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**MONITORING OF EMERGING VIRUSES AT THE POULTRY-WILDLIFE
INTERFACE**

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Abstract

This thesis delves into the dynamics of pathogen transmission at the poultry-wildlife interface, emphasizing interspecies transmission's significant impact on animal and public health. Employing a multidisciplinary approach involving fieldwork, molecular biology techniques, sequence and phylogenetic analyses, and statistical analysis and modeling, these studies focus on significant poultry pathogens, namely avian influenza virus (AIV), avian metapneumovirus (aMPV), and infectious bursal disease virus (IBDV), within the context of their circulation in wild birds.

A comprehensive camera-trap survey conducted in Northern Italy enabled the identification of key wild and domestic animal species frequenting the vicinity of poultry houses. The spatiotemporal patterns of these visits were thoroughly characterized, providing essential groundwork for the implementation of biosecurity measures to prevent pathogen spillover to poultry. In the same study area, a combination of sampling strategies and advanced genomic analyses revealed 11 low pathogenic (LP) AIVs circulating in wild hosts during the 2021-2022 AI epidemic in Italian poultry. The identification of diverse gene constellations and molecular markers potentially linked to an increased AIV zoonotic risk underscored the necessity for Whole Genome Sequencing techniques in characterizing LPAIVs in wild hosts.

While the role of wild birds in the AIV epidemiology has long been recognized, the involvement of free-ranging avifauna in the aMPV and IBDV transmission to poultry has been questioned. A systematic review and meta-analysis of aMPV in wild species identified the potential virus reservoirs or vectors, such as wild ducks, geese, gulls, and pheasants. Notably, the first detection of an aMPV subtype C virus in a novel migratory duck species, the Eurasian wigeon, further emphasized the likely wild duck's role in aMPV virus circulation and long-distance spread along migratory routes. Additionally, a systematic review and meta-analysis of IBDV prevalence in wild birds provided valuable insights into the virus's epidemiology, although conclusive evidence on specific species' roles remains elusive.

Overall, this thesis has offered novel insights for evidence-based epidemiological studies and targeted viral surveillance in wild hosts concerning AIV, aMPV and IBDV, avian viruses of great economic importance for the global poultry industry. The findings not only deepen our understanding of pathogen transmission dynamics at the poultry-wildlife interface, but also pave the way for designing innovative strategies to prevent cross-species disease transmission.

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INTRODUCTION

Transboundary emerging and re-emerging infectious diseases are being increasingly recognized as interconnected threats to public health, domestic animals, and wildlife communities (Gilchrist *et al.*, 2007; Miller *et al.*, 2017). In this context, the convergence of activities associated to farmed animal production with wildlife habitats creates optimal circumstances for multi-host pathogen emergence and virus spillover (Gortazar *et al.*, 2014; Gortázar *et al.*, 2007). From a One Health perspective, it is noteworthy that around 60% of globally reported emerging infectious diseases in humans are zoonotic, of which more than 70% come from wild species (Cleaveland *et al.*, 2001; Jones *et al.*, 2008). The wildlife-domestic animal-human interfaces, through direct or indirect interactions between different hosts, can indeed lead to interspecies disease transmission (Craft, 2015). Consequences of such events include health issues in domestic or wild animals, economic losses for the livestock or poultry sector and threats to public health (Daszak, 2000; Gortázar *et al.*, 2007; Wiethoelter *et al.*, 2015). Understanding and characterizing the domestic-wildlife animal interface is therefore crucial in preventing and controlling both animal and zoonotic infectious diseases (Lloyd-Smith *et al.*, 2009).

The Italian poultry industry stands as one of the most productive in Europe, with over 1.3 million tons of poultry meat and approximately 12 billion eggs produced in 2021 (ISMEA Mercati, 2022). This fully integrated industry supports the intensive production of 148 million birds annually across 6,800 poultry farms. However, introduction and spread of infectious diseases in poultry holdings inflict direct losses, with increased morbidity, mortality, and condemnation rates, thus significantly impacting the poultry industry. With respect to disease transmitted at the poultry-wildlife interface, wild birds take center stage, as they serve as vector or reservoir for a wide range of bacterial and viral pathogens for poultry (Ayala *et al.*, 2020; Franklin *et al.*, 2021). On the other hand, mammals can also introduce and spread infectious diseases to domestic birds. However, the understanding of their epidemiological role remains relatively limited, with most studies focusing on specific taxa (Domanska-Blicharz *et al.*, 2023; Root & Shriner, 2020).

The presented thesis delves into the role of the poultry-wildlife interface in potentially shaping the transmission dynamics of three economically significant viral diseases in poultry, namely the avian influenza (AI), avian metapneumovirus (aMPV) infections and the infectious bursal disease (IBD).

The **first section** of this work lies on the characterization of the poultry-wildlife interface and on exploring the low pathogenic AI virus (LPAIV) genetic diversity in wild birds. AIV finds its reservoir in waterbirds belonging to the Anseriformes (ducks, swans, and geese) and Charadriiformes orders (shorebirds, terns, and gulls) (Webster *et al.*, 1992). Since their first detection in China and South Korea in early 2010s, highly pathogenic AI (HPAI) viruses of clade 2.3.4.4b of the H5 goose/Guangdong (Gs/Gd) lineage have been persistently circulating in wild aquatic birds and spread globally (Lee *et al.*, 2017; Pohlmann *et al.*, 2022). The

transmission of these viruses at the wild-domestic bird interface, coupled with secondary spread between farms, has resulted in numerous HPAI virus outbreaks in rural and commercial poultry across Eurasia, Africa, and America. The Italian poultry sector faced a significant challenge during the 2021-2022 HPAI epidemic season, with over 300 outbreaks, especially concentrated in densely populated poultry areas (DPPAs) of Northern Italy, causing a production loss of 12% (European Food Safety Authority *et al.*, 2022; ISMEA Mercati, 2022). Uncontrolled worldwide HPAI virus circulation in birds has also led to a rising number of spillover events in non-human mammals globally, raising concerns about potential pandemics (Carnaccini & Perez, 2020; European Food Safety Authority *et al.*, 2023; Kuiken *et al.*, 2023). Given this background, the overall situation emphasizes the urgent need to address infections at the poultry-wildlife interface, where virus spillover can happen. **Chapter 1** and **Chapter 2** provide a comprehensive characterization of this interface, employing remote sensing cameras in a yearlong study conducted in three commercial laying hen farms in a DPPA of Northern Italy, located in a high-risk area for avian influenza introduction. Chapter 1 focuses on wild birds approaching the vicinity of poultry houses, while Chapter 2 investigates domestic and wild mammals. Evidence of the susceptibility of wild birds and non-human mammals to the novel HPAIV H5Nx viruses of clade 2.3.4.4b infection is then summarized in **Chapter 3**, which provides a comprehensive review on the clinical signs and pathological lesions. Finally, **Chapter 4** delves into the genetic diversity of LPAIVs obtained from active viral monitoring conducted in wild birds during the 2021-2022 HPAI epidemic in poultry. Through a multi-site survey employing different sampling strategies in DPPAs of the Emilia-Romagna region, 11 LPAIV subtypes were identified across different wild host. Five of these viruses were isolated and genetically and phylogenetically characterized following whole genome sequencing, revealing diverse gene constellations.

The **second section** of the thesis focuses on the aMPV epidemiology in wild birds. aMPV is a widespread poultry pathogen linked to respiratory and reproductive disorders characterized by high morbidity and contained mortality (Suarez *et al.*, 2020). Currently, four aMPV subtypes (A, B, C and D) have been identified based on genetic or antigenic characteristics (Bäyon-Auboyer *et al.*, 2000; Juhasz & Easton, 1994; Seal, 2000). Additionally, and two aMPV of unknown subtype have been detected in wild bird species in North America (Canuti *et al.*, 2019; Retallack *et al.*, 2019). In recent years, the impact of respiratory disease resulting from aMPV infections has been generally on the rise in the European broiler industry (Franzo *et al.*, 2017; Tucciarone *et al.*, 2018). Field studies in Italy have revealed the widespread circulation of aMPV-B subtype and, to a lesser extent, of the aMPV-A, in poultry from DPPAs in the northeastern part of the country (Listorti *et al.*, 2014; Lupini *et al.*, 2011; Tucciarone *et al.*, 2018). Additionally, aMPV-C specific antibodies were detected in farmed domestic mallards in the Veneto region (Legnardi *et al.*, 2021). Given the global disease burden in poultry and the evidence of a potential role played by wild birds in the aMPV epidemiology, **Chapter 5**

synthesizes global serological and molecular data on virus prevalence in wild birds through a systematic review and meta-analysis, identifying key targets of future epidemiological studies. **Chapter 6** reports a molecular survey conducted on samples collected from wild birds in DPPAs in Northern Italy, aiming at shedding light on the status of migratory and resident free-living birds with respect to aMPV. A novel wild duck species tested positive for aMPV-C virus detection, and the strain was characterized through Sanger sequencing of the matrix, fusion, and glycoprotein genes.

In the **third and final section** of this thesis, the epidemiology of the infectious bursal disease virus (IBDV) in wild birds is examined. IBD is an acute and highly contagious viral disease of chickens, associated with a significant economic impact on the global poultry industry due to the immunosuppression it causes in infected individuals (Müller *et al.*, 2003). New genetic lineages of IBDV continue to emerge worldwide (Michel & Jackwood, 2017), exhibiting different antigenic and pathogenetic properties (Felice *et al.*, 2017; Lupini *et al.*, 2016; Tomás *et al.*, 2019). Despite an increasing awareness of the role of wildlife in poultry pathogens' ecology, little is known about the involvement of wild birds in IBDV epidemiology and their potential role in virus transmission. **Chapter 7** therefore provides an up-to-date overview of IBDV serological and virological prevalence in wild birds, summarizing existing literature through a systematic review and further analyzing the data through a meta-analysis of proportion.

References

- Ayala, A. J., Yabsley, M. J., & Hernandez, S. M. (2020). A Review of Pathogen Transmission at the Backyard Chicken-Wild Bird Interface. *Front Vet Sci*, 7, 539925. doi:10.3389/fvets.2020.539925
- Bäyon-Auboyer, M.-H., Arnauld, C., Toquin, D., & Eterradosi, N. (2000). Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. *J Gen Virol*, 81(11), 2723-2733. doi:https://doi.org/10.1099/0022-1317-81-11-2723
- Canuti, M., Kroyer, A. N. K., Ojkic, D., Whitney, H. G., Robertson, G. J., & Lang, A. S. (2019). Discovery and Characterization of Novel RNA Viruses in Aquatic North American Wild Birds. *Viruses*, 11(9), 768. Retrieved from https://www.mdpi.com/1999-4915/11/9/768
- Carnaccini, S., & Perez, D. R. (2020). H9 Influenza Viruses: An Emerging Challenge. *Cold Spring Harb Perspect Med*, 10(6). doi:10.1101/cshperspect.a038588
- Cleaveland, S., Laurenson, M. K., & Taylor, L. H. (2001). Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philos Trans R Soc Lond B Biol Sci*, 356(1411), 991-999. doi:10.1098/rstb.2001.0889
- Craft, M. E. (2015). Infectious disease transmission and contact networks in wildlife and livestock. *Philos Trans R Soc Lond B Biol Sci*, 370(1669). doi:10.1098/rstb.2014.0107
- Daszak, P. (2000). Emerging infectious diseases of wildlife - threats to biodiversity and human health. *Science*, 287.
- Domanska-Blicharz, K., Opolska, J., Lisowska, A., & Szczotka-Bochniarz, A. (2023). Bacterial and Viral Rodent-borne Infections on Poultry Farms. An Attempt at a Systematic Review. *J Vet Res*, 67(1), 1-10. doi:10.2478/jvetres-2023-0012
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Munoz Guajardo, I., & Baldinelli, F. (2022). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., Staubach, C., Terregino, C., Baldinelli, F., Rusinà, A., & Kohnle, L. (2023). Avian influenza overview June–September 2023. *EFSA J*, 21(10), e08328. doi:https://doi.org/10.2903/j.efsa.2023.8328
- Felice, V., Franzo, G., Catelli, E., Di Francesco, A., Bonci, M., Cecchinato, M., Mescolini, G., Giovanardi, D., Pesente, P., & Lupini, C. (2017). Genome sequence analysis of a

- distinctive Italian infectious bursal disease virus. *Poult Sci*, 96(12), 4370-4377. doi:10.3382/ps/pex278
- Franklin, A. B., Bevins, S. N., & Shriner, S. A. (2021). Pathogens from Wild Birds at the Wildlife–Agriculture Interface. In *Infectious Disease Ecology of Wild Birds* (pp. 207-228).
- Franzo, G., Tucciarone, C. M., Enache, M., Bejan, V., Ramon, G., Koutoulis, K. C., & Cecchinato, M. (2017). First Report of Avian Metapneumovirus Subtype B Field Strain in a Romanian Broiler Flock During an Outbreak of Respiratory Disease. *Avian Dis*, 61(2), 250-254. doi:10.1637/11557-121216-ResNote.1
- Gilchrist, M. J., Greko, C., Wallinga, D. B., Beran, G. W., Riley, D. G., & Thorne, P. S. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ Health Perspect*, 115(2), 313-316. doi:10.1289/ehp.8837
- Gortazar, C., Diez-Delgado, I., Barasona, J. A., Vicente, J., De La Fuente, J., & Boadella, M. (2014). The Wild Side of Disease Control at the Wildlife-Livestock-Human Interface: A Review. *Front Vet Sci*, 1, 27. doi:10.3389/fvets.2014.00027
- Gortázar, C., Ferroglio, E., Höfle, U., Frölich, K., & Vicente, J. (2007). Diseases shared between wildlife and livestock: a European perspective. *Eur J Wildl Res*, 53(4). doi:10.1007/s10344-007-0098-y
- ISMEA Mercati. (2022). Avicoli e uova - Ultime dal settore. Retrieved from <https://www.ismeamercati.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/12289>
- Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, 451(7181), 990-993. doi:10.1038/nature06536
- Juhasz, K., & Easton, A. J. (1994). Extensive Sequence Variation in the Attachment (G) Protein Gene of Avian Pneumovirus: Evidence for Two Distinct Subgroups. *J Gen Virol*, 75(11), 2873-2880. doi:https://doi.org/10.1099/0022-1317-75-11-2873
- Kuiken, T., Fouchier, R. A. M., & Koopmans, M. P. G. (2023). Being ready for the next influenza pandemic? *Lancet Infect Dis*, 23(4), 398-399. doi:10.1016/S1473-3099(23)00117-2
- Lee, D. H., Bertran, K., Kwon, J. H., & Swayne, D. E. (2017). Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. *J Vet Sci*, 18(S1), 269-280. doi:10.4142/jvs.2017.18.S1.269
- Legnardi, M., Allée, C., Franzo, G., Cecchinato, M., & Brown, P. (2021). Research Note: Detection of Avian metapneumovirus subgroup C specific antibodies in a mallard flock in Italy. *Poult Sci*, 100(7), 101186. doi:https://doi.org/10.1016/j.psj.2021.101186
- Listorti, V., Lupini, C., Cecchinato, M., Pesente, P., Rossi, G., Giovanardi, D., Naylor, C. J., & Catelli, E. (2014). Rapid detection of subtype B avian metapneumoviruses using RT-

- PCR restriction endonuclease digestion indicates field circulation of vaccine-derived viruses in older turkeys. *Avian Pathol*, 43(1), 51-56. doi:10.1080/03079457.2013.866212
- Lloyd-Smith, J. O., George, D., Pepin, K. M., Pitzer, V. E., Pulliam, J. R., Dobson, A. P., Hudson, P. J., & Grenfell, B. T. (2009). Epidemic dynamics at the human-animal interface. *Science*, 326(5958), 1362-1367. doi:10.1126/science.1177345
- Lupini, C., Cecchinato, M., Ricchizzi, E., Naylor, C. J., & Catelli, E. (2011). A turkey rhinotracheitis outbreak caused by the environmental spread of a vaccine-derived avian metapneumovirus. *Avian Pathol*, 40(5), 525-530. doi:10.1080/03079457.2011.607428
- Lupini, C., Giovanardi, D., Pesente, P., Bonci, M., Felice, V., Rossi, G., Morandini, E., Cecchinato, M., & Catelli, E. (2016). A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. *Avian Pathol*, 45(4), 458-464. doi:10.1080/03079457.2016.1165792
- Michel, L. O., & Jackwood, D. J. (2017). Classification of infectious bursal disease virus into genogroups. *Arch Virol*, 162(12), 3661-3670. doi:10.1007/s00705-017-3500-4
- Miller, R. S., Sweeney, S. J., Sloomaker, C., Grear, D. A., Di Salvo, P. A., Kiser, D., & Shwiff, S. A. (2017). Cross-species transmission potential between wild pigs, livestock, poultry, wildlife, and humans: implications for disease risk management in North America. *Sci Rep*, 7(1), 7821. doi:10.1038/s41598-017-07336-z
- Müller, H., Islam, M. R., & Raue, R. (2003). Research on infectious bursal disease--the past, the present and the future. *Vet Microbiol*, 97(1-2), 153-165. doi:10.1016/j.vetmic.2003.08.005
- Pohlmann, A., King, J., Fusaro, A., Zecchin, B., Banyard, A. C., Brown, I. H., Byrne, A. M. P., Beerens, N., Liang, Y., Heutink, R., Harders, F., James, J., Reid, S. M., Hansen, R. D. E., Lewis, N. S., Hjulsgaard, C., Larsen, L. E., Zohari, S., Anderson, K., Bröjer, C., Nagy, A., Savič, V., Borm, S. v., Steensels, M., Briand, F.-X., Swieton, E., Smietanka, K., Grund, C., Beer, M., & Harder, T. (2022). Has Epizootic Become Enzoitic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. *mBio*, 13(4), e00609-00622. doi:doi:10.1128/mbio.00609-22
- Retallack, H., Clubb, S., & DeRisi, J. L. (2019). Genome Sequence of a Divergent Avian Metapneumovirus from a Monk Parakeet *Myiopsitta monachus*. *Microbiol Resour Announc*, 8(16), 10.1128/mra.00284-00219. doi:doi:10.1128/mra.00284-19
- Root, J., & Shriner, S. (2020). Avian Influenza A Virus Associations in Wild, Terrestrial Mammals: A Review of Potential Synanthropic Vectors to Poultry Facilities. *Viruses*, 12(12). doi:10.3390/v12121352
- Seal, B. S. (2000). Avian pneumoviruses and emergence of a new type in the United States of America. *Anim Health Res Rev*, 1(1), 67-72. doi:10.1017/S1466252300000062

- Suarez, D. L., Miller, P. J., Koch, G., Mundt, E., & Rautenschlein, S. (2020). Newcastle Disease, Other Avian Paramyxoviruses, and Avian Metapneumovirus Infections. In *Diseases of Poultry* (pp. 109-166).
- Tomás, G., Marandino, A., Courtillon, C., Amelot, M., Keita, A., Pikula, A., Hernández, M., Hernández, D., Vagnozzi, A., Panzera, Y., Domańska-Blicharz, K., Eterradossi, N., Pérez, R., & Soubies, S. M. (2019). Antigenicity, pathogenicity and immunosuppressive effect caused by a South American isolate of infectious bursal disease virus belonging to the "distinct" genetic lineage. *Avian Pathol*, 48(3), 245-254. doi:10.1080/03079457.2019.1572867
- Tucciarone, C. M., Franzo, G., Lupini, C., Alejo, C. T., Listorti, V., Mescolini, G., Brandão, P. E., Martini, M., Catelli, E., & Cecchinato, M. (2018). Avian Metapneumovirus circulation in Italian broiler farms. *Poult Sci*, 97(2), 503-509. doi:https://doi.org/10.3382/ps/pex350
- Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev*, 56(1), 152-179. doi:10.1128/mr.56.1.152-179.1992
- Wiethoelter, A. K., Beltran-Alcrudo, D., Kock, R., & Mor, S. M. (2015). Global trends in infectious diseases at the wildlife-livestock interface. *Proc Natl Acad Sci U S A*, 112(31), 9662-9667. doi:10.1073/pnas.1422741112

**SECTION ONE: BRIDGING THE GAP ON AVIAN
INFLUENZA VIRUS EPIDEMIOLOGY OUTSIDE POULTRY
HOUSES**

**I. Characterizing the domestic-wild bird interface through
camera traps in a high-risk area for avian influenza
introduction in Northern Italy**

Abstract

Direct or indirect interactions between sympatric wildlife and poultry can lead to interspecies disease transmission. Particularly, avian influenza (AI) is a viral epidemic disease for which the poultry-wild bird interface shapes the risks of new viral introductions into poultry holdings. Given this background, the study hereby presented aimed to identify wild bird species in poultry house surroundings and characterize the spatiotemporal patterns of these visits. Eight camera traps were deployed for a year (January to December 2021) in three commercial chicken layer farms, including free-range and barn-type setups, located in a densely populated poultry area in Northern Italy at high risk for AI introduction via wild birds. Camera traps' positions were chosen based on wildlife signs identified during preliminary visits to the establishments studied. Various methods, including time series analysis, correspondence analysis, and generalized linear models, were employed to analyze the daily wild bird visits. A total of 1,958 camera trap days yielded 5,978 videos of wild birds from 27 different species and 16 taxonomic families. The animals were predominantly engaged in foraging activities nearby poultry houses. Eurasian magpies (*Pica pica*), ring-necked pheasants (*Phasianus colchicus*) and Eurasian collared doves (*Streptopelia decaocto*) were the most frequent visitors. Mallards (*Anas platyrhynchos*), an AI reservoir species, were observed only in a farm located next to a fishing sport lake. Time series analysis indicated that wild bird visits increased during spring and winter. Farm and camera trap location also influenced visit frequencies. Overall, the results highlighted specific species that could be prioritized for future AI epidemiological surveys. However, further research is required to assess their susceptibility to currently circulating AI viruses, essential for identifying novel bridge hosts.

1.1. Introduction

Direct or indirect interactions between wildlife and domestic animals may lead to interspecies disease transmission (Craft, 2015), resulting in animal health issues and economic losses for the livestock and poultry sector (Daszak, 2000; Gortázar *et al.*, 2007; Wiethoelter *et al.*, 2015). Factors such as human population growth, spatial overlap between hosts and vectors, land-use, or environmental changes play crucial roles in increasing the risk of disease emergence at the wildlife-livestock interface (Hassell *et al.*, 2017; Vanwambeke *et al.*, 2019). From a One Health perspective, 60% of globally reported emerging infectious diseases in humans are zoonoses, of which 70% originates from wild animals (Cleaveland *et al.*, 2001; Jones *et al.*, 2008). Therefore, characterizing the wildlife-domestic animal-human interface is pivotal in preventing and controlling both animal and zoonotic infectious diseases (Lloyd-Smith *et al.*, 2009).

For poultry, wild birds serve as vectors or reservoirs for a wide range of bacterial and viral pathogens (Ayala *et al.*, 2020; Franklin *et al.*, 2021; Graziosi *et al.*, 2022a; Graziosi *et al.*, 2022b; Smoglica *et al.*, 2023; Tucciarone *et al.*, 2022). Among these, the avian influenza virus (AIV) finds its reservoir in waterbirds belonging to the Anseriformes (ducks, swans, and geese) and Charadriiformes orders (shorebirds, terns, and gulls) (Webster *et al.*, 1992). Since 2020, highly pathogenic AI (HPAI) H5Nx viruses of clade 2.3.4.4b of the H5 goose/Guangdong (Gs/Gd) lineage have been persistently circulating in wild aquatic birds, leading to their global spread (V. Caliendo *et al.*, 2022; European Food Safety Authority *et al.*, 2023d). Primary introductions from wild birds, as well as secondary spread between farms, have caused an unprecedented number of HPAI virus outbreaks in rural and commercial poultry in Eurasia, Africa, and North and South America (European Food Safety Authority *et al.*, 2022b, 2023a; European Food Safety Authority *et al.*, 2023c; European Food Safety Authority *et al.*, 2022d). HPAI viruses of clade 2.3.4.4b are also of public health concern due to the presence of molecular signatures in their genomes that indicate adaptation to mammals, making them a pandemic risk (Kuiken *et al.*, 2023).

In Italy, multiple incursions of H5Nx HPAIVs of clade 2.3.4.4b have resulted in 358 outbreaks in poultry between October 2021 and July 2023. The most affected region is the northeastern part of the Country, where Densely Populated Poultry Areas (DPPAs) coexist with numerous wetlands and lagoons. Interestingly, recent H5Nx HPAI outbreaks in Italy and Europe have been linked to primary introductions from wild birds (European Food Safety Authority *et al.*, 2023b; European Food Safety Authority *et al.*, 2023c). Waterfowl and shorebirds, which have distinct ecological requirements, are considered to have limited contact with domestic birds (Caron *et al.*, 2014). However, the role of synanthropic wildlife as bridge hosts in transmitting AI from reservoir species to poultry or between poultry farms has recently been called into question with respect to the virus epidemiology (Caron *et al.*, 2014; Root & Shriner, 2020; Shriner & Root,

2020). Nevertheless, there is a lack of comprehensive information on wild birds visiting patterns in poultry farms, particularly in Italy. To date, such assessments have only been conducted through visual bird counts carried out between January and May on seven mixed-species backyard farms and two commercial poultry holdings (Veen *et al.*, 2007).

To address this knowledge gap, the present study aimed to characterize the domestic-wild bird interface in three commercial layer farms located within a DPPA of Northern Italy. This was achieved through a year-long camera trap survey. The study specifically focused on identifying the wild bird species that visited the surroundings of the poultry houses and describing their behavior and detection patterns over time. Additionally, the study examined whether these visits varied according to season and type of areas monitored. Overall, the data hereby obtained could be utilized to parameterize disease transmission models for pathogens that have a reservoir in free-range avifauna, such as the avian influenza virus.

1.2. Materials and methods

1.2.1 Study design

The study was conducted on three commercial layer farms, located in the Bologna province, Emilia-Romagna region, namely Farm 1, Farm 2 and Farm 3 (Figure 1). These were selected after conducting preliminary on-site visits to assess the presence of wildlife in the poultry farm areas. The assessment involved conducting short interviews with farmers, making direct observations of wildlife, and identifying indirect signs such as tracks, scats, fecal droppings. Prior permission was obtained from the owner to install camera-traps before commencing the study.

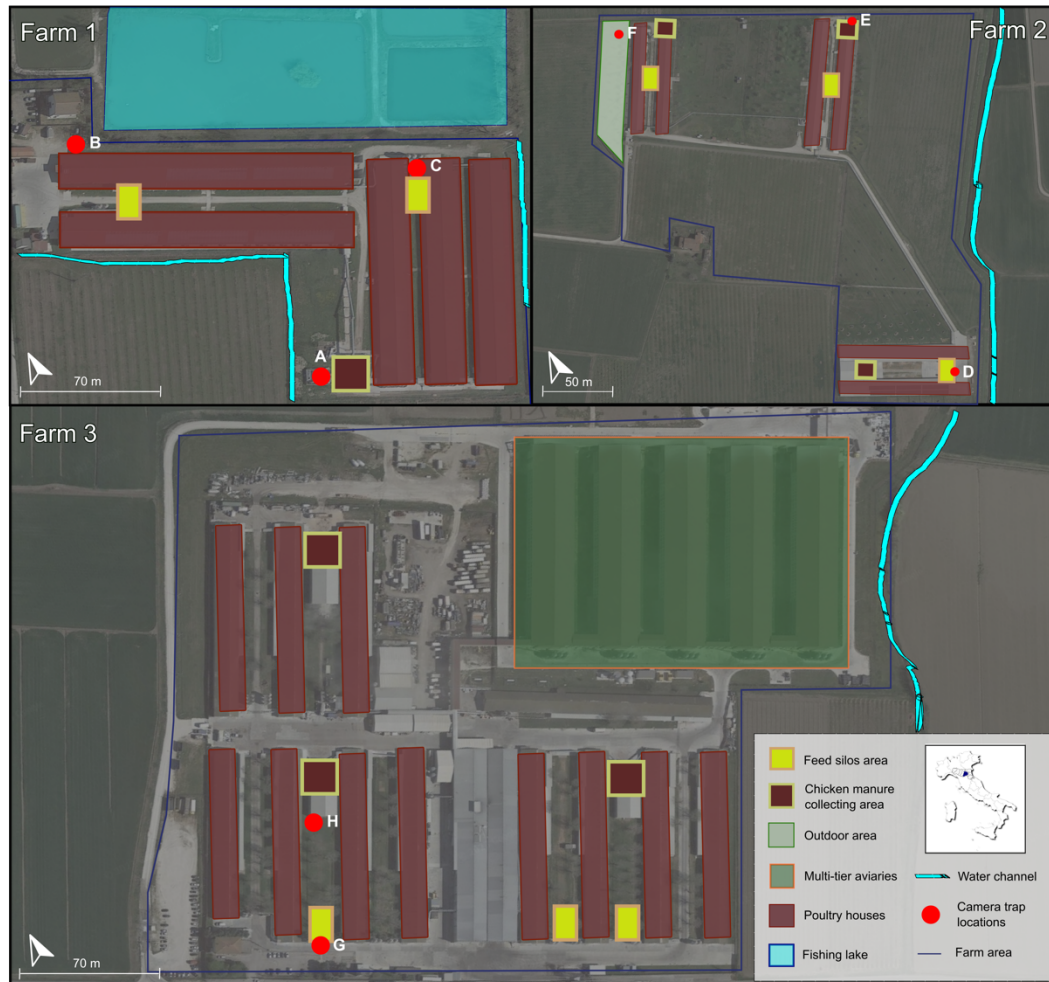


Figure 1. Schematic representation of Farm 1, 2 and 3. The red circles represent the camera trap locations (A to H). The figure also indicates the positions of poultry houses, chicken manure collection areas, and feed silos for each farm. Satellite images modified from Google Earth Pro.

Farm 1 housed approximately 130,000 hens distributed across five multi-tier aviaries and included an egg packing plant. The farm was located in close proximity to a fishing sport lake (100 meters away) and quarry lakes formed from dismissed cave systems (less than 1 kilometers away). Chicken manure was collected every three days without being stored on-site. Waterfowl were sporadically observed in the farm area. Moreover, during the 2016/2017 H5Nx HPAI epidemic in Italy, an H5N8 HPAIV outbreak was reported in Farm 1, and it was determined that the virus had been introduced through direct or indirect contacts with infected wild aquatic birds (Istituto Zooprofilattico Sperimentale delle Venezie, 2018).

Farm 2 was an organic layer farm that housed 140,000 hens in six poultry houses. The farm had outdoor spaces accessible to the animals, which were surrounded by water channels and arable

fields. In these outdoor areas, there were no poultry feeding points, and water was sheltered to prevent access by wild animals.

Farm 3 consisted of five multi-floor sheds and 11 multi-tier aviaries, making it one of the largest poultry holdings in the Emilia-Romagna region, with 1.4 million raised hens. The farm included an egg packing facility and a poultry manure pelleting facility. The farm area was surrounded on three sides by arable fields and on one side by a small waterway. In Farm 2 and 3, specific sites were used for manure storage before further processing. During the study period, the layers in the three farms were tested once for the detection of antibodies against AIV, as part of the Avian Influenza Surveillance Plan 2021 (<https://www.izsvenezie.it/documenti/temi/influenza-aviaria/piani-sorveglianza/piano-nazionale-influenza-aviaria-2021.pdf>) and the Commission Delegated Regulation (EU) 2020/689. All tests resulted negative.

Overall, the three facilities are located in the Po Valley, within a DPPA with a high risk of introduction of HPAI from wild birds due to the presence of waterways and natural or artificial wetlands used for purposes such as water storage for cropland irrigation, gamebirds hunting grounds, or wastewater plants. Furthermore, within a 20-kilometer radius of the three sites, the Argenta valleys of the Delta Po Regional Park is situated. These valleys are among the largest freshwater wetlands in northern Italy and included in the Ramsar Convention of Wetlands of International Importance (<https://www.ramsar.org/sites/default/files/documents/library/sitelist.pdf>). The area encompasses the Campotto/Bassarone and Vallesanta expansion reservoirs, as well as the Traversante hygrophilous woodland, covering a total of approximately 1600 hectares and providing a suitable habitat for over 300 resident, wintering, or breeding wild bird species (<https://ambiente.regione.emilia-romagna.it/it/parchi-natura2000/rete-natura-2000/siti/it4060001>).

1.2.2 Camera trap survey

The study was conducted from January 2021 until December 2021. As various variables, including detection distance, body mass, temperature, and the frontal or lateral approach of the animal, could influence positive triggers (Randler & Kalb, 2018), a 4-week preliminary test was carried out to evaluate the detection probability of birds, using two motion-sensing infrared digital cameras (Dark-Ops Pro XD Dual Lens, Browning Trail Cameras, Utah, USA). Based on the results of the pilot study, a total of nine infrared motion-triggered camera traps were deployed in the studied layer farms.

The camera trap model was chosen based on information available on a reference website (<https://www.trailcampro.com>) and considering the cost-effectiveness of the instrument. The

main requirements for selection included fast triggers and good video quality. The chosen camera trap system had a detection range of 27 m, a fast video trigger (0.72 s), a quick recovery speed (1.9 s) and a wide detection angle (41.7°; field of view of 41.8°). Additionally, the system featured two lenses, one for day recordings and one for night recordings, ensuring good overall footage quality that was crucial for identifying animals at the species level, even in low-light conditions.

Camera traps were deployed at sites where signs of wild birds were seen during preliminary visits or at locations likely to attract wild birds, such as chicken manure collecting sites, drainage ditches nearby poultry houses or feed silos areas.

On Farm 1, three cameras (locations A to C in Figure 1) were deployed from January 2021 to December 2021. One camera was positioned near a feed silo (A), another was placed near the air inlets of a poultry house (B), facing the area adjacent to the fishing sport lake, and the third camera was placed in front of the chicken manure collection point (C). On Farm 2, two cameras (locations D and E in Figure 1) were deployed from January 2021 to December 2021. One camera was positioned to capture the feed silos area (D), and the other was placed at a chicken manure collection point (E). Since Farm 2 was an organic farm, an additional camera was placed within 10 meters from the poultry house entrance, facing the fenced outdoor area accessible to hens and located near the outdoor poultry drinking station (location F in Figure 1). The camera at location F was operational only from July 2021 to mid-October 2021, which coincided with the period when Italian poultry was re-allowed to outdoor access after restriction implemented during the H5N1 HPAI epidemic in poultry (European Food Safety Authority *et al.*, 2022c). On Farm 3, two cameras (locations G and H in Figure 1) were deployed from February 2021 to November 2021. One camera was positioned at the feed silos area, and the other was placed near one of the farm's chicken manure collection point, nearby drainage ditches.

The camera traps at locations A to E and G and H were set to operate from 6 a.m. to 6 p.m. and programmed to record 30-second-long videos after detecting movement, with a lag period of 30 seconds to avoid continuous triggers. The camera at location F was operational from 5 p.m. to 6 a.m., with the same setup, to avoid continuous detections of chickens during daylight. Prior to deploying the camera traps, all instruments were synchronized to the correct time and date, and a unique code was assigned to identify each camera location ID. Throughout the study, batteries and SD cards were replaced every three weeks. Camera trap operations, such as setup, battery and SD card replacements, and malfunctions, were recorded in a data sheet using Microsoft Excel 2021, version 16.49.

1.2.3 Data processing and analysis

1.2.3.1 Temporal pattern of wild bird visits and behaviors

Data were stored and managed in accordance with previously published guidelines on camera trap studies (Wearn & Glover-Kapfer, 2017). Footages analysis was performed using Timelapse Image Analyzer (Greenberg & Godin, 2012). Data visualization and further analyses were conducted using GraphPad Prism (version 9) and R software, version 4.0.4. (Team, 2020). After each SD card collection, videos were renamed based on the camera location ID and the date of recording using the imageRename function from the 'camtrapR' package (version 2.2.0) (Niedballa *et al.*, 2016).

A wild bird visit to poultry farms was defined as an observation of at least one individual of a given wild bird species within a single 1-minute time interval (1 minute was the smallest non-zero value allowed in camtrapR) on the camera's field of view. Two authors (G.G. and F.D.F.) screened the videos, and the following information was recorded from each footage clip: date, time (hh:mm:ss), type of visitor (human, poultry, wild bird, wild mammal, cat or dog), species, behavior, and time spent in front of the camera. Subsequent footages with the same species, number of animals recorded, date, and time (hh:mm) up to 4 minutes from the first detection were considered duplicates and therefore removed from the counts but included in the recording of behavior. In cases where more than one species of wild bird was present in the same footage, the event was identified as a separate visit (Scott *et al.*, 2018). Footage clips with humans or domestic animals were excluded from further analysis and removed.

The behaviors exhibited by wild birds were classified into several categories including moving through, observing surroundings, vocalizing, foraging, grooming, territorial behavior, and excreting (Table 1).

Table 1. Category of behaviors exhibited by wild birds observed in the poultry farms studied.

Activity	Description
Moving through	Moving from one side to another of the camera's field of view, on land or by flight, without exhibiting other behaviors
Observing surroundings	Explorative behavior expressed as observing surroundings
Vocalizing	Emitting sounds to communicate with other individuals of the same species or other species
Grooming	Rubbing the beak to remove dirt and dust from feathers or dust bathing
Excreting	Defecating
Foraging	Eating or drinking

Territorial behavior	Attacking or charging an intruder of the same species or other species
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Daily mean detection rates (MDR) and 95% confidence interval (CI) were calculated for each species for the overall farms studied, each farm, camera trap location (except for location F, as described later), and for seasons defined as spring (from March 1st), summer (from June 1st), autumn (from September 1st), and winter (from December 1st). Since wild bird counts showed overdispersion and aggregation over time, an intercept-only model was fitted using a negative binomial distribution to estimate the 95% confidence interval.

The number of visits by calendar month was further formatted into a time-series object, and the seasonal pattern of visits was assessed by inspecting the 3-month rolling averages using the 'zoo' package (version 1.8-12) (Zeileis *et al.*, 2014). Activity patterns of wild birds on poultry farms were computed through the 'camtrapR' package only when ≥ 40 visits/year were recorded for a given species.

1.2.3.2 Influence of farm, location, and seasons on wild bird counts

Examination of MDRs along with their 95% confidence interval and the results of time-series analysis revealed a potential link between the number of wild bird counts and various explanatory variables. To investigate whether the number of wild birds varied across farms (Farm 1, 2 and 3) and camera trap locations (silos area and chicken manure collecting point) during different season, generalized linear models (GLMs) were applied. All the statistical analyses were performed using the R software, version 4.0.4. (Team, 2020). Two camera trap locations, specifically the one positioned nearby the air inlets in Farm 1 (location B) and the one in the outdoor area in Farm 2 (location F), were excluded from the analysis as they were not present in all three farms, making them non-comparable. Before conducting the analysis, the data from each camera trap were aggregated by date, camera trap location (chicken manure collection point or feed silos area), farm monitored, and season, using the 'aggregate' function. Regarding the season variable, after inspecting the overall 3-month rolling averages, the ones with the highest number of counts, namely winter and spring, were aggregated as season 1, and summer and autumn as season 0. A negative binomial distribution of counts ('glm.nb' function of the 'MASS' package) was employed to account for over-dispersion and aggregation of counts over time (Zuur *et al.*, 2009). The most complex model utilized in the analysis included camera trap locations, farms, and seasons as predictors, along with two-way interactions between farms and camera trap locations, farms and seasons, and camera trap locations and seasons. A total of 6 models were

initially fit, one per response variable and for each one of the four datasets. The selection of the best combination of fixed variables to be retained was made following the parsimony principle, selecting the model with the lowest Akaike information criterion (AIC) (Zuur *et al.*, 2009). For this purpose, the dredge function of the 'MuMIn' package was used to generate a selection table of models with different combinations of fixed terms. Results are expressed as rate ratios (RR) with their 95% confidence intervals (95% CI), after the exponentiation of the estimated parameters. Lastly, to better understand the model outcome with respect to the seasonal pattern of observations, a correspondence analysis (CA) was performed (Elbers & Gonzales, 2020). The R packages 'FactoMineR' and 'factoextra' were used to evaluate the dependency of the three bird orders most frequently observed (Passeriformes, Galliformes and Columbiformes) and the month of the year.

1.2.3.3 Observations in the outdoor area of the free-range hen farm

Since the camera trap at location F in Farm 2 was operational for a shorter duration and was the only one situated in an outdoor area accessible to poultry, the data from this camera were analyzed separately. An indirect contact between wild birds and poultry was defined as the presence of a wild species in the outdoor area. Overall mean indirect contact rate (ICR) between wild birds and free-range hens and their 95% confidence interval were calculated as abovementioned for the MDR. Direct contacts between wild birds and poultry were not considered for statistical analysis, as the camera was only active from sunrise to sunset to avoid continuous chicken detections.

Behaviors observed during the analysis of the footages were categorized as previously described (Table 1).

1.3. Results

1.3.1. Overview of the survey, wild bird population and behaviors

Throughout the study period, the camera traps were operational for a total of 877 trap nights on Farm 1 (with a monthly camera trap effort ranging from 20 to 26 trap nights), 623 trap nights on Farm 2 (with a monthly camera trap effort ranging from 17 to 24 trap nights), and 458 trap nights on Farm 3 (with a monthly camera trap effort ranging from 17 to 31 trap nights). A total number of 33,519 footages were recorded, as shown in Table 2, and a cumulative review time of 492 hours was spent analyzing the recordings.

Table 2. Number of videos recorded by each camera trap during the study period.

Farm	Location monitored	Total number of videos recorded (N)	Wild bird counts (% of N)
1	CMan [^] (A)	3,863	583 (15)
	PHouse [§] (B)	3,910	225 (5.8)
	FSilo ^{&} (C)	3,755	684 (18)
2	FSilo (D)	5,072	1,571 (31)
	CMan (E)	8,551	2,446 (28.6)
	Out [*] (F)	1,745	22 (1.3)
3	FSilo (G)	2,363	380 (1.6)
	CMan (H)	4,260	69 (1.6)

[^]Chicken manure collection point.

[§]Side of the poultry house, adjacent to air inlets.

[&]Feed silos area.

^{*}Outdoor area.

Among the videos, 5,978 (17.8%) displayed wild birds, with a range of 0-46 visits per day. In total, 27 different species of birds were detected, belonging to 16 taxonomic families: Anatidae, Ardeidae, Columbidae, Corvidae, Falconidae, Fringillidae, Motacillidae, Muscicapidae, Passeridae, Phasianidae, Rallidae, Strigidae, Sturnidae, Threskiornithidae, Turdidae and Upupidae. The majority of species observed were synanthropic corvids and passerines, with pheasants, pigeons, and doves also being observed, albeit to a lesser extent (Figure 2). Among these observations, there were relatively fewer sightings of aquatic wild birds. The Eurasian magpie (*Pica pica* (LINNAEUS, 1758)) was the most frequently observed species in the poultry farm area across all the facilities, with a total of 3,249 recorded observations. It was followed by the Eurasian collared dove (*Streptopelia decaocto* (FRIVALDSZKY, 1838)) which was observed 887 times, and the common pheasant (*Phasianus colchicus* LINNAEUS, 1758), which was observed 589 times. The number of individuals per species detected per month is illustrated in Figure 2G and further discussed for each bird order in the respective specific sections. The highest diversity of wild bird species was observed in the chicken manure collection area for Farm 1 (13 species) and Farm 2 (11 species), and nearby feed silos (7 species) for Farm 3.

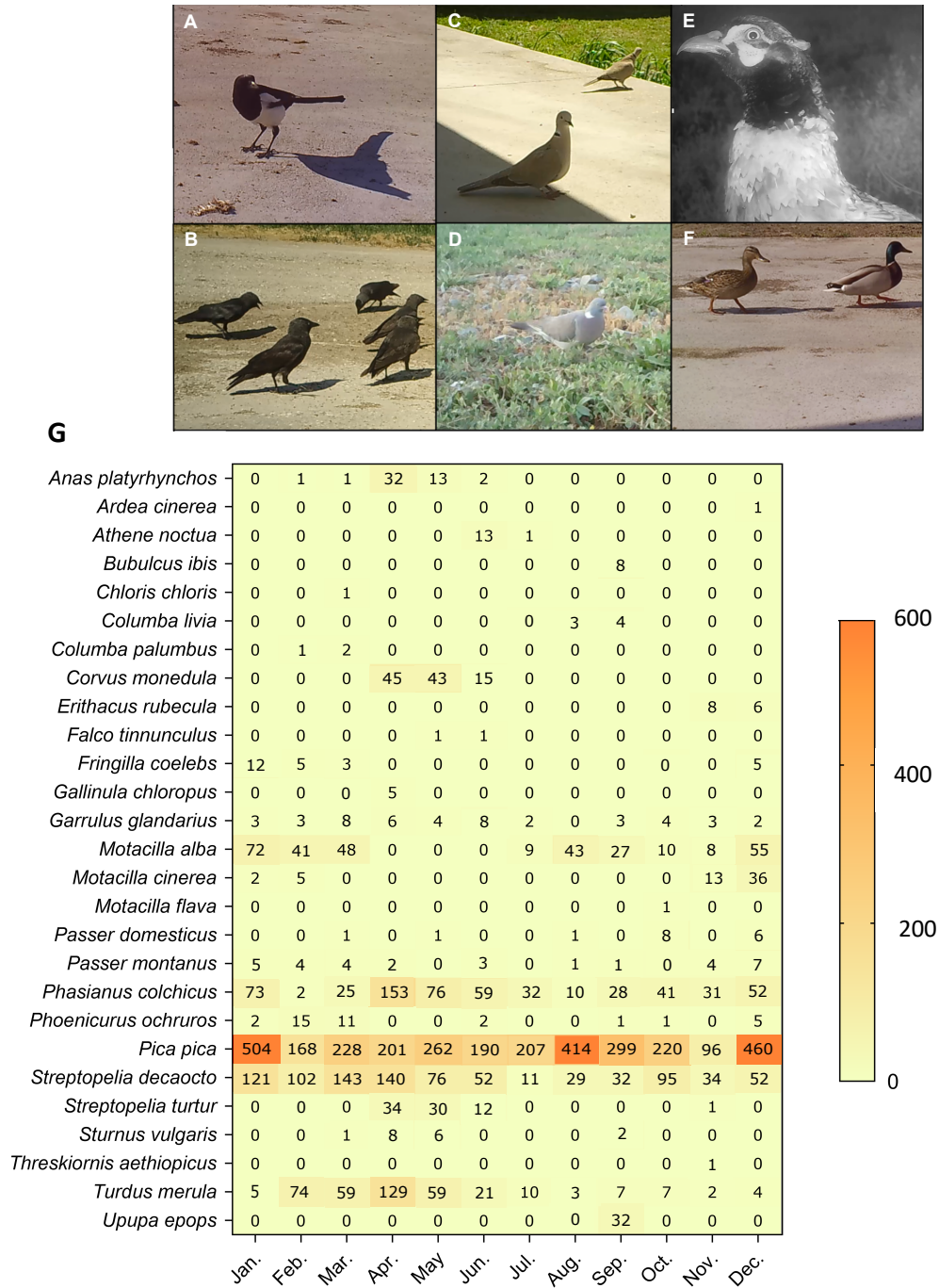


Figure 2. A selection of wild bird species observed in the poultry farms studied (A to F) and overall wild bird visits detected per month (G). (A) Eurasian magpie (*Pica pica*), (B) European jackdaw (*Corvus monedula*), (C) Eurasian-collared dove (*Streptopelia decaocto*), (D) wood pigeon (*Columba palumbus*), (E) wild pheasant (*Phasianus colchicus*), (F) and a male (right) and female (left) mallards (*Anas platyrhynchos*). (G) Number of wild birds per species per month detected by camera traps on the three farms studied.

1.3.1.1. Corvids and other passerines (Passeriformes order)

The wild birds most observed belonged to the Passeriformes order. A total of 14 species were recorded, listed from most to least frequent: Eurasian magpie (3,249 visits), common blackbird (*Turdus merula* LINNAEUS, 1758; 379 visits), white wagtail (*Motacilla alba* LINNAEUS, 1758; 310 visits), Western jackdaw (*Corvus monedula* (LINNAEUS, 1758); 103 visits), grey wagtail (*Motacilla cinerea* TUNSTALL, 1771; 56 visits), Eurasian jay (*Garrulus glandarius* (LINNAEUS, 1758); 46 visits), black redstart (*Phoenicurus ochruros* (S.G. GMELIN, 1774); 37 visits), Eurasian tree sparrow (*Passer montanus* (LINNAEUS, 1758); 31 visits), common chaffinch (*Fringilla coelebs* LINNAEUS, 1758; 25 visits), house sparrow (*Passer domesticus* (LINNAEUS, 1758); 18 visits), common starling (*Sturnus vulgaris* LINNAEUS, 1758; 18 visits), European robin (*Erithacus rubecula* (LINNAEUS, 1758); 14 visits), European greenfinch (*Chloris chloris* (LINNAEUS, 1758); 1 visit) and Western yellow wagtail (*Motacilla flava* LINNAEUS, 1758; 1 visit). Among the corvids, Eurasian magpies and Western jackdaw were the species most frequently observed. Regarding other passerines, the common blackbird and white wagtail were the two most frequent visitors observed in the surroundings of the poultry houses. The activity patterns of these four species are presented in Figure 3 (3A-3D). Eurasian magpies were regularly observed from 5:30 a.m. to 7:30 p.m., while common blackbirds, white wagtails, and Western jackdaws mostly visited the poultry farms between midday and 6:00 p.m.

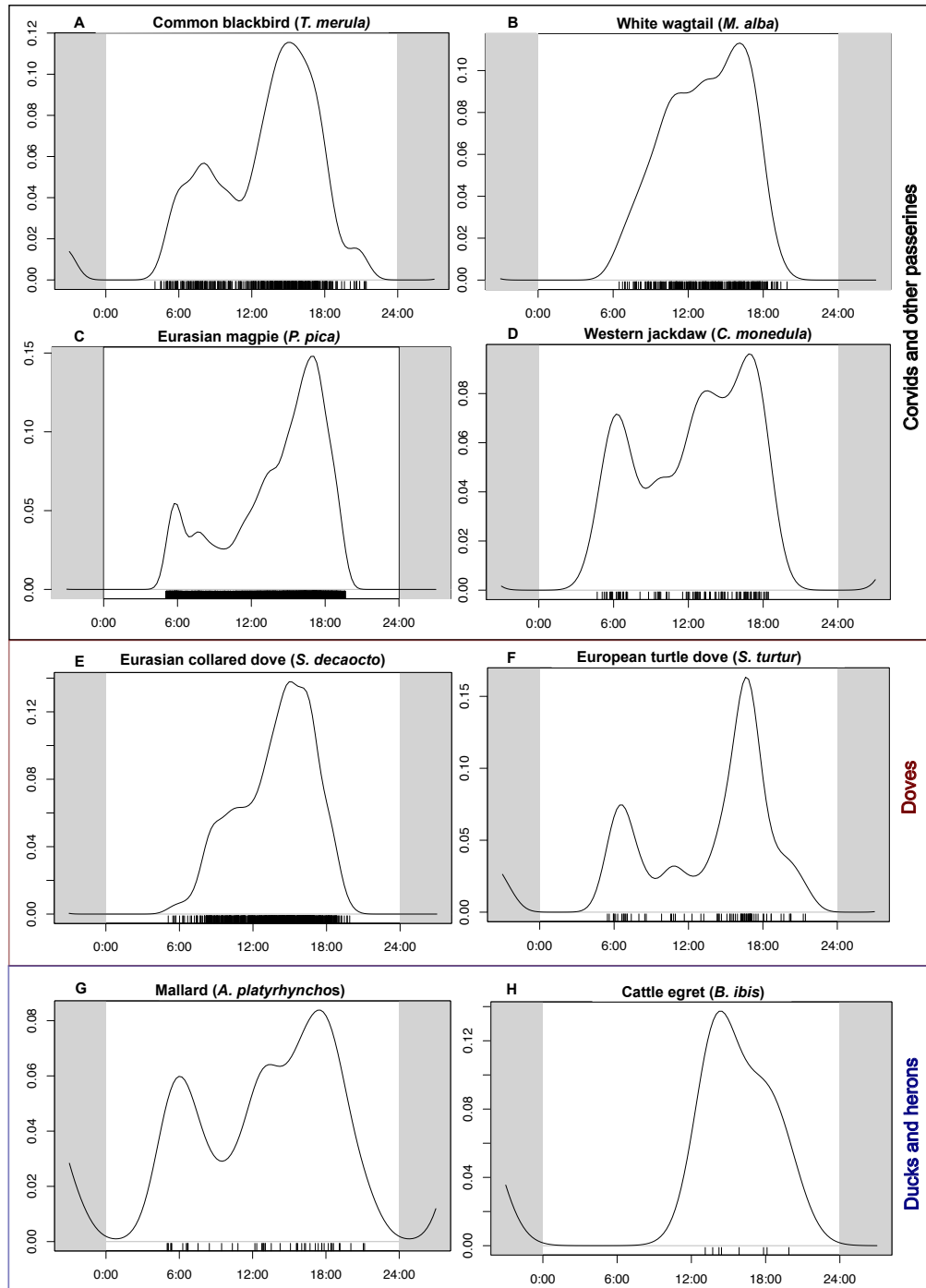


Figure 3. Activity patterns (relative frequency by time) of selected wild bird species visiting the poultry farms studied. (A) Common blackbird (*Turdus merula*), (B) white wagtail (*Motacilla alba*), (C) Eurasian magpie (*Pica pica*), (D) Western jackdaw (*Corvus monedula*), (E) Eurasian collared dove (*Streptopelia decaocto*), (F) European turtle dove (*Streptopelia turtur*), (G) mallard (*Anas platyrhynchos*), and (H) cattle egret (*Bubulcus ibis*).

In terms of the temporal distribution of the visits (Figure 2G), Eurasian magpies were observed throughout the year, common blackbirds were frequently observed during the spring and early summer months, white wagtails were mainly seen from December to March, and Western jackdaws were recorded only from April to May. The predominant behaviors displayed were feeding (46.3% of total observations for common blackbirds, 56% for Eurasian magpies, and 70.9% for Western jackdaws) or moving through the camera's field of view (73.9% for white wagtails) (Figure 4). Furthermore, magpies were observed foraging on eggs fallen from the longitudinal belt of the automated egg transport system to convey eggs from the hen houses to the egg packing plant of Farm 1.

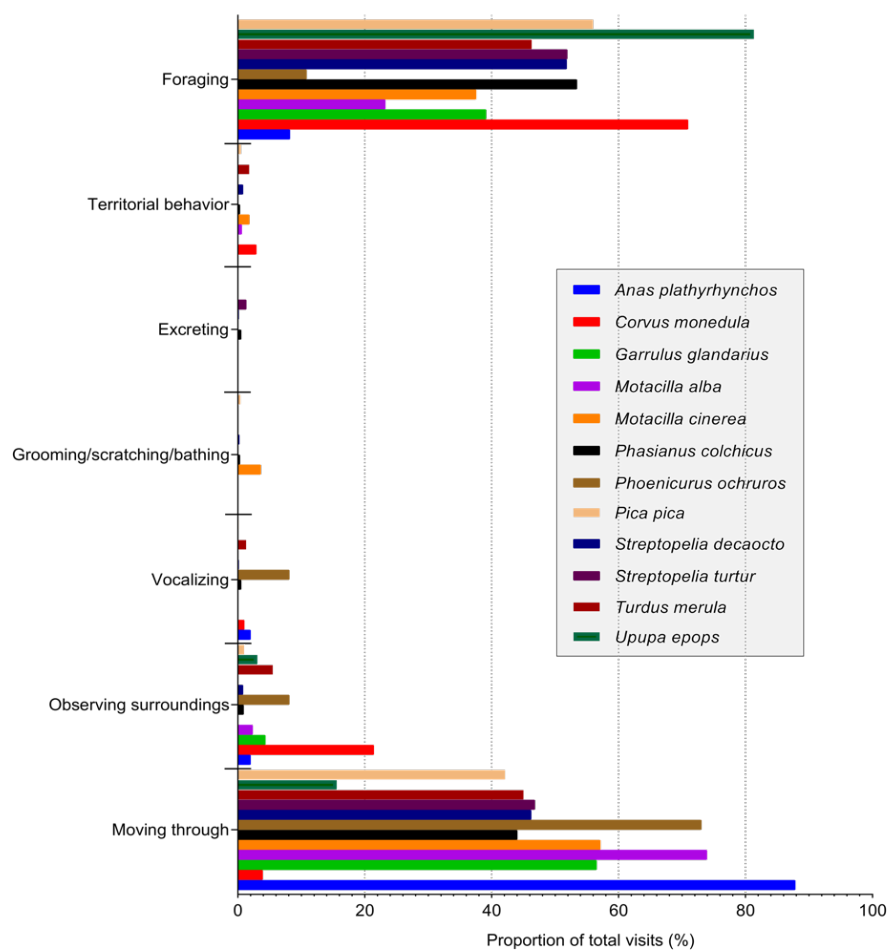


Figure 4. Behaviors displayed by selected wild bird species during the period of study.

1.3.1.2. Pigeons, doves (Columbiformes order), and pheasants (Galliformes order)

Among columbids, Eurasian collared doves were regularly present in the surroundings of the poultry houses throughout the year (887 visits), except for the month of June. During the spring migration and breeding season, the migratory European turtle dove (*Streptopelia turtur* LINNAEUS, 1758; 77 visits) was also seen (Figure 2G). Both species of doves were primarily observed engaging in feeding behavior (51.8% and 51.9% respectively) or moving through the camera's field of view (46.2% and 46.8%) (Figure 4). A small proportion of the recorded footages captured instances of Eurasian collared doves (0.1%) and European turtle doves (1.3%) in the act of defecating. The activity pattern of the doves exhibited consistent occurrences throughout daylight hours for the Eurasian collared dove, while the European turtle dove was predominantly seen in the afternoon, specifically from 4 to 6 p.m. (Figure 3E-3F). Conversely, sightings of rock pigeons (*Columba livia* GMELIN, 1774) and wood pigeons (*Columba palumbus* LINNAEUS, 1758) were infrequent (7 and 3 visits, respectively).

The common pheasant stood out as one of the most frequently observed species across all the sites monitored, except for the feed silos area (location G) on Farm 3. Pheasant visits displayed a consistent distribution throughout the entire year, with a notable peak in April (Figure 2G). The observed behaviors primarily encompassed foraging activities (53.4%) and moving through the camera's field of view (44%), while 0.5% of the recorded instances captured pheasants in the act of defecating (Figure 4). The daily activity pattern of pheasants exhibited two peaks in counts: a smaller one around sunrise (4:30 a.m. to 7:00 a.m.) and a more pronounced one from 4 p.m. to 8 p.m. (Figure 3E-3F).

1.3.1.3. Wild aquatic birds (Anseriformes, Gruiformes and Pelecaniformes orders)

Among the farms studied, the presence of wild aquatic birds was primarily evident in Farm 1, situated in close proximity to a fishing sport lake. However, there were also sightings of cattle egrets (*Bubulcus ibis* LINNAEUS, 1758) in Farm 2. The observed bird species, listed in descending order of frequency (Figure 2G), included mallards (*Anas platyrhynchos* LINNAEUS, 1758; 49 visits), cattle egrets (8 visits), common moorhens (*Gallinula chloropus* (LINNAEUS, 1758); 5 visits), a grey heron (*Ardea cinerea* LINNAEUS, 1758; 1 visit) and an African sacred ibis (*Threskiornis aethiopicus* (LATHAM, 1790); 1 visit).

Mallards were spotted in Farm 1 during the late winter and breeding season (February to June) across all camera trap locations (A to C), primarily moving through the camera's field of view (87.8% of observations) (Figure 4). Notably, ducklings were observed in late June near

camera location B, which is close to the air inlets of a poultry house facing the area adjacent to the fishing sport lake. About 8.2% of the recorded mallard observations showed them foraging near feed silos or drinking from rainwater ponds, with peak observations occurring around 6:00 a.m. and 6:00 p.m. (Figure 3G).

On the other hand, cattle egret observations were restricted to the chicken manure collection area (location E) in Farm 2. Their behavior was predominantly categorized as either moving through the camera's field of view (62.5%) or feeding (37.5%) in groups (Figure 4), primarily during midday (Figure 3H).

1.3.2. Temporal and spatial patterns of visits

The MDRs (95% CI) of total wild bird counts across the three farms are presented in Table 3. Among the locations, the chicken manure collection point of Farm 2 (location E) exhibited the highest MDR (8.70; 6.64-10.77 95% CI), followed by the feed silos area (location D) (4.59; 2.53-6.65 95% CI), and the chicken manure collection point (location C) of Farm 1 (2.20; 0.11-4.29 95% CI).

Table 3. Detection rates of wild bird visits on Farm 1, 2 and 3.

Farm	Camera trap days	Overall mean detection rate (95% CI)	Location	Mean detection rate (95% CI)
1	309	4.10 (2.03 - 6.17)	CMan [^] (A)	1.89 (0 - 4.01)
	257		PHouse [§] (B)	0.88 (0 - 3.10)
	311		FSilo ^{&} (C)	2.20 (0.11 - 4.29)
2	342	11.64 (9.61 - 13.68)	FSilo (D)	4.59 (2.53 - 6.65)
	281		CMan (E)	8.70 (6.64 - 10.77)
3	211	1.87 (0 - 4.14)	FSilo (G)	1.73 (0 - 4.00)
	247		CMan (H)	0.32 (0 - 2.80)

[^]Chicken manure collection point.

[§]Side of the poultry house, adjacent to air inlets.

[&]Feed silos area.

^{*}Outdoor area.

As illustrated in the time-series analysis in Figure 5A, the occurrence of daily wild bird visits exhibited an increase during late autumn and early winter, reaching its peak in December

and January. The spring season displayed the highest number of consecutive wild bird detections, while the lowest peaks were observed during the summer and autumn months. In the case of Farm 1, the periods with the highest daily visitation rates were spring (20 visits/day) and late autumn/December (12 visits/day) (Figure 5B). For Farm 2 (Figure 5B), notable observation peaks occurred in January 2021 (37 visits/day), December 2021 (30 visits/day), and late summer-early autumn (~20 visits/day). Despite being monitored for a shorter duration, Farm 3 (Figure 5B) displayed significant peaks in daily wild bird counts during February (11 visits/day) and March (15 visits/day). Overall, Farm 2 consistently recorded a higher frequency of daily wild bird observations compared to Farm 1 and Farm 3.

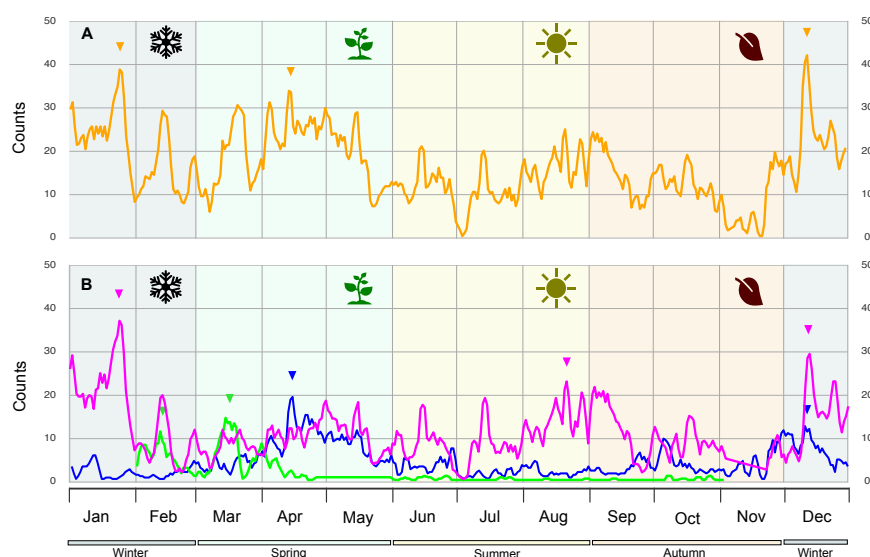


Figure 5. Temporal distribution of daily wild bird visits throughout the period studied (camera traps at location A to E, G and H). (A) Overall daily counts for Farms 1, 2 and 3; (B) Daily counts displayed separately for the three farms (Farm 1, light blue line; Farm 2, magenta line; Farm 3, green line). Color-coded arrows indicate peaks of wild bird observations.

MDRs and 95% confidence interval for each season are presented in Figure 6A. Farm 1 exhibited the highest average counts during the spring season (MDR 7.39; 5.29 – 9.50), followed by autumn (3.44; 1.28 – 5.61), winter (3.38; 1.14 – 5.62) and summer (2.15; 0 – 4.34). For Farm 2, the peak mean detection rate occurred in winter (16.14; 14.04 – 18.25), followed by summer (10.21; 8.10 – 12.31), autumn (10; 7.88 – 12.12) and spring (9.95; 7.88 – 12.01). Lastly, Farm 3 displayed the highest mean detection rate during winter (6.24; 4.04 – 8.44), followed by spring (4.24; 1.95 – 6.53), summer (0.19; 0 – 2.75) and autumn (0.15; 0 – 3.19). The CA results, depicted in Figure 6B, indicate

a significant association between the presence of Passeriformes species and the months of August, September, and December. The presence of wild Galliformes, represented solely by pheasants, was linked to a higher number of visits in April and June. Additionally, the Columbiformes showed a connection to the month of March.

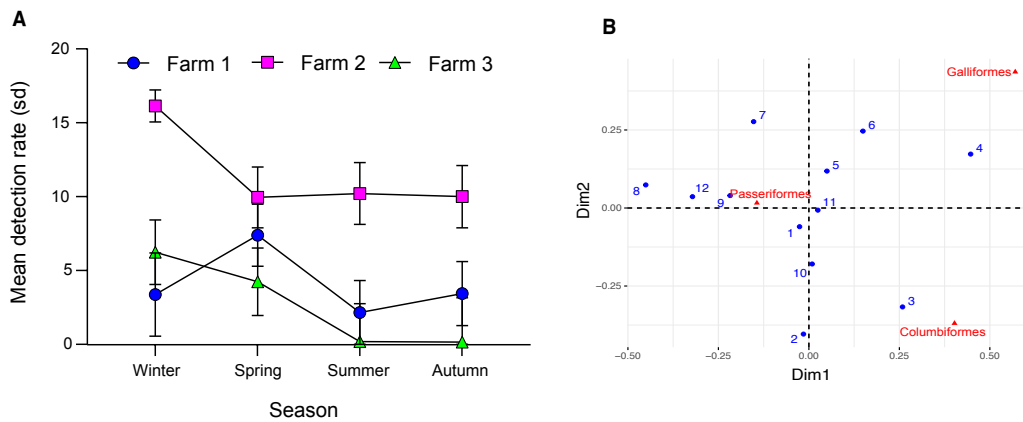


Figure 6. Seasonal mean detection rates of daily counts of wild birds visiting the three poultry farms studied (A) and correspondence analysis plot (B). Farm 1, blue circle; Farm 2, magenta square; Farm 3, green triangle.

The frequency of the wild bird visits was statistically analyzed in relation to the monitored farm, camera trap location, and the season. The fitted model accounted for 42.6% of the data variance based on the pseudo R^2 (Zuur *et al.*, 2009). A summary of the results is provided in Table 4. The effect of the camera trap location on wild bird counts differed according to the farm considered. Specifically, at the feed silos area, Farm 1 displayed a higher number of bird visits in comparison to chicken manure collection point of Farm 2. Similarly, when Farm 3 was monitored at the feed silos area, the RR was 10.8 (95% CI: 7.24 - 16.13, $p = <0.001$), indicating an increased number of bird visits. Although the effect of season alone was modest and not statistically significant, its significance increased when considering the farm monitored. Particularly, a decrease in wild bird visits during season 0 (autumn and summer) was observed for Farm 1 (RR: 0.75, 95% CI: 0.58-0.95, $p = <0.05$) and Farm 3 (RR: 0.04, 95% CI: 0.02-0.07, $p = <0.001$), in comparison to counts at Farm 1 during season 1 (winter and spring).

1 **Table 4.** Generalized linear model selected and explanatory variables considered. The percentage of variation explained (V.E.) by fixed
2 terms, the exponentiated estimates (95% CI), and the *p*-value of Wald test for contrasts between the reference level and the level
3 considered are displayed.

Dataset tested	V.E.	Explanatory variable and levels		Rate ratio (95% CI)	Estimate	p-value	
Wild bird counts	42.6%	Farm monitored ¹	Farm 1	0.22 (0.18 - 0.27)	-1.49	<0.001***	
			Farm 3	0.08 (0.05-0.11)	-2.51	<0.001***	
		Camera trap location ²		Feed silos area	0.51 (0.42 - 0.61)	-0.67	<0.001***
		Season ³		Season 0	1.13 (0.71 - 1.08)	-0.12	0.225
		Farm monitored × Camera trap location	Farm 1 × Feed silos area	2.39 (1.88 - 3.04)	0.87	<0.001***	
			Farm 3 × Feed silos area	10.8 (7.24 - 16.13)	2.38	<0.001***	
		Farm monitored × Season	Farm 1 × Season 0	0.75 (0.58 - 0.95)	-0.28	< 0.05*	
			Farm 3 × Season 0	0.04 (0.02 - 0.07)	-3.15	<0.001***	
		Camera trap location × Season	Feed silos area × Season 0	1.09 (0.89 - 1.34)	0.09	0.446	

4 ¹ Farm 2 taken as a reference; ² Chicken manure collection point as a reference; ³ Season 1 (winter and spring seasons) taken as a reference; season 0
5 refers to summer and autumn grouped together; * *p*<0.05; ** *p*<0.01; *** *p*<0.001.

1.3.3. Wild birds in the free-range area

The camera trap located in the outdoor area of Farm 2 (location F) was operational from July 2021 to mid-October 2021, running from 6 p.m. to 6 a.m. during 88 camera trap nights. Throughout this timeframe, a total of 1745 video-clips were captured. Among these, 1,711 videos captured the presence of poultry, which accessed the area between 5:30 p.m. and 8:30 p.m. daily or every two days, depending on weather conditions. Occasionally, a small group of 2-3 hens remained outdoors during the night. Instances of wild bird detection numbered 21 (1.2% of the total number of recordings) and an ICR of 0.50 (0 – 3.04 95% CI) was calculated. The most frequently observed species included the house sparrow and the common pheasant, both observed 7 times. The Eurasian magpie was spotted 6 times, while the common chaffinch 5 times. The Eurasian collared dove appeared 3 times, while the Eurasian hoopoe (*Upupa epops* LINNAEUS, 1758), wood pigeon, and common blackbird were observed 2, 1, and 1 time(s) respectively.

Behaviors exhibited by these species primarily involved moving through the camera field and foraging, as depicted in Figure 8. Specifically, wood pigeons (100%), house sparrows (100%), Eurasian magpies (60%), and Eurasian collared doves (100%) were predominantly engaged in foraging activities.

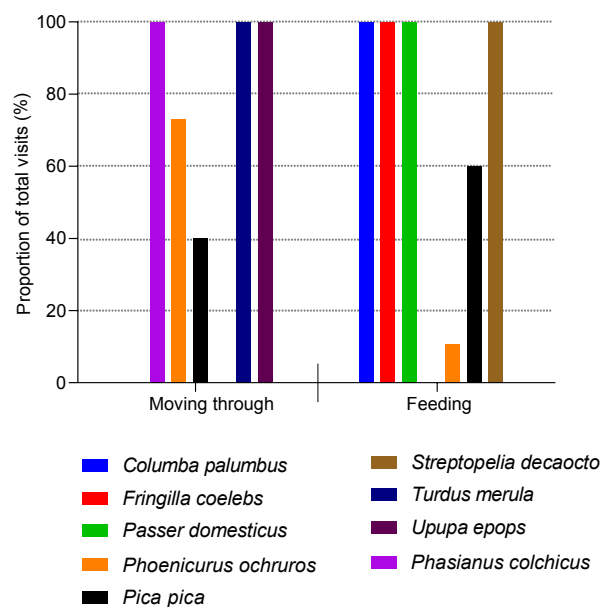


Figure 8. Different behaviors displayed by wild birds in the outdoor area of Farm 2.

Despite the camera trap's primary activity during the period when laying hens were indoors, a total of 8 instances of co-occurrence between wild birds and poultry were recorded, constituting 38% of the observed wild bird visits in the outdoor area. Notably, three instances were documented where Eurasian magpies were observed foraging in the meadow. Additionally, two separate recordings captured common chaffinches engaging in foraging

activities within the area. One footage depicted a common pheasant moving through the camera's field of view, while another clip recorded a Eurasian dove foraging. Lastly, occurrences were noted where both common pheasants and Eurasian doves were observed moving through the camera's field of view and engaging in foraging, respectively.

