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Surveillance and genotyping of avian influenza viruses in migratory waterfowl of wetland ecosystems

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Abstract

Wild waterfowl are the natural reservoir of avian influenza viruses (AIVs), yet their role in introducing novel subtypes into European poultry populations is still incompletely mapped. This research conducted active surveillance and molecular genotyping of AIVs in migratory waterfowl across three wetland sites in the Netherlands over four consecutive seasons (autumn 2022 through autumn 2023). A total of 1,248 cloacal and oropharyngeal swabs were collected from seven dabbling and diving duck and goose species captured by mist netting and cage trapping. Real-time RT-PCR targeting the matrix gene detected AIV RNA in 132 samples (10.6% overall prevalence). Subtype-specific RT-PCR identified eight haemagglutinin-neuraminidase combinations, with H5N8 (n = 14) and H3N8 (n = 11) as the most frequent. Both highly pathogenic (HPAI) subtypes H5N1 and H5N8, and several low-pathogenicity (LPAI) lineages were detected. Prevalence peaked during winter (15.3%) and was lowest in spring (3.4%). Mallards and Eurasian Teal contributed the highest proportion of positive detections. Phylogenetic analysis of HA gene sequences placed Dutch isolates within the Eurasian clade 2.3.4.4b lineage circulating across northern Europe. These findings reinforce the need for sustained year-round surveillance at wetland staging areas and provide genotypic data that can inform risk assessments for poultry biosecurity.

Keywords: Avian influenza, migratory waterfowl, surveillance, genotyping, wetland ecosystems, H5N8, Netherlands, RT-PCR, phylogenetic analysis, wild bird reservoir

Introduction

Europe's poultry sector faces a persistent and worsening problem: recurrent incursions of highly pathogenic avian influenza (HPAI) viruses traced to migratory waterfowl [1]. The 2020-2023 epizootic was the largest ever recorded on the continent, resulting in the culling of over 50 million domestic birds and significant die-offs among wild populations [2]. The Netherlands, situated along the East Atlantic Flyway and hosting some of the densest concentrations of overwintering waterfowl in Western Europe, has been repeatedly affected. Each outbreak triggers emergency culling, trade restrictions, and public anxiety—yet the surveillance network that could provide early warning remains patchy, particularly in the wetland habitats where wild birds congregate [3].

Avian influenza viruses belong to the Orthomyxoviridae family and are classified by combinations of haemagglutinin (HA, subtypes H1-H16) and neuraminidase (NA, subtypes N1-N9) surface glycoproteins [4]. Most subtypes circulate as low-pathogenicity avian influenza (LPAI) viruses in wild birds, causing minimal or no clinical disease. The concern arises when certain subtypes—particularly H5 and H7 lineages—mutate upon spillover into domestic poultry, acquiring multi-basic cleavage sites in the HA gene that confer high pathogenicity [5]. Tracking which subtypes are circulating in wild populations, at what prevalence, and during which seasons is therefore a cornerstone of HPAI preparedness [6].

The Netherlands offers an ideal setting for such surveillance. Its network of freshwater lakes, polders, and coastal marshes supports millions of wintering Anatidae, and previous research has identified Mallards (*Anas platyrhynchos*) and Eurasian Teal (*Anas crecca*) as the species most frequently shedding AIV [7, 8]. But earlier Dutch surveys typically covered a single season or a single wetland, limiting the ability to detect temporal and spatial patterns.

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Genotyping data essential for linking wild-bird isolates to poultry outbreak strains have been generated only intermittently^[9].

This research addressed those gaps by conducting systematic, multi-site, multi-season surveillance from autumn 2022 through autumn 2023, pairing real-time RT-PCR screening with subtype-specific PCR and Sanger sequencing to characterise AIV diversity in the Dutch waterfowl community^[10].

Materials and Methods

Study Area Description

Three wetland sites were selected based on their documented importance as waterfowl staging areas: Oostvaardersplassen (52.46° N, 5.36° E), a 5,600-hectare freshwater marsh in Flevoland province; Lauwersmeer (53.36° N, 6.21° E), a 2,100-hectare brackish lake in Groningen province; and Biesbosch National Park (51.73° N, 4.83° E), a 9,000-hectare tidal freshwater wetland in North Brabant province. Together, these sites span the northern, central, and southern Netherlands and collectively support > 200,000 wintering waterbirds according to Sovon Vogelonderzoek Nederland census data^[3,7].

