elusive, die-off events

among wild sea lions along the Pacific coast of South America, as well as outbreaks in fur farms across Europe, suggested the potential for avian-independent transmission of the virus [81,84,233,234].

Even pets have not been spared from HPAI virus of clade 2.3.4.4b infection. In Poland, domestic cats (*Felis catus*) were found to be HPAI H5N1-infected around mid-2023, likely as a result of consuming contaminated poultry meat [237]. In Italy, dogs (*Canis lupus familiaris*) and a cat were found to have seroconverted following exposure to H5N1 virus during a poultry outbreak [118].

In terms of human cases of HPAI infections with clade 2.3.4.4b, eleven sporadic cases have been reported from January 2022 to November 2023 following infected-poultry exposure. However, as of now, there has been no confirmed evidence of human-to-human transmission [238].

8.1. Farmed Animals and Pets

Among farmed animals, domestic pigs (*Sus scrofa*) have always been spotlighted in the context of AIV infection due to their role as a “mixing vessel” in the virus epidemiology, enabling further reassortment between human, avian, and swine strains [239]. Serological detections of HPAI H5Nx clade 2.3.4.4b exposure have been identified in asymptomatic pigs across various farms in France [235], as well as in healthy pigs sampled during an HPAI H5N1 outbreak on a rural mixed-species poultry farm in Italy [236]. From literature, to date, a single experimental challenge in pigs has been reported by Graaf, *et al.* [240]. In this study, four-month-old pigs were nasally exposed to an HPAI H5N1 virus (A/chicken/Germany/AI04286/2022), yet no clinical manifestations or pathological changes were observed. Among the eight pigs, only one seroconverted at 14 dpi, and minimal levels of viral RNA were detected in the respiratory, alimentary, and brain tissues of this individual. The authors concluded that pigs are unlikely to serve as significant carriers for transmitting this specific genotype of HPAI virus H5N1 clade 2.3.4.4b among pigs and across interfaces [240].

More recently, The U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) has confirmed the detection of highly pathogenic avian influenza (HPAI) in goats in Minnesota (1) and dairy herds in Texas (7) Kansas (2), Michigan (1), and New Mexico (1). Adult bovines presented decreased lactation, low appetite, and other non-specific clinical signs [23]. Affected dairy farms have reported about 10% morbidity of their milking cows, but there have been no confirmed deaths associated with HPAI in dairy cattle yet [241]. In addition to that, one person in Texas has tested positive for HPAI A(H5N1) virus after exposure to the infected ruminants [242]. In Minnesota, detection was prompted by unusual deaths of newly kidded goats on the property where a backyard poultry flock had been depopulated due to HPAI in February 2024. The goats and poultry had access to the same areas and included a shared water source [243].

Different outcomes have been noted in farmed animals of the Carnivora order. Specifically, American minks (*Neovison vison*) raised for fur production in Spain exhibited a sudden increase in mortality rate (0.77% versus 0.2-0.3% during the initial phase of the outbreak) due to HPAI H5N1 infection [233]. Clinical signs included loss of appetite, hypersalivation, depression, bloody snouts, and neurological manifestations like ataxia and tremors. PME revealed either hemorrhagic pneumonia or red hepatization of the lungs. Elevated viral loads were detected through rRT-PCR analysis of oropharyngeal and rectal swabs. As the infection spread across the holding and affected more minks, onward transmission of the virus within the population was suspected [233]. Since mid-July 2023, HPAI H5N1 outbreaks have impacted American minks, raccoon dogs (*Nyctereutes procyonoides*), arctic foxes (*Vulpes lagopus*), red foxes (*Vulpes vulpes*) and hybrid foxes on fur farms in Finland [234]. An elevated mortality rate, ranging from 2 to 10 times higher than normal, was observed, accompanied by symptoms characteristic of HPAI H5N1 virus infection in mammals. These included lethargy, neurological signs, diarrhea, and rapid death. PMEs revealed systemic disease signs and lung lesions. Phylogenetic analyses indicated multiple introductions of the virus from birds to farmed fur animals, while also suggesting potential intra-species transmission, given the higher number of cases and farms involved in the outbreaks [234].