1.4. Discussion

A year-long camera-trap study was conducted on three laying-hen farms located in an area with a high risk of AIV introduction from wild birds. Through individual observations of the footages recorded, the diversity of wild bird species and the quantification and characterization of their visits were achieved. This work is notably one of the few that focuses on studying the domestic-wild bird interface also in conventional indoor farms (Burns *et al.*, 2012; Scott *et al.*, 2018), as prior research mainly concentrated on free-range facilities (Bestman *et al.*, 2018; Elbers & Gonzales, 2020; Le Gall-Ladevèze *et al.*, 2022).

Throughout the year, a total of 5,978 wild bird visits from 14 taxonomic families were recorded. The free-range farm (Farm 2) showed a higher number of visits compared to the conventional indoor farms (Farm 1 and 3). This was in contrast with the findings of Scott *et al.* (2018), where indoor poultry farms (cage layer type) had a higher number of wildlife visits. Risk factors and reasons for the difference in wild bird visits between the two farm type, although significant, were not obvious. Potential factors influencing this variation could be environmental (Bestman *et al.*, 2018), such as surrounding landscape characteristics, or anthropogenic practices affecting wild bird presence, like cleanliness practices (Hinsley *et al.*, 1995). Furthermore, the interaction effect between the monitored farm and the camera trap location significantly impacted wild bird counts, highlighting the importance of studying the domestic-wild bird interface in different contexts. Seasons also played a role in wild bird presence, with fewer visits in Farm 1 and 3 during summer and autumn, in contrast to Farm 2 during winter and spring. This could be related to increased feeding requirements of wild species during the breeding spring period and colder months (Kuenzel *et al.*, 1999). Given that camera locations near feed silos and chicken manure collection points predominantly captured wild birds during foraging, this underscored the attractiveness of these sites to wild species. The feed silos area, which can experience feed spills, and the chicken manure collection point, with uncleaned litter rich in organic material, offer direct food sources and potentially favor insect populations, thus attracting both granivorous and omnivorous/carnivorous birds (Scott *et al.*, 2018). Despite the farms' proximity to lowland areas with nearby irrigation canals and one farm being close to a fishing sport lake, there were relatively few sightings of known AIV reservoirs. This differed from results presented by Elbers and Gonzales (2020), who frequently observed dabbling ducks and gulls in a video-monitored free-range layer farm located near waterways. Mallards were the sole duck species visiting Farm 1, mainly during spring and

April, with peaks of observations at sunrise and sunset. This seasonal pattern of mallards' visits was in contrast with findings from Welby *et al.* (2010) and Elbers and Gonzales (2020), where waterfowl visits peaked from December to February. Furthermore, in 8.2% of instances recorded in this study, mallards were observed foraging near feed silos or drinking from rainwater ponds. Since waterfowl tend to exhibit strong site fidelity even toward livestock facilities (McDuie *et al.*, 2022), it is crucial to minimize the likelihood of attracting these species to the farm through regular cleaning and maintenance. Additionally, other wild aquatic birds were sporadically identified, including a grey heron and an African sacred ibis, each observed once on Farm 1 likely due to its proximity to a fishing sport lake where these species used to reside.

The wild bird species most frequently observed in this survey were synanthropic corvids, namely Eurasian magpies and Western Jackdaws, as well as small passerines like white wagtails and common blackbirds. Foraging activities were predominant, with Eurasian magpies even observed while foraging on eggs fallen from the automated egg-transport system. The frequency of visits of Passeriformes birds strongly and positively correlated with September and December, months when migratory wild ducks, known AIV reservoirs, populate nearby wetlands during wintering seasons (Spina & Volponi, 2008b). Due to their scavenging behavior, HPAI viral isolation in corvids has been linked to AI outbreaks in poultry farms (Shriner & Root, 2020). Eurasian magpie and Western jackdaw have both been assessed as susceptible to HPAI infection (Department for Environment Food & Rural Affairs, 2022), with Eurasian magpies even included in the target list of wild birds for passive surveillance of H5 HPAI viruses (European Food Safety Authority *et al.*, 2017). Moreover, immunohistochemistry analyses of the digestive tracts of deceased HPAI H5N8-infected Eurasian magpies revealed widespread virus antigen presence, suggesting cloacal virus shedding and environmental contamination (Valentina Caliendo *et al.*, 2020). Given their activity on poultry farms and their resident behavior (Spina & Volponi, 2008a), magpies and jackdaws could potentially act as bridge hosts (Caron *et al.*, 2014) in the local scale of AIV epidemiology. Regarding other passerines, different species have been shown to be susceptible to natural (European Food Safety Authority *et al.*, 2022a; Gronesova *et al.*, 2008; Peterson *et al.*, 2008; Slusher *et al.*, 2014), or experimental AIV infection (Brown *et al.*, 2009; Fujimoto *et al.*, 2015). Since both white wagtails and common blackbirds have been commonly observed on European poultry farms (Elbers & Gonzales, 2020; Le Gall-Ladevèze *et al.*, 2022; Veen *et al.*, 2007), their specific role in the context of HPAI virus epidemiology requires further investigation.

Pheasants and doves were also frequently seen in the farms monitored. Pheasants' visits were positively associated with spring months (April, May, and June). Tammiranta *et al.* (2023) highlighted how mass mortalities in HPAI H5N1-infected wild pheasants were spatially and temporally linked to AI cases in mammals, illustrating rapid disease spread among these

birds. Being territorial birds with a small home range (maximum 4.5 km) (Turner, 2007) and highly susceptible to HPAI infection (Brookes *et al.*, 2022), pheasants might therefore act as AI bridge hosts, particularly in DPPAs. Given their limited flight ability, strategies such as fencing the farm area and maintaining fences to prevent their access could reduce pheasant occurrence on poultry farms.

Lastly, columbids have been proved susceptible to HPAIV infection, with different clinical outcomes according to the virus considered (Abolnik, 2014; Liu *et al.*, 2020; Perkins & Swayne, 2002; Peters *et al.*, 2022). Experimental study with different AI subtypes (H5N8 LPAI, H5N8 HPAI, H5N2, H4N6) or human influenza viruses (H7N9) (Bosco-Lauth *et al.*, 2019; Jeong *et al.*, 2021; Kwon *et al.*, 2017; Shriner *et al.*, 2016; Uchida *et al.*, 2017) in pigeons resulted in limited or absent viral shedding, with no transmission to contact individuals. In contrast, an experimental study with H5N6 HPAIVs of clade 2.3.4.4b revealed a high viral shedding from the oropharynx and cloaca (Liu *et al.*, 2020). Eurasian collared doves represented the columbid species most frequently observed in this camera trap survey, and their presence resulted strongly associated with the month of April. While their susceptibility to H5Nx HPAIV strains has not been studied experimentally yet, dead individuals have tested positive for HPAI infection during surveillance activities (Capua *et al.*, 2000; Istituto Zooprofilattico Sperimentale delle Venezie, 2023). Considering their frequent presence nearby poultry houses, further research is recommended to clarify their actual role in the transmission of currently circulating HPAI viruses at the interface between domestic and wild birds.

Regarding the camera placed in the free-range area of the organic farm, only 1.2% of the collected recordings featured wild birds, yielding a mean ICR of 0.50 (0–3.04 95% CI). This observation rate was lower than those reported in other European free-range poultry facilities (Elbers & Gonzales, 2020; Le Gall-Ladevèze *et al.*, 2022), potentially due to the constrained period surveyed (July to mid-October, from 6 a.m. to 6 a.m) and the utilization of a single camera. The species most frequently observed in the free-range setting were small passerines like house sparrows and common chaffinches, along with Eurasian magpies, aligning with previous observations (Elbers & Gonzales, 2020; Veen *et al.*, 2007). Furthermore, 38% of the wild bird recordings displayed the concurrent presence of wild species, particularly Eurasian magpies, alongside the laying hens. This underscored the heightened risk of AIV introduction via wild birds in organic or free-range farms, given the increased potential for contacts between infectious wild birds and domestic poultry (Bouwstra *et al.*, 2017; Holt, 2021). Hence, it is crucial to diminish the attractiveness of free-range areas to wild birds. Achieving this may involve regular inspections of the outdoor space, swift removal of carcasses and eggs, and implementation of measures to discourage wild bird habituation (Elbers & Gonzales, 2020). The use of cover panels and netted sides in the outdoor space could serve a dual purpose: preventing wild bird access to the area and providing enrichment to encourage hens to explore a broader part of the range. Indeed, hens tend to congregate in a limited area nearby the

poultry house if supplementary artificial shelters or vegetation are absent (Gilani *et al.*, 2014; Zeltner & Hirt, 2003).

Effective farming practices hinge not only on the management protocols of the poultry company but also on the farmers' understanding of the repercussions of risky behaviors on animal health (Hinjoy *et al.*, 2023). The utilization of visual evidence depicting wild birds around poultry houses, such as the footages recorded through camera traps, could facilitate health authorities in educating and raising awareness between stakeholders regarding wild bird presence and potential pathogen spillover opportunities.

As far as our knowledge extends, this study marks the first instance of utilizing camera traps to document wild bird activities and behaviors on commercial poultry farms in Italy. The collected data could help the development of transmission models of diseases at the domestic-wild bird interface, such as through the integration of seasonal MDRs in social network analyses. However, several limitations inherent to this study should be acknowledged. Primarily, the camera-trap monitoring encompassed a limited number of poultry farms and sites, possibly limiting the generalizability of the conclusions to other farm settings. Additionally, the placement of cameras in areas of heightened human activity (e.g., feed silos; chicken manure collection points) resulted in numerous non-relevant triggers, contributing to battery depletion and camera trap failures, thereby reducing the camera trap effort over the study period, a phenomenon documented in other studies as well (Bacigalupo *et al.*, 2022; Engeman *et al.*, 2011). Lastly, for a comprehensive grasp of the factors influencing wild bird activity on poultry farms, future research should encompass additional variables such as habitat characteristics and environmental factors, aspects that were not considered in the models presented within this study.

1.5. Conclusions

The findings presented in this study laid a groundwork for understanding disease transmission at the domestic-wild bird interface. Such information is essential for informed scientific guidance in designing surveillance and intervention strategies, aiming to predict the emergence of pathogens and thwart cross-species transmission (Hassell *et al.*, 2017). Among the wild birds more frequently observed, the assessment of their susceptibility to infection with currently circulating HPAI viruses is a necessary step to fully evaluate their actual role as bridge hosts (Caron *et al.*, 2014). Given the continuous evolution of HPAIV through genetic drift and reassortment events between different subtypes and genotypes, potentially giving rise to strains with novel biological traits (European Food Safety Authority *et al.*, 2023d; Lycett *et al.*, 2019), it is paramount for farmers to be cognizant of the presence of potentially high-risk wild birds around poultry houses. Coupled with the diligent application and enforcement of

biosecurity measures, this awareness stands as one of the most effective preventive measures to avert spillover or spillback at the interface between domestic and wild bird populations.

1.6. References

- Abolnik, C. (2014). A current review of avian influenza in pigeons and doves (Columbidae). *Vet Microbiol*, 170(3-4), 181-196. doi:10.1016/j.vetmic.2014.02.042
- Ayala, A. J., Yabsley, M. J., & Hernandez, S. M. (2020). A Review of Pathogen Transmission at the Backyard Chicken-Wild Bird Interface. *Front Vet Sci*, 7, 539925. doi:10.3389/fvets.2020.539925
- Bacigalupo, S. A., Dixon, L. K., Gubbins, S., Kucharski, A. J., & Drewe, J. A. (2022). Wild boar visits to commercial pig farms in southwest England: implications for disease transmission. *Eur J Wildl Res*, 68(6), 69. doi:10.1007/s10344-022-01618-2
- Bestman, M., de Jong, W., Wagenaar, J.-P., & Weerts, T. (2018). Presence of avian influenza risk birds in and around poultry free-range areas in relation to range vegetation and openness of surrounding landscape. *Agrofor Syst*, 92(4), 1001-1008. doi:10.1007/s10457-017-0117-2
- Bosco-Lauth, A. M., Marlenee, N. L., Hartwig, A. E., Bowen, R. A., & Root, J. J. (2019). Shedding of clade 2.3.4.4 H5N8 and H5N2 highly pathogenic avian influenza viruses in peridomestic wild birds in the U.S. *Transbound Emerg Dis*, 66(3), 1301-1305. doi:10.1111/tbed.13147
- Bouwstra, R., Gonzales, J., de Wit, S., Stahl, J., Fouchier, R. A. M., & Elbers, A. R. W. (2017). Risk for Low Pathogenicity Avian Influenza Virus on Poultry Farms, the Netherlands, 2007–2013. *Emerg Inf Dis*, 23(9), 1510. doi:10.3201/eid2309.170276
- Brookes, S. M., Mansfield, K. L., Reid, S. M., Coward, V., Warren, C., Seekings, J., Brough, T., Gray, D., Núñez, A., & Brown, I. H. (2022). Incursion of H5N8 high pathogenicity avian influenza virus (HPAIV) into gamebirds in England. *Epidemiol Infect*, 150, e51. doi:10.1017/s0950268821002740
- Brown, J. D., Stallknecht, D. E., Berghaus, R. D., & Swayne, D. E. (2009). Infectious and Lethal Doses of H5N1 Highly Pathogenic Avian Influenza Virus for House Sparrows (*Passer Domesticus*) and Rock Pigeons (*Columbia Livia*). *J Vet Diagn Invest*, 21(4), 437-445. doi:10.1177/104063870902100404
- Burns, T. E., Ribble, C., Stephen, C., Kelton, D., Toews, L., Osterhold, J., & Wheeler, H. (2012). Use of observed wild bird activity on poultry farms and a literature review to target species as high priority for avian influenza testing in 2 regions of Canada. *Can Vet J*, 53(2), 158-166.
- Caliendo, V., Leijten, L., Begeman, L., Poen, M. J., Fouchier, R. A. M., Beerens, N., & Kuiken, T. (2020). Enterotropism of highly pathogenic avian influenza virus H5N8 from the 2016/2017 epidemic in some wild bird species. *Vet Res*, 51(1), 117. doi:10.1186/s13567-020-00841-6

- Caliendo, V., Lewis, N. S., Pohlmann, A., Baillie, S. R., Banyard, A. C., Beer, M., Brown, I. H., Fouchier, R. A. M., Hansen, R. D. E., Lameris, T. K., Lang, A. S., Laurendeau, S., Lung, O., Robertson, G., van der Jeugd, H., Alkie, T. N., Thorup, K., van Toor, M. L., Waldenström, J., Yason, C., Kuiken, T., & Berhane, Y. (2022). Transatlantic spread of highly pathogenic avian influenza H5N1 by wild birds from Europe to North America in 2021. *Sci Rep*, 12(1), 11729. doi:10.1038/s41598-022-13447-z
- Capua, I., Grosse, B., Bertoli, E., & Cordioli, P. (2000). Monitoring for highly pathogenic avian influenza in wild birds in Italy. *Vet Rec*, 147(22).
- Caron, A., Grosbois, V., Etter, E., Gaidet, N., & de Garine-Wichatitsky, M. (2014). Bridge hosts for avian influenza viruses at the wildlife/domestic interface: an eco-epidemiological framework implemented in southern Africa. *Prev Vet Med*, 117(3-4), 590-600. doi:10.1016/j.prevetmed.2014.09.014
- Cleaveland, S., Laurenson, M. K., & Taylor, L. H. (2001). Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philos Trans R Soc Lond B Biol Sci*, 356(1411), 991-999. doi:10.1098/rstb.2001.0889
- Craft, M. E. (2015). Infectious disease transmission and contact networks in wildlife and livestock. *Philos Trans R Soc Lond B Biol Sci*, 370(1669). doi:10.1098/rstb.2014.0107
- Daszak, P. (2000). Emerging infectious diseases of wildlife - threats to biodiversity and human health. *Science*, 287.
- Department for Environment Food & Rural Affairs, A. a. P. H. A. (2022). Highly pathogenic avian influenza (HPAI) in the UK and Europe. Update outbreak assessment #23. Retrieved from https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1070246/Highly_pathogenic_avian_influenza__HPAI__in_the_UK_and_Europe__outbreak_update_23.pdf
- Elbers, A. R. W., & Gonzales, J. L. (2020). Quantification of visits of wild fauna to a commercial free-range layer farm in the Netherlands located in an avian influenza hot-spot area assessed by video-camera monitoring. *Transbound Emerg Dis*, 67(2), 661-677. doi:10.1111/tbed.13382
- Engeman, R., Betsill, C., & Ray, T. (2011). Making Contact: Rooting Out the Potential for Exposure of Commercial Production Swine Facilities to Feral Swine in North Carolina. *EcoHealth*, 8(1), 76-81. doi:10.1007/s10393-011-0688-8
- European Food Safety Authority, Aznar, I., Baldinelli, F., Stoicescu, A., & Kohnle, L. (2022a). Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2021. *EFSA J*, 20(9), e07554. doi:https://doi.org/10.2903/j.efsa.2022.7554
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A.,

- Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2022b). Avian influenza overview March - June 2022. *EFSA J*, 20(8), e07415. doi:10.2903/j.efsa.2022.7415
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2023a). Avian influenza overview September - December 2022. *EFSA J*, 21(1), e07786. doi:10.2903/j.efsa.2023.7786
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Munoz Guajardo, I., & Baldinelli, F. (2022c). Avian influenza overview May - September 2021. *EFSA J*, 20(1), e07122. doi:10.2903/j.efsa.2022.7122
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinaviciute, G., Niqueux, E., Stahl, K., Staubach, C., Terregino, C., Broglia, A., & Baldinelli, F. (2023b). Avian influenza overview March - April 2023. *EFSA J*, 21(5), 45. doi:10.2903/j.efsa.2023.8039
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Brown, I., Kuiken, T., Mulatti, P., Smietanka, K., Staubach, C., Stroud, D., Therkildsen, O. R., Willeberg, P., Baldinelli, F., Verdonck, F., & Adlhoch, C. (2017). Avian influenza overview September - November 2017. *EFSA J*, 15(12), e05141. doi:10.2903/j.efsa.2017.5141
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Mirinaviciute, G., Niqueux, E., Stahl, K., Staubach, C., Terregino, C., Broglia, A., & Baldinelli, F. (2023c). Avian influenza overview December 2022 - March 2023. *EFSA J*, 21(3), e07917. doi:10.2903/j.efsa.2023.7917
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Munoz Guajardo, I., & Baldinelli, F. (2022d). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- European Food Safety Authority, European Centre for Disease Prevention Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Melidou, A., Mirinavičiūtė, G., Niqueux, É., Ståhl, K., Staubach, C., Terregino, C., Baldinelli, F., Broglia, A., & Kohnle, L. (2023d). Avian influenza

- overview April – June 2023. *EFSA J*, 21(7), e08191. doi:<https://doi.org/10.2903/j.efsa.2023.8191>
- Franklin, A. B., Bevins, S. N., & Shriner, S. A. (2021). Pathogens from Wild Birds at the Wildlife–Agriculture Interface. In *Infectious Disease Ecology of Wild Birds* (pp. 207-228).
- Fujimoto, Y., Usui, T., Ito, H., Ono, E., & Ito, T. (2015). Susceptibility of wild passerines to subtype H5N1 highly pathogenic avian influenza viruses. *Avian Pathol*, 44(4), 243-247. doi:10.1080/03079457.2015.1043235
- Gilani, A. M., Knowles, T. G., & Nicol, C. J. (2014). Factors affecting ranging behaviour in young and adult laying hens. *Br Poult Sci*, 55(2), 127-135. doi:10.1080/00071668.2014.889279
- Gortázar, C., Ferroglio, E., Höfle, U., Frölich, K., & Vicente, J. (2007). Diseases shared between wildlife and livestock: a European perspective. *Eur J Wildl Res*, 53(4). doi:10.1007/s10344-007-0098-y
- Graziosi, G., Catelli, E., Fanelli, A., & Lupini, C. (2022a). Infectious bursal disease virus in free-living wild birds: A systematic review and meta-analysis of its sero-viroprevalence on a global scale. *Transbound Emerg Dis*, 69(5), 2800-2815. doi:10.1111/tbed.14433
- Graziosi, G., Lupini, C., & Catelli, E. (2022b). Disentangling the role of wild birds in avian metapneumovirus (aMPV) epidemiology: A systematic review and meta-analysis. *Transbound Emerg Dis*, 69(6), 3285-3299. doi:10.1111/tbed.14680
- Greenberg, S., & Godin, T. (2012). Timelapse Image Analysis Manual. Technical Report 2012-1028-11. doi:<https://grouplab.cpsc.ucalgary.ca/grouplab/uploads/Publications/Publications/2012-TimelapseImageAnalysis.Report2012-1028-11.pdf>
- Gronesova, P., Kabat, P., Trnka, A., & Betakova, T. (2008). Using nested RT-PCR analyses to determine the prevalence of avian influenza viruses in passerines in western Slovakia, during summer 2007. *Scand J Infect Dis*, 40(11-12), 954-957. doi:10.1080/00365540802400576
- Hassell, J. M., Begon, M., Ward, M. J., & Fevre, E. M. (2017). Urbanization and Disease Emergence: Dynamics at the Wildlife-Livestock-Human Interface. *Trends Ecol Evol*, 32(1), 55-67. doi:10.1016/j.tree.2016.09.012
- Hinjoy, S., Thumrin, P., Sridet, J., Chaiyasuwan, P., Rodchangphuen, J., Thukngamdee, Y., & Suddee, W. (2023). Risk perceptions of avian influenza among poultry farmers on smallholder farms along border areas of Thailand. *Front Vet Sci*, 10, 1075308. doi:10.3389/fvets.2023.1075308
- Hinsley, S. A., Bellamy, P. E., Newton, I., & Sparks, T. H. (1995). Habitat and Landscape Factors Influencing the Presence of Individual Breeding Bird Species in Woodland Fragments. *Journal of Avian Biology*, 26(2), 94-104. doi:10.2307/3677057

- Holt, P. S. (2021). Centennial Review: A revisiting of hen welfare and egg safety consequences of mandatory outdoor access for organic egg production. *Poul Sci*, 100(12), 101436. doi:<https://doi.org/10.1016/j.psj.2021.101436>
- Istituto Zooprofilattico Sperimentale delle Venezie. (2018). *Situazione epidemiologica 2016-2018*. Retrieved from <https://www.izsvenezie.it/documenti/temi/influenza-aviaria/situazione-epidemiologica-HPAI/HPAI-archivio-situazioni/2016-2-H5N5-H5N8/HPAI-focolai.pdf>
- Istituto Zooprofilattico Sperimentale delle Venezie. (2023). *Highly Pathogenic Avian Influenza in birds other than poultry in Italy. Epidemiological situation 20 July 2023*. Retrieved from <https://www.izsvenezie.com/documents/reference-laboratories/avian-influenza/italy-updates/HPAI/2022-1/italy-wildbirds.pdf>
- Jeong, S., Kwon, J. H., Lee, S. H., Kim, Y. J., Jeong, J. H., Park, J. E., Jheong, W. H., Lee, D. H., & Song, C. S. (2021). Subclinical Infection and Transmission of Clade 2.3.4.4 H5N6 Highly Pathogenic Avian Influenza Virus in Mandarin Duck (*Aix galericulata*) and Domestic Pigeon (*Columbia livia domestica*). *Viruses*, 13(6). doi:10.3390/v13061069
- Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, 451(7181), 990-993. doi:10.1038/nature06536
- Kuenzel, W. J., Beck, M. M., & Teruyama, R. (1999). Neural sites and pathways regulating food intake in birds: A comparative analysis to mammalian systems. *J Exp Zool*, 283(4-5), 348-364. doi:[https://doi.org/10.1002/\(SICI\)1097-010X\(19990301/01\)283:4/5<348::AID-JEZ5>3.0.CO;2-5](https://doi.org/10.1002/(SICI)1097-010X(19990301/01)283:4/5<348::AID-JEZ5>3.0.CO;2-5)
- Kuiken, T., Fouchier, R. A. M., & Koopmans, M. P. G. (2023). Being ready for the next influenza pandemic? *Lancet Infect Dis*, 23(4), 398-399. doi:10.1016/s1473-3099(23)00117-2
- Kwon, J. H., Noh, Y. K., Lee, D. H., Yuk, S. S., Erdene-Ochir, T. O., Noh, J. Y., Hong, W. T., Jeong, J. H., Jeong, S., Gwon, G. B., Song, C. S., & Nahm, S. S. (2017). Experimental infection with highly pathogenic H5N8 avian influenza viruses in the Mandarin duck (*Aix galericulata*) and domestic pigeon (*Columba livia domestica*). *Vet Microbiol*, 203, 95-102. doi:10.1016/j.vetmic.2017.03.003
- Le Gall-Ladevèze, C., Guinat, C., Fievet, P., Vollot, B., Guérin, J. L., Cappelle, J., & Le Loc'h, G. (2022). Quantification and characterisation of commensal wild birds and their interactions with domestic ducks on a free-range farm in southwest France. *Sci Rep*, 12(1), 9764. doi:10.1038/s41598-022-13846-2
- Liu, K., Gao, R., Wang, X., Han, W., Ji, Z., Zheng, H., Gu, M., Hu, J., Liu, X., Hu, S., Chen, S., Gao, S., Peng, D., Jiao, X. A., & Liu, X. (2020). Pathogenicity and transmissibility of clade 2.3.4.4 highly pathogenic avian influenza virus subtype H5N6 in pigeons. *Vet Microbiol*, 247, 108776. doi:10.1016/j.vetmic.2020.108776

- Lloyd-Smith, J. O., George, D., Pepin, K. M., Pitzer, V. E., Pulliam, J. R., Dobson, A. P., Hudson, P. J., & Grenfell, B. T. (2009). Epidemic dynamics at the human-animal interface. *Science*, 326(5958), 1362-1367. doi:10.1126/science.1177345
- Lycett, S. J., Duchatel, F., & Digard, P. (2019). A brief history of bird flu. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1775), 20180257. doi:10.1098/rstb.2018.0257
- McDuie, F., Matchett, E. L., Prosser, D. J., Takekawa, J. Y., Pitesky, M. E., Lorenz, A. A., McCuen, M. M., T, O. C., Ackerman, J. T., De La Cruz, S. E. W., & Casazza, M. L. (2022). Pathways for avian influenza virus spread: GPS reveals wild waterfowl in commercial livestock facilities and connectivity with the natural wetland landscape. *Transbound Emerg Dis*, 69(5), 2898-2912. doi:10.1111/tbed.14445
- Niedballa, J., Sollmann, R., Courtiol, A., & Wilting, A. (2016). camtrapR: an R package for efficient camera trap data management. *Methods Ecol Evol*, 7(12), 1457-1462. doi:https://doi.org/10.1111/2041-210X.12600
- Perkins, L. E., & Swayne, D. E. (2002). Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Dis*, 46(1), 53-63. doi:10.1637/0005-2086(2002)046[0053:Poahko]2.0.Co;2
- Peters, M., King, J., Wohlsein, P., Grund, C., & Harder, T. (2022). Genuine lethal infection of a wood pigeon (*Columba palumbus*) with high pathogenicity avian influenza H5N1, clade 2.3.4.4b, in Germany, 2022. *Vet Microbiol*, 270, 109461. doi:10.1016/j.vetmic.2022.109461
- Peterson, A. T., Bush, S. E., Spackman, E., Swayne, D. E., & Ip, H. S. (2008). Influenza A virus infections in land birds, People's Republic of China. *Emerg Infect Dis*, 14(10), 1644-1646. doi:10.3201/eid1410.080169
- Randler, C., & Kalb, N. (2018). Distance and size matters: A comparison of six wildlife camera traps and their usefulness for wild birds. *Ecology and evolution*, 8(14), 7151-7163. doi:https://doi.org/10.1002/ece3.4240
- Root, J., & Shriner, S. (2020). Avian Influenza A Virus Associations in Wild, Terrestrial Mammals: A Review of Potential Synanthropic Vectors to Poultry Facilities. *Viruses*, 12(12). doi:10.3390/v12121352
- Scott, A. B., Phalen, D., Hernandez-Jover, M., Singh, M., Groves, P., & Toribio, J. (2018). Wildlife Presence and Interactions with Chickens on Australian Commercial Chicken Farms Assessed by Camera Traps. *Avian Dis*, 62(1), 65-72. doi:10.1637/11761-101917-Reg.1
- Shriner, S. A., & Root, J. J. (2020). A Review of Avian Influenza A Virus Associations in Synanthropic Birds. *Viruses*, 12(11). doi:10.3390/v12111209
- Shriner, S. A., Root, J. J., Mooers, N. L., Ellis, J. W., Stopak, S. R., Sullivan, H. J., VanDalen, K. K., & Franklin, A. B. (2016). Susceptibility of rock doves to low-pathogenic avian influenza A viruses. *Arch Virol*, 161(3), 715-720. doi:10.1007/s00705-015-2685-7

- Slusher, M. J., Wilcox, B. R., Lutrell, M. P., Poulson, R. L., Brown, J. D., Yabsley, M. J., & Stallknecht, D. E. (2014). Are passerine birds reservoirs for influenza A viruses? *J Wildl Dis*, 50(4), 792-809. doi:10.7589/2014-02-043
- Smoglica, C., Graziosi, G., De Angelis, D., Lupini, C., Festino, A., Catelli, E., Vergara, A., & Di Francesco, A. (2023). Wild birds as drivers of *Salmonella* Braenderup and multidrug resistant bacteria in wetlands of Northern Italy. *Submitted to Transbound Emerg Dis*.
- Spina, F., & Volponi, S. (2008a). *Atlante della migrazione degli uccelli in Italia: 2. Passeriformi*. Roma, Italy: Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA).
- Spina, F., & Volponi, S. (2008b). *Atlante della migrazione degli uccelli in Italia: Non-passeriformi*. Roma: Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA).
- Tammiranta, N., Isomursu, M., Fusaro, A., Nylund, M., Nokireki, T., Giussani, E., Zecchin, B., Terregino, C., & Gadd, T. (2023). Highly pathogenic avian influenza A (H5N1) virus infections in wild carnivores connected to mass mortalities of pheasants in Finland. *Infect Genet Evol*, 111, 105423. doi:10.1016/j.meegid.2023.105423
- Team, R. C. (2020). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>
- Tucciarone, C. M., Franzo, G., Legnardi, M., Pasotto, D., Lupini, C., Catelli, E., Quaglia, G., Graziosi, G., Dal Molin, E., Gobbo, F., & Cecchinato, M. (2022). Molecular Survey on A, B, C and New Avian Metapneumovirus (aMPV) Subtypes in Wild Birds of Northern-Central Italy. *Vet Sci*, 9(7). doi:10.3390/vetsci9070373
- Turner, C. V. (2007). *The fate and management of pheasants (Phasianus colchicus) released in the UK*. (Doctor of Philosophy). Imperial College, University of London, Retrieved from <https://spiral.imperial.ac.uk/bitstream/10044/1/11939/2/Turner-CV-2008-PhD-Thesis.pdf>
- Uchida, Y., Kanehira, K., Takemae, N., Hikono, H., & Saito, T. (2017). Susceptibility of chickens, quail, and pigeons to an H7N9 human influenza virus and subsequent egg-passaged strains. *Arch Virol*, 162(1), 103-116. doi:10.1007/s00705-016-3090-6
- Vanwambeke, S. O., Linard, C., & Gilbert, M. (2019). Emerging challenges of infectious diseases as a feature of land systems. *Curr Opin Environ Sustain*, 38, 31-36. doi:10.1016/j.cosust.2019.05.005
- Veen, J., Brouwer, J., Atkinson, P., Bilgin, C., Blew, J., Eksioglu, S., Hoffmann, M., Nardelli, R., Spina, F., Tendi, C., & Delany, S. (2007). Ornithological data relevant to the spread of Avian Influenza in Europe (phase 2): further identification and first field assessment of Higher Risk Species. *Wetlands International, Wageningen, The Netherlands*.
- Wearn, O. R., & Glover-Kapfer, P. (2017). *Camera-trapping for conservation: a guide to best-practices* (Vol. 1). Woking, United Kingdom: WWF-UK.

- Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev*, 56(1), 152-179. doi:10.1128/mr.56.1.152-179.1992
- Welby, S., Poncin, O., Claes, G., Van der Stede, Y., Vangeluwe, D., Marché, S., Lambrecht, B., & Van den Berg, T. (2010). Empirical approach for risk based model to enable detection and measures against spread of low pathogenic avian influenza, preliminary results of wild bird contacts with outdoor poultry pens. *Sci Rep*, 2009–2010, 36-38.
- Wiethoelter, A. K., Beltran-Alcrudo, D., Kock, R., & Mor, S. M. (2015). Global trends in infectious diseases at the wildlife-livestock interface. *Proc Natl Acad Sci U S A*, 112(31), 9662-9667. doi:10.1073/pnas.1422741112
- Zeileis, A., Grothendieck, G., Ryan, J. A., Andrews, F., & Zeileis, M. A. (2014). Package ‘zoo’. *R package version*, 1.7-12.
- Zeltner, E., & Hirt, H. (2003). Effect of artificial structuring on the use of laying hen runs in a free-range system. *Br Poult Sci*, 44(4), 533-537. doi:10.1080/00071660310001616264
- Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer

II. Potential biosecurity breaches in poultry farms: presence of free-ranging mammals near laying-hen houses assessed through a camera-trap study

Abstract

Diligent application and enforcement of biosecurity measures stand as one of the most effective measures to prevent disease transmission through direct or indirect interactions between poultry and free-ranging animals. Among these, free-ranging mammals can be hosts or disseminators of several pathogens relevant for poultry and of public health concern. Moreover, evidence of susceptibility to avian influenza virus infection in non-human mammals has raised questions about their potential role in the virus's epidemiology at the domestic animals-wildlife interface. Given this background, this study aimed to identify key mammal species occurring in the surroundings of laying-hen houses and characterize the spatiotemporal patterns of these visits. Seven camera traps were deployed for a year-long period in three commercial poultry farms located in a densely populated poultry area in Northern Italy. Camera traps' positions were chosen based on wildlife signs identified during preliminary visits to the studied establishments. Various methods, including time series analysis and generalized linear models, were employed to analyze daily mammal visits. A total of 1,867 camera trap nights yielded 567 videos of seven species of wild mammals, and 1,866 videos showed domestic pet species (cats and dogs). Coypus (*Myocastor coypus*) and cats were the two mammals more frequently observed near poultry houses. For wild mammals, visits significantly increased at night, and slightly decreased during the spring season. Overall, the data hereby provided lay the groundwork for designing novel surveillance and intervention strategies to prevent cross-species disease transmission. Moreover, the utilization of visual evidence depicting free-ranging animals approaching poultry houses could assist health authorities in educating and raising awareness among stakeholders about potential risks of pathogen spillover.

2.1. Introduction

During recent years, highly pathogenic avian influenza (HPAI) have had devastating consequences to the poultry industry in multiple countries. Since 2020, an unprecedented number of outbreaks due to the H5Nx HPAI viruses of clade 2.3.4.4b has been reported worldwide, following the 2021 intercontinental spread of this Eurasian lineage to the American continent (Bevins *et al.*, 2022; Caliendo *et al.*, 2022; Mariana *et al.*, 2023). A high number of HPAI infections in wild bird has also been observed in the same period (European Food Safety Authority *et al.*, 2023b). While some wild aquatic birds, especially dabbling ducks, are considered as more resistant to the HPAI infection (Koethe *et al.*, 2020; Soda *et al.*, 2023), mortality events and die-offs have been reported for a broad range of bird species (Kleyheeg *et al.*, 2017; Rijks *et al.*, 2022). Besides the detections in avian species, H5Nx HPAI virus infections of clade 2.3.4.4b have also affected non-human wild or domestic mammals in Europe, U.S., Canada, South America, and Japan (Animal and Plant Health Inspection Service, 2023; European Food Safety Authority *et al.*, 2023b). The majority of the species affected belonged to the Carnivora order, which came into contact with naturally infected wild birds or poultry through a predator-prey relationship. This is the case for terrestrial mammals such as foxes (*Vulpes vulpes* (LINNAEUS, 1758)) (Bordes *et al.*, 2023; Floyd *et al.*, 2021; Rijks *et al.*, 2022), otters (*Lutra lutra* (LINNAEUS, 1758)) (Tammiranta *et al.*, 2023), lynxes (*Lynx lynx* (LINNAEUS, 1758)) (Tammiranta *et al.*, 2023), but also of marine mammals, like seals (Floyd *et al.*, 2021; Puryear *et al.*, 2023; Shin *et al.*, 2019), sea lions (*Otaria flavescens* (SHAW, 1800)) (Gamarra-Toledo *et al.*, 2023; Mariana *et al.*, 2023), and cetaceans (Murawski *et al.*, 2023; Thorsson *et al.*, 2023). Farmed animals have also been found infected, such as American minks (*Neovison vison* (SCHREBER, 1777)) and others raised for fur production in Europe (Agüero *et al.*, 2023; Lindh *et al.*, 2023). Recent H5N1 HPAI virus detection in domestic pets also raised concerns for public health, due to the close contacts of cats and dogs with humans (European Food Safety Authority *et al.*, 2023a).

The circulation of HPAIV genotypes with specific amino acid substitutions in the virus genome has been associated to increased virus replication in mammals (European Food Safety Authority *et al.*, 2023b; Vreman *et al.*, 2023). Evidence of susceptibility to infection has therefore raised concerns about the potential involvement of wild or domestic mammals in the epidemiology of avian influenza viruses (AIVs) in or near poultry farms (Root & Shriner, 2020). Given the rising number of HPAI detections in mammals, an increased passive surveillance in wild and free-roaming domestic carnivores is therefore recommended (European Food Safety Authority *et al.*, 2023b). However, also knowledge gained from a deeper understanding of the domestic bird-wildlife interface could enhance targeted mammal species selection for improved AIV epidemiological surveys, and surveillance and prevention strategies (Barasona *et al.*, 2013).

Previous research on this topic has primarily concentrated on wild bird data (Burns *et al.*, 2012; Le Gall-Ladevèze *et al.*, 2022; Veen *et al.*, 2007). Regarding mammals, the occurrence mostly of rodents and wild carnivores in poultry farms has been reported (Elbers & Gonzales, 2020; Scott *et al.*, 2018). This study aimed to characterize visits of domestic and wild mammal to three commercial poultry facilities in Northern Italy, through a year-long camera trap survey. Specifically, the research focused on identifying the species frequenting the vicinity of the poultry houses and describing their behavior and detection patterns over time.

2.2. Materials and methods

2.2.1. Camera trap survey

The data presented were gathered during a study focused on characterizing the domestic-wildlife interface. The dataset regarding mammals is the solely taken in consideration for this work, whereas wild bird visits were detailed in Chapter 1. Although the study area remained the same as in Chapter 1, there were several differences in the study design. Three commercial layer farms, located in the Bologna province, Emilia-Romagna region, namely Farm 1, Farm 2 and Farm 3 were selected after conducting preliminary on-site visits to assess the presence of wildlife, as described in Chapter 1. Seven motion-sensing infrared digital cameras (Dark-Ops Pro XD Dual Lens, Browning Trail Cameras, Utah, USA) were deployed in the three above-mentioned facilities at sites where signs of wildlife were observed. Cameras were set to operate from 6 a.m. to 6 p.m. and programmed to record 30-second-long videos after detecting movement, with a lag period of 30 seconds to avoid continuous triggers. Cameras were deployed across the three farms (Figure 1) at feed silos area (location C, D, F) and chicken manure collection point (location A, E, G). Drainage ditches were located nearby location G, within the camera's field of view. On Farm 1 and 2, the study run from January 2021 to December 2021, whereas for Farm 3 from February 2021 to November 2021. On Farm 1 an additional camera was placed near the air inlets of a poultry house (location B), facing the area adjacent to the fishing sport lake. Prior to deployment, time and date displayed on the camera traps were synchronized, and a unique code was assigned to identify each camera location ID. Throughout the study, camera traps' batteries and SD cards were replaced every three weeks. The operations performed, such as setup, battery and SD card replacements, and malfunctions, were recorded in a data sheet using Microsoft Excel 2021, version 16.49.



Figure 1. Schematic representation of Farm 1, 2 and 3. The red circles represent the camera trap locations (A to G). Positions of poultry houses, chicken manure collection areas and feed silos, and presence of waterways are shown. Satellite images modified from Google Earth Pro.

2.2.2. Data processing and analysis

This study exclusively considered videos capturing wild or domestic mammals, while wild bird visits were documented in Chapter 1. Visual analysis of the recordings was conducted using the Timelapse Image Analyzer (Greenberg & Godin, 2012) by two authors (G.G. and F.D.F). Duplicate footage, defined as recordings of the same species and number of animals within 30 minutes of the first detection, was removed from the analysis (Scott et al., 2018). A wild or domestic mammal visit to poultry farms was defined as an observation of at least one individual of a given species within a 30-minute time interval from previous observation of the same species. The 30-minute interval was introduced to avoid continuous detection of the same individual (Payne *et al.*, 2016). Clips featuring humans were removed and excluded from further analysis. Metadata extraction, data visualization and time-series analysis were performed utilizing R software version 4.0.4. (Team, 2020), according to methods described in Chapter 1. Briefly, the ‘camtrapR’ package was used for dataset exploration and computation of activity patterns (relative frequency by time) for selected wild mammals visiting the poultry farms, namely coypus (*Myocastor coypus* (MOLINA, 1782)), wild rats (*Rattus* spp.), and the European hedgehog (*Erinaceus europaeus* LINNAEUS, 1758). Daily mean detection rates (MDR) of visits were calculated based on

data collected from three farms over a year, with 95% confidence intervals (95% CI) computed using the 'poisson.test' function. Daily visit counts were transformed into a time-series object, and seasonal patterns were analyzed by examining three-month rolling averages through the 'zoo' package (version 1.8-12) (Zeileis *et al.*, 2014). Observed behaviors were categorized as per Table 1, and the occurrence percentages of each behavior concerning the total visits of a species were calculated.

Data recorded by different cameras were considered as independent. Prior to further analysis, data from each camera trap were aggregated based on date, monitored farm, season, and time of the day (Day: from 6.00 a.m. to 5:59 p.m.; Sunset: from 6 p.m. to 8:59 p.m.; Night: from 9 p.m. to 5:59 a.m) using the 'aggregate' function. Normality testing using the Shapiro-Wilk test revealed a non-normal distribution in the aggregated dataset (Shapiro & Wilk, 1965). To assess potential variations in mammal visits among farms, camera trap locations (chicken manure collection point and feed silos area), seasons, and times of the day, a Kruskal-Wallis test was employed. Generalized linear models (GLMs) utilizing a Poisson distribution, implemented via the 'MASS' package ('glm' function), were employed for further analysis of daily wild mammal visit frequencies. Domestic mammal presence on poultry farms, predominantly linked to cat colonies and local dogs, was not included in the modeling. Explanatory variables considered were the monitored farm, season (defined as spring from March 1st, summer from June 1st, autumn from September 1st, and winter from December 1st), the presence of a fishing sport lake, and the time of the day.

Table 1. Behaviors exhibited by wild or domestic mammals observed.

Activity	Description
Moving through	Passing from one side to another of the camera fields, without exhibiting other behaviors.
Observing surroundings	Explorative behavior expressed as observing surroundings.
Vocalizing	Emitting sounds to communicate with other individuals of the same species or other species.
Grooming/scratching	Applying paws to the body in repetitive movements, scratching.
Excreting	Urinating or defecating.

Foraging	Eating or drinking.
Territorial behavior	Attacking or charging an intruder of the same species or other species.

2.3. Results

2.3.1. Overview of the survey

During the study period, the camera traps were operated for a combined total of 877 trap nights on Farm 1, with a monthly camera trap effort ranging from 20 to 26 trap nights. On Farm 2, there were 532 trap nights, with a monthly camera trap effort varying from 17 to 24 trap nights. Farm 3 had 458 trap nights, with monthly camera trap effort ranging from 17 to 31 trap nights. In total, 31,774 recordings were captured, as detailed in Table 2, requiring a cumulative review time of 442 hours for individual analysis.

Table 2. Number of videos recorded by each camera trap during the study period.

Farm	Location monitored	Nights surveyed	Total number of videos recorded (N)	Wild mammals (% of N)	Domestic mammals (% of N)
1	CMan [^] (A)	309	3,863	296 (7.6)	101 (2.6)
	PHouse [§] (B)	267	3,910	11 (0.3)	125 (3.2)
	FSilo ^{&} (C)	301	3,755	72 (1.9)	256 (6.8)
2	FSilo (D)	301	5,072	53 (1.0)	28 (0.5)
	CMan (E)	231	8,551	13 (0.15)	24 (0.3)
3	FSilo (F)	211	2,363	37 (1.56)	224 (9.5)
	CMan (G)	247	4,260	85 (2.0)	1108)

[^]Chicken manure collection point.

[§]Side of the poultry house, adjacent to air inlets.

[&]Feed silos area.

Among the recorded videos, 567 (1.8%) featured wild mammals with a daily visit range of 0-31, while 1866 videos (5.8%) showed domestic mammals, with a daily visit range of 0-99. Seven wild mammal species were identified in the poultry farm areas during the study period

(Figure 2A). These were ranked in descending order of frequency: coypu (439 visits), European hedgehog (57 visits), wild rats (56 visits), European hare (*Lepus europaeus* PALLAS, 1778, 8 visits), red fox (6 visits), wood mouse (*Apodemus sylvaticus* (LINNAEUS, 1758), 5 visits) and beech marten (*Martes foina* (ERXLEBEN, 1777), 1 visit). Examining the temporal pattern of visits (Figure 2B), coypus were observed year-round, wild rats during autumn and winter, and European hedgehogs were predominantly observed from July to October. Other mammals were sporadically observed in different months. Regarding domestic mammals, cats were more frequently recorded than dogs (1815 times *versus* 51), with observations spanning the entire study year. Notably, the majority of cat sightings occurred on Farm 3, constituting 73.4% of all cat observations, attributed to the presence of a feral cat colony.

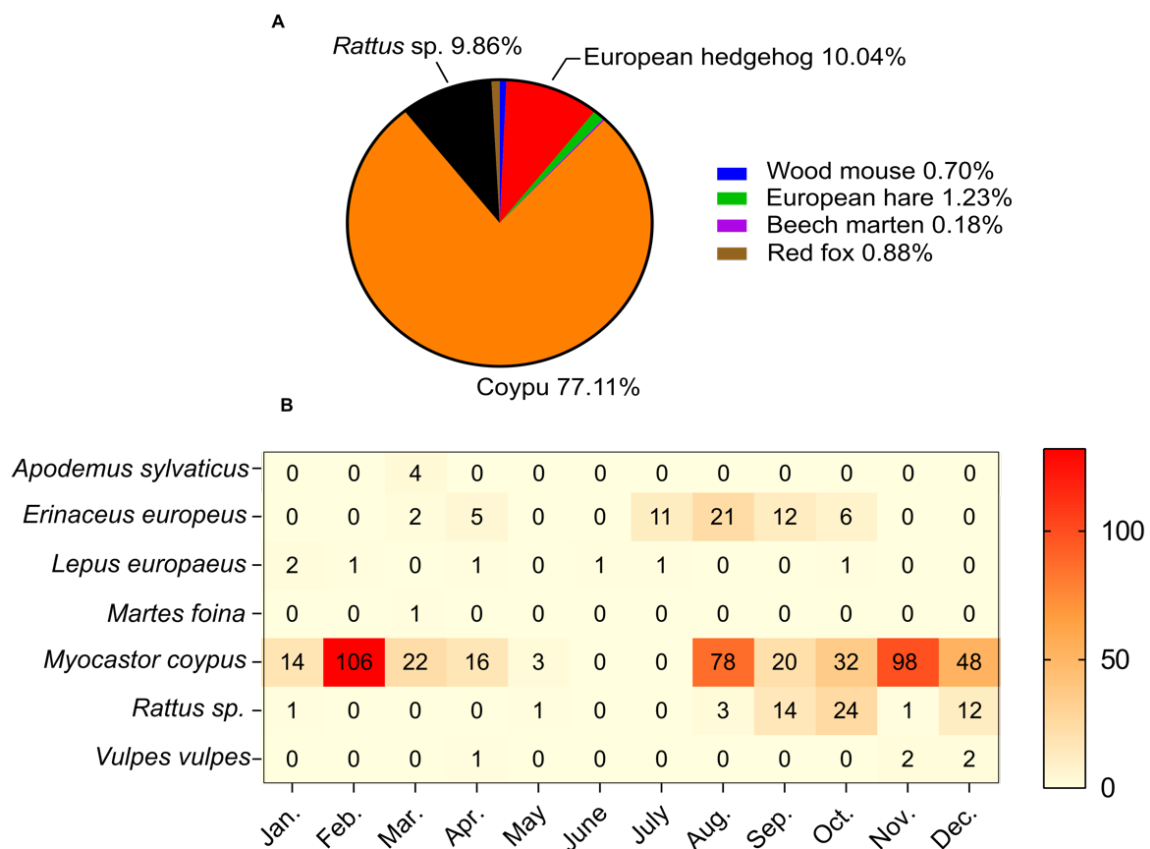


Figure 2. Wild mammals observed in the poultry farms studied. (A) Pie chart of wild mammal species; (B) number of wild mammals observed per month.

The primary behavior exhibited by wild mammals was moving through the camera's field of view, constituting an average of 88.5% of all observations. Feeding behavior was specifically noted in coypus (43% of observations), wood mice (2%), and European hedgehogs (2%) (Figure

3). Among these, coypus, being herbivores, directed their feeding behavior towards terrestrial vegetation in meadow areas within the camera's range.

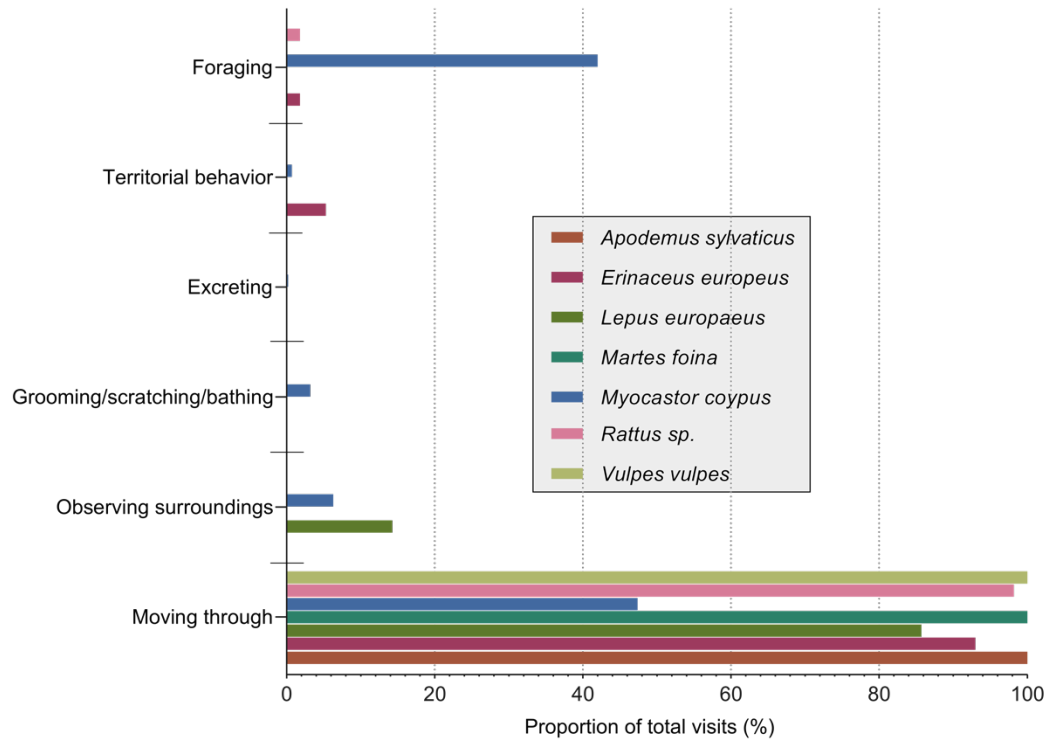


Figure 3. Behaviors displayed by wild mammals during the period of study.

2.3.2. Temporal and spatial patterns of observations

The characteristics of visits from coypus, wild rats, hedgehogs, cats, and dogs on farm facilities are summarized in **Table 3**.

Table 3. Summary of the characteristics of coypus, wild rats, hedgehogs, cats, and dogs on farm facilities.

	Coypu	Wild rats	Hedgehog	Cat	Dog
Daily mean detection rate (95% CI)	0.23 (0.21 - 0.26)	0.03 (0.02 – 0.04)	0.03 (0.02 – 0.04)	0.97 (0.93 – 1.02)	0.03 (0.02 – 0.03)
Mean number of individuals per visit (range)	1.13 (1-6)	1.05 (1-2)	1.00 (1)	1.00 (1)	1.00 (1)

Favorite season	Winter	Autumn	Summer	n.a. ¹	n.a.
Favorite site	Chicken manure collection point	Feed silos area	Chicken manure collection point	Feed silos area	Poultry house side

¹n.a. not applicable - favorite season variable was not considered for domestic mammals due to their presence on poultry farms not related to natural sources.

Wild mammals were predominantly sighted at the chicken manure collection area (location A) of Farm 1 (296 visits), followed by the chicken manure collection area (location G) of Farm 3 (85 visits) and the silos area (location C) of Farm 1 (72 visits). The silos area of Farm 3 (location G) had the highest number of visits (1108) for domestic mammals, succeeded by the silos area (location C) of Farm 2 (256 visits) and another silos area (location F) of Farm 3 (224 visits). The daily mean detection rate (MDR) of wild mammal visits across the three farms was 0.30 (95% CI: 0.28-0.33) (Table 4), while for domestic mammals, it was 0.99 (95% CI: 0.95-1.04) (Table 5). The number of mammal visits significantly varied between farms (Kruskal-Wallis $\chi^2 = 127.36$, $df = 2$, $p < 0.001$) and between cameras located at silos or chicken manure collection areas (Kruskal-Wallis $\chi^2 = 75.284$, $df = 1$, $p < 0.001$). Among the three most frequently observed wild mammal species, the visits of coypus did not differ significantly between farms but did vary significantly based on the monitored locations (Kruskal-Wallis $\chi^2 = 34.97$, $df = 1$, $p < 0.001$), seasons (Kruskal-Wallis $\chi^2 = 12.295$, $df = 3$, $p = 0.006$), and times of the day (Kruskal-Wallis $\chi^2 = 20.059$, $df = 2$, $p < 0.001$). Observations of wild rats and hedgehogs did not show significant differences regarding farms, locations, seasons, or times.

Table 4. Detection rates (95% CI) of wild mammal visits on Farm 1, 2, and 3.

Farm	Camera days	trap	Overall mean detection rate (95% CI)	Location	Mean detection rate (95% CI)
1	309		0.43 (0.39 – 0.47)	CMan [^] (A)	0.95 (0.85 – 1.07)
	267			PHouse ^s (B)	0.04 (0.02 – 0.07)
	301			FSilo ^{&} (C)	0.24 (0.19 – 0.30)
2	301		0.19 (0.16 – 0.23)	FSilo (D)	0.18 (0.13 – 0.23)
	231			CMan (E)	0.05 (0.03 – 0.97)

3	211	0.18 (0.15 – 0.23)	FSilo (F)	0.17 (0.12 – 0.24)
	247		CMan (G)	0.34 (0.27 – 0.42)

[^]Chicken manure collection point.

[§]Side of the poultry house, adjacent to air inlets.

[&]Feed silos area.

Table 5. Detection rates (95% CI) of domestic mammal visits on Farm 1, 2, and 3.

Farm	Camera days	trap	Overall mean detection rate (95% CI)	Location	Mean detection rate (95% CI)
1	309	0.55 (0.50 – 0.60)		CMan [^] (A)	0.33 (0.27 – 0.40)
	267			PHouse [§] (B)	0.47 (0.39 – 0.55)
	301			FSilo ^{&} (C)	0.85 (0.75 – 0.96)
2	301	0.09 (0.07 – 0.13)		FSilo (D)	0.09 (0.61 – 0.13)
	231			CMan (E)	0.10 (0.06 – 0.15)
3	211	2.91 (2.75 – 3.06)		FSilo (F)	1.06 (0.92 – 1.21)
	247			CMan (G)	4.48 (4.22 – 4.76)

[^]Chicken manure collection point.

[§]Side of the poultry house, adjacent to air inlets.

[&]Feed silos area.

The time-series analysis in Figure 4A demonstrates a notable increase in daily domestic mammal visits during winter, reaching its maximum in February. Conversely, the spring, summer, and autumn seasons exhibited a lower average number of detections. For wild mammals, the rolling averages of daily visits are provided only for the three most frequently observed species, constituting 97% of wild mammal observations (Figure 4B). This analysis reaffirmed the monthly patterns of visits observed in Figure 2B. Specifically, coypus displayed peak daily observations in February, August, and late October to November. Wild rats and hedgehogs were predominantly observed between July and December.

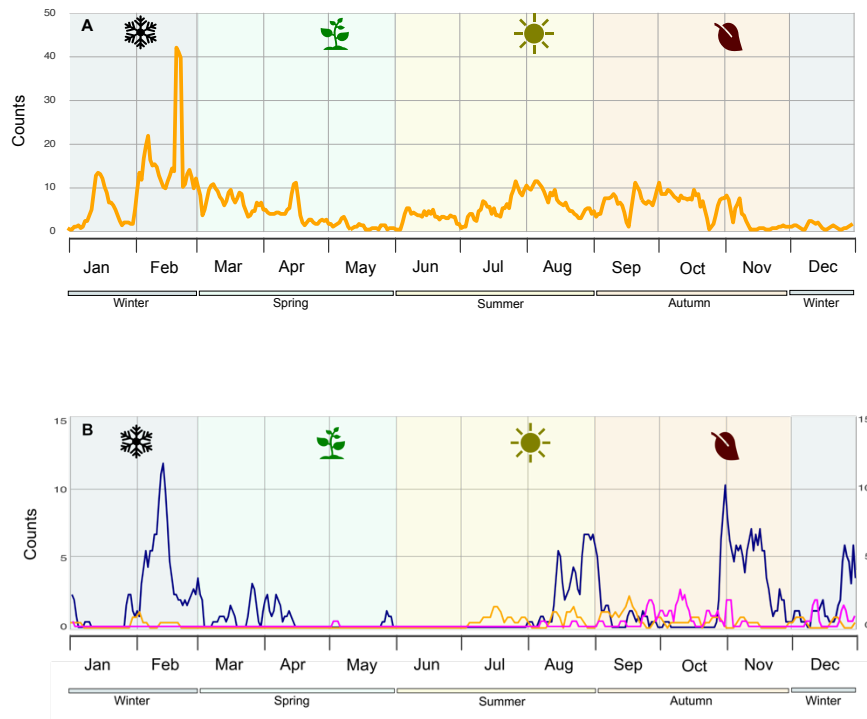


Figure 4. Temporal distribution of mammal visits across study period (camera traps at location A to G). (A) Three-month rolling average of daily counts for domestic mammals; (B) Three-month rolling average of selected wild mammals. Coypus represented by the blue line, wild rats by the pink line, and hedgehogs by the other line.

The activity patterns of coypus, European hedgehogs, and wild rats are presented in Figure 5 (5A-5C). Coypus were observed consistently throughout the day, with a slight decrease in frequency around mid-day. Hedgehogs and wild rats, being nocturnal species, were observed exclusively from 9:00 p.m. to 7:00 a.m.

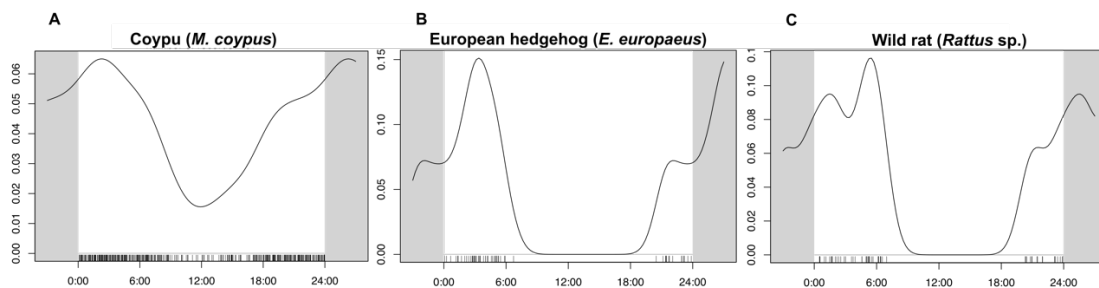


Figure 5. Activity patterns (relative frequency by time) of coypus, European hedgehogs, and wild rats.

The frequency of the wild mammal visits was statistically analyzed in relation to the monitored farm, camera trap location, season, and time of the day. The fitted model accounted for 14.1% of the data variance based, as indicated by the pseudo R^2 , and showed an overdispersion value of 0.93 (Zuur *et al.*, 2009). A summary of the results is provided in Table 6. Specifically, the feed silos area displayed a significantly lower number of wild mammal visits compared to the chicken manure collection point (RR: 0.58, 95% CI: 0.46 - 0.73, $p < 0.001$). When comparing the number of visits during spring to autumn, the observations during spring were 0.64 times lower (95% CI: 0.45 - 0.91, $p = 0.01$). Lastly, observations during nighttime were 1.35 times higher than during the daytime (95% CI: 1.04 - 1.76, $p < 0.05$).

Table 6. Generalized linear model to explain the frequency of visits of wild mammals to poultry farms. The estimates, rate ratios (exponentiated estimates) (95% CI), and the p -values of Wald test for contrasts between the reference level and the level considered are displayed.

Explanatory variable and levels		Estimate	Rate ratio (95% CI)	p -value
Farm monitored ¹	Farm 2	-0.25	0.78 (0.56 – 1.07)	0.1251
	Farm 3	-0.03	0.97 (0.73 - 1.29)	0.8320
Camera trap location ²	Feed silos area	-0.54	0.51 (0.46 - 0.73)	<0.001***
Season ³	Spring	-0.43	0.64 (0.45 - 0.91)	0.0145*
	Summer	-0.06	0.93 (0.70 – 1.25)	0.6582
	Winter	0.09	1.10 (0.86 - 1.42)	0.4473
Time of the day ⁴	Night	0.31	1.35 (1.05 - 1.76)	0.0219*
	Sunset	-0.18	0.83 (0.60 - 1.14)	0.2680

¹ Farm 1 taken as a reference.

² Chicken manure collection point as a reference.

³ Autumn taken as a reference.

⁴ Day time taken as a reference.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.4. Discussion

A year-long camera-trap study was conducted on three laying-hen farms located in a densely populated poultry area at high risk of AIV introduction. This study focused exclusively on videos capturing mammals and represents the most detailed report in this regard, following only a few publications that primarily focused on wild birds (Bestman *et al.*, 2018; Burns *et al.*, 2012; Le Gall-Ladevèze *et al.*, 2022; Scherer *et al.*, 2013). Through individual observations of clips obtained, we achieved species diversity data of both wild and domestic mammals, as well as the quantification and characterization of their visits.

A total of 567 wild mammal visits and 1,866 cat and dog visits were recorded during 1,867 camera trap nights. The results from GLM showed that the monitored farms did not influence the frequency of visits, but the location of the camera traps did affect the presence of wild animals. Specifically, the chicken manure collection point had a positive influence on the frequency of wild mammal visits. While wild bird observations within the same farms, as detailed in Chapter 1, predominantly showed the animals engaged in foraging activities around the feed silos area and chicken manure collection point, underscoring the attractiveness of the resources offered by these sites, mammals were rarely observed foraging. Therefore, wild mammal observations near the chicken manure collection point were more likely associated with the spatial use of the farm area by wild individuals. Since the chicken manure collection point was located near perimeter areas (Farm 1 and Farm 2) or close to drainage ditches (Farm 3), the animals were observed moving through the camera's field of view to further explore the farm area or shelter in the drainage ditches. Visits increased at night, primarily due to the nocturnal activity of the most frequently observed mammals, namely coypus, European hedgehogs, and wild rats. However, coypus were also observed during daylight hours (from 6:00 a.m. to 6:00 p.m.), likely due to the absence of large wild predators (Mori *et al.*, 2020) and minimal human disturbance affecting their activity. The coypu, a large semiaquatic rodent native to the subtropical regions of South America (Woods *et al.*, 1992), is now widespread in Northern and Central Italy, classified as an invasive alien species subject to population control (Cocchi & Bertolino, 2021). To the authors' knowledge, this study represents the first documented instance of coypu's activity on commercial animal farms. Coypus were observed throughout the year in all three facilities, and they were engaged in feeding behavior in 43% of the total observations. Unlike wild birds, which are attracted to the food resources offered by the farm (Scott *et al.*, 2018), coypus were only observed foraging on terrestrial vegetation in meadow areas within the camera's range. The presence of these animals was facilitated by breaches in the farms' fences, allowing individuals to enter the farm area. Implementing control measures such as reducing the coypu population in areas surrounding the poultry farms and ensuring proper maintenance of the fences by the farm staff could reduce the

risk of coypus near poultry houses. Free-ranging coypus have tested positive to various pathogens of poultry, including zoonotic agents, such as *Streptococcus equi* subspecies *zooepidemicus* (Martino *et al.*, 2014), *Chlamydia psittaci* (Howerth *et al.*, 1994; Martino *et al.*, 2014), and *Toxoplasma gondii* (Bollo *et al.*, 2003; Howerth *et al.*, 1994). With respect to AIV, a major viral threat to poultry health and welfare and of public health concern, there have been no reports of coypus being susceptible to infection. Field or experimental studies are therefore needed in this regard, considering the aquatic habits of this rodent and the potential overlap in ecological niche with AIV hosts.

Hedgehogs and wild rats were observed far less frequently than coypus, with 57 and 56 observations, respectively. Epidemiological studies on wild hedgehogs found high prevalence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium or Enteritidis (Handeland *et al.*, 2002; Lawson *et al.*, 2018), are major food-borne bacteria posing a threat to public health worldwide (EFSA Panel on Biological Hazards *et al.*, 2019). With respect to AIV, to date, hedgehogs have never been reported as being infected, and their susceptibility to the infection remains unknown. On the other hand, AIV molecular detection in wild rats has been reported in the United States (Cummings *et al.*, 2019) and China (Shao *et al.*, 2023). Serological evidence of AIV infection in rats has been found in individuals sampled on a gamebird farm which experienced an LPAI outbreak (Shriner *et al.*, 2012). Moreover, other rodent species not observed in this study, such as mice (*Mus musculus* LINNAEUS, 1758) and bank voles (*Myodes glareolus* (SCHREBER, 1780)) have been experimentally infected with non-rodent adapted LPAI and HPAI viruses that efficiently replicated in these hosts (Romero Tejeda *et al.*, 2015; Shriner *et al.*, 2012). Rats, which are highly synanthropic animals, have been abundantly detected around poultry houses during camera surveys (Elbers & Gonzales, 2020; Scott *et al.*, 2018), although surprisingly not in the study hereby presented. Given the small home range of wild rats (Davis *et al.*, 1948), they could potentially influence the local-scale AIV epidemiology by transitioning from the external environment into poultry houses and actively shed the virus. Additionally, they could act as mechanical vectors through AIV-contaminated coats (Velkers *et al.*, 2017). However, their actual role in the virus epidemiology requires further investigation. Furthermore, a recent paper has reviewed bacterial infections of poultry that can be spread through murids and included *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., *Yersinia pseudotuberculosis* and *Y. enterocolitica* (Domanska-Blicharz *et al.*, 2023). This evidence emphasizes the importance of implementing efficient on-farm rodent control strategies.

Other wild mammals observed in this study included red foxes, European hares, wood mice, and beech martens, but their visits were rare. Overall, the 0.30 (95% CI: 0.28-0.33) MDR of wild mammals suggest that their presence nearby poultry houses is limited. To further deter wild mammals from farm areas, strategies such as fencing the farm and maintaining the fences in good

condition are crucial, especially for medium-sized mammals. Implementing pest control through a combination of trapping methods and rodenticide application has been associated with a decreased risk of selecting resistant individuals, which could reproduce and replenish the population (Guidobono *et al.*, 2010). Understanding the behavioral reactions of rodents to baits is also an important element for efficient numerical control of rats and mice on the farm (Pelz & Klemann, 2004).

Results of the camera trap survey hereby presented revealed a high frequency of domestic mammal visits, with a 1.0 (95% CI: 0.95-1.04) MDR and 97% of observations of cats. Rural cats can be often found in animal farm areas (Coleman & Temple, 1993). These were mostly present on Farm 3, where a cat colony was established. Notably, H5 HPAI virus detection or serological evidence of infection have been recently reported in cats and dogs (Briand *et al.*, 2023; Domańska-Blicharz *et al.*, 2023; European Food Safety Authority *et al.*, 2023a), sometime epidemiologically linked to AI outbreaks in poultry farms (Briand *et al.*, 2023; Moreno *et al.*, 2023). The outcomes of HPAI infection in domestic pets can vary from asymptomatic (Moreno *et al.*, 2023) to fatal disease (Briand *et al.*, 2023; Canadian Food Inspection Agency, 2023; Domańska-Blicharz *et al.*, 2023; Klopfleisch *et al.*, 2007; Songserm *et al.*, 2006). Considering the high number of cat observations reported in our study and their susceptibility to HPAI infection, it is crucial to limit the presence of these mammals on poultry farms to minimize the risk of disease transmission also to humans.

To the best of our knowledge, this study represents the first instance of thoroughly characterizing mammal visits on commercial poultry farms using camera trap data (Bestman *et al.*, 2018; Burns *et al.*, 2012; Le Gall-Ladevèze *et al.*, 2022; Scherer *et al.*, 2013). The utilization of visual evidence depicting mammals' activity around poultry houses, such as the footages recorded through camera traps, could assist health authorities in educating and raising awareness of stakeholders about wildlife presence and potential pathogen spillover risks. In combination with the diligent application and enforcement of biosecurity measures, this awareness stands as one of the most effective preventive measures to prevent pathogens spillover or spillback at the interface between domestic animals, wildlife, and humans.

Regarding the limits of the study hereby presented, the monitoring covered a limited number of poultry farms and sites. While it provided essential information on the free-ranging mammals-poultry interface in a densely populated poultry area in Northern Italy, this approach potentially reduces the generalizability of the conclusions to other farm settings and locations. The deployment of cameras was carried out based on the observation of wildlife signs, aiming to maximize chances of detecting free-ranging animals in the vicinity of poultry houses. A potential selection bias could have been therefore introduced and led to overestimation of the results obtained. Additionally, camera placement in areas of high human activity (e.g., feed silos; chicken manure collection points) caused numerous non-relevant triggers, contributing to battery

depletion and camera trap failures. This reduced the camera trap effectiveness over the study period, as also reported in other similar studies (Bacigalupo *et al.*, 2022; Engeman *et al.*, 2011). Lastly, for a comprehensive understanding of the factors influencing wild mammal presence and activity on poultry farms, future research should consider additional variables such as quantitative farm-biosecurity scores, habitat characteristics and environmental factors, not included in the models presented within this study.

2.5. Conclusions

The findings presented in this study offer valuable insights into the poultry-wildlife interface. These are important prerequisites for providing scientific guidance in designing disease surveillance and intervention strategies, with the aim of preventing cross-species pathogen transmission (Hassell *et al.*, 2017). In the context of AI, the primary current threat to worldwide poultry production, studies on the susceptibility to infection with currently circulating HPAI viruses are necessary steps to fully evaluate the actual role of the observed wild and domestic mammals species as bridge hosts in the virus's epidemiology (Caron *et al.*, 2014). Lastly, the study of AIV infections in non-human mammals is critical for understanding the genetic evolution of the virus in novel hosts, and epidemiological surveys are encouraged in this regard.

