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Histopathology and clinical signs in black-headed gulls (*Chroicocephalus ridibundus*) attributed to natural infection with highly pathogenic avian influenza virus (BB H5N1)

Piotr Indykiewicz[1], Joanna Bogucka[2], Agnieszka Chłodowska[2]✉

[1]Department of Biology and Animal Environment, Faculty of Animal Breeding and Biology, Bydgoszcz University of Science and Technology, 85-084 Bydgoszcz, Poland
[2]Department of Basic and Preclinical Sciences, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, 87-100 Toruń, Poland
chlo@umk.pl

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Abstract

Introduction: The highly pathogenic avian influenza virus (HPAIV) H5N1 clade 2.3.4.4b causes global mass mortality in wild birds, yet detailed clinicopathological data from cases of infection in adult (*Chroicocephalus ridibundus*) remain scarce. Black-headed gull HPAI data have usually been acquired in experimental infection studies in juveniles. The aim of this study was to investigate histopathological changes and clinical signs in adult birds which were naturally infected with HPAI. **Material and Methods:** Adult black-headed gulls were comprehensively assessed in Poland during the 2023 breeding season, encompassing 112 clinical observations, 383 gross post-mortem examinations and histopathological analyses of tissues from 40 birds. **Results:** Infected individuals manifested a severe systemic disease involving all major internal organs, particularly the nervous and musculoskeletal systems. Clinical signs included lethargy, ataxia and localised cutaneous cyanosis (notably of the pelvic limbs and abdominal integument), while respiratory distress was absent. Post-mortem examinations revealed a substantial reduction of body mass (17% in males and 9% in females) relative to multi-year averages. Histopathology confirmed viral pantropism, with the myocardium, liver, and lungs being the most frequently affected tissues. Notably, extensive inflammatory infiltration and congestion within the ovaries suggest that HPAIV may severely compromise reproductive cycles. **Conclusion:** The H5N1 strain of HPAIV induces debilitating systemic infection in adult black-headed gulls. Significant gonadal pathology suggests potential long-term impacts on the reproductive success and population dynamics of this reservoir species.

Keywords: black-headed gull, H5N1, highly pathogenic avian influenza virus, histopathology, wild birds.

Introduction

The highly pathogenic avian influenza virus (HPAIV) clade 2.3.4.4b, subtype A(H5Nx), was initially isolated from domestic waterfowl in China in 2013 and South Korea in 2014 (23). Since its emergence in gulls, herons, terns, grebes, and cormorants in China and along the Russia–Mongolia border in 2016, this clade carried by migratory birds has achieved global distribution, also reaching Europe, North and South America, Canada (7), Antarctica, Africa, and the Middle East. This expansion has been characterised by continuous viral evolution driven by genetic reassortment with indigenous low-pathogenic avian influenza virus strains.

By the end of 2023, HPAIV H5Nx had been documented in 502 wild bird species and 61 mammalian species (24). Furthermore, 90 laboratory-confirmed human cases had been

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reported globally, of which 21 were fatal, highlighting the escalating zoonotic threat posed by this lineage (9). The outbreaks have also resulted in substantial economic disruption ensuing from the depopulation of over 400 million poultry (19) and approximately 300,000 fur-bearing animals in Finland and Spain (2).

During the October 2016–August 2017 and December 2021–March 2022 epizootics in Europe, HPAIV was most frequently detected in Charadriiformes, particularly gulls (1). The highest HPAIV mortality rate was observed in black-headed gulls breeding in Europe. In 2023 in the Netherlands, HPAIV was responsible for the death of at least 9,500 adult individuals (5.3% of the population). In the colonies across the continent, the overall mortality rate ranged from 18.1% to 49.8%, while the rates in 3–4-week-old chicks were up to 80–100% (4). In Poland, adult mortality rates ranged from 1.7% to 77.8%, depending on the colony, and a minimum of 51,000 individuals died as a result of the infection (11).

These data clearly demonstrate the threat posed by HPAIV and its ongoing evolution to wild animals, especially birds, as well as to the poultry industry and public health. Therefore, it is of the utmost importance to continue studying the effects of HPAIV on individual animals and populations, including natural reservoir hosts such as gulls.