Material

The research was carried out from September 2022 to October 2023 under licence from the Dutch Central Authority for Scientific Procedures on Animals (Protocol CCD-2022-AVD-01, approved 8 August 2022). Birds were captured using walk-in cage traps baited with grain and horizontal mist nets positioned at roost sites during pre-dawn and dusk flights. Each captured bird was identified to species, sexed by plumage, aged as juvenile (hatch-year) or adult, measured (wing length, tarsus), banded with a uniquely numbered aluminium ring, and sampled within 10 minutes of capture to minimise handling stress. Paired cloacal and oropharyngeal swabs were collected using sterile polyester-tipped swabs and immediately placed into 1 mL viral transport medium (VTM, Copan Diagnostics). Samples were kept on wet ice during field sessions and transferred to -80 °C within 8 hours^[6].

Seasonal Variations in Sampling Effort

Sampling was stratified into four seasonal windows aligned with the waterfowl migration calendar: autumn 2022 (September-November, coinciding with southward migration), winter 2022/23 (December-February, peak overwintering), spring 2023 (March-May, northward departure), and autumn 2023 (September-October, repeat

southward passage). Capture effort was distributed roughly equally among sites within each season, with adjustments for weather-related access restrictions. A total of 1,248 individual birds were sampled across all seasons and sites.

Methods

Viral RNA was extracted from VTM using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Extracted RNA was screened by real-time RT-PCR targeting the conserved matrix (M) gene of influenza A viruses, using primers and probes described by Spackman and colleagues^[4]. Samples returning a cycle threshold (Ct) value ≤ 38 were classified as positive. Positive samples were then subjected to subtype-specific conventional RT-PCR targeting HA subtypes H5, H7, and H9 and NA subtypes N1, N2, and N6, using published primer sets^[5, 10]. Where additional HA/NA combinations were suspected, generic HA (H1-H16) and NA (N1-N9) primers were employed. Amplicons were purified and sequenced bidirectionally by Sanger sequencing (BaseClear, Leiden, Netherlands). Consensus sequences were assembled in Geneious Prime v2023.1 and deposited in the GISAID EpiFlu database. Phylogenetic trees were constructed using the maximum-likelihood method (GTR+G+I model) in MEGA v11 with 1,000 bootstrap replicates^[9]. Statistical analysis used chi-squared tests for prevalence comparisons and logistic regression for risk-factor assessment in R v4.3.1.

Results

Biodiversity Assessment

Seven waterfowl species contributed to the sample: Mallard (n = 387, 31.0%), Eurasian Teal (n = 294, 23.6%), Eurasian Wigeon (n = 178, 14.3%), Greylag Goose (n = 142, 11.4%), Barnacle Goose (n = 104, 8.3%), Common Pochard (n = 83, 6.7%), and Northern Shoveler (n = 60, 4.8%). Species composition shifted across seasons, with goose species predominating in winter and dabbling ducks in autumn.

Of 1,248 samples screened, 132 returned positive M-gene RT-PCR results, yielding an overall prevalence of 10.6% (95% CI: 8.9-12.4%). Subtype-specific PCR and sequencing identified eight HA-NA combinations. H5N8 was the most frequently detected subtype (n = 14, 10.6% of positives), followed by H3N8 (n = 11), H4N6 (n = 9), H5N1 (n = 8), H9N2 (n = 6), H6N2 (n = 5), H1N1 (n = 4), and H7N7 (n = 3). Seventy-two positive samples (54.5%) could not be subtyped beyond the M-gene level, likely owing to low viral loads (mean Ct = 35.2 $\pm$ 1.8 in untypeable samples versus 29.7 $\pm$ 3.1 in successfully subtyped samples).