With respect to pets, while there is serological evidence of exposure to infection with H5 viruses of clade 2.3.4.4b [118], only a limited number of reports exists on direct virus detection. An instance during an HPAI H5N1 outbreak in a breeding duck farm in France in 2022 involved a cat exhibiting

general condition disturbances, including apathy and mild hyperthermia [244]. As the clinical condition worsened, with the onset of neurological and respiratory symptoms, a compassionate euthanasia was administered. Notably, negative results from serologic and rRT-PCR analyses of samples from the dog and another cat in the same household indicated an absence of inter-mammal transmission [244]. In the year 2023, an HPAI H5N1 virus outbreak impacted 25 domestic cats across various regions in Poland for a period of three weeks [237]. Distinct clinical signs were documented, encompassing loss of appetite, apathy, hypersalivation, fever, dyspnea, a firm and tender abdomen, and occasional urinary incontinence. Additional symptoms included reddened mucous membranes, trismus, and subsequently, neurological manifestations such as epileptic seizures, heightened muscle tension, and occasional limb stiffness. Given the rapid deterioration of the animals' condition within one or two days, euthanasia was commonly employed. PMEs of 11 individuals unveiled systemic inflammation accompanied by congestion and enlargement of internal organs, including the kidneys, liver, intestine, spleen, and pancreas. Breathing impairment was attributed to turbid fluid in the respiratory portion of the pharynx, and the lungs displayed congestion along with alveolar hemorrhages, interspersed with areas of both lighter emphysema and darker atelectasis. The cranial cavity and subdural space contained bloody fluid, with congested superficial brain vessels. Notably, the highest AIV viral loads were detected in the brain, lungs, and bronchi [237].

8.2. Wild Mammals

8.2.1. Marine Mammals

Spill-over events involving HPAI H5Nx viruses of clade 2.3.4.4b to marine mammals are widely observed in seals. Specifically, along the Baltic coast of Poland during 2016-2017, an HPAI H5N8 virus was isolated from lung samples taken from two gray seals (*Halichoerus grypus*) [245]. Examination of the lungs by gross pathology and histopathology did not reveal any suspicious lesions that indicated an influenza virus infection, that was diagnosed by PCR analysis [245]. Subsequent reports emerged in the UK in late 2020, involving harbor seals (*Phoca vitulina*) and a gray seal that either perished or were humanely euthanized within a 2-day span from onset of clinical signs at a wildlife rehabilitation center [171]. Affected seals had been housed in the proximity of swans which were the first to succumb to HPAI H5N8 clade 2.3.4.4b infection at the center. Both species of seal exhibited an abrupt onset of systemic or neurological clinical signs. Harbor seals manifested seizures followed by death, while the gray seal displayed fever, facial twitching, and stupor, necessitating euthanasia on animal welfare grounds. PME revealed generalized lymphadenomegaly, occasionally coupled with multiple pale foci in the lungs, as well as congested meninges. Microscopic evaluation unveiled severe necrotizing nonsuppurative polioencephalitis associated with abundant viral antigens in neurons and ependymal cells. Surprisingly, PCR testing yielded negative results for AIV antigens in nasal and rectal swab samples of the three seals. In another occurrence, adult harbor seals were reported deceased in mid-August 2021 along the German North Sea due to HPAI H5N8 infection [246]. Histopathological assessments revealed mild to moderate lymphohistiocytic meningoencephalitis, accompanied by limited neutrophils and hemorrhage, indicating a fatal CNS infection. Further lesions encompassed moderate multifocal lymphohistiocytic vasculitis, along with single-cell necrosis affecting primarily glial cells. Eosinophilic, shrunken, necrotic neurons, as well as fading neurons, were observed within the cerebrum and cerebellum. One individual exhibited a severe, diffuse, acute lung hyperemia, alongside alveolar emphysema, and oedema. Intriguingly, AIV antigen was not detected in the lungs, although it was present in brain tissue. Notably, tracheal swabs from the affected seals tested negative for influenza virus genomes, and lung tissues displayed notably lower viral genome loads compared to brain tissue (cycle threshold Ct value = 30-38 *versus* Ct = 18-22, respectively) [246]. Following the introduction of HPAI H5Nx clade 2.3.4.4b viruses into North America through migratory wild birds in 2021 [71,72], non-reassortant Eurasian 2.3.4.4b viruses were detected in harbor and gray seals stranded in Maine in June 2022, as reported by Puryear, *et al.* [247]. Although most seals were found

deceased, respiratory symptoms and, less frequently, neurological impairment were noticed prior death. The respiratory tract was the most consistent source of AIV PCR-positive samples [247].