Despite extensive research, the pathogenetic mechanisms of HPAIV in black-headed gulls remain only partially elucidated. This knowledge gap is particularly significant, given that this species is the most abundant gull in Europe, migrates over long distances, breeds in colonies and forages synanthropically. These are traits that facilitate rapid viral transmission and heighten epidemiological risk.

The primary objective of the present study was to systematically characterise the clinical manifestations (encompassing behavioural, systemic, macroscopic, and histopathological alterations) observed in adult black-headed gulls following natural infection with HPAIV (subtype H5N1 and genotype BB). Additionally, the research sought to determine whether the pathological profile identified in this species correlates with observations in other susceptible wild avian taxa.

Material and Methods

Study material. The study was based on 112 live black-headed gulls and 383 carcasses collected from three breeding colonies located in north-central Poland: islands in Lake Pakoskie North (N 52.782972; E 18.08419), a gravel mine reservoir in Wojdal (N 52.850325; E 18.076299) and Koronowo Reservoir (N 53.334406; E 17.964956). Totals of 248, 66, and 69 dead birds from each respective location were examined. From May 15 to June 10, 2023, the breeding colonies were monitored daily. The carcasses of 40 adult black-headed gulls (24 males and 16 females) were collected from the colonies on the day of death, between May 25 and June 2, 2023, and subjected to pathological evaluation.

Field study – documentation of behavioural changes in the infected birds. Clinical signs of infection were described in 112 birds, and examples were recorded on video (*e.g.* lethargy, failed flight attempts, swimming in circles, partial submersion and seizures). During the inspection of the total of 383 carcasses, particular attention was paid to bulging of the orbit; contamination of the feathers around the cloaca with urates and faecal matter; and changes in skin colour of the abdomen, pelvic limbs, and beak.

Virus identification and phylogenetic analyses. Results of the studies on virus identification and phylogenetic analyses were described by Indykiewicz *et al.* (11).

Necropsy, tissue collection and preparation of histology slides. During necropsy, the body mass and sex of each individual were recorded, as well as the amount of subcutaneous and visceral fat (scored 0–3). The heart, liver, spleen, pancreas, air sacs, proventriculus, gizzard, kidneys, testes, and ovaries were dissected for evaluation of the macroscopic changes.

Each collected specimen was fixed in 10% neutral buffered formalin for six weeks. Subsequently, they were routinely processed using an automated vacuum tissue processor (ASP6025 S, Leica Biosystems, Nussloch, Germany). Tissues were embedded in paraffin (HistoCore Arcadia H, Leica Biosystems) and sectioned on a microtome (HistoCore MULTICUT, Leica Biosystems). The 3.5 µm sections were stained with HE (ST Infinity Kit, Leica Biosystems).

Histopathological evaluation. Histopathological examination was conducted on samples of the heart, liver, lungs, proventriculus, ovaries, testes, and kidneys from 40 birds. It was not possible to conduct a histological analysis of the cerebral tissue because all available brain tissue had been used for virus detection. Microscopic analysis was conducted using a Nikon Ci-L light microscope with a Nikon DS-Fi3 5.9 MPix digital camera, and image analysis was performed with the NIS-Elements software (all Nikon Corporation, Tokyo, Japan).

To assess the severity of histopathological changes (congestion, necrosis, parenchymal degeneration, vacuolation, connective tissue proliferation and inflammatory infiltrate), a classification protocol was adapted following

Lean *et al.* (14). Ten randomly selected microscopic fields per sample were assessed, and lesions were graded as absent (–), minimal when present in fewer than 25% of fields (+), mild when present in fewer than 50% of fields (++), moderate when present in fewer than 75% of fields (+++) or severe when observed in more than 75% of fields (++++).

Results

Clinical findings. Mortality rates in the three studied black-headed gull breeding colonies ranged from 6.6% to 17.8%. All carcasses tested positive for HPAIV, with phylogenetic analysis identifying genotype BB (H5N1, A/Herring_gull/France/22P015977/2022-like). The phylogenetic results and phylogenetic tree were presented by Indykiewicz *et al.* (11).