2.6. References

- Agüero, M., Monne, I., Sánchez, A., Zecchin, B., Fusaro, A., Ruano, M. J., del Valle Arrojo, M., Fernández-Antonio, R., Souto, A. M., Tordable, P., Cañas, J., Bonfante, F., Giussani, E., Terregino, C., & Orejas, J. J. (2023). Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. *Euro Surveill*, 28(3), 2300001. doi:https://doi.org/10.2807/1560-7917.ES.2023.28.3.2300001
- Animal and Plant Health Inspection Service, U.S. Department of Agriculture. (2023). 2022-2023 Detections of Highly Pathogenic Avian Influenza in Mammals. Retrieved from <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-mammals>
- Bacigalupo, S. A., Dixon, L. K., Gubbins, S., Kucharski, A. J., & Drewe, J. A. (2022). Wild boar visits to commercial pig farms in southwest England: implications for disease transmission. *Eur J Wildl Res*, 68(6), 69. doi:10.1007/s10344-022-01618-2
- Barasona, J. A., VerCauteren, K. C., Saklou, N., Gortazar, C., & Vicente, J. (2013). Effectiveness of cattle operated bump gates and exclusion fences in preventing ungulate multi-host sanitary interaction. *Prev Vet Med*, 111(1-2), 42-50. doi:10.1016/j.prevetmed.2013.03.009
- Bestman, M., de Jong, W., Wagenaar, J.-P., & Weerts, T. (2018). Presence of avian influenza risk birds in and around poultry free-range areas in relation to range vegetation and openness of surrounding landscape. *Agrofor Syst*, 92(4), 1001-1008. doi:10.1007/s10457-017-0117-2
- Bevins, S., Shriner, S., Cumbee, J., Dilione, K., Douglass, K., Ellis, J., Killian, M. L., Torchetti, M., & Lenocho, J. (2022). Intercontinental Movement of Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4 Virus to the United States, 2021. *Emerg Inf Dis*, 28(5), 1006. doi:10.3201/eid2805.220318
- Bollo, E., Pregel, P., Gennero, S., Pizzoni, E., Rosati, S., Nebbia, P., & Biolatti, B. (2003). Health status of a population of nutria (*Myocastor coypus*) living in a protected area in Italy. *Res Vet Sci*, 75(1), 21-25. doi:https://doi.org/10.1016/S0034-5288(03)00035-3
- Bordes, L., Vreman, S., Heutink, R., Roose, M., Venema, S., Pritz-Verschuren, S. B. E., Rijks, J. M., Gonzales, J. L., Germeraad, E. A., Engelsma, M., & Beerens, N. (2023). Highly Pathogenic Avian Influenza H5N1 Virus Infections in Wild Red Foxes (*Vulpes vulpes*) Show Neurotropism and Adaptive Virus Mutations. *Microbiol Spectr*, 11(1), e0286722. doi:10.1128/spectrum.02867-22
- Briand, F. X., Souchaud, F., Pierre, I., Beven, V., Hirchaud, E., Hérault, F., Planel, R., Rigau, A., Bernard-Stoecklin, S., Van der Werf, S., Lina, B., Gerbier, G., Eterradossi, N., Schmitz, A., Niqueux, E., & Grasland, B. (2023). Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus in Domestic Cat, France, 2022. *Emerg Infect Dis*, 29(8), 1696-1698. doi:10.3201/eid2908.230188

- Burns, T. E., Ribble, C., Stephen, C., Kelton, D., Toews, L., Osterhold, J., & Wheeler, H. (2012). Use of observed wild bird activity on poultry farms and a literature review to target species as high priority for avian influenza testing in 2 regions of Canada. *Can Vet J*, 53(2), 158-166.
- Caliendo, V., Lewis, N. S., Pohlmann, A., Baillie, S. R., Banyard, A. C., Beer, M., Brown, I. H., Fouchier, R. A. M., Hansen, R. D. E., Lameris, T. K., Lang, A. S., Laurendeau, S., Lung, O., Robertson, G., van der Jeugd, H., Alkie, T. N., Thorup, K., van Toor, M. L., Waldenström, J., Yason, C., Kuiken, T., & Berhane, Y. (2022). Transatlantic spread of highly pathogenic avian influenza H5N1 by wild birds from Europe to North America in 2021. *Sci Rep*, 12(1), 11729. doi:10.1038/s41598-022-13447-z
- Canadian Food Inspection Agency (2023). Domestic dog tests positive for avian influenza in Canada. Retrieved from <https://www.canada.ca/en/food-inspection-agency/news/2023/04/domestic-dog-tests-positive-for-avian-influenza-in-canada.html>
- Caron, A., Grosbois, V., Etter, E., Gaidet, N., & de Garine-Wichatitsky, M. (2014). Bridge hosts for avian influenza viruses at the wildlife/domestic interface: an eco-epidemiological framework implemented in southern Africa. *Prev Vet Med*, 117(3-4), 590-600. doi:10.1016/j.prevetmed.2014.09.014
- Cocchi, R., & Bertolino, S. (2021). Piano di gestione nazionale della Nutria *Myocastor coypus*. Retrieved from https://www.mase.gov.it/sites/default/files/archivio/allegati/trasparenza_valutazione_merito/ATTIGENERALI/2021/piano_gestione_nutria_10-2021.pdf
- Coleman, J. S., & Temple, S. A. (1993). Rural Residents' Free-Ranging Domestic Cats: A Survey. *Wildl Soc Bull (1973-2006)*, 21(4), 381-390.
- Cummings, C. O., Hill, N. J., Puryear, W. B., Rogers, B., Mukherjee, J., Leibler, J. H., Rosenbaum, M. H., & Runstadler, J. A. (2019). Evidence of Influenza A in Wild Norway Rats (*Rattus norvegicus*) in Boston, Massachusetts. *Front Ecol Evol*, 7. doi:10.3389/fevo.2019.00036
- Davis, D. E., Emlen, J. T., & Stokes, A. W. (1948). Studies on Home Range in the Brown Rat1. *Journal of Mammalogy*, 29(3), 207-225. doi:10.2307/1375387
- Domanska-Blicharz, K., Opolska, J., Lisowska, A., & Szczotka-Bochniarz, A. (2023). Bacterial and Viral Rodent-borne Infections on Poultry Farms. An Attempt at a Systematic Review. *J Vet Res*, 67(1), 1-10. doi:10.2478/jvetres-2023-0012
- Domańska-Blicharz, K., Świętoń, E., Świątalska, A., Monne, I., Fusaro, A., Tarasiuk, K., Wyrostek, K., Styś-Fijoł, N., Giza, A., Pietruk, M., Zechchin, B., Pastori, A., Adaszek, Ł., Pomorska-Mól, M., Tomczyk, G., Terregino, C., & Winiarczyk, S. (2023). Outbreak of highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b virus in cats, Poland, June to July 2023. *Euro Surveill*, 28(31). doi:10.2807/1560-7917.Es.2023.28.31.2300366

- EFSA Panel on Biological Hazards, Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Dewulf, J., Hald, T., Michel, V., Niskanen, T., Ricci, A., Snary, E., Boelaert, F., Messens, W., & Davies, R. (2019). Salmonella control in poultry flocks and its public health impact. *EFSA J*, 17(2), e05596. doi:<https://doi.org/10.2903/j.efsa.2019.5596>
- Elbers, A. R. W., & Gonzales, J. L. (2020). Quantification of visits of wild fauna to a commercial free-range layer farm in the Netherlands located in an avian influenza hot-spot area assessed by video-camera monitoring. *Transbound Emerg Dis*, 67(2), 661-677. doi:10.1111/tbed.13382
- Engeman, R., Betsill, C., & Ray, T. (2011). Making Contact: Rooting Out the Potential for Exposure of Commercial Production Swine Facilities to Feral Swine in North Carolina. *EcoHealth*, 8(1), 76-81. doi:10.1007/s10393-011-0688-8
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., Staubach, C., Terregino, C., Baldinelli, F., Rusinà, A., & Kohnle, L. (2023a). Avian influenza overview June–September 2023. *EFSA J*, 21(10), e08328. doi:<https://doi.org/10.2903/j.efsa.2023.8328>
- European Food Safety Authority, European Centre for Disease Prevention Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Melidou, A., Mirinavičiūtė, G., Niqueux, É., Ståhl, K., Staubach, C., Terregino, C., Baldinelli, F., Broglia, A., & Kohnle, L. (2023b). Avian influenza overview April – June 2023. *EFSA J*, 21(7), e08191. doi:<https://doi.org/10.2903/j.efsa.2023.8191>
- Floyd, T., Banyard, A. C., Lean, F. Z. X., Byrne, A. M. P., Fullick, E., Whittard, E., Mollett, B. C., Bexton, S., Swinson, V., Macrelli, M., Lewis, N. S., Reid, S. M., Núñez, A., Duff, J. P., Hansen, R., & Brown, I. H. (2021). Encephalitis and Death in Wild Mammals at a Rehabilitation Center after Infection with Highly Pathogenic Avian Influenza A(H5N8) Virus, United Kingdom. *Emerg Infect Dis*, 27(11), 2856-2863. doi:10.3201/eid2711.211225
- Gamarra-Toledo, V., Plaza, P. I., Gutiérrez, R., Inga-Díaz, G., Saravia-Guevara, P., Pereyra-Meza, O., Coronado-Flores, E., Calderón-Cerrón, A., Quiroz-Jiménez, G., Martínez, P., Huamán-Mendoza, D., Nieto-Navarrete, J. C., Ventura, S., & Lambertucci, S. A. (2023). Mass Mortality of Marine Mammals Associated to Highly Pathogenic Influenza Virus (H5N1) in South America. *bioRxiv*, 2023.2002.2008.527769. doi:10.1101/2023.02.08.527769
- Greenberg, S., & Godin, T. (2012). Timelapse Image Analysis Manual. Technical Report 2012-1028-11.

- doi:<https://grouplab.cpsc.ucalgary.ca/grouplab/uploads/Publications/Publications/2012-TimelapseImageAnalysis.Report2012-1028-11.pdf>
- Guidobono, J. S., León, V., Gómez Villafañe, I. E., & Busch, M. (2010). Bromadiolone susceptibility in wild and laboratory *Mus musculus* L.(house mice) in Buenos Aires, Argentina. *Pest Manag Sci*, 66(2), 162-167. doi:10.1002/ps.1850
- Handeland, K., Refsum, T., Johansen, B. S., Holstad, G., Knutsen, G., Solberg, I., Schulze, J., & Kapperud, G. (2002). Prevalence of *Salmonella* typhimurium infection in Norwegian hedgehog populations associated with two human disease outbreaks. *Epidemiol Infect*, 128(3), 523-527. doi:10.1017/s0950268802007021
- Hassell, J. M., Begon, M., Ward, M. J., & Fevre, E. M. (2017). Urbanization and Disease Emergence: Dynamics at the Wildlife-Livestock-Human Interface. *Trends Ecol Evol*, 32(1), 55-67. doi:10.1016/j.tree.2016.09.012
- Howerth, E. W., Reeves, A. J., McElveen, M. R., & Austin, F. W. (1994). Survey for selected diseases in nutria (*Myocastor coypus*) from Louisiana. *J Wildl Dis*, 30(3), 450-453. doi:10.7589/0090-3558-30.3.450
- Kleyheeg, E., Slaterus, R., Bodewes, R., Rijks, J. M., Spierenburg, M. A. H., Beerens, N., Kelder, L., Poen, M. J., Stegeman, J. A., Fouchier, R. A. M., Kuiken, T., & van der Jeugd, H. P. (2017). Deaths among Wild Birds during Highly Pathogenic Avian Influenza A(H5N8) Virus Outbreak, the Netherlands. *Emerg Infect Dis*, 23(12), 2050-2054. doi:10.3201/eid2312.171086
- Klopfleisch, R., Wolf, P. U., Uhl, W., Gerst, S., Harder, T., Starick, E., Vahlenkamp, T. W., Mettenleiter, T. C., & Teifke, J. P. (2007). Distribution of Lesions and Antigen of Highly Pathogenic Avian Influenza Virus A/Swan/Germany/R65/06 (H5N1) in Domestic Cats after Presumptive Infection by Wild Birds. *Vet Pathol*, 44(3), 261-268. doi:10.1354/vp.44-3-261
- Koethe, S., Ulrich, L., Ulrich, R., Amler, S., Graaf, A., Harder, T. C., Grund, C., Mettenleiter, T. C., Conraths, F. J., Beer, M., & Globig, A. (2020). Modulation of lethal HPAIV H5N8 clade 2.3.4.4B infection in AIV pre-exposed mallards. *Emerg Microbes Infect*, 9(1), 180-193. doi:10.1080/22221751.2020.1713706
- Lawson, B., Franklinos, L. H. V., Rodriguez-Ramos Fernandez, J., Wend-Hansen, C., Nair, S., Macgregor, S. K., John, S. K., Pizzi, R., Núñez, A., Ashton, P. M., Cunningham, A. A., & E, M. d. P. (2018). Salmonella Enteritidis ST183: emerging and endemic biotypes affecting western European hedgehogs (*Erinaceus europaeus*) and people in Great Britain. *Sci Rep*, 8(1), 2449. doi:10.1038/s41598-017-18667-2
- Le Gall-Ladevèze, C., Guinat, C., Fievet, P., Vollot, B., Guérin, J. L., Cappelletti, J., & Le Loc'h, G. (2022). Quantification and characterisation of commensal wild birds and their interactions with domestic ducks on a free-range farm in southwest France. *Sci Rep*, 12(1), 9764. doi:10.1038/s41598-022-13846-2

- Lindh, E., Lounela, H., Ikonen, N., Kantala, T., Savolainen-Kopra, C., Kauppinen, A., Österlund, P., Kareinen, L., Katz, A., Nokireki, T., Jalava, J., London, L., Pitkäpaasi, M., Vuolle, J., Punto-Luoma, A. L., Kaarto, R., Voutilainen, L., Holopainen, R., Kalin-Mänttari, L., Laaksonen, T., Kiviranta, H., Pennanen, A., Helve, O., Laamanen, I., Melin, M., Tammiranta, N., Rimhanen-Finne, R., Gadd, T., & Salminen, M. (2023). Highly pathogenic avian influenza A(H5N1) virus infection on multiple fur farms in the South and Central Ostrobothnia regions of Finland, July 2023. *Euro Surveill*, 28(31). doi:10.2807/1560-7917.Es.2023.28.31.2300400
- Mariana, L., Alejandra, G.-G., Breno, M.-S., Diana, J., Patricia, B., Carlos, C.-M., Javier, J., Walter, S., Karl, P., Lady, A., Paulo, C.-C., Marcela, M. U., Martha, I. N., & Jesus, L. (2023). Highly pathogenic avian influenza A (H5N1) in marine mammals and seabirds in Peru. *bioRxiv*, 2023.2003.2003.531008. doi:10.1101/2023.03.03.531008
- Martino, P. E., Stanchi, N. O., Silvestrini, M., Brihuega, B., Samartino, L., & Parrado, E. (2014). Seroprevalence for selected pathogens of zoonotic importance in wild nutria (*Myocastor coypus*). *Eur J Wildl Res*, 60(3), 551-554. doi:10.1007/s10344-014-0805-4
- Moreno, A., Bonfante, F., Bortolami, A., Cassaniti, I., Caruana, A., Cottini, V., Cereda, D., Farioli, M., Fusaro, A., Lavazza, A., Lecchini, P., Lelli, D., Maroni Ponti, A., Nassuato, C., Pastori, A., Rovida, F., Ruocco, L., Sordilli, M., Baldanti, F., & Terregino, C. (2023). Asymptomatic infection with clade 2.3.4.4b highly pathogenic avian influenza A(H5N1) in carnivore pets, Italy, April 2023. *Euro Surveill*, 28(35). doi:10.2807/1560-7917.Es.2023.28.35.2300441
- Mori, E., Andreoni, A., Cecere, F., Magi, M., & Lazzeri, L. (2020). Patterns of activity rhythms of invasive coypus *Myocastor coypus* inferred through camera-trapping. *Mamm Biol*, 100(6), 591-599. doi:10.1007/s42991-020-00052-8
- Murawski, A., Fabrizio, T., Ossiboff, R., Kackos, C., Jeevan, T., Jones, J., Kandeil, A., Walker, D., Turner, J., Patton, C., Govorkova, E., Hauck, H., Mickey, S., Barbeau, B., Bommineni, R., Torchetti, M., Lantz, K., Kercher, L., Allison, A., Vogel, P., Walsh, M., & Webby, R. (2023). Highly pathogenic avian influenza A(H5N1) virus in a common bottlenose dolphin (*Tursiops truncatus*) in Florida. In: Research Square.
- Payne, A., Chappa, S., Hars, J., Dufour, B., & Gilot-Fromont, E. (2016). Wildlife visits to farm facilities assessed by camera traps in a bovine tuberculosis-infected area in France. *European Journal of Wildlife Research*, 62(1), 33-42. doi:10.1007/s10344-015-0970-0
- Pelz, H. J., & Klemann, N. (2004). Rat control strategies in organic pig and poultry production with special reference to rodenticide resistance and feeding behaviour. *NJAS – WAGEN J LIFE SC*, 52(2), 173-184. doi:https://doi.org/10.1016/S1573-5214(04)80012-5
- Puryear, W., Sawatzki, K., Hill, N., Foss, A., Stone, J., Doughty, L., Walk, D., Gilbert, K., Murray, M., Cox, E., Patel, P., Mertz, Z., Ellis, S., Taylor, J., Fauquier, D., Smith, A., DiGiovanni, R., van de Guchte, A., Gonzalez-Reiche, A. S., Khalil, Z., van Bakel, H.,

- Torchetti, M., Lantz, K., Lenocho, J., & Runstadler, J. (2023). Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerg Infect Dis*, 29(4), 786. doi:10.3201/eid2904.221538
- Rijks, J. M., Leopold, M. F., Kühn, S., In 't Veld, R., Schenk, F., Brenninkmeijer, A., Lilipaly, S. J., Ballmann, M. Z., Kelder, L., de Jong, J. W., Courtens, W., Slaterus, R., Kleyheeg, E., Vreman, S., Kik, M. J. L., Gröne, A., Fouchier, R. A. M., Engelsma, M., de Jong, M. C. M., Kuiken, T., & Beerens, N. (2022). Mass Mortality Caused by Highly Pathogenic Influenza A(H5N1) Virus in Sandwich Terns, the Netherlands, 2022. *Emerg Infect Dis*, 28(12), 2538-2542. doi:10.3201/eid2812.221292
- Romero Tejeda, A., Aiello, R., Salomoni, A., Berton, V., Vascellari, M., & Cattoli, G. (2015). Susceptibility to and transmission of H5N1 and H7N1 highly pathogenic avian influenza viruses in bank voles (*Myodes glareolus*). *Vet Res*, 46(1), 51. doi:10.1186/s13567-015-0184-1
- Root, J., & Shriner, S. (2020). Avian Influenza A Virus Associations in Wild, Terrestrial Mammals: A Review of Potential Synanthropic Vectors to Poultry Facilities. *Viruses*, 12(12). doi:10.3390/v12121352
- Scherer, A. L., Martins Scherer, J. d. F., Petry, M. V., & Sander, M. (2013). *Occurrence and interaction of wild birds at poultry houses in southern Brazil* (Vol. 19).
- Scott, A. B., Phalen, D., Hernandez-Jover, M., Singh, M., Groves, P., & Toribio, J. (2018). Wildlife Presence and Interactions with Chickens on Australian Commercial Chicken Farms Assessed by Camera Traps. *Avian Dis*, 62(1), 65-72. doi:10.1637/11761-101917-Reg.1
- Shao, J. W., Zhang, X. L., Sun, J., Liu, H., & Chen, J. M. (2023). Infection of wild rats with H5N6 subtype highly pathogenic avian influenza virus in China. *J Infect*, 86(5), e117-e119. doi:10.1016/j.jinf.2023.03.007
- Shapiro, S. S., & Wilk, M. B. (1965). An Analysis of Variance Test for Normality (Complete Samples). *Biometrika*, 52(3/4), 591-611. doi:10.2307/2333709
- Shin, D. L., Siebert, U., Lakemeyer, J., Grilo, M., Pawliczka, I., Wu, N. H., Valentin-Weigand, P., Haas, L., & Herrler, G. (2019). Highly Pathogenic Avian Influenza A(H5N8) Virus in Gray Seals, Baltic Sea. *Emerg Infect Dis*, 25(12), 2295-2298. doi:10.3201/eid2512.181472
- Shriner, S. A., VanDalen, K. K., Mooers, N. L., Ellis, J. W., Sullivan, H. J., Root, J. J., Pelzel, A. M., & Franklin, A. B. (2012). Low-pathogenic avian influenza viruses in wild house mice. *PLoS One*, 7(6), e39206. doi:10.1371/journal.pone.0039206
- Soda, K., Usui, T., Yamaguchi, T., & Ito, T. (2023). Multiple infections with H5N8 subtype high pathogenicity avian influenza viruses in a feral mallard. *J Vet Med Sci*, 85(8), 849-852. doi:10.1292/jvms.23-0124

- Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., & Poovorawan, Y. (2006). Avian Influenza H5N1 in Naturally Infected Domestic Cat. *Emerg Inf Dis*, 12(4), 681. doi:10.3201/eid1204.051396
- Tammiranta, N., Isomursu, M., Fusaro, A., Nylund, M., Nokireki, T., Giussani, E., Zecchin, B., Terregino, C., & Gadd, T. (2023). Highly pathogenic avian influenza A (H5N1) virus infections in wild carnivores connected to mass mortalities of pheasants in Finland. *Infect Genet Evol*, 111, 105423. doi:10.1016/j.meegid.2023.105423
- Team, R. C. (2020). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>
- Thorsson, E., Zohari, S., Roos, A., Banihashem, F., Bröjer, C., & Neimanis, A. (2023). Highly Pathogenic Avian Influenza A(H5N1) Virus in a Harbor Porpoise, Sweden. *Emerg Infect Dis*, 29(4), 852-855. doi:10.3201/eid2904.221426
- Veen, J., Brouwer, J., Atkinson, P., Bilgin, C., Blew, J., Eksioglu, S., Hoffmann, M., Nardelli, R., Spina, F., Tendi, C., & Delany, S. (2007). Ornithological data relevant to the spread of Avian Influenza in Europe (phase 2): further identification and first field assessment of Higher Risk Species. *Wetlands International, Wageningen, The Netherlands*.
- Velkers, F. C., Blokhuis, S. J., Veldhuis Kroeze, E. J. B., & Burt, S. A. (2017). The role of rodents in avian influenza outbreaks in poultry farms: a review. *Vet Q*, 37(1), 182-194. doi:10.1080/01652176.2017.1325537
- Vreman, S., Kik, M., Germeraad, E., Heutink, R., Harders, F., Spierenburg, M., Engelsma, M., Rijks, J., van den Brand, J., & Beerens, N. (2023). Zoonotic Mutation of Highly Pathogenic Avian Influenza H5N1 Virus Identified in the Brain of Multiple Wild Carnivore Species. *Pathogens*, 12(2). doi:10.3390/pathogens12020168
- Woods, C. A., Contreras, L., Willner-Chapman, G., & Whidden, H. P. (1992). *Myocastor coypus*. *Mamm Species* 398, 1-8.
- Zeileis, A., Grothendieck, G., Ryan, J. A., Andrews, F., & Zeileis, M. A. (2014). Package ‘zoo’. *R package version*, 1.7-12.
- Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer

III. A review of infections with highly pathogenic avian influenza virus of clade 2.3.4.4b in wild birds and mammals

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 highly pathogenic avian influenza viruses (HPAIV) H5 of clade 2.3.4.4b have undergone unprecedented reassortment of internal genes 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 low pathogenic AIVs, are frequently found infected with HPAIVs of clade 2.3.4.4b, which can also cause high morbidity and mortality. The sustained circulation of these HPAI viruses in waterfowl has also led to virus infection in other wild bird species, with implications for the conservation of endangered species. Furthermore, due to critical mutations related to virus adaptation to mammalian species, HPAIVs of clade 2.3.4.4b have been isolated in various wild and domestic mammals worldwide, raising concerns about virus spillover to humans. The main clinical signs and gross and histopathological findings associated to infection with HPAIVs of clade 2.3.4.4b in wild birds and non-human mammals are hereby summarized.

3.1. Introduction

Avian influenza viruses (AIVs), genus *Alphainfluenzavirus*, family *Orthomyxoviridae* (Schoch *et al.*, 2020), are highly contagious respiratory viruses of birds, responsible of high morbidity and mortality worldwide. AIVs are single-stranded, negative-sense, segmented and enveloped RNA viruses which include eight gene segments coding for 11+ structural and non-structural/regulatory proteins (Hutchinson *et al.*, 2014; McCrone *et al.*, 2018; Muramoto *et al.*, 2013). The polymerase complex, composed of the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA), 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 in the envelope. The antigenic specificities of HA and NA proteins determine AIV classification into subtypes (Lamb & Krug, 2001). A total of 18 HA (H1-H18) and 11 NA (N1-N11) are recognized (Alexander, 2007a; Tong *et al.*, 2013; Wu *et al.*, 2014b), and all but the bats-related H17N10 and the H18N11 are found in avian species. Based on the disease severity that AIVs can cause in chickens, they are classified as either low or highly pathogenic (LPAI or HPAI). While these traits are likely polygenic, modifications to the HA protein are crucial, as it mediates the initiation of the virus cycle and determines the virus host range (Swayne, 2007). The HA is a homotrimer whose globular domain contains a receptor binding site (RBS) that recognizes avian-like $\alpha 2,3$ - and/or human-like $\alpha 2,6$ -linked sialylated glycans of the host's cell, playing a pivotal role in host's cell receptor binding and entry (Long *et al.*, 2019). Upon endocytic uptake, HA triggers fusion between the viral envelope and the endosomal membrane and the virus RNA is released into the cytoplasm (Steinhauer, 1999). 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 the above-mentioned membrane fusion (Steinhauer, 1999; Wiley & Skehel, 1987; Wilson *et al.*, 1981). Based on the sequence of the CS, different enzymes can mediate the HA cleavage and affects the viral virulence in chickens and other gallinaceous birds (Swayne, 2009). For most AIVs, the CS contains a single arginine residue (monobasic CS) and is cleaved extracellularly by trypsin or trypsin-like proteases

(plasmin, blood clotting factor-like proteases, tryptase Clara, bacterial proteases), only secreted in lung epithelium (Chen *et al.*, 1998) and intestinal tract of birds (Gotoh *et al.*, 1990), therefore mediating localized infections and hence milder disease (Klenk & Garten, 1994). However, when the CS is longer and contains multiple basic amino acids (polybasic CS), it is cleaved by a family of subtilisin-like proteases (Stieneke-Gröber *et al.*, 1992), which are ubiquitously expressed and allow virus replication in different cell types, causing an often-fatal systemic disease (Hulse *et al.*, 2004; Swayne & Pantin-Jackwood, 2006). The HA protein also contains the major antigenic determinants targeted by the humoral host's immune response, which also recognizes, to a lesser extent, the antigenic sites on the NA (Cattoli *et al.*, 2011; Johansson *et al.*, 1998; Marcelin *et al.*, 2012; Suarez & Schultz-Cherry, 2000; Wilson & Cox, 1990). These antigenic regions on the HA, however, can undergo frequent amino acid changes due to a selective antibody pressure from the host (Plotkin & Dushoff, 2003). When nucleotide substitutions accumulate in a process called *antigenic drift*, these can impair the effective neutralization of the virus by host antibodies (Bouvier & Palese, 2008). Genetic variation, according to the viral protein considered, can result in changes in viral properties such as receptor binding, replicative capacity, and transmission (Suttie *et al.*, 2019). Being AIVs characterized by a segment genome, this enables the so-called *antigenic shift*, a phenomenon that occurs through reassortment events between two or more viruses that infect the same cell. This has paved the way to pandemic influenza in humans (Pappas *et al.*, 2008; Webster & Govorkova, 2014). Considering poultry, reassortment events between different AIVs has contributed to the emergence of several novel emerging zoonotic subtypes with internal gene cassettes from other circulating subtypes (Carnaccini & Perez, 2020).

Wild waterfowl (Anseriformes, Charadriiformes) are considered the natural reservoir of all 16 HA (H1-16) and 9 NA (N1-9) subtype combinations, and they carry AIVs mostly sub-clinically (Alexander, 2000). Spillover events of AIVs from wild birds to domestic poultry typically produce a variety of outcomes, from asymptomatic infection to respiratory disease, diarrhea and drop in egg production. On occasion, the recirculation of LPAI H5 and H7 virus subtypes in poultry lead to virus evolution and increased disease severity with up to 100% fatality (HPAI). Cases associated with increased pathogenicity of AIVs are restricted to the H5 and H7 subtypes, which are reportable diseases to the World Organisation for Animal Health (WOAH) (Swayne & Suarez,

2000; World Organisation for Animal Health, 2023; World Organization for Animal Health, 2002). Eurasian H5 HPAIVs are a serious concern for the WOA, since these viruses are zoonotic, potentially pandemic, and manifest increased host range and tissue tropism. These are considered 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 (Guo *et al.*, 1998; Xu *et al.*, 1999). 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 (Lee *et al.*, 2017a; Marandino *et al.*, 2023). Certain strains have also been reported to cause severe systemic infections and high mortality rates among waterfowl (Pantin-Jackwood & Swayne, 2007).

3.2. H5 Genetic Evolution and Epidemiology

In recent years, due to the persistent circulation of HPAI viruses in poultry in Asia, viruses have 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 (Lee *et al.*, 2021). This has led the HPAI H5Nx viruses of the Gs/Gd lineage 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 (Smith & Donis, 2015; World Health Organization, 2020). 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 (Kuiken *et al.*, 2023; Lee *et al.*, 2017a). Initially isolated from a Chinese live poultry market in 2010 (Zhao *et al.*, 2013), a subtype H5N8 virus of clade 2.3.4.4 caused multiple outbreaks in poultry and wild birds in South Korea (Lee *et al.*, 2014). 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 (European Food Safety Authority *et al.*, 2023a). The first intercontinental spread of the H5 clade 2.3.4.4 to North America dates back to 2014, with an H5N8 HPAI virus introduced most likely via migratory birds through the Bering Strait to breeding grounds in Alaska (Lee *et al.*, 2015). After reassorting with LPAI gene segments from North American wild birds (Lee *et al.*, 2016), an H5N2 HPAI reassortant virus of clade 2.3.4.4c became predominant and affected over 50 million birds in the U.S. in spring 2015 (Lee *et al.*, 2018). After sporadic detections in wild birds, the H5 clade 2.3.4.4c viruses in North America were lastly isolated in late 2016 (United States Department of Agriculture & USGS, 2017). Since their first detection in domestic ducks in Eastern China (2013) and South Korea (2014) (Lee *et al.*, 2014; Wu *et al.*, 2014a), 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 (Lee *et al.*, 2017a; Lee *et al.*, 2017b; Li *et al.*, 2017). In Europe, 1,207 H5Nx HPAI poultry outbreaks and 1,590 wild bird mortality events were reported during the 2016/2017 virus epidemic, mostly due to clade 2.3.4.4b (European Food Safety Authority *et al.*, 2017; Lycett *et al.*, 2020). Sporadic outbreaks of H5N6 or H5N8 HPAI 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 (European Food Safety Authority *et al.*, 2021b; European Food Safety Authority *et al.*, 2018a; European Food Safety Authority *et al.*, 2018b; European Food Safety Authority *et al.*, 2020). Since fall 2020, a surge in H5Nx HPAI cases due to 2.3.4.4b viruses has been observed across the Eurasian continent, with an unprecedented number of outbreaks in poultry and cases in wild birds continuing to be reported (European Food Safety Authority *et al.*, 2021d). 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 2020-2021 (European Food Safety Authority *et al.*, 2022c). At least 20 different H5 clade 2.3.4.4b genotypes were characterized in Europe, originating from multiple inter- and intra-subtype reassortment events (Adlhoch *et al.*, 2022), and three of these, namely the AB (A/duck/Saratov/29-02/2021-like (H5N1)), CH (A/Mallard/Netherlands/18/2022-like (H5N1)) and BB (A/Herring gull_France/22P015977/2022-like (H5N1)) have then become predominantly isolated (European

Food Safety Authority *et al.*, 2023c). The transatlantic spread to North America of Eurasian-origin viruses has 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* LINNAEUS, 1758). 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 (Bevins *et al.*, 2022; Caliendo *et al.*, 2022d), possibly via Iceland (Günther *et al.*, 2022). A further intercontinental virus spread to North America then occurred via the Pacific and the North Atlantic-linked migratory flyways later on in 2022 (Alkie *et al.*, 2022). 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 (Youk *et al.*, 2023b). 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 (Animal and Plant Health Inspection Service) and the detection of the virus in 7,152 wild birds (Animal and Plant Health Inspection Service). The subsequent spread to South America (Mariana *et al.*, 2023) has solidified the global dominance of HPAI H5 viruses clade 2.3.4.4b (Figure 1). With respect to other 2.3.4.4 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 (Bi *et al.*, 2016; Kwon *et al.*, 2017; Takemae *et al.*, 2017). Clade 2.3.4.4a H5N6 virus has only been detected in Asia, where it circulates enzootically since 2013, and distant geographical spread to other regions has not been reported yet (Bi *et al.*, 2016).

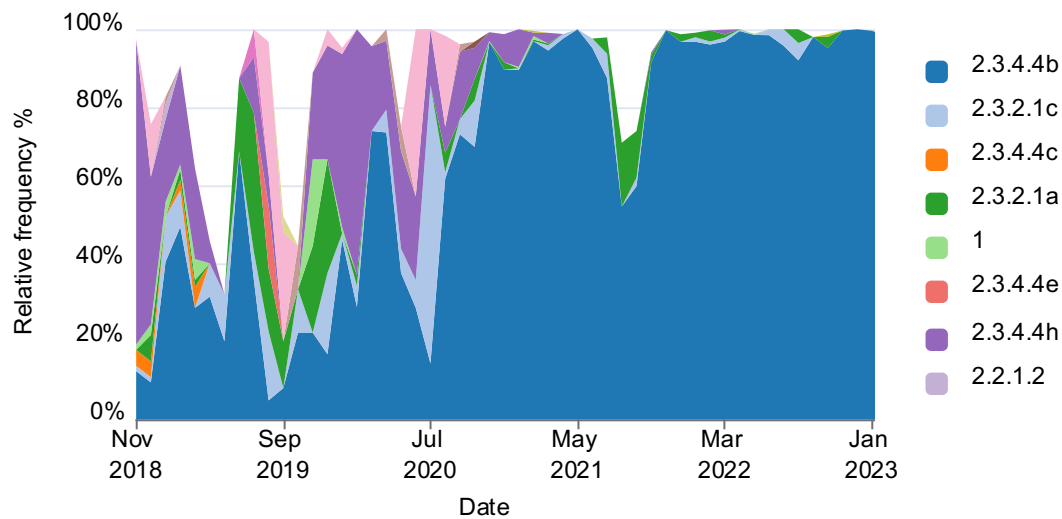


Figure 1. Relative frequency (%) over time of highly pathogenic avian influenza H5 Gs/Gd virus clades global isolation from November 2018 to September 2023. From GISAID's EpiFlu Database (Elbe & Buckland-Merrett, 2017).

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 (Koethe *et al.*, 2020; Leyson *et al.*, 2019), considered as the AIV natural hosts, accompanied by asymptomatic infections in certain dabbling ducks (Gobbo *et al.*, 2021; Kim *et al.*, 2014; Tarasiuk *et al.*, 2023). Frequent die-offs have been observed, particularly in colony-breeding seabirds (European Food Safety Authority *et al.*, 2021a; European Food Safety Authority *et al.*, 2022a; European Food Safety Authority *et al.*, 2023b; Kleyheeg *et al.*, 2017; Pohlmann *et al.*, 2023), which have been attributed to a shift in the HPAI virus epidemiology among waterbirds, resulting in endemic circulation of these strains (Bodewes & Kuiken, 2018; Pohlmann *et al.*, 2022). Due to its sustained circulation in the free-ranging avifauna, H5N1 HPAI is therefore considered as a new threat to wild bird conservation (Co-Convened Scientific Task Force on Avian Influenza and Wild Birds, 2023). Globally threatened species, as defined by the IUCN Red List version 2022-2 (IUCN, 2023), have been already decimated due to the infection, such as the above-mentioned seabirds (Gamarra-Toledo *et al.*, 2023a), cranes (the hooded cranes, *Grus monacha* TEMMINCK, 1835; the white-naped crane, *Antigone vipio* (PALLAS, 1811); and red-crowned crane, *Grus japonensis* (STATIUS MÜLLER, 1776)), Dalmatian pelicans (*Pelicanus crispus* BRUCH, 1832), Bald eagles (*Haliaeetus leucocephalus* (LINNAEUS, 1766)) and California condors (*Gymnogyps californianus* (SHAW, 1797)) (Alexandrou *et al.*, 2022; Cunningham *et al.*, 2022; EAAFP, 2022; United States Fish

& Wildlife Service, 2023). With respect to California condors, given the impact of H5N1 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 (Animal and Plant Health Inspection Service, 2023). Detection of an 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 mendiculus*) (Stokstad, 2023). 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 (Gobbo *et al.*, 2022; Song *et al.*, 2015), with transmissibility rates varying within infected flocks (Kwon *et al.*, 2023; Takadate *et al.*, 2023). 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 (European Food Safety Authority *et al.*, 2022b; European Food Safety Authority *et al.*, 2023b; European Food Safety Authority *et al.*, 2021c; European Food Safety Authority *et al.*, 2021d), raising concerns for public health due to the identification molecular markers of virus adaptation to mammalian hosts (European Food Safety Authority *et al.*, 2023d; Vreman *et al.*, 2023).

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 the 9th of August 2023. 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’.

3.3. Wild aquatic birds

3.3.1. 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.

Table 1. Experimental infections of highly pathogenic avian influenza (HPAI) clade 2.3.4.4b in wild waterbird species included in this review. Symptomatic (S) or asymptomatic (A) infection; death time (DT) in days-post inoculation.

Ref.	Species (age)	Virus	Route of infection (dose)	Morbidity* (%)	Mortality (%)	S or A	DT	Transmission
(Leyson <i>et al.</i> , 2019)	Mallard (2 wk)	A/Tufted- duck/Denmark/11470/LW PL/2016 (H5N8)	IC [^] (10 ³ EID ₅₀ /mL)	0/5 (0)	0/5 (0)	n.a.	n.a.	No
			IC (10 ⁵ EID ₅₀ /mL)	5/5	4/5 (80)	S	5.8	Yes
			IC (10 ⁷ EID ₅₀ /mL)	5/5	4/5(80)	S	3.2	Yes
(Tarasiuk <i>et al.</i> , 2023)	Mallard (6 wk)	A/herring gull/Poland/MB082B/2016 (H5N8)	IO ^{&} and IN [§] (10 ⁶ EID ₅₀ /mL)	12/12 (100)	7/12 (58)	S	3-14	n.d.
(Tanikawa <i>et al.</i> , 2022) ^o	Mallard (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.d.
(Caliendo <i>et al.</i> , 2022c)	Mallard (>9mo)	A/EurasianWigeon/NL/4/2 016 (H5N8)**	IC (10 ⁷ TCID ₅₀ /mL)	7/7 (100)	0/7 (0)	A	n.a.	n.d.

	Tufted duck	A/Eurasian Wigeon/NL/4/	IC	7/7 (100)	1/7 (14)	S	4	n.d.
	(>9mo)	2016 (H5N8)**	(10 ⁷ TCID ₅₀ /mL)					
(Beerens <i>et al.</i> , 2021)	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.d.
		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.d.
(Tanikawa <i>et al.</i> , 2021)	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.d.
(Tarasiuk <i>et al.</i> , 2022)	Herring gull (8 wk)	A/herring gull/Poland/MB082B/2016 (H5N8)	IO and IN (10 ⁷ EID ₅₀ /mL)	12/12	11/12 (90)	S	1-6	Yes

[^]IC, intrachoanal route; [§]IN, intranasal route; ^{IO}, intraocular route; [†]IT, intratracheal route; n.a., not applicable; n.d., not defined.

*Infected individuals based on RT-PCR or virus isolation or HI assay.

[°]Partial data included.

**Data from subsequent challenge following pre-exposure to LPAIV or HPAIV were excluded.

Dabbling ducks (Anatidae, Anatinae subfamily) have conventionally been regarded as more resistant to overt HPAI clinical disease (Campbell & Magor, 2020; Kim *et al.*, 2009). Notably, a sustained viral circulation in these species has been detected by surveillance efforts (European Food Safety Authority *et al.*, 2022b; European Food Safety Authority *et al.*, 2021b). Among these, the mallard (*Anas platyrhynchos* LINNAEUS, 1758) stands out as the most abundant and widely distributed duck species, serving as a crucial reservoir for LPAIVs (Webster *et al.*, 1992). Mallards are generally considered as more resistant to chickens to HPAI infection, through RIG-I receptor-mediated IFN- β signaling in infected cells and the rapid induction of ISGs that critically limit HPAI virus spread and viremia (Barber *et al.*, 2010; Evseev & Magor, 2019). 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 (Tanikawa *et al.*, 2022). However, when considering experimental infections with H5Nx HPAI 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 clade 2.3.4.4c (Beerens *et al.*, 2021; Grund *et al.*, 2018). 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 (Kang *et al.*, 2013; Uchida *et al.*, 2019). 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 (Leyson *et al.*, 2021). 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, synspecific equivalent to mallards (Appleby *et al.*, 2004). 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 (Beerens *et al.*, 2021; Leyson *et al.*, 2019; Slomka *et al.*, 2019; Tarasiuk *et al.*, 2023). A notable weight loss has been associated to intratracheal experimental

inoculation of mallards with 10^7 TCID₅₀/mL of A /Eurasian Wigeon/NL/4/2016 (H5N8) or 10^7 EID₅₀/mL of A/Tufted-duck/Denmark/11470/LWPL/2016 (H5N8), compared to sham-inoculated control groups (Caliendo *et al.*, 2022c; Leyson *et al.*, 2019), with a dose-dependent effect (Leyson *et al.*, 2019). 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 (Caliendo *et al.*, 2022c; Grund *et al.*, 2018; Leyson *et al.*, 2019; Tarasiuk *et al.*, 2023). Microscopic lesions associated with the infection differed according to the day of sampling (Tarasiuk *et al.*, 2023). 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 (Tarasiuk *et al.*, 2023). Virus shedding was predominantly linked to the respiratory route (Caliendo *et al.*, 2022c; Grund *et al.*, 2018; Koethe *et al.*, 2020; Slomka *et al.*, 2019), consistent with previous experimental infections of ducks with other HPAIV clades (Berhane *et al.*, 2016; Lee *et al.*, 2017c; Li *et al.*, 2017; Pantin-Jackwood *et al.*, 2017; Pantin-Jackwood *et al.*, 2016), but in contrast to LPAIVs preferential replication in the gastrointestinal tract of ducks (Hinshaw *et al.*, 1980; Webster *et al.*, 1978). 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 a 2014-H5N8 HPAIV (Beerens *et al.*, 2021). The phenomenon of augmented enterotropism within recently circulating clade 2.3.4.4b viruses has been supported by immunohistochemical analysis in naturally infected individuals (Caliendo *et al.*, 2020; Caliendo *et al.*, 2022a). 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 (Caliendo *et al.*, 2022a). 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

(Lean *et al.*, 2022b), while H5N8-infected ducks, on the contrary, rarely exhibited mild pancreatic necrosis (Lean *et al.*, 2022a).

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 (Animal & Plant Health Agency, 2018; European Food Safety Authority *et al.*, 2022b; European Food Safety Authority *et al.*, 2023b; European Food Safety Authority *et al.*, 2021c). This observation led to the hypothesis that pre-exposure to homologous or heterologous AIV could potentially modulate the outcome of the disease. Such a phenomenon has been previously demonstrated for mallards and wood ducks (*Aix sponsa* (LINNAEUS, 1758)) challenged with other Gs/Gd strains isolated during 2005 and belonging to clade 2.2 (Costa *et al.*, 2011; Fereidouni *et al.*, 2009). 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 (Caliendo *et al.*, 2022c; Koethe *et al.*, 2020; Tarasiuk *et al.*, 2023). Following a less-virulent HPAIV challenge in wild ducks, a long-term immunity (> one year) has been assessed (Caliendo *et al.*, 2022c). 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 (Caliendo *et al.*, 2022c; Koethe *et al.*, 2020; Tarasiuk *et al.*, 2023). 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 (Tarasiuk *et al.*, 2023).

Although HPAI H5N8-infected satellite-tracked mallards were still capable of migration, suggesting a potential wide dispersion of the virus (Lv *et al.*, 2022), 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 from fecal samples collected from healthy Eurasian wigeons (*Mareca penelope* (LINNAEUS, 1758)) during the years 2014 and 2015 in the Netherlands (Poen *et al.*, 2016; Verhagen *et al.*, 2015), as well as in Russia (Marchenko *et al.*, 2015). 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 (Gobbo *et al.*, 2021). 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 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 (Beerens *et al.*, 2021). 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 (Beerens *et al.*, 2021; van den Brand *et al.*, 2018). 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 (Kleyheeg *et al.*, 2017). 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 (Beerens *et al.*, 2021). Interestingly, IHC results of naturally infected individuals with H5N8 HPAI viruses circulating in Europe during 2016 confirmed virus replication in the intestinal tract (Caliendo *et al.*, 2022a). However, in the case of individuals infected with the 2020-H5N8 HPAI 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 (Caliendo *et al.*, 2022a).

Among dabbling ducks, Eurasian teals (*Anas crecca* LINNAEUS, 1758) have also been proposed as potential long-distance vectors of HPAI infections due to asymptomatic disease (Gobbo *et al.*, 2021; Harder *et al.*, 2015; van den Brand *et al.*, 2018). Experimental infection of common teals with 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 (Tanikawa *et al.*, 2021).

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 (Alexander, 2007b; Fouchier *et al.*, 2003; Germundsson *et al.*, 2010; Hall *et al.*, 2015; Krauss *et al.*, 2007; Krauss *et al.*, 2004; Wilson *et al.*, 2013). Following natural H5N8 HPAIV of 2.3.4.4b clade infection, a significant number of tufted ducks (*Aythya fuligula* (LINNAEUS, 1758)) were found deceased in Germany, displaying macroscopic changes such as severe hepatic necrosis, multifocal petechiae, and varying degrees of lung hyperemia and oedema (Pohlmann *et al.*, 2017). IHC examination revealed the presence of AIV nucleoprotein (NP) antigen associated with necrotic lesions in the liver, heart, brain, spleen, pancreas, and thymus (Pohlmann *et al.*, 2017). 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 H5N8-HPAI virus before exposure to a more virulent strain of clade 2.3.4.4b remained asymptomatic, avoiding body weight loss and mortality, in contrast to immunologically naïve control individuals (Caliendo *et al.*, 2022c). Depending on the clade virus considered, HPAIV infection can however manifest different levels of pathogenicity. For instance, experimentally challenged common pochards (*Aythya ferina* (LINNAEUS, 1758)) 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 (van den Brand *et al.*, 2018). 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 (Keawcharoen *et al.*, 2008).

Geese and swans (Anatidae, Anserinae subfamily) are highly susceptible to HPAI clade 2.3.4.4b infection, with wild populations experiencing severe impact (Co-Convened Scientific Task Force on Avian Influenza and Wild Birds, 2022; Food and Agriculture Organization of the United Nations, 2023; European Food Safety Authority *et al.*, 2022c; Waldenström *et al.*, 2022).

IHC results from naturally infected graylag goose (*Anser anser* (LINNAEUS, 1758)) revealed 2016-H5N8 of clade 2.3.4.4b virus replication not only within the respiratory tract but also in the intestinal epithelium of deceased individuals (Caliendo *et al.*, 2020). Grossly, the primary pathological changes attributed to H5 HPAI natural infection encompassed multifocal necrosis in the liver and pancreas, pin-point hemorrhages in the brain, sub-pericardial hemorrhages, and multifocal lung hemorrhages (Caliendo *et al.*, 2022a). Notably, gross findings in H5N8-infected black swans (*Cygnus atratus* (LATHAM, 1790)) were scarcely undetectable, with occasional mild pancreatic or splenic necrosis (Lean *et al.*, 2022a). Hydropericardium was identified in a mute swan (*Cygnus olor* (GMELIN, JF, 1789)) that underwent necropsy and tested positive for HPAI H5N1 clade 2.3.4.4b virus through rRT-PCR detection (Lean *et al.*, 2022b). As reported by Floyd *et al.* (2021), 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 (Floyd *et al.*, 2021).

3.3.2. *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 (European Food Safety Authority *et al.*, 2023b). Recent instances of mass mortality among adult seabirds have been documented across various European countries including Belgium, France, Germany, Italy, and the Netherlands (European Food Safety Authority *et al.*, 2023b; Globig *et al.*, 2017). 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* (PONTOPPIDAN, 1763)) challenged with an HPAI H5N8 virus 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

(Tarasiuk *et al.*, 2022). 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 (Tarasiuk *et al.*, 2022). Similar to ducks, a greater survival rate was noted when birds had prior exposure to H5N1 LPAIV infection. However, differently from ducks, pre-exposed exhibited abundant viral shedding from both the oral cavity and cloaca. The post-mortem findings 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 (Tarasiuk *et al.*, 2022).

Within the Charadriiformes order, HPAI viruses have been also detected in various species of wild shorebirds (Adlhoch *et al.*, 2022; European Food Safety Authority *et al.*, 2023a). These birds hold a central role in the ecology of LPAIV in North America (Olsen *et al.*, 2006), though they are less frequently sampled during HPAI surveillance efforts in Europe (Authority *et al.*, 2022; European Food Safety Authority *et al.*, 2021a). 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.* (2019) reported the presence of antibodies against this H5 clade in red-necked stints (*Calidris ruficollis* (PALLAS, 1776)) sampled during the 2016-2017 austral summer, thereby indicating prior virus exposure in this long-distance migratory wader.

3.3.3. Other aquatic wild birds

HPAI infection in non-reservoir aquatic hosts is linked to a high incidence of morbidity and mortality, which becomes a wildlife conservation concern, particularly when endangered species are affected. During 2021-2022, the HPAI H5N1 virus of clade 2.3.4.4b epidemic in Europe has been unprecedented in terms of number of wild birds found dead (European Food Safety Authority *et al.*, 2022b, 2023a). In July 2021 in Scotland, notable die-offs of great skuas (*Stercorarius skua* (BRÜNNICH, 1764)) were observed, with instances escalating to the extent that mass mortality

events were recorded among various breeding populations (Banyard *et al.*, 2022). 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 (Camphuysen *et al.*, 2022). 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 (Camphuysen *et al.*, 2022). Also sandwich terns (*Thalasseus sandvicensis* (LATHAM, 1787)) were severely affected by HPAI H5N1 clade 2.3.4.4b infection, with instances of die-offs and nests being abandoned during the same time frame in the Netherlands (Rijks *et al.*, 2022). The afflicted birds 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 (Rijks *et al.*, 2022).

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 (Fusaro *et al.*, 2019). In January 2019, notable mortalities were documented among African penguins (*Spheniscus demersus* (LINNAEUS, 1758)), an endangered penguin species, in Namibia due to H5N8 HPAI infection (Molini *et al.*, 2020). 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 HPAI H5N8 infection had been previously documented in the Western and Eastern Cape Provinces of South Africa, alongside other migratory aquatic species, including greater crested terns (*Thalasseus bergii* (LICHTENSTEIN, MHC, 1823)), common terns (*Sterna hirundo* LINNAEUS, 1758), sandwich terns, Cape gannets (*Sula capensis* (LICHTENSTEIN, MHC, 1823)), Cape cormorants (*Phalacrocorax capensis* (SPARRMAN, 1788)), and Hartlaub's gulls (*Choroicocephalus hartlaubis* (BRUCH, 1855)) (Abolnik *et al.*, 2019), in absence of further clinic-pathological examination. Additionally, Cape cormorants, an endangered bird species endemic to the southwestern African coasts, recently experienced H5N1 clade 2.3.4.4b outbreaks resulting in a significant decline in a Namibian colony in 2022 (Molini *et al.*, 2023). Lastly great white pelicans (*Pelecanus onocrotalus* LINNAEUS, 1758) infected with H5N1 HPAI displayed paralysis, dyspnea, diarrhea, and subcutaneous hemorrhages (Beyit *et al.*, 2023).

3.4. 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 (Bertran *et al.*, 2014). Furthermore, game birds have been considered as potential bridge host for AIV incursion in poultry farms (Bertran *et al.*, 2014). In the context of H5 HPAI clade 2.3.4.4b, the literature offers scant reports. Wild ring-necked pheasants (*Phasianus colchicus* LINNAEUS, 1758) 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 (European Food Safety Authority *et al.*, 2023a; Grant *et al.*, 2022). 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 (Tammiranta *et al.*, 2023). 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 (Liang *et al.*, 2022). Similarly to experimental infection, naturally infected pheasants with 2017-HPAI viruses of clade 2.3.4.4b displayed severe clinical signs with neurological disorders (Brookes *et al.*, 2022; Stoimenov *et al.*, 2019). PME findings aligned with prior observations in other gallinaceous species, characterized by necrotic and hemorrhagic lesions in visceral organs (Bertran *et al.*, 2014; Brookes *et al.*, 2022; Stoimenov *et al.*, 2019). 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 (Brookes *et al.*, 2022). 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 (Brookes *et al.*, 2022).

Evidence from necropsied naturally H5N1 HPAIV clade 2.3.4.4b-infected pheasants, peafowl (*Pavo cristatus* LINNAEUS, 1758), and guinea fowls (*Numida meleagris* LINNAEUS, 1758) in the UK has primarily revealed pancreatic and splenic necrosis, accompanied by hydropericardium and epicardial petechiae (Lean *et al.*, 2022b).

3.5. 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 (Abolnik, 2014). Nevertheless, varying outcomes of infection can be observed depending on the viral clade used for experimental infection (Jeong *et al.*, 2021; Kwon *et al.*, 2017). 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 (Abolnik *et al.*, 2019). A noteworthy instance of natural HPAI H5N1 clade 2.3.4.4b infection was documented in a wood pigeon (*Columba palumbus* LINNAEUS, 1758) that was found dead in a German wildlife sanctuary (Peters *et al.*, 2022). 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 (Peters *et al.*, 2022).

3.6. 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 (Shriner & Root, 2020). Consequently, HPAI epizootics involving clade 2.3.4.4b viruses have had severe impacts on these bird populations (European Food Safety Authority *et al.*, 2022c; Günther *et al.*, 2023; Kleyheeg *et al.*, 2017; Krone *et al.*, 2018). In the context of the Accipitriformes order, natural H5N1 HPAIV of clade 2.3.4.4b infection in an African fish eagle (*Ichthyophaga vocifer* (DAUDIN, 1800)) resulted in gross findings of carcass dehydration and oedematous brain (Letsholo *et al.*, 2022). In 2017, die offs in white-tailed eagles (*Haliaeetus albicilla* (LINNAEUS, 1758)) were attributed to H5N8 clade 2.3.4.4b infection (Krone *et al.*, 2018). 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 (Krone *et al.*, 2018). PMEs of HPAI H5N8-naturally infected common buzzards (*Buteo buteo* (LINNAEUS, 1758)) 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 (Caliendo *et al.*, 2022b). 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 cardiotropism 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 (Caliendo *et al.*, 2020).

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* (LINNAEUS, 1758)) (Caliendo *et al.*, 2020), which are often found around poultry houses and have been regarded as bridge hosts for avian influenza transmission between domestic and wild birds (Shriner & Root, 2020).

3.7. Mammals

Since 2016, HPAI H5NI 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 (Animal and Plant Health Inspection Service, 2023; European Food Safety Authority *et al.*, 2023d). Among wild mammal species, the infection with HPAI H5Nx 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 (Agüero *et al.*, 2023; Hervé *et al.*, 2021; Lindh *et al.*, 2023), or through exposure to infected poultry (Rosone *et al.*, 2023). 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 (Tammiranta *et al.*, 2023). 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 (Agüero *et al.*, 2023; Gamarra-Toledo *et al.*, 2023b; Lindh *et al.*, 2023; Mariana *et al.*, 2023).

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 (Domańska-Blicharz *et al.*, 2023). In Italy, dogs (*Canis lupus familiaris*) and a cat were found to have seroconverted following exposure to the virus during a poultry outbreak (European Food Safety Authority *et al.*, 2023d).

In terms of human cases of HPAI infection with clade 2.3.4.4b, eleven sporadic cases have been reported since January 2022 following infected-poultry exposure. However, as of now, there has been no confirmed evidence of human-to-human transmission (Centers for Disease Prevention and Control, 2023).

3.7.1. *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”, enabling further reassortment between human, avian, and swine viruses (Ma *et al.*, 2008). Serological detections of HPAI H5Nx clade 2.3.4.4b exposure have been identified in asymptomatic pigs across various farms in France (Hervé *et al.*, 2021), as well as in healthy pigs sampled during an HPAI H5N1 outbreak on a rural mixed-species poultry farm (Rosone *et al.*, 2023). From literature, to date, a single experimental challenge in pigs has been reported by Graaf *et al.* (2023). In this study, four-month-old pigs were 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 (Graaf *et al.*, 2023).

Contrastingly, different outcomes have been noted in farmed animals of the Carnivora order. Specifically, American minks (*Neovison vison* (SCHREBER, 1777)) raised for fur production in Spain exhibited a sudden increase in mortality rate (0.77% *versus* 0.2-0.3% during the initial outbreak phase) due to HPAI H5N1 infection (Agüero *et al.*, 2023). 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 (Agüero *et al.*, 2023). Since mid-July 2023, H5N1 HPAI

outbreaks have impacted American minks, raccoon dogs (*Nyctereutes procyonoides* (GRAY, 1834)), arctic foxes (*Vulpes lagopus* (LINNAEUS, 1758)), red foxes (*Vulpes vulpes* (LINNAEUS, 1758)) and hybrid foxes on fur farms in Finland (Lindh *et al.*, 2023). 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 symptoms included lethargy, neurological signs, diarrhea, and rapid death. PME revealed systemic disease signs and lung lesions. Phylogenetic analyses indicated multiple introductions of the virus from birds to fur animals, while also suggesting potential inter-species and intra-species transmission, given the higher number of cases and farms involved in the outbreak (Lindh *et al.*, 2023).

With respect to pets, while there is serological evidence of exposure to infection, only a limited number of reports exist on direct virus detection (European Food Safety Authority *et al.*, 2023d). 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 (Briand *et al.*, 2023). As the clinical condition worsened, with the onset of neurological and respiratory symptoms (such as dyspnea), 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 (Briand *et al.*, 2023). In the year 2023, an HPAI H5N1 virus outbreak impacted 25 domestic cats across various regions in Poland for a period of three weeks (Domańska-Blicharz *et al.*, 2023). Distinct clinical signs were documented, encompassing loss of appetite, apathy, hypersalivation, fever, dyspnea (characterized by shallow and rapid breathing), 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. Clinical examinations revealed an exacerbated vesicular murmur and non-responsive constricted pupils in the presence of light. Given the rapid deterioration of the animals' condition within one or two days, euthanasia was commonly employed. PME 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 viral loads were detected in the brain, lungs, and bronchi (Domańska-Blicharz *et al.*, 2023).

3.7.2. Wild mammals

3.7.2.1. Marine mammals

Spill-over events involving HPAI H5Nx viruses of clade 2.3.4.4b to mammals were initially 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* (O. FABRICIUS, 1791)) (Shin *et al.*, 2019). 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 (Shin *et al.*, 2019). Subsequent reports emerged in the UK in late 2020, involving harbor seals (*Phoca vitulina* LINNAEUS, 1758) and a gray seal that either perished or were euthanized within a 2-day span at a quarantine unit within a wildlife rehabilitation center (Floyd *et al.*, 2021). These events occurred approximately a week subsequent to the demise of five swans within the same unit, all succumbing to HPAI H5N8 virus infections of clade 2.3.4.4b. Notably, both marine mammal species exhibited an abrupt onset of general 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, with viral antigens present in the brain upon IHC examination. 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 (Postel *et al.*, 2022). 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 IAV 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) (Postel *et al.*, 2022). Following the introduction of HPAI H5Nx clade 2.3.4.4b viruses into North America through migratory wild birds in 2021 (Bevins *et al.*, 2022; Caliendo *et al.*, 2022d), 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.*, 2023b). 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 PCR-positive samples (Puryear *et al.*, 2023a), however organ specimens were not collected for PCR analysis.

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 (Gamarra-Toledo *et al.*, 2023c). After instances of mass die-offs among wild aquatic birds, mass mortalities in sea lions have been documented along Peruvian coastlines (Gamarra-Toledo *et al.*, 2023b; Mariana *et al.*, 2023). Dying individuals displayed predominantly neurological clinical signs, including tremors, convulsions, and paralysis (Gamarra-Toledo *et al.*, 2023b). Additionally, respiratory indicators such as dyspnea, tachypnea, nasal and buccal secretions, as well as pulmonary edema, were observed. PME highlighted the rapid nature of death, a conclusion supported by the overall good body condition of the necropsied individuals. Examination of the respiratory tract revealed copious whitish secretions filling the trachea and pharynx, and the lungs exhibited congestion alongside hemorrhagic foci indicative of interstitial pneumonia. Congestion and hemorrhagic foci in the brain further indicated the presence of encephalitis (Gamarra-Toledo *et al.*, 2023b).

Infections of cetacean species with HPAI H5Nx viruses of clade 2.3.4.4b have also been documented, although less frequently than in other mammal species (European Food Safety Authority *et al.*, 2023d). Murawski *et al.* (2023) conducted a pathological examination of a common bottlenose dolphin (*Tursiops truncatus* (MONTAGU, 1821)) found to be infected with an H5N1 HPAI virus in Florida, US. Apart from gross findings unrelated to the virus infection, microscopic analysis revealed a moderate to marked mononuclear inflammation of the brain and leptomeninges, dominated by lymphocytes and histiocytes, accompanied by neuronal necrosis and neuropil malacia. The brain exhibited the highest viral load (Ct = 23.6), followed by the spinal cord, lungs, and stomach. IHC results demonstrated widespread virus antigen distribution within the CNS, as confirmed by *in situ* hybridization (ISH). Some alveolar surfaces and the pulmonary interstitium displayed weak IHC labeling, yet the absence of virus infection in the lungs was indicated by negative ISH results (Murawski *et al.*, 2023). In June 2022, a harbor porpoise (*Phocoena phocoena* (LINNAEUS, 1758)) was found stranded in shallow waters off the west coast of Sweden (Thorsson *et al.*, 2023). The animal was observed swimming in circles, unable to right itself, and succumbed shortly after its discovery. Macroscopic examinations revealed minimal findings, with pulmonary oedema indicative of drowning being the notable feature. Microscopically, a moderate lymphoplasmacytic meningoencephalitis was observed in the brain, together with neuronal necrosis, gliosis, perivascular cuffing, and vasculitis. Results of from the rRT-PCR indicated the highest viral load within the brain (Ct 20.57), followed by the lungs, liver, and spleen (Ct values > 30). IHC results demonstrated the presence of virus antigen primarily in the brain, located in nuclei and cytoplasm of neurons, glial cells, and epithelial cells of the choroid plexus. In scattered cells within alveoli (presumably macrophages or sloughed epithelium), scant intranuclear and intracytoplasmic viral antigen was detected, while no viral antigen was observed in other examined tissues, consistent with findings by Murawski *et al.* (2023). Interestingly, the genome of the detected HPAI H5N1 virus lacked any recognized genetic markers of mammal adaptation, the clinical manifestations and virus presence across diverse organs, including the brain, underscore the potential risk of HPAI viruses to mammalian hosts even in the absence of previous adaptation (Thorsson *et al.*, 2023).

3.7.2.2. Terrestrial mammals

Then 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 (Floyd *et al.*, 2021). 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 in the brain, 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 (Rijks *et al.*, 2021). 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* (LINNAEUS, 1758)) in the same region during that period (Rijks *et al.*, 2022). Bordes *et al.* (2023) 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 (Floyd *et al.*, 2021; Reperant *et al.*, 2008).

Multiple carnivore species were found HPAI H5N1-infected in Finland following mass mortalities of infected pheasants (Tammiranta *et al.*, 2023). An otter, two red foxes and a 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 (Tammiranta *et al.*, 2023).

Further cases of infection were identified among carnivores in Asia. An Ezo red fox (*Vulpes vulpes schrencki* (KISHIDA, 1924)) was found dead in a public garden in Japan in March 2022, coinciding with a crow die-off (Hiono *et al.*, 2023). Additionally, a severely emaciated Japanese raccoon dog (*Nyctereutes procyonoides albus* (TEMMINCK, 1834)) was euthanized due to its critical clinical condition (Hiono *et al.*, 2023). 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 (Floyd *et al.*, 2021), 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 (Hiono *et al.*, 2023).

3.8. Conclusions

Since 2016, the Asian-origin H5Nx clade 2.3.4.4b viruses have spread extensively to Europe, Africa, Asia, and America via migratory birds, undergoing significant genetic evolution and reassortment of internal genes with locally circulating LPAIVs (Verhagen *et al.*, 2021; Youk *et al.*, 2023a). Through mutations and reassortments, HPAIVs of clade 2.3.4.4b have acquired novel pathobiological characteristics (Kwon *et al.*, 2023; Leyson *et al.*, 2019). 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 not only through the oropharyngeal route, in line with other HPAIVs, but also through the cloacal route, key factor for LPAIVs circulation, contributing to HPAIVs environmental contamination (Beerens *et al.*, 2021; Caliendo *et al.*, 2020). Besides waterbirds, a wide range of wild bird species and both wild and domestic mammals seem to be permissive to infection with HPAIVs of clade 2.3.4.4b, 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 of clade 2.3.4.4b, further research is necessary to develop efficient strategies for preventing future outbreaks in poultry and reducing the zoonotic risk.

3.9. References

- Abolnik, C. (2014). A current review of avian influenza in pigeons and doves (Columbidae). *Vet Microbiol*, 170(3-4), 181-196. doi:10.1016/j.vetmic.2014.02.042
- Abolnik, C., Pieterse, R., Peyrot, B. M., Choma, P., Phiri, T. P., Ebersohn, K., Heerden, C. J. V., Vorster, A. A., Zel, G. V., Geertsma, P. J., Laleye, A. T., Govindasamy, K., & Rauff, D. L. (2019). The Incursion and Spread of Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4 Within South Africa. *Avian Dis*, 63(sp1), 149-156. doi:10.1637/11869-042518-Reg.1
- Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Aznar, I., Muñoz Guajardo, I., & Baldinelli, F. (2022). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- Agüero, M., Monne, I., Sánchez, A., Zecchin, B., Fusaro, A., Ruano, M. J., del Valle Arrojo, M., Fernández-Antonio, R., Souto, A. M., Tordable, P., Cañas, J., Bonfante, F., Giussani, E., Terregino, C., & Orejas, J. J. (2023). Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. *Euro Surveill*, 28(3), 2300001. doi:https://doi.org/10.2807/1560-7917.ES.2023.28.3.2300001
- Alexander, D. J. (2000). A review of avian influenza in different bird species. *Vet Microbiol*, 74(1-2), 3-13. doi:10.1016/s0378-1135(00)00160-7
- Alexander, D. J. (2007a). An overview of the epidemiology of avian influenza. *Vaccine*, 25(30), 5637-5644. doi:https://doi.org/10.1016/j.vaccine.2006.10.051
- Alexander, D. J. (2007b). Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. *Avian Dis*, 51(1 Suppl), 161-166. doi:10.1637/7602-041306r.1
- Alexandrou, O., Malakou, M., & Catsadorakis, G. (2022). The impact of avian influenza 2022 on Dalmatian pelicans was the worst ever wildlife disaster in Greece. *Oryx*, 56(6), 813-813. doi:10.1017/S0030605322001041
- Alkie, T. N., Lopes, S., Hisanaga, T., Xu, W., Suderman, M., Koziuk, J., Fisher, M., Redford, T., Lung, O., Joseph, T., Himsworth, C. G., Brown, I. H., Bowes, V., Lewis, N. S., & Berhane, Y. (2022). A threat from both sides: Multiple introductions of genetically distinct H5 HPAI viruses into Canada via both East Asia-Australasia/Pacific and Atlantic flyways. *Virus Evol*, 8(2). doi:10.1093/ve/veac077
- Animal & Plant Health Agency. (2018). *Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2017*. Retrieved from https://food.ec.europa.eu/system/files/2018-11/ad_control-measures_ai_surv-rslt_pltry-wld-brds_2017.pdf
- Animal and Plant Health Inspection Service, U.S. Department of Agriculture. 2022-2023 Detections of Highly Pathogenic Avian Influenza in Wild Birds. Retrieved from

- <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-wild-birds>
- Animal and Plant Health Inspection Service, U.S. Department of Agriculture. 2022-2023 confirmations of highly pathogenic avian influenza in commercial and backyard flocks. Retrieved from <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-commercial-backyard-flocks>
- Animal and Plant Health Inspection Service, U.S. Department of Agriculture. (2023a). USDA Takes Action to Help Protect Endangered California Condors From Highly Pathogenic Avian Influenza. Retrieved from https://www.aphis.usda.gov/aphis/newsroom/stakeholder-info/sa_by_date/sa-2023/ca-condor-hpai
- Animal and Plant Health Inspection Service, U.S. Department of Agriculture. (2023). 2022-2023 Detections of Highly Pathogenic Avian Influenza in Mammals. Retrieved from <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-mammals>
- Appleby, M. C., Mench, J. A., & Hughes, B. O. (2004). *Poultry Behaviour and Welfare* United Kingdom: CABI Publishing.
- Authority, E. F. S., Aznar, I., Baldinelli, F., Stoicescu, A., & Kohnle, L. (2022). Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2021. *EFSA J*, 20(9), e07554. doi:<https://doi.org/10.2903/j.efsa.2022.7554>
- Banyard, A. C., Lean, F. Z. X., Robinson, C., Howie, F., Tyler, G., Nisbet, C., Seekings, J., Meyer, S., Whittard, E., Ashpitel, H. F., Bas, M., Byrne, A. M. P., Lewis, T., James, J., Stephan, L., Lewis, N. S., Brown, I. H., Hansen, R. D. E., & Reid, S. M. (2022). Detection of Highly Pathogenic Avian Influenza Virus H5N1 Clade 2.3.4.4b in Great Skuas: A Species of Conservation Concern in Great Britain. *Viruses*, 14(2). doi:10.3390/v14020212
- Barber, M. R. W., Aldridge, J. R., Webster, R. G., & Magor, K. E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U.S.A.*, 107(13), 5913-5918. doi:[doi:10.1073/pnas.1001755107](https://doi.org/10.1073/pnas.1001755107)
- Beerens, N., Germeraad, E. A., Venema, S., Verheij, E., Pritz-Verschuren, S. B. E., & Gonzales, J. L. (2021). Comparative pathogenicity and environmental transmission of recent highly pathogenic avian influenza H5 viruses. *Emerg Microbes Infect*, 10(1), 97-108. doi:[10.1080/22221751.2020.1868274](https://doi.org/10.1080/22221751.2020.1868274)
- Berhane, Y., Kobasa, D., Embury-Hyatt, C., Pickering, B., Babiuk, S., Joseph, T., Bowes, V., Suderman, M., Leung, A., Cottam-Birt, C., Hisanaga, T., & Pasick, J. (2016). Pathobiological Characterization of a Novel Reassortant Highly Pathogenic H5N1

- Virus Isolated in British Columbia, Canada, 2015. *Sci Rep*, 6, 23380. doi:10.1038/srep23380
- Bertran, K., Dolz, R., & Majó, N. (2014). Pathobiology of avian influenza virus infection in minor gallinaceous species: a review. *Avian Pathol*, 43(1), 9-25. doi:10.1080/03079457.2013.876529
- Bevins, S. N., Shriner, S. A., Cumbee, J. C., Jr., Dilione, K. E., Douglass, K. E., Ellis, J. W., Killian, M. L., Torchetti, M. K., & Lenocho, J. B. (2022). Intercontinental Movement of Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4 Virus to the United States, 2021. *Emerg Infect Dis*, 28(5), 1006-1011. doi:10.3201/eid2805.220318
- Beyit, A. D., Meki, I. K., Barry, Y., Haki, M. L., El Ghassem, A., Hamma, S. M., Abdelwahab, N., Doumbia, B., Ahmed Benane, H., Daf, D. S., Sidatt, Z. E. A., Ould Mekhalla, L., El Mamy, B., Gueya, M. O. B., Settypalli, T. B. K., Ouled Ahmed Ben Ali, H., Datta, S., Cattoli, G., Lamien, C. E., & Dundon, W. G. (2023). Avian influenza H5N1 in a great white pelican (*Pelecanus onocrotalus*), Mauritania 2022. *Vet Res Commun*. doi:10.1007/s11259-023-10100-6
- Bi, Y., Chen, Q., Wang, Q., Chen, J., Jin, T., Wong, G., Quan, C., Liu, J., Wu, J., Yin, R., Zhao, L., Li, M., Ding, Z., Zou, R., Xu, W., Li, H., Wang, H., Tian, K., Fu, G., Huang, Y., Shestopalov, A., Li, S., Xu, B., Yu, H., Luo, T., Lu, L., Xu, X., Luo, Y., Liu, Y., Shi, W., Liu, D., & Gao, G. F. (2016). Genesis, Evolution and Prevalence of H5N6 Avian Influenza Viruses in China. *Cell Host Microbe*, 20(6), 810-821. doi:10.1016/j.chom.2016.10.022
- Co-Convened Scientific Task Force on Avian Influenza and Wild Birds. (2022). *Scientific Task Force on Avian Influenza and Wild Birds statement. H5N1 Highly Pathogenic Avian Influenza in poultry and wild birds: Winter of 2021/2022 with focus on mass mortality of wild birds in UK and Israel*. Retrieved from <https://www.woah.org/app/uploads/2022/03/avian-influenza-0.pdf>
- Co-Convened Scientific Task Force on Avian Influenza and Wild Birds. (2023). Scientific Task Force on Avian Influenza and Wild Birds Statement - July 2023. Retrieved from https://www.cms.int/sites/default/files/publication/avian_influenza_2023_aug.pdf
- Bodewes, R., & Kuiken, T. (2018). Changing Role of Wild Birds in the Epidemiology of Avian Influenza A Viruses. *Adv Virus Res*, 100, 279-307. doi:10.1016/bs.aivir.2017.10.007
- Bordes, L., Vreman, S., Heutink, R., Roose, M., Venema, S., Pritz-Verschuren, S. B. E., Rijks, J. M., Gonzales, J. L., Germeraad, E. A., Engelsma, M., & Beerens, N. (2023). Highly Pathogenic Avian Influenza H5N1 Virus Infections in Wild Red Foxes (*Vulpes vulpes*) Show Neurotropism and Adaptive Virus Mutations. *Microbiol Spectr*, 11(1), e0286722. doi:10.1128/spectrum.02867-22

- Bouvier, N. M., & Palese, P. (2008). The biology of influenza viruses. *Vaccine*, 26, D49-D53. doi:<https://doi.org/10.1016/j.vaccine.2008.07.039>
- Briand, F. X., Souchaud, F., Pierre, I., Beven, V., Hirchaud, E., Hérault, F., Planel, R., Rigau, A., Bernard-Stoecklin, S., Van der Werf, S., Lina, B., Gerbier, G., Etteradossi, N., Schmitz, A., Niqueux, E., & Grasland, B. (2023). Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus in Domestic Cat, France, 2022. *Emerg Infect Dis*, 29(8), 1696-1698. doi:10.3201/eid2908.230188
- Brookes, S. M., Mansfield, K. L., Reid, S. M., Coward, V., Warren, C., Seekings, J., Brough, T., Gray, D., Núñez, A., & Brown, I. H. (2022). Incursion of H5N8 high pathogenicity avian influenza virus (HPAIV) into gamebirds in England. *Epidemiol Infect*, 150, e51. doi:10.1017/S0950268821002740
- Caliendo, V., Leijten, L., Begeman, L., Poen, M. J., Fouchier, R. A. M., Beerens, N., & Kuiken, T. (2020). Enterotropism of highly pathogenic avian influenza virus H5N8 from the 2016/2017 epidemic in some wild bird species. *Vet Res*, 51(1), 117. doi:10.1186/s13567-020-00841-6
- Caliendo, V., Leijten, L., van de Bildt, M., Germeraad, E., Fouchier, R. A. M., Beerens, N., & Kuiken, T. (2022a). Tropism of Highly Pathogenic Avian Influenza H5 Viruses from the 2020/2021 Epizootic in Wild Ducks and Geese. *Viruses*, 14(2). doi:10.3390/v14020280
- Caliendo, V., Leijten, L., van de Bildt, M. W. G., Fouchier, R. A. M., Rijks, J. M., & Kuiken, T. (2022b). Pathology and virology of natural highly pathogenic avian influenza H5N8 infection in wild Common buzzards (*Buteo buteo*). *Sci Rep*, 12(1), 920. doi:10.1038/s41598-022-04896-7
- Caliendo, V., Leijten, L., van de Bildt, M. W. G., Poen, M. J., Kok, A., Bestebroer, T., Richard, M., Fouchier, R. A. M., & Kuiken, T. (2022c). Long-Term Protective Effect of Serial Infections with H5N8 Highly Pathogenic Avian Influenza Virus in Wild Ducks. *J Virol*, 96(18), e0123322. doi:10.1128/jvi.01233-22
- Caliendo, V., Lewis, N. S., Pohlmann, A., Baillie, S. R., Banyard, A. C., Beer, M., Brown, I. H., Fouchier, R. A. M., Hansen, R. D. E., Lameris, T. K., Lang, A. S., Laurendeau, S., Lung, O., Robertson, G., van der Jeugd, H., Alkie, T. N., Thorup, K., van Toor, M. L., Waldenström, J., Yason, C., Kuiken, T., & Berhane, Y. (2022d). Transatlantic spread of highly pathogenic avian influenza H5N1 by wild birds from Europe to North America in 2021. *Sci Rep*, 12(1), 11729. doi:10.1038/s41598-022-13447-z
- Campbell, L. K., & Magor, K. E. (2020). Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens. *Front Cell Infect Microbiol*, 10, 209. doi:10.3389/fcimb.2020.00209
- Camphuysen, C., Gear, S., & Furness, B. (2022). Avian influenza leads to mass mortality of adult Great Skuas in Foula in summer 2022. 42, 312-323.

