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RESEARCH ARTICLE

Genomic Surveillance of Influenza Virus Evolution in Wild Bird Populations

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ABSTRACT

Wild waterfowl remain the principal reservoir from which highly pathogenic avian influenza (HPAI) viruses periodically spill into poultry and, less often, mammals, yet routine surveillance at wetland stopover sites still under-samples the genomic diversity circulating in these hosts between outbreak seasons. We carried out an eighteen-month active surveillance programme at four wetland complexes along the Scheldt-Meuse estuarine system in Belgium, collecting 2,847 fresh faecal samples from mixed waterfowl roosts and combining real-time RT-PCR screening with whole-genome nanopore sequencing of positive samples. Of 312 influenza A-positive samples, 268 yielded genomes of sufficient completeness for phylogenetic and reassortment analysis. HPAI H5N1 clade 2.3.4.4b dominated detections during the fourth quarter of each surveillance year (October-December), coinciding with the arrival of Arctic-breeding Anser and Anas species, while low-pathogenicity subtypes, principally H3N8, H4N6 and H11N9, circulated at a low background prevalence throughout the study. Whole-genome analysis identified eleven independent reassortment events involving exchange of internal gene segments between clade 2.3.4.4b and co-circulating low-pathogenicity viruses, four of which produced viruses carrying a PB2 segment with the mammalian-adaptation-associated marker M631L in a haemagglutinin and neuraminidase backbone otherwise typical of wild-bird lineages. Bayesian phylodynamic reconstruction placed the most recent common ancestor of the local H5N1 lineage substantially earlier than its first detection in regional poultry, implying a period of cryptic circulation in wild hosts. These findings argue for surveillance designs that sample wild bird reservoirs continuously across the annual cycle rather than reactively following poultry outbreaks, since the genomic events most relevant to spillover risk -- reassortment and the accrual of mammalian-adaptation markers -- were detectable in wild birds weeks to months before any associated event in domestic or captive animals.

Keywords: avian influenza; genomic surveillance; wild waterfowl; reassortment; phylodynamics; H5N1 clade 2.3.4.4b; Scheldt-Meuse estuary

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1. INTRODUCTION

Influenza A viruses maintain their full genetic and antigenic diversity in wild aquatic birds, principally

members of the orders Anseriformes and Charadriiformes, which harbour virtually all known haemagglutinin and neuraminidase subtype combinations and have done so for as long as the virus

has been studied [2,3]. Domestic poultry, swine and, occasionally, humans acquire infection episodically from this reservoir, and it is within the reservoir itself, rather than in any single spillover event, that the reassortment and mutation processes that eventually generate viruses of pandemic or panzootic concern actually take place [16,17]. The emergence and subsequent near-global spread of highly pathogenic avian influenza (HPAI) H5 clade 2.3.4.4b since 2020 has made this reservoir-centred view difficult to ignore: the clade has caused unprecedented mortality in wild seabird colonies, crossed into a wide range of mammalian species including marine mammals and dairy cattle, and shows no sign of resolving into the multi-year quiescent periods that characterised earlier H5 panzootics [5,12,13].

Much of what is known about clade 2.3.4.4b's genomic evolution comes from sequences generated during or shortly after poultry outbreaks, when regulatory reporting requirements make sampling comparatively easy [6,14]. This creates an observational bias: the wild bird genomes that make it into public databases are disproportionately drawn from birds found dead near affected farms, rather than from a representative cross-section of the wild reservoir sampled irrespective of outbreak status. Reassortment between HPAI and co-circulating low-pathogenicity avian influenza (LPAI) lineages, which supplies the internal gene segments in which mammalian-adaptation markers most often first appear, is thought to occur predominantly in wild birds at high-density mixed-species roosts, yet is rarely captured directly because such roosts are not the locations where outbreak-triggered sampling occurs [15,29,44].