Among the 383 dead gulls, the majority (87.9%) were found in sternal recumbency with contracted pelvic limbs, while 7.3% were in dorsal or lateral recumbency with limbs extended. Torticollis was prevalent (84.6%), manifesting as neck muscle paralysis directed to the right (56.4%) or left (28.2%). Wings were extended in 51.3% of individuals (Fig. 1). External findings included cloacal soiling (37.6%), periorbital oedema (8.1%) and localised cyanosis of the pectoral and abdominal skin (10.7%) or plantar metatarsal pads (5.2%). Feathers remained generally clean and smooth.

In live HPAIV-infected individuals, predominant signs included lethargy (98.2%), aphonia (100%) and inability to fly (100%). Neurological manifestations included stereotypic head movements, ataxia and circular swimming (typically clockwise). The agonal phase was characterised by involuntary muscle contractions, tremors, and head-tilting. Notably, no respiratory distress (*e.g.* dyspnoea or sneezing) was observed. Suborbital sinus swelling occurred occasionally (3.6%).

Post-mortem findings. Mean body mass at the time of death was 222.6 g for males (n = 24, range 165–262 g) and 214.1 g for females (n = 16, range 171–261 g). All individuals had empty gastrointestinal tracts. Subcutaneous and visceral fat were absent or negligible (scores 0–1) in 79.2% of males and 65.6% of females, while abundant fat (score 3) was present in only 12.5% and 18.8%, respectively.

In more than half (57.5%) of the studied gulls, congestion and petechiae were noted in the proventriculus and in cardiac muscle. Similar lesions were observed in the liver (42.5%) and kidneys (35.0%). Additionally, 25.0% of the birds presented with renomegaly and 12.5% of females exhibited ovarian oedema (Table 1).

Histopathological changes. Histopathological lesions were identified in all examined organs, with variable frequency and severity depending on the tissue affected. Within the heart, the most frequent lesion was cardiomyocyte necrosis, observed in all studied birds. Multifocal degenerative changes of cardiomyocytes were also present, including cellular swelling, cytoplasmic granularity and loss of cross-striation. These changes ranged from minimal to severe in over 70% of analysed specimens. Vacuolar degeneration, characterised by cells filled with large, pale hydropic vacuoles, was observed in 57% of birds. The severity of the degeneration was mostly minimal to mild, with only one case graded as moderate. Other, less pronounced cardiac lesions included mild-to-moderate perivascular inflammatory infiltrates composed predominantly of heterophils and mononuclear cells, as well as circulatory disturbances, including congestion and multifocal haemorrhages (Tables 2 and 3, Fig. 2a).

In the liver, focal hepatocellular necrosis accompanied by sparse infiltrates of heterophils and lymphocytes was observed in 95% of the examined black-headed gulls. In 28% of cases, hepatocyte necrosis was extensive, occasionally multifocal or coalescing. Kupffer cell hypertrophy was observed as a non-specific response to liver damage. Congestion was noted in 70% of cases (Tables 2 and 3, Fig. 2b).

Within the lungs, the predominant lesions included pulmonary oedema, congestion, necrosis of the air capillary epithelium (pneumocytes) and associated inflammatory infiltrates. Type-II pneumocyte damage mainly consisted of desquamation and regressive changes (cytoplasmic swelling and necrosis), sometimes accompanied by cellular hyperplasia with enlarged nuclei. Damage to type-I pneumocytes manifested as erosion of the air capillary epithelium or loss of cellular nuclei. Detached pneumocytes were observed in the air capillary lumen. In all examined birds, pulmonary congestion was manifested, with 46% of specimens presenting a severe form. Fibrinous exudate within the air capillaries, accompanied by lymphocytic infiltrates, was observed in 62% of the studied animals, with 28% of cases graded as moderate to severe. In 56% of individuals, mild interstitial widening and connective tissue proliferation associated with inflammatory changes were also noted (Tables 2 and 3, Fig. 2c).

In the proventriculus, the most commonly observed lesions included necrosis of glandular epithelial cells, multifocal necrosis of smooth muscle cells within the muscular layer, and focal mucosal necrosis accompanied by infiltrates of heterophils and mononuclear inflammatory cells. Mild interstitial widening and focal connective-

tissue proliferation associated with inflammatory changes were occasionally observed. The severity of the lesions was generally minimal to mild (Tables 2 and 3, Fig. 2d).