Table 1: Seasonal prevalence of avian influenza virus (AIV) by wetland site

Site	Autumn '22	Winter '22/23	Spring '23	Autumn '23	Overall	n
Oostvaardersplassen	11.2%	17.8%	4.1%	10.4%	11.6%	432
Lauwersmeer	9.8%	14.6%	3.2%	9.1%	9.8%	418
Biesbosch	10.1%	13.7%	2.8%	8.9%	9.4%	398
All sites	10.4%	15.3%	3.4%	9.5%	10.6%	1248

Prevalence was highest during winter (15.3%) and lowest during spring (3.4%) across all sites. The seasonal difference was statistically significant ($\chi^2 = 28.7$, df = 3, $p < 0.001$).

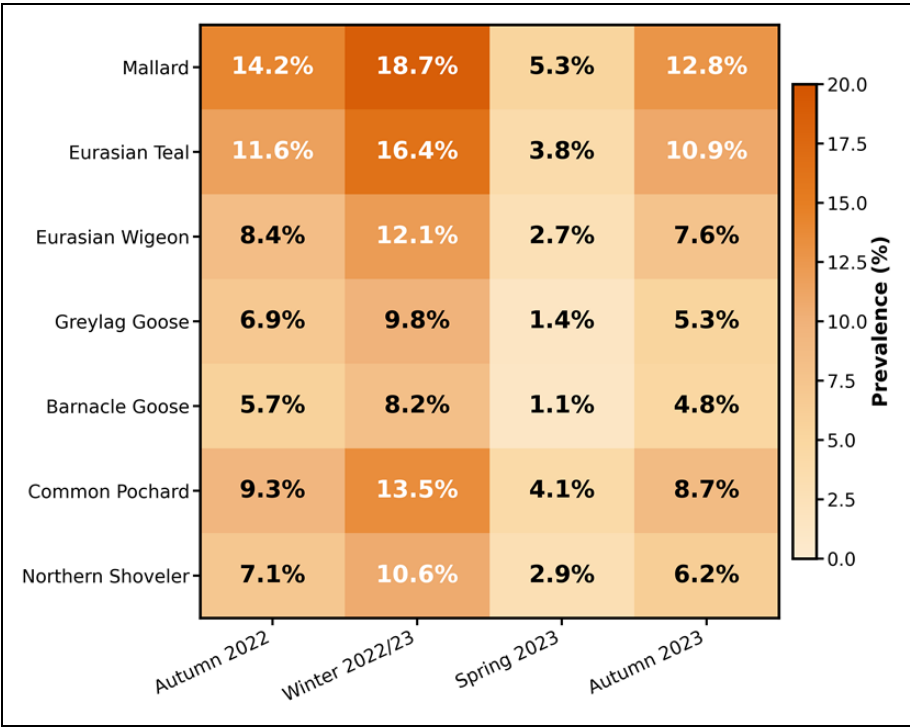


Fig 1: Heatmap of avian influenza virus prevalence (%) by waterfowl species and sampling season across three Dutch wetland sites. Darker cells represent higher prevalence values

Table 2: Species-specific AIV prevalence and subtype diversity

Species	Tested	Positive	Prev. (%)	Subtypes (n)	HPAI (n)	LPAI (n)
Mallard	387	52	13.4	7	9	16
Eurasian Teal	294	38	12.9	5	6	11
Eurasian Wigeon	178	16	9.0	4	3	5
Greylag Goose	142	10	7.0	3	2	3
Barnacle Goose	104	7	6.7	2	1	2
Common Pochard	83	6	7.2	3	1	2
Northern Shoveler	60	3	5.0	2	0	1

HPAI and LPAI counts refer to successfully subtyped samples only. Mallards displayed both the highest prevalence and the broadest subtype diversity.