- Carnaccini, S., & Perez, D. R. (2020). H9 Influenza Viruses: An Emerging Challenge. *Cold Spring Harb Perspect Med*, 10(6). doi:10.1101/cshperspect.a038588
- Cattoli, G., Milani, A., Temperton, N., Zecchin, B., Buratin, A., Molesti, E., Aly, M. M., Arafa, A., & Capua, I. (2011). Antigenic drift in H5N1 avian influenza virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. *J Virol*, 85(17), 8718-8724. doi:10.1128/jvi.02403-10
- Centers for Disease Prevention and Control (2023). Ask the Expert: Highly Pathogenic Avian Influenza A(H5N1) Viruses. Retrieved from <https://www.cdc.gov/flu/avianflu/spotlights/2022-2023/avian-flu-highly-pathogenic.htm>
- Chen, J., Lee, K. H., Steinhauer, D. A., Stevens, D. J., Skehel, J. J., & Wiley, D. C. (1998). Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell*, 95(3), 409-417. doi:10.1016/s0092-8674(00)81771-7
- Costa, T. P., Brown, J. D., Howerth, E. W., Stallknecht, D. E., & Swayne, D. E. (2011). Homo- and heterosubtypic low pathogenic avian influenza exposure on H5N1 highly pathogenic avian influenza virus infection in wood ducks (*Aix sponsa*). *PLoS One*, 6(1), e15987. doi:10.1371/journal.pone.0015987
- Cunningham, E. J. A., Gamble, A., Hart, T., Humphreys, E. M., Philip, E., Tyler, G., & Wood, M. J. (2022). The incursion of Highly Pathogenic Avian Influenza (HPAI) into North Atlantic seabird populations: an interim report from the 15th International Seabird Group conference. *Seabird*, 34, 67-73. Retrieved from <http://www.seabirdgroup.org.uk/journals/seabird-34/seabird-34-67.pdf>
- Food and Agriculture Organization of the United Nations. (2023). *Global Avian Influenza Viruses with Zoonotic Potential situation update*. Retrieved from <https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/en#:~:text=LPAI%20events%20in%20animals%20officially,2%20new%20events%20were%20reported.>
- Domańska-Blicharz, K., Świętoń, E., Świątalska, A., Monne, I., Fusaro, A., Tarasiuk, K., Wyrostek, K., Styś-Fijoł, N., Giza, A., Pietruk, M., Zechchin, B., Pastori, A., Adaszek, Ł., Pomorska-Mól, M., Tomczyk, G., Terregino, C., & Winiarczyk, S. (2023). Outbreak of highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b virus in cats, Poland, June to July 2023. *Euro Surveill*, 28(31). doi:10.2807/1560-7917.Es.2023.28.31.2300366
- EAAFP. (2022). Updates of HPAI recorded in East Asian – Australasian Flyway. Retrieved from <https://www.eaaflyway.net/updates-hpai-eaaf/>
- Elbe, S., & Buckland-Merrett, G. (2017). Data, disease and diplomacy: GISAID's innovative contribution to global health. *Glob Chall*, 1(1), 33-46. doi:10.1002/gch2.1018

- European Food Safety Authority, Aznar, I., Baldinelli, F., Papanikolaou, A., Stoicescu, A., & Van der Stede, Y. (2021a). Annual Report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2020. *EFSA J*, 19(12), e06953. doi:<https://doi.org/10.2903/j.efsa.2021.6953>
- European Food Safety Authority, Aznar, I., Baldinelli, F., Stoicescu, A., & Kohnle, L. (2022a). Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2021. *EFSA J*, 20(9), e07554. doi:<https://doi.org/10.2903/j.efsa.2022.7554>
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2022b). Avian influenza overview March - June 2022. *EFSA J*, 20(8), e07415. doi:10.2903/j.efsa.2022.7415
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2023a). Avian influenza overview September - December 2022. *EFSA J*, 21(1), e07786. doi:10.2903/j.efsa.2023.7786
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Mirinaviciute, G., Niqueux, E., Stahl, K., Staubach, C., Terregino, C., Broglia, A., & Baldinelli, F. (2023b). Avian influenza overview December 2022 - March 2023. *EFSA J*, 21(3), e07917. doi:10.2903/j.efsa.2023.7917
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Munoz Guajardo, I., & Baldinelli, F. (2022c). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., Staubach, C., Terregino, C., Baldinelli, F., Rusinà, A., & Kohnle, L. (2023c). Avian influenza overview June–September 2023. *EFSA J*, 21(10), e08328. doi:<https://doi.org/10.2903/j.efsa.2023.8328>
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian influenza, Brown, I., Kuiken, T., Mulatti, P., Smietanka, K., Staubach, C., Stroud, D., Therkildsen, O. R., Willeberg, P.,

- Baldinelli, F., Verdonck, F., & Adlhoch, C. (2017). Avian influenza overview September – November 2017. *EFSA J*, 15(12), e05141. doi:<https://doi.org/10.2903/j.efsa.2017.5141>
- European Food Safety Authority, European Centre for Disease Prevention Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Aznar, I., Muñoz Guajardo, I., & Baldinelli, F. (2021b). Avian influenza overview September – December 2021. *EFSA J*, 19(12), e07108. doi:<https://doi.org/10.2903/j.efsa.2021.7108>
- European Food Safety Authority, European Centre for Disease Prevention Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Melidou, A., Mirinavičiūtė, G., Niqueux, É., Ståhl, K., Staubach, C., Terregino, C., Baldinelli, F., Broglia, A., & Kohnle, L. (2023d). Avian influenza overview April – June 2023. *EFSA J*, 21(7), e08191. doi:<https://doi.org/10.2903/j.efsa.2023.8191>
- European Food Safety Authority, Prevention, E. C. f. D., Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Brouwer, A., Kuiken, T., Miteva, A., Mulatti, P., Smietanka, K., Staubach, C., Gogin, A., Muñoz Guajardo, I., & Baldinelli, F. (2018a). Avian influenza overview August – November 2018. *EFSA J*, 16(12), e05573. doi:<https://doi.org/10.2903/j.efsa.2018.5573>
- European Food Safety Authority, Prevention, E. C. f. D., Control, European Union Reference Laboratory for Avian influenza, Adlhoch, C., Brouwer, A., Kuiken, T., Mulatti, P., Smietanka, K., Staubach, C., Willeberg, P., Barrucci, F., Verdonck, F., Amato, L., & Baldinelli, F. (2018b). Avian influenza overview November 2017 - February 2018. *EFSA Journal*, 16(3), e05240. doi:<https://doi.org/10.2903/j.efsa.2018.5240>
- European Food Safety Authority, Prevention, E. C. f. D., Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., Lima, E., & Baldinelli, F. (2021c). Avian influenza overview February – May 2021. *EFSA J*, 19(12), e06951. doi:<https://doi.org/10.2903/j.efsa.2021.6951>
- European Food Safety Authority, Prevention, E. C. f. D., Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Muñoz Guajardo, I., Lima, E., & Baldinelli, F. (2021d). Avian influenza overview December 2020 – February 2021. *EFSA J*, 19(3), e06497. doi:<https://doi.org/10.2903/j.efsa.2021.6497>
- European Food Safety Authority, Prevention, E. C. f. D., Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Kuiken, T., Niqueux, E., Staubach, C., Terregino, C., Guajardo, I. M., & Baldinelli, F. (2020). Avian influenza overview November 2019– February 2020. *EFSA J*, 18(3), e06096. doi:<https://doi.org/10.2903/j.efsa.2020.6096>

- Evseev, D., & Magor, K. E. (2019). Innate Immune Responses to Avian Influenza Viruses in Ducks and Chickens. *Vet Sci*, 6(1), 5.
- Fereidouni, S. R., Starick, E., Beer, M., Wilking, H., Kalthoff, D., Grund, C., Häuslaigner, R., Breithaupt, A., Lange, E., & Harder, T. C. (2009). Highly Pathogenic Avian Influenza Virus Infection of Mallards with Homo- and Heterosubtypic Immunity Induced by Low Pathogenic Avian Influenza Viruses. *PLoS One*, 4(8), e6706. doi:10.1371/journal.pone.0006706
- Floyd, T., Banyard, A. C., Lean, F. Z. X., Byrne, A. M. P., Fullick, E., Whittard, E., Mollett, B. C., Bexton, S., Swinson, V., Macrelli, M., Lewis, N. S., Reid, S. M., Núñez, A., Duff, J. P., Hansen, R., & Brown, I. H. (2021). Encephalitis and Death in Wild Mammals at a Rehabilitation Center after Infection with Highly Pathogenic Avian Influenza A(H5N8) Virus, United Kingdom. *Emerg Infect Dis*, 27(11), 2856-2863. doi:10.3201/eid2711.211225
- Fouchier, R. A., Olsen, B., Bestebroer, T. M., Herfst, S., van der Kemp, L., Rimmelzwaan, G. F., & Osterhaus, A. D. (2003). Influenza A virus surveillance in wild birds in Northern Europe in 1999 and 2000. *Avian Dis*, 47(3 Suppl), 857-860. doi:10.1637/0005-2086-47.s3.857
- Fusaro, A., Zecchin, B., Vrancken, B., Abolnik, C., Ademun, R., Alassane, A., Arafa, A., Awuni, J. A., Couacy-Hymann, E., Coulibaly, M. B., Gaidet, N., Go-Maró, E., Joannis, T., Jumbo, S. D., Minoungou, G., Meseko, C., Souley, M. M., Ndumu, D. B., Shittu, I., Twabela, A., Wade, A., Wiersma, L., Akpeli, Y. P., Zamperin, G., Milani, A., Lemey, P., & Monne, I. (2019). Disentangling the role of Africa in the global spread of H5 highly pathogenic avian influenza. *Nat Commun*, 10(1), 5310. doi:10.1038/s41467-019-13287-y
- Gamarra-Toledo, V., Plaza, P. I., Angulo, F., Gutiérrez, R., García-Tello, O., Saravia-Guevara, P., Mejía-Vargas, F., Epiqueñ-Rivera, M., Quiroz-Jiménez, G., Martínez, P., Huamán-Mendoza, D., Inga-Díaz, G., La Madrid, L. E., Luyo, P., Ventura, S., & Lambertucci, S. A. (2023a). Highly Pathogenic Avian Influenza (HPAI) strongly impacts wild birds in Peru. *Biol Conserv*, 286, 110272. doi:https://doi.org/10.1016/j.biocon.2023.110272
- Gamarra-Toledo, V., Plaza, P. I., Gutiérrez, R., Inga-Díaz, G., Saravia-Guevara, P., Pereyra-Meza, O., Coronado-Flores, E., Calderón-Cerrón, A., Quiroz-Jiménez, G., Martínez, P., Huamán-Mendoza, D., Nieto-Navarrete, J. C., Ventura, S., & Lambertucci, S. A. (2023b). Mass Mortality of Marine Mammals Associated to Highly Pathogenic Influenza Virus (H5N1) in South America. *bioRxiv*, 2023.2002.2008.527769. doi:10.1101/2023.02.08.527769
- Gamarra-Toledo, V., Plaza, P. I., Gutiérrez, R., Luyo, P., Hernani, L., Angulo, F., & Lambertucci, S. A. (2023c). Avian flu threatens Neotropical birds. *Science*, 379(6629), 246. doi:10.1126/science.adg2271

- Germundsson, A., Madslien, K. I., Hjortaas, M. J., Handeland, K., & Jonassen, C. M. (2010). Prevalence and subtypes of influenza A viruses in wild waterfowl in Norway 2006-2007. *Acta Vet Scand*, 52(1), 28. doi:10.1186/1751-0147-52-28
- Globig, A., Staubach, C., Sauter-Louis, C., Dietze, K., Homeier-Bachmann, T., Probst, C., Gethmann, J., Depner, K. R., Grund, C., Harder, T. C., Starick, E., Pohlmann, A., Höper, D., Beer, M., Mettenleiter, T. C., & Conraths, F. J. (2017). Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4b in Germany in 2016/2017. *Front Vet Sci*, 4, 240. doi:10.3389/fvets.2017.00240
- Gobbo, F., Fornasiero, D., De Marco, M. A., Zecchin, B., Mulatti, P., Delogu, M., & Terregino, C. (2021). Active Surveillance for Highly Pathogenic Avian Influenza Viruses in Wintering Waterbirds in Northeast Italy, 2020-2021. *Microorganisms*, 9(11). doi:10.3390/microorganisms9112188
- Gobbo, F., Zanardello, C., Bottinelli, M., Budai, J., Bruno, F., De Nardi, R., Patreggiani, T., Catania, S., & Terregino, C. (2022). Silent Infection of Highly Pathogenic Avian Influenza Virus (H5N1) Clade 2.3.4.4b in a Commercial Chicken Broiler Flock in Italy. *Viruses*, 14(8). doi:10.3390/v14081600
- Gotoh, B., Ogasawara, T., Toyoda, T., Inocencio, N. M., Hamaguchi, M., & Nagai, Y. (1990). An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. *Embo j*, 9(12), 4189-4195. doi:10.1002/j.1460-2075.1990.tb07643.x
- Graaf, A., Piesche, R., Sehl-Ewert, J., Grund, C., Pohlmann, A., Beer, M., & Harder, T. (2023). Low Susceptibility of Pigs against Experimental Infection with HPAI Virus H5N1 Clade 2.3.4.4b. *Emerg Infect Dis*, 29(7), 1492-1495. doi:10.3201/eid2907.230296
- Grant, M., Bröjer, C., Zohari, S., Nöremark, M., Uhlhorn, H., & Jansson, D. S. (2022). Highly Pathogenic Avian Influenza (HPAI H5Nx, Clade 2.3.4.4.b) in Poultry and Wild Birds in Sweden: Synopsis of the 2020-2021 Season. *Vet Sci*, 9(7). doi:10.3390/vetsci9070344
- Grund, C., Hoffmann, D., Ulrich, R., Naguib, M., Schinköthe, J., Hoffmann, B., Harder, T., Saenger, S., Zscheppang, K., Tönnies, M., Hippenstiel, S., Hocke, A., Wolff, T., & Beer, M. (2018). A novel European H5N8 influenza A virus has increased virulence in ducks but low zoonotic potential. *Emerg*, 7(1), 1-14. doi:10.1038/s41426-018-0130-1
- Günther, A., Krone, O., Svansson, V., Pohlmann, A., King, J., Hallgrímsson, G. T., Skarphéðinsson, K. H., Sigurðardóttir, H., Jónsson, S. R., Beer, M., Brugger, B., & Harder, T. (2022). Iceland as Stepping Stone for Spread of Highly Pathogenic Avian Influenza Virus between Europe and North America. *Emerg Infect Dis*, 28(12), 2383-2388. doi:10.3201/eid2812.221086
- Günther, A., Pohlmann, A., Globig, A., Ziegler, U., Calvelage, S., Keller, M., Fischer, D., Staubach, C., Groschup, M. H., Harder, T., & Beer, M. (2023). Continuous surveillance of potentially zoonotic avian pathogens detects contemporaneous occurrence of highly

- pathogenic avian influenza viruses (HPAIV H5) and flaviviruses (USUV, WNV) in several wild and captive birds. *Emerg Microbes Infect*, 12(2), 2231561. doi:10.1080/22221751.2023.2231561
- Guo, Y., Xu, X., & Wan, X. (1998). Genetic characterization of an avian influenza A (H5N1) virus isolated from a sick goose in China. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, 12(4), 322-325.
- Hall, J. S., Russell, R. E., Franson, J. C., Soos, C., Dusek, R. J., Allen, R. B., Nashold, S. W., TeSlaa, J. L., Jónsson, J. E., Ballard, J. R., Harms, N. J., & Brown, J. D. (2015). Avian Influenza Ecology in North Atlantic Sea Ducks: Not All Ducks Are Created Equal. *PLoS One*, 10(12), e0144524. doi:10.1371/journal.pone.0144524
- Harder, T., Maurer-Stroh, S., Pohlmann, A., Starick, E., Höreth-Böntgen, D., Albrecht, K., Pannwitz, G., Teifke, J., Gunalan, V., Lee, R. T., Sauter-Louis, C., Homeier, T., Staubach, C., Wolf, C., Strebelow, G., Höper, D., Grund, C., Conraths, F. J., Mettenleiter, T. C., & Beer, M. (2015). Influenza A(H5N8) Virus Similar to Strain in Korea Causing Highly Pathogenic Avian Influenza in Germany. *Emerg Infect Dis*, 21(5), 860-863. doi:10.3201/eid2105.141897
- Hervé, S., Schmitz, A., Briand, F. X., Gorin, S., Quéguiner, S., Niqueux, É., Paboeuf, F., Scoizec, A., Le Bouquin-Leneveu, S., Etteradossi, N., & Simon, G. (2021). Serological Evidence of Backyard Pig Exposure to Highly Pathogenic Avian Influenza H5N8 Virus during 2016-2017 Epizootic in France. *Pathogens*, 10(5). doi:10.3390/pathogens10050621
- Hinshaw, V. S., Webster, R. G., & Turner, B. (1980). The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. *Can J Microbiol*, 26(5), 622-629. doi:10.1139/m80-108
- Hiono, T., Kobayashi, D., Kobayashi, A., Suzuki, T., Satake, Y., Harada, R., Matsuno, K., Sashika, M., Ban, H., Kobayashi, M., Takaya, F., Fujita, H., Isoda, N., Kimura, T., & Sakoda, Y. (2023). Virological, pathological, and glycovirological investigations of an Ezo red fox and a tanuki naturally infected with H5N1 high pathogenicity avian influenza viruses in Hokkaido, Japan. *Virology*, 578, 35-44. doi:10.1016/j.virol.2022.11.008
- Hulse, D. J., Webster, R. G., Russell, R. J., & Perez, D. R. (2004). Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. *J Virol*, 78(18), 9954-9964. doi:10.1128/jvi.78.18.9954-9964.2004
- Hutchinson, E. C., Charles, P. D., Hester, S. S., Thomas, B., Trudgian, D., Martínez-Alonso, M., & Fodor, E. (2014). Conserved and host-specific features of influenza virion architecture. *Nat Commun*, 5, 4816. doi:10.1038/ncomms5816
- IUCN. (2023). The IUCN Red List of Threatened Species. Version 2022-2. Retrieved from <https://www.iucnredlist.org>

- Jeong, S., Kwon, J. H., Lee, S. H., Kim, Y. J., Jeong, J. H., Park, J. E., Jheong, W. H., Lee, D. H., & Song, C. S. (2021). Subclinical Infection and Transmission of Clade 2.3.4.4 H5N6 Highly Pathogenic Avian Influenza Virus in Mandarin Duck (*Aix galericulata*) and Domestic Pigeon (*Columbia livia domestica*). *Viruses*, 13(6). doi:10.3390/v13061069
- Johansson, B. E., Matthews, J. T., & Kilbourne, E. D. (1998). Supplementation of conventional influenza A vaccine with purified viral neuraminidase results in a balanced and broadened immune response. *Vaccine*, 16(9-10), 1009-1015. doi:10.1016/s0264-410x(97)00279-x
- Kang, H. M., Choi, J. G., Kim, K. I., Kim, B. S., Batchuluun, D., Erdene-Ochir, T. O., Kim, M. C., Kwon, J. H., Park, C. K., & Lee, Y. J. (2013). Pathogenicity in domestic ducks and mice of highly pathogenic H5N1 clade 2.3.2.1 influenza viruses recently circulating in Eastern Asia. *Vet Microbiol*, 167(3-4), 327-333. doi:10.1016/j.vetmic.2013.09.013
- Keawcharoen, J., van Riel, D., van Amerongen, G., Bestebroer, T., Beyer, W. E., van Lavieren, R., Osterhaus, A. D., Fouchier, R. A., & Kuiken, T. (2008). Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). *Emerg Infect Dis*, 14(4), 600-607. doi:10.3201/eid1404.071016
- Kim, J. K., Negovetich, N. J., Forrest, H. L., & Webster, R. G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza Other Respir Viruses*, 3(4), 121-128. doi:10.1111/j.1750-2659.2009.00084.x
- Kim, Y. I., Pascua, P. N., Kwon, H. I., Lim, G. J., Kim, E. H., Yoon, S. W., Park, S. J., Kim, S. M., Choi, E. J., Si, Y. J., Lee, O. J., Shim, W. S., Kim, S. W., Mo, I. P., Bae, Y., Lim, Y. T., Sung, M. H., Kim, C. J., Webby, R. J., Webster, R. G., & Choi, Y. K. (2014). Pathobiological features of a novel, highly pathogenic avian influenza A(H5N8) virus. *Emerg Microbes Infect*, 3(10), e75. doi:10.1038/emi.2014.75
- Klenk, H. D., & Garten, W. (1994). Host cell proteases controlling virus pathogenicity. *Trends Microbiol*, 2(2), 39-43. doi:https://doi.org/10.1016/0966-842X(94)90123-6
- Kleyheeg, E., Slaterus, R., Bodewes, R., Rijks, J. M., Spierenburg, M. A. H., Beerens, N., Kelder, L., Poen, M. J., Stegeman, J. A., Fouchier, R. A. M., Kuiken, T., & van der Jeugd, H. P. (2017). Deaths among Wild Birds during Highly Pathogenic Avian Influenza A(H5N8) Virus Outbreak, the Netherlands. *Emerg Infect Dis*, 23(12), 2050-2054. doi:10.3201/eid2312.171086
- Koethe, S., Ulrich, L., Ulrich, R., Amler, S., Graaf, A., Harder, T. C., Grund, C., Mettenleiter, T. C., Conraths, F. J., Beer, M., & Globig, A. (2020). Modulation of lethal HPAIV H5N8 clade 2.3.4.4B infection in AIV pre-exposed mallards. *Emerg Microbes Infect*, 9(1), 180-193. doi:10.1080/22221751.2020.1713706
- Krauss, S., Obert, C. A., Franks, J., Walker, D., Jones, K., Seiler, P., Niles, L., Pryor, S. P., Obenauer, J. C., Naeve, C. W., Widjaja, L., Webby, R. J., & Webster, R. G. (2007).

- Influenza in migratory birds and evidence of limited intercontinental virus exchange. *PLoS Pathog*, 3(11), e167. doi:10.1371/journal.ppat.0030167
- Krauss, S., Walker, D., Pryor, S. P., Niles, L., Chenghong, L., Hinshaw, V. S., & Webster, R. G. (2004). Influenza A viruses of migrating wild aquatic birds in North America. *Vector Borne Zoonotic Dis*, 4(3), 177-189. doi:10.1089/vbz.2004.4.177
- Krone, O., Globig, A., Ulrich, R., Harder, T., Schinköthe, J., Herrmann, C., Gerst, S., Conraths, F. J., & Beer, M. (2018). White-Tailed Sea Eagle (*Haliaeetus albicilla*) Die-Off Due to Infection with Highly Pathogenic Avian Influenza Virus, Subtype H5N8, in Germany. *Viruses*, 10(9). doi:10.3390/v10090478
- Kuiken, T., Fouchier, R. A. M., & Koopmans, M. P. G. (2023). Being ready for the next influenza pandemic? *Lancet Infect Dis*, 23(4), 398-399. doi:10.1016/s1473-3099(23)00117-2
- Kwon, J.-H., Bertran, K., Lee, D.-H., Criado, M. F., Killmaster, L., Pantin-Jackwood, M. J., & Swayne, D. E. (2023). Diverse infectivity, transmissibility, and pathobiology of clade 2.3.4.4 H5Nx highly pathogenic avian influenza viruses in chickens. *Emerging Microbes & Infections*, 12(1), 2218945. doi:10.1080/22221751.2023.2218945
- Kwon, J. H., Noh, Y. K., Lee, D. H., Yuk, S. S., Erdene-Ochir, T. O., Noh, J. Y., Hong, W. T., Jeong, J. H., Jeong, S., Gwon, G. B., Song, C. S., & Nahm, S. S. (2017). Experimental infection with highly pathogenic H5N8 avian influenza viruses in the Mandarin duck (*Aix galericulata*) and domestic pigeon (*Columba livia domestica*). *Vet Microbiol*, 203, 95-102. doi:10.1016/j.vetmic.2017.03.003
- Lamb, R., & Krug, R. (2001). Orthomyxoviridae: The Viruses and Their Replication. In D. M. Knipe & P. M. Howley (Eds.), *Fields Virology* (Vol. 1). Philadelphia: Lippincott Williams & Wilkins.
- Lean, F. Z. X., Núñez, A., Banyard, A. C., Reid, S. M., Brown, I. H., & Hansen, R. D. E. (2022a). Gross pathology associated with highly pathogenic avian influenza H5N8 and H5N1 in naturally infected birds in the UK (2020–2021). *Vet Rec*, 190(1), e731. doi:https://doi.org/10.1002/vetr.731
- Lean, F. Z. X., Vitores, A. G., Reid, S. M., Banyard, A. C., Brown, I. H., Núñez, A., & Hansen, R. D. E. (2022b). Gross pathology of high pathogenicity avian influenza virus H5N1 2021–2022 epizootic in naturally infected birds in the United Kingdom. *One Health*, 14, 100392. doi:https://doi.org/10.1016/j.onehlt.2022.100392
- Lee, D. H., Bahl, J., Torchetti, M. K., Killian, M. L., Ip, H. S., DeLiberto, T. J., & Swayne, D. E. (2016). Highly Pathogenic Avian Influenza Viruses and Generation of Novel Reassortants, United States, 2014–2015. *Emerg Infect Dis*, 22(7), 1283-1285. doi:10.3201/eid2207.160048
- Lee, D. H., Bertran, K., Kwon, J. H., & Swayne, D. E. (2017a). Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. *J Vet Sci*, 18(S1), 269-280. doi:10.4142/jvs.2017.18.S1.269

- Lee, D. H., Criado, M. F., & Swayne, D. E. (2021). Pathobiological Origins and Evolutionary History of Highly Pathogenic Avian Influenza Viruses. *Cold Spring Harb Perspect Med*, 11(2). doi:10.1101/cshperspect.a038679
- Lee, D. H., Sharshov, K., Swayne, D. E., Kurskaya, O., Sobolev, I., Kabilov, M., Alekseev, A., Irza, V., & Shestopalov, A. (2017b). Novel Reassortant Clade 2.3.4.4 Avian Influenza A(H5N8) Virus in Wild Aquatic Birds, Russia, 2016. *Emerg Infect Dis*, 23(2), 359-360. doi:10.3201/eid2302.161252
- Lee, D. H., Torchetti, M. K., Hicks, J., Killian, M. L., Bahl, J., Pantin-Jackwood, M., & Swayne, D. E. (2018). Transmission Dynamics of Highly Pathogenic Avian Influenza Virus A(H5Nx) Clade 2.3.4.4, North America, 2014-2015. *Emerg Infect Dis*, 24(10), 1840-1848. doi:10.3201/eid2410.171891
- Lee, D. H., Torchetti, M. K., Winker, K., Ip, H. S., Song, C. S., & Swayne, D. E. (2015). Intercontinental Spread of Asian-Origin H5N8 to North America through Beringia by Migratory Birds. *J Virol*, 89(12), 6521-6524. doi:10.1128/jvi.00728-15
- Lee, I. H., Jin, S. Y., & Seo, S. H. (2017c). Genetic and pathogenic analysis of a novel reassortant H5N6 influenza virus isolated from waterfowl in South Korea in 2016. *Arch Virol*, 162(11), 3507-3510. doi:10.1007/s00705-017-3488-9
- Lee, Y. J., Kang, H. M., Lee, E. K., Song, B. M., Jeong, J., Kwon, Y. K., Kim, H. R., Lee, K. J., Hong, M. S., Jang, I., Choi, K. S., Kim, J. Y., Lee, H. J., Kang, M. S., Jeong, O. M., Baek, J. H., Joo, Y. S., Park, Y. H., & Lee, H. S. (2014). Novel reassortant influenza A(H5N8) viruses, South Korea, 2014. *Emerg Infect Dis*, 20(6), 1087-1089. doi:10.3201/eid2006.140233
- Letsholo, S. L., James, J., Meyer, S. M., Byrne, A. M. P., Reid, S. M., Settypalli, T. B. K., Datta, S., Oarabile, L., Kemolatlhe, O., Pebe, K. T., Mafonko, B. R., Kgotlele, T. J., Kumile, K., Modise, B., Thanda, C., Nyange, J. F. C., Marobela-Raborokgwe, C., Cattoli, G., Lamien, C. E., Brown, I. H., Dundon, W. G., & Banyard, A. C. (2022). Emergence of High Pathogenicity Avian Influenza Virus H5N1 Clade 2.3.4.4b in Wild Birds and Poultry in Botswana. *Viruses*, 14(12). doi:10.3390/v14122601
- Leyson, C., Youk, S.-s., Smith, D., Dimitrov, K., Lee, D.-H., Larsen, L. E., Swayne, D. E., & Pantin-Jackwood, M. J. (2019). Pathogenicity and genomic changes of a 2016 European H5N8 highly pathogenic avian influenza virus (clade 2.3.4.4) in experimentally infected mallards and chickens. *Virology*, 537, 172-185. doi:https://doi.org/10.1016/j.virol.2019.08.020
- Leyson, C. M., Youk, S., Ferreira, H. L., Suarez, D. L., & Pantin-Jackwood, M. (2021). Multiple Gene Segments Are Associated with Enhanced Virulence of Clade 2.3.4.4 H5N8 Highly Pathogenic Avian Influenza Virus in Mallards. *J Virol*, 95(18), e0095521. doi:10.1128/jvi.00955-21

- Li, M., Liu, H., Bi, Y., Sun, J., Wong, G., Liu, D., Li, L., Liu, J., Chen, Q., Wang, H., He, Y., Shi, W., Gao, G. F., & Chen, J. (2017). Highly Pathogenic Avian Influenza A(H5N8) Virus in Wild Migratory Birds, Qinghai Lake, China. *Emerg Infect Dis*, 23(4), 637-641. doi:10.3201/eid2304.161866
- Liang, Y., Hjulsgaard, C. K., Seekings, A. H., Warren, C. J., Lean, F. Z. X., Núñez, A., James, J., Thomas, S. S., Banyard, A. C., Slomka, M. J., Brown, I. H., & Larsen, L. E. (2022). Pathogenesis and infection dynamics of high pathogenicity avian influenza virus (HPAIV) H5N6 (clade 2.3.4.4b) in pheasants and onward transmission to chickens. *Virology*, 577, 138-148. doi:https://doi.org/10.1016/j.virol.2022.10.009
- Lindh, E., Lounela, H., Ikonen, N., Kantala, T., Savolainen-Kopra, C., Kauppinen, A., Österlund, P., Kareinen, L., Katz, A., Nokireki, T., Jalava, J., London, L., Pitkäpaasi, M., Vuolle, J., Punto-Luoma, A.-L., Kaarto, R., Voutilainen, L., Holopainen, R., Kalin-Mänttari, L., Laaksonen, T., Kiviranta, H., Pennanen, A., Helve, O., Laamanen, I., Melin, M., Tammiranta, N., Rimhanen-Finne, R., Gadd, T., & Salminen, M. (2023). Highly pathogenic avian influenza A(H5N1) virus infection on multiple fur farms in the South and Central Ostrobothnia regions of Finland, July 2023. *Euro Surveill*, 28(31), 2300400. doi:https://doi.org/10.2807/1560-7917.ES.2023.28.31.2300400
- Long, J. S., Mistry, B., Haslam, S. M., & Barclay, W. S. (2019). Host and viral determinants of influenza A virus species specificity. *Nat Rev Microbiol*, 17(2), 67-81. doi:10.1038/s41579-018-0115-z
- Lv, X., Li, X., Sun, H., Li, Y., Peng, P., Qin, S., Wang, W., Li, Y., An, Q., Fu, T., Qu, F., Xu, Q., Qin, R., Zhao, Z., Wang, M., Wang, Y., Wang, Y., Zeng, X., Hou, Z., Lei, C., Chu, D., Li, Y., & Chai, H. (2022). Highly Pathogenic Avian Influenza A(H5N8) Clade 2.3.4.4b Viruses in Satellite-Tracked Wild Ducks, Ningxia, China, 2020. *Emerg Inf Dis*, 28(5), 1039. doi:10.3201/eid2805.211580
- Lycett, S. J., Pohlmann, A., Staubach, C., Caliendo, V., Woolhouse, M., Beer, M., & Kuiken, T. (2020). Genesis and spread of multiple reassortants during the 2016/2017 H5 avian influenza epidemic in Eurasia. *Proc Natl Acad Sci U S A*, 117(34), 20814-20825. doi:10.1073/pnas.2001813117
- Ma, W., Kahn, R. E., & Richt, J. A. (2008). The pig as a mixing vessel for influenza viruses: Human and veterinary implications. *J Mol Genet Med*, 3(1), 158-166.
- Marandino, A., Tomás, G., Panzera, Y., Leizagoyen, C., Pérez, R., Bassetti, L., Negro, R., Rodríguez, S., & Pérez, R. (2023). Spreading of the High-Pathogenicity Avian Influenza (H5N1) Virus of Clade 2.3.4.4b into Uruguay. *Viruses*, 15(9). doi:10.3390/v15091906
- Marcelin, G., Sandbulte, M. R., & Webby, R. J. (2012). Contribution of antibody production against neuraminidase to the protection afforded by influenza vaccines. *Rev Med Virol*, 22(4), 267-279. doi:10.1002/rmv.1713

- Marchenko, V. Y., Susloparov, I. M., Kolosova, N. P., Goncharova, N. I., Shipovalov, A. V., Durymanov, A. G., Ilyicheva, T. N., Budatsirenova, L. V., Ivanova, V. K., Ignatyev, G. A., Ershova, S. N., Tulyahova, V. S., Mikheev, V. N., & Ryzhikov, A. B. (2015). Influenza A(H5N8) virus isolation in Russia, 2014. *Arch Virol*, 160(11), 2857-2860. doi:10.1007/s00705-015-2570-4
- Mariana, L., Alejandra, G.-G., Breno, M.-S., Diana, J., Patricia, B., Carlos, C.-M., Javier, J., Walter, S., Karl, P., Lady, A., Paulo, C.-C., Marcela, M. U., Martha, I. N., & Jesus, L. (2023). Highly pathogenic avian influenza A (H5N1) in marine mammals and seabirds in Peru. *bioRxiv*, 2023.2003.2003.531008. doi:10.1101/2023.03.03.531008
- McCrone, J. T., Woods, R. J., Martin, E. T., Malosh, R. E., Monto, A. S., & Luring, A. S. (2018). Stochastic processes constrain the within and between host evolution of influenza virus. *Elife*, 7. doi:10.7554/eLife.35962
- Molini, U., Aikukutu, G., Roux, J. P., Kemper, J., Ntahonshikira, C., Marruchella, G., Khaiseb, S., Cattoli, G., & Dundon, W. G. (2020). Avian Influenza H5N8 Outbreak in African Penguins (*Spheniscus demersus*), Namibia, 2019. *J Wildl Dis*, 56(1), 214-218.
- Molini, U., Yabe, J., Meki, I. K., Ouled Ahmed Ben Ali, H., Settypalli, T. B. K., Datta, S., Coetzee, L. M., Hamunyela, E., Khaiseb, S., Cattoli, G., Lamien, C. E., & Dundon, W. G. (2023). Highly pathogenic avian influenza H5N1 virus outbreak among Cape cormorants (*Phalacrocorax capensis*) in Namibia, 2022. *Emerg*, 12(1), 2167610. doi:10.1080/22221751.2023.2167610
- Muramoto, Y., Noda, T., Kawakami, E., Akkina, R., & Kawaoka, Y. (2013). Identification of novel influenza A virus proteins translated from PA mRNA. *J Virol*, 87(5), 2455-2462. doi:10.1128/jvi.02656-12
- Murawski, A., Fabrizio, T., Ossiboff, R., Kackos, C., Jeevan, T., Jones, J., Kandeil, A., Walker, D., Turner, J., Patton, C., Govorkova, E., Hauck, H., Mickey, S., Barbeau, B., Bommineni, R., Torchetti, M., Lantz, K., Kercher, L., Allison, A., Vogel, P., Walsh, M., & Webby, R. (2023). Highly pathogenic avian influenza A(H5N1) virus in a common bottlenose dolphin (*Tursiops truncatus*) in Florida. In: Research Square.
- Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D., & Fouchier, R. A. (2006). Global patterns of influenza a virus in wild birds. *Science*, 312(5772), 384-388. doi:10.1126/science.1122438
- Pantin-Jackwood, M. J., Costa-Hurtado, M., Bertran, K., DeJesus, E., Smith, D., & Swayne, D. E. (2017). Infectivity, transmission and pathogenicity of H5 highly pathogenic avian influenza clade 2.3.4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese. *Vet Res*, 48(1), 33. doi:10.1186/s13567-017-0435-4
- Pantin-Jackwood, M. J., Costa-Hurtado, M., Shepherd, E., DeJesus, E., Smith, D., Spackman, E., Kapczynski, D. R., Suarez, D. L., Stallknecht, D. E., & Swayne, D. E. (2016).

- Pathogenicity and Transmission of H5 and H7 Highly Pathogenic Avian Influenza Viruses in Mallards. *J Virol*, 90(21), 9967-9982. doi:10.1128/jvi.01165-16
- Pantin-Jackwood, M. J., & Swayne, D. E. (2007). Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. *Avian Dis*, 51(1 Suppl), 250-259. doi:10.1637/7710-090606r.1
- Pappas, C., Aguilar, P. V., Basler, C. F., Solórzano, A., Zeng, H., Perrone, L. A., Palese, P., García-Sastre, A., Katz, J. M., & Tumpey, T. M. (2008). Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. *Proc Natl Acad Sci U.S.A.*, 105(8), 3064-3069. doi:doi:10.1073/pnas.0711815105
- Peters, M., King, J., Wohlsein, P., Grund, C., & Harder, T. (2022). Genuine lethal infection of a wood pigeon (*Columba palumbus*) with high pathogenicity avian influenza H5N1, clade 2.3.4.4b, in Germany, 2022. *Vet Microbiol*, 270, 109461. doi:10.1016/j.vetmic.2022.109461
- Plotkin, J. B., & Dushoff, J. (2003). Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc Natl Acad Sci U S A*, 100(12), 7152-7157. doi:10.1073/pnas.1132114100
- Poen, M. J., Verhagen, J. H., Manvell, R. J., Brown, I., Bestebroer, T. M., van der Vliet, S., Vuong, O., Scheuer, R. D., van der Jeugd, H. P., Nolet, B. A., Kleyheeg, E., Müskens, G. J., Majoor, F. A., Grund, C., & Fouchier, R. A. (2016). Lack of virological and serological evidence for continued circulation of highly pathogenic avian influenza H5N8 virus in wild birds in the Netherlands, 14 November 2014 to 31 January 2016. *Euro Surveill*, 21(38). doi:10.2807/1560-7917.Es.2016.21.38.30349
- Pohlmann, A., King, J., Fusaro, A., Zecchin, B., Banyard, A. C., Brown, I. H., Byrne, A. M. P., Beerens, N., Liang, Y., Heutink, R., Harders, F., James, J., Reid, S. M., Hansen, R. D. E., Lewis, N. S., Hjulsgager, C., Larsen, L. E., Zohari, S., Anderson, K., Bröjer, C., Nagy, A., Savič, V., Borm, S. v., Steensels, M., Briand, F.-X., Swieton, E., Smietanka, K., Grund, C., Beer, M., & Harder, T. (2022). Has Epizootic Become Enzoitic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. *mBio*, 13(4), e00609-00622. doi:doi:10.1128/mbio.00609-22
- Pohlmann, A., Starick, E., Harder, T., Grund, C., Höper, D., Globig, A., Staubach, C., Dietze, K., Strebelow, G., Ulrich, R. G., Schinköthe, J., Teifke, J. P., Conraths, F. J., Mettenleiter, T. C., & Beer, M. (2017). Outbreaks among Wild Birds and Domestic Poultry Caused by Reassorted Influenza A(H5N8) Clade 2.3.4.4 Viruses, Germany, 2016. *Emerg Infect Dis*, 23(4), 633-636. doi:10.3201/eid2304.161949
- Pohlmann, A., Stejskal, O., King, J., Bouwhuis, S., Packmor, F., Ballstaedt, E., Hälterlein, B., Hennig, V., Stacker, L., Graaf, A., Hennig, C., Günther, A., Liang, Y., Hjulsgager, C., Beer, M., & Harder, T. (2023). Mass mortality among colony-breeding seabirds in the

- German Wadden Sea in 2022 due to distinct genotypes of HPAIV H5N1 clade 2.3.4.4b. *J Gen Virol*, 104(4). doi:10.1099/jgv.0.001834
- Postel, A., King, J., Kaiser, F. K., Kennedy, J., Lombardo, M. S., Reineking, W., de le Roi, M., Harder, T., Pohlmann, A., Gerlach, T., Rimmelzwaan, G., Rohner, S., Striewe, L. C., Gross, S., Schick, L. A., Klink, J. C., Kramer, K., Osterhaus, A., Beer, M., Baumgärtner, W., Siebert, U., & Becher, P. (2022). Infections with highly pathogenic avian influenza A virus (HPAIV) H5N8 in harbor seals at the German North Sea coast, 2021. *Emerg Microbes Infect*, 11(1), 725-729. doi:10.1080/22221751.2022.2043726
- Puryear, W., Sawatzki, K., Hill, N., Foss, A., Stone, J., Doughty, L., Walk, D., Gilbert, K., Murray, M., Cox, E., Patel, P., Mertz, Z., Ellis, S., Taylor, J., Fauquier, D., Smith, A., DiGiovanni, R., van de Guchte, A., Gonzalez-Reiche, A. S., Khalil, Z., van Bakel, H., Torchetti, M., Lantz, K., Lenocho, J., & Runstadler, J. (2023a). Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerg Infect Dis*, 29(4), 786. doi:10.3201/eid2904.221538
- Puryear, W., Sawatzki, K., Hill, N., Foss, A., Stone, J. J., Doughty, L., Walk, D., Gilbert, K., Murray, M., Cox, E., Patel, P., Mertz, Z., Ellis, S., Taylor, J., Fauquier, D., Smith, A., DiGiovanni, R. A., Jr., van de Guchte, A., Gonzalez-Reiche, A. S., Khalil, Z., van Bakel, H., Torchetti, M. K., Lantz, K., Lenocho, J. B., & Runstadler, J. (2023b). Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States. *Emerg Infect Dis*, 29(4), 786-791. doi:10.3201/eid2904.221538
- Reperant, L. A., van Amerongen, G., van de Bildt, M. W., Rimmelzwaan, G. F., Dobson, A. P., Osterhaus, A. D., & Kuiken, T. (2008). Highly pathogenic avian influenza virus (H5N1) infection in red foxes fed infected bird carcasses. *Emerg Infect Dis*, 14(12), 1835-1841. doi:10.3201/eid1412.080470
- Rijks, J. M., Leopold, M. F., Kühn, S., In 't Veld, R., Schenk, F., Brenninkmeijer, A., Lilipaly, S. J., Ballmann, M. Z., Kelder, L., de Jong, J. W., Courtens, W., Slaterus, R., Kleyheeg, E., Vreman, S., Kik, M. J. L., Gröne, A., Fouchier, R. A. M., Engelsma, M., de Jong, M. C. M., Kuiken, T., & Beerens, N. (2022). Mass Mortality Caused by Highly Pathogenic Influenza A(H5N1) Virus in Sandwich Terns, the Netherlands, 2022. *Emerg Infect Dis*, 28(12), 2538-2542. doi:10.3201/eid2812.221292
- Rosone, F., Bonfante, F., Sala, M. G., Maniero, S., Cersini, A., Ricci, I., Garofalo, L., Caciolo, D., Denisi, A., Napolitan, A., Parente, M., Zecchin, B., Terregino, C., & Scicluna, M. T. (2023). Seroconversion of a Swine Herd in a Free-Range Rural Multi-Species Farm against HPAI H5N1 2.3.4.4b Clade Virus. *Microorganisms*, 11(5), 1162. Retrieved from <https://www.mdpi.com/2076-2607/11/5/1162>
- Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., Leipe, D., McVeigh, R., O'Neill, K., Robbertse, B., Sharma, S., Soussov, V., Sullivan, J. P., Sun, L.,

- Turner, S., & Karsch-Mizrachi, I. (2020). NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database (Oxford)*, 2020. doi:10.1093/database/baaa062
- Shin, D. L., Siebert, U., Lakemeyer, J., Grilo, M., Pawliczka, I., Wu, N. H., Valentin-Weigand, P., Haas, L., & Herrler, G. (2019). Highly Pathogenic Avian Influenza A(H5N8) Virus in Gray Seals, Baltic Sea. *Emerg Infect Dis*, 25(12), 2295-2298. doi:10.3201/eid2512.181472
- Shriner, S. A., & Root, J. J. (2020). A Review of Avian Influenza A Virus Associations in Synanthropic Birds. *Viruses*, 12(11). doi:10.3390/v12111209
- Slomka, M. J., Puranik, A., Mahmood, S., Thomas, S. S., Seekings, A. H., Byrne, A. M. P., Núñez, A., Bianco, C., Mollett, B. C., Watson, S., Brown, I. H., & Brookes, S. M. (2019). Ducks Are Susceptible to Infection with a Range of Doses of H5N8 Highly Pathogenic Avian Influenza Virus (2016, Clade 2.3.4.4b) and Are Largely Resistant to Virus-Specific Mortality, but Efficiently Transmit Infection to Contact Turkeys. *Avian Dis*, 63(sp1), 172-180. doi:10.1637/11905-052518-Reg.1
- Smith, G. J., & Donis, R. O. (2015). Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. *Influenza Other Respir Viruses*, 9(5), 271-276. doi:10.1111/irv.12324
- Song, B. M., Kang, H. M., Lee, E. K., Jung, J., Kang, Y., Lee, H. S., & Lee, Y. J. (2015). Pathogenicity of H5N8 virus in chickens from Korea in 2014. *J Vet Sci*, 16(2), 237-240. doi:10.4142/jvs.2015.16.2.237
- Steinhauer, D. A. (1999). Role of Hemagglutinin Cleavage for the Pathogenicity of Influenza Virus. *Virology*, 258(1), 1-20. doi:https://doi.org/10.1006/viro.1999.9716
- Stieneke-Gröber, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H. D., & Garten, W. (1992). Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *Embo j*, 11(7), 2407-2414. doi:10.1002/j.1460-2075.1992.tb05305.x
- Stoimenov, G. M., Goujgoulova, G. V., Nikolov, B., Hristov, K., & Teneva, A. (2019). Pathological Changes in Natural Infection of Pheasants with Highly Pathogenic Avian Influenza A (H5N8) in Bulgaria. *J Vet Res*, 63(4), 497-502. doi:10.2478/jvetres-2019-0073
- Stokstad, E. (2023). Deadly avian flu reaches Galápagos Islands. *Science*. Retrieved from <https://www.science.org/content/article/deadly-avian-flu-reaches-galapagos-islands>
- Suarez, D. L., & Schultz-Cherry, S. (2000). Immunology of avian influenza virus: a review. *Dev Comp Immunol*, 24(2), 269-283. doi:https://doi.org/10.1016/S0145-305X(99)00078-6
- Suttie, A., Deng, Y. M., Greenhill, A. R., Dussart, P., Horwood, P. F., & Karlsson, E. A. (2019). Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. *Virus Genes*, 55(6), 739-768. doi:10.1007/s11262-019-01700-z

- Swayne, D. E. (2007). Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. *Avian Dis*, 51(1 Suppl), 242-249. doi:10.1637/7763-110706-regr.1
- Swayne, D. E. (2009). *Avian influenza*: John Wiley & Sons.
- Swayne, D. E., & Pantin-Jackwood, M. (2006). Pathogenicity of avian influenza viruses in poultry. *Dev Biol (Basel)*, 124, 61-67.
- Swayne, D. E., & Suarez, D. L. (2000). Highly pathogenic avian influenza. *Rev Sci Tech*, 19(2), 463-482. doi:10.20506/rst.19.2.1230
- Takadate, Y., Tsunekuni, R., Kumagai, A., Mine, J., Kikutani, Y., Sakuma, S., Miyazawa, K., & Uchida, Y. (2023). Different Infectivity and Transmissibility of H5N8 and H5N1 High Pathogenicity Avian Influenza Viruses Isolated from Chickens in Japan in the 2021/2022 Season. *Viruses*, 15(2). doi:10.3390/v15020265
- Takemae, N., Tsunekuni, R., Sharshov, K., Tanikawa, T., Uchida, Y., Ito, H., Soda, K., Usui, T., Sobolev, I., Shestopalov, A., Yamaguchi, T., Mine, J., Ito, T., & Saito, T. (2017). Five distinct reassortants of H5N6 highly pathogenic avian influenza A viruses affected Japan during the winter of 2016-2017. *Virology*, 512, 8-20. doi:10.1016/j.virol.2017.08.035
- Tammiranta, N., Isomursu, M., Fusaro, A., Nylund, M., Nokireki, T., Giussani, E., Zecchin, B., Terregino, C., & Gadd, T. (2023). Highly pathogenic avian influenza A (H5N1) virus infections in wild carnivores connected to mass mortalities of pheasants in Finland. *Infect Genet Evol*, 111, 105423. doi:10.1016/j.meegid.2023.105423
- Tanikawa, T., Fujii, K., Sugie, Y., Tsunekuni, R., Nakayama, M., & Kobayashi, S. (2022). Comparative susceptibility of mallard (*Anas platyrhynchos*) to infection with high pathogenicity avian influenza virus strains (Gs/Gd lineage) isolated in Japan in 2004-2017. *Vet Microbiol*, 272, 109496. doi:10.1016/j.vetmic.2022.109496
- Tanikawa, T., Sakuma, S., Yoshida, E., Tsunekuni, R., Nakayama, M., & Kobayashi, S. (2021). Comparative susceptibility of the common teal (*Anas crecca*) to infection with high pathogenic avian influenza virus strains isolated in Japan in 2004–2017. *Vet Microbiol*, 263, 109266. doi:https://doi.org/10.1016/j.vetmic.2021.109266
- Tarasiuk, K., Kycko, A., Knitter, M., Świętoń, E., Wyrostek, K., Domańska-Blicharz, K., Bocian, Ł., Meissner, W., & Śmietanka, K. (2022). Pathogenicity of highly pathogenic avian influenza H5N8 subtype for herring gulls (*Larus argentatus*): impact of homo- and heterosubtypic immunity on the outcome of infection. *Veterinary Research*, 53(1), 108. doi:10.1186/s13567-022-01125-x
- Tarasiuk, K., Kycko, A., Świętoń, E., Bocian, Ł., Wyrostek, K., & Śmietanka, K. (2023). Homo- and Heterosubtypic Immunity to Low Pathogenic Avian Influenza Virus Mitigates the Clinical Outcome of Infection with Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4.b in Captive Mallards (*Anas platyrhynchos*). *Pathogens*, 12(2), 217.

- Thorsson, E., Zohari, S., Roos, A., Banihashem, F., Bröjer, C., & Neimanis, A. (2023). Highly Pathogenic Avian Influenza A(H5N1) Virus in a Harbor Porpoise, Sweden. *Emerg Infect Dis*, 29(4), 852-855. doi:10.3201/eid2904.221426
- Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L. M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P. J., Gilbert, A. T., Chang, J., Guo, Z., Davis, C. T., Paulson, J. C., Stevens, J., Rupprecht, C. E., Holmes, E. C., Wilson, I. A., & Donis, R. O. (2013). New world bats harbor diverse influenza A viruses. *PLoS Pathog*, 9(10), e1003657. doi:10.1371/journal.ppat.1003657
- Uchida, Y., Mine, J., Takemae, N., Tanikawa, T., Tsunekuni, R., & Saito, T. (2019). Comparative pathogenicity of H5N6 subtype highly pathogenic avian influenza viruses in chicken, Pekin duck and Muscovy duck. *Transbound Emerg Dis*, 66(3), 1227-1251. doi:10.1111/tbed.13141
- United States Fish & Wildlife Service. (2023). California condors & HPAI update. Retrieved from <https://www.fws.gov/program/california-condor-recovery/southwest-california-condor-flock-hpai-information-updates-2023>
- United States Department of Agriculture & USGS. (2017). July 2016 - June 2017 wild bird highly pathogenic avian influenza cases in the United States. Retrieved from https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/ai/uspo_sitivecases17.pdf.
- van den Brand, J. M. A., Verhagen, J. H., Veldhuis Kroeze, E. J. B., van de Bildt, M. W. G., Bodewes, R., Herfst, S., Richard, M., Lexmond, P., Bestebroer, T. M., Fouchier, R. A. M., & Kuiken, T. (2018). Wild ducks excrete highly pathogenic avian influenza virus H5N8 (2014-2015) without clinical or pathological evidence of disease. *Emerg Microbes Infect*, 7(1), 67. doi:10.1038/s41426-018-0070-9
- Verhagen, J. H., Fouchier, R. A. M., & Lewis, N. (2021). Highly Pathogenic Avian Influenza Viruses at the Wild-Domestic Bird Interface in Europe: Future Directions for Research and Surveillance. *Viruses*, 13(2). doi:10.3390/v13020212
- Verhagen, J. H., van der Jeugd, H. P., Nolet, B. A., Slaterus, R., Kharitonov, S. P., de Vries, P. P., Vuong, O., Majoor, F., Kuiken, T., & Fouchier, R. A. (2015). Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways. *Euro Surveill*, 20(12), 21069. doi:https://doi.org/10.2807/1560-7917.ES2015.20.12.21069
- Vreman, S., Kik, M., Germeraad, E., Heutink, R., Harders, F., Spierenburg, M., Engelsma, M., Rijks, J., van den Brand, J., & Beerens, N. (2023). Zoonotic Mutation of Highly Pathogenic Avian Influenza H5N1 Virus Identified in the Brain of Multiple Wild Carnivore Species. *Pathogens*, 12(2). doi:10.3390/pathogens12020168

- Waldenström, J., van Toor, M., Lewis, N., Lopes, S., Javakhishvili, Z., Muzika, D., Fouchier, R. A. M., & Brouwer, A. (2022). Active wild bird surveillance of avian influenza viruses, a report. *EFSA support. publ.*, 19(12). doi:10.2903/sp.efsa.2022.EN-7791
- Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev*, 56(1), 152-179. doi:10.1128/mr.56.1.152-179.1992
- Webster, R. G., & Govorkova, E. A. (2014). Continuing challenges in influenza. *An. N. Y. Acad Sci*, 1323(1), 115-139. doi:https://doi.org/10.1111/nyas.12462
- Webster, R. G., Yakhno, M., Hinshaw, V. S., Bean, W. J., & Copal Murti, K. (1978). Intestinal influenza: Replication and characterization of influenza viruses in ducks. *Virology*, 84(2), 268-278. doi:https://doi.org/10.1016/0042-6822(78)90247-7
- Wiley, D. C., & Skehel, J. J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem*, 56(1), 365-394. doi:10.1146/annurev.bi.56.070187.002053
- Wille, M., Lisovski, S., Risely, A., Ferenczi, M., Roshier, D., Wong, F. Y. K., Breed, A. C., Klaassen, M., & Hurt, A. C. (2019). Serologic Evidence of Exposure to Highly Pathogenic Avian Influenza H5 Viruses in Migratory Shorebirds, Australia. *Emerg Infect Dis*, 25(10), 1903-1910. doi:10.3201/eid2510.190699
- Wilson, H. M., Hall, J. S., Flint, P. L., Franson, J. C., Ely, C. R., Schmutz, J. A., & Samuel, M. D. (2013). High seroprevalence of antibodies to avian influenza viruses among wild waterfowl in Alaska: implications for surveillance. *PLoS One*, 8(3), e58308. doi:10.1371/journal.pone.0058308
- Wilson, I. A., & Cox, N. J. (1990). Structural basis of immune recognition of influenza virus hemagglutinin. *Annu Rev Immunol*, 8, 737-771. doi:10.1146/annurev.iy.08.040190.003513
- Wilson, I. A., Skehel, J. J., & Wiley, D. C. (1981). Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature*, 289(5796), 366-373. doi:10.1038/289366a0
- World Health Organization. (2020). *Antigenic and genetic characteristics of zoonotic influenza A viruses and development of candidate vaccine viruses for pandemic preparedness*. Geneva: WHO.
- World Organisation for Animal Health. (2023). Infection with high pathogenicity avian influenza viruses. *Terrestrial Animal Health Code*. Retrieved from https://www.woah.org/fileadmin/Home/eng/Health_standards/tahc/2023/chapitre_avian_influenza_viruses.pdf
- World Organization for Animal Health. (2002). International animal health code. Retrieved from www.oieint/eng/normes/MCode/A_00003htm

- Wu, H., Peng, X., Xu, L., Jin, C., Cheng, L., Lu, X., Xie, T., Yao, H., & Wu, N. (2014a). Novel reassortant influenza A(H5N8) viruses in domestic ducks, eastern China. *Emerg Infect Dis*, 20(8), 1315-1318. doi:10.3201/eid2008.140339
- Wu, Y., Wu, Y., Tefsen, B., Shi, Y., & Gao, G. F. (2014b). Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol*, 22(4), 183-191. doi:10.1016/j.tim.2014.01.010
- Xu, X., Subbarao, Cox, N. J., & Guo, Y. (1999). Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology*, 261(1), 15-19. doi:10.1006/viro.1999.9820
- Youk, S., Torchetti, M. K., Lantz, K., Lenocho, J. B., Killian, M. L., Leyson, C., Bevins, S. N., Dilione, K., Ip, H. S., Stallknecht, D. E., Poulson, R. L., Suarez, D. L., Swayne, D. E., & Pantin-Jackwood, M. J. (2023a). H5N1 highly pathogenic avian influenza clade 2.3.4.4b in wild and domestic birds: Introductions into the United States and reassortments, December 2021–April 2022. *Virology*, 587, 109860. doi:https://doi.org/10.1016/j.virol.2023.109860
- Youk, S., Torchetti, M. K., Lantz, K., Lenocho, J. B., Killian, M. L., Leyson, C., Bevins, S. N., Dilione, K. E., Ip, H. S., Stallknecht, D. E., Poulson, R. L., Suarez, D. L., Swayne, D. E., & Pantin-Jackwood, M. J. (2023b). H5N1 highly pathogenic avian influenza clade 2.3.4.4b in wild and domestic birds: Introductions into the United States and reassortments, December 2021–April 2022. *Virology*, 587, 109860. doi:https://doi.org/10.1016/j.virol.2023.109860
- Zhao, K., Gu, M., Zhong, L., Duan, Z., Zhang, Y., Zhu, Y., Zhao, G., Zhao, M., Chen, Z., Hu, S., Liu, W., Liu, X., Peng, D., & Liu, X. (2013). Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China. *Vet Microbiol*, 163(3-4), 351-357. doi:10.1016/j.vetmic.2012.12.025

IV. Circulation of diverse low pathogenic avian influenza (AI) viruses in hunted wild birds and wetlands during the 2021-2022 AI epidemic season in Italy

Abstract

Avian influenza viruses (AIVs), which circulate endemically in wild aquatic birds, pose a significant threat to poultry and raise concerns for their zoonotic potential. From August 2021 to April 2022, a multi-site cross-sectional study involving active AIV epidemiological monitoring was conducted in wetlands of the Emilia-Romagna region, Northern Italy, adjacent to densely populated poultry areas. A total of 129 cloacal swab samples (CSs) and 407 avian fecal droppings samples (FDs) were collected, with seven CSs (5.4%) and four FDs (1%) testing positive for AIV matrix gene through rRT-PCR. A COI-barcoding protocol was applied to recognize the species of origin of AIV-positive FDs. Multiple LPAIV subtypes were identified, and five of these were isolated, including an H5N3 LPAIV, an H1N1 and three H9N2 in wild ducks. Following whole-genome sequencing, phylogenetic analyses of the hereby obtained strains showed close genetic relationships with AIVs collected in countries along the Black Sea/Mediterranean migratory flyway. Notably, none of the analyzed gene segments was genetically related to HPAI H5N1 viruses of clade 2.3.4.4b isolated from Italian poultry during the concurrent 2021-2022 epidemic. Overall, the detected AIV genetic diversity emphasizes the necessity for ongoing monitoring in wild hosts using diverse sampling strategies and whole-genome sequencing.

4.1. Introduction

Avian influenza viruses (AIVs, family *Orthomyxoviridae*, genus *Alphainfluenzavirus*) (Walker *et al.*, 2020) infect birds and, occasionally mammals, including humans, posing a great risk for animal and public health (Horman *et al.*, 2018). Wild aquatic birds of the Anseriformes and Charadriiformes orders (ducks, geese, gulls, and shorebirds) serve as the natural reservoir for low pathogenic (LP) AIVs (Stallknecht & Brown, 2008). Through flyways, AIV-infected migratory birds facilitate the global spread of the virus and contribute to its genetic evolution and genomic reassortment (Bevins *et al.*, 2022; Lo *et al.*, 2022; The Global Consortium for H5N8 and Related Influenza Viruses, 2016). In poultry, the outcomes of AIV infections vary based on the host and virus-related factors. Gallinaceous birds typically exhibit mild or no disease when infected with LPAIVs (Pantin-Jackwood & Swayne, 2009). However, if H5 or H7 AIV subtypes are involved, the virus can evolve into high pathogenic (HP) AI and cause severe outbreaks with high mortality rates in both wild birds and poultry (Banks *et al.*, 2001; Röhm *et al.*, 1995). Since their first detection in 1996 in China (Xu *et al.*, 1999), HPAIVs belonging to the A/goose/Guangdong/1/1996 (Gs/Gd) (H5N1) genetic lineage have found their way back to their natural reservoir, the wild waterfowl, and have exhibited unprecedented global spread, genetic drift, and tendency to reassort with LPAIVs (Chen *et al.*, 2005; Ellis *et al.*, 2004; Vijaykrishna *et al.*, 2008). Over time, the HPAIVs of H5 clade 2.3.4.4 have become dominant, and various subclades (a-h) have been identified according to the World Health Organization (World Health Organization, 2018). In October 2020, a novel H5N8 HPAIV of clade 2.3.4.4b was detected in the Netherlands in wild mute swans (Beerens *et al.*, 2021) and subsequently spread throughout Europe, causing the most extensive and severe AI epizootic ever recorded in wild birds, poultry, and captive birds (European Food Safety Authority *et al.*, 2023a; European Food Safety Authority *et al.*, 2022c). In Italy, between October 2021 and April 2022, 317 outbreaks of H5N1 HPAIVs of clade 2.3.4.4b were reported, resulting in the culling of over 8 million birds culled and impacting all poultry categories (European Food Safety Authority *et al.*, 2022c). Most of these outbreaks were concentrated in the northeastern part of the country, where densely populated poultry areas (DPPAs) are situated nearby wetlands, posing a high risk of AIV introduction via migratory aquatic birds (Gobbo *et al.*, 2021). Recent detections of HPAIVs in healthy migratory and resident wild ducks suggest a potential enzootic circulation of the virus in wild populations (Pohlmann *et al.*, 2022). Field and experimental studies have pinpointed Eurasian wigeons (*Mareca penelope*), mallards (*Anas platyrhynchos*) and common teals (*Anas crecca*) as the most suitable long-distance vectors of HPAIVs along migratory flyways (Gobbo *et al.*, 2021; Lewis *et al.*, 2021; Tarasiuk *et al.*, 2023). The shift in HPAIVs ecology within wild birds has led to the generation of a wide range of

viral genotypes, with gene segments frequently exchanged with LPAIVs circulating in waterbirds (Lycett *et al.*, 2019). Cases of HPAIV infection in wild and domestic mammals are also being reported, posing a potential risk of AI transmission to humans (Animal and Plant Health Inspection Service, 2023a; European Food Safety Authority *et al.*, 2023c; Wang *et al.*, 2021). Additionally, global human population growth and natural habitat loss have created new opportunities for spillover events at the wildlife-domestic animal-human interface (Ellwanger & Chies, 2021). Therefore, understanding AIV circulation patterns in local ecosystem is crucial, providing fundamental information for AIV risk assessment and identifying potential hotspots for novel the emergence of novel strains (Hoye *et al.*, 2021).

This cross-sectional study investigated the occurrence of AIVs in hunted or found death wild birds and avian fecal droppings collected in wetlands located in the Emilia-Romagna region, Northern Italy, during the 2021-2022 epidemic waves of H5 HPAI in poultry. The study area, characterized by DPPAs adjacent to critical wetlands for waterfowl migrating along the Black Sea/Mediterranean flyway, was selected for AIV monitoring using a combination of diverse sampling strategies. The detected AIVs were isolated and characterized utilizing whole-genome sequencing in order to perform sequence analysis and investigate phylogenetic relationships with previously detected isolates.

4.2. Materials and methods

4.2.1. Background

A cross-sectional study on active epidemiological monitoring was conducted from August 2021 to April 2022. Cloacal swab samples (CSs) were collected from hunted or found dead wild birds, and avian fecal droppings (FDs) were obtained in wetlands located in Northern Italy, near DPPAs. The sampling sites comprised three inland wetlands and one coastal wetland, all situated in the Emilia-Romagna region (Figure 1). These sites were chosen due to the presence of numerous overwintering and breeding waterfowl species migrating along the Eurasian/Mediterranean flyway that congregate and intermingle with resident populations of wild birds. Specifically, the "Campotto-Bassarone wetland" (44.589983, 11.826349) is a 600-hectare inland artificial freshwater impoundment at the confluence of the Idice and Reno rivers, located within the Delta Po Regional Park in the Ferrara province. It is considered as one of Italy's most important areas for aquatic birdlife, especially for breeding populations. It was designated as a Wetland of International Importance under the Ramsar Convention in 1949 and is part of the Site of Community Importance "Valli di Argenta" within the European Natura 2000 Network. The "Boscoforte peninsula" (44.550177, 12.154855) is a coastal wetland also within the Delta Po

Regional Park in the Ferrara province. This area is densely populated by migratory and resident shorebirds due to its proximity to the Adriatic Sea. It was chosen specifically to tentatively broaden the range of AIV hosts sampled, considering that shorebirds are often underrepresented in AIV monitoring activities in Europe (European Food Safety Authority *et al.*, 2021a; European Food Safety Authority *et al.*, 2022a). Additionally, a private waterfowl hunting preserve in the Bologna province was included in the study as the third location. FDs were collected from “Campotto-Bassarone wetland”, “Boscoforte peninsula”, and the private hunting ground from August 2021 to April 2022, while CSs were obtained from October 2021 to January 2022 from hunted or found death wild birds in the hunting preserve or within the Delta Po Regional Park.