The Scheldt and Meuse estuarine wetlands of Belgium and the adjoining Netherlands sit on the boundary of the East Atlantic Flyway and host some of the largest mixed overwintering waterfowl aggregations in western Europe, with tens of thousands of *Anser*, *Anas*, *Cygnus* and *Branta* individuals from breeding populations across Scandinavia, Svalbard and the Russian Arctic converging with locally resident mute swans, mallards and coots [30,31]. The region has recorded recurrent HPAI H5 detections in wild birds and poultry over the past decade, but, as elsewhere, surveillance has historically been reactive: carcasses are tested when found, and active sampling of clinically normal birds is intermittent and unevenly distributed across the year [4,45]. This leaves the reassortment dynamics and phylodynamic history of the local viral population poorly resolved precisely during the low-mortality intervals when reassortment is thought to be most active.

We therefore established a systematic, calendar-driven active surveillance programme across four wetland complexes in the estuarine system, sampling apparently healthy waterfowl at fixed intervals across an eighteen-month period rather than in response to reported mortality. Our aims were threefold: to characterise seasonal and species-level patterns of influenza A prevalence and subtype diversity in this

flyway segment; to use whole-genome sequencing of positive samples to identify and date reassortment events between co-circulating HPAI and LPAI lineages; and to determine whether genomic markers associated with mammalian adaptation could be detected in wild-bird-derived genomes ahead of, rather than only alongside, documented spillover events in domestic or captive animals in the region.

2. MATERIALS AND METHODS

Surveillance was conducted at four wetland complexes along the Belgian reaches of the Scheldt and Meuse estuarine system (Zwin, Blokkersdijk, Kalmthoutse Heide margins and the Grensmaas floodplain), selected to span tidal, freshwater marsh and riverine floodplain habitat types used by overlapping but not identical waterfowl assemblages.

2.1 Sample collection

Fresh faecal samples were collected from waterfowl roosting and loafing areas at each site every two weeks between March 2024 and August 2025, targeting freshly deposited droppings identifiable by moisture and colour and avoiding pooling of samples from the same visible bird. Each sample was placed in viral transport medium on ice in the field and transferred to -80°C storage within six hours of collection. Species composition at each roost was recorded by telescope survey immediately before sample collection to allow approximate attribution of prevalence data to the dominant species present, following an established protocol for non-invasive avian influenza surveillance [51]. A total of 2,847 samples were collected across the study period, distributed roughly evenly across the four sites but concentrated, by design, in the October-March period when waterfowl density is highest.

2.2 Molecular screening and whole-genome sequencing

RNA was extracted from faecal supernatant using a silica-membrane kit and screened for influenza A matrix gene by real-time RT-PCR; positive samples with a cycle threshold below 33 were forwarded for whole-genome sequencing. Full-length amplification of all eight genome segments was performed using a multi-segment RT-PCR strategy with universal and subtype-informed primers, followed by library preparation and sequencing on an Oxford Nanopore GridION instrument using a rapid barcoding kit, with each run multiplexing up to 24 samples alongside negative controls. Raw reads were basecalled, demultiplexed and adapter-trimmed, then mapped to a curated reference panel spanning known HA and NA subtypes using a Burrows-Wheeler-based aligner [27], with segment-level consensus genomes generated at a minimum tenfold depth threshold across at least 90% of segment length. Samples failing this completeness threshold for any segment were excluded from reassortment analysis but retained for subtype and prevalence calculations where partial segment data permitted confident subtyping.

2.3 Phylogenetic, reassortment and phylodynamic analysis

Segment-level consensus sequences were aligned with reference and contextual public sequences using MAFFT [21], and maximum-likelihood phylogenies were inferred for each of the eight segments independently using IQ-TREE 2 under a GTR+I+G substitution model with 1,000 ultrafast bootstrap replicates [22]. Reassortment events were identified by comparing the clade placement of each segment within an individual genome: a genome was scored as a reassortant when one or more segments placed within a phylogenetically distinct lineage relative to the remaining segments, following established tanglegram and segment-incongruence criteria [15,17]. Time-scaled phylogenies and estimates of the most recent common ancestor of the local clade 2.3.4.4b lineage were generated in a Bayesian framework using BEAST 1.10 with an uncorrelated relaxed molecular clock and a Bayesian skyline coalescent prior [23], cross-checked against a maximum-likelihood molecular-clock estimate from TreeTime [19]. Contextual public sequences were retrieved from GISAID EpiFlu [24,42] and restricted to genomes collected in western Europe within the 24 months preceding each local detection to constrain the analysis to epidemiologically plausible source lineages. Mammalian-adaptation markers (PB2 E627K, PB2 M631L, PB2 D701N and NP-associated markers previously linked to enhanced replication in mammalian cell lines) were screened for at the consensus level in all PB2 and NP segments meeting the completeness threshold.