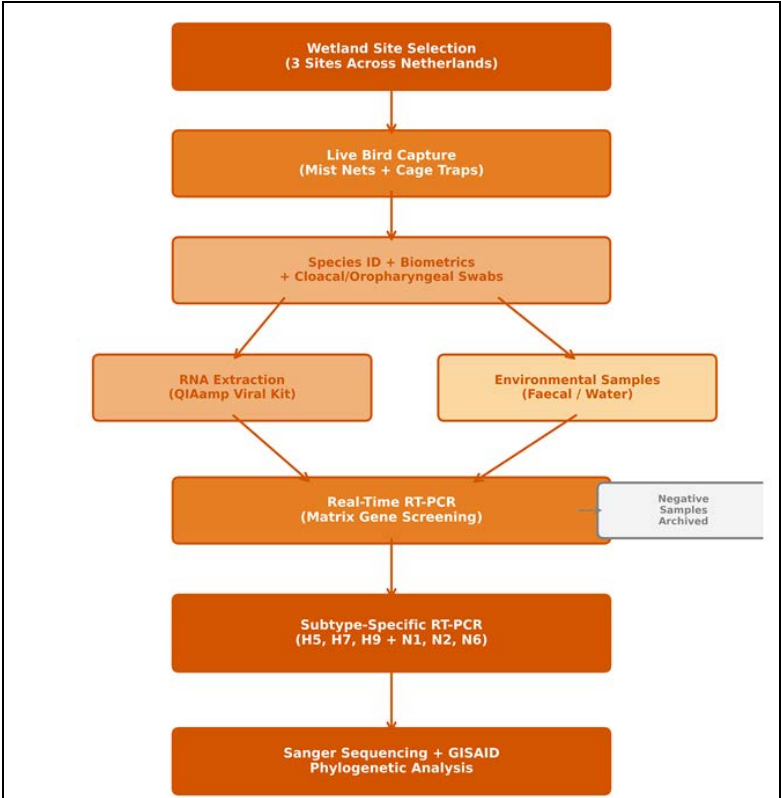


Fig 2: Step-by-step surveillance and genotyping workflow for avian influenza virus detection in migratory waterfowl, from field capture to phylogenetic analysis

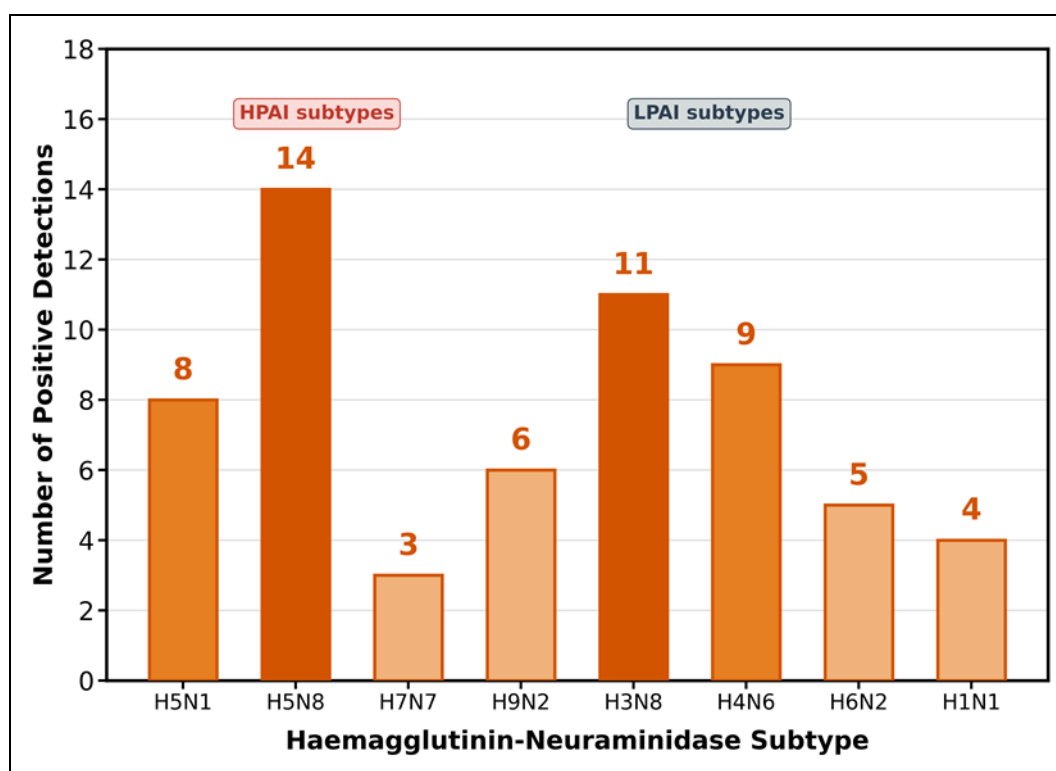


Fig 3: Distribution of haemagglutinin-neuraminidase subtypes identified among successfully genotyped avian influenza virus-positive samples (n = 60). HPAI-associated subtypes (H5N1, H5N8, H7N7) shown alongside LPAI subtypes