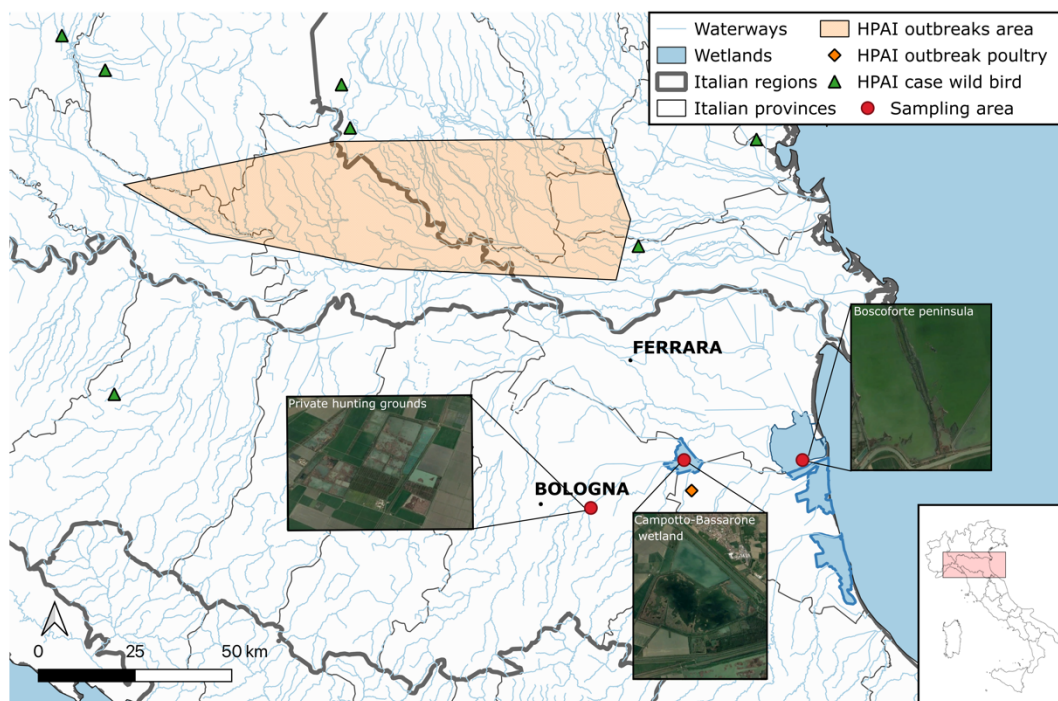


Figure 1. Study area designated for monitoring of avian influenza (AI) viruses in wild birds and wetlands in the Emilia Romagna region, Northern Italy. Poultry outbreaks (orange area and orange diamond) and cases in wild birds during the 2021-2022 AI epidemic are reported. Created with QGIS v.3.6.0.

Access to all sampling locations was granted by relevant authorities or owners. The wild aquatic birds hunted or found dead included in this study were sampled within the application of the National AI Surveillance Plan 2021 (<https://www.izsvenezie.it/documenti/temi/influenza-aviaria/piani-sorveglianza/piano->

nazionale-influenza-aviaria-2021.pdf, accessed on October 2021) and the Commission Delegated Regulation (EU) 2020/689. AI surveillance activities were carried out on the behalf of the Veterinary Services of the Local Health Unit of Imola (A.U.S.L. of Imola) by sampling birds provided by local hunters or found dead by local ornithologists. Game species were hunted according to the National Hunting law 157/1992, without the necessity of any additional permits.

4.2.2. *Fecal dropping samples collection*

To estimate the positivity rate of AIVs during different phases of the bird migration phenology, samplings were conducted every two weeks during the southward autumn migration (August – October) and upward spring migration (February – April). Additionally, samples were collected once a month during the remaining months, totaling of 13 field trips. Collection of fresh FDs was carried out at roosting sites of waterbirds (e.g. ducks, ibises, geese, flamingos, gulls, herons, shorebirds), identified with the assistance of local ornithologists, along 5 to 10 m in length on banks or scattered islets, depending on the location. The avian species of origin of the FDs was determined through direct observation and/or photographic records of birds in act of defecation. When identification by direct observation was not possible, phenotypic characteristics of the fecal samples were utilized to recognize the bird group without species-level identification. FDs were collected with a minimum distance of 15 cm from one to another to avoid sampling the same bird more than once. Samples were collected in duplicate using sterile rayon-tipped wooden swabs (Kaltex, Saonara, Italy). The portion of the FD with visible uric acids was excluded and, whenever feasible, only the upper-central portion of the feces was collected to prevent potential contamination by PCR inhibitors from the soil. The swabs were then immersed in a sterile tube containing 1.0 mL of PBS/glycerol (9/1) buffer supplemented with antibiotics and antimycotic agents (10000 IU/mL penicillin, 10 mg/mL streptomycin, 50 mg/mL gentamycin, and 10000 IU/mL nystatin). FDs were kept on ice during the sampling activities and transportation, then stored at -80°C until analysis.

4.2.3. *Cloacal swab samples collection*

Due to the unpredictable number of water birds hunted or found dead, an opportunistic sampling approach without a predefined sample size estimation was set. For each sampled bird, information on species, sex, and age classes (juvenile of the year or adult) was recorded. Cloacal swabs were collected using a sterile rayon-tipped wooden swab then immersed in 600 µL of PBS/glycerol buffer supplemented with antibiotics and antimycotic agents, as previously

detailed. Samples were kept on ice during the sampling activities and transportation, then stored at -80°C until analysis.

4.2.4. Sample processing and molecular detection of AIVs

FDs were pre-processed to increase nucleic acids yields. Avian feces have been described as challenging matrices for nucleic acids extraction due to the presence of molecules that can interfere with the procedure, such as uric acid, bile salts, complex polysaccharides, nucleases (Regnaut *et al.*, 2006; Vo & Jedlicka, 2014). After thawing on ice, FDs were vigorously mixed using a vortex mixer for an average of one minute per sample or until the fecal pellet was homogeneously re-suspended in the PBS/ glycerol buffer. The samples were then pooled per sampling date and, whenever possible, per taxa, in groups of five, by aliquoting 100 µL of the suspension into a new 1.5 mL tube. The pooled FD was further diluted in 1 mL of PBS. Similarly, CSs were thawed on ice, vortexed for one minute, and pooled in groups of 5 per species by aliquoting 100 µL from each CS into a new 1.5 mL tube. Each FD and CS pooled sample was then centrifuged at 11,000 *g* for 30 seconds at 4°C.

RNA extraction was performed on 100 µL of supernatant from each pool using NucleoSpin® RNA (MACHEREY-NAGEL, Düren, Germany), following manufacturer's instruction for cultured cells and tissue. Intype® IC-RNA (INDICAL BIOSCIENCE GmbH, Leipzig, Germany), an exogenous RNA template, was employed as an internal control for RNA extraction. This control was used to detect the presence of potential RNA inhibitors and therefore to exclude false-negative results. A multiplex real-time RT-PCR (rRT-PCR) protocol was applied for the detection of the matrix (M) gene of AIV (Spackman *et al.*, 2002) and the RNA template (Istituto Zooprofilattico Sperimentale delle Venezie). QuantiTect® Multiplex RT-PCR kit (QIAGEN®, Hilden, Germany) was used on a Corbett Research Rotor-Gene™ RG-3000 platform. If the RNA template was not detected by the rRT-PCR, the RNA extraction process was repeated by adding an additional 500 µL to the supernatant of the inhibited pooled sample. Pools positive for AIVs were traced back to the original samples, which underwent individual RNA extraction and subsequent multiplex rRT-PCR analysis. Further characterization of AIV-positive samples was performed at the Istituto Zooprofilattico delle Venezie, the EU/WOAH/National Reference Laboratory for Avian Influenza and Newcastle disease virus. For HA typing, rRT-PCRs targeting H5, H7 and H9 were applied (Panzarin *et al.*, 2022; Slomka *et al.*, 2007b; Slomka *et al.*, 2009). A conventional One-Step RT-PCR was further used to subtype the hemagglutinin cleavage site by Sanger sequencing (Slomka *et al.*, 2007a). For other HA typing and for NA typing, rRT-PCRs were conducted using multiple oligonucleotide sets (Hassan *et al.*, 2022; James *et al.*, 2018).

4.2.5. *Virus isolation in specific pathogen free (SPF) embryonated eggs*

To assess the viability of the detected AIVs, samples that tested positive to viral molecular detection were inoculated in 9 to 11-day-old SPF embryonated chicken eggs (Istituto Zooprofilattico Sperimentale delle Venezie, 2022; World Organisation for Animal Health, 2021). For FDs, whenever possible, virus isolation was attempted using the duplicate sample that had not been previously thawed for molecular biology analyses. In brief, 500 μ L of FD and CS supernatants were filtered through 0.22- μ m Millipore filters and diluted in 1 ml of PBS with antibiotics and antimycotic agents. After centrifugation at 8,000 *rpm* for 10 minutes, 200 μ L of the supernatant was inoculated into the allantoic cavity of 9 to 11-day-old SPF embryonated chicken eggs (five eggs per sample). The eggs were incubated at 37°C until embryo death was observed or up to seven days. Inoculated SPF eggs were then chilled for a minimum of four hours; the harvested allantoic fluids were submitted to hemagglutination assay (HA) and hemagglutination inhibition (HI) testing.

4.2.6. *Genome sequencing and phylogenetic analysis*

The obtained AIV isolates underwent further RNA extraction using the NucleoSpin® RNA (MACHEREY-NAGEL, Düren, Germany), following the manufacturer's instruction for cultured cells and tissue. The extracted RNA was subjected to whole genome sequencing using a Next-Generation Sequencing (NGS) technology on the Illumina MiSeq System (Illumina Inc., San Diego, U.S.A.). The preparation of libraries, assessment of their quality and quantity, and sequencing reactions were conducted as described elsewhere (Gobbo *et al.*, 2021).

For phylogenetic analyses, the viral sequences were aligned against and compared with previously published AIV sequences available on the Global Initiative on Sharing All Influenza Data (GISAID) Epi-Flu Database (<https://platform.epicov.org/>). Viruses showing high sequence identity to the isolates hereby obtained were included in the analysis. Multiple sequence alignment was performed using the Multiple Alignment with Fast Fourier Transformation (MAFFT) online service with default parameters (Kato & Standley, 2013). Alignments were manually trimmed in Geneious Prime® v.2022.2.2, and gene segment lengths used for phylogenetic analysis were as follows: PB2 (2277), PB1 (2271), PA (2148), HA1 (1648), HA5 (1645), HA9 (1627), NP (1495), NA1 (1336), NA2 (1408), NA3 (1408), M1 (757), and NS1 (691). The best partition scheme, substitution model selection based on the Bayesian information criterion (BIC), and maximum likelihood phylogenetic reconstruction was performed on the IQ-TREE web server (Kalyaanamoorthy *et al.*, 2017; Trifinopoulos *et al.*, 2016) separately for each gene (PB2, PB1, PA, HA, NP, NA, M, NS). The robustness of inferred clades was evaluated using 1,000 ultrafast

bootstrap replicates. Branches with bootstrap (BS) values ≥ 70 were considered reliable. The phylogenetic tree was visualized by using FigTree v.1.4.2.

4.2.7. Sequence analysis

To assess the presence of molecular markers associated with AIV zoonotic risk, the full-length sequences of all eight AIV gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) were analyzed. The sequences were submitted to the FluSurver mutations app of the GISAID Initiative (Khare *et al.*, 2021) and manually screened based on inventories of markers affecting AIV biological properties (Cáceres *et al.*, 2021; Suttie *et al.*, 2019). The identified amino acid substitutions are referred to using H3 numbering. Nucleotide similarity searches were performed on the GISAID Epi-Flu database using Basic Local Alignment Search Tool (BLAST nucleotide - BLASTn) to compare the gene segments obtained in this study with those of previously published strains. Following MAFFT alignment, amino acid substitutions of the HA and NA gene segments of the H1N1, H5N3 LPAI, and the H9N2 strains were annotated in comparison to their closest relatives.

4.2.8. Cytochrome oxidase I (COI) barcoding for host identification

FDs that tested positive to AIV molecular detection were analyzed for identification or confirmation of the avian species of origin using a PCR targeting the COI mitochondrial DNA (Cheung *et al.*, 2009). After thawing the sample on ice, the fecal pellet was resuspended into PBS/glycerol using a vortex mixer. Genomic DNA from the fecal suspension was extracted using the QIAamp® Fast DNA Stool mini kit (QIAGEN®, Hilden, Germany) following the manufacturer's instructions and eluted in a final volume of 100 μ L. A 749-bp region of the COI gene was amplified by PCR using primers BirdF1 (5'-TTCTCCAACCACAAAGACATTGGCAC-3') and BirdR1 (5'-ACGTGGGAGATAATTCCAAATCCTG-3') (Hebert *et al.*, 2004). PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Waltham, MA, USA) and sequencing was performed by a commercial sequencing service (Macrogen Europe, Amsterdam, the Netherlands). Nucleotide sequences were edited and assembled using Geneious Prime® v.2022.2.2, and then queried against the Barcode of Life Data System database (<http://boldsystems.org>, accessed on 19th October 2023) to attempt taxonomic species identification (Ratnasingham & Hebert, 2007).

4.3. Results

4.3.1. Wild bird population sampled

Overall, 536 samples were collected for successive AIV molecular detection. Of these, 407 were FDs collected between August 2021 and April 2022. As identified through direct or photographic observation of birds in act of defecation, the majority of the FDs belonged to ducks (249/407, 61.2%), followed by geese (46/407, 11.3%), shorebirds (34/407, 8.3%), herons (30/407, 7.3%), gulls (19/407, 4.6%), ibises (18/407, 4.4%), rails (10/407, 2.4%) and flamingos (1/407, 0.2%) (Figure 2A). A total of 129 CSs were obtained from waterbirds hunted or found dead, from October 2021 to January 2022. Eighty-five Eurasian teals (*A. crecca*), 22 Northern shovelers (*Spatula clypeata*), 8 mallards (*A. platyrhynchos*), 5 Northern lapwings (*Vanellus vanellus*), 3 greylag geese (*Anser anser*), 2 gadwalls (*Mareca strepera*), 2 common snipes (*Gallinago gallinago*), 1 wigeon (*M. penelope*), and 1 spotted redshank (*Tringa erythropus*) were sampled. Of these, 59.7% were identified as females (77/129), 38% (49/129) as males, and 3 (2.3%) were undetermined (Figure 2B). Concerning age classes, 47.3% (61/129) of the birds were aged as juveniles (first calendar year) and 52.7% (68/129) as adults (Figure 2C).

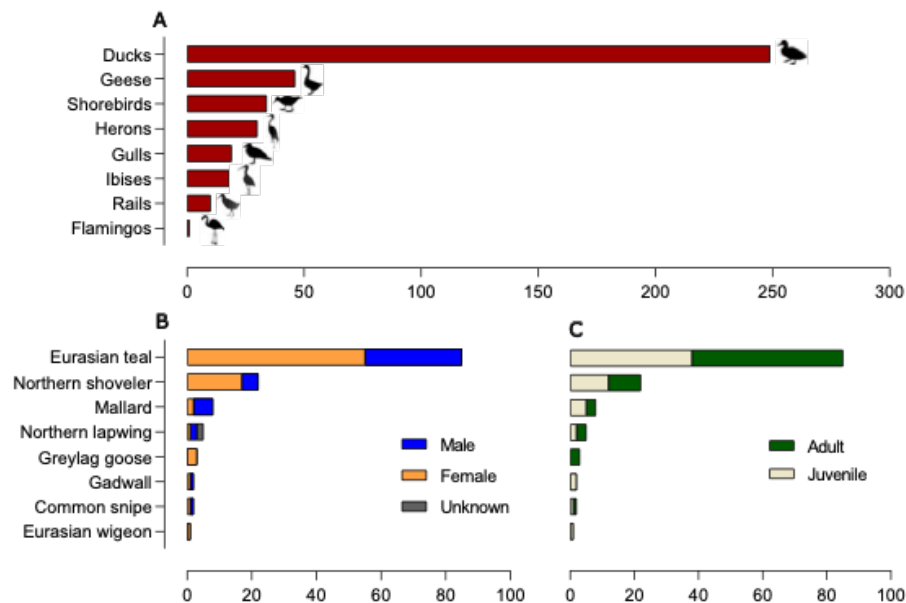


Figure 2. Wild bird population sampled. (a) Bird taxa/number of fecal droppings collected in wetlands; (b) Species/number of hunted or found dead wild birds included in the study according to sex; (c) Species/number of hunted or found dead wild birds included in the study according to age classes.

4.3.2. AIVs detection in fecal droppings and cloacal swabs

Overall, 11 out of 536 samples (2%) tested positive for AIV at rRT-PCR for M gene. Of these, four AIVs were detected in FDs (4/407, 1%) (Table 1) and seven in CSs (7/129, 5.4%) (Table 2). Positive samples were collected between October and November 2021, during the late autumn migration and wintering period. AIV detections were further subtyped, virus isolation was attempted, and FDs species of origin were successfully identified through COI barcoding. With respect to FDs (Table 1), the following AIVs were detected: H5N3 LPAIV and HxN9 in two wild mallards (*A. platyrhynchos*); H9N2 in a graylag goose (*A. anser*); HxN2 in a common snipe (*G. gallinago*). FDs of ibises, gulls, herons, flamingos, and rails did not yield positive results for AIV molecular detection. Among AIVs from FDs, only the H5N3 LPAIV strain was successfully isolated (A/mallard/Italy/22VIR4203-2/2021 (H5N3)) and subsequently subjected to NGS. With respect to CSs (Table 2), the following AIVs subtypes were detected in Eurasian teals (*A. crecca*): H1N1 (in one juvenile female), HxN9 (in one juvenile male), H9N2 (in one juvenile male and three juvenile females), H6N1 (in one adult male). Of these, four viruses were successfully isolated and subjected to NGS, namely A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1), A/teal/Italy/1856-7_22VIR4622-7/2021 (H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021 (H9N2), and A/teal/Italy/1821-14_22VIR4622-3/2021 (H9N2).

Table 1. Sample size, positivity rate, and AIV subtypes identified in fecal dropping samples in three wetlands of the Emilia-Romagna region, Northern Italy. Phases of the bird migration phenology and bird taxa of origin of the samples are detailed.

Variable	Positive/Sample size (Positivity rate %)	AIV subtype detected
Bird migration phenology period		
Autumn migration [†]	2/203 (1)	HxN9, H5N3
Wintering ^{††}	2/134 (1.5)	H9N2, HxN2
Spring migration ^{†††}	0/70 (0)	n.a. [°]
Bird of origin of the fecal dropping		
Ducks	2/249 (0.8)	HxN9, H5N3
Geese	1/46 (2.1)	H9N2
Ibises	0/18 (0)	n.a.
Gulls	0/19 (0)	n.a.
Hérons	0/30 (0)	n.a.
Flamingos	0/1 (0)	n.a.
Shorebirds	1/34 (3.0)	HxN2
Rails	0/10 (0)	n.a.
Total	4/407 (1.0)	HxN9, H5N3, H9N2, HxN2

[†]August – October; ^{††}November-January; ^{†††}February-April.

[°]n.a., not applicable.

Table 2. Sample size, positivity rate, and AIV subtypes identified in cloacal swabs in waterbirds hunted or found dead in wetlands of the Emilia-Romagna region, Northern Italy.

Bird species	Positive/Sample size (Positivity rate)	AIV subtype detected
<i>Anseriformes order</i>		
Common teal (<i>Anas crecca</i>)	7/85 (8.2)	H1N1, HxN9, H9N2, H6N1
Mallard (<i>Anas platyrhynchos</i>)	0/8 (0)	n.a. ^o
Greylag goose (<i>Anser anser</i>)	0/3 (0)	n.a.
Eurasian wigeon (<i>Mareca penelope</i>)	0/1 (0)	n.a.
Gadwall (<i>Mareca strepera</i>)	0/2 (0)	n.a.
Northern shoveler (<i>Spatula clypeata</i>)	0/22 (0)	n.a.
<i>Charadriiformes order</i>		
Common snipe (<i>Gallinago gallinago</i>)	0/2 (0)	n.a.
Spotted redshank (<i>Tringa erythropus</i>)	0/1 (0)	n.a.
Northern lapwing (<i>Vanellus vanellus</i>)	0/5 (0)	n.a.
Total	7/129 (5.4)	H1N1, HxN9, H9N2, H6N1

^on.a., not applicable.

4.3.3. Sequence analysis of the isolates and nucleotide similarity searches

The HA cleavage site of the H5N3 strain (amino acid residues at positions 321-329: PQRETR↓GLF) did not contain multi-basic amino acids (arginine, R, or lysine, K), therefore the virus was classified as LPAI. Full-length sequences analysis of the three isolated H9N2 AIVs showed a 100% identity between them and a cleavage site motif of low pathogenicity for chickens (amino acid residues at positions 324-332: PAASDR↓GLF) (Horimoto & Kawaoka, 1994; Senne *et al.*, 1996; Veits *et al.*, 2012). With respect to molecular markers associated to increased AIV zoonotic risk, the HA H5 gene segment carried the S159N aminoacidic substitution, a reported marker for potential virus binding to mammal-like receptors (Wang *et al.*, 2010). Other markers related to increased transmission and/or replication in mammals, particularly the I155T and D225G amino acid substitutions, were identified in the three HA gene segments of the H9N2 isolates (Li *et al.*, 2014; Mancera Gracia *et al.*, 2017). Both the NA genes of the H1N1 and H5N3 LPAIVs did not contain any amino acid changes relevant for reduced antiviral activity (Suttie *et al.*, 2019), while the H9N2 isolates contained an Y155H amino acid substitution that has been

associated with resistance to neuraminidase inhibitors (Monto *et al.*, 2006). The N2 gene segments of the H9N2 viruses also carried the A30T substitution, previously observed in AIV airborne transmission in ferrets (Kimble *et al.*, 2011). Regarding internal genes, sequence analysis of the PB2 gene segments of the H5N3, H9N2 and H1N1 isolates did not detect the E627K and the D710N amino acid substitutions, which play an important role in increased polymerase activity in mammalian cell line and increased virulence in mice (Sang *et al.*, 2015; Sediri *et al.*, 2016). However, the PB2 gene segment of the H9N2 isolates carried the T58I amino acid change, observed in AIV airborne transmission in ferrets (Kimble *et al.*, 2011). The V44I amino acid substitution in the PA gene sequence was detected in two of the H9N2 isolates, namely A/teal/Italy/1856-7_22VIR4622-7/2021 (H9N2) and A/teal/Italy/1828-6_22VIR4622-5/2021 (H9N2). This substitution, in combination with the V127A, C241Y, A343T, and I573V amino acid changes that were absent in the PA gene segment of the H9N2 isolates of this study, has been associated with increased replication in mammalian cell line and increased virulence in mice (Yamaji *et al.*, 2015).

The results of the BLAST nucleotide sequence similarity searches are summarized in Table 3. The H1N1, H5N3 LPAIV and the three H9N2 isolates shared 98-100% nucleotide sequence identity with AIVs circulating in Eurasia. Specifically, the PB2, PB1, PA, HA, NP, and NA gene segments of the H1N1 isolate exhibited high nucleotide similarity (98-100%) to H1N1, H4N6, and H5N2 LPAIVs isolated from Northern Europe. Additionally, the M and NS genes shared a 99-100% nucleotide sequence similarity with H3N2 and H3N8 viruses isolated from Russia and South Korea in 2021, respectively. The eight gene segments of the H5N3 LPAIV showed the highest nucleotide sequence similarity (99%) to other H5N3 LPAIVs concomitantly isolated in wild birds in Italy, based on recent sequences available on GISAID. Regarding the H9N2 viruses, the PB2, PB1, HA, NP, and the NA genes exhibited the highest nucleotide sequence similarity (98-99%) to LPAIVs from Germany, Italy, Poland, the Netherlands, and England, respectively. The PA and the NS gene segments displayed a 98-99% nucleotide sequence similarity with H3N8 isolates from wild bird from western Siberia and South Korea, respectively. Lastly, the M gene segment showed the highest genetic similarity (98%) to a 2015-H3N6 isolate from Egypt. Amino acid substitutions for each HA and NA segments of the H1N1, H5N3 LPAI, and the H9N2 viruses in comparison to their closest relatives are presented in Table 4.

Table 3. Highest nucleotide sequence similarities to the AIVs obtained in the current study, according to BLAST search tool on the GISAID Epi-Flu Database.

Virus subtype	Gene segment	Highest homology AIV	Accession number	% homology
A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1)	PB2	A/ <i>Anas platyrhynchos</i> /Belgium/1098_2/2020(H1N1)	EPI1942951	99
	PB1	A/ <i>Anas platyrhynchos</i> /Belgium/2213_0006/2021(H11N6)	EPI2122874	99
	PA	A/ <i>Anas platyrhynchos</i> /Belgium/1098_2/2020(H1N1)	EPI1942953	98
	HA	A/ <i>Anas platyrhynchos</i> /Belgium/1098_2/2020(H1N1)	EPI1942954	99
	NP	A/ <i>Anas platyrhynchos</i> /Belgium/11025_44/2017 (H11N1)	EPI1774315	99
	NA	A/ chicken/Denmark/S02750-3/2020(H5N1)	EPI1694135	98
	M	A/ duck/Moscow/5881/2021(H3N2)	EPI2175875	99
	NS	A/ Mallard /South Korea/ KNU2021-17/2021(H3N8)	EPI2153475	100

Table 3. *Cont.*

Virus subtype	Gene segment	Highest homology AIV	Accession number	% homology
A / mallard / Italy / 22V IR4203- 2 / 2021 (H5N3)	PB2	A / teal / Italy / 21VIR8923-73 / 2021(H5N3)	EPI 7987343	99
	PB1	A / duck / Italy / 21VIR8022-1 / 2021(H5N3)	EPI1946718	99
	PA	A / duck / Italy / 21VIR8022-1 / 2021(H5N3)	EPI1946719	99
	HA	A / duck / Italy / 21VIR8022-1 / 2021(H5N3)	EPI1946720	99
	NP	A / duck / Italy / 21VIR8022-1 / 2021(H5N3)	EPI1946721	99
	NA	A / duck / Italy / 21VIR8022-1 / 2021(H5N3)	EPI1946726	99
	M	A / teal / Italy / 21VIR8923-73 / 2021(H5N3)	EPI1947344	99
	NS	A / duck / Italy / 21VIR8022-1 / 2021 (H5N3)	EPI1946723	99

Table 3. Cont.

Virus subtype	Gene segment	Highest homology AIV	Accession number	% homology
A/ teal/ Italy / 1856- 7_22VIR4622 -7/ 2021 (H9N2); A/ teal/ Italy / 1828- 6_22VIR4622 -5/ 2021 (H9N2); A/ teal/ Italy / 1821- 14_22VIR462 2-3/ 2021 (H9N2).	PB2	A/ <i>Anas platyrhynchos</i> / Belgium/ 10402_H195386/ 2017 (H1N1)	EPI1775505	98
	PB1	A/ duck/ Italy/ 21VIR8024-4/ 2021(H5N3)	EPI1947293	99
	PA	A/ shoveler/ Novosibirsk region/ 3465k/ 2020 (H3N8)	EPI1849977	98
	HA	A/ mallard/ Poland/ P096/ 2020(H9N2)	EPI2618292	98
	NP	A/ Mallard/ Netherlands/ 37/ 2015(H3N8)	EPI1530590	98
	NA	A/ environment/ England/ 030642/ 2020 (H5N2)	EPI2062134	98
	M	A/ pintail/ Egypt/ MB-D-384C/ 2015(H3N6)	EPI1581277	98
	NS	A/ Mallard/ South Korea/ KNU2021-17/ 2021(H3N8)	EPI2153475	97

Table 4. Amino acid substitutions of the HA and NA gene segments of AIVs isolated in the current study, in comparison with the highest homologous strains obtained by BLAST search tool on GISAID Epi-Flu database.

Gene	Strain		Subtype		Amino acid substitutions*	Source	
	This study	Best similar	This study	Best similar		This study	Best similar
HA	A/teal/Italy/1821-10_22VIR4622-1/2021	A/ <i>Anas platyrhynchos</i> /Belgium/1098_2/2020	H1N1	H1N1	456 (W → R)	Cloacal swab; Common teal; Bologna province, 15 th of October 2021	Mallard; Belgium; 12 th of September 2020
NA	A/teal/Italy/1821-10_22VIR4622-1/2021	A/chicken/Denmark/S02750-3/2020	H1N1	H5N1 (LPAIV)	79 (I → V) 83 (T → A) 283 (M → I)	Cloacal swab; Common teal; Bologna province, 15 th of October 2021	Chicken; Denmark; 28 th of January 2020
HA	A/mallard/Italy/22VIR4203-2/2021	A/duck/Italy/21VIR8022-1/2021	H5N3 (LPAIV)	H5N3 (LPAIV)	81 (K → N) 395 (I → V)	Fecal dropping; Mallard; Emilia-Romagna region; 13 th of October 2021	Duck; Italy, Lombardy region; 23 rd September 2021
NA	A/mallard/Italy/22VIR4203-2/2021	A/duck/Italy/21VIR8022-1/2021	H5N3 (LPAIV)	H5N3 (LPAIV)	169 (V → I)	Fecal dropping; Mallard; Emilia-Romagna region; 13 th of October 2021	Duck; Italy, Lombardy region; 23 rd September 2021

HA	A / teal / Italy / 1856-7_22VIR4622-7/2021(H9N2) ^o	A / mallard / Poland / P096/2020	H9N2	H9N2	245 (I → V) 419 (D → N)	Cloacal swab; Common teal; Bologna province, October - November 2021	Duck; Poland; 2 nd of November 2020
NA	A / teal / Italy / 1856-7_22VIR4622-7/2021(H9N2) ^o	A / environment / England / 030642/2020	H9N2	H5N2 (LPAIV)	43 (S → N) 216 (G → V) 262 (V → I) 302 (I → V) 313 (D → G)	Cloacal swab; Common teal; Bologna province, October - November 2021	Feces; England; 31 st of October 2020

^oThe HA and NA gene segments of A / teal / Italy / 1828-6_22VIR4622-5/2021(H9N2) and A / teal / Italy / 1821-14_22VIR4622-3/2021(H9N2) were identical.

*H3 amino acid numbering; N2 amino acid numbering.

4.3.4. *Phylogenetic analyses*

Phylogenetic analyses of the HA and NA gene sequences revealed that the viruses isolated in the current study were closely related to AIVs circulating in wild birds or, to a lesser extent, in domestic birds in Europe during 2020-2022. The H1 hemagglutinin gene of A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1) was in sister group relationship with another H1N1 isolate from Belgium (BS 100), and these two viruses formed a monophyletic clade (BS 99) with A/mallard/Netherlands/18015513-001/2018 (H1N1) (Figure 3).

The H5 HA gene of A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI was in sister group relationship (BS 64) with A/mallard/Netherlands/7/2022, and these were included in a monophyletic clade with other H5 LPAI isolates from the Netherlands and Italy obtained from wild birds during 2021-2022 (Figure 4). The H9 viruses obtained in this study clustered within the Y439-like sub-lineage in the Eurasian lineage of H9 hemagglutinin gene (Figure 5). These viruses formed a monophyletic clade with maximum support, which was in sister relationship with an H9N2 isolate from a mallard in Poland obtained in 2020. The N1 gene segment of the H1N1 isolate clustered together (BS 98) with A/chicken/Denmark/S0275-3/2020 (H5N1) LPAIV isolated in chickens (Figure 6). The N3 NA gene of the H5N3 LPAIV was in sister group relationship (BS 72) with a clade composed of NA genes of H5N3 LPAI Italian isolates from wild ducks (Figure 7). The N2 NA gene segments of the three H9N2 isolates formed a maximum-supported monophyletic clade, in sister group relationship with a clade including N2 NA genes of German and Polish isolates of H5N2 HPAIVs from swans (A/swan/Germany-BW/AI00996/2022 and A/swan/Germany-BW/AI00997/2022), and from chickens (A/chicken/Poland/H182_22VIR2515-1/2022) (Figure 8).

The phylogenetic analyses of the PB2, PB1, PA, M, NS, and NP gene segments showed that, for each AIV subtype hereby detected, these genes arose from different ancestors and the closest relatives have been isolated both in Europe and in Asia (Supplemental Material S1).



Figure 3. Phylogenetic analysis of the H1 hemagglutinin subtype of the A/teal/Italy/1821-10_22VIR4622-1/2021(H1N1) isolate. The assembled full H1 sequence hereby obtained (red, bold) is compared with full coding sequences available at GenBank and/or GISAID. Human isolates of the pandemic influenza A(H1N1)2009 virus lineage and swine A(H1N1) viruses were included. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was GTR+F+I+G4.

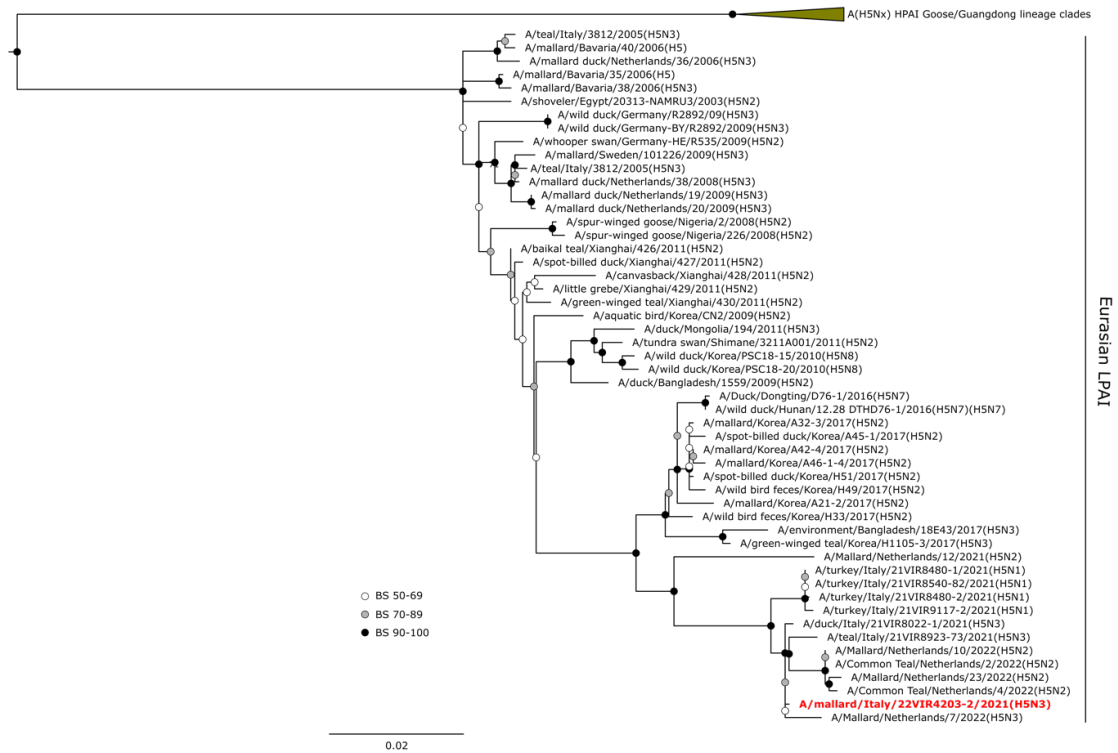


Figure 4. Phylogenetic analysis of the H5 hemagglutinin subtype of the A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI isolate. The assembled full H5 sequence hereby obtained (red, bold) is compared with full coding sequences available at GenBank and/or GISAID. The collapsed clade in the upper part of the phylogenetic tree shows the HPAIVs isolated in Europe during 2020-2023. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was GTR+F+I+G4.

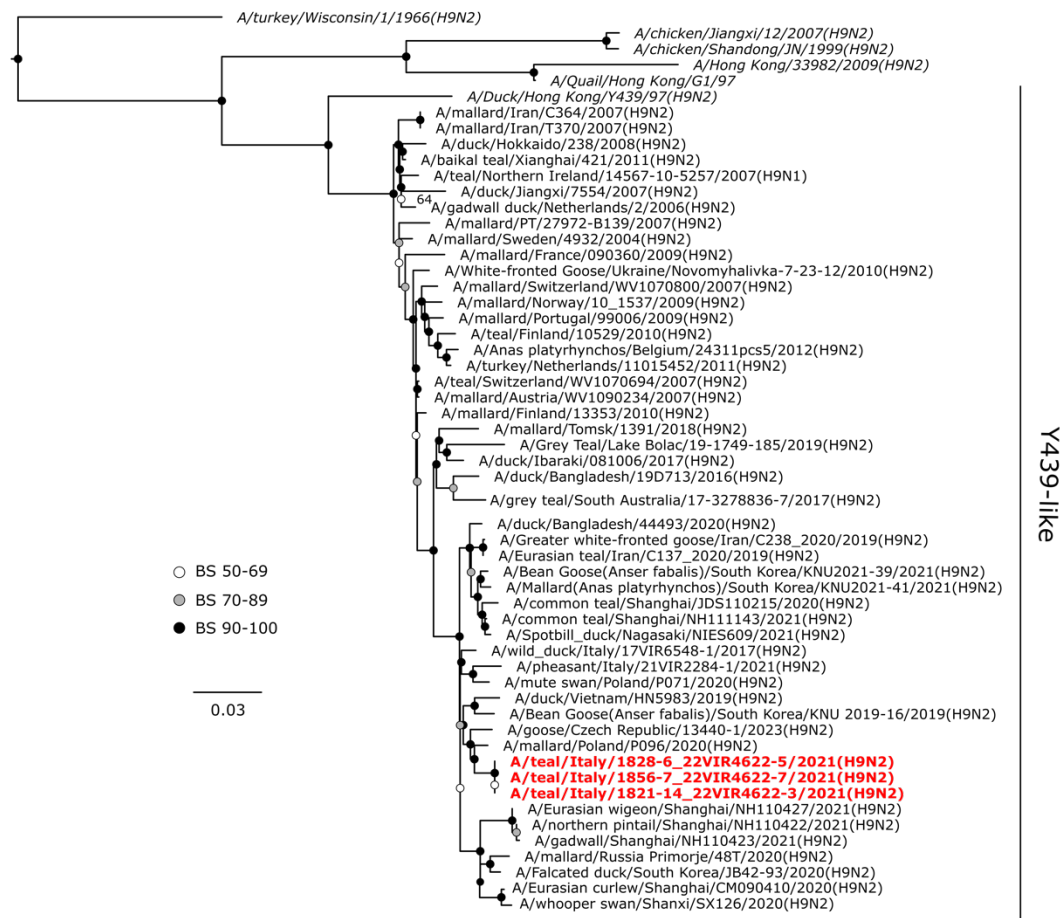


Figure 5. Phylogenetic analysis of the H9 hemagglutinin subtype of the *A/teal/Italy/1856-7_22VIR4622-7/2021(H9N2)*, *A/teal/Italy/1828-6_22VIR4622-5/2021(H9N2)*, *A/teal/Italy/1821-14_22VIR4622-3/2021(H9N2)* isolates. The assembled full H9 sequences hereby obtained (red, bold) are compared with full coding sequences available at GenBank and/or GISAID. Reference sequences are shown in italics. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was GTR+F+G4.

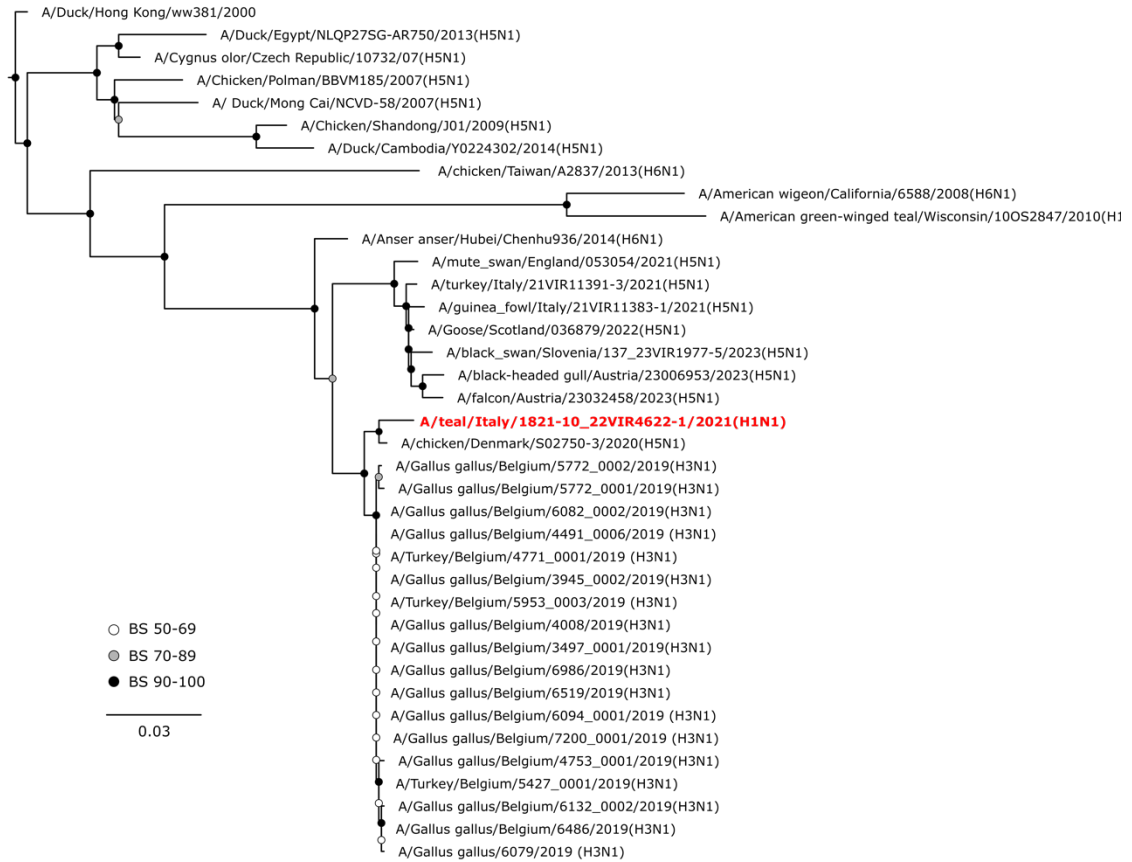


Figure 6. Phylogenetic analysis of the N1 neuraminidase subtype of the A/teal/Italy/1821-10_22VIR4622-1/2021(H1N1) isolate. The assembled full N1 sequence hereby obtained (red, bold) is compared with full coding sequences available at GenBank and/or GISAID. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was TIM2+F+G4.

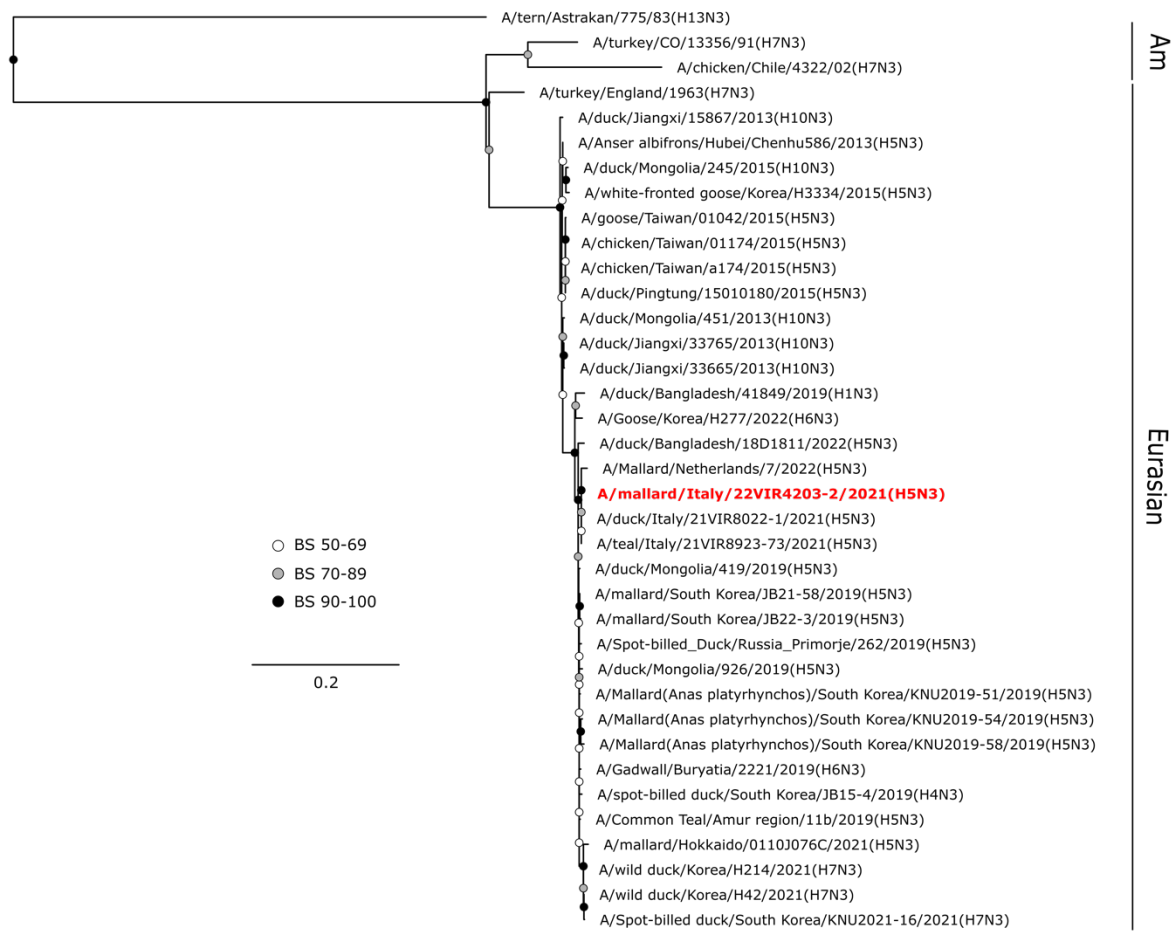


Figure 7. Phylogenetic analysis of the N3 neuraminidase gene of the A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI. The assembled full N3 sequence hereby obtained (red, bold) is compared with full coding sequences available at GenBank and/or GISAID. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was TIM2+F+G4.

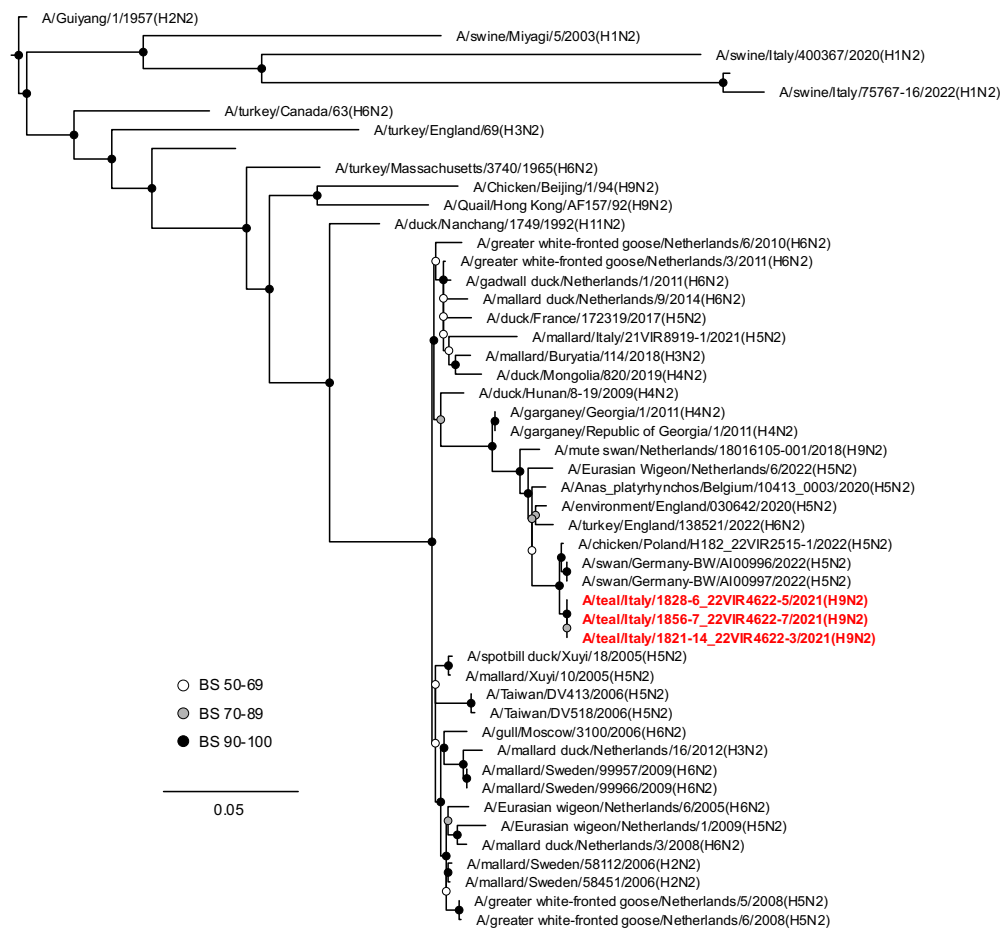


Figure 8. Phylogenetic analysis of the N2 neuraminidase genes of the A/teal/Italy/1856-7_22VIR4622-7/2021(H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021(H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021(H9N2) isolates. The assembled full N2 sequences hereby obtained (red, bold) are compared with full coding sequences available at GenBank and/or GISAID. Circles at nodes indicate the bootstrap (BS) score range, and are labelled in white (BS 50-69), grey (BS 70-89), and black (BS 90-100). The substitution model for the maximum likelihood phylogenetic reconstruction was TVM+F+G4.

4.4. Discussion

To actively monitor AIVs occurring in wild birds in Northern Italy, where densely populated poultry areas are located, a cross-sectional study was conducted from August 2021 to April 2022. This survey included the collection and testing of cloacal swabs and avian fecal droppings for AIV molecular detection. A total of 11 AIVs were obtained, belonging to subtypes H1N1, H5N3, H6N1, H9N2, HxN9 and HxN2; five of these (H1N1, H5N3 LPAI, and three H9N2 virus strains) were isolated and fully sequenced. HPAIVs were not detected although the concurrent widespread circulation of HPAI H5N1 viruses of clade 2.3.4.4b in Italian poultry, linked to 317 outbreaks and over 8 million birds culled from October 2021 to April 2022 (Adlhoch *et al.*, 2022). AI outbreaks were concentrated in poultry farms in the Po valley of the Veneto and Lombardy regions, Northeast Italy, while the Emilia-Romagna region, where this study was conducted, reported only two HPAI cases in non-commercial poultry farms in the Rimini and Ravenna province, near wetlands (European Food Safety Authority *et al.*, 2021b). With respect to wild birds, during the 2021-2022 surveillance activities, a total of 23 positive cases of HPAIV of clade 2.3.4.4b infections were reported (Istituto Zooprofilattico Sperimentale delle Venezie, 2022). These were primarily found in scavenging or predator birds, such as raptors, owls, corvids, and gulls, indicating a wider host range than previous years (Adlhoch *et al.*, 2022; European Food Safety Authority *et al.*, 2021b).

Species belonging to the Anseriformes and Charadriiformes orders, specifically the mallard, greylag goose, and common snipe, as identified through COI-barcoding, tested positive for LPAIVs molecular detection in fecal droppings. Shorebirds exhibited the highest positivity rate (3%, 1/34), followed by geese (2.1%, 1/46), and ducks (0.8%, 2/249). Nevertheless, the convenience sampling strategy employed might have resulted in an overestimation of the positivity rate for shorebirds and geese, as these groups were represented by smaller samples sizes compared to the ducks. While mallards and common snipes are listed as huntable according to Italian legislation (National Hunting Law 157/1992) and could be monitored in our study through cloacal swabbing, the majority of other bird groups of sampled via FD collection included non-huntable species. The use of an environmental sampling strategy in this study proved to be a valuable tool for broadening the host range in AIV monitoring. While active surveillance in wild birds is considered ideal, the utilization of avian fecal droppings from environment may offer a less biased and cost-effective approach to AIV surveillance (Verhagen *et al.*, 2021). Both LPAI and HPAI viruses can indeed remain viable in bird feces for weeks, even months, at low temperatures. This non-invasive sampling strategy might be particularly valuable amid the current widespread circulation of HPAIVs H5N1 of clade 2.3.4.4b among different wild bird species (Animal and Plant Health Inspection Service, 2023b; European Food Safety Authority *et al.*, 2023c). Furthermore, strategically located wetlands, such as those included in this study near DPPAs,

where numerous migratory birds feed and rest, could serve as potential sites for the emergence of novel AI strains. Consequently, these areas should be prioritized for year-long environmental monitoring. Successful detection of AIVs in fecal dropping samples, coupled with the identification of the bird species of origin through COI-barcoding, paves the way for employing this sampling strategy to enhance wild animal surveillance in the vicinity of poultry farms within HPAI restriction zones, as useful tool for monitoring potential virus spillover at the domestic bird-wildlife interface. Environmental matrices, however, might yield lower viral loads than swabs collected from wild birds, potentially limiting the chance of AIV subtyping and isolation.

With respect to the sampling activity on hunted or found dead waterbirds, Eurasian teals constituted the majority (65.9%, 85/129) of birds tested for AIV molecular detection. An 8.2% LPAIV positivity rate was recorded for this species, aligning with previous studies on AIV molecular surveys conducted in teals where prevalence ranged from 3.6% to 15% (Busquets *et al.*, 2010; Henriques *et al.*, 2011; Lebarbenchon *et al.*, 2009; Lebarbenchon *et al.*, 2007; Munster *et al.*, 2007). Notably, Eurasian teals have been found positive for HPAIVs H5Nx of clade 2.3.4.4b, displaying asymptomatic infection associated with oropharyngeal and cloacal shedding (Gobbo *et al.*, 2021; Tanikawa *et al.*, 2021; van den Brand *et al.*, 2018). Absent viral detection in the other species hereby sampled, especially in the other dabbling ducks, could be related to a low sample size achieved.

In this study, LPAIVs were exclusively detected in October and November 2021, in coincidence with waterbirds' autumn migration and early wintering periods. No virus circulation was observed in the remaining months. However, during the spring migration period, results could have been biased due to the smaller sample size of FDs and the absence of cloacal swab collection due to the suspension of hunting. A higher AIV prevalence during autumn and early winter was also reported in surveys conducted on wild bird fecal droppings collected in wetlands in Spain and Mongolia (Barkhasbaatar *et al.*, 2023; Torrontegi *et al.*, 2019). During the autumn migration, the congregation of birds from diverse origins within the same area, mingling with the local avian community, facilitates multiple-strain AIV transmission. This, coupled with the presence of immunologically naïve juveniles THAT amplify the infection, contributes to increased AIV prevalence in reservoir species (Hill *et al.*, 2016; Latorre-Margalef *et al.*, 2013; van Dijk *et al.*, 2014). The subsequent onset of homo- and heterosubtypic immunity likely protect birds against infection from phylogenetically close AIV strains, reducing viral circulation during the remaining winter season (Latorre-Margalef *et al.*, 2017; Latorre-Margalef *et al.*, 2013; Tarasiuk *et al.*, 2023).

Considering AIV's high mutation rate and the diverse gene constellations resulting from reassortment events in wild birds, WGS plays a pivotal in characterizing the isolated strains through sequence and phylogenetic analyses (Verhagen *et al.*, 2021). The gene segments of the LPAIVs obtained in this study exhibited close genetic relationship with viruses isolated from

wild birds, and, to a lesser extent, domestic birds from Europe, Northern Africa, and Asia. Strains identified in the same flyway, such as the Black sea/Mediterranean flyway hereby considered, are indeed more similar to each other than those found in other flyways during a specific period (Venkatesh *et al.*, 2018). Interestingly, none of internal genes of the isolates obtained in this study clustered together with gene segments belonging to HPAIVs involved in the concurrent poultry epidemic occurring in Northeast Italy. This allows us to speculate that the AIV genetic diversity found in our study primarily arose from reassortment events occurred in wild bird populations. Among the HA and NA gene segments of the strains hereby isolated, one to five amino acid substitutions were identified in comparison to AIVs with the highest nucleotide sequence similarity; however, none of these mutations was associated to molecular markers affecting viral biological properties (Suttie *et al.*, 2019). Through sequence analysis, several other amino acid residues have been identified as potentially relevant within the context of AIV increased zoonotic risk. With respect to the H5N3 LPAIV, the 159N amino acid residue identified in the HA gene segment has been linked to increased binding to mammal-like receptors, as demonstrated for a live attenuated influenza A/Vietnam/1203/2004 (H5N1) vaccine virus in challenged ferrets (Wang *et al.*, 2010). This substitution has been sporadically observed in H5 gene segments of strains circulating in birds in Europe from 2015 on (Briand *et al.*, 2017; European Food Safety Authority *et al.*, 2022b; European Food Safety Authority *et al.*, 2023b). The I155T amino acid substitutions in the H9 HA gene segment of the isolates hereby obtained has been reported as linked to increased binding affinity for mammal-like receptor in H5N1 HPAIV (Auewarakul *et al.*, 2007; Watanabe *et al.*, 2011). The effect of this amino acid change in the H9 subtype has however not been assessed yet. The H9 HA sequences also carried the D225G amino acid substitution, associated to a broader tissue tropism and increased post-transmission replication in swine during an experimental study involving an H9N2-2009 pandemic H1N1 reassortant virus generated through reverse genetics (Mancera Gracia *et al.*, 2017). Two amino acid substitutions found in the NA (A30T) and PB2 (T58I) gene segments of the H9N2 viruses have been detected in ferrets following airborne transmission in an experiment involving H9N2-2009 pandemic H1N1 reassortant viruses (Kimble *et al.*, 2011). Also, the Y155H amino acid substitution in the N2 gene segments has been associated to resistance to oseltamivir in A/Hokkaido/15/2002 (H1N1) virus population in humans (Monto *et al.*, 2006). In terms of the PA gene segments, the V44I amino acid substitution found in the H9 isolates has been associated with enhanced replication of an HPAI H5N1 virus in A549 cells and increased pathogenicity in mice, proved in association with other substitutions not identified in the H9N2 viruses' PA gene segments examined (Yamaji *et al.*, 2015). Interestingly, this substitution was absent in any of the other PA genes of the viruses included in the phylogenetic analysis. The H9N2 virus subtype has been endemically circulating in poultry in Asia, the Middle East, and parts of Africa and has a high prevalence of infection in wild birds (Carnaccini & Perez, 2020). Given the H9N2 virus

isolations also from swine and humans, these AIVs are considered involved potentially in the emergence of the next influenza pandemic and should therefore be closely monitored in birds (Carnaccini & Perez, 2020).

4.5. Conclusions

Results of this study revealed the active circulation of multiple LPAIV subtypes in wild birds in Northern Italy. The survey involved the collection of cloacal swabs from waterbirds and avian fecal droppings from wetlands, thus detecting a broader range of AIV subtypes than each sampling strategy could have achieved individually. Despite the close genetic relationship with other common AIV isolates previously found in Eurasia and Northern Africa, the HA and NA gene segments of the viruses obtained in this study displayed several unique amino acid changes. Potential markers associated with increased AIV zoonotic risk were also identified, but the actual effect of these mutations on the biological characteristics of the obtained viruses is unknown. Further *in vitro* and *in vivo* experiments are needed in this regard to improve existing knowledge. Wild waterfowl in Eurasia harbor a vast genetic pool of AIVs, contributing to the emergence of novel strains even capable of infecting new hosts, such as domestic poultry and mammals. The genetic diversity hereby detected emphasizes the need for continued surveillance in wild hosts using different sampling strategies and whole genome sequencing characterization of the isolates.

[illegible]

Supplemental Material S1. Phylogenetic analyses of PB2, PB1, PA, M, NP, and NS gene segments.



Figure 2. PB1 sequences phylogenetic maximum-likelihood reconstruction. The PB1 gene sequences of the isolates hereby obtained, namely A/teal/Italy/1821-10_22VIR4622-1/2021(H1N1), A/mallard/Italy/22VIR4203-2/2021(H5N3) LPAI, A/teal/Italy/1856-7_22VIR4622-7/2021(H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021(H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021(H9N2) are shown in red. The substitution model for the maximum likelihood phylogenetic reconstruction was TIM+G4.

Supplemental Material S1. Phylogenetic analyses of PB2, PB1, PA, M, NP, and NS gene segments.

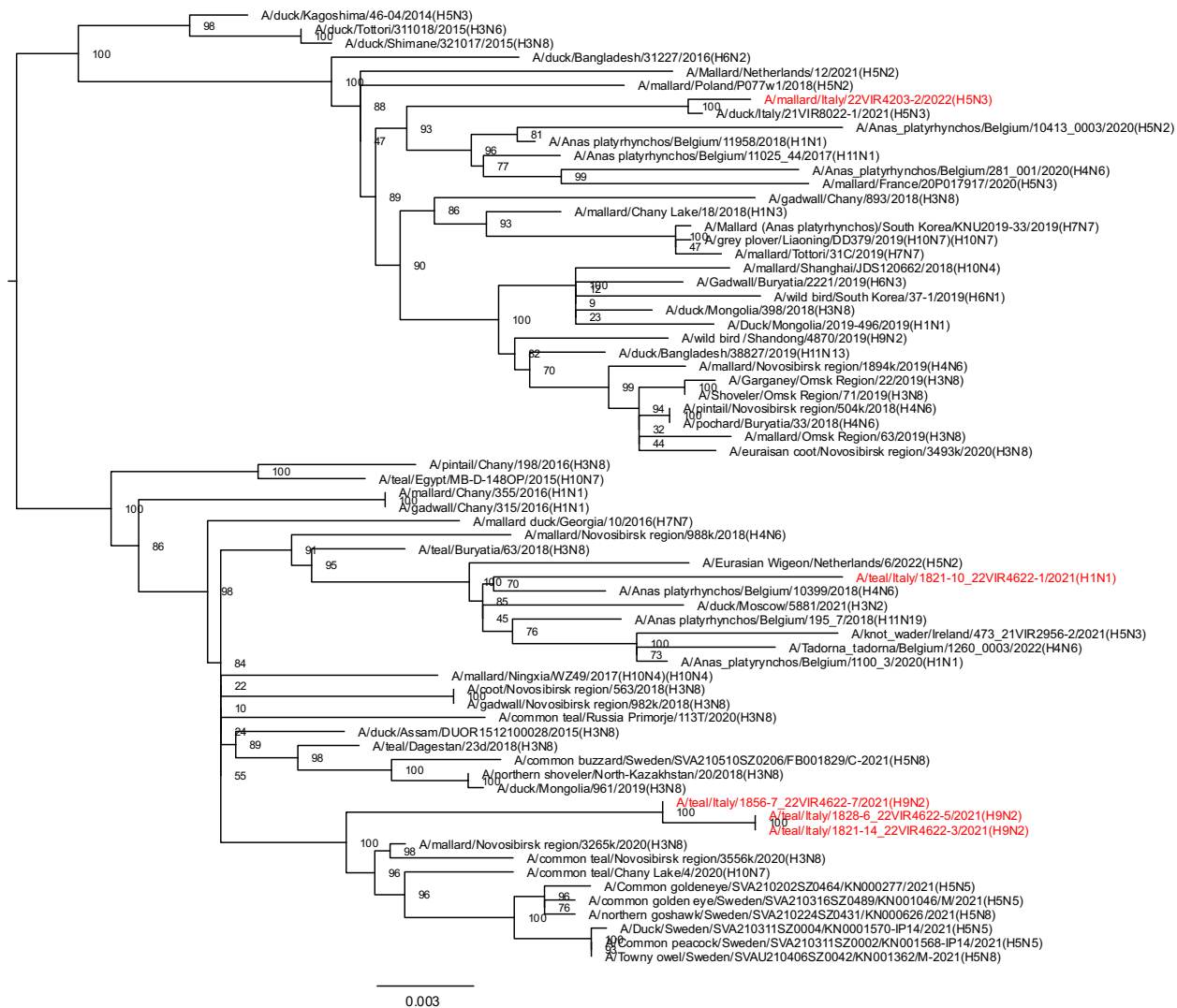


Figure 3. PA sequences phylogenetic maximum-likelihood reconstruction. The PA gene sequences of the isolates hereby obtained, namely A/teal/Italy/1821-10_22VIR4622-1/2021(H1N1), A/mallard/Italy/22VIR4203-2/2021(H5N3) LPAI, A/teal/Italy/1856-7_22VIR4622-7/2021(H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021(H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021(H9N2) are shown in red. The substitution model for the maximum likelihood phylogenetic reconstruction was K3Pu+F+G4.

Supplemental Material S1. Phylogenetic analyses of PB2, PB1, PA, M, NP, and NS gene segments.

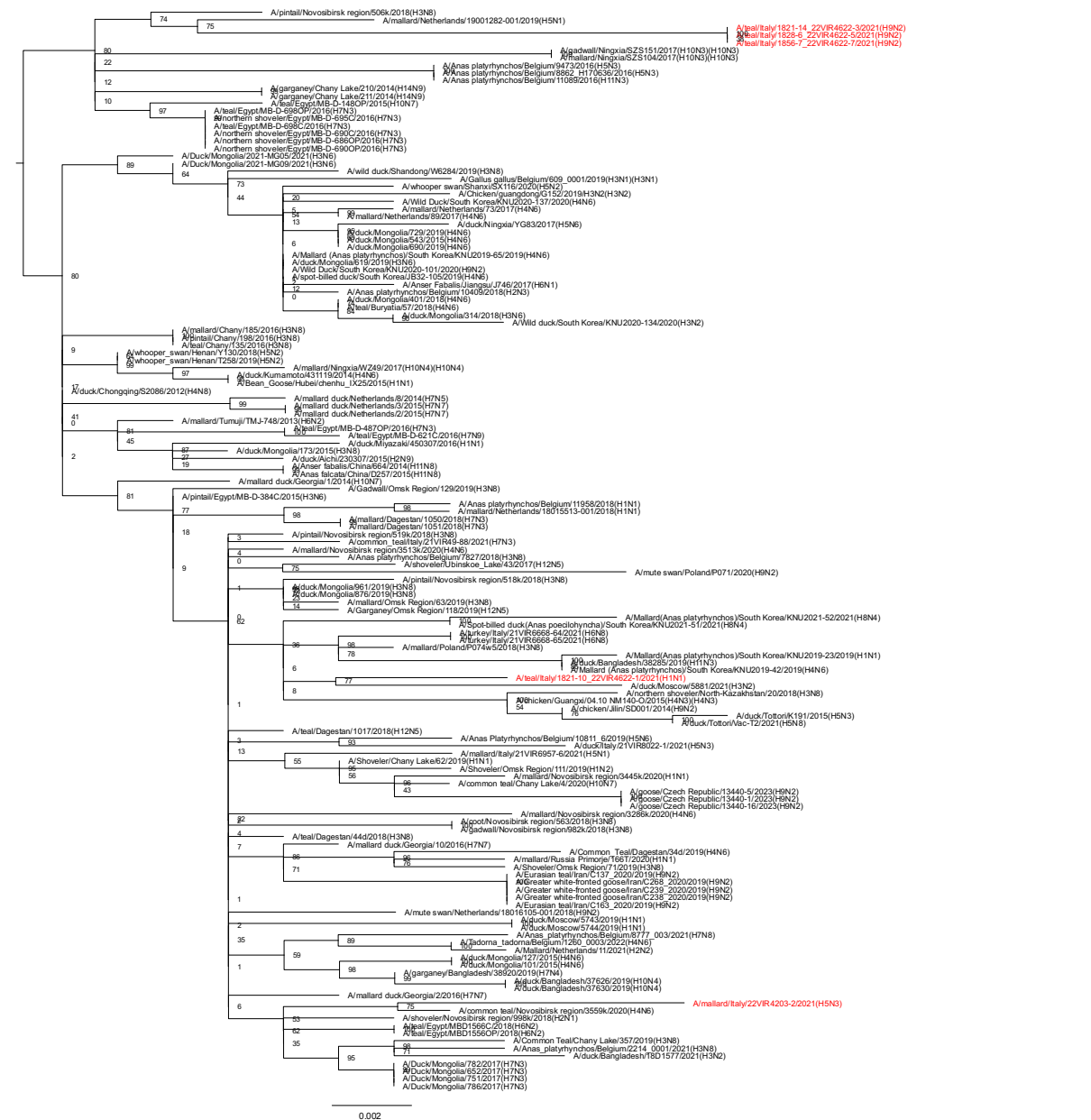


Figure 4. M1 sequences phylogenetic maximum-likelihood reconstruction. The M1 gene sequences of the isolates hereby obtained, namely A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1), A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI, A/teal/Italy/1856-7_22VIR4622-7/2021 (H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021 (H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021 (H9N2) are shown in red. The substitution model for the maximum likelihood phylogenetic reconstruction was K3P+I+G4.

Figure 5. NP sequences phylogenetic maximum-likelihood reconstruction. The NP gene sequences of the isolates hereby obtained, namely A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1), A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI, A/teal/Italy/1856-7_22VIR4622-7/2021 (H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021 (H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021 (H9N2) are shown in red. The substitution model for the maximum likelihood phylogenetic reconstruction was K3Pu+F+I+G4.



Figure 6. NS1 sequences phylogenetic maximum-likelihood reconstruction. The NS1 gene sequences of the isolates hereby obtained, namely A/teal/Italy/1821-10_22VIR4622-1/2021 (H1N1), A/mallard/Italy/22VIR4203-2/2021 (H5N3) LPAI, A/teal/Italy/1856-7_22VIR4622-7/2021 (H9N2), A/teal/Italy/1828-6_22VIR4622-5/2021 (H9N2), A/teal/Italy/1821-14_22VIR4622-3/2021 (H9N2) are shown in red. The substitution model for the maximum likelihood phylogenetic reconstruction was TPM2u+F+I.