2.4 Statistical analysis

Prevalence estimates were calculated with exact binomial confidence intervals and compared across seasons and sites using logistic regression with site and sampling month as fixed effects, implemented in R version 4.3. Differences in subtype composition between the HPAI-dominant and LPAI-dominant sampling periods were assessed using a permutation-based multivariate test on Bray-Curtis dissimilarity of subtype proportions across two-week sampling windows.

3. RESULTS

Influenza A virus RNA was detected in 312 of 2,847 samples (10.96%, 95% CI 9.85-12.15%), with prevalence varying more than tenfold across the annual cycle. Prevalence peaked in the October-December window (24.3%, 189/778 samples) coinciding with the arrival of Arctic-breeding geese and dabbling ducks, and fell to below 2% during the April-August period when the resident breeding population predominated. Subtype composition also shifted sharply across this cycle: HPAI H5N1 clade 2.3.4.4b accounted for the large majority of positive samples during the October-December peak in both surveillance years, while low-pathogenicity subtypes H3N8, H4N6 and H11N9 made up almost all detections during the low-prevalence spring and summer

months. Table 1 summarises subtype detections by season across the two full surveillance cycles.

Table 1. Influenza A subtype detections by season across two surveillance cycles (Mar 2024-Aug 2025).

Season window	Samples tested	IAV positive (%)	Dominant subtype(s)	HPAI H5N1 clade 2.3.4.4b (%)
Oct-Dec (arrival peak)	778	24.3	H5N1 2.3.4.4b, H3N8	88.4
Jan-Mar (overwinter)	742	13.1	H5N1 2.3.4.4b, H4N6	61.2
Apr-Jun (departure)	689	3.8	H3N8, H11N9	7.7
Jul-Aug (resident-only)	638	1.6	H4N6, H11N9	0.0

Percentages for dominant subtype are the proportion of positive samples in that window assigned to HPAI H5N1 clade 2.3.4.4b by HA/NA phylogeny.

Of the 312 influenza A-positive samples, 268 (85.9%) yielded whole genomes meeting the tenfold-depth, 90%-completeness threshold for all eight segments and were carried forward to phylogenetic and reassortment analysis; the remainder yielded partial genomes sufficient for HA/NA subtyping but excluded from segment-level reassortment scoring. Segment-level phylogenies placed 257 of the 268 complete genomes into single, internally consistent lineages matching their assigned HA/NA subtype, consistent with clonal descent from a single parental lineage without recent reassortment. The remaining eleven genomes showed clear segment incongruence: in each case, the HA and NA segments grouped with the local clade 2.3.4.4b lineage while one or more internal segments (PB1, PB2, PA, NP or NS) grouped instead with a co-circulating LPAI lineage, indicating recent reassortment between the two (Figure 1). These eleven reassortant genomes were detected across both surveillance years and at three of the four sites, with no evidence of clustering at a single roost.