In the kidneys, interstitial nephritis composed predominantly of lymphocytic infiltrates was observed. These lesions were frequently accompanied by mild degenerative and necrotic changes of the tubular epithelium. In one case, moderate tubular dilatation containing mucus, urate crystals, and urinary casts were identified (Tables 2 and 3, Fig. 2e).

In reproductive organs, multifocal necrosis of ovarian stromal and follicular cells and infiltrates of heterophils and mononuclear inflammatory cells were observed. Most females presented with leukocyte infiltrates in the ovary, alongside follicular necrosis and atresia. Males also presented with necrotic foci and inflammatory cell infiltration. Although the overall tubular architecture of the testes was preserved, degeneration, atrophy and necrosis of the germinal epithelium were observed, together with interstitial widening and focal connective tissue proliferation associated with inflammatory changes (Tables 2 and 3, Fig. 2f).



Fig. 1. Black-headed gulls (*Chroicocephalus ridibundus*) found dead and diagnosed with highly pathogenic avian influenza caused by type A(H5N1) virus. a) in dorsal recumbency with legs stretched toward the tail and wings folded alongside the body; b) in sternal recumbency with both wings extended; c) left wing extended with opisthotonos; d) right wing extended; e) cyanosis of hind limbs; f) abdominal cyanosis

Table 1. Frequency of gross lesions observed in selected organs of adult black-headed gulls (*Chroicocephalus ridibundus*) naturally infected with highly pathogenic avian influenza A(H5N1) virus, n = 40

Organ	Congestion		Petechiae		Discolouration		Inflammation		Oedema	
	N	%	N	%	N	%	N	%	N	%
Heart	9	22.5	2	5.0	12	30.0	NL	NL	2	5.0
Liver	7	17.5	1	2.5	9	22.5	NL	NL	NL	NL
Kidney	9	22.5	NL	NL	5	12.5	NL	NL	10	25.0
Gizzard	2	5.0	11	27.5	10	25.0	NL	NL	2	5.0
Lung	3	7.5	NL	NL	4	10.0	2	5.0	3	7.5
Testis	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
Ovary	1	12.5	NL	NL	1	6.3	NL	NL	2	12.5