4.6. References

- Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Aznar, I., Muñoz Guajardo, I., & Baldinelli, F. (2022). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- Animal and Plant Health Inspection Service, U. S. D. o. A. (2023a). 2022-2023 Detections of Highly Pathogenic Avian Influenza in Mammals. Retrieved from <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-mammals>
- Animal and Plant Health Inspection Service, U. S. D. o. A. (2023b). 2022-2023 Detections of Highly Pathogenic Avian Influenza in Wild Birds. Retrieved from <https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-wild-birds>
- Auewarakul, P., Suptawiwat, O., Kongchanagul, A., Sangma, C., Suzuki, Y., Ungchusak, K., Louisirirothanakul, S., Lerdsamran, H., Pooruk, P., Thitithanyanont, A., Pittayawonganon, C., Guo, C. T., Hiramatsu, H., Jampangern, W., Chunsutthiwat, S., & Puthavathana, P. (2007). An avian influenza H5N1 virus that binds to a human-type receptor. *J Virol*, 81(18), 9950-9955. doi:10.1128/jvi.00468-07
- Banks, J., Speidel, E. S., Moore, E., Plowright, L., Piccirillo, A., Capua, I., Cordioli, P., Fioretti, A., & Alexander, D. J. (2001). Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. *Arch Virol*, 146(5), 963-973. doi:10.1007/s007050170128
- Barkhasbaatar, A., Gilbert, M., Fine, A. E., Shiilegdamba, E., Damdinjav, B., Buuveibaatar, B., Khishgee, B., Johnson, C. K., Leung, C. Y. H., Ankhambaatar, U., Purevtseren, D., Tuttle, J. M., Mazet, J. A. K., Peiris, J. S. M., Jambal, L., Shatar, M., Sukhbaatar, T., & Olson, S. H. (2023). Ecological characterization of 175 low-pathogenicity avian influenza viruses isolated from wild birds in Mongolia, 2009–2013 and 2016–2018. *Vet Med Sci*. doi:10.1002/vms3.1281
- Beerens, N., Heutink, R., Harders, F., Roose, M., Pritz-Verschuren, S. B. E., Germeraad, E. A., & Engelsma, M. (2021). Incursion of Novel Highly Pathogenic Avian Influenza A(H5N8) Virus, the Netherlands, October 2020. *Emerg Infect Dis*, 27(6), 1750-1753. doi:10.3201/eid2706.204464
- Bevins, S., Shriner, S., Cumbee, J., Dilione, K., Douglass, K., Ellis, J., Killian, M. L., Torchetti, M., & Lenocho, J. (2022). Intercontinental Movement of Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4 Virus to the United States, 2021. *Emerg Infect Dis*, 28(5), 1006. doi:10.3201/eid2805.220318

- Briand, F. X., Schmitz, A., Ogor, K., Le Prioux, A., Guillou-Cloarec, C., Guillemoto, C., Allée, C., Le Bras, M. O., Hirchaud, E., Quenault, H., Touzain, F., Cherbonnel-Pansart, M., Lemaitre, E., Courtillon, C., Gares, H., Daniel, P., Fediaevsky, A., Massin, P., Blanchard, Y., Eterradossi, N., van der Werf, S., Jestin, V., & Niqueux, E. (2017). Emerging highly pathogenic H5 avian influenza viruses in France during winter 2015/16: phylogenetic analyses and markers for zoonotic potential. *Euro Surveill*, 22(9). doi:10.2807/1560-7917.Es.2017.22.9.30473
- Busquets, N., Alba, A., Napp, S., Sánchez, A., Serrano, E., Rivas, R., Núñez, J. I., & Majó, N. (2010). Influenza A virus subtypes in wild birds in North-Eastern Spain (Catalonia). *Virus Res*, 149(1), 10-18. doi:https://doi.org/10.1016/j.virusres.2009.12.005
- Cáceres, C. J., Rajao, D. S., & Perez, D. R. (2021). Airborne Transmission of Avian Origin H9N2 Influenza A Viruses in Mammals. *Viruses*, 13(10). doi:10.3390/v13101919
- Carnaccini, S., & Perez, D. R. (2020). H9 Influenza Viruses: An Emerging Challenge. *Cold Spring Harb Perspect Med*, 10(6). doi:10.1101/cshperspect.a038588
- Chen, H., Smith, G. J. D., Zhang, S. Y., Qin, K., Wang, J., Li, K. S., Webster, R. G., Peiris, J. S. M., & Guan, Y. (2005). H5N1 virus outbreak in migratory waterfowl. *Nature*, 436(7048), 191-192. doi:10.1038/nature03974
- Cheung, P. P., Leung, Y. H., Chow, C. K., Ng, C. F., Tsang, C. L., Wu, Y. O., Ma, S. K., Sia, S. F., Guan, Y., & Peiris, J. S. (2009). Identifying the species-origin of faecal droppings used for avian influenza virus surveillance in wild-birds. *J Clin Virol*, 46(1), 90-93. doi:10.1016/j.jcv.2009.06.016
- Ellis, T. M., Bousfield, R. B., Bissett, L. A., Dyrting, K. C., Luk, G. S., Tsim, S. T., Sturm-Ramirez, K., Webster, R. G., Guan, Y., & Malik Peiris, J. S. (2004). Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol*, 33(5), 492-505. doi:10.1080/03079450400003601
- Ellwanger, J. H., & Chies, J. A. B. (2021). Zoonotic spillover: Understanding basic aspects for better prevention. *Genet Mol Biol*, 44(1 Suppl 1), e20200355. doi:10.1590/1678-4685-gmb-2020-0355
- European Food Safety Authority, Aznar, I., Baldinelli, F., Papanikolaou, A., Stoicescu, A., & Van der Stede, Y. (2021a). Annual Report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2020. *EFSA J*, 19(12), e06953. doi:https://doi.org/10.2903/j.efsa.2021.6953
- European Food Safety Authority, Aznar, I., Baldinelli, F., Stoicescu, A., & Kohnle, L. (2022a). Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2021. *EFSA J*, 20(9), e07554. doi:https://doi.org/10.2903/j.efsa.2022.7554
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A.,

- Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2022b). Avian influenza overview March - June 2022. *EFSA J*, 20(8), e07415. doi:10.2903/j.efsa.2022.7415
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Guajardo, I. M., & Baldinelli, F. (2023a). Avian influenza overview September - December 2022. *EFSA J*, 21(1), e07786. doi:10.2903/j.efsa.2023.7786
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Mirinaviciute, G., Niqueux, E., Stahl, K., Staubach, C., Terregino, C., Broglia, A., & Baldinelli, F. (2023b). Avian influenza overview December 2022 - March 2023. *EFSA J*, 21(3), e07917. doi:10.2903/j.efsa.2023.7917
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, E., Staubach, C., Terregino, C., Aznar, I., Munoz Guajardo, I., & Baldinelli, F. (2022c). Avian influenza overview December 2021 - March 2022. *EFSA J*, 20(4), e07289. doi:10.2903/j.efsa.2022.7289
- European Food Safety Authority, European Centre for Disease Prevention and Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Mirinavičiūtė, G., Niqueux, É., Staubach, C., Terregino, C., Baldinelli, F., Rusinà, A., & Kohnle, L. (2023c). Avian influenza overview June–September 2023. *EFSA J*, 21(10), e08328. doi:https://doi.org/10.2903/j.efsa.2023.8328
- European Food Safety Authority, European Centre for Disease Prevention Control, European Union Reference Laboratory for Avian Influenza, Adlhoch, C., Fusaro, A., Gonzales, J. L., Kuiken, T., Marangon, S., Niqueux, É., Staubach, C., Terregino, C., Aznar, I., Muñoz Guajardo, I., & Baldinelli, F. (2021b). Avian influenza overview September – December 2021. *EFSA Journal*, 19(12), e07108. doi:https://doi.org/10.2903/j.efsa.2021.7108
- Gobbo, F., Fornasiero, D., De Marco, M. A., Zecchin, B., Mulatti, P., Delogu, M., & Terregino, C. (2021). Active Surveillance for Highly Pathogenic Avian Influenza Viruses in Wintering Waterbirds in Northeast Italy, 2020-2021. *Microorganisms*, 9(11). doi:10.3390/microorganisms9112188
- Hassan, K. E., Ahrens, A. K., Ali, A., El-Kady, M. F., Hafez, H. M., Mettenleiter, T. C., Beer, M., & Harder, T. (2022). Improved Subtyping of Avian Influenza Viruses Using an RT-qPCR-Based Low Density Array: ‘Riems Influenza a Typing’, Version 2 (RITA-2). *Viruses*, 14(2), 415. Retrieved from https://www.mdpi.com/1999-4915/14/2/415

- Hebert, P. D., Stoeckle, M. Y., Zemplak, T. S., & Francis, C. M. (2004). Identification of Birds through DNA Barcodes. *PLoS Biol*, 2(10), e312. doi:10.1371/journal.pbio.0020312
- Henriques, A. M., Fagulha, T., Barros, S. C., Ramos, F., Duarte, M., Luís, T., & Fevereiro, M. (2011). Multiyear surveillance of influenza A virus in wild birds in Portugal. *Avian Pathol*, 40(6), 597-602. doi:10.1080/03079457.2011.618943
- Hill, N. J., Ma, E. J., Meixell, B. W., Lindberg, M. S., Boyce, W. M., & Runstadler, J. A. (2016). Transmission of influenza reflects seasonality of wild birds across the annual cycle. *Ecol Lett*, 19(8), 915-925. doi:https://doi.org/10.1111/ele.12629
- Horimoto, T., & Kawaoka, Y. (1994). Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *Virol J*, 68(5), 3120-3128. doi:doi:10.1128/jvi.68.5.3120-3128.1994
- Horman, W. S. J., Nguyen, T. H. O., Kedzierska, K., Bean, A. G. D., & Layton, D. S. (2018). The Drivers of Pathology in Zoonotic Avian Influenza: The Interplay Between Host and Pathogen. *Front Immunol*, 9, 1812. doi:10.3389/fimmu.2018.01812
- Hoye, B. J., Donato, C. M., Lisovski, S., Deng, Y. M., Warner, S., Hurt, A. C., Klaassen, M., & Vijaykrishna, D. (2021). Reassortment and Persistence of Influenza A Viruses from Diverse Geographic Origins within Australian Wild Birds: Evidence from a Small, Isolated Population of Ruddy Turnstones. *J Virol*, 95(9). doi:10.1128/jvi.02193-20
- Istituto Zooprofilattico Sperimentale delle Venezie. Diagnostic Protocol. Retrieved from <https://www.izsvenezie.it/documenti/temi/influenza-aviaria/procedure-prova/PDP-VIR-143.pdf>
- Istituto Zooprofilattico Sperimentale delle Venezie. Diagnostic Protocols. Retrieved from <https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/diagnostic-protocols/>
- Istituto Zooprofilattico Sperimentale delle Venezie. (2021). Highly Pathogenic Avian Influenza in wild birds in Italy. Last update 13 April 2021. Retrieved from <https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/italy-update/>
- Istituto Zooprofilattico Sperimentale delle Venezie. (2022). Highly Pathogenic Avian Influenza in birds other than poultry in Italy. Epidemiological situation 01 March 2022. Retrieved from <https://www.izsvenezie.com/documents/reference-laboratories/avian-influenza/italy-updates/HPAI/2021-1/italy-wildbirds.pdf>
- James, J., Slomka, M. J., Reid, S. M., Thomas, S. S., Mahmood, S., Byrne, A. M. P., Cooper, J., Russell, C., Mollett, B. C., Agyeman-Dua, E., Essen, S., Brown, I. H., & Brookes, S. M. (2018). Development and Application of Real-Time PCR Assays for Specific Detection of Contemporary Avian Influenza Virus Subtypes N5, N6, N7, N8, and N9. *Avian Dis*, 63(sp1), 209-218, 210. Retrieved from <https://doi.org/10.1637/11900-051518-Reg.1>

- Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*, 14(6), 587-589. doi:10.1038/nmeth.4285
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*, 30(4), 772-780. doi:10.1093/molbev/mst010
- Khare, S., Gurry, C., Freitas, L., Schultz, M. B., Bach, G., Diallo, A., Akite, N., Ho, J., Lee, R. T., Yeo, W., Curation Team, G. C., & Maurer-Stroh, S. (2021). GISAID's Role in Pandemic Response. *China CDC Wkly*, 3(49), 1049-1051. doi:10.46234/ccdcw2021.255
- Kimble, J. B., Sorrell, E., Shao, H., Martin, P. L., & Perez, D. R. (2011). Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model. *Proc Natl Acad Sci U.S.A.*, 108(29), 12084-12088. doi:doi:10.1073/pnas.1108058108
- Latorre-Margalef, N., Brown, J. D., Fojtik, A., Poulson, R. L., Carter, D., Franca, M., & Stallknecht, D. E. (2017). Competition between influenza A virus subtypes through heterosubtypic immunity modulates re-infection and antibody dynamics in the mallard duck. *PLoS Pathog*, 13(6), e1006419. doi:10.1371/journal.ppat.1006419
- Latorre-Margalef, N., Grosbois, V., Wahlgren, J., Munster, V. J., Tolf, C., Fouchier, R. A. M., Osterhaus, A. D. M. E., Olsen, B., & Waldenström, J. (2013). Heterosubtypic Immunity to Influenza A Virus Infections in Mallards May Explain Existence of Multiple Virus Subtypes. *PLOS Pathog*, 9(6), e1003443. doi:10.1371/journal.ppat.1003443
- Lebarbenchon, C., Albespy, F., Brochet, A. L., Grandhomme, V., Renaud, F., Fritz, H., Green, A. J., Thomas, F., van der Werf, S., Aubry, P., Guillemain, M., & Gauthier-Clerc, M. (2009). Spread of avian influenza viruses by common teal (*Anas crecca*) in Europe. *PLoS One*, 4(10), e7289. doi:10.1371/journal.pone.0007289
- Lebarbenchon, C., van der Werf, S., Thomas, F., Aubin, J. T., Azebi, S., Cuvelier, F., Jeannin, P., Roca, V., Chang, C. M., Kayser, Y., Roche, B., Guégan, J. F., Renaud, F., & Gauthier-Clerc, M. (2007). Absence of detection of highly pathogenic H5N1 in migratory waterfowl in southern France in 2005–2006. *Infect Genet Evol*, 7(5), 604-608. doi:https://doi.org/10.1016/j.meegid.2007.05.009
- Lewis, N. S., Banyard, A. C., Whittard, E., Karibayev, T., Al Kafagi, T., Chvala, I., Byrne, A., Meruyert Akberovna, S., King, J., Harder, T., Grund, C., Essen, S., Reid, S. M., Brouwer, A., Zinyakov, N. G., Tegzhanov, A., Irza, V., Pohlmann, A., Beer, M., Fouchier, R. A. M., Akhmetzhan Akievich, S., & Brown, I. H. (2021). Emergence and spread of novel H5N8, H5N5 and H5N1 clade 2.3.4.4 highly pathogenic avian influenza in 2020. *Emerg Microbes Infect*, 10(1), 148-151. doi:10.1080/22221751.2021.1872355
- Li, X., Shi, J., Guo, J., Deng, G., Zhang, Q., Wang, J., He, X., Wang, K., Chen, J., Li, Y., Fan, J., Kong, H., Gu, C., Guan, Y., Suzuki, Y., Kawaoka, Y., Liu, L., Jiang, Y., Tian, G., Li, Y.,

- Bu, Z., & Chen, H. (2014). Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 Avian Influenza viruses. *PLoS Pathog*, 10(11), e1004508. doi:10.1371/journal.ppat.1004508
- Lo, F. T., Zecchin, B., Diallo, A. A., Racky, O., Tassoni, L., Diop, A., Diouf, M., Diouf, M., Samb, Y. N., Pastori, A., Gobbo, F., Ellero, F., Diop, M., Lo, M. M., Diouf, M. N., Fall, M., Ndiaye, A. A., Gaye, A. M., Badiane, M., Lo, M., Youm, B. N., Ndao, I., Niaga, M., Terregino, C., Diop, B., Ndiaye, Y., Angot, A., Seck, I., Niang, M., Soumare, B., Fusaro, A., & Monne, I. (2022). Intercontinental Spread of Eurasian Highly Pathogenic Avian Influenza A(H5N1) to Senegal. *Emerg Infect Dis*, 28(1), 234-237. doi:10.3201/eid2801.211401
- Lycett, S. J., Duchatel, F., & Digard, P. (2019). A brief history of bird flu. *PHILOS T R SOC B*, 374(1775), 20180257. doi:doi:10.1098/rstb.2018.0257
- Mancera Gracia, J. C., Van den Hoecke, S., Richt, J. A., Ma, W., Saelens, X., & Van Reeth, K. (2017). A reassortant H9N2 influenza virus containing 2009 pandemic H1N1 internal-protein genes acquired enhanced pig-to-pig transmission after serial passages in swine. *Sci Rep*, 7(1), 1323. doi:10.1038/s41598-017-01512-x
- Monto, A. S., McKimm-Breschkin, J. L., Macken, C., Hampson, A. W., Hay, A., Klimov, A., Tashiro, M., Webster, R. G., Aymard, M., Hayden, F. G., & Zambon, M. (2006). Detection of Influenza Viruses Resistant to Neuraminidase Inhibitors in Global Surveillance during the First 3 Years of Their Use. *Antimicrob Agents Chemother*, 50(7), 2395-2402. doi:doi:10.1128/aac.01339-05
- Munster, V. J., Baas, C., Lexmond, P., Waldenström, J., Wallensten, A., Fransson, T., Rimmelzwaan, G. F., Beyer, W. E. P., Schutten, M., Olsen, B., Osterhaus, A. D. M. E., & Fouchier, R. A. M. (2007). Spatial, Temporal, and Species Variation in Prevalence of Influenza A Viruses in Wild Migratory Birds. *PLOS Pathog*, 3(5), e61. doi:10.1371/journal.ppat.0030061
- Pantin-Jackwood, M. J., & Swayne, D. E. (2009). Pathogenesis and pathobiology of avian influenza virus infection in birds. *Rev Sci Tech*, 28(1), 113-136.
- Panzarin, V., Marciano, S., Fortin, A., Brian, I., D'Amico, V., Gobbo, F., Bonfante, F., Palumbo, E., Sakoda, Y., Le, K. T., Chu, D.-H., Shittu, I., Meseko, C., Haido, A. M., Odoom, T., Diouf, M. N., Djegui, F., Steensels, M., Terregino, C., & Monne, I. (2022). Redesign and Validation of a Real-Time RT-PCR to Improve Surveillance for Avian Influenza Viruses of the H9 Subtype. *Viruses*, 14(6), 1263. Retrieved from <https://www.mdpi.com/1999-4915/14/6/1263>
- Pohlmann, A., King, J., Fusaro, A., Zecchin, B., Banyard, A. C., Brown, I. H., Byrne, A. M. P., Beerens, N., Liang, Y., Heutink, R., Harders, F., James, J., Reid, S. M., Hansen, R. D. E., Lewis, N. S., Hjulsgaard, C., Larsen, L. E., Zohari, S., Anderson, K., Bröjer, C., Nagy, A., Savič, V., van Borm, S., Steensels, M., Briand, F. X., Swieton, E., Smietanka, K., Grund,

- C., Beer, M., & Harder, T. (2022). Has Epizootic Become Enzootic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. *mBio*, 13(4), e0060922. doi:10.1128/mbio.00609-22
- Ratnasingham, S., & Hebert, P. D. (2007). bold: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Mol Ecol Notes*, 7(3), 355-364. doi:10.1111/j.1471-8286.2007.01678.x
- Regnaut, S., Lucas, F. S., & Fumagalli, L. (2006). DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations. *Conserv Genet*, 7(3), 449-453. doi:10.1007/s10592-005-9023-7
- Röhm, C., Horimoto, T., Kawaoka, Y., Süß, J., & Webster, R. G. (1995). Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? *Virology*, 209(2), 664-670. doi:10.1006/viro.1995.1301
- Sang, X., Wang, A., Chai, T., He, X., Ding, J., Gao, X., Li, Y., Zhang, K., Ren, Z., Li, L., Yu, Z., Wang, T., Feng, N., Zheng, X., Wang, H., Zhao, Y., Yang, S., Gao, Y., & Xia, X. (2015). Rapid emergence of a PB2-E627K substitution confers a virulent phenotype to an H9N2 avian influenza virus during adoption in mice. *Arch Virol*, 160(5), 1267-1277. doi:10.1007/s00705-015-2383-5
- Sediri, H., Thiele, S., Schwalm, F., Gabriel, G., & Klenk, H. D. (2016). PB2 subunit of avian influenza virus subtype H9N2: a pandemic risk factor. *J Gen Virol*, 97(1), 39-48. doi:10.1099/jgv.0.000333
- Senne, D. A., Panigrahy, B., Kawaoka, Y., Pearson, J. E., Süß, J., Lipkind, M., Kida, H., & Webster, R. G. (1996). Survey of the Hemagglutinin (HA) Cleavage Site Sequence of H5 and H7 Avian Influenza Viruses: Amino Acid Sequence at the HA Cleavage Site as a Marker of Pathogenicity Potential. *Avian Dis*, 40(2), 425-437. doi:10.2307/1592241
- Slomka, M. J., Coward, V. J., Banks, J., Löndt, B. Z., Brown, I. H., Voermans, J., Koch, G., Handberg, K. J., Jørgensen, P. H., Cherbonnel-Pansart, M., Jestin, V., Cattoli, G., Capua, I., Ejdersund, A., Thorén, P., & Czifra, G. (2007a). Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. *Avian Dis*, 51(1 Suppl), 227-234. doi:10.1637/7674-063006r1.1
- Slomka, M. J., Pavlidis, T., Banks, J., Shell, W., McNally, A., Essen, S., & Brown, I. H. (2007b). Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. *Avian Dis*, 51(1 Suppl), 373-377. doi:10.1637/7664-060906r1.1
- Slomka, M. J., Pavlidis, T., Coward, V. J., Voermans, J., Koch, G., Hanna, A., Banks, J., & Brown, I. H. (2009). Validated RealTime reverse transcriptase PCR methods for the diagnosis and pathotyping of Eurasian H7 avian influenza viruses. *Influenza Other Respir Viruses*, 3(4), 151-164. doi:10.1111/j.1750-2659.2009.00083.x

- Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T., & Suarez, D. L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol*, 40(9), 3256-3260. doi:10.1128/jcm.40.9.3256-3260.2002
- Stallknecht, D. E., & Brown, J. D. (2008). Ecology of Avian Influenza in Wild Birds. In *Avian Influenza* (pp. 43-58).
- Suttie, A., Deng, Y. M., Greenhill, A. R., Dussart, P., Horwood, P. F., & Karlsson, E. A. (2019). Inventory of molecular markers affecting biological characteristics of avian influenza A viruses. *Virus Genes*, 55(6), 739-768. doi:10.1007/s11262-019-01700-z
- Tanikawa, T., Sakuma, S., Yoshida, E., Tsunekuni, R., Nakayama, M., & Kobayashi, S. (2021). Comparative susceptibility of the common teal (*Anas crecca*) to infection with high pathogenic avian influenza virus strains isolated in Japan in 2004–2017. *Veterinary Microbiology*, 263, 109266. doi:https://doi.org/10.1016/j.vetmic.2021.109266
- Tarasiuk, K., Kycko, A., Świętoń, E., Bocian, Ł., Wyrstek, K., & Śmietanka, K. (2023). Homo- and Heterosubtypic Immunity to Low Pathogenic Avian Influenza Virus Mitigates the Clinical Outcome of Infection with Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4.b in Captive Mallards (*Anas platyrhynchos*). *Pathogens*, 12(2), 217. Retrieved from https://www.mdpi.com/2076-0817/12/2/217
- The Global Consortium for H5N8 and Related Influenza Viruses. (2016). Role for migratory wild birds in the global spread of avian influenza H5N8. *Science*, 354(6309), 213-217. doi:10.1126/science.aaf8852
- Torrontegi, O., Alvarez, V., Acevedo, P., Gerrikagoitia, X., Höfle, U., & Barral, M. (2019). Long-term avian influenza virus epidemiology in a small Spanish wetland ecosystem is driven by the breeding Anseriformes community. *Vet Res*, 50(1), 4. doi:10.1186/s13567-019-0623-5
- Trifinopoulos, J., Nguyen, L. T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res*, 44(W1), W232-235. doi:10.1093/nar/gkw256
- van den Brand, J. M. A., Verhagen, J. H., Veldhuis Kroeze, E. J. B., van de Bildt, M. W. G., Bodewes, R., Herfst, S., Richard, M., Lexmond, P., Bestebroer, T. M., Fouchier, R. A. M., & Kuiken, T. (2018). Wild ducks excrete highly pathogenic avian influenza virus H5N8 (2014-2015) without clinical or pathological evidence of disease. *Emerg Microbes Infect*, 7(1), 67. doi:10.1038/s41426-018-0070-9
- van Dijk, J. G., Hoyer, B. J., Verhagen, J. H., Nolet, B. A., Fouchier, R. A., & Klaassen, M. (2014). Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus. *J Anim Ecol*, 83(1), 266-275. doi:10.1111/1365-2656.12131
- Veits, J., Weber, S., Stech, O., Breithaupt, A., Gräber, M., Gohrbandt, S., Bogs, J., Hundt, J., Teifke, J. P., Mettenleiter, T. C., & Stech, J. (2012). Avian influenza virus hemagglutinins

- H2, H4, H8, and H14 support a highly pathogenic phenotype. *Proc Natl Acad Sci U.S.A.*, 109(7), 2579-2584. doi:doi:10.1073/pnas.1109397109
- Venkatesh, D., Poen, M. J., Bestebroer, T. M., Scheuer, R. D., Vuong, O., Chkhaidze, M., Machabishvili, A., Mamuchadze, J., Ninua, L., Fedorova, N. B., Halpin, R. A., Lin, X., Ransier, A., Stockwell, T. B., Wentworth, D. E., Kriti, D., Dutta, J., van Bakel, H., Puranik, A., Slomka, M. J., Essen, S., Brown, I. H., Fouchier, R. A. M., & Lewis, N. S. (2018). Avian Influenza Viruses in Wild Birds: Virus Evolution in a Multihost Ecosystem. *J Virol*, 92(15). doi:10.1128/jvi.00433-18
- Verhagen, J. H., Fouchier, R. A. M., & Lewis, N. (2021). Highly Pathogenic Avian Influenza Viruses at the Wild-Domestic Bird Interface in Europe: Future Directions for Research and Surveillance. *Viruses*, 13(2). doi:10.3390/v13020212
- Vijaykrishna, D., Bahl, J., Riley, S., Duan, L., Zhang, J. X., Chen, H., Peiris, J. S. M., Smith, G. J. D., & Guan, Y. (2008). Evolutionary Dynamics and Emergence of Panzootic H5N1 Influenza Viruses. *PLOS Pathog*, 4(9), e1000161. doi:10.1371/journal.ppat.1000161
- Vo, A.-T. E., & Jedlicka, J. A. (2014). Protocols for metagenomic DNA extraction and Illumina amplicon library preparation for faecal and swab samples. *Mol Ecol Resour*, 14(6), 1183-1197. doi:https://doi.org/10.1111/1755-0998.12269
- Walker, P. J., Siddell, S. G., Lefkowitz, E. J., Mushegian, A. R., Adriaenssens, E. M., Dempsey, D. M., Dutilh, B. E., Harrach, B., Harrison, R. L., Hendrickson, R. C., Junglen, S., Knowles, N. J., Kropinski, A. M., Krupovic, M., Kuhn, J. H., Nibert, M., Orton, R. J., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D. B., Varsani, A., Zerbini, F. M., & Davison, A. J. (2020). Changes to virus taxonomy and the Statutes ratified by the International Committee on Taxonomy of Viruses (2020). *Arch Virol*, 165(11), 2737-2748. doi:10.1007/s00705-020-04752-x
- Wang, D., Zhu, W., Yang, L., & Shu, Y. (2021). The Epidemiology, Virology, and Pathogenicity of Human Infections with Avian Influenza Viruses. *Cold Spring Harb Perspect Med*, 11(4). doi:10.1101/cshperspect.a038620
- Wang, W., Lu, B., Zhou, H., Suguitan, A. L., Jr., Cheng, X., Subbarao, K., Kemble, G., & Jin, H. (2010). Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. *J Virol*, 84(13), 6570-6577. doi:10.1128/jvi.00221-10
- Watanabe, Y., Ibrahim, M. S., Ellakany, H. F., Kawashita, N., Mizuike, R., Hiramatsu, H., Sriwilaijaroen, N., Takagi, T., Suzuki, Y., & Ikuta, K. (2011). Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. *PLoS Pathog*, 7(5), e1002068. doi:10.1371/journal.ppat.1002068

- World Health Organization. (2018). Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. *Wkly Epidemiol Rec*, 93(12), 142-152.
- World Organisation for Animal Health. (2021). OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Retrieved from https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf
- Xu, X., Subbarao, Cox, N. J., & Guo, Y. (1999). Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology*, 261(1), 15-19. doi:10.1006/viro.1999.9820
- Yamaji, R., Yamada, S., Le, M. Q., Ito, M., Sakai-Tagawa, Y., & Kawaoka, Y. (2015). Mammalian adaptive mutations of the PA protein of highly pathogenic avian H5N1 influenza virus. *J Virol*, 89(8), 4117-4125. doi:10.1128/jvi.03532-14

SECTION TWO: LOOKING FOR AVIAN METAPNEUMOVIRUS'S WILD HOSTS

V. Disentangling the role of wild birds in Avian Metapneumovirus (AMPV) epidemiology: a systematic review and meta-analysis

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Given the Avian metapneumovirus (aMPV) disease burden in poultry worldwide and the evidence of a possible role played by wild birds in the virus epidemiology, the present study summarises aMPV serological and molecular data on free-ranging avifauna available in the literature by conducting a systematic review and meta-analysis. A computerised literature research was performed on PubMed, Scopus, CAB Direct and Web of Science to identify relevant publications across the period 1990–2021, along with the screening of reference lists. A random-effect model was applied to calculate pooled prevalence estimates with 95% confidence intervals. The inconsistency index statistic (I^2) was applied to assess between-study heterogeneity. Subgroup analyses for molecular studies only were performed according to geographical area of samplings, taxonomic order, genus and migration patterns of the birds surveyed. A total of 11 publications on molecular surveys and 6 on serological ones were retained for analysis. The pooled molecular prevalence was 6% (95% CI: 1–13%) and a high between-study heterogeneity was detected ($I^2 = 96\%$, $p < 0.01$). Moderator analyses showed statistically significant differences according to geographical area studied, taxonomic order and genus. Concerning serological prevalence, a pooled estimate of 14% (95% CI: 1–39%), along with a high between-study heterogeneity, was obtained ($I^2 = 98\%$, $p < 0.01$). Moderator analysis was not performed due to the scarcity of eligible serological studies included. Overall, molecular and serological evidence suggests that some wild bird taxa could play a role in aMPV epidemiology. Particularly, wild ducks, geese, gulls and pheasants, according to scientific contributions hereby considered, proved to be susceptible to aMPV, and due to host ecology, may act as a viral carrier or reservoir. Further surveys of wild birds are encouraged for a better comprehension of the poultry/wild bird interface in aMPV epidemiology and for better characterising the virus host breadth.

5.1. Introduction

5.1.1. Rationale

Avian metapneumovirus (aMPV) is an enveloped negative-sense RNA virus included in the genus *Metapneumovirus* of the Pneumoviridae family (Rima *et al.*, 2017). aMPV is directly transmitted and causes upper respiratory tract disease and reproductive disorders in turkeys, chickens, domestic ducks and Guinea fowls, with subsequent economic and welfare issues for the worldwide poultry industry (Cecchinato *et al.*, 2018; Rautenschlein, 2020).

To date, four aMPV subtypes (A, B, C and D) have been recognised according to genetic or antigenic characteristics (Bäyon-Auboyer *et al.*, 2000; Juhasz & Easton, 1994; Seal, 2000). Recent detections of phylogenetically distinct aMPV strains in North America suggest the existence of additional viral subtypes. Particularly, a divergent aMPV strain was detected in a great black-backed gull (*Larus marinus* LINNAEUS, 1758) (Canuti *et al.*, 2019) and another in a monk parakeet chick (*Myiopsitta monachus* BODDAERT, 1783) (Retallack *et al.*, 2019).

With respect to viral spreading, aMPV-A and B are distributed almost worldwide and are especially found in Asia, Africa, Europe and South America; aMPV-C, which has been further recognised in a North American and a Eurasian genetic lineage, has occurred in the United States (North American lineage) (Senne *et al.*, 1997) and in France and China (Eurasian lineage) (Sun *et al.*, 2014; Toquin *et al.*, 1999, 2006; Wei *et al.*, 2013). aMPV subtypes also differ in terms of host breadth. aMPV-A, B and C (North American lineage) mainly infect turkeys or chickens, whilst the Eurasian subtype C lineage infects ducks, and the subtype D (detected only once in France) turkeys (Rautenschlein, 2020). However, experimental evidence suggests that the subtypes' host-range may be more intricate (Brown *et al.*, 2019).

Since aMPV's first appearance in South Africa in the late 1970s (Buys & du Preez, 1980), its origin and subsequent spread worldwide have been repeatedly ascribed, among other factors, to wild birds and to their migratory movements along their natural flight paths (Jones, 1996; Panigrahy *et al.*, 2000; Shin *et al.*, 2002). Particularly, aMPV's sudden appearance in Minnesota (USA), a state with a large migratory bird population, strongly contributed to this hypothesis (Cook, 2000b). Further evidence of a seasonal pattern observed during aMPV outbreaks in North American turkeys and viral detections of aMPV in wild birds outside endemic territories, suggested wild species as a viral carrier or reservoir host (Shin *et al.*, 2002; Turpin *et al.*, 2008). Additional viral or serological evidence of aMPV occurrence in free-living birds, as reported in America and Europe over the last two decades, proved the actual viral circulation in non-domesticated species.

Considering that studies providing a wide overview of diseases at the poultry/wild bird interface have provided valuable information in directing future research (Chen *et al.*, 2019; Graziosi *et al.*, 2021; Sawicka *et al.*, 2020; Sukon *et al.*, 2021), the present work aims to assess current knowledge on aMPV occurrence in wild birds.

5.1.2. Objectives

Given the aMPV disease burden in poultry worldwide and the evidence of a possible role played by wild birds in aMPV epidemiology, the present study summarises aMPV serological and molecular data available in the literature on free-ranging avifauna by conducting a systematic review and meta-analysis. Although aMPV infection has been widely reviewed (Cook, 2000a, 2000b; Cook & Cavanagh, 2002; Jones, 1996; Kaboudi & Lachheb, 2021; Naylor & Jones, 1993; Njenga *et al.*, 2003), to the best of our knowledge this is the first systematic review and meta-analysis which focuses on aMPV occurrence in wild birds.

5.2. Materials and methods

5.2.1. Protocol

A systematic review and metanalysis were applied to summarise data on aMPV infection in wild birds. To build our protocol, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA-P) (Moher *et al.*, 2015) and the PRISMA 2020 Statement recommendations (Page *et al.*, 2020) were followed.

5.2.2. Information sources and search strategy

A literature search for scientific contributions on aMPV in wild birds was conducted from 16/02/2021 to 16/12/2021. Four electronic databases were accessed including PubMed (<https://pubmed.ncbi.nlm.nih.gov/>), the Web of Science (<https://apps.webofknowledge.com/>), Scopus (<https://www.scopus.com/>) and CAB Direct (<https://www.cabdirect.org>). Advanced search builders and two separate search strategies were applied, one for searching molecular studies and another for serological ones. The following keywords were used: 'avian metapneumovirus' or 'avian pneumovirus' and 'wild birds' or 'free-living birds' together with molecular or serological method related terms (Table 1 and Table 2). Filters on language (English) and timespan (studies published after 1990) were used. Manual screening of citations and reference lists of the articles retrieved were also performed to increase the chance of finding relevant publications (Higgins *et al.*, 2021).

Table 1. Search lines used for the literature research of aMPV molecular studies in wild birds present in PubMed, Scopus, CAB Direct and Web of Science databases. Boolean operators ‘OR’ and ‘AND’ were applied. Number of scientific contributions retrieved before duplicate removal is reported.

Database	Search line	No. of studies retrieved
PubMed	((("pneumovirus"[Title/Abstract] OR "avian pneumovirus" OR "avian metapneumovirus" OR "ampv"[Title/Abstract] OR "avian Metapneumovirus"[Title/Abstract]) OR ("metapneumovirus/genetics"[MeSH Terms] OR "metapneumovirus/isolation and purification"[MeSH Terms] OR "avian metapneumovirus"[MeSH Terms])) AND ("animals, wild"[MeSH Terms] OR "wild bird"[MeSH Terms] OR ("wild"[Title/Abstract] AND "bird"[Title/Abstract]))	28
Scopus	TITLE-ABS ("avian metapneumovirus") OR TITLE-ABS ("pneumovirus") OR TITLE-ABS("avian pneumovirus") AND (TITLE-ABS ("wild") AND TITLE-ABS (*bird*)) OR TITLE-ABS (*wild AND bird*) AND (TITLE-ABS (*detect*) OR TITLE-ABS ("infection") OR TITLE-ABS (* isolat*))	22
CAB Direct	(title:(Avian Pneumovirus) OR ab:(Avian Pneumovirus) OR up:(Avian Pneumovirus) OR id:(Avian Pneumovirus) OR cabicode:(Avian Pneumovirus) OR (title:(Avian metapneumovirus) OR ab:(Avian metapneumovirus) OR up:(Avian metapneumovirus) OR id:(Avian metapneumovirus) OR cabicode:(Avian metapneumovirus)) AND (title:(detection) OR ab:(detection) OR up:(detection) OR id:(detection) OR cabicode:(detection) OR title:(isolation) OR ab:(isolation) OR up:(isolation) OR id:(isolation) OR cabicode:(isolation)) AND (title:(wild bird*) OR ab:(wild bird*) OR up:(wild bird*) OR id:(wild bird*) OR cabicode:(wild bird*))	10
Web of Science	(ALL=(("avian metapneumovirus" OR "avian pneumovirus" OR "pneumovirus") AND (((wild) OR (free-living)) AND (bird*)) AND (infection* OR detection* OR isolat*))	22

Table 2. Search lines used for the literature research of aMPV serological studies in wild birds present in PubMed, Scopus, CAB Direct and Web of Science databases. Boolean operators ‘OR’ and ‘AND’ were applied. Number of scientific contributions retrieved before duplicate removal is reported.

Database	Search line	No. of studies retrieved
PubMed	((("pneumovirus"[Title/ Abstract]) OR "avian pneumovirus"[Title/ Abstract]) OR metapneumovirus"[Title/ Abstract])) AND (((((((Antibodies, Viral[MeSH Terms]) OR *antibody*[Title/ Abstract]) OR *serolog*[Title/ Abstract]) OR prevalence*[Title/ Abstract]) OR Study*[Title/ Abstract]) OR Survey*[Title/ Abstract]) OR Survey[MeSH Terms])) AND ("animals, wild"[MeSH Terms] OR wild bird*[MeSH Terms] OR (wild[Title/ Abstract] AND bird*[Title/ Abstract]))	16
Scopus	TITLE-ABS("avian metapneumovirus") OR TITLE-ABS("pneumovirus") OR TITLE-ABS("avian pneumovirus") AND (TITLE-ABS("wild") AND TITLE-ABS(*bird*)) OR TITLE-ABS (*wild AND bird*) AND (TITLE-ABS (*sero*) OR TITLE-ABS (*survey*) OR TITLE-ABS (*antibod*))	14
CAB Direct	(title:(Avian Pneumovirus) OR ab:(Avian Pneumovirus) OR up:(Avian Pneumovirus) OR id:(Avian Pneumovirus) OR cabicode:(Avian Pneumovirus) OR (title:(Avian metapneumovirus) OR ab:(Avian metapneumovirus) OR up:(Avian metapneumovirus) OR id:(Avian metapneumovirus) OR cabicode:(Avian metapneumovirus)) AND (title:(sero*) OR ab:(sero*) OR up:(sero*) OR id:(sero*) OR cabicode:(sero*) OR title:(prevalence) OR ab:(prevalence) OR up:(prevalence) OR id:(prevalence) OR cabicode:(prevalence)) AND (title:(wild bird*) OR ab:(wild bird*) OR up:(wild bird*) OR id:(wild bird*) OR cabicode:(wild bird*))	11
Web of Science	ALL=((("avian metapneumovirus" OR "avian pneumovirus" OR "pneumovirus") AND (((wild) OR (free-living)) AND (bird*)) AND (antibod* OR sero* OR survey*))	14

5.2.3. Selection criteria

Literature was screened by two independent investigators (G.G. and C.L.). Considering the paucity of information on aMPV occurrence in wild birds, different types of scientific contributions were considered, such as original articles, book chapters, scientific correspondence, conference proceedings, conference contributions and short communications. After duplicate removal, titles and abstracts were screened to exclude non-relevant articles with respect to our research question (i.e. studies regarding experimental trials, studies on poultry or on intensive-reared ducks, studies on farmed wild species). The full texts of the articles which passed the initial screening were

downloaded and independently assessed for eligibility, data analysis and extraction by G.G. and C.L. If disagreements occurred, a third experienced author in the avian pathology field was consulted (E.C.). In particular, an article was considered eligible if the following requirements were met: 1) the study reported information on the occurrence of aMPV in wild bird species both free-living or captivity kept (i.e. from wildlife rescue centres); 2) the population tested was included in two studies; 3) the species of bird tested was not identified; 4) the aMPV sero-prevalence or viro-prevalence outcome was not reported. If pre-established criteria were partially met, partial data consistent with our standards were considered. Whenever the same population was surveyed in more than one publication, we would consider the contribution with the most exhaustive information. Reasons for exclusion of any study were appropriately recorded and discussed. Whenever necessary, additional information on the survey was directly sought from the respective authors.

5.2.4. *Data managing and pre-processing*

The following information was extracted from each study and included in a data extraction sheet (Microsoft Excel 2021, version 16.49): first author, year of the publication, title, country, region, sampling period, taxonomic order of the host, taxonomic genus of the birds, species, age classes and sex, number of birds sampled, number of birds testing positive and diagnostic method applied. All the species were also categorised as 'migrant', 'migrant/resident' and 'resident' according to the region of study and the information provided by distribution maps on BirdLife International (<http://datazone.birdlife.org/species/factsheet>). Whenever prevalence data were expressed as percentages, raw numbers were obtained converting the percentages to the closest integers. As taxonomic inconsistencies were found, common names and species were adapted to current standards following an international online database (Gill, Donsker & Rasmussen, 2021). To facilitate data analysis, countries of each study were grouped into continents.

5.2.5. *Study risk of bias and quality assessment*

Despite current tools able to evaluate publication bias, conventional funnel plots are believed to be inaccurate for meta-analyses of proportion with low outcomes (Hunter *et al.*, 2014). G.G. and C.L. independently assessed the quality of the eligible studies applying the Joanna Briggs Institute (JBI) critical appraisal checklist for prevalence studies (<https://jbi.global/critical-appraisal-tools>). If the JBI checklist's outcome resulted in 'seek for further info', the corresponding author of a given study was contacted. Although the JBI checklist was intended for human-related studies, it perceivably suited the dataset of the study hereby presented.

5.2.6. *Statistical analysis*

Collected data were analysed using R software, version 4.0.4. (R Core Team, 2019) using the *meta* package. Primary outcomes of interest were the estimated overall molecular prevalence and the serological prevalence of aMPV in wild birds, calculated using a double-arcsine transformation of data and a random-effects model (Wang, 2018). For the pooled estimates, the Cochran's Q and the inconsistency index (I^2) were used to estimate the between-study heterogeneity. The I^2 statistic was interpreted as small, medium or high according to < 25%, 25–50% and > 75% values, respectively (Higgins & Thompson, 2002). Subgroup analysis was planned to explore the potential sources of heterogeneity including the following variables: continent where the study was conducted; migration pattern of the species according to the region of the study; taxonomy of the birds (order and genus). Two independent meta-analysis were performed, one for serological studies and another for molecular ones. Eventually, given the paucity of data for serological surveys, we performed the moderator analysis on molecular surveys only.

5.3. Results

5.3.1. Literature searches

As shown in Figure 1, 98 publications on aMPV molecular surveys were retrieved through database research and reading of relevant reference lists. After duplicate records' removal ($n = 41$), the initial screening of titles and abstracts according to pre-established criteria brought about the exclusion of 30 more works. Altogether, 27 scientific contributions were accessed as full texts. In a secondary assessment, 16 further articles were excluded due to: data already included in other publications; surveys regarding farmed wild species; prevalence data not available; preliminary results presented. Eventually, 11 publications were retained for qualitative synthesis and meta-analysis as shown in Table 3. With regards to geographical distribution, the majority of the studies were conducted in North America ($n = 5$), followed by Europe ($n = 4$) and South America ($n = 2$). Particularly, the rank order of countries based on the number of aMPV molecular studies was United States ($n = 3$) > Canada ($n = 2$) ~ Brazil ($n = 2$) ~ Germany ($n = 2$) > Italy ($n = 1$) ~ Netherlands ($n = 1$). A total of 3011 wild birds were molecularly tested for aMPV and 160 individuals tested positive.

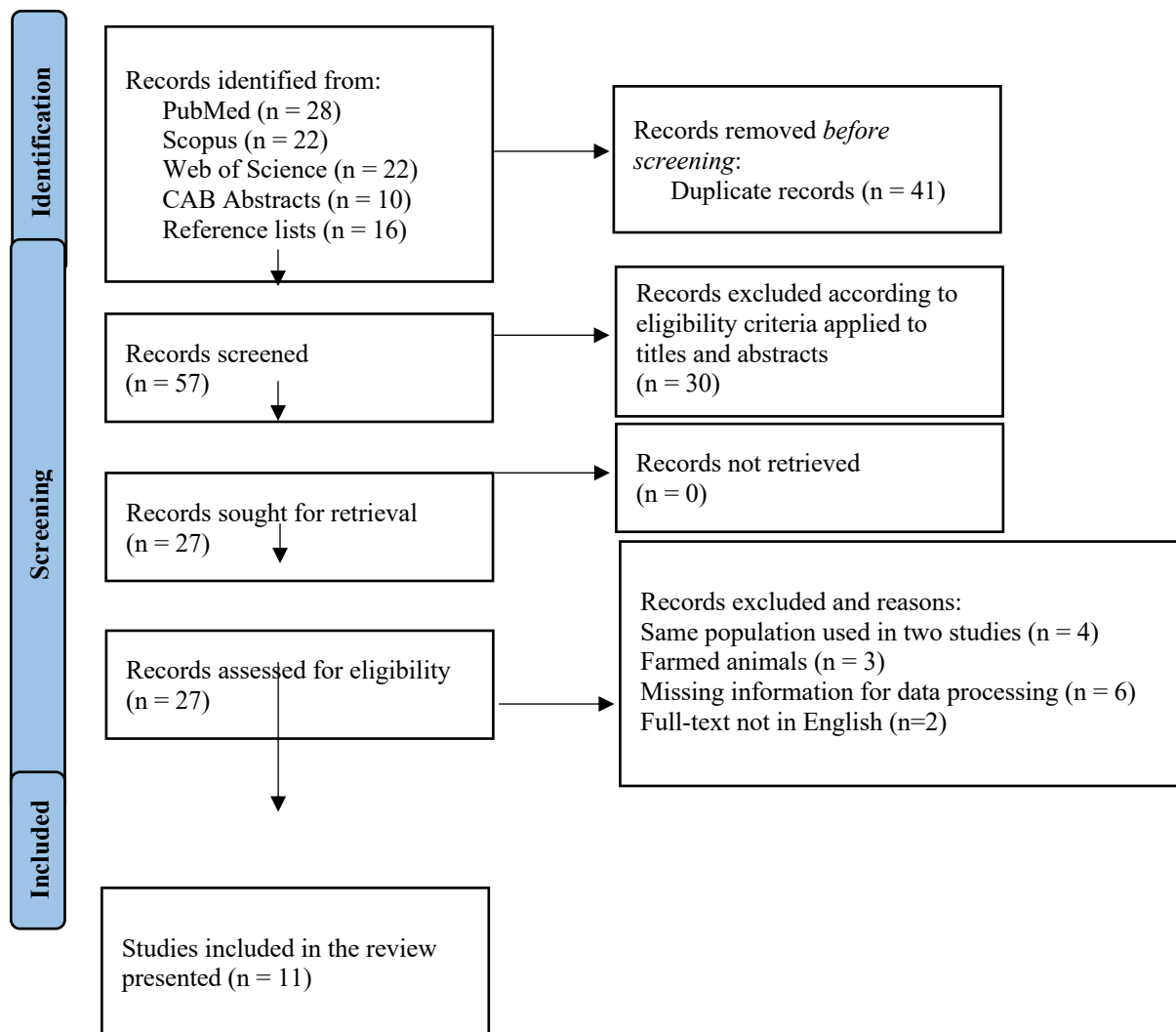


Figure 1. Flow diagram of the selection process of molecular studies on aMPV in free-living wild birds, identified via databases and reference list reading.

1 **Table 3.** Details of the eligible studies on the molecular prevalence of aMPV in wild birds, sorted by continent.
2

Reference	Location	Period	Sample type	Taxonomic order of birds	Test	Gene targeted (Subtype specificity of the test)	Sample size	Number of positives (%)	GenBank accession number	Subtype detected
NORTH AMERICA										
Canuti <i>et al.</i> 2019	Canada (Newfoundland and Labrador)	2014-2015	Oropharyngeal swab; cloacal swab	Charadriiformes; Anseriformes	Nested-PCR on Ion Torrent sequencing products	n.a.	83	1 (1.2)	MN175553	aMPV - <i>Gull Metapneumovirus</i> [†]
Jardine <i>et al.</i> 2018	Canada (Ontario)	2016	Oropharyngeal swab; cloacal swab	Anseriformes	qRT-PCR	M gene (C subtype)	374	84 (22.4)	n.a. [‡]	aMPV-C
Cha <i>et al.</i> 2013	United States (n.s. [§])	n.r.	Oropharyngeal swab	Anseriformes; Gruiformes	RT-PCR	G genes (C subtype)	50	12 (24)	n.r. [¶]	aMPV-C
Turpin <i>et al.</i> 2008	United States (Georgia, South Carolina, Arkansas, Ohio)	2000	Choanal swab	Anseriformes; Gruiformes	RT-PCR	M gene (A, B and C subtypes)	706	17 (2.4)	FJ195329.1 - FJ195345.1	aMPV-C
Bennett <i>et al.</i> 2004	United States (Minnesota)	2001-2002	Choanal swab; nasal turbinates tissue	Charadriiformes; Passeriformes	RT-PCR	M gene (C subtype)	272 ^{††}	13 (4.8)	DQ009484.1 ; Q2Y2M2-Q2Y2M6	aMPV-C
SOUTH AMERICA										
Rizotto <i>et al.</i> 2019	Brazil (São Paulo State)	2013-2015	Oropharyngeal swab; cloacal swab	Anseriformes; Columbiformes; Strigiformes;	RT-PCR	N gene (A and B subtypes)	67	6 (8.9)	n.r.	aMPV-A

				Falconiformes; Piciformes						
Felippe <i>et al.</i> 2011	Brazil (São Paulo State)	2005- 2008	Tracheal swab; cloacal swab	Anseriformes; Columbiformes	RT-PCR	G gene (A and B subtypes)	67	14 (20.9)	JF758852 - JF758858; JF758830 - JF758836	aMPV-A; aMPV-B
EUROPE										
Curland <i>et al.</i> 2018	Germany (Lower Saxony)	2011- 2014	Tracheal sample	Galliformes	RT-PCR	G gene (A and B subtypes)	121	8 (6.6)	n.r.	aMPV-A or B
van Boheemen <i>et al.</i> 2012	The Netherlands	n.s.	Oropharyngeal swab	Anseriformes; Charadriiformes	qRT-PCR	L gene (A, B and C subtypes)	847	5 (0.6)	n.r. ^{††}	aMPV-C
Delogu <i>et al.</i> 2004	Italy	2001	Choanal swab	Charadriiformes; Phenicopteriformes; Pelecaniformes	RT-PCR	G gene (A and B subtypes)	394	0 (0.0)	n.a.	n.a.
Heffels- Redmann <i>et al.</i> 1998	Germany	1990	Conchae and tracheal sample	Charadriiformes	RT-PCR	G gene (A and B subtypes)	30	0 (0)	n.a.	n.a.

3 [†]as proposed by Canuti *et al.* 2019

4 [†]n.a., not applicable

5 [§]n.s., not specified

6 [¶]n.r., not reported

7 ^{††}Pooled samples were excluded from counts

8 ^{##}Sequences were made readily available after request to the Authors.

36 free (SPF) eggs (Rizotto *et al.*, 2019) or chicken embryo tracheal organ cultures (Heffels-Redmann *et*
37 *al.*, 1998). In one study, virus isolation was attempted through oculo-nasal inoculation of SPF three-
38 day old turkeys (Cha *et al.*, 2013). Viral isolation was successfully achieved by Turpin *et al.* (2008)
39 and Cha *et al.* (2013).

40 As shown in Figure 3, a total of 57 contributions on aMPV serological surveys were retrieved
41 through database research and reference list reading. After duplicate records' removal (n = 30), titles
42 and abstracts of 27 articles were screened according to the pre-established criteria. Finally, six full
43 texts, whose details are shown in Table 4, were included in the qualitative synthesis and meta-
44 analysis. Regarding geographic distribution, n = 2 studies were conducted in Germany, n = 1 in Italy,
45 n = 1 in the United States and n = 1 in South Africa. In total, 1646 sera of wild birds were tested and
46 213 resulted positive. Eight different taxonomic orders were surveyed: Anseriformes (n = 1 paper;
47 310 birds tested; 103 positives), Charadriiformes (n = 3 papers; 421 birds tested; 45 positives),
48 Columbiformes (n = 1 paper; 195 birds tested; 1 positive), Coraciiformes (n = 1 paper; 1 bird tested;
49 0 positive), Galliformes (n = 3 papers; 432 birds tested; 37 positives), Gruiformes (n = 1 paper; 114
50 birds tested; 20 positives), Passeriformes (n = 1 paper; 69 birds tested; 6 positives), Pelecaniformes
51 (n = 2 papers; 69 birds tested; 1 positive), Phoenicopteriformes (n = 1 paper; 30 birds tested; 0
52 positive), Strigiformes (n = 1 paper; 3 birds tested; 0 positive).

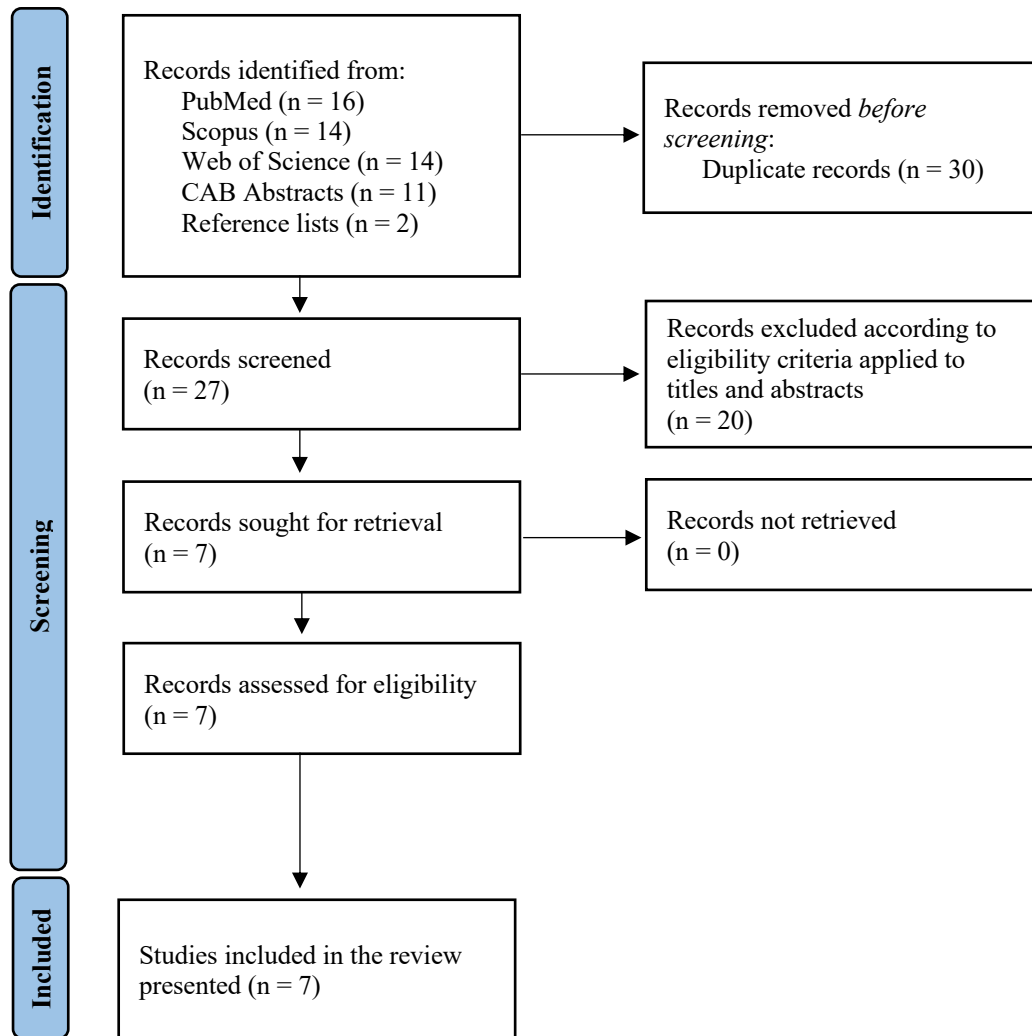
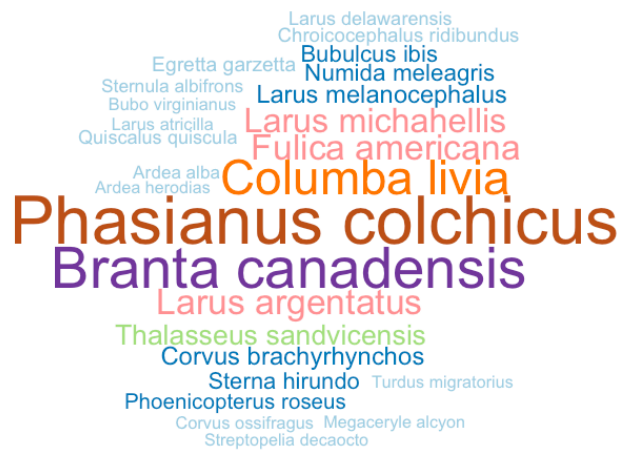


Figure 3. Flow diagram of the selection process of serological studies on aMPV in free-living wild birds, identified via databases and reference list reading.



91 **Figure 4.** Word cloud showing the sample size of wild bird species serologically tested for aMPV.
 92 Data are ordered from low to high, with light colours and a smaller font size for lower number of
 93 studies on a species, darker colour and bigger font size for higher number of studies.
 94
 95

96 **Table 4.** Details of the eligible studies on the serological prevalence of aMPV in wild birds, sorted by continent.
97

Reference	Location	Period	Taxonomic order of birds	Test	Antigen subtype used in the method	Sample size	Number of positives (%)
NORTH AMERICA							
Turpin <i>et al.</i> 2008	United States (Georgia, South Carolina, Arkansas, Ohio)	2000-2001	Anseriformes; Charadriiformes; Columbiformes; Coraciiformes; Pelecaniformes; Passeriformes; Strigiformes; Gruiformes	ELISA [†]	Subtype C – North American lineage	732	131 (17.9)
AFRICA							
Ratcliffe <i>et al.</i> 2000	South Africa	1997-2000	Galliformes	ELISA [‡]	n.a. [§]	17	7 (41.2)
EUROPE							
Gethöffer <i>et al.</i> 2021	Germany	2011-2015	Galliformes	VNT [¶]	n.a.	152	21 (13.8)
Delogu <i>et al.</i> 2004	Italy	2001	Charadriiformes; Phenicopteriformes; Pelecaniformes	ELISA ^{††}	Subtype B	368	0
Catelli <i>et al.</i> 2001	Italy	1992-1994	Galliformes	ELISA ^{††}	Subtype B	263	9 (3.4)
Heffels-Redmann <i>et al.</i> 1998	Germany	1990	Charadriiformes	VNT	Subtype A	114	45 (39.5)

98 [†]in-house Enzyme Linked Immunosorbent Assay (ELISA) test
99 [‡]commercial ELISA kit (Pathasure, Cambridge Veterinary Sciences Ltd., Ely, UK)
100 [§]n.a.: not available
101 [¶]VNT, virus neutralization test
102 ^{††}commercial ELISA kit (Svanovir Avian Pneumovirus-Ab EIA Test, SVANOVA, Biotech, Uppsala, Sweden).

5.3.2. Quality assessment

According to the JBI critical appraisal checklist for prevalence studies, all the contributions met the required standard.

5.3.3. Statistical analyses

The pooled molecular prevalence of aMPV in wild birds was 6% (95% CI: 1–13%) ($I^2 = 96\%$, $p < 0.01$) (Figure 5). Subgroup analyses were performed according to geographical area, taxonomic order and genus of the birds surveyed, and migration patterns. Results suggest a significant difference between the pooled effect estimates for each geographic subgroup with the highest prevalence for South America (P: 14%, 95% CI: 1–39%), followed by North America (P: 8%, 95% CI: 2–20%) and Europe (P: 8%, 95% CI: 0–4%). Taxonomic orders and genera both appear to influence the effect estimates ($p < 0.0001$ each, respectively). Regarding the orders, the highest viro-prevalence was detected in the Passeriformes order (P: 66%, 95% CI: 38–91%), followed by the Columbiformes order (P: 55%, 95% CI: 30–80%) and the Falconiformes order (P: 50%, 95% CI: 0–100%). According to genera, *Passer* showed the highest prevalence (P: 66%, 95% CI: 37–91%), followed by *Columba* (P: 56%, 95% CI: 30–80%) and *Falco* (P: 50%, 95% CI: 0–100%). Finally, migration patterns of the species surveyed did not appear to be significant ($p = 0.1234$).

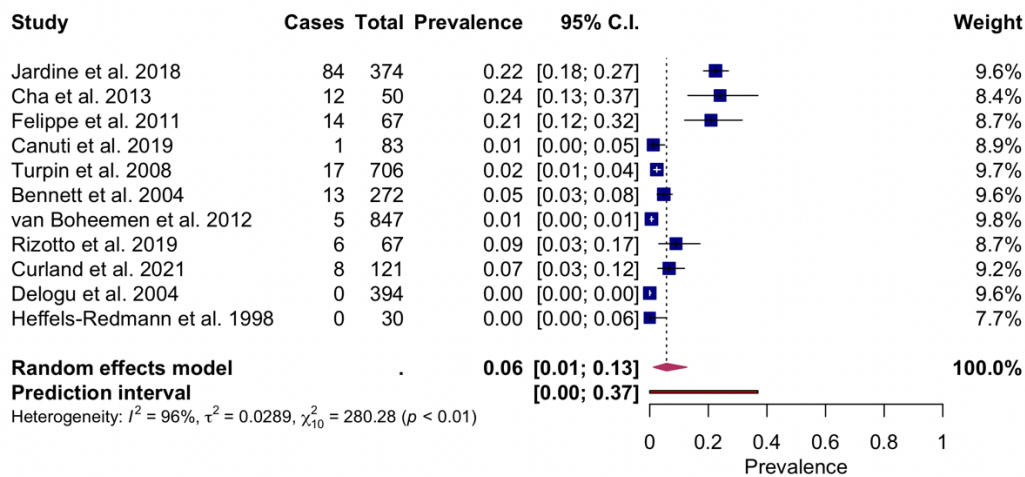


Figure 5. Forest plot of the random-effects meta-analysis of aMPV molecular prevalence. I^2 (inverse variance index), τ^2 = the between-study variance, χ^2 and p-value of the Cochran's Q test for heterogeneity.

With respect to the pooled seroprevalence of aMPV in wild birds, it was estimated as 14% (95% CI: 1–39%) ($I^2 = 98\%$, $p < 0.01$) (Figure 6). Given the lack of data available, subgroup analyses

according to geographical area, taxonomic order of birds surveyed, genera and migration patterns were not performed for serological studies.

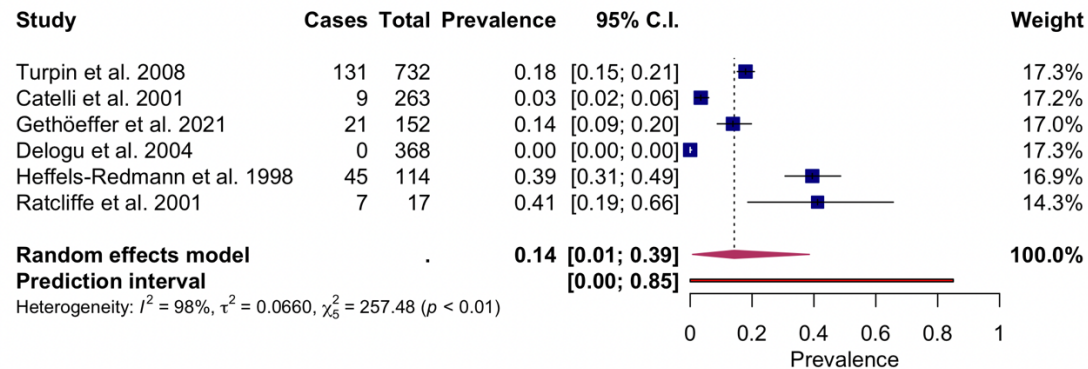


Figure 6. Forest plot of the random-effects meta-analysis of aMPV serological prevalence. I^2 (inverse variance index), τ^2 = the between-study variance, χ^2 and p-value of the Cochran's Q test for heterogeneity.

5.4. Discussion

5.4.1. Summary of evidence

The potential role of wild birds as reservoir hosts or epiphenomena with respect to aMPV epidemiology have not been fully disclosed yet. Despite limited data available, a systematic revision of literature and meta-analysis were, for the first time, applied in our study to the abovementioned question to summarise the sero-viroprevalence of aMPV in wild birds, as a necessary step to identify research gaps and encourage future study directions.

Literature search strategies hereby applied retrieved more molecular studies than serological ones, probably due to the increase in the use of PCR-based technologies with respect to serology for the detection of infectious agents. The overall results of the meta-analyses showed a moderate exposure of wild birds to aMPV (aMPV molecular prevalence: 6%; aMPV serological prevalence: 14%). Given the high degree of between-study heterogeneity evidenced by the meta-analyses conducted, the pooled estimates need to be considered together with the 95% CI.

Possible sources of heterogeneity may be related to the different sensitivities and specificities of the assays used, the area studied, the different sample type analysed, and the different ecology and habits of the wild birds sampled.

The targeting of different gene segments in molecular studies likely influenced the results obtained when testing wild birds, due to the different subtype-specificity of the RT-PCRs assays

used (Jones & Rautenschlein, 2013). With respect to serological surveys, the use of tests having different sensitivity and specificity levels could have further influenced the accuracy of the results obtained.

According to subgroup analyses, our results showed a statistically significant association between positive aMPV molecular findings and the location of the samplings ($p = 0.01$). However, considering that only three categorical variables were extracted from our dataset with respect to the geographical distribution of the studies (North America, South America and Europe), we believe this to be poorly representative of global trends. With respect to North America, initial aMPV detections were linked to spillover events from poultry to free-living wild species. The first aMPV-C molecular detections (Shin *et al.*, 2000) and successful viral isolation in a healthy live-captured Canada goose (*B. canadensis*) in Minnesota (Bennett *et al.*, 2002) led the authors to consider wild birds as natural viral reservoirs. Subsequently, aMPV-C occurrence outside poultry USA endemic territories was reported by Turpin (2008), who detected both antibodies and viral RNA of aMPV-C in American coots (*Fulica americana* GMELIN, 1789) and in Canada geese (*B. canadensis*) in Georgia, South Carolina, Arkansas and Ohio. aMPV-C direct evidence in wild species was also identified in aquatic birds by Jardine *et al.* (2018) in Canada where aMPV infection has never been reported for poultry. Considering South America, aMPV subtypes A and B were detected in numerous free-living wild species and in individuals hosted in rehabilitation centres (Felippe *et al.*, 2011; Rizotto *et al.*, 2019), probably implying aMPV spread between the birds sampled in captivity. Concerning Europe, viral subtype C was molecularly detected in wild mallards (*A. platyrhynchos*), graylag geese (*Anser anser* (LINNAEUS, 1758)) and common gulls (*Larus canus* LINNAEUS, 1758) sampled in the Netherlands (van Boheemen *et al.*, 2012) and in wild anatids sampled in Italy (Graziosi *et al.*, 2022; Tucciarone *et al.*, 2021). aMPV was also detected by RT-PCR in free-living pheasants (*P. colchicus*) in Germany (Curland *et al.*, 2018), without further viral subtyping.

Statistical comparison among orders and genera of molecularly tested wild birds revealed a significant association ($p < 0.0001$) between these moderators and the aMPV molecular prevalence. Nevertheless, not all the taxa included in the subgroup analysis were represented in more than one study. The orders Passeriformes, Columbiformes and Falconiformes showed the highest molecular/viroprevalence outcomes, although sampling bias due to under-sampling probably influenced the results. In fact, the Passeriformes order, represented solely by the house sparrow (*Passer domesticus* (LINNAEUS, 1758)), and the Falconiformes order, represented solely by the American kestrel (*Falco sparverius* LINNAEUS, 1758), were both sampled once (Bennett *et al.*, 2004; Rizotto *et al.*, 2019). Considering Columbiformes, this order also included a single species sampled, the pigeon (*Columba livia* GMELIN, 1789), which was surveyed twice (Felippe *et al.*, 2011; Rizotto *et al.*, 2019). House sparrows, American kestrels and pigeons are common rural species inhabiting agroecosystems, which could therefore encounter aMPV by frequenting poultry farm surroundings. Regarding house sparrows and/or pigeons, results of experimental infection with aMPV are controversial (Catelli *et al.*, 2012; Gharaibeh & Shamoun, 2012; Gough *et al.*, 1988). Current evidence

does not allow a clear epidemiological role to be assigned to these species, although they appear more likely to be epiphenomena.

Taken as a whole, molecular and serological evidence suggests that some wild bird taxa could play a role in aMPV epidemiology. Particularly, wild ducks, geese, pheasants and gulls, according to scientific contributions hereby considered, proved to be susceptible to aMPV infection. Considering the Eurasian lineage of aMPV-C as well adapted to ducks (Brown *et al.*, 2019), further investigation on wild anatids may lead to disclosure of potential aMPV reservoir species. Although moderator analysis on the association between aMPV molecular detection and migration patterns resulted in a non-statistical significance, migratory or non-migratory attitudes of species still deserve to be considered for further consideration. It can be fairly hypothesised that migratory birds may condition the spread of aMPV along migratory flight paths, especially for the aMPV subtype C, as already proven for Avian influenza (Global Consortium for H5N8 and Related Influenza Viruses, 2016). In particular, the detection of aMPV-C in wild migratory species sampled in territories where this subtype has never been reported for poultry (van Boheemen *et al.*, 2012; Graziosi *et al.*, 2022; Jardine *et al.*, 2018; Turpin *et al.*, 2008) corroborates this hypothesis. Separate migratory attitudes of Eurasian and American wildfowl could also be relevant in shaping aMPV-C genetic diversity, which already recognises two distinct lineages.

On the other hand, with respect to resident wild bird species, these could possibly play a bridging role in between-poultry farm or between-potential natural reservoir species and poultry. Considering aMPV-A, B or C (North American lineage) as more adapted to gallinaceous birds (Brown *et al.*, 2019), wild Galliformes such as pheasants could therefore be involved in the viral epidemiology. Evidence of aMPV-A or B subtype infection in free-living or farmed pheasants (Catelli *et al.*, 2001; Curland *et al.*, 2018; Gough *et al.*, 2001; Welchman *et al.*, 2002), and the molecular detection of aMPV-C in pheasants sampled in a live-bird market (Lee *et al.*, 2007) in South Korea, enlighten the potential impact of this territorial species with respect to local aMPV circulation at the poultry/wildlife interface with free-range pheasants being frequently observed in rural areas or around poultry farms.

Finally, with respect to gulls, their trophic plasticity and the evidence of their susceptibility to aMPV (Canuti *et al.*, 2019; Heffels-Redmann *et al.*, 1998; van Boheemen *et al.*, 2012) suggest their possible involvement in viral epidemiology. *Larus* are indeed regarded as generalist carnivores, which use different habitats as nesting sites and consume both marine and terrestrial food resources (Belant *et al.*, 1998; Shaffer *et al.*, 2017).

5.4.2. Limitations

Among the constraints identified regarding the systematic review and meta-analysis hereby presented, we acknowledge the scarcity of publications on aMPV occurrence in free-ranging birds in comparison with the abundance of studies available for poultry. In that sense, a possible limitation of our study might be due to a lack of data from Africa and Asiatic countries, which may generate a

misinterpretation of the actual geographic distribution and prevalence of known aMPV subtypes in wild birds.

We also suggest the existence of possible research which may have not been accessible through the search strategy adopted. With respect to the statistical analysis, the scarcity of eligible articles concerning aMPV serological surveys prevented us from performing subgroup analyses and exploring the sources of the between serological study heterogeneity recorded. Moreover, outlier data were not statistically identified to avoid further decreasing of the overall number of studies considered.

Finally, any statistical test to quantify publication bias was not applied due to the absence of specific tools applicable to studies on proportions (Murad *et al.*, 2018; Olsen *et al.*, 2019).

5.5. Conclusions

Further aMPV surveys in wild birds are encouraged for a better comprehension of aMPV epidemiology and to better characterise the virus host breadth. Considering aMPV as transmitted by direct contact, the gregariousness of wild species could be an important trait to be considered in selecting the species to be tested. Furthermore, accurate recording of taxonomic and demographic information of individuals tested, especially sex and age classes, would be essential to notice specific aMPV infection patterns. Moreover, sampling location data would support the understanding of epidemiological links between wild free-ranging avifauna and aMPV occurrence on poultry farms. Whenever positive results are discussed, a thorough examination of the host ecology may allow further epidemiological considerations. With respect to the diagnostic methods applied, in order to avoid underestimation of the circulation of aMPV in new bird taxa and reveal unobvious aspects of viral distribution in wild hosts, it is strongly recommended to choose molecular protocols that are not subtype-specific and to proceed with further characterisation of positive findings by sequencing.