By the end of 2022, HPAI H5Nx viruses of clade 2.3.4.4b reached South America, posing a threat to both marine and terrestrial wild birds [248]. After instances of mass die-offs among wild aquatic birds, mass mortalities in sea lions (*Otaria flavescens*) have been documented along Peruvian coastlines [81,84]. Dying individuals displayed predominantly neurological clinical signs, including tremors, convulsions, and paralysis [84]. Additionally, respiratory indicators such as dyspnea, tachypnea, nasal and buccal secretions, as well as pulmonary edema, were observed. The overall good body condition of the necropsied individuals highlighted the rapid nature of death. Copious whitish secretions were filling the trachea and pharynx, alongside pulmonary congestion, and hemorrhages indicative of interstitial pneumonia. Meningoencephalitis was suspected upon observation of diffuse congestion and hemorrhage [84].

Infections of cetacean species with HPAI H5Nx viruses of clade 2.3.4.4b have seldom been documented [118]. Reports include a common bottlenose dolphin (*Tursiops truncatus*) out of the coast of Florida (US) and a harbor porpoise (*Phocoena phocoena*) found stranded with overt neurological signs in shallow waters off the west coast of Sweden, in 2022. In both cases, macroscopic findings were minimal, while microscopically they presented moderate to marked lymphohistiocytic meningoencephalitis accompanied by neuronal necrosis, gliosis, perivascular cuffing, and vasculitis [249] [250]. The brain exhibited the highest viral load (Ct = 23.6 and Ct 20.57, in dolphin and porpoise respectively), followed by the spinal cord, lungs, and stomach in the dolphin, and by lungs, liver, and spleen (Ct values > 30) in the harbor porpoise [250]. IHC and *in situ* hybridization (ISH) confirmed widespread virus distribution within the CNS of the bottlenose dolphin [249]. In the harbor porpoise, IHC demonstrated the presence of virus antigen primarily in the brain, located in the nuclei and cytoplasm of neurons, glial cells, and epithelial cells of the choroid plexus. Scarce amount of virus antigen was detected in alveolar macrophages and sloughed epithelium of the harbor porpoise, while no viral antigen was observed in other examined tissues, consistent with findings by Murawski *et al.* [249]. Interestingly, sequence analysis of the HPAI H5N1 clade 2.3.4.4b virus isolates affecting these cetaceans revealed that the only genetic marker associated to mammalian adaptation was the HA-T192I (H3 numbering) amino acid substitution, linked to increased α 2,6-sialic acid binding [249,251]. The overt neurotropism of this virus in marine mammals in absence of most common markers of mammalian adaptation underscores the potential threat of HPAI viruses to mammalian hosts [250].

8.2.2. Terrestrial Mammals

First virological confirmation of HPAI H5 of clade 2.3.4.4b infection in a terrestrial mammal, specifically a red fox, was reported in the UK in late 2020 [171]. The animal was discovered dead after the sudden onset of malaise and inappetence, in a wildlife rehabilitation center where HPAI H5N8 infected swans were kept. Histopathological examination unveiled a severe acute necrotizing nonsuppurative polioencephalitis, accompanied by a mild acute nonsuppurative myocarditis and interstitial pneumonia, suggesting systemic virus replication. During the spring of 2021, additional cases of HPAI infection in red foxes emerged in the Netherlands [175]. Specifically, two cubs exhibited neurological symptoms and, after ruling out lyssavirus infection, both tested positive for HPAI H5N1 virus detection in brain samples. Genetic analysis indicated these cases might have been linked to the extensive HPAIV outbreak in barnacle geese (*Branta canadensis*) in the same region during that period [175]. Bordes, *et al.* [252] also reported further instances of HPAI H5N1 detection in deceased red foxes in the Netherlands. While pathological examination revealed respiratory lesions due to concurrent parasitic infection in all three foxes, strong virus protein expression was observed in the brain through IHC, most notably in the cerebrum. Neurons and microglia cells in the gray matter exhibited virus protein expression, which correlated with a nonsuppurative encephalitis featuring perivascular cuffing. Additionally, one individual displayed subacute lymphoplasmacytic myocarditis with myocardial degeneration and necrosis, along with virus protein expression in cardiomyocytes. Concerning the respiratory tract, virus protein expression was only identified in the olfactory epithelial cells of one fox, concomitant with necrosis of these cells. This predominant

neurotropism of the virus represented a novel finding, diverging from previous HPAI H5 virus infections in foxes which displayed a diffuse tropism with heightened virus replication in the respiratory system, akin to HPAI infections in poultry [171,253].