NL – no lesions

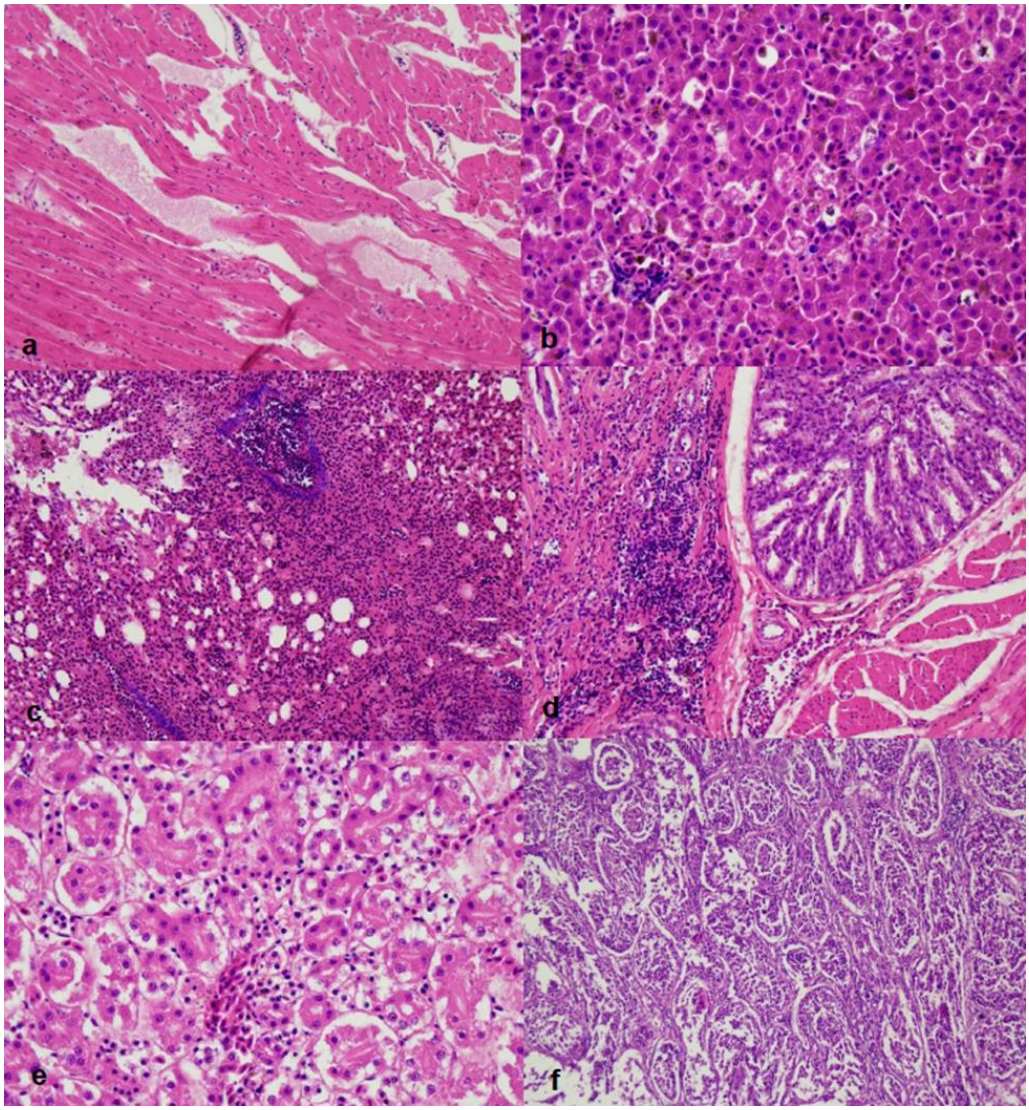


Fig. 2. Microscopic findings in tissue samples from black-headed gulls (*Chroicocephalus ridibundus*) naturally infected with highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b. a) cardiac myocyte degeneration with interstitial oedema; b) hepatocellular necrosis; c) pulmonary congestion; d) inflammatory infiltrate in proventricular *lamina propria*; e) renal parenchymal degeneration; f) damage and degeneration of the seminiferous tubules

Table 2. Histopathological changes as tissue injuries in black-headed gulls (*Chroicocephalus ridibundus*) naturally infected with highly pathogenic avian influenza virus A(H5N1) clade 2.3.4.4b

Organ	Congestion %	Congestion grade*	Necrosis %	Necrosis grade*	Parenchymal degeneration %	Parenchymal degeneration grade*	Vacuolation %	Vacuolation grade*
Heart	54	+ to +++	100	+ to ++++	74	+ to ++++	57	+ to +++
Liver	70	+ to ++++	95	+ to ++++	63	+ to ++++	48	+ to +++
Lung	100	+ to ++++	8	+ to ++	0	–	0	–
Proventriculus	3	+	69	+ to ++	0	–	0	–
Kidney	0	–	39	+ to ++	78	+ to ++	14	+ to ++
Ovary	8	+	25	++ to +++	83	+ to ++++	0	–
Testis	0	–	25	++ to +++	91	+ to ++++	0	–

*Histopathology grade: absent –; minimal +; mild ++; moderate +++; severe ++++

Table 3. Histopathological changes as tissue responses in black-headed gulls (*Chroicocephalus ridibundus*) naturally infected with highly pathogenic avian influenza virus A(H5N1) clade 2.3.4.4b

Organ	Connective tissue proliferation %	Connective tissue proliferation grade*	Inflammatory infiltrates %	Inflammatory infiltrates grade*
Heart	86	+ to ++++	54	+ to ++++
Liver	54	+ to +++	15	++ to ++++
Lung	56	+ to ++++	62	+ to ++++
Proventriculus	11	+	13	+
Kidney	0	–	42	+ to ++
Ovary	50	+ to +++	75	+ to ++++
Testis	23	+ to +++	41	+ to +++