5.6. References

- Bäyon-Auboyer, M. H., Arnould, C., Toquin, D., & Eterradossi, N. (2000). Nucleotide sequences of the F, L and G protein genes of two non-A / non-B avian pneumoviruses (APV) reveal a novel APV subtype. *J Gen Vir*, 81, 2723–2733. doi:10.1099/0022-1317-81-11-2723
- Belant, J. L., Ickes, S. K., & Seamans, T. W. (1998). Importance of landfills to urban-nesting herring and ring-billed gulls. *Landsc Urban Plan*, 43. doi:10.1016/S0169-2046(98)00100-5
- Bennett, R. S., McComb, B., Shin, H. J., Njenga, M. K., Nagaraja, K. V., & Halvorson, D. A. (2002). Detection of avian pneumovirus in wild Canada (*Branta canadensis*) and blue-winged teal (*Anas discors*) geese. *Avian Dis*, 46, 1025–1029. doi:10.1637/0005-2086(2002)046[1025:DOAPIW]2.0.CO;2
- Bennett, R. S., Nezworski, J., Velayudhan, B. T., Nagaraja, K. V., Zeman, D. H., Dyer, N., Graham, T., Lauer, D. C., Njenga, M. K., & Halvorson, D. A. (2004). Evidence of avian pneumovirus spread beyond Minnesota among wild and domestic birds in central North America. *Avian Dis*, 48, 902–908. doi:10.1637/7208-051804R
- Brown, P.A., Allée, C., Courtillon, C., Szerman, N., Lemaitre, E., Toquin, D., Mangart, J. M., Amelot, M., & Eterradossi, N. (2019). Host specificity of avian metapneumoviruses. *Avian Pathol*, 48, 311–318. doi:10.1080/03079457.2019.1584390
- Buys, S. B., & du Preez, J. H. (1980). A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, 36.
- Cannon, R.M., & Roe, R.T. (1982). *Livestock Disease Surveys. A Field Manual for Veterinarians*. Canberra: Australian Government Publishing Service.
- Verhoeven, J., Canuti, M., Munro, H. J., Dufour, S. C., & Lang, A. S. (2018). ViDiT-CACTUS: an inexpensive and versatile library preparation and sequence analysis method for virus discovery and other microbiology applications. *Can J Microbiol*, 64, 761–773. doi:10.1139/cjm-2018-0097
- Canuti, M., Kroyer, A., Ojkic, D., Whitney, H. G., Robertson, G. J., & Lang, A. S. (2019). Discovery and Characterization of Novel RNA Viruses in Aquatic North American Wild Birds. *Viruses*, 11, 768. doi:10.3390/v11090768
- Catelli, E., De Marco, M.A., Delogu, M., Terregino, C., & Guberti, V. (2001). Serological evidence of avian pneumovirus infection in reared and free-living pheasants. *Vet Rec*, 149, 56-57.
- Catelli, E., Lupini, C., Listorti, V., Marziali, A., De Matteo, P., Naylor, C. J., & Cecchinato, M. (2012). The pigeon (*Columba livia*) is not sensitive to avian metapneumovirus subtype b and does not play any role in virus spread in experimental conditions. In U. Heffels-Redmann & D. Sommer (Eds.), *Proceedings of the 7th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens* (pp. 285-291). Rauischholzhausen, Germany: WPAVA and Justus-Liebig-Universität.

- Cecchinato, M., Lupini, C., Silveira, F., Listorti, V., Mescolini, G., Morandini, E., Franzo, G., & Catelli, E. (2018). Molecular characterization of avian metapneumovirus from Guinea fowls (*Numida meleagris*). *Pak Vet J*, 38, 419-423. doi:10.29261/pakvetj/2018.088
- Cha, R. M., Qingzhong, Y., & Zsak, L. (2013). The pathogenicity of avian metapneumovirus subtype C wild bird isolates in domestic turkeys. *Virol J*, 38. doi:10.1186/1743-422X-10-38
- Chen, X., Li, C., Sun, H. T., Ma, J., Qi, Y., & Qin, S. Y. (2019). Prevalence of avian influenza viruses and their associated antibodies in wild birds in China: A systematic review and meta-analysis. *Microb pathog*, 135, 103613. doi:10.1016/j.micpath.2019.103613
- Cook, J. K. (2000a). Avian pneumovirus infections of turkeys and chickens. *Vet J*, 160, 118–125. doi:10.1053/tvj.2000.0486
- Cook, J. K. (2000b). Avian rhinotracheitis. *REV SCI TECH OIE*, 19, 602–613. <https://doi.org/10.20506/rst.19.2.1233>
- Cook, J. K., & Cavanagh, D. (2002). Detection and differentiation of avian pneumoviruses (metapneumoviruses). *Avian Pathol*, 31, 117–132. doi:10.1080/03079450120118603
- Curland, N., Gethöffer, F., van Neer, A., Ziegler, L., Heffels-Redmann, U., Lierz, M., Baumgärtner, W., Wohlsein, P., Völker, I., Lapp, S., Bello, A., Pfankuche, V. M., Braune, S., Runge, M., Moss, A., Rautenschlein, S., Jung, A., Teske, L., Strube, C., Schulz, J., Bodewes, R., Osterhaus, A. D. M. E., & Siebert, U. (2018). Investigation into diseases in free-ranging ring-necked pheasants (*Phasianus colchicus*) in northwestern Germany during population decline with special reference to infectious pathogens. *Eur J Wildl Res*, 64. doi:10.1007/s10344-018-1173-2
- Delogu, M., De Marco, M. A., Catelli, E., Cecchinato, M., Sperati Ruffoni, L., Pesente, P., & Franciosi, C. (2004). Molecular and serological survey of avian pneumovirus infection in wild aquatic birds in Italy. In E. Kaleta & U. Heffels-Redmann (Eds.), *Proceedings of the 4th International Symposium on Avian Corona- and pneumovirus infection* (pp. 282-286). Rauischholzhausen, Germany: WPAVA and Justus-Liebig-Universität.
- Felippe, P. A., Silva, L. H., Santos, M. B., Sakata, S. T., & Arns, C. W. (2011). Detection of and phylogenetic studies with avian metapneumovirus recovered from feral pigeons and wild birds in Brazil. *Avian Pathol*, 40, 445–452. doi:10.1080/03079457.2011.596812
- Gethöffer, F., Curland, N., Voigt, U., Woelfing, B., Ludwig, T., Heffels-Redmann, U., Hafez, H. M., Lierz, M., & Siebert, U. (2021). Seroprevalences of specific antibodies against avian pathogens in free-ranging ring-necked pheasants (*Phasianus colchicus*) in Northwestern Germany. *PloS one*, 16, e0255434. doi:10.1371/journal.pone.0255434
- Gharaibeh, S., & Shamoun, M. (2012). Avian metapneumovirus subtype B experimental infection and tissue distribution in chickens, sparrows, and pigeons. *Vet Pathol*, 49, 704–709. doi:10.1177/0300985811402845
- Gill, F., Donsker, D., & Rasmussen, P. (2021). *IOC World Bird List* (v11.2). doi:10.14344/IOC.ML.11.2
- Global Consortium for H5N8 and Related Influenza Viruses (2016). Role for migratory wild birds in the global spread of avian influenza H5N8. *Science*, 354, 213–217. doi:10.1126/science.aaf8852

- Gough, R. E, Collins, M. S., Cox, W. J., & Chettle, N. J. (1988). Experimental infection of turkeys, chickens, ducks, geese, guinea fowl, pheasants and pigeons with turkey rhinotracheitis virus. *Vet Rec*, 123, 58-59. doi:10.1136/vr.123.2.58
- Gough, R. E., Drury, S. E., Aldous, E., & Laing, P. W. (2001). Isolation and identification of avian pneumovirus from pheasants. *Vet Rec*, 149, 312.
- Graziosi, G., Catelli, E., Fanelli, A., & Lupini, C. (2021). Infectious bursal disease virus in free-living wild birds: A systematic review and meta-analysis of its sero-viroprevalence on a global scale. *Transbound Emerg Dis*. Advance online publication. doi:10.1111/tbed.14433
- Graziosi, G., Mescolini, G., Silveira, F., Lupini, C., Tucciarone, C. M., Franzo, G., Cecchinato, M., Legnardi, M., Gobbo, F., Terregino C., & Catelli, E. (2022). First detection of Avian metapneumovirus subtype C Eurasian Lineage in a Eurasian wigeon (*Mareca penelope*) wintering in Northeastern Italy: an additional hint on the role of migrating birds in the viral epidemiology. *Avian Pathol*, 51, 283-290. doi:10.1080/03079457.2022.2051429
- Heffels-Redmann, U., Neumann, U., Braune, S., Cook, J. K. A., & Prüter, J. (1998). Serological evidence for susceptibility of sea gulls to avian pneumovirus (APV) infection. In E. Kaleta & U. Heffels-Redmann (Eds.), *Proceedings of International Symposium on Infectious Bronchitis and Pneumovirus Infections in Poultry* (pp. 23-25). Rauischholzhausen, Germany: WPAVA and Justus-Liebig-Universität.
- Higgins, J. P. T., & Thompson, S. G. (2002). Quantifying heterogeneity in a meta-analysis. *Stat Med*, 21, 1539–1558. doi:10.1002/sim.1186
- Higgins, J. P. T., Thomas, J., Chandler, J., Cumpston, M., Li, T., Page, M. J., & Welch, V. A. (2021). *Cochrane handbook for systematic reviews of interventions version 6.2* (updated February 2021). Retrieved from www.training.cochrane.org/handbook
- Hunter, J. P., Saratzis, A., Sutton, A. J., Boucher, R. H., Sayers, R. D., & Bown, M. J. (2014). In meta-analyses of proportion studies, funnel plots were found to be an inaccurate method of assessing publication bias. *J Clin Epidemiol*, 67, 897–903. doi:10.1016/j.jclinepi.2014.03.003
- Jardine, C. M., Parmley, E. J., Buchanan, T., Nituch, L., & Ojkic, D. (2018). Avian metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. *Transbound Emerg Dis*, 65, 1098–1102. <https://doi.org/10.1111/tbed.12832>
- Jones, R. C. (1996). Avian pneumovirus infection: Questions still unanswered. *Avian Pathol*, 25, 639–648. doi: 10.1080/03079459608419171
- Jones, R., and S. Rautenschlein. (2013). Avian metapneumovirus. In D.E. Swayne, L.R. McDougald, L. Nolan, K., D.L. Suarez, & V. Nair. (Eds), *Diseases of Poultry*. 13th edn (pp. 112-138). Iowa: Wiley-Blackwell.
- Juhasz, K., & Easton, A. J. (1994). Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. *J Gen Virol*, 75, 2873–2880. doi:10.1099/0022-1317-75-11-2873
- Kaboudi, K., & Lachheb, J. (2021). Avian metapneumovirus infection in turkeys: a review on turkey rhinotracheitis. *J Appl Poult Res*, 30. doi:10.1016/j.japr.2021.100211

- Lee, E. H., Song, M. S., Shin, J. Y., Lee, Y. M., Kim, C. J., Lee, Y. S., Kim, H., & Choi, Y. K. (2007). Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. *Virus Res*, 128, 18–25. doi:10.1016/j.virusres.2007.03.029
- Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., & Stewart, L. A. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Syst Rev*, 4. doi:10.1186/2046-4053-4-1
- Murad, M., Chu, H., Lin, L., & Wang, Z. (2018). The effect of publication bias magnitude and direction on the certainty in evidence. *Evid Based Med*, 23, 84–86. doi:10.1136/bmjebm-2018-110891.
- Naylor, C. J., & Jones, R. C. (1993). Turkey rhinotracheitis: a review. *Vet Bull*, 63, 339-349.
- Njenga, M. K., Lwamba, H. M., & Seal, B. S. (2003). Metapneumoviruses in birds and humans. *Virus Res*, 91, 163–169. doi:10.1016/s0168-1702(02)00256-3
- Olsen, A., Berg, R., Tagel, M., Must, K., Deksnė, G., Enemark, H., Alban, L., Johansen, M., Nielsen, H., Sandberg, M., Lundén, A., Stensvold, C., Pires, S., & Jokelainen, P. (2019). Seroprevalence of *Toxoplasma gondii* in domestic pigs, sheep, cattle, wild boars, and moose in the Nordic-Baltic region: a systematic review and meta-analysis. *Parasite Epidemiology Control*, 5. doi:10.1016/j.parepi.2019.e00100.
- Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hrobjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., . . . & Moher, D. (2021). The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ-BRIT MED J*, 372. doi:10.1136/bmj.n71
- Panigrahy, B., Senne, D. A., Pedersen, J. C., Gidlewski, T., & Edson, R. K. (2000). Experimental and serologic observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. *Avian Dis*, 44, 17–22.
- R Core Team. (2018). A language and environment for statistical computing. *R Foundation for Statistical Computing* (version 3.5.2). Retrieved from <https://www.r-project.org/>
- Ratcliffe, C. S. (2000). Factors which may be preventing the recovery of populations of helmeted guineafowl in the midland of KwaZulu-Natal. (Master's thesis, University of Cape Town, Cape Town, South Africa). Retrieved from <https://open.uct.ac.za/handle/11427/9275>
- Rautenschlein, S. (2020). Avian metapneumovirus. In D. E. Swayne, M. Boulianne, C. M. Logue, L. R. McDougald, V. Nair, D. L. Suarez, S. de Wit, T. Grimes, D. Johnson, M. Kromm, T. Y. Prajitno, I. Rubinoff, & G. Zavala. (Eds.), *Diseases of Poultry* 14th edn (pp. 135-143). Hoboken: Wiley Blackwell.
- Retallack, H., Clubb, S., & DeRisi, J. L. (2019). Genome Sequence of a Divergent Avian Metapneumovirus from a Monk Parakeet (*Myiopsitta monachus*). *Microbiol Resour Announc*, 8, e00284-19. doi:10.1128/MRA.00284-19

- Rima, B., Collins, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R. A., Lee, B., Maisner, A., Rota, P., Wang, L., & ICTV Report Consortium (2017). ICTV Virus Taxonomy Profile: Pneumoviridae. *J Gen Virol*, 98, 2912–2913. doi:10.1099/jgv.0.000959
- Rizotto, L. S., Simão, R. M., Scagion, G. P., Simasaki, A. A., Caserta, L. C., Benassi, J. C., Arns, C. W., & Ferreira, H. L. (2019). Detection of avian metapneumovirus subtype A from wild birds in the State of São Paulo, Brazil. *Pesqui Vet Bras*, 39, 209–213. doi:10.1590/1678-5150-PVB-6021
- Sawicka, A., Durkalec, M., Tomczyk, G., & Kurs, O. (2020). Occurrence of Mycoplasma gallisepticum in wild birds: A systematic review and meta-analysis. *PloS one*, 15, e0231545. doi:10.1371/journal.pone.0231545
- Seal, B. S. (2000). Avian pneumoviruses and emergence of a new type in the United States of America. *Anim Health Res Rev*, 1, 67–72. https://doi.org/10.1017/s1466252300000062
- Senne, D., Edson, J. C., & Pederson, B. P. (1997). Avian pneumovirus update. In *Proceedings of the 134th annual convention of the American Veterinary Medical Association* (p. 190). Reno, Nevada.
- Shaffer, S. A., Cockerham, S., Warzybok, P., Bradley, R. W., Jahncke, J., Clatterbuck, C. A., Lucia, M., Jelincic, J. A., Cassel, A. L., Kelsey, E. C., & Adams, J. (2017). Population-level plasticity in foraging behavior of western gulls (*Larus occidentalis*). *Mov Ecol*, 5. doi:10.1186/s40462-017-0118-9
- Shin, H. J., Njenga, M. K., McComb, B., Halvorson, D. A., & Nagaraja, K. V. (2000). Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic homology with RNA from APV isolates from domestic turkeys. *J Clin Microbiol*, 38, 4282–4284. doi:10.1128/JCM.38.11.4282-4284.2000
- Shin, H. J., Cameron, K. T., Jacobs, J. A., Turpin, E. A., Halvorson, D. A., Goyal, S. M., Nagaraja, K. V., Kumar, M. C., Lauer, D. C., Seal, B. S., & Njenga, M. K. (2002). Molecular epidemiology of subtype C avian pneumoviruses isolated in the United States and comparison with subtype a and B viruses. *J Clin Microbiol*, 40, 1687–1693. doi:10.1128/JCM.40.5.1687-1693.2002
- Sun, S., Chen, F., Cao, S., Liu, J., Lei, W., Li, G., Song, Y., Lu, J., Liu, C., Qin, J., & Li, H. (2014). Isolation and characterization of a subtype C avian metapneumovirus circulating in Muscovy ducks in China. *Vet Res*, 45, 74. doi:10.1186/s13567-014-0074-y
- Sukon, P., Nam, N. H., Kittipreeya, P., Sara-In, A., Wawilai, P., Inchuai, R., & Weerakhun, S. (2021). Global prevalence of chlamydial infections in birds: A systematic review and meta-analysis. *Prev Vet Med*, 192, 105370. doi:10.1016/j.prevetmed.2021.105370
- Toquin, D., Băyon-Auboyer, M.H., Eterradossi, N., Jestin, V., & Morin, H. (1999). Isolation of a pneumovirus from a Muscovy duck. *Vet Rec*, 145, 680.
- Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C., & Eterradossi, N. (2006). European and American subtype C isolates of avian metapneumovirus belong to different genetic lineages. *Virus genes*, 32, 97–103. doi:10.1007/s11262-005-5850-3
- Tucciarone, C. M., Franzo, G., Legnardi, M., Lupini, C., Catelli, E., Quaglia, G., Dal Molin, E., Gobbo, F., Cecchinato, M. (2021). Ricerca di Avian metapneumovirus (aMPV) in volatili selvatici. In

Atti della Società Italiana di Patologia Aviaria 2021, LX Convegno Annuale e VI Simposio Scientifico (pp. 183-189). Bologna, Italy: Società Italiana di Patologia Aviaria (SIPA).

- Turpin, E. A., Stallknecht, D. E., Slemons, R. D., Zsak, L., & Swayne, D. E. (2008). Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South Carolina, Arkansas and Ohio, USA. *Avian Pathol*, 37, 343–351. doi:10.1080/03079450802068566
- van Boheemen, S., Bestebroer, T. M., Verhagen, J. H., Osterhaus, A.D.M.E., Pas, S.D., Herfst, S., & Fouchier, R.A. (2012). A family-wide RT-PCR assay for detection of paramyxoviruses and application to a large-scale surveillance study. *PloS one*, 7. doi:10.1371/journal.pone.0034961
- Wang, N. (2018). How to conduct a meta-analysis of proportions in R: A Comprehensive Tutorial. doi:10.13140/RG.2.2.27199. 00161
- Wei, L., Zhu, S., Yan, X., Wang, J., Zhang, C., Liu, S., She, R., Hu, F., Quan, R., & Liu, J. (2013). Avian metapneumovirus subtype C infection in chickens, China. *Emerg Infect Dis*, 19, 1092–1094. doi:10.3201/eid1907.121126
- Welchman, D., Bradbury, J. M., Cavanagh, D., & Aebischer, N. J. (2002). Infectious agents associated with respiratory disease in pheasants. *Vet Rec*, 150, 658–664. doi:10.1136/vr.150.21.658

VI. First detection of avian metapneumovirus subtype C Eurasian lineage in a Eurasian wigeon (*Mareca penelope*) wintering in Northeastern Italy: an additional hint on the role of migrating birds in the viral epidemiology

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Abstract

Avian metapneumovirus (aMPV) economically affects the global poultry industry causing respiratory and reproductive disorders. Considering the paucity of data on the aMPV occurrence in European free-ranging avifauna, a molecular survey was conducted on wild birds of 23 species belonging to the orders Anseriformes, Charadriiformes or Passeriformes, captured alive and sampled in Northeast Italy as part of the national Avian influenza virus (AIV) surveillance activities. A total of 492 oropharyngeal swabs, collected from 2007 to 2010, all AIV negative, were screened from aMPV by subtype-specific qRT-PCR. An aMPV-C strain, named aMPV/C/IT/Wigeon/758/07, was found in a wintering young Eurasian wigeon (*Mareca penelope*) sampled in November 2007. The matrix, fusion, and attachment glycoprotein genes of the detected strain were subsequently amplified by specific independent RT-PCRs, then sequenced, and compared in a phylogenetic framework with known aMPV homologous sequences retrieved from GenBank. Close genetic relationships were found between the aMPV/C/IT/Wigeon/758/07 strain and subtype C Eurasian lineage strains isolated in the late 1990s in French domestic ducks, suggesting epidemiological links. Eurasian wigeons are indeed medium to long-range migrant dabbling ducks that move along the Black Sea/Mediterranean flyway, our finding might therefore be related to migratory bridges between countries. To our knowledge, this is the first molecular evidence of the occurrence of a subtype C in Italy and backdates the aMPV-C circulation to 2007. Moreover, results suggest the susceptibility of Eurasian wigeons to aMPV. Broader investigations are needed to assess the role of wild ducks and the significance of the wildfowl/poultry interface in the aMPV-C epidemiology.

6.1. Introduction

The *Metapneumovirus* (MPV) genus belongs to the *Pneumoviridae* family, and includes non-segmented, single-stranded, negative-sense RNA viruses. To date, two MPVs species are recognized: *Avian metapneumovirus* (aMPV) and *Human metapneumovirus* (hMPV) (Rima *et al.*, 2017). *Avian metapneumovirus* is an emerging poultry pathogen mainly associated to respiratory and reproductive disorders in turkeys and chickens, resulting in severe economic losses to domestic poultry industry.

To date, different aMPV-subtypes have been recognized based on genetic and antigenic profiles (aMPV-A, -B, -C, -D) further showing diverse spatial distribution (Rautenschlein, 2020) and host range (Brown *et al.*, 2019). aMPV-A and -B subtypes have been detected in Asia, Africa, Europe, and South America, whereas subtype D it was only detected once in France (Bäyon-Auboyer *et al.*, 2000). Subtype C, firstly reported in the US (Senne *et al.*, 1997), genetically diverges from aMPV-A, -B and -D, and is closely related to hMPV, suggesting a possible common origin (Yunus *et al.*, 2003). Two aMPV-C lineages have been recognized so far, according to the attachment glycoprotein (G) gene sequence and named North American or Eurasian lineages (Toquin *et al.*, 2006). Moreover, two divergent aMPV strains have been recently detected in North America, one in a great black-backed gull (*Larus marinus* LINNAEUS, 1758) (Canuti *et al.*, 2019), and another in a monk parakeet chick (*Myiopsitta monachus* BODDAERT 1783) (Retallack *et al.*, 2019), tentatively increasing aMPV known subtypes to six.

In Italy, field studies revealed an aMPV widespread circulation in poultry mainly from densely populated poultry areas in the Northeastern part of the country. Even though the infection is mostly sustained by subtype B (Catelli *et al.*, 2004; Listorti *et al.*, 2014; Cecchinato *et al.*, 2018; Tucciarone *et al.*, 2018), aMPV-A has also been sporadically detected (Catelli *et al.*, 2006; Lupini *et al.*, 2011). Eventually, aMPV-C specific antibodies were demonstrated in intensively raised domestic mallards (Legnardi *et al.*, 2021).

Since aMPV first appearance in South Africa in 1978 (Buys & du Preez, 1980) followed by an initial spread to Europe and Israel (Jones, 1996), migratory birds and respective flyways have been hypothetically linked to aMPV global transmission. Repeated detections of aMPV subtype C in free-living birds have been reported in North America (Shin *et al.*, 2000; Bennett *et al.*, 2002, 2004; Turpin *et al.*, 2008; Cha *et al.*, 2013; Jardine *et al.*, 2018) and the periodic pattern of aMPV outbreaks in poultry has been linked to migratory movements (Shin *et al.*, 2000). As regards to previous aMPV detections in European countries, aMPV has been detected in wild mallards (*Anas platyrhynchos* LINNAEUS, 1758), graylag geese (*Anser anser* (LINNAEUS, 1758)) and common gulls (*Larus canus* LINNAEUS, 1758) in the Netherlands and characterized as subtype C according to partial polymerase gene (L) sequence analysis (van Boheemen *et al.*, 2012). Serological (Catelli *et al.*, 2001; Gethöffer *et al.*, 2021) and molecular evidence (Curland *et al.*, 2018) of aMPV in free-living pheasants (*Phasianus colchicus* LINNAEUS, 1758) has been

reported in Italy and Germany, without providing further indication regarding the subtype involved. Taken as a whole, all this evidence increasingly supports the possible role of wild birds as aMPV carriers or reservoir hosts.

To enlighten the status of migratory and resident free-living birds with respect to the aMPV epidemiology, a molecular survey was performed in wild species sampled from 2007 to 2010 in northeast Italy for the Avian influenza (AI) National Surveillance Plan.

6.2. Materials and methods

6.2.1. Ethical Statement

All the samples were collected as part of Italy's live wild bird Avian Influenza (AI) surveillance activities carried out by the Istituto Zooprofilattico delle Venezie, the Italian National Reference Centre and European Union Reference Laboratory for AI and Newcastle disease. Particularly, wild birds were captured alive during the authorized ringing activities conducted from 2007 to 2010 in accordance with the Italian Institute for Environmental Protection and Research (ISPRA—Higher Institute for Environmental Protection and Research). No supplementary permits or approvals were needed for sampling from wild birds captured alive for ringing activities, the sampling was indeed conducted as part of the National Avian influenza surveillance program. All birds were handled in accordance with “Guidelines to the Use of Wild Birds in Research” (Fair *et al.*, 2010).

6.2.2. Background and sample collection

Oropharyngeal swabs were obtained from 23 species of wild birds (Table 1) sampled within the National Surveillance Plan for AI performed from spring to autumn 2007-2010. Swabs were individually collected and immersed in 1 ml of phosphate-buffered saline (PBS) with antibiotics and stored at -80°C until processing. Sample size was defined according to a previously published aMPV survey on wild birds (van Boheemen *et al.*, 2012). Setting the average aMPV prevalence at 1%, a minimum of 299 samples was required to find at least one positive sample in an infinite population with a confidence of 95% (Cannon & Roe, 1982). A total number of 492 samples was eventually analyzed.

Of the birds studied, 53.9% (265/492) belonged to the order Anseriformes; 19.5% (96/492) to the order Charadriiformes, and 26.6% (131/492) to the order Passeriformes. According to age classes as defined by trained ornithologists, 27.8% (137/492) of the birds were aged as adults, 40% (197/492) as juveniles (1st calendar year), and 32.1% (158/492) were not categorized. The study area comprised wetlands of the Veneto and Emilia-Romagna regions located in the Po river delta, considered as strategic resting, wintering, and breeding

sites for Anseriformes, Charadriiformes and Passeriformes species. All the samples screened for aMPV molecular detection resulted negative for AI.

1 **Table 1.** Wild birds sampled from 2007 to 2010 during national Avian Influenza surveillance efforts in northeastern Italian wetlands and tested
2 for aMPV detection. Age classes were assessed by trained ornithologists attending sampling sessions.

3

Taxonomic identification	Age classes ^a			Sampling period				Total	Migratory status ^b
	juv.	ad.	n.s.	2007	2008	2009	2010		
Anseriformes									
Common teal (<i>Anas crecca</i>)	21	9	-	6	3	21	-	30	W; B
Northern pintail (<i>Anas acuta</i>)	3	3	-	-	-	6	-	6	W
Mallard (<i>Anas platyrhynchos</i>)	40	44	-	10	40	21	13	84	W; B; R
Garganey (<i>Anas querquedula</i>)	9	9	-	-	-	18	-	18	B
Common pochard (<i>Aythya ferina</i>)	17	4	-	-	7	14	-	21	W; B
Eurasian wigeon (<i>Mareca penelope</i>)	36	44	-	42	5	9	24	80	W
Gadwall (<i>Mareca strepera</i>)	12	1	-	-	5	6	2	13	W; B
Northern shoveler (<i>Spatula clypeata</i>)	11	2	-	3	4	4	2	13	W; B
Charadriiformes									
Yellow-legged gull (<i>Larus michahellis</i>)	-	-	96	-	-	96	-	96	W; B; R

Table 1. Cont.

Taxonomic identification	Age classes ^a			Sampling period				Total	Migratory status ^b
	juv.	ad.	n.s.	2007	2008	2009	2010		
Passeriformes									
Eurasian blackcap (<i>Sylvia atricapilla</i>)	7	1	1	-	-	9	-	9	W; B
European goldfinch (<i>Carduelis carduelis</i>)	-	1	-	-	-	1	-	1	W; B; R
Great tit (<i>Parus major</i>)	11	3	1	-	-	15	-	15	W; B; R
Common chaffinch (<i>Fringilla coelebs</i>)	-	-	4	-	-	4	-	4	W; B; R
Common blackbird (<i>Turdus merula</i>)	-	12	14	-	-	20	6	26	W; B; R
Dunnock (<i>Prunella modularis</i>)	2	2	4	-	-	8	-	8	W; B
European robin (<i>Erithacus rubecula</i>)	25	1	1	-	-	27	-	27	W; B; R
Eurasian wren (<i>Troglodytes troglodytes</i>)	1	-	-	-	-	1	-	1	W; B
Song thrush (<i>Turdus philomelos</i>)	1	-	35	-	-	20	16	36	W; B
Redwing (<i>Turdus iliacus</i>)	1	1	-	-	-	-	2	2	W
Cetti's warbler (<i>Cettia cetti</i>)	-	-	1	-	-	1	-	1	B
European greenfinch (<i>Carduelis chloris</i>)	-	-	1	-	-	1	-	1	W; B; R
Total	197	137	158	61	64	302	65	492	

^aAge classes: juv. = juvenile; ad. = adult; n.s. = not specified. ^bMigratory status in Italy: W = wintering; B = breeding; R = resident.

6.2.3. Sample processing and RNA extraction

For elution, each sample was centrifuged at 1.500 g for 5 minutes at 4°C and processed in pools of five. Samples were pooled, whenever possible, according to the species. Total RNA was extracted from each pool using a protocol based on guanidine-thiocyanate method (Chomczynski & Sacchi, 1987).

6.2.4. aMPV detection and subtyping by qRT-PCR

A total of 99 pools was screened by qRT-PCR, with minor modifications of the method described by Lemaitre *et al.* (2018), for aMPV detection and subtyping. The reaction was performed with SuperScript™ III Platinum™ SYBR™ Green One-Step (Invitrogen, Waltham, MA, USA) on Lightcycler® 96 Instrument (Roche, Basel, Switzerland), using the primer pair PanMPV/N1fwdA and PanMPV/N1RevB (Lemaitre *et al.*, 2018) targeting the nucleoprotein (N) gene. For each reaction, 2 µl of extracted RNA were added to a standard reaction mix composed of 1X SYBR® Green Reaction Mix, 0.2 µl of SuperScript III RT/Platinum Taq Mix, and 0.6 µM of each primer. Molecular biology grade water was added up to a final volume of 10 µl. The thermal protocol was set as follows: 50° C for 3 minutes for the reverse transcription phase, 95° C for 5 minutes for the initial activation step, 45 cycles at 95° C for 15 seconds for the denaturation phase and 60° C for 30 seconds for annealing and extension. Melting curve analysis was performed by progressively increasing the temperature from 40° C to 90° C and continuously monitoring the fluorescence data. Tentative strain subtyping was performed by melting temperature (T_m) evaluation and comparison with reference strains.

Pools positive for aMPV were traced back to the original samples, which underwent individual RNA extraction and subsequent qRT-PCR analysis.

6.2.4.1. aMPV subtype C molecular characterization

For molecular characterization of the aMPV subtype C positive samples, a RT-PCR targeting partial matrix (M) viral gene was performed as previously described (Seal, 1998). Furthermore, additional primers were designed on a previously published aMPV-C complete sequence (Brown *et al.*, 2014) targeting the fusion (F) and attachment glycoprotein (G) viral genes (Table 2).

Table 2. Sequence of primers used for PCR amplifications of F and G genes of aMPV subtype C.

Primer name	Sequence (5' – 3')	Gene	Position ^a	Product size (bp)
FC3+	GGTATACAAATGTGTTTCAC	F	3130	591
FC3-	GTCCTGAAGATGTGGGCATG	F	3721	
FC5+	GACAGTTTCTGACAATGC	F	3616	597

FC5-	CCAAAGGTCTGATTATTCC	F	4213	
FC7+	CGTCACCCAATAAGCATGG	F	4102	691
FC7-	CTCAGTAATTGATTGAGCAGG	M2	4793	
SHC2+	GGGATTGTGGTTATGCTTGAG	SH	5910	615
GC2-	CTGTCTGTCCTTGGGTGCTG	G	6525	
GC2+	GAGCAACAACAGGGGCAGAGC	G	6505	981
GC4-	TTGCTCTGGGCTGTTTTGG	G	7486	
GC4+	GAGGCAGACACGAGAACACCC	G	7314	820
LC2-	GTTCTTCTCCTTGATCCTAGTC	L	8134	

^a Anti-genome position of primer 5' terminus based on subtype C sequence of the 99178 viral strain (GenBank accession number: HG934338).

F and G genes were amplified using one reverse transcription (RT) and 3 overlapping independent PCRs for each gene. RT reactions were performed using a OneScript® Plus cDNA Synthesis Kit according to manufacturer instructions (Applied Biological Materials Inc., Richmond, BC, Canada). Random primers (1 μ M) were used for total RNA transcription. The reactions were performed at 50°C for 15 min followed by RT inactivation at 85°C for 5 min.

The following reaction mix was used in each PCR: 0.25 μ L of GoTaq® DNA Polymerase (Promega, Madison, WI, United States), 10 pmol of the appropriate primer pair, 1 μ L of dNTPs (Promega, Madison, WI, United States), 10 μ L of PCR GoTaq® 5x reaction buffer, 3.5 μ L MgCl₂ (3 mM), 31.25 μ L of nuclease free water (Promega, Madison, WI, United States) and 3 μ L of cDNA. The PCR cycling parameters for both F and G gene PCR assays were as follows. A pre-cycle step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 50°C for 40 s and 72°C for 65 s, followed by a final extension at 72°C for 5 min.

PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States). Sequencing was performed by a commercial sequencing service (Macrogen Europe, Amsterdam, The Netherlands).

Nucleotide sequences were edited and assembled using BioEdit Sequence Alignment Editor version 7.2 (Hall *et al.*, 2011), then aligned against and compared with previously published aMPV sequences available on NCBI GenBank database, using MAFFT version 7.397 online service which applied automatic detection of the parameter set (Kato *et al.*, 2002). Best partition scheme, substitution model selection according to Bayesian information criterion (BIC), and maximum likelihood phylogenetic reconstruction for M, F and G genes were performed separately on the IQ-TREE web server (Trifinopoulos *et al.* 2016; Kalyaanamoorthy *et al.*, 2017). The robustness of inferred clades was evaluated using 1000 ultrafast bootstrap replicates. Branches with bootstrap values ≥ 70 were considered reliable. Pairwise genetic p-distance and between group mean distance were estimated using MEGA software version 11.0.10 (Kumar *et al.* 2018).

6.5. Results

Among all the tested samples, a young male Eurasian wigeon (*Mareca penelope* (LINNAEUS, 1758)) captured alive in November 2007 in the Po river delta area (Rovigo province, Veneto) was positive at qRT-PCR for aMPV subtype C (qRT-PCR cycle threshold value of the positive pooled sample: 35; qRT-PCR cycle threshold value of the positive individual sample: 33.5). The strain, named aMPV/C/IT/Wigeon/758/07 following the nomenclature reported by Mescolini *et al.* (2021), was molecularly characterized. Partial M and F gene sequences of 400 and 1620 bases respectively, and the complete sequence of the G gene (2160 bases) were obtained by RT-PCR and sequencing. Sequence data were submitted to the NCBI GenBank database under accession numbers OM021855, OM021856, and OM021857.

The phylogenetic tree constructed on the G gene sequence (Figure 1) demonstrated the clustering of the aMPV/C/IT/Wigeon/758/07 strain within the Eurasian lineage of aMPV-C, which includes French and Chinese subtype C field strains isolated from domestic duck flocks (Toquin *et al.*, 1999, 2006; Sun *et al.*, 2014). Specifically, the aMPV/C/IT/Wigeon/758/07 strain was closely related to a clade including strains isolated from Muscovy ducks (99178 and 99350 strains) and White Pekin ducks (00094 strain) in France (Toquin *et al.*, 2006). Between group mean distance, measured among the G gene sequence obtained in the present study and the French clade, was 0.042.

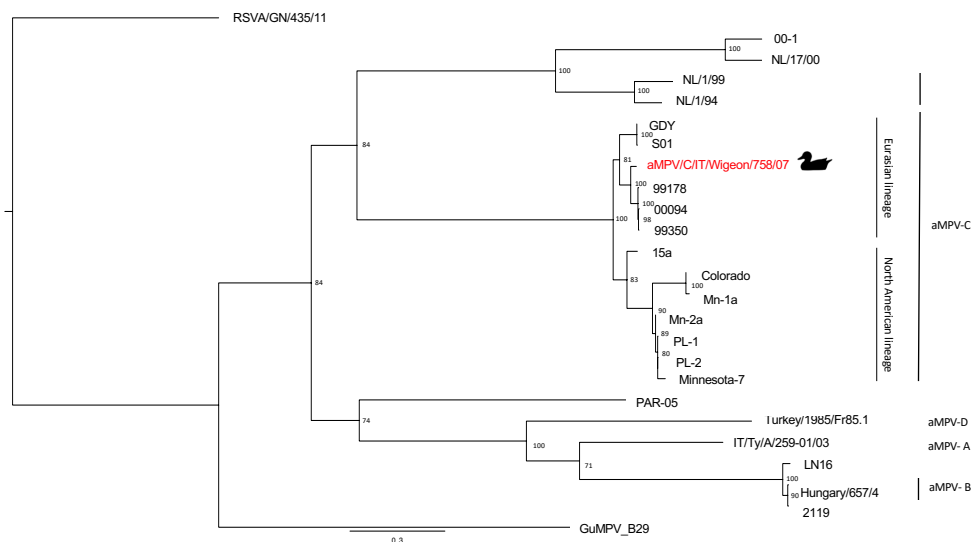
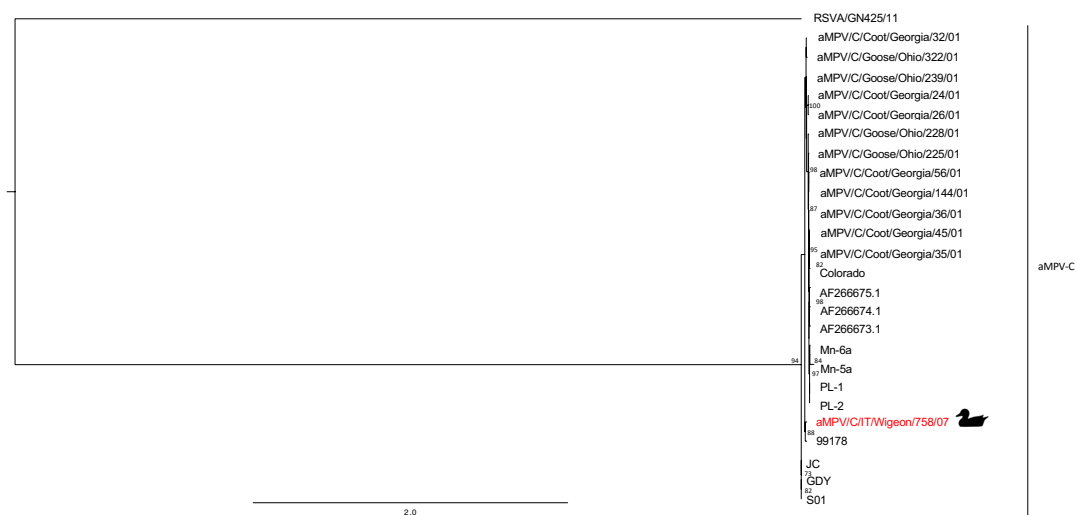


Figure 1. Phylogenetic tree based on G gene nucleotide sequences of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red), aMPV and hMPV reference strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The G sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup.

M and F genes phylogenetic trees (Figure 2 and Figure 3, respectively), confirming the results obtained with the G gene, showed a close relationship between the aMPV/C/IT/Wigeon/758/07 strain and the 99178 strain (Brown *et al.*, 2014). Pairwise p-distance values obtained comparing the above-mentioned strains were of 0.020 in M gene and 0.014 in F gene, respectively.

Figure 2. Phylogenetic tree based on partial M gene nucleotide sequences (from nucleotide 2514 to 2913 in the genome) of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red) and



aMPV-C strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The partial M sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup.

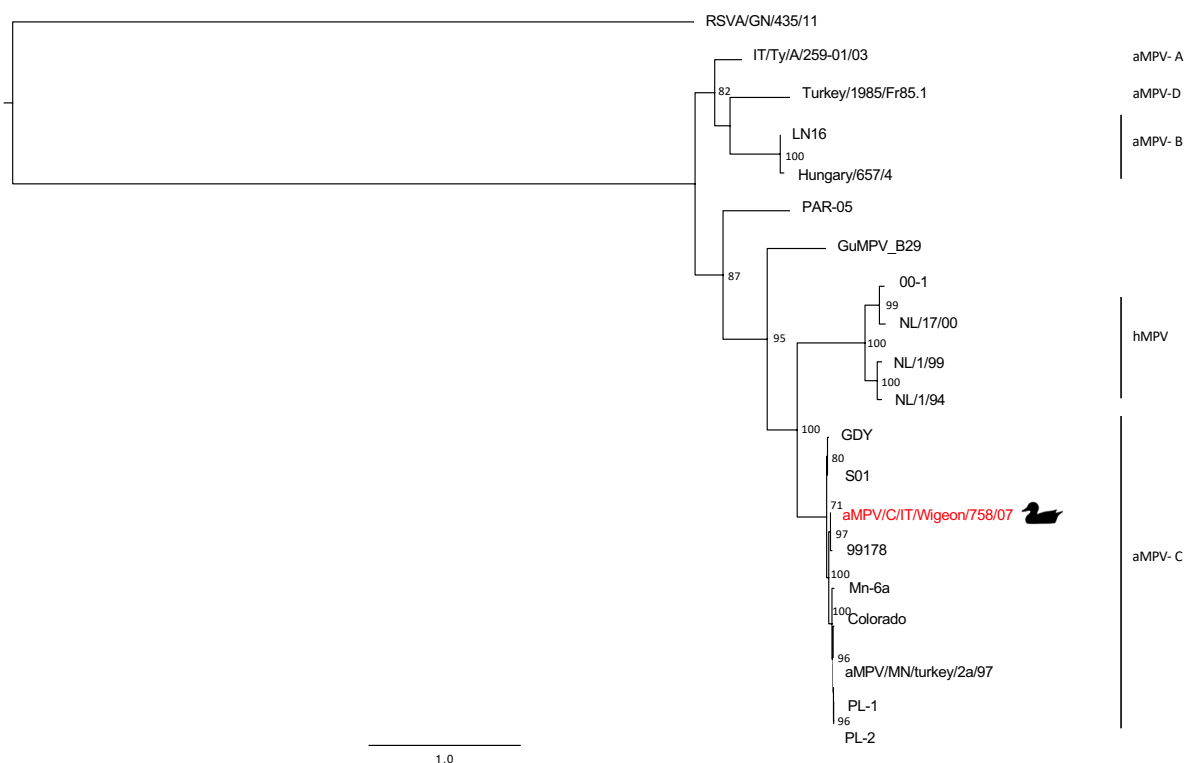


Figure 3. Phylogenetic tree based on partial F gene nucleotide sequences (from nucleotide 3319 to 4650 in the genome) of aMPV/C/IT/Wigeon/758/07 strain detected in the study (in red), aMPV and hMPV reference strains obtained from NCBI GenBank database. Only bootstrap values ≥ 70 are shown. The partial F sequence of the Human respiratory syncytial virus strain RSVA/GN/435/11 (Accession number JX627336.1) was included and used as an outgroup.

6.6. Discussion

We hereby report the detection of an aMPV subtype C in a juvenile Eurasian wigeon (*M. penelope*) wintering in northeast Italy. To our knowledge, this is the first molecular evidence of the occurrence of a subtype C in Italy and it backdates the aMPV-C circulation to 2007. Eventually, our finding suggests for the first time the susceptibility to aMPV infection of Eurasian wigeons.

Close genetic relationships were found between the detected strain and strains of the subtype C Eurasian genetic lineage, particularly with those isolated in the late 1990s in French domestic ducks. This might be related to migratory bridges between countries, along the Black Sea/Mediterranean flyway. Eurasian wigeons are indeed considered medium to long-range migrant dabbling ducks. Whilst major concentrations of wintering individuals are found in Northwestern Europe and Eastern Asia, remarkable numbers are also reported for France and other Mediterranean countries, including Italy (Atkinson *et al.*, 2006). Furthermore, ringed birds' recoveries showed a direct connection between Italian and French wetlands (Atkinson *et al.*, 2007; Spina & Volponi, 2008). Diverse factors such as migratory movements, dense birds' congregations, and presence of

immunologically naïve juveniles are considered relevant drivers in disease dynamics as already known for a major avian viral disease such as AI (Gaidet *et al.*, 2010; van Dijk *et al.*, 2014). Our finding suggests that these factors might also be relevant for the aMPV-C epidemiology. Considering that the geographical separation of wild hosts undertaking seasonal movements along different migration routes was linked to the initial diversification of AI viruses into Eurasian and American clades (Krauss *et al.*, 2007), the presence of two distinct genetic lineages of aMPV-C could also be associated to wild hosts' migratory movements.

Recent serological findings of aMPV-C infection in Italian asymptomatic intensively raised domestic mallards (Legnardi *et al.*, 2021) further underline the potential epidemiological role of ducks for aMPV-C circulation. Considering that the Eurasian lineage of aMPV-C is well adapted to domestic ducks as demonstrated by *in vivo* experimental trials (Brown *et al.*, 2019), and that domestic duck breeds were domesticated from wild mallards (Qu *et al.*, 2009), it is plausible that wild duck species might act as carrier and reservoir hosts for this viral lineage. Our detection of aMPV-C in an additional wild anatid species further supports the latter hypothesis. However, given the paucity of aMPV-C Eurasian-origin strains found, the inferred relationship might be affected by a sampling bias and only partially represents the actual scenario. Thus, broader investigations are needed to assess the role of wild ducks and the significance of the wildfowl/poultry interface in the aMPV-C epidemiology.

Eventually, to evaluate the biological features of aMPV strains circulating in wild species through *in vitro* and *in vivo* studies and to facilitate whole genome sequencing, viral isolation, hereby not performed, would be essential.

6.7. References

- Atkinson, P.W., Clark, J.A., Delany, S., Diagana, C.H., du Feu, C., Fiedler, W., Fransson, T., Gauthier-Clerc, M., Grantham, M.J., Gschweng, M., Hagemeijer, W., Helmink, T., Johnson, A., Khomenko, S., Martakis, G., Overdijk, O., Robinson, R.A., Solokha, A., Spina, F., Sylla, S.I., Veen, J. & Visser, D. (2006). Urgent preliminary assessment of ornithological data relevant to the spread of Avian Influenza in Europe. Retrieved from https://ec.europa.eu/environment/nature/conservation/wildbirds/birdflue/docs/rep_spread_avian_influenza_report.pdf
- Atkinson, P.W., Robinson, R.A., Clark, J.A., Miyar, T., Downie, I.S., du Feu, C.R., Fiedler, W., Fransson, T., Grantham, M.J., Gschweng, M., Spina, F. & Crick, H.Q.P. (2007) Migratory movements of waterfowl: a web-based mapping tool. *EURING report to the EU Commission*. Retrieved from <http://blx1.bto.org/ai-eu/>
- Bäyon-Auboyer, M. H., Arnauld, C., Toquin, D. & Etteradossi, N. (2000). Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subtype. *J Gen Virol*, 81, 2723–2733. doi:10.1099/0022-1317-81-11-2723
- Bennett, R.S., McComb, B., Shin, H.J., Njenga, M.K., Nagaraja, K.V. & Halvorson, D.A. (2002). Detection of avian pneumovirus in wild Canada (*Branta canadensis*) and blue-winged teal (*Anas discors*) geese. *Avian Dis*, 46, 1025–1029. doi:10.1637/0005-2086(2002)046[1025:DOAPIW]2.0.CO;2
- Bennett, R.S., Nezworski, J., Velayudhan, B.T., Nagaraja, K.V., Zeman, D.H., Dyer, N., Graham, T., Lauer, D.C., Njenga, M.K. & Halvorson, D.A. (2004). Evidence of avian pneumovirus spread beyond Minnesota among wild and domestic birds in central North America. *Avian Dis*, 48, 902–908. doi:10.1637/7208-051804R
- Brown, P.A., Lemaitre, E., Briand, F. X., Courtillon, C., Guionie, O., Allée, C., Toquin, D., Bayon-Auboyer, M-H., Jestin, V. & Etteradossi, N. (2014). Molecular Comparisons of Full Length Metapneumovirus (MPV) Genomes, Including Newly Determined French AMPV-C and -D Isolates, Further Supports Possible Subclassification within the MPV Genus. *PLoS ONE*, 9, e102740. doi:10.1371/journal.pone.0102740
- Brown, P.A., Allée, C., Courtillon, C., Szerman, N., Lemaitre, E., Toquin, D., Mangart, J.M., Amelot, M. & Etteradossi, N. (2019). Host specificity of avian metapneumoviruses. *Avian Pathol*, 48, 311–318. doi:10.1080/03079457.2019.1584390
- Buys, S.B. & du Preez, J.H. (1980). A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, 36.
- Cannon, R.M. & Roe, R.T. (1982). *Livestock Disease Surveys. A Field Manual for Veterinarians*. Canberra: Australian Government Publishing Service.
- Catelli, E., De Marco, M.A., Delogu, M., Terregino, C. & Guberti, V. (2001). Serological evidence of avian pneumovirus infection in reared and free-living pheasants. *Vet Rec*, 149, 56-57.

- Catelli, E., Cecchinato, M., Delogu, M., De Matteo, P., Ortali, G., Franciosi, C., De Marco, M.A. & Naylor, C.J. (2004). Avian Pneumovirus infection in turkey and broiler farms in Italy: A virological, molecular, and serological field survey. *Ital J Anim Sci*, 3, 287–292. doi:10.4081/ijas.2004.287
- Catelli, E., Cecchinato, M., Savage, C.E., Jones, R.C. & Naylor, C.J. (2006). Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. *Vaccine*, 24, 6476–6482. doi:10.1016/j.vaccine.2006.06.076
- Cecchinato, M., Lupini, C., Silveira, F., Listorti, V., Mescolini, G., Morandini, E., Franzo, G. & Catelli, E. (2018). Molecular characterization of avian metapneumovirus from guinea fowls (*Numida meleagris*). *Pak Vet J*, 38, 419–423. doi:10.29261/pakvetj/2018.088
- Canuti, M., Kroyer, A., Ojkic, D., Whitney, H.G., Robertson, G.J. & Lang, A.S. (2019). Discovery and Characterization of Novel RNA Viruses in Aquatic North American Wild Birds. *Viruses*, 11, 768. doi:10.3390/v11090768
- Cha, R.M., Qingzhong, Y. & Zsak, L. (2013). The pathogenicity of avian metapneumovirus subtype C wild bird isolates in domestic turkeys. *Virol J*, 38. <https://doi.org/10.1186/1743-422X-10-38>
- Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 162, 156–159. doi:10.1006/abio.1987.9999
- Curland, N., Gethöffer, F., van Neer, A., Ziegler, L., Heffels-Redmann, U., Lierz, M., Baumgärtner, W., Wohlsein, P., Völker, I., Lapp, S., Bello, A., Pfankuche, V.M., Braune, S., Runge, M., Moss, A., Rautenschlein, S., Jung, A., Teske, L., Strube, C., Schulz, J., Bodewes, R., Osterhaus, A.D.M.E. & Siebert, U. (2018). Investigation into diseases in free-ranging ring-necked pheasants (*Phasianus colchicus*) in northwestern Germany during population decline with special reference to infectious pathogens. *Eur J Wildl*, 64. doi:10.1007/s10344-018-1173-2
- Fair, J., Paul, E. & Jones, J. (2010). Guidelines to the Use of Wild Birds in Research. Washington: Ornithological Council. Retrieved from <https://birdnet.org/info-for-ornithologists/guidelines-english-3rd-edition-2010/>
- Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, J., Iverson, S.A., Douglas, D.C., Perry, W.M., Mundkur, T. & Newman, S.H. (2010). Potential spread of highly pathogenic avian influenza H5N1 by wildfowl: dispersal ranges and rates determined from large-scale satellite telemetry. *J Appl Ecol*, 47, 1147–1157.
- Gethöffer, F., Curland, N., Voigt, U., Woelfing, B., Ludwig, T., Heffels-Redmann, U., Hafez, H. M., Lierz, M. & Siebert, U. (2021). Seroprevalences of specific antibodies against avian pathogens in free-ranging ring-necked pheasants (*Phasianus colchicus*) in Northwestern Germany. *PloS one*, 16, e0255434. doi:10.1371/journal.pone.0255434
- Hall, T., Ibis Biosciences & Carlsbad, C. (2011). BioEdit: an important software for molecular biology. *GERF bull biosci*, 2, 60–61.

- Jardine, C.M., Parmley, E.J., Buchanan, T., Nituch, L. & Ojkic, D. (2018). Avian metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. *Transbound Emerg Dis*, 65, 1098–1102. doi:10.1111/tbed.12832
- Jones, R.C. (1996). Avian pneumovirus infection: Questions still unanswered. *Avian Pathol*, 25, 639–648. doi:10.1080/03079459608419171
- Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A. & Jermini, L.S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat Methods*, 14, 587–589. doi:10.1038/nmeth.4285
- Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*, 30, 3059–3066. doi:10.1093/nar/gkf436
- Krauss, S., Obert, C. A., Franks, J., Walker, D., Jones, K., Seiler, P., Niles, L., Pryor, S.P., Obenauer, J.C., Naeve, C.W., Widjaja, L., Webby, R.J. & Webster, R.G. (2007). Influenza in migratory birds and evidence of limited intercontinental virus exchange. *PLoS Pathog*, 3, e167. doi:10.1371/journal.ppat.0030167
- Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mo. Biol Evol*, 35, 1547–1549.
- Legnardi, M., Allée, C., Franzo, G., Cecchinato, M. & Brown, P. (2021). Research Note: Detection of Avian metapneumovirus subtype C specific antibodies in a mallard flock in Italy. *Poult Sci J*, 100, 101186. doi:10.1016/j.psj.2021.101186
- Lemaitre, E., Allée, C., Vabret, A., Eterradossi, N. & Brown, P.A. (2018). Single reaction, real time RT-PCR detection of all known avian and human metapneumoviruses. *J Virol Methods*, 251, 61–68. doi:10.1016/j.jviromet.2017.10.010
- Listorti, V., Lupini, C., Cecchinato, M., Pesente, P., Rossi, G., Giovanardi, D., Naylor, C.J. & Catelli, E. (2014). Rapid detection of subtype B avian metapneumoviruses using RT-PCR restriction endonuclease digestion indicates field circulation of vaccine-derived viruses in older turkeys. *Avian Pathol*, 43, 51–56. doi:10.1080/03079457.2013.866212
- Lupini, C., Cecchinato, M., Ricchizzi, E., Naylor, C.J. & Catelli, E. (2011). A turkey rhinotracheitis outbreak caused by the environmental spread of a vaccine-derived avian metapneumovirus. *Avian Pathol*, 40, 525–530. doi:10.1080/03079457.2011.607428
- Mescolini, G., Lupini, C., Franzo, G., Quaglia, G., Legnardi, M., Cecchinato, M., Tucciarone, C. M., Blanco, A., Turblin, V., Biarnés, M., Tatone, F., Falchieri, M. & Catelli, E. (2021). What is new on molecular characteristics of Avian metapneumovirus strains circulating in Europe? *Transbound Emerg Dis*, 68, 1314–1322. doi:10.1111/tbed.13788
- Qu, L., Liu, W., Yang, F., Hou, Z., Zheng, J., Xu, G. & Yang, N. (2009). Origin and domestication history of Peking ducks determined through microsatellite and mitochondrial marker analysis. *Sci China Life Sci*, 52, 1030–1035 doi:10.1007/s11427-009-0145-x
- Rautenschlein, S. (2020). Avian metapneumovirus. In D.E. Swayne, M. Boulianne, C.M. Logue, L.R. McDougald, V. Nair, D.L. Suarez, S. de Wit, T. Grimes, D. Johnson, M. Kromm, T.Y.

- Prajitno, I. Rubinoﬀ & G. Zavala (Eds.). *Diseases of Poultry* 14th edn (pp. 135-143). Hoboken: Wiley Blackwell.
- Retallack, H., Clubb, S. & DeRisi, J.L. (2019). Genome Sequence of a Divergent Avian Metapneumovirus from a Monk Parakeet (*Myiopsitta monachus*). *Microbiol Resour Announc*, 8, e00284-19. doi:10.1128/MRA.00284-19
- Rima, B., Collins, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R.A., Lee, B., Maisner, A., Rota, P., Wang, L. & ICTV Report Consortium. (2017). ICTV Virus Taxonomy Profile: Pneumoviridae. *J Gen Virol*, 98, 2912–2913. doi:10.1099/jgv.0.000959
- Seal, B.S. (1998). Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. *Virus Res*, 58, 45–52. doi:10.1016/s0168-1702(98)00098-7
- Senne, D., Edson, J.C. & Pederson, B.P. (1997). Avian pneumovirus update. In *Proceedings of the 134th annual convention of the American Veterinary Medical Association* (p. 190). Reno, Nevada.
- Shin, H.J., Njenga, M.K., McComb, B., Halvorson, D.A. & Nagaraja, K.V. (2000). Avian pneumovirus (APV) RNA from wild and sentinel birds in the United States has genetic homology with RNA from APV isolates from domestic turkeys. *J Clin Microbiol*, 38, 4282–4284. doi:10.1128/JCM.38.11.4282-4284.2000
- Spina, F. & Volponi, S. (2008). *Atlante della Migrazione Degli Uccelli in Italia: Non-Passeriformi*. Roma: Ministero dell’Ambiente e della Tutela del Territorio e del Mare, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA).
- Sun, S., Chen, F., Cao, S., Liu, J., Lei, W., Li, G., Song, Y., Lu, J., Liu, C., Qin, J. & Li, H. (2014). Isolation and characterization of a subtype C avian metapneumovirus circulating in Muscovy ducks in China. *Vet Res*, 45. doi:10.1186/s13567-014-0074-y
- Trifinopoulos, J., Nguyen, L.T., von Haeseler, A. & Minh, B.Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res*, 44. doi:10.1093/nar/gkw256
- Toquin, D., Băyon-Auboyer, M.H., Eterradossi, N., Jestin, V. & Morin, H. (1999). Isolation of a pneumovirus from a Muscovy duck. *Vet Rec*, 145, 680.
- Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C. & Eterradossi, N. (2006). European and American subtype C isolates of avian metapneumovirus belong to different genetic lineages. *Virus genes*, 32, 97–103. doi:10.1007/s11262-005-5850-3
- Tucciarone, C.M., Franzo, G., Lupini, C., Torres Alejo, C., Listorti, V., Mescolini, G., Branão, P.E., Martini, M., Catelli, E. & Cecchinato, M. (2018). Avian Metapneumovirus circulation in Italian broiler farms. *Poul Sci J*, 97, 503-509. doi:10.3382/ps/pex350.
- Turpin, E.A., Stallknecht, D.E., Slemons, R.D., Zsak, L. & Swayne, D.E. (2008). Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South Carolina, Arkansas and Ohio, USA. *Avian Pathol*, 37, 343–351. doi:10.1080/03079450802068566

- van Boheemen, S., Bestebroer, T. M., Verhagen, J. H., Osterhaus, A.D.M.E., Pas, S.D., Herfst, S. & Fouchier, R.A. (2012). A family-wide RT-PCR assay for detection of paramyxoviruses and application to a large-scale surveillance study. *PloS one*, 7. doi:10.1371/journal.pone.0034961
- van Dijk, J.G., Hoyer, B.J., Verhagen, J.H., Nolet, B.A., Fouchier, R.A. & Klaassen, M. (2014). Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus. *J Anim Ecol*, 83, 266–275. doi:10.1111/1365-2656.12131
- Yunus, A.S., Govindarajan, D., Huang, Z. & Samal, S.K. (2003). Deduced amino acid sequence of the small hydrophobic protein of US avian pneumovirus has greater identity with that of human metapneumovirus than those of non-US avian pneumoviruses. *Virus Res*, 93, 91–97. doi:10.1016/s0168-1702(03)00074-1

SECTION THREE: EXPLORING THE WILD SIDE OF THE INFECTIOUS BURSAL DISEASE VIRUS

VII. Infectious bursal disease virus in free-living wild birds: a systematic review and meta-analysis of its sero-viroprevalence on a global scale

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Infectious bursal disease virus (IBDV) is an economically important pathogen for poultry, whereas knowledge of its occurrence in non-poultry hosts is limited. The objective of this systematic review and meta-analysis is to summarize the up-to-date knowledge about the sero-viroprevalence of IBDV in wild birds on a global scale. A computerized literature research was performed on PubMed, Scopus, CAB Direct and Web of Science to find relevant publications, along with the screening of reference lists. Journal articles, book chapters, scientific correspondences, conference proceedings and short communications on IBDV virological and/or serological surveys in free-living wild birds published between 1970 and 2021 were considered as eligible. Among 184 studies found, 36 original contributions met the pre-established criteria. A random-effect model was applied to calculate pooled seroprevalence estimates with 95% confidence intervals, whereas the paucity of virological studies ($n = 6$) only allowed a qualitative description of the data. The pooled seroprevalence was estimated to be 6% (95% C.I.: 3%-9%) and a high heterogeneity was detected ($I^2=96\%$). Sub-group analyses were not performed due to the scarcity of available information about hypothetical moderators. With respect to virological studies, IBDV was detected in Anseriformes, Columbiformes, Galliformes, Passeriformes and Pelecaniformes and different strains related to poultry infection were isolated.

Our estimates of serological data showed a moderate exposure of wild birds to IBDV. The susceptibility of different species to IBDV infection underlines their potential role in its epidemiology at least as carriers or spreaders. Indeed, the isolation of IBDV in healthy wild birds with a migratory attitude might contribute to a long-distance spread of the virus and to strain diversity. Whilst a wild reservoir host could not be clearly identified, we believe our work provides useful insights for conducting future surveys which are needed to broaden our knowledge of IBDV occurrence in wild birds.

7.1. Introduction

Infectious bursal disease (IBD) or Gumboro disease (GD) is an economically impacting disease of the global poultry industry caused by Infectious bursal disease virus (IBDV), a highly contagious bi-segmented double stranded RNA virus. IBDV belongs to the genus *Avibirnavirus* within the family *Birnaviridae* (Lefkowitz *et al.*, 2018) and two serotypes can be identified by cross-neutralization assays, namely serotype 1 and serotype 2. Serotype 1 includes pathogenic strains for chickens and serotype 2 includes non-pathogenic strains which naturally occurs in turkeys (McNulty *et al.*, 1979; McFerran *et al.*, 1980) and are also detected in other avian species (Candelora *et al.*, 2010; Gough *et al.*, 2002). IBDV transmission commonly happens through the fecal-oral route (Benton *et al.*, 1967). Being a non-enveloped RNA virus, IBDV is also extremely resistant in the environment and has the potential to be spread by different fomites (Crespo *et al.*, 2016) and mechanical vectors (Howie & Thorsen, 1981; McAllister *et al.*, 1995; Pagès-Manté *et al.*, 2004; Park *et al.*, 2010).

Regardless of the pathogenicity of the strain, IBDV infection damages the bursa of Fabricius and causes an immunosuppression which is more severe the younger the animals affected (Lupini *et al.*, 2020; Rautenschlein *et al.*, 2003; Silveira *et al.*, 2019; Sharma *et al.*, 2000). IBDV-related immunodeficiency indeed leads a flock to higher susceptibility to secondary bacterial infections and decreases the efficiency of vaccination programs routinely applied (Aricibasi *et al.*, 2010). Because of viral evolution through mutation (Aliyu *et al.*, 2021), reassortment (Jackwood *et al.*, 2016) and recombination (Jackwood, 2012; He *et al.*, 2009), diverse IBDV genotypes are detected and classified in numerous genogroups (Michel & Jackwood, 2017; Islam *et al.*, 2021). Infections with antigenically different strains can significantly impact the poultry production system due to the potential limited efficacy of implemented vaccination plans. Furthermore, the origin of newly emergent IBDV strains can be unobvious and epidemiological surveys can lead to inconclusive epidemiological links (Felice *et al.*, 2017; Lupini *et al.*, 2016; Thai *et al.*, 2021).

Despite an increasing awareness of the role of wildlife in poultry pathogens' ecology, little is known about the role played by wild birds in the IBDV epidemiology. Since 1980, scientific papers have demonstrated that other avian species apart from chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) are susceptible to IBDV (Gough *et al.*, 1998; McFerran *et al.*, 1980; Wang *et al.*, 1997; Zhou *et al.*, 1998). It has therefore been hypothesized that wild birds could be epidemiologically relevant to the genetic evolution of circulating IBDV strains (Hon *et al.*, 2006; Tammiranta *et al.*, 2018; Yamaguchi *et al.*, 1997) or could act as spreaders between infected farms (Gilchrist, 2005).

Wild birds can exhibit extremely heterogeneous patterns of movement according to species and populations. Given their ability to fly over long distances, wild migratory birds interconnect different parts of the globe and can deliver pathogens from one country to another (Jourdain *et al.*, 2007). In addition to natural habitats, some species are well adapted to human-driven environments and can act as bridges between pristine ecosystems and anthropogenic ones (Patankar *et al.*, 2021; Wille *et al.*, 2020).

Considering the continuous detection of diverse strains of IBDV, disentangling the potential role of wild birds in the epidemiology of this pathogen is pivotal. In this study we summarize the up-to-date evidence of IBDV sero-virological prevalence in wild birds on a global scale conducting a systematic review and meta-analysis. To the best of our knowledge, this is the first review that focuses on IBDV in wild species rather than in poultry (Alkie & Rautenschlein, 2016; Dey *et al.*, 2019; Berg, 2000; Mahgoub *et al.*, 2012).

7.2. Materials and methods

A systematic review and meta-analysis were performed to estimate the seroprevalence of the IBDV in free-living wild birds. Given the small number of studies using molecular or virological methods to define the viral prevalence, it was determined that these could not be considered together. Thus, the analysis was limited to a systematic review and the studies' characteristics were displayed in a descriptive table.

Our work followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA-P) (Moher *et al.*, 2015) and the PRISMA 2020 Statement (Page *et al.*, 2020)

7.2.1. Information sources and search strategy

Four databases were accessed from 01/10/2020 to 24/04/2021. These included: PubMed (<https://pubmed.ncbi.nlm.nih.gov/>), Scopus (<https://www.scopus.com/>), CAB Direct (<https://www.cabdirect.org>) and Web of Science (<https://apps.webofknowledge.com/>). The reference lists of eligible studies were also screened to find other relevant contributions by hand (Higgins *et al.*, 2021).

The search strategy used for each database is reported in Table 1. Boolean operators 'OR' and 'AND' were used in all the computerized databases and MeSH Terms related to IBDV were used in PubMed. Eligible studies were merged and managed in a Microsoft Excel 2021 sheet (Version 16.49).

Table 1. Search lines used for the computerized literature research and number of studies retrieved from each database (PubMed, Scopus, CAB Direct, Web of Science) before duplicates removal.

Database	Search line	No. of studies retrieved
PubMed	(((((infectious bursal disease*[Title/ Abstract]) OR "infectious bursal disease virus"[MeSH Terms]) OR "gumboro disease"[Title/ Abstract]) OR "ibdv"[Title/ Abstract]) OR "birnaviridae infections/ veterinary"[MeSH Terms]) AND ((animal, wild[MeSH Terms]) OR "free living"[Title/ Abstract]) AND (((“birds”[MeSH Terms]) OR bird*[Title/ Abstract]) OR "wild birds"[Title/ Abstract])	14
Scopus	TITLE-ABS("infectious bursal disease virus") OR TITLE-ABS("infectious bursal disease") OR ("Gumboro") OR TITLE-ABS("IBDV") AND (TITLE-ABS("wild") OR TITLE-ABS(*free-living*)) AND TITLE-ABS(*bird*)	49
CAB Direct	(title:("infectious bursal disease virus") OR ab:("infectious bursal disease virus") OR title:("Gumboro disease") OR ab:("gumboro disease") OR title:("IBDV") OR ab:("IBDV")) AND ((title:("wild") OR ab:("wild")) OR (title:(free-living) OR ab(free-living)) AND (title:("bird") OR ab:("bird"))	42
Web of Science	ALL=((("infectious bursal disease" OR "infectious bursal disease virus" OR "avian infectious bursitis" OR "gumboro disease" OR "IBDV") AND (((wild) OR (free-living)) AND (bird*)))	60

7.2.2. Selection process

Literature searches and the screening process of the papers were independently performed by two reviewers (G.G. and A.F.). Eligible studies were selected by applying pre-established inclusion criteria to title and abstract of each work (Table 2). Portuguese and Chinese manuscripts were screened after translation into English through Google Translate (<https://translate.google.com>). After removing duplicate papers, the reasons for exclusion of any other study were recorded. If disagreements occurred, a third experienced author in the avian pathology field was consulted (E.C.).

Table 2. Inclusion criteria applied for screening titles and abstracts of the publications retrieved.

All studies (journal articles, book chapter, scientific correspondence, conference proceedings and contributions, short communication) on natural infections of IBDV published after 1970
All languages
Full text access
Virological/ molecular study (direct diagnostic methods)
Serological study (indirect diagnostic methods)
Samples: blood, tissue samples, swabs
Free-living wild birds: species of free-range wild birds not living in captivity when the study occurred, exception for wild birds admitted to rehabilitation center
Sample size > 10
Population not included in more than one study

7.2.3. Data collection process

Data extraction from each eligible study was performed by two independent reviewers (G.G. and A.F.) and the quality of the data was double checked by a third author (C.L.). Whenever two different diagnostic methods were applied, only the outcomes from the test used as confirmatory were considered. If prevalence data retrieved from original articles was expressed as percentage, raw numbers were obtained converting the percentages to the closest integers.

Two data extraction sheets were created, one for the seroprevalence surveys and another for viral prevalence ones. The first was filled with the following information: title, first author last name, year of publication, country, language, sampling period, host identification (order, family and species), age classes and sex, sample type, total number of animals tested, total number of positive cases, serological method, serological cut-off used to define a sample as positive, specificity and sensitivity of the method applied and serotype characterization (serotype 1 or serotype 2). The second with: title, first author last name, year of publication, country, language, sampling period, host identification (order, family and species), age classes and sex, sample type, total number of animals tested, total number of positive cases, molecular method, viral isolation method, specificity and sensitivity of the methods applied and GenBank accession number (<http://www.ncbi.nlm.nih.gov/>).

7.2.4. Study risk of bias assessment

Despite the availability of different tools to evaluate the publication bias, the authors could not find a suitable one to the given dataset (Hunter *et al.*, 2014). Considering the heterogenous study designs, diversity of species tested, different sample sizes and zero positive cases reported in some original papers it was decided to include all the eligible full texts retrieved. A comment section was added to the data extraction sheets to report anything that could affect the interpretation of the

results (Fanelli *et al.* 2020). Moreover, outliers were identified through leave-one-out analysis and excluded from the meta-analysis (Viechtbauer & Cheung, 2010).

7.2.5. *Summary outcomes*

The principal summary outcome was the overall seroprevalence of IBDV in free-living wild birds without restriction to any geographical area. As mentioned before, data from virological studies were not pooled due to the paucity of original articles available and virological prevalence was therefore calculated for each individual study.

7.2.6. *Synthesis methods*

7.2.6.1. Statistical synthesis methods

All statistical calculations were performed on R software version 3.5.2 (R Core Team, 2018). Visual representation of search results was realized with the *wordcloud* package. The *meta* version 4.18-2 and *metaphor* version 2.4.0 packages were used to perform the meta-analysis of serologic data as described in the comprehensive tutorial of Wang (2018). Briefly, the observed proportions were transformed using the double arcsine method to normalize the data (Freeman & Tukey, 1950; Miller, 1978). For reporting, the pooled transformed proportions and its 95% confidence interval (C.I.) was converted back to the observed proportions. The between-study variance was estimated through the random effects method using the restricted maximum-likelihood estimator (REML). The heterogeneity between studies was quantified using the inconsistency index (I^2) and interpreted as small, medium or high according to <25%, 25-50% and >75% values respectively (Higgins & Thompson, 2002). After visual inspection of the pooled seroprevalence forest plot, outliers were identified performing leave-one-out analysis and therefore removed.

Considering the virological prevalence, the *prevalence* package version 0.4.0 was applied to calculate this estimate and its 95% C.I. using the “exact” method.

7.2.6.2. Other analyses

Countries were grouped into sub-regions according to the standards of the Statistics Division of the United Nations Secretariat (<https://unstats.un.org/unsd/methodology/m49/>). Eventual inconsistencies in the taxonomic nomenclature used by the Authors were harmonized according to current nomenclature following the latest Avibase version (Lepage & Warnier, 2014). The taxonomic order of the hosts, the age classes and the geographical area of the studies were hypothesized as possible moderators. However, we decided not to perform sub-group analyses because of the scarcity of data available and heterologous age attribution techniques. Moreover, the studies were

not pooled according to serological methods given the lack of any information about the sensitivity and specificity of the different tests applied.

With respect to virological studies, sequence analysis of IBDV Viral Protein 2 from wild birds was performed. Reference strains reported in Aliyu *et al.* (2021) were obtained from NCBI GenBank and used to represent IBDV genogroups (Michel & Jackwood, 2017). Sequences were aligned by MUSCLE algorithm. Best partition scheme, substitution models and maximum likelihood phylogenetic reconstruction were performed on the IQ-TREE web server using 1000 ultrafast bootstrap replicates (Trifinopoulos *et al.* 2016).