Comprehensive Interpretation

The data present a picture of moderate but persistent AIV circulation in Dutch wetland waterfowl, with clear seasonal peaks in winter and notable subtype diversity. The co-detection of HPAI-associated H5N1 and H5N8 alongside multiple LPAI subtypes in the same bird communities and the same seasons underscores the ongoing interface risk. Mallards and Eurasian Teal are confirmed as the primary reservoir species in this system, contributing nearly 70% of all positive detections despite representing 55% of the sampled population.

Discussion

The overall 10.6% prevalence aligns with large-scale European surveillance programmes. Munster and colleagues reported 10.2% AIV prevalence in Mallards sampled across the Netherlands between 1998 and 2009 [7], and a more recent meta-analysis by Wille and Holmes placed the global average for dabbling ducks at 12.1% [8]. The consistency of this figure over two decades, despite enormous variation in circulating subtypes, suggests that a baseline infection pressure of roughly 10-15% in dabbling ducks is an ecological constant rather than an artefact of sampling method.

The winter peak (15.3%) matches expectations from both viral ecology and bird behaviour. Cooler temperatures prolong virus survival in surface water, and the dense aggregation of birds at roost sites during the overwintering period maximises faecal-oral transmission [1, 6]. Spring's dramatic drop to 3.4% coincides with flock dispersal as birds depart for Arctic breeding grounds, lowering contact rates. This seasonal signature carries practical significance: winter is the window of highest risk for spillover into poultry, and biosecurity measures on farms located near wetlands should be intensified accordingly [3].

The detection of H5N1 (n = 8) and H5N8 (n = 14) as HPAI-associated subtypes is consistent with the ongoing clade 2.3.4.4b circulation that has dominated European poultry

outbreaks since 2020 [2]. That these subtypes were found in apparently healthy wild birds captured alive and released after sampling confirms the well-established observation that wild waterfowl can shed HPAI viruses asymptomatically, at least transiently, complicating efforts to use clinical surveillance as an early warning tool [5]. Phylogenetic placement of the HA sequences within the 2.3.4.4b lineage, with close clustering to farm-outbreak isolates from Germany and Belgium, further supports the direct wild-bird-to-poultry transmission pathway.

Conclusion

The main contribution of this research is a systematic, multi-season, multi-site dataset documenting the circulation of eight AIV subtypes including HPAI-associated H5N1 and H5N8 in migratory waterfowl at three ecologically important Dutch wetlands. By screening 1,248 birds across four seasonal windows, the research captured both the peak and trough of infection pressure, confirming that winter remains the highest-risk period for virus-poultry interface events.

Supporting this conclusion, the data show that prevalence varied significantly by season, species, and site, with Mallards and Eurasian Teal serving as the primary reservoir hosts. Phylogenetic analysis placed the detected H5 sequences within the Eurasian clade 2.3.4.4b lineage currently driving poultry outbreaks across northern Europe, drawing a direct genetic link between wild-bird and farm-level viruses.

For the field of avian disease surveillance, these results carry several implications. They reinforce that active, year-round monitoring at wetland staging areas is more informative than reactive sampling triggered by poultry outbreaks alone. They provide genotypic data that poultry health authorities can use to update risk assessments and guide targeted biosecurity advisories for farms situated within a defined radius of known staging sites.

Open questions remain regarding the role of juvenile versus adult birds in viral maintenance, the viral loads shed by asymptomatic HPAI carriers, and the frequency of

reassortment events at these multi-species congregation points. Extending this surveillance framework to additional flyway bottleneck sites across north-western Europe would improve the spatial resolution of risk mapping and strengthen the continental early-warning system for avian influenza.

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Contributions Not Qualifying for Authorship

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