*Histopathology grade: absent –; minimal +; mild ++; moderate +++; severe ++++

Discussion

This study describes the behavioural changes and gross and microscopic lesions in black-headed gulls naturally infected with HPAIV (H5N1 genotype BB). Previously, knowledge of HPAIV infection in black-headed gulls in their natural environment was limited to only four reports involving a total of 20 individuals across three continents. The previously reported cases included one in Hong Kong (8), three individuals from North America (7) and four from the UK (14) typically characterised by severe autolysis or restricted to the autumn–winter period, and twelve from the Netherlands (12). Our results address this gap by providing comprehensive data from adult individuals infected during the May–June breeding season, including 112 clinical observations, 383 gross examinations and histopathological examinations of tissues collected from 40 birds. Unlike prior research on experimentally infected chicks (6, 18), this study focuses on naturally infected adult birds in their breeding habitat.

Clinical signs, gross observations, histopathological data, and the confirmed HPAIV infections suggested systemic disease affecting all internal organs, including the nervous and musculoskeletal systems. Early clinical signs included lethargy, anorexia, lack of vocalisation, ataxia, abnormal head movements, and local skin cyanosis. Similar clinical signs have been previously reported in wild birds and poultry (6, 22). The gulls we observed did not show any signs of respiratory distress, consistent with the observations of 4–5-week-old experimentally infected chicks (18). However, impaired respiration and dyspnoea have been reported in other gull species. In contrast to wild-bird and poultry cases reported previously in the literature, our studied black-headed gulls did not present ruffled feathers. However, similarly to the earlier reports on poultry, we also reported cyanosis of the skin of the pelvic limbs and abdomen (3).

Post-mortem examinations showed below-average body mass with little to no subcutaneous and visceral fat and empty gastrointestinal tracts. Presumably, these were effects of impaired nervous and locomotor systems, resulting in impaired feeding. Males were approximately 45 g (17%) and females 21 g (9%) lighter than the averages from numerous previous breeding seasons (11). Lower weights may also have been partly due to empty gastrointestinal tracts, as their contents typically weigh 14–19 g (10). Similar weight deficiency (of approximately 26 g) was also recorded in infected 4–5-week-old black-headed gull chicks (17) and four-week-old common gull fledglings. In the latter case, a lower amount of subcutaneous and perienteric fat was also observed (17). While one study reported HPAIV-infected wild birds to be in good condition, even during the winter period (8), this report lacked quantitative support for many of its included outbreaks. The frequency and severity of macroscopic lesions varied across both internal organs and external structures. The most frequently observed findings were circulatory disturbances:

congestion, petechiae, oedema, and cyanosis. Inflammation accompanied by exudate was also noted occasionally. Similar anatomopathological changes have previously been described in both wild and farmed bird species (5).

Histopathologically, the heart, liver, and lungs were the most frequently affected organs. Necrotic changes in cardiomyocytes and hepatocytes underscore the pantropic nature of the H5N1 virus and are consistent with previous reports demonstrating systemic dissemination of HPAIVs beyond the respiratory tract. Multiorgan involvement, including cardiovascular, hepatic, renal, and nervous tissues, is considered a hallmark of severe H5N1 infection in susceptible avian species (6, 13, 16). Although histopathological examination of the brain was not performed, the neurological signs observed in affected birds were consistent with previous studies describing viral replication in the central nervous system and associated encephalitic lesions. Neurotropism has been recognised as an important feature of contemporary H5N1 strains and is frequently associated with severe clinical disease and increased mortality (5, 12). Renal lesions, including tubular necrosis and epithelial degeneration, were also observed and were consistent with previous reports in chickens, waterfowl, and gulls. Such lesions may reflect direct viral replication within renal tissues or secondary systemic effects associated with severe infection (12, 16, 29). Nevertheless, considerable interspecies variability has been reported regarding lesion distribution and severity. While renal involvement is common in some species, studies in mute swans and domestic roosters have documented limited or absent kidney lesions. These clinicopathological differences may reflect species-specific susceptibility; variation in viral tissue tropism or host immune responses; differences in clade virulence among circulating viral genotypes; longer or shorter infection durations or more or less favourable environmental conditions (6, 13, 19, 20, 21).