7.3. Results

7.3.1. Search summary

A total of 165 articles were identified through the computerized literature research on PubMed, CAB Direct, Scopus and Web of Science. After duplicates removal ($n = 67$), 98 articles were assessed for eligibility through the screening of titles and abstracts. Eventually, 23 studies met the pre-established criteria and were full-text accessed. Of these, one article out of 23 was afterwards excluded due to lack of necessary information on the species tested (Pawar *et al.*, 2009). 22 eligible studies were therefore obtained from literature research on selected databases.

Another 19 articles were added after reference lists reading. Two of these studies were excluded due to unsuccessful retrieval of the full texts (Oña *et al.*, 2000; Edgar & Cho, 1965). Three more studies were rejected after full text reading: 1) Wang *et al.* (1997) and 2) Zhou *et al.* (1998) surveyed the same population that was raised and held in captivity, 3) Candelora *et al.* (2008) presented the preliminary results of Candelora *et al.* (2010), only the latter was therefore considered. As result, 14 studies retrieved through reference lists reading were considered eligible.

To conclude, 36 original articles about virological and serological surveys of IBDV in wild birds were included in the systematic review here presented (Figure 1). After full-text retrieval, articles applying direct ($n = 3$) or indirect ($n = 30$) diagnostic methods were sorted into two different Microsoft Excel sheets as previously described. Watts *et al.* (2009), Vargas-Castillo *et al.* (2019) and Kasanga *et al.* (2008) which applied both serological and virological diagnostic methods were included in both databases.

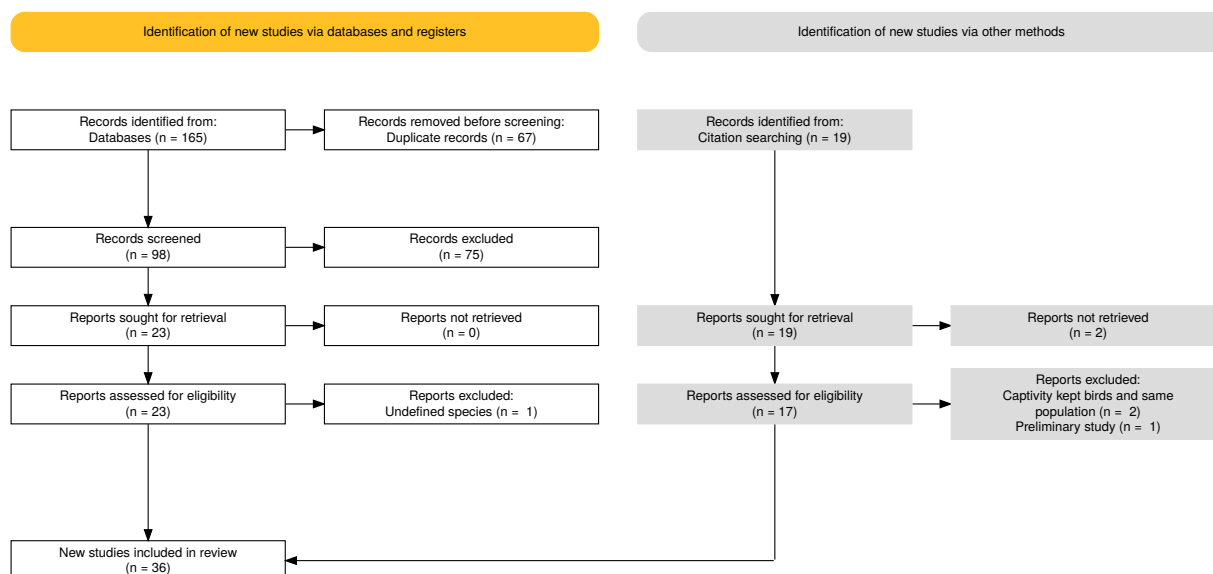


Figure 1. PRISMA flow chart of the systematic review method applied to the occurrence of IBDV in free-living wild birds on a global scale (https://estech.shinyapps.io/prisma_flowdiagram/).

7.3.2. Study characteristics

We identified 36 eligible articles on IBDV surveys in free-living wild birds published between 1978 and 2020 and covering 22 countries (Figure 2). An overview of the scientific publications is provided in Table 3 and Table 4, respectively for the use of indirect or direct methods for IBDV diagnosis.

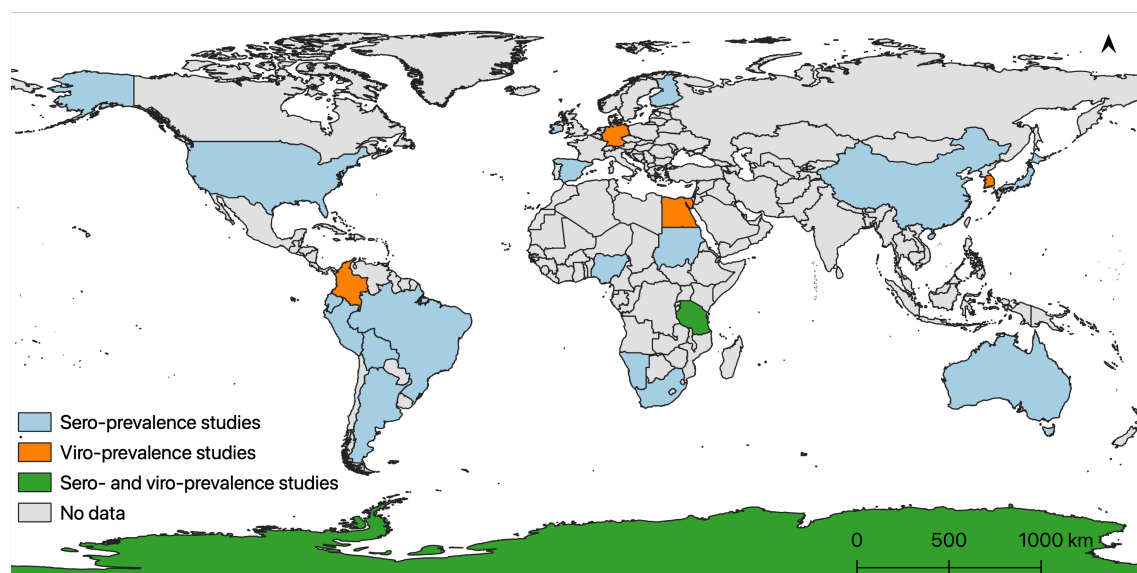


Figure 2. Global distribution of IBDV publications in free-living wild birds considered as “eligible” according to pre-established criteria applied as screening method of literature. Green = both direct and indirect diagnostic methods applied in the study; orange = direct diagnostic methods; blue = indirect diagnostic methods. Created with QGIS 3.6.0.

1 **Table 3.** Overview of the serological studies on IBDV in wild birds included in the qualitative synthesis of results and in the meta-analysis.
2

Authors and publication year	Geographic area [†]	Study period	Method	Host identification	Total no. birds (total cases)	Apparent prevalence (%)	Sero- type
Leite <i>et al.</i> , 2020	South America	n.r.	ELISA ^a	Columbiformes, Pas- seriformes	30(0)	0	n.a.
Uhart <i>et al.</i> , 2020	South America	1994-2008	AGID	Sphenisciformes	337(0)	0	n.a.
Orakpoghenor <i>et al.</i> , 2020	Western Africa	2017	ELISA ^b	Columbiformes, Pele- caniformes, Pas- seriformes	150(3)	2	n.a.
Vargas-Castillo <i>et al.</i> , 2019	South America	2012-2013	ELISA ^a	Apodiformes, Colum- biformes, Passeri- formes	29(0)	0	n.a.
Dwight <i>et al.</i> , 2018	Northern America	2014-2015	ELISA ^a	Galliformes	33(23)	69.7	n.a.
Grimaldi <i>et al.</i> , 2018	Antarctica	2010-2013	ELISA and VNT	Sphenisciformes	424(10)	2.4	n.r.
Adamu <i>et al.</i> , 2017	Western Africa	2014	ELISA ^a	Columbiformes, Pas- seriformes, Pelecani- formes	195(12)	6.2	n.a.
Parsons <i>et al.</i> , 2016	Southern Africa	2007; 2009; 2010-2013	ELISA ^a	Sphenisciformes	443(12)	2.7	n.a.
Assam <i>et al.</i> , 2014	Western Africa	2012	AGID	Accipitriformes, Anseriformes, Chara- driiformes, Ciconii-	95(0)	0	n.a.

				formes, Columbi- formes, Falconi- formes, Galliformes, Gruiformes, Passeri- formes, Peleca- niformes, Piciformes			
Iman <i>et al.</i> , 2013	Northern Africa	2011-2012	AGID	Anseriformes, Charadriiformes, Columbiformes, Coraciiformes, Gruiformes, Passeriformes, Pelecaniformes	41(0)	0	n.a.
Nunes <i>et al.</i> , 2012	South America	2012	ELISA ^a and AGID	Sphenisciformes	89(42)	47.2	n.a.
Candelora <i>et al.</i> , 2010	Northern America	2004-2007; 1992-1998	VNT	Galliformes, Gruiformes	757(87)	11.5	2
Miller & Shellam, 2010	Antarctica	2008	VNT	Sphenisciformes	560(187)	33.4	n.r.
Watts <i>et al.</i> , 2009	Antarctica	1996-2002	VNT	Charadriiformes, Sphenisciformes	1395(190)	13.6	1
Kasanga <i>et al.</i> , 2008	Eastern Africa	2005	VNT	Columbiformes, Galliformes	11(0)	0	1
Miller <i>et al.</i> , 2008	Antarctica	1999	VNT	Charadriiformes	118(20)	16.9	1
Smith <i>et al.</i> , 2008	South America	1992-1994	AGID	Sphenisciformes	20(0)	0	n.a.

Miller & Shellam, 2007	Antarctica	2006	VNT	Sphenisciformes	313(214)	68.4	n.r.
Travis <i>et al.</i> , 2006a	South America	2003	AGID	Suliformes	68(0)	0	n.a.
Travis <i>et al.</i> , 2006b	South America	2003-2004	AGID	Sphenisciformes	75(0)	0	n.a.
Deem <i>et al.</i> , 2005	South America	2000	AGID	Psittaciformes	22(0)	0	n.a.
Gauthier-Clerc <i>et al.</i> , 2002	Antarctica	1996-1997	VNT	Sphenisciformes	302(14)	4.6	1; 2
Höfle <i>et al.</i> , 2001	Southern Europe	n.r.	VNT	Accipitriformes, Falconiformes, Strigiformes	37(4)	10.8	n.r.
Campbell, 2001	Northern Europe	n.r.	AGID and/or ELISA ^a	Anseriformes, Colum- biformes, Galliformes, Passeriformes	41(10)	24.4	n.a.
Hollmén <i>et al.</i> , 2000	Northern Europe; Northern America	1998	VNT	Anseriformes, Charadriiformes	211(146)	69.2	1
Fagbohun <i>et al.</i> , 2000	Western Africa	n.r.	ELISA	Columbiformes, Pele- caniformes	75(14)	18.7	n.a.
Karesh <i>et al.</i> , 1999	South America	1994	AGID	Sphenisciformes	30(0)	0	n.a.
Ogawa <i>et al.</i> , 1998	Eastern Asia	1989-1997	VNT	Anseriformes, Chara- driiformes, Columbi- formes, Falco- niformes, Passeri-	739(51)	6.9	1; 2

				formes, Pelecaniformes, Strigiformes			
Gu <i>et al.</i> , 1998	Eastern Asia	n.r.	AGID	Galliformes	70(14)	20	n.a.
Gardner <i>et al.</i> , 1997	Antarctica	1991; 1995-1996	VNT	Sphenisciformes	364(39)	10.7	n.r.
Ezeifeke <i>et al.</i> , 1992	Western Africa	n.r.	AGID	Passeriformes, Piciformes	35(0)	0	n.a.
Wilcox <i>et al.</i> , 1983	Australia and New Zealand	1977-1979	AGID and VNT	Anseriformes, Charadriiformes, Procellariiformes	397(14)	3.5	1; 2
Nawathe <i>et al.</i> , 1978	Western Africa	1977	AGID	Passeriformes, Piciformes	50(6)	12	n.r.

1 [†]Countries of origin of the studies were grouped into sub-regions as stated by the Statistics Division of the United Nations Secretariat
2 (<https://unstats.un.org/unsd/methodology/m49/overview/>)

3 ^aELISA kit: IDEXX IBD[®], IDEXX Laboratories – specificity and sensitivity on chicken serum (%): 100 and 88, respectively. (De Wit *et al.*, 2001)

4 ^bELISA kit: IDEXX IBD-XR[®], IDEXX Laboratories – specificity and sensitivity on chicken serum (%): 95.4 and 100, respectively (De Wit *et al.*, 2001)

5 n.a.: not applicable; n.r.: not reported

1 **Table 4.** Overview of the molecular / virological studies on IBDV in wild birds included in the qualitative synthesis of results.

2

Authors and publication year	Geographic area	Study period	Sample type	Molecular method	Viral isolation	GenBank accession number	Host identification	Total no. birds (total cases)	Prevalence % (95% C.I.)
Naggar <i>et al.</i> , 2020	Northern Africa	2019	cloacal swab	RT-PCR	CAMs (qRT-PCR [‡])	MT304668 MT304669 MT304670	<i>Anseriformes</i> , <i>Pelecaniformes</i> , <i>Galliformes</i>	28(3)	10.7 (0%-20%)
Vargas-Castillo <i>et al.</i> , 2019	South America	2012-2013	cloacal swab	RT-PCR	-	-	<i>Apodiformes</i> , <i>Columbiformes</i> , <i>Passeriformes</i>	48(0)	0
Curland <i>et al.</i> , 2018	Western Europe	2011-2014	bursa of Fabricius	RT-PCR	-	-	<i>Galliformes</i>	27(1)	3.8 (0%-11%)
Watts <i>et al.</i> , 2009	Antarctica	2000	bursa of Fabricius	RT nested PCR	CAMs	-	<i>Sphenisciformes</i>	23(3) [†]	0 [†]
Kasanga <i>et al.</i> , 2008	Eastern Africa	2005	bursa of Fabricius	RT-PCR	-	AB306716	<i>Galliformes</i>	11(1)	9.1 (8%-27%)
Jeon <i>et al.</i> , 2008	Eastern Asia	2006-2007	cecal tonsil	RT-PCR	CAMs (RT-PCR [‡] ; AGP [‡])	EU493342 EU493343 EU493345 EU493344 EU493341	<i>Anseriformes</i> , <i>Charadriiformes</i> , <i>Columbiformes</i> , <i>Passeriformes</i>	107(5)	4.7 (0.7%-9%)

3 [†] Watts *et al.* (2009) reported 3 IBDV positive samples interpreted as a possible positive-control contamination after genetic characterization therefore the
4 prevalence (%) was considered as 0.[‡] Diagnostic tests used to confirm IBDV isolation with CAMs.

Regarding serological surveys, the studies retrieved (n = 33) were published between 1978 and 2020 and covered 20 countries of the world. A total amount of 7556 sera of free-living wild birds were examined and 1112 resulted positive. The serological methods applied included the enzyme-linked immunosorbent assay (ELISA) performed with both in-house and commercial kits, the viral neutralization test (VNT) and the agar gel immunodiffusion assay (AGID). None of the studies reported the sensitivity and specificity of the methods with regards to the species tested. The most common technique was the VNT (n = 13) whose details on strains and cell cultures applied are listed in Table 5, followed by the ELISA (n = 10). Four studies (Campbell, 2001; Grimaldi *et al.*, 2018; Nunes *et al.*, 2012; Wilcox *et al.*, 1983) used two different serological tests in parallel or subsequently. Only eight out of 33 studies distinguished the IBDV serotype as serotype 1 or 2 for the positive cases (Candelora *et al.*, 2010; Gauthier-Clerc *et al.*, 2002; Hollmén *et al.*, 2000; Kasanga *et al.*, 2008; Miller *et al.*, 2008; Ogawa *et al.*, 1998; Watts *et al.*, 2009; Wilcox *et al.*, 1983).

Table 5. IBDV strains and cell cultures used for virus neutralization test (VNT) as serological screening of IBDV in wild bird samples in the eligible studies which reported the information.

Reference	IBDV strain (serotype)	Cell culture
Grimaldi <i>et al.</i> , 2018	GT101 strain (1)	Chicken embryo fibroblast (CEF)
Candelora <i>et al.</i> , 2010	Ohio strain (2)	Chicken embryo fibroblast (CEF)
Watts <i>et al.</i> , 2009	GT101 strain (1)	Chicken embryo fibroblast (CEF)
Kasanga <i>et al.</i> , 2008	GBF-1E strain (1)	Chicken embryo fibroblast (CEF)
Miller <i>et al.</i> , 2008	GT101 strain (1)	Chicken embryo fibroblast (CEF)
Gauthier-Clerc <i>et al.</i> , 2002	CT strain (1); TY89 strain (2)	Chicken embryo fibroblast (CEF)
Höfle <i>et al.</i> , 2001	D78 strain (1)	Chicken embryo liver (CEL)
Ogawa <i>et al.</i> , 2008	GBF-1E strain (1); OH strain (2)	Chicken embryo fibroblast (CEF)
Wilcox <i>et al.</i> 1983	PBG98 strain (1); TY89 strain (2)	Chicken embryo fibroblast (CEF)

Regarding the host, a total of 17 orders of birds were sampled. Most studies concerned Sphenisciformes (12/33 papers), Passeriformes (10/33), Columbiformes (9/33), and Charadriiformes (7/33) (Figure 3). Sphenisciformes (n = 4050 birds), Galliformes (n = 753 birds) and

Anseriformes (n = 459 birds) were the groups most frequently sampled. The highest rate of positive cases per order was retrieved for Falconiformes (33.3%), Anseriformes (32.9%); Gruiformes (32.7%), Sphenisciformes (16.8%), Accipitriformes (12.9%) and Charadriiformes (11.1%).



Figure 3. Word cloud showing the number of serological studies on IBDV according to different taxonomic orders of wild birds. Data are ordered from low to high, with light colors for lower number of studies on a specific order and darker color for higher values (Sphenisciformes n = 12 studies; Columbiformes n = 9 studies; Passeriformes n = 8 studies; Charadriiformes n = 7 studies; Pelecaniiformes, Galliformes n = 6 studies each; Anseriformes n = 5 studies; Accipitriformes, Falconiformes n = 4 studies each; Procellariiformes, Strigiformes n = 3 studies each; Gruiformes, Piciformes n = 2 studies each. Ciconiiformes, Psittaciformes and Suliformes with n = 1 study each are not represented). After statistical analyses, the following studies were identified as outliers and therefore removed from the summary of results: Dwight *et al.* (2018), Hollmén *et al.* (2000) and Miller & Shellam (2007).

The six molecular / virological studies retrieved were published between 2009 and 2020 and covered 6 countries (Figure 2, reported above).

Bursa of Fabricius (n = 3), cloacal swabs (n = 2) and cecal tonsils (n = 1) were sampled for attempting IBDV diagnosis. The total number of animals sampled were 244 of which 10 resulted positives in four different studies (Curland *et al.*, 2018; Jeon *et al.*, 2008; Kasanga *et al.*, 2008; Naggar *et al.*, 2020). The results from Watts *et al.* (2009) were not included in the count of the positive cases due to a hypothetical positive control contamination of the reverse-transcriptase PCR (RT-PCR) as assessed by the Authors of the paper. Regarding the technique applied, the RT-PCR or RT nested PCR assays were used as a screening method in all the studies. Primers and genes amplified are listed in Table 6. Naggar *et al.* (2020) and Jeon *et al.* (2008) both attempted viral isolation *via* chorioallantoic membrane (CAM) inoculation of embryonated specific pathogen free (SPF) chicken eggs. Whereas the CAM harvested material in Naggar *et al.* (2020) was confirmed of IBDV isolation through a Real-Time quantitative RT-PCR (qRT-PCR), Jeon *et al.* (2008) used both RT-PCR and AGP (agar gel immunoprecipitation test) for confirmation. Strains isolated by Jeon *et al.* (2008) and Naggar

et al. (2020) and detected by Kasanga *et al.* (2008) were sequenced in Viral Protein 2 (VP2) gene, whereas Curland *et al.* (2018) did not further characterize the IBDV positive RT-PCR products.

Table 6. Primers and genes amplified for molecular screening of IBDV in wild bird samples in the eligible studies considered.

Reference	Gene(s) amplified	Primers 5'-3'	Location of 5' nucleotide
Naggar <i>et al.</i> , 2020	Viral Protein 2	Forward: GCCCAGAGTCTACACCAT Reverse: CCCGGATTATGTCTTTGA	717 1459
Vargas-Castillo <i>et al.</i> , 2019	Viral Protein 3; Viral Protein 4	Forward: GTRACRATCACACTGTTCTCAGC Reverse: GATGTRAYTGGCTGGGTTATCTC	804 1050
Curland <i>et al.</i> , 2018	Viral Protein 2	Forward: GCCCAGAGTCTACACCAT Reverse: CCCGGATTATGTCTTTGA	717 1459
Watts <i>et al.</i> , 2009	Viral Protein 2	Outer set of primers: Forward: TCACCGTCCTCAGCTTA Reverse: TCAGGATTTGGGATCAGC Inner set of primers: Forward: GCCCAGAGTCTACACCATAACTGC Reverse: GCGACCGTAACGACAGATC	587 1212 717 1174
Kasanga <i>et al.</i> , 2008	Viral Protein 2	Forward: CCAGAGTCTACACCATAA Reverse: CCTGTTGCCACTCTTTCGTA	719 1189
Jeon <i>et al.</i> , 2008	Viral Protein 2	Forward: GCCCAGAGTCTACACCAT Reverse: CCCGGATTATGTCTTTGA	717 1459

With respect to the host, eight different orders of birds were investigated and the Columbiformes and Galliformes were surveyed in 3 out of 6 papers. The most frequently sampled groups were Anseriformes (n = 92 birds), Passeriformes (n = 43 birds) and Galliformes (n = 37 birds). The highest rate of positive cases per order was retrieved for Pelecaniformes (33.3%) and Anseriformes (5.4%).

7.3.3. Results of individual serological and virological studies

Effect estimates and confidence intervals for each seroprevalence study are reported in the forest plot in Figure 4. The results from individual studies on serological and molecular/virological prevalence are shown in Table 3 and Table 4 reported above, respectively.

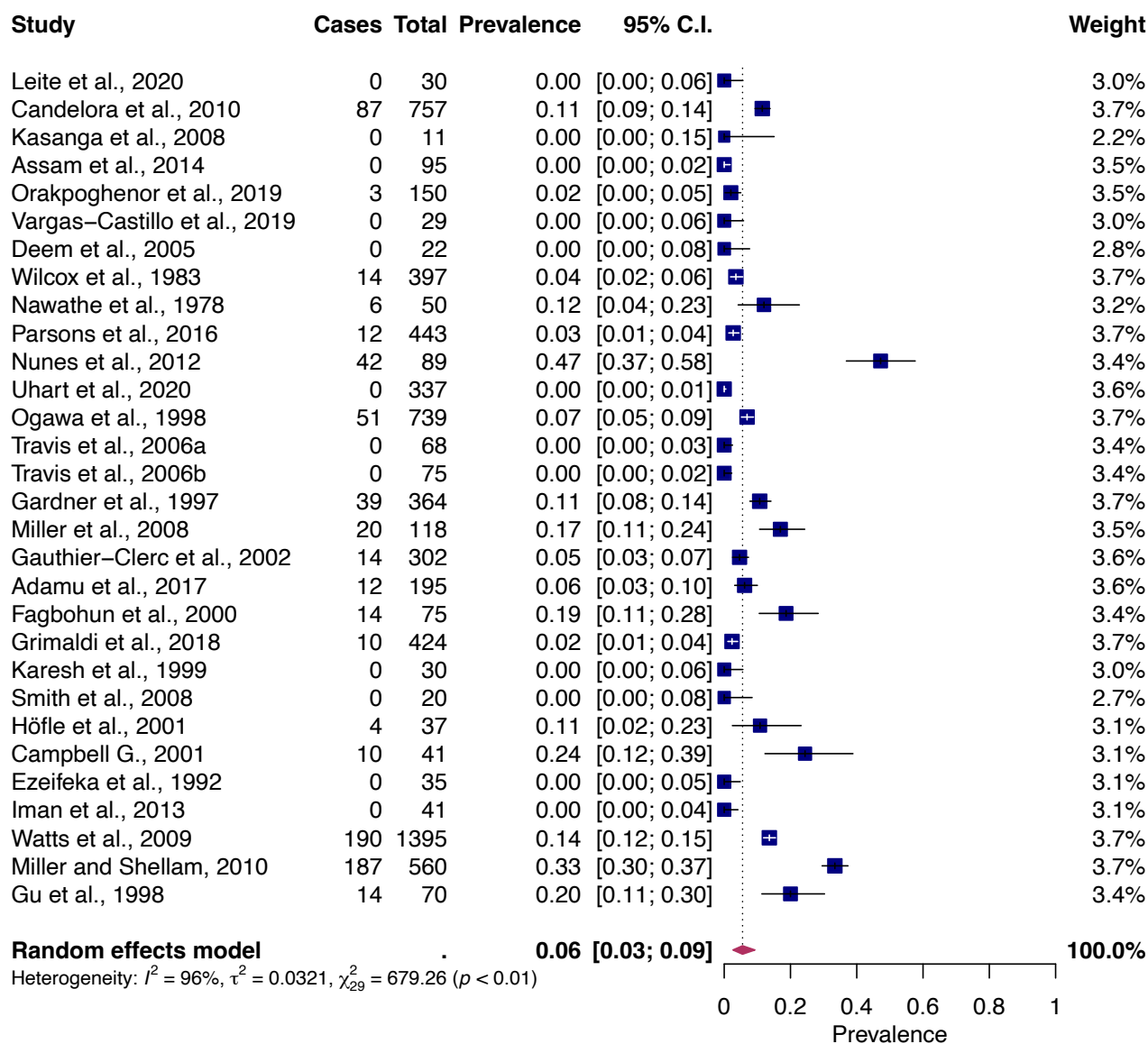


Figure 4. Forest plot of the random-effects meta-analysis of IBDV serological prevalence. I^2 (inverse variance index), τ^2 = the between study variance, χ^2 and p-value of the Cochran's Q test for heterogeneity

7.3.4. Risk of bias in serological and virological studies

No relevant risk of bias was identified by reviewers.

7.3.5. Pooled seroprevalence of serological studies

The pooled seroprevalence of IBDV in free-living wild birds was 6% (C.I. 95%: 3-9%). The I^2 statistic showed high heterogeneity ($I^2 = 96\%$, $p < 0.01$) (Figure 4, reported above).

7.3.6. Phylogenetic analysis

Phylogenetic reconstruction was performed using VP2 sequences from wild bird strains by applying a TIM2+F+G4 substitution model to the first codon position and a TIM2e+G4 substitution model to the second and third codon positions, as implemented on the IQ-TREE web server. As represented in Figure 6, IBDV strains detected in wild birds clustered with IBDV genogroup 1 and genogroup 3 strains.

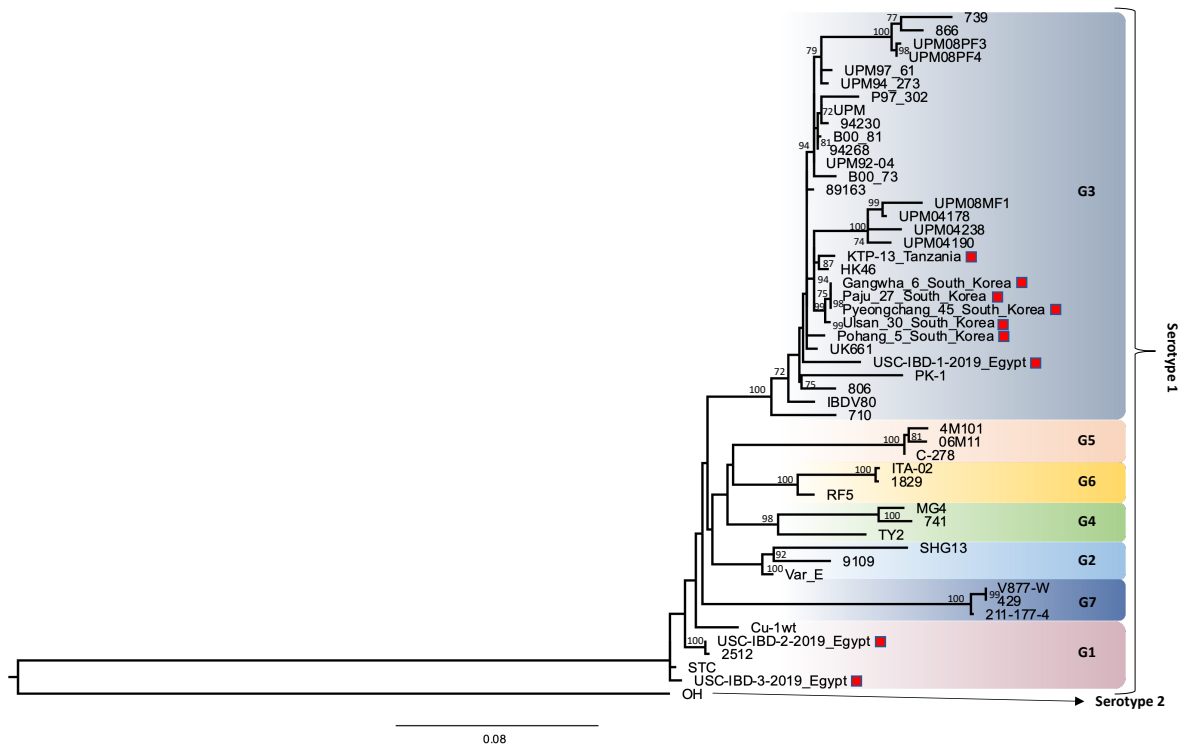


Figure 5. Phylogenetic tree of IBDV reference strains including wild bird sequences retrieved from eligible virological studies (red square). IBDV strains are grouped into genogroups from G1 to G7 and into serotype 1 and serotype 2. Only nodes with bootstrap values >70 are shown.

7.4. Discussion

7.4.1. Summary of evidence

To the best of our knowledge this is the first systematic review and meta-analysis of the seroprevalence of IBDV in wild birds on a global scale. Considering the persistence of serum antibodies over time and the ease of revealing both past and current infection, the majority of relevant papers retrieved from literature concerned serological surveys rather than virological ones. With respect to seroprevalence, the results showed that wild birds from different taxonomic groups were exposed to IBDV with an overall pooled prevalence estimate of 6% (95% C.I.: 3-9%). The meta-analysis also highlighted a high degree of heterogeneity ($I^2 = 96\%$, $p < 0.01$) thus our estimate is important to be considered together with its 95% C.I.. Hollmén *et al.* (2000), Dwight *et al.* (2018), and Miller & Shellam (2007) were identified as outliers. Although these studies reported the highest percentages of wild birds with antibody titers against IBDV (69.2%, 69.7% and 63.6% respectively), none of the positive cases were epidemiologically linked to outbreaks in poultry. Hollmén *et al.* (2000) found a high seroprevalence of IBDV in spectacled eiders (*Somateria fischeri*) living in remote areas of western Alaska, speculating that they might act as carrier hosts of IBDV. Dwight *et al.* (2018) carried out a sero-survey on wild and pen-reared pheasants (*Phasianus colchicus*). High seroprevalence found in both populations was interpreted by the Authors as a potential false positivity due to unvalidated diagnostic test applied or as a potential unexploited epidemiological role of pheasants in the ecology of IBDV. Miller & Shellam (2007) detected IBDV antibodies in adults and 1 year-old king penguins (*Aptenodytes patagonicus*) and adult royal penguins (*Eudyptes schlegeli*) on a sub-Antarctic island, thus implying an endemic circulation of IBDV in these populations.

Despite the rigorous statistical methods applied, our findings need to be cautiously interpreted. Several variables could have been hypothetically considered as moderators (e.g. geographic areas, host characteristics, diagnostic methods), however we could not perform any subgroup analyses due to the scarcity of available data. This can be considered a limit of the review hereby presented. With regards to the spatial distribution of data, we included 33 eligible studies from 11 different world sub-regions. More than one sero-survey per area were retrieved only for South America, Western Africa, Eastern Asia and Antarctica therefore we were not able to pool the data according to this factor. Concerning the avian host characteristics, free-living wild birds can be frequently found around rural and commercial poultry farms (Burns *et al.*, 2012). Moreover, some species could be considered as more exposed to possible IBDV infection due to their ecology and feeding habits. Higher taxonomic categories could have been considered as a possible grouping factor to investigate the heterogeneous results generated from the diversity of species tested. Unfortunately, several orders of birds were underrepresented or included exclusively in one survey. Still, we hereby discuss relevant findings for guiding future surveys. Sphenisciformes was the most studied order (12/33 papers) due to the increasing attention of men-driven threats to the Antarctic ecosystem. IBDV seropositivity was frequently observed in wild penguins (16.8%), possibly implying an endemic circulation (Miller & Shellam, 2010; Watts *et al.*, 2009) or a diagnostic cross-reactivity with closely related viruses (Gauthier-Clerc *et al.*, 2002; Gilbert *et al.*, 2013; Grimaldi *et al.*, 2018). Currently available data though is not sufficient for considering these birds as a possible natural IBDV reservoir. Falconiformes and Anseriformes appear to be the orders with the highest

rates of seropositive animals (33.3% and 32.9%, respectively), however: 1) in Falconiformes only 3 animals were sampled; 2) the major contribution to positive cases in Anseriformes (127 cases over 151 total positives) arose from spectacled eiders tested solely by Hollmén *et al.* (2000). Gruiformes also showed an high rate of seropositive animals (32.7%), although this order was investigated in just two out of the 33 sero-surveys retrieved (Candelora *et al.*, 2010; Assam *et al.*, 2014). Candelora *et al.* (2010) found IBDV antibodies against serotype 2 in sandhill cranes (*Grus canadensis*) in Florida, USA. The Authors hypothesized that these infected cranes could interact with the endangered whooping cranes (*Grus americana*) living in the same habitat and be a potential carrier of infection for the latter, with unknown consequences for the species' conservation. A 12.9% overall seropositivity was found in Accipitriformes, with positive cases originated from only one (Höfle *et al.*, 2001) of the two studies where representatives of this order were included (Assam *et al.*, 2014). This taxon comprises diurnal predators with feeding behaviors that might expose them to IBDV spillover from poultry farms with low biosecurity levels, however current data is insufficient to drive any conclusion. Lastly, Charadriiformes showed an 11.1% overall seropositive IBDV rate. This order includes aquatic birds as gulls, terns, plovers, and other shorebirds which are often gregarious and migrants. Hollmén *et al.* (2000) hypothesized that herring gulls (*Larus argentatus*) might be IBDV carriers due to their exposure to poultry waste and their opportunistic feeding behaviors. The trophic plasticity of Laridae species might indeed be a factor to be taken into account when arranging epidemiological surveys in wild birds. Furthermore, skuas (Stercorariidae) might migrate and introduce pathogens in Antarctica by stealing food resources from other birds and scavenging around fishing boats (Miller *et al.*, 2008; Nunes *et al.*, 2012).

Another possible source of the heterogeneity observed in our meta-analysis could be found in the age or sex of the tested birds. Demographic data is indeed believed to be an important tool for comparing the outcomes from different studies. Unfortunately, we could not investigate age or sex related patterns of seroprevalence since many studies lacked information or applied incomparable criteria of age classification.

Also, the use of different serological methods with different performances could have affected the obtained results. None of the studies reported about the sensitivity and the specificity of the test used with regards to the species. ELISA is frequently chosen for conducting sero-epidemiological surveys and is considered as a sensitive and rapid diagnostic tool, however the commercial kits validated for poultry could yield false-positives when applied to non-poultry hosts (Karesh *et al.*, 1999; Nunes *et al.*, 2012; Parsons *et al.*, 2016; Smith *et al.*, 2008; Travis *et al.*, 2006b; Uhart *et al.*, 2020). The virus neutralization test is recognized as a very sensitive and very specific serological test and has the advantage of being possibly used with the sera of any species (Phalen, 2002). VNT can also differentiate antibodies from exposure to IBDV serotype 1 or 2. The latter is considered apathogenic for fowl, however high titers against IBDV serotype 2 had been associated with high mortality and morbidity events in captive-reared whooping cranes (*G. americana*) during a reintroduction program in Florida, USA (Spalding *et al.*, 2008). Only few studies distinguished between IBDV serotypes (8/33 articles), still this differentiation is advised to give a more exhaustive

epidemiological picture. Grimaldi *et al.* (2018) and Nunes *et al.* (2012) combined two different serologic methods (an in-house competitive ELISA and the VNT and a commercial ELISA kit and the AGID, respectively) to confirm the positivity observed with the ELISA test and overcome methodological limitations.

With respect to viro-prevalence studies, we could not perform a meta-analysis due to the limited number of relevant articles retrieved ($n = 6$). Virus detection proves active viral shedding and allows further characterization of the isolated strains, we therefore consider it relevant to review the available data even if scarce. As reported by Naggar *et al.* (2020), the isolation of IBDVs in alive wild birds with a migratory attitude highlights the chance of a long-distance spread of the virus. It is also relevant to notice that these positive individuals did not show any sign of disease. IBDV strains were also detected in a black-billed magpie (*Pica hudsonia*) (Jeon *et al.*, 2008), a wild pigeon (*Columba livia*) (Kasanga *et al.*, 2008) and a wild pheasant (*P. colchicus*) (Curland *et al.*, 2018) which are common species around poultry farms. According to sequence analyses, wild bird isolates of IBDVs are closely related to IBDV genogroup 3 strains (Jeon *et al.*, 2008; Kasanga *et al.*, 2008; Naggar *et al.*, 2020) or IBDV genogroup 1 strains (Naggar *et al.*, 2020), suggesting epidemiological links between domestic chicken and wild birds. Unique aminoacidic residues found in the VP2 hypervariable region (HVR) of some isolates also indicates that IBDV can undergo mutational changes in the wild hosts (Kasanga *et al.*, 2008, Naggar *et al.*, 2020). Furthermore, wild birds might harbor strains that could eventually reassort with poultry ones leading to the emergence of novel IBDV outbreaks in the future.

7.4.2. Limitations

Among the constrains identified regarding the systematic review presented, we acknowledge the scarcity of publications on IBDV in free-living wild birds in comparison with the abundance of studies available for poultry. We also imply the existence of possible research which may not be accessible through the search strategy here adopted. With respect to the statistical analysis, the scarcity of relevant articles about IBDV virological prevalence prevented us from pooling the data and performing a meta-analysis. Furthermore, we did not apply any test neither to assess nor to quantify the publication bias because of the absence of specific tools applicable to our research question (Fanelli *et al.*, 2021).

7.5. Conclusions

Considering the continuous evolution of IBDV strains and their spatial distribution over time, we feel that the role of free-living wild birds has implications that remain unexplored. Our systematic review and meta-analysis provide an up-to-date synthesis of the available literature related to IBDV sero-viroprevalence in wild hosts. Whereas it is not possible to currently identify any wild bird species as a reservoir of IBDV, it is still important to assess the role that free-living

birds could have in the epidemiology of this virus considering their movements and aggregation patterns. An effective screening strategy should combine, whenever possible, serological and virological methods of diagnosis to increase the significance of the outcomes. Increasing attention has been placed on synanthropic wildlife as bridge hosts, potentially vehiculating viruses from natural maintenance hosts to poultry, between different poultry farms (Shriner *et al.*, 2016) or from poultry to other wild birds. To better interpret the epidemiological links existing among different IBDV host species, it is crucial to perform a genetic characterization of the isolates. We also consider of main importance collecting exhaustive information on the individuals tested, such as species, sex and age classes according to international standards. Whenever positive results are presented, a thorough examination of the host ecology may help for further epidemiological considerations. Due to man-driven environmental changes, pathogens' ecology at the wildlife-domestic interface is evolving fast. Further research is necessary to better understand the role that wild birds might play in the eco-epidemiology of this virus and in driving changes in the way IBDV impacts poultry production.

7.6. References

- Adamu, H. U., Balami, A. G., & Abdu, P. A. (2017). Avian influenza, Gumboro and Newcastle disease antibodies and antigens in apparently healthy wild birds in Kano Metropolis, Nigeria. *Niger Vet J*, 38, 69-77.
- Aliyu, H. B., Hair-Bejo, M., Omar, A. R., & Ideris, A. (2021). Genetic diversity of recent Infectious Bursal Disease Viruses isolated from vaccinated poultry flocks in Malaysia. *Front Vet Sci*, 8, 643976. doi:10.3389/fvets.2021.64396
- Alkie, T. N., & Rautenschlein, S. (2016). Infectious bursal disease virus in poultry: current status and future prospects. *Vet Med (Auckland, New Zealand)*, 7, 9-18. doi:10.2147/VMRR.S68905
- Aricibasi, M., Jung, A., Heller, E. D., & Rautenschlein, S. (2010). Differences in genetic background influence the induction of innate and acquired immune responses in chickens depending on the virulence of the infecting infectious bursal disease virus (IBDV) strain. *Vet Immunol Immunopathol*, 35, 79-92. doi:10.1016/j.vetimm.2009.11.005
- Assam, A., Abdu, P. A., Ademola, A. O., Augustine, E., & Lawal, S. (2014). Avian influenza, Newcastle and Gumboro disease antibodies and antigens in apparently healthy wild birds in Kaduna state, Nigeria. *Bull anim health prod Afr*, 62.
- Benton, W. J., Cover, M. S., & Rosenberger, J. K. (1967). Studies on the transmission of the infectious bursal agent (IBA) of chickens. *Avian Dis*, 11, 430-438.
- Berg, T. P. (2000) Acute infectious bursal disease in poultry: a review. *Avian Pathol*, 29, 175-194. doi:10.1080/03079450050045431
- Burns, T. E., Ribble, C., Stephen, C., Kelton, D., Toews, L., Osterhold, J., & Wheeler, H. (2012). Use of observed wild bird activity on poultry farms and a literature review to target species as high priority for avian influenza testing in 2 regions of Canada. *Can Vet J*, 53, 158-166.
- Campbell, G. (2001). Investigation into evidence of exposure to infectious bursal disease virus and infectious anaemia virus in wild birds in Ireland. In E. F. Kaleta, H. R. Ursula, & H. In Lange-Herbs (Eds.), *Proceedings of the II International Symposium on Infectious Bursal Disease and Chicken Infectious Anaemia* (pp. 230-235). Gießen, Germany: Justus-Liebig University.
- Candelora, K. L., Spalding, M. G., Nesbitt, S. A., Sellers, H. S., Olson, J., Perrin, L., & Parker, J. (2008). Infectious bursal disease in wild populations of turkeys and sandhill cranes: preliminary findings. In M. J. Folk, & S. A. Nesbitt (Eds.) *Proceedings of the Tenth North American Crane Workshop* (pp. 171-172). Zacatecas, Mexico: North American Crane Working Group.
- Candelora, K. L., Spalding, M. G., & Sellers, H. S. (2010). Survey for antibodies to infectious bursal disease virus serotype 2 in wild turkeys and Sandhill Cranes of Florida, USA. *J Wildl Dis*, 46, 742-52. doi:10.7589/0090-3558-46.3.742
- Crespo, R., Badcoe, L., Williams, C., & Bary, A. (2016). Inactivation of Infectious Bursal Disease Virus Through Composting of Litter from Poultry Houses. *Avian Dis*, 60, 506-510. doi:10.1637/11341-120615-ResNote

- Curland, N., Gethöffer, F., van Neer, A., Ziegler, L., Heffels-Redmann, U., Lierz, M., Baumgärtner, W., Wohlsein, P., Völker, I., Lapp, S., Bello, A., Pfankuche, V. M., Braune, S., Runge, M., Moss, A., Rautenschlein, S., Jung, A., Teske, L., Strube, C., Schulz, J., Bodewes, R., Osterhaus, A. D. M. E., & Siebert, U. (2018). Investigation into diseases in free-ranging ring-necked pheasants (*Phasianus colchicus*) in northwestern Germany during population decline with special reference to infectious pathogens. *Eur J Wildl Res*, 64. doi:10.1007/s10344-018-1173-2
- De Wit, J. J., Heijmans, J. F., Mekkes, D. R., & Van Loon, A. A. (2001). Validation of five commercially available ELISAs for the detection of antibodies against infectious bursal disease virus (serotype 1). *Avian Pathol*, 30, 543–549. doi:10.1080/03079450120078743
- Deem, S. L., Noss, A. J., Cuéllar, R. L., & Karesh, W. B. (2005). Health evaluation of free-ranging and captive blue-fronted Amazon parrots (*Amazona aestiva*) in the Gran chaco, Bolivia. *J Zoo Wildl Med*, 36, 598–605.
- Dey, S., Pathak, D. C., Ramamurthy, N., Maity, H. K., & Chellappa, M. M. (2019). Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. *Vet Med (Auckland, New Zealand)*, 10, 85–97. doi:10.2147/VMRR.S185159
- Dwight, I. A., Coates, P. S., Stoute, S. T., Senties-Cue, C. G., Gharpure, R. V., & Pitesky, M. E. (2018). Serologic Surveillance of Wild and Pen-reared Ring-necked Pheasants (*Phasianus colchicus*) as a method of understanding disease reservoirs. *J Wildl Dis*, 54, 414–418. doi:10.7589/2017-08-191
- Edgar, S. A., & Cho, Y. (1965). Avian nephrosis (Gumboro disease) and its control by immunization. *Poult Sci*, 44, 1366.
- Ezeifeke, G. O., Dowoh, S. K., & Umoh, J. U. (1992). Involvement of wild and domestic birds in the epidemiology of Newcastle disease and Infectious bursal disease in Zaria, Nigeria. *Bull anim health prod Afr*, 40(2), 125-127.
- Fagbohun, O. A., Owoade, A. A., Oluwayelu, D. O., & Olayemi, F. O. (2000). Serological survey of infectious bursal diseases virus antibodies in cattle egrets, pigeons and Nigerian laughing doves. *Afr J Biomed Res*, 3.
- Fanelli, A., Battisti, E., Zanet, S., Trisciuglio, A., & Ferroglio, E. (2021). A systematic review and meta-analysis of *Toxoplasma gondii* in roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) in Europe. *Zoonoses Public Health*, 68, 182–193. doi:10.1111/zph.12780
- Felice, V., Franzo, G., Catelli, E., Di Francesco, A., Bonci, M., Cecchinato, M., Mescolini, G., Giovanardi, D., Pesente, P., & Lupini, C. (2017). Genome sequence analysis of a distinctive Italian infectious bursal disease virus. *Poult Sci*, 96, 4370-4377. doi:10.3382/ps/pex278.
- Freeman, M., & Tukey, J. (1950). Transformations related to the angular and the square root. *Ann Math Stat*, 21, 607-611. doi:10.1214/aoms/1177729756
- Gardner, H., Kerry, K., Riddle, M., Brouwer, S., & Gleeson, L. (1997). Poultry virus infection in Antarctic penguins. *Nature*, 387, 245. doi:10.1038/387245a0
- Gauthier-Clerc, M., Eterradossi, N., Toquin, D., Guittet, M., Kuntz, G., & Le Maho, Y. (2002). Serological survey of the king penguin, *Aptenodytes patagonicus*, in Crozet

- Archipelago for antibodies to infectious bursal disease, influenza A and Newcastle disease viruses. *Polar Biol*, 25, 316–319. doi:10.1007/s00300-001-0346-7
- Gilbert, A. T., Fooks, A. R., Hayman, D. T., Horton, D. L., Müller, T., Plowright, R., Peel, A. J., Bowen, R., Wood, J. L., Mills, J., Cunningham, A. A., & Rupprecht, C. E. (2013). Deciphering serology to understand the ecology of infectious diseases in wildlife. *Ecohealth*, 10(3), 298-313. doi:10.1007/s10393-013-0856-0
- Gilchrist, P. (2005). Involvement of free-flying wild birds in the spread of the viruses of avian influenza, Newcastle disease and infectious bursal disease from poultry products to commercial poultry. *Worlds Poult Sci J*, 61(2), 198-214. <https://doi.org/10.1079/WPS200451>
- Gough, R. E., Drury, S. E., Cox, W. J., Johnson, C. T., & Courtenay, A. E. (1998). Isolation and identification of birnaviruses from ostriches (*Struthio camelus*). *Vet Rec*, 142(5), 115-116. doi:10.1136/vr.142.5.115
- Gough, R. E., Drury, S. E., Welchman, D. de B., Chitty, J. R., & Summerhays, G. E. (2002). Isolation of birnavirus and reovirus-like agents from penguins in the United Kingdom. *Vet Rec*, 151(14), 422-424. doi:10.1136/vr.151.14.422
- Grimaldi, W., Ainley, D. G., & Massaro, M. (2018). Multi-year serological evaluation of three viral agents in the Adélie Penguin (*Pygoscelis adeliae*) on Ross Island, Antarctica. *Polar Biol*, 41. doi:10.1007/s00300-018-2342-1
- Gu, F., Li, S., Ye, X., & Liu, R. (1998) Serological investigation and artificial infection of guinea-fowls and pheasants with infectious bursal disease. *J Vet Sci Technol*, 28(1), 22-23.
- He, C. Q., Ma, L. Y., Wang, D., Li, G. R., & Ding, N. Z. (2009). Homologous recombination is apparent in infectious bursal disease virus. *Virology*, 384(1), 51-58. doi:10.1016/j.virol.2008.11.009
- Higgins, J. P. T., & Thompson, S. G. (2002). Quantifying heterogeneity in a meta-analysis. *Stat Med*, 21, 1539-1558. doi:10.1002/sim.1186
- Higgins, J. P. T., Thomas, J., Chandler, J., Cumpston, M., Li, T., Page, M. J., & Welch, V. A. (2021). *Cochrane handbook for systematic reviews of interventions version 6.2* (updated February 2021). The Cochrane Collaboration, 2011.
- Höfle, U., Blanco, J. M., & Kaleta, E. F. (2001). Neutralizing antibodies against infectious bursal disease virus in sera of free-living and captive birds of prey from central Spain (preliminary results). In E. F. Kaleta, H. R. Ursula, & H. Lange-Herbs (Eds.), *Proceedings of the II International Symposium on Infectious Bursal Disease and Chicken Infectious Anaemia* (pp. 247–251). Gießen, Germany: Justus-Liebig University.
- Hollmén, T., Franson, J. C., Docherty, D. E., Kilpi, M., Hario, M., Creekmore, L. H., & Petersen, M. R. (2000). Infectious Bursal Disease Virus antibodies in Eider Ducks and Herring Gulls. *Condor*, 102, 688–691. doi:10.1093/condor/102.3.688
- Hon, C. C., Lam, T. Y., Drummond, A., Rambaut, A., Lee, Y. F., Yip, C. W., Zeng, F., Lam, P. Y., Ng, P. T., & Leung, F. C. (2006). Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. *Virol J*, 80, 8503-8509. doi:10.1128/JVI.00585-06

- Howie, R. I., & Thorsen, J. (1981). Identification of a strain of infectious bursal disease virus isolated from mosquitoes. *Can j comp med*, 45, 315-320.
- Hunter, J. P., Saratzis, A., Sutton, A. J., Boucher, R. H., Sayers, R. D., & Bown, M. J. (2014). In meta-analyses of proportion studies, funnel plots were found to be an inaccurate method of assessing publication bias. *J Clin Epidemiol*, 67, 897–903. doi:10.1016/j.jclinepi.2014.03.003
- Iman, M. E., Faki, A. E., AlHassan, A. M., Selma, O. A., Egbal, S. A., Rahim, J. I., & Esmat, E. (2013). Microbiological and serological studies of some poultry pathogens in wild birds in Sudan. *Bull anim health prod Afr*, 61.
- Islam, M. R., Nooruzzaman, M., Rahman, T., Mumu, T. T., Rahman, M. M., Chowdhury, E. H., Eterradosi, N., & Müller, H. A. (2021). Unified genotypic classification of infectious bursal disease virus based on both genome segments. *Avian Pathol*, 50, 190-206. doi:10.1080/03079457.2021.1873245
- Jackwood, D. J. (2012). Molecular epidemiologic evidence of homologous recombination in infectious bursal disease viruses. *Avian Dis*, 56(3), 574-577. doi:10.1637/10053-010912-ResNote.1
- Jackwood, D. J., Stoute, S. T., & Crossley, B. M. (2016). Pathogenicity of genome reassortant Infectious Bursal Disease Viruses in chickens and turkeys. *Avian Dis*, 60, 765-772. doi:10.1637/11409-031116-Reg
- Jeon, W. J., Lee, E. K., Joh, S. J., Kwon, J. H., Yang, C. B., Yoon, Y. S., & Choi, K. S. (2008). Very virulent infectious bursal disease virus isolated from wild birds in Korea: epidemiological implications. *Virus Res*, 137(1), 153-156. doi:10.1016/j.virusres.2008.06.013
- Jourdain, E., Gauthier-Clerc, M., Bicout, D. J., & Sabatier, P. (2007). Bird migration routes and risk for pathogen dispersion into western Mediterranean wetlands. *Emerg Infect Dis*, 13, 365–372. doi:10.3201/eid1303.060301
- Karesh, W. B., Uhart, M. M., Frere, E., Gandini, P., Braselton, W. E., Puche, H., & Cook, R. A. (1999). Health evaluation of free-ranging rockhopper penguins (*Eudyptes chrysocomes*) in Argentina. *J Zoo Wildl Med*, 30(1), 25-31.
- Kasanga, C. J., Yamaguchi, T., Wambura, P. N., Munang'andu, H. M., Ohya, K., & Fukushi, H. (2008). Detection of infectious bursal disease virus (IBDV) genome in free-living pigeon and guinea fowl in Africa suggests involvement of wild birds in the epidemiology of IBDV. *Virus genes*, 36, 521–529. doi:10.1007/s11262-008-0219-z
- Lefkowitz, E. J., Dempsey, D. M., Hendrickson, R. C., Orton, R. J., Siddell, S. G., & Smith, D. B. (2018). Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). *Nucleic Acids Res*, 46. doi:10.1093/nar/gkx932
- Leite, A. T. M., Fischer, G., Hübner, S. de O., Lima, M. de., & Vargas, G. D. (2020). Investigation of impact viruses in poultry health of wild birds near a free range poultry farm in Pelotas, RS. *Sci Anim Health*, 8, 73-87. doi:10.15210/sah.v8i1.17601
- Lepage, D., & Warnier, J. (2014). The Peters' Checklist of the Birds of the World (1931-1987) Database. Retrieved from <https://avibase.bsc-eoc.org/peterschecklist.jsp>

- Lupini, C., Giovanardi, D., Pesente, P., Bonci, M., Felice, V., Rossi, G., Morandini, E., Cecchinato, M., & Catelli, E. (2016). A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. *Avian Pathol*, 45, 458–464. doi:10.1080/03079457.2016.1165792
- Lupini, C., Felice, V., Silveira, F., Mescolini, G., Berto, G., Listorti, V., Cecchinato, M., & Catelli, E. (2020). Comparative in vivo pathogenicity study of an ITA genotype isolate (G6) of infectious bursal disease virus. *Transbound Emerg Dis*, 67, 1025–1031. doi:10.1111/tbed.13421
- Mahgoub, H. A., Bailey, M., & Kaiser, P. (2012). An overview of infectious bursal disease. *Arch Virol*, 157, 2047–57. doi:10.1007/s00705-012-1377-9
- McAllister, J. C., Steelman, C. D., Newberry, L. A., & Skeeles, J. K. (1995). Isolation of infectious bursal disease virus from the lesser mealworm, *Alphitobius diaperinus*. *Poult Sci*, 74(1), 45–49. doi:10.3382/ps.0740045
- McFerran, J. B., McNulty, M. S., McKillop, E. R., Connor, T. J., McCracken, R. M., Collins, D. S. & Allan, G. M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. *Avian Pathol*, 9, 395–404. doi:10.1080/03079458008418423
- McNulty, M. S., Allan, G. M., & McFerran, J. B. (1979). Isolation of infectious bursal disease virus from turkeys. *Avian Pathol*, 8, 205–212. doi:10.1080/03079457908418346
- Michel, L. O., & Jackwood, D. J. (2017). Classification of infectious bursal disease virus into genogroups. *Arch Virol*, 162, 3661–3670. <https://doi.org/10.1007/s00705-017-3500-4>
- Miller, G. D., & Shellam, G. R. (2007, September). Disease status of penguins on Macquarie Island. *Poster presented at the 6th international penguin conference, Hobart*.
- Miller, G. D., Watts, J., & Shellam, G. (2008). Viral antibodies in south polar skuas around Davis Station, Antarctica. *Antarct Sci*, 20, 455–461. <https://doi.org/10.1017/S0954102008001259>
- Miller, G. D., & Shellam, G. R. (2010, August–September). Seasonal prevalence of viral antibodies in emperor penguins at Auster Colony, Antarctica. *Poster presented at the 7th international penguin conference, Boston*.
- Miller, J. J. (1978). The Inverse of the Freeman – Tukey Double Arcsine Transformation. *Am Stat*, 32, 138. doi:10.1080/00031305.1978.10479283
- Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., & Stewart, L.A. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Sys Rev*, 4. doi:10.1186/2046-4053-4-1
- Naggar, R. F. E., Rohaim, M. A., & Munir, M. (2020). Potential reverse spillover of infectious bursal disease virus at the interface of commercial poultry and wild birds. *Virus Genes*, 56, 705–711. doi:10.1007/s11262-020-01793-x
- Nawathe, D. R., Onunkwo, O., & Smith, I. M. (1978). Serological evidence of infection with the virus of infectious bursal disease in wild and domestic birds in Nigeria. *Vet Rec*, 102, 444. doi:10.1136/vr.102.20.444

- Nunes, C. F., Fonseca, F., Leite, A. T. M., da Silva Filho, R. P., Fonseca Finger, P., Castro, C. C., Fischer, G., D'ávila Vargas, G., & de Oliveira, H. S. (2012). Investigation on Newcastle Disease Virus (NDV), Infectious Bursal Disease Virus (IBDV) and Avian Poxvirus (APV) in magellanic penguins in Southern region of Brazil. *Braz Arch Biol Technol*, 55. doi:10.1590/S1516-89132012000400008
- Oña, A., Martinez, J., Berg, T. P., Casal, I., Negro, J. J., & Rodriguez, J. F. (2000). Epidemiological survey of infectious bursal disease virus in wild birds. In *Proceedings of the 4th Meeting of the European Wildlife Disease Association*, (p 39). Zaragoza, Spain: European Wildlife Disease Association.
- Ogawa, M., Wakuda, T., Yamaguchi, T., Murata, K., Setiyono, A., Fukushi, H., & Hirai, K. (1998). Seroprevalence of infectious bursal disease virus in free-living wild birds in Japan. *J Vet Med Sci*, 60, 1277-1279. doi:10.1292/jvms.60.1277
- Orakpoghenor, O., Oladele, S., & Abdu, P. (2020). Detection of infectious bursal disease virus antibodies in free-living wild birds in Zaria, Nigeria. *Poult Sci*, 99, 1975-1977.
- Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hróbjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., McGuinness, L. A., Stewart, L. A., Thomas, J., Tricco, A. C., Welch, V. A., Whiting, P., & Moher, D. (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *Br Med J*, 372. doi:10.1136/bmj.n71
- Pagès-Manté, A., Torrents, D., Maldonado, J., & Saubi, N. (2004). Dogs as potential carriers of infectious bursal disease virus. *Avian Pathol*, 33, 205-209. doi:10.1080/0307945042000195821
- Patankar, S., Jambhekar, R., Suryawanshi, K. R., & Nagendra, H. (2021). Which Traits Influence Bird Survival in the City? A Review. *Land*, 10(2), 92. <https://doi.org/10.3390/land10020092>
- Park, M. J., Park, J. H., & Kwon, H. M. (2010). Mice as potential carriers of infectious bursal disease virus in chickens. *Vet J*, 183, 352-354. doi:10.1016/j.tvjl.2008.12.005
- Parsons, N. J., Gous, T. A., Schaefer, A. M., & Vanstreels, R. E. (2016). Health evaluation of African penguins (*Spheniscus demersus*) in southern Africa. *Onderstepoort J Vet Res*, 83. doi:10.4102/ojvr.v83i1.1147
- Pawar, S., Pande, S., Jamgaonkar, A., Koratkar, S., Pal, B., Raut, S., Nanaware, S., Ray, K., Chackrabarti, A., Kode, S., Thite, V., Khude, M., Randive, S., Basu, A., Pawashe, A., Ponkshe, Pandit, P., & Deshpande, P. (2009). Avian influenza surveillance in wild migratory, resident, domestic birds and in poultry in Maharashtra and Manipur, India, during avian migratory season 2006–07. *Curr Sci*, 97, 550-554.
- Phalen, D. N. (2002). Virus neutralization assays used in exotic bird medicine. *Semin avian exot pet med*, 11(1), 19-24.
- R Core Team. (2018). A Language and Environment for Statistical Computing. R Foundation for Statistical Computing (version 3.5.2). Retrieved from <https://www.r-project.org/>

- Rautenschlein, S., von Samson-Himmelstjerna, G., & Haase, C. (2007). A comparison of immune responses to infection with virulent (IBDV) between specific-pathogen-free chickens infected at 12 and 28 days of age. *Vet Immunol Immunopathol*, 115, 251–260. doi:10.1016/j.vetimm.2006.11.002
- Sharma, J. M., Kim, I. J., Rautenschlein, S., & Yeh, H. Y. (2000). Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. *Dev Comp Immunol*, 24, 223–235. doi:10.1016/s0145-305x(99)00074-9
- Shriner, S., Root, J., Lutman, M., Kloft, J. M., VanDalen, K. K., Sullivan, H. J., White, T. S., Milleson, M. P., Hairston, J. L., Chandler, S. C., Wolf, P. C., Turnage, T. C., McCluskey, B. J., Vincent, A. L., Torchetti, M. K., Gidlewski, T., & DeLiberto, T. (2016). Surveillance for highly pathogenic H5 avian influenza virus in synanthropic wildlife associated with poultry farms during an acute outbreak. *Sci Rep*, 6. doi:10.1038/srep36237
- Silveira, F., Felice, V., Franzo, G., Mescolini, G., Catelli, E., Cecchinato, M., Berto, G., Listorti, V., & Lupini, C. (2019). Inoculation of specific pathogen-free chickens with an of the ITA genotype (G6) leads to a high and persistent viral load in lymphoid tissues and to a delayed antiviral response. *Vet Microbiol*, 235, 136–142. <https://doi.org/10.1016/j.vetmic.2019.06.014>
- Smith, K. M., Karesh, W. B., Majluf, P., Paredes, R., Zavalaga, C., Reul, A. H., Stetter, M., Braselton, W. E., Puche, H., & Cook, R. A. (2008). Health evaluation of free-ranging Humboldt penguins (*Spheniscus humboldti*) in Peru. *Avian Dis*, 52(1), 130–135. doi:10.1637/8265-071007-Reg
- Spalding, M. G., Sellers, H. S., Hartup, B. K., & Olsen, G. H. (2008). A wasting syndrome in released Whooping Cranes in Florida associated with infectious bursal disease titers. In: M. J. Folk, & S. A. Nesbitt (Eds.), *Proceedings of the Tenth North American Crane Workshop* (p. 202). Zacatecas, Mexico: North American Crane Working Group.
- Tamiranta, N., Ek-Kommonen, C., Rossow, L., & Huovilainen, A. (2018). Circulation of very virulent avian infectious bursal disease virus in Finland. *Avian Pathol*, 47, 520–525. doi:10.1080/03079457.2018.1503642
- Thai, T. N., Jang, I., Kim, H. A., Kim, H. S., Kwon, Y. K., & Kim, H. R. (2021). Characterization of antigenic variant infectious bursal disease virus strains identified in South Korea. *Avian Pathol*, 50, 174–181. doi:10.1080/03079457.2020.1869698
- Travis, E. K., Vargas, F. H., Merkel, J., Gottdenker, N., Miller, R. E., & Parker, P. G. (2006a). Hematology, plasma chemistry, and serology of the flightless cormorant (*Phalacrocorax harrisi*) in the Galapagos Islands, Ecuador. *J Wildl Dis*, 42, 133–141. doi:10.7589/0090-3558-42.1.133
- Travis, E. K., Vargas, F. H., Merkel, J., Gottdenker, N., Miller, R. E., & Parker, P. G. (2006b). Hematology, serum chemistry, and serology of Galápagos penguins (*Spheniscus mendiculus*) in the Galápagos Islands, Ecuador. *J Wildl Dis*, 42, 625–632. doi:10.7589/0090-3558-42.3.625
- Trifinopoulos, J., Nguyen, L. T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res*, 44. doi:10.1093/nar/gkw256

- Uhart, M., Thijl Vanstreels, R. E., Gallo, L., Cook, R. A., & Karesh, W. B. (2020). Serological survey for select infectious agents in wild magellanic penguins (*Spheniscus magellanicus*) in Argentina, 1994-2008. *J Wildl Dis*, 56, 66-81. doi:10.7589/2019-01-022
- Vargas-Castillo, L., Soler-Tovar, D., Gómez, A. P., & Santander, A. F., Benavides, E., & Villamil Jiménez, L. C. (2019). Evaluation of viral agents in wild birds of the Middle-East of Colombia. *Ornitol Colomb*, 17.
- Viechtbauer, W., & Cheung M. W. L. (2010). Outlier and Influence Diagnostics for Meta-Analysis. *Res Synth Methods*, 1, 112–25. doi:10.1002/jrsm.11
- Wang, Y., Zhou, Z., Chunsheng, Z., Luo, H., Ding, C., & Fang, Y. (1997). Studies on the ecology of infectious bursal disease virus: Serological surveys of non-chicken avian species naturally infected with IBDV. *J Vet Sci Technol*, 27, 15-16.
- Wang, N. (2018). How to conduct a meta-analysis of proportions in R: A Comprehensive Tutorial. doi:10.13140/RG.2.2.27199.00161
- Watts, J. M., Miller, G. D., & Shellam, G. R. (2009). Infectious Bursal Disease Virus and Antarctic Birds. In K. R. Kerry, & M. Riddle (Eds), *Health of Antarctic Wildlife* (pp. 95-105). Berlin, Heidelberg: Springer. doi:10.1007/978-3-540-93923-8_5
- Wilcox, G. E., Flower, R. L., Baxendale, W., & Mackenzie, J. S. (1983). Serological survey of wild birds in Australia for the prevalence of antibodies to egg drop syndrome 1976 (EDS-76) and infectious bursal disease viruses. *Avian Pathol*, 12, 135-139. doi:10.1080/03079458308436155
- Wille, M., & Holmes, E. C. (2020). Wild birds as reservoirs for diverse and abundant gamma- and deltacoronaviruses. *FEMS Microbiol Reviews*, 44, 631-644. doi:10.1093/femsre/fuaa026
- Yamaguchi, T., Ogawa, M., Miyoshi, M., Inoshima, Y., Fukushi, H., & Hirai, K. (1997). Sequence and phylogenetic analyses of highly virulent infectious bursal disease virus. *Arch Virol*, 142, 1441-1458. doi:10.1007/s007050050171
- Zhou, Z., Wang, Y., Deng, X., Diao, Z., Gao, J., Shi, Z., Hanlu, S., & Fang, Y. (1998). Survey on the Ecology and Epidemiology of Infectious Bursal Disease Virus (IBDV). *Chin J Vet Sci*, 18, 430-433.

CONCLUSIONS

Direct or indirect contacts at the poultry-wildlife interface mediate interspecies pathogen transmission and can significantly impact animal and public health, depending on the disease considered. By employing a multidisciplinary approach, combining fieldwork and molecular biology techniques, along with sequence and phylogenetic analyses, as well as statistical analysis and modeling, the present thesis sheds light on the epidemiology of selected economically impactful poultry pathogens, namely AIV, aMPV, and IBDV, within the context of their circulation in wild birds.

As a first step towards characterizing mechanisms facilitating virus spillover or spillback at the domestic bird-wildlife interface, the main species frequenting the surroundings of poultry houses were identified through a yearlong camera-trap survey in three commercial laying hen farms located in Northern Italy, within a DPPA at high risk for AI introduction. In the studies reported in **Chapter 1 and 2**, animal species more likely to be found nearby poultry houses have been identified, such as Eurasian magpies, ring-necked pheasants, Eurasian collared doves, coypus, and domestic cats. However, identifying novel bridge hosts in the context of AIV epidemiology depends also on assessing their susceptibility to AI. An increasing number of bird and mammal species are being found infected with HPAIV of clade 2.3.4.4b, and the main evidence reported in literature have been reviewed in **Chapter 3**. Based on current knowledge, magpies and pheasants are susceptible to the infection. Their frequent detection in poultry farms suggests they may play a bridging role between AIV reservoirs and poultry, as well as between AIV-infected farms. With respect to coypus and doves, although frequently observed nearby poultry houses, further studies are needed to assess their susceptibility to currently circulating AIVs. Overall, the data hereby provided regarding the characteristics of the poultry-wildlife interface lay the groundwork for designing novel surveillance and intervention strategies to prevent cross-species disease transmission.

In **Chapter 4**, two sampling strategies were combined for monitoring the AIV circulation in wild hosts in a DPPA of the Emilia-Romagna region. This involved collecting avian fecal droppings in selected wetlands and cloacal swabs from hunted waterbirds. The identification of 11 LPAIVs, representing at least six different viral subtypes, underscores how integrating these two sampling methods facilitated the detection of diverse viruses concurrently circulating within the same study area. Furthermore, the successful detection of AIVs in fecal dropping samples, coupled with the identification of the bird species of origin through COI-barcoding, paves the way for employing this sampling strategy to enhance wild animal surveillance in the vicinity of poultry farms within HPAI restriction zones, as useful tool for monitoring potential virus spillover at the domestic bird-wildlife interface. The phylogenetic analyses of the eight gene segments of five isolates (H1N1, H5N3 LPAI and three H9N2 viruses) revealed their close genetic relationship with AIVs previously isolated in Italy, Northern Europe, Northern Africa, and Asia. Furthermore, employing WGS techniques to characterize the isolated LPAI strains of H5N3 and H9N2 subtypes enabled the identification of amino acid substitutions potentially relevant for virus adaptation to mammals in the HA, PB2, and PA gene segments. This underscores the significant role of WGS in mapping the potential zoonotic risk associated with AIV.

Results of the systematic review of literature and meta-analysis of molecular and serological prevalence of aMPV in wild birds, reported in **Chapter 5**, suggest that certain taxa could play a role in the virus's epidemiology at the domestic-wild bird interface, such as wild ducks, geese, gulls, and pheasants. In particular, pheasants could potentially mediate the circulation of aMPV in-between farms, being territorial and resident birds often observed in the surroundings of poultry houses. On the other hand, migratory birds such as ducks, geese, or some gulls, could facilitate the long-distance spread of the virus. Considering that the Eurasian lineage of aMPV-C is well-adapted to domestic ducks, further investigations on wild anatids are encouraged and may disclose potential aMPV vector or even reservoir species. The discovery of an aMPV-C virus in an additional wild migratory duck, the Eurasian wigeon, as reported in **Chapter 6**, further supports this hypothesis. Following nucleotide sequencing of the F, G, and M genes, phylogenetic analyses revealed a close genetic relationship between the aMPV-C virus detected in the present study and a clade of viruses isolated in the 1990s in French domestic ducks, within the Eurasian lineage of the aMPV-C subtype. This finding could be related to migratory bridges between countries along the Black Sea/Mediterranean flyway. However, given the paucity of aMPV-C Eurasian-lineage strains found, the inferred phylogenetic relationship might be influenced by a sampling bias and might only partially represent the actual scenario. To fully understand the role of wild ducks in aMPV-C virus circulation, further evaluation of the biological features of aMPV strains circulating in wild species would be essential.

In **Chapter 7**, a systematic review and meta-analysis of the IBDV prevalence in wild birds was presented. Serological surveys indicated that wild penguins, sea ducks, pheasants, and gulls frequently tested positive for IBDV antibodies, while virus detection was achieved in ducks, geese, magpies, pigeons, and pheasants. Although conclusive evidence on specific species' roles remains elusive, the reported findings represent a foundational basis for future epidemiological studies related to IBDV in wild birds.

Overall, this thesis work has explored the complex dynamics of pathogen transmission at the poultry-wildlife interface. In a One Health paradigm, these results offer valuable insights for future evidence-based epidemiological studies, targeted viral surveillance, preemptive interventions, and strategies to control cross-species disease transmission.