Multiple carnivore species were found HPAI H5N1-infected in Finland following mass mortalities of infected pheasants [188]. An otter (*Lutra lutra*), two red foxes and a lynx (*Lynx lynx*) were examined. The observed organ lesions aligned with acute infectious diseases, featuring petechial hemorrhages in muscles, subcutaneous tissues, and multiple organs, as well as dark red, swollen, and edematous lungs. Microscopic examination of the otter revealed moderate nonsuppurative necrotizing meningoencephalitis in the cerebrum and brain stem, along with severe multifocal hemorrhagic and necrotizing pneumonia in the lungs, and severe multifocal necrotizing pancreatitis. In the case of the foxes, severe multifocal necrotizing pneumonia and acute hemorrhages in the liver and kidneys were evident; however, notably, no abnormalities were observed in the brain. Lastly, the lynx exhibited multifocal acute hemorrhages in the heart and lungs, accompanied by severe necrotizing pneumonia [188].

Further cases of infection were identified among carnivores in Asia. An Ezo red fox (*Vulpes vulpes schrencki*) was found dead in a public garden in Japan in March 2022, coinciding with a crow die-off [254]. Additionally, a severely emaciated Japanese raccoon dog (*Nyctereutes procyonoides albus*) was euthanized due to its critical clinical condition [254]. Both animals tested positive for HPAI H5N1 infection, and the fox exhibited a higher virus titer in brain tissue homogenates than in the respiratory tract, as assessed by rRT-PCR (Ct = 16.29 versus Ct = 25, respectively). PME of the Ezo red fox did not reveal the presence of severe pneumonia, in contrast to previous findings in foxes [171], and no gross lesions were observed in the brain. However, localized meningoencephalitis with neutrophil infiltration and focal necrosis around the lesion were detected, albeit at a mild-to-moderate level. IHC results unveiled AIV antigen presence in the brain, bronchi, bronchioles, and trachea, indicating the virus's neurotropism and preference for the upper respiratory tract. In the case of the raccoon dog, similar to the fox, viral antigens were detected in ciliated tracheal epithelia and tracheal glands. However, rRT-PCR analysis revealed lower viral loads in tissue homogenates from the brain, lung, and trachea. This suggested virus clearance by the immune system and implies that death resulted from parasitic infestation and secondary bacterial infections, which were identified during PME [254].

9. Conclusions

Since 2016, the Eurasian-origin H5Nx clade 2.3.4.4b viruses have spread extensively to Europe, Africa, Asia, and the Americas via migratory birds, undergoing significant genetic evolution and reassortment of internal genes with locally circulating LPAIVs [77,255]. Through mutations and reassortments, viruses of clade 2.3.4.4b have acquired novel pathobiological characteristics [94,116]. In the current epidemic, the persistent isolation of HPAIVs in migratory waterfowl and the geographic extent of these findings are unprecedented. Notably, some H5Nx strains of clade 2.3.4.4b in waterfowl are abundantly excreted both through the oropharyngeal and cloacal routes, in line with other HPAIVs, thus contributing to HPAIVs environmental contamination [124,143]. In poultry, HPAIV clade 2.3.4.4b infection can lead to various clinical outcomes, challenging early disease recognition and intervention. Besides poultry and waterbirds, a wide range of wild bird species and both wild and domestic mammals seem to be permissive to HPAIV clade 2.3.4.4b infection, raising concerns about zoonotic events and public health implications. The severity of clinical disease, mortality, and pathological changes varies according to the host. Several factors, virus (e.g. strain, genotype) and host-related (e.g. species/breed, age, immune system), play a key role in HPAIV disease severity and mortality, and have been, to date, only partially understood. Given the recent unprecedented widespread circulation of the HPAIVs clade 2.3.4.4b, further research is necessary to develop efficient strategies for preventing future outbreaks in poultry and reducing the zoonotic risk.

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