The predominance of lesions in the heart, liver, and lungs supports the concept that these organs represent major targets of HPAIV replication across diverse avian taxa. The consistency of this lesion pattern among Charadriiformes, Anseriformes and domestic poultry suggests that, despite species-specific differences in susceptibility, the systemic pathogenesis of HPAI remains largely conserved (6, 13, 16). Kroeze *et al.* (12) reported that naturally HPAI H5N1-infected black-headed gulls exhibited severe multifocal necrotic lesions, particularly in the brain, pancreas, lungs, and preen gland, frequently associated with abundant viral antigen expression. Histopathological examination revealed pronounced pulmonary inflammation and necrosis, as well as multifocal parenchymal necrosis in multiple organs, supporting the pantropic nature of the genotype BB virus. Conversely, histopathological changes in the proventriculus were minimal. This corresponds with findings in long-tailed skuas, where viral antigens are often detected without significant tissue damage in the glandular and muscular stomach (13).

A particularly significant finding in this study was the identification of extensive inflammatory changes and ovarian congestion in infected females. The ovarian lesions observed in the present study may indicate that the reproductive tract is more susceptible to HPAIV-associated injury in black-headed gulls than previously recognised. The coexistence of congestion, inflammatory infiltrates, follicular degeneration, and necrosis suggests active tissue damage rather than passive vascular disturbance alone. Such findings may reflect direct viral replication within ovarian tissues, although confirmation would require immunohistochemical or molecular analyses (5, 12). However, this contrasts with previous reports in which HPAIV presence in black-headed gull ovaries was not accompanied by histopathological lesions (8, 13). The discrepancy between viral antigen detection and the absence of significant histological damage may indicate that viral replication alone is insufficient to induce tissue injury. Local differences in cellular susceptibility, innate immune responses or regenerative capacity may contribute to the preservation of tissue architecture despite viral presence (12, 13). However, sporadic gonadal lesions, including multifocal necrosis and ovarian degeneration, have been documented in various poultry species, great skuas (*Stercorarius skua*), and experimentally infected passerines (5, 12). These findings are critical, as these pathological changes could severely impair future reproductive cycles, similar to the egg-laying diminishment induced by HPAIV infection observed in poultry (7). Furthermore, the ovarian lesions observed in the present study may potentially affect future reproductive performance in surviving females, although the long-term consequences for reproductive success in wild black-headed gull populations remain unknown.

This study has several limitations. Firstly, although HPAIV was confirmed in all analysed gulls (11), the coexistence of other pathogens such as *Salmonella* or *Clostridium botulinum* cannot be excluded, as these have been previously documented in wild populations. Secondly, data regarding the age of the birds and their prior exposure to LPAI or HPAI viruses were unavailable. This is a significant limitation, as host age and

acquired adaptive immunity are known to influence disease progression and AIV prevalence in wild birds. Lastly, histopathological analysis of the brain tissue could not be performed, as the brains were used in their entirety for virological testing.

Conclusion

Our research demonstrated that in naturally infected adult black-headed gulls, HPAIV (BB H5N1 A/Herring_gull/France/22P015977/2022-like) induces systemic disease. The clinical signs and macroscopic and histopathological changes observed in the heart, liver, lungs, proventriculus, kidneys, and reproductive organs partially resemble those previously detected in poultry and some experimentally infected wild bird species.

Our results can serve as a supplementary source of knowledge for veterinarians, ornithologists, and biologists on the specific clinical changes occurring in adult black-headed gulls naturally infected with HPAIV. This should not only facilitate the diagnosis of HPAI but also enable faster, more effective reporting of outbreaks to the authorities. It could ease the management of outbreaks and help prevent epidemics. Such proceedings are crucial to minimise the negative impact of HPAIV on human and on wild, domesticated, companion, and zoo-housed species' health.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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CRediT Authorship Contribution Statement: Piotr Indykiewicz: research concept and design, collection and assembly of data, data analysis and interpretation, writing the article, critical revision of the article, final approval of the article. **Joanna Bogucka:** collection and assembly of data, data analysis and interpretation, writing the article, critical revision of the article, final approval of the article. **Agnieszka Chłodowska:** data analysis and interpretation, writing the article, critical revision of the article, final approval of the article.

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