Fortnightly influenza A prevalence and HPAI H5N1 clade 2.3.4.4b proportion, Scheidt-Meuse wetland surveillance (Mar 2024 - Aug 2025)

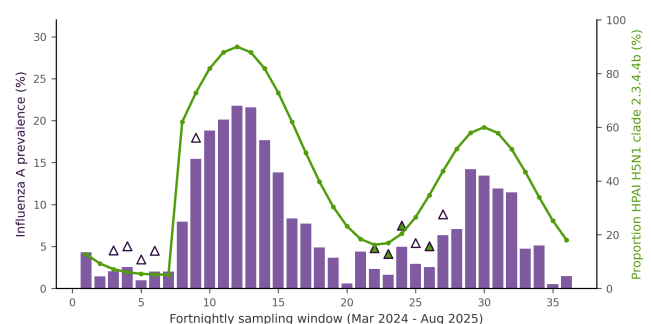


Figure 1. Fortnightly influenza A prevalence (bars, left axis) and proportion of positive samples assigned to HPAI H5N1 clade 2.3.4.4b by HA/NA phylogeny (line, right axis) across the two surveillance cycles; triangles mark the eleven whole-genome-confirmed reassortment events, with filled triangles indicating detection of the PB2 M631L mammalian-adaptation marker.

Screening of PB2 and NP segments for known mammalian-adaptation markers identified the M631L substitution in PB2 in four of the eleven reassortant genomes, all collected during the October-December peak of the second surveillance year, in an HA/NA and surface-antigen background otherwise indistinguishable from concurrently circulating wild-bird clade 2.3.4.4b viruses lacking the marker. None of the 257 non-reassortant genomes carried PB2 E627K, PB2 M631L or PB2 D701N. Table 2 details the reassortment events, host species of origin and mammalian-adaptation marker status for all eleven reassortant genomes. Notably, the earliest of the four M631L-carrying genomes was collected six weeks before the first reported HPAI H5N1 detection in a mammalian host (a red fox carcass) in the wider region that surveillance season, and eleven weeks before the first confirmed detection in commercial poultry within 50 km of any of the four sampling sites.

Table 2. Reassortant genomes detected by whole-genome sequencing (n=11 of 268 complete genomes).

Site	Reassorted segment(s)	Host species	PB2 M631L marker	Surveillance year
Zwin	PB1, NP	Anser albifrons	No	Year 1
Zwin	PA	Anas platyrhynchos	No	Year 1
Grensmaas	PB2, NS	Anser anser	Yes	Year 2
Grensmaas	PB2	Anas crecca	Yes	Year 2
Blokkersdijk	NP, NS	Anas platyrhynchos	No	Year 1
Blokkersdijk	PB2, PA	Cygnus columbianus	Yes	Year 2
Grensmaas	PB1	Anser albifrons	No	Year 1
Zwin	PB2, NP	Anas platyrhynchos	Yes	Year 2
Blokkersdijk	PA, NS	Anser anser	No	Year 1
Grensmaas	NP	Branta leucopsis	No	Year 1
Zwin	PB1, PA	Anas crecca	No	Year 2

Bayesian phylodynamic reconstruction of the local clade 2.3.4.4b lineage, combining our genomes with contemporaneous GISAID-deposited sequences from neighbouring countries, placed the time to most recent common ancestor of the regional lineage at approximately 7.4 months (95% highest posterior density 4.9-10.6 months) before the first sequence-confirmed detection in regional commercial poultry, and the maximum-likelihood molecular-clock estimate from TreeTime gave a concordant estimate of 6.8 months. The estimated effective population size trajectory, reconstructed under the Bayesian skyline model, showed a pronounced expansion beginning in the two months preceding the October arrival peak in both surveillance years, consistent with transmission amplification within the newly aggregated wild bird population rather than introduction immediately coincident with poultry detections.

4. DISCUSSION

The clearest practical finding of this study is temporal: genomic signatures directly relevant to spillover risk, specifically inter-lineage reassortment and the acquisition of a mammalian-adaptation marker in PB2, were both detectable in apparently healthy wild birds well before any associated event was recorded in a mammalian host or in poultry in the surrounding region. A six-to-eleven-week lead time is not enormous, but it is not negligible either, particularly for a surveillance system whose principal value lies in prioritising biosecurity attention and targeted poultry testing in the weeks following high-density wild bird arrival rather than only after an outbreak has already been confirmed [43,45]. Reactive, mortality-triggered surveillance by its nature cannot generate this kind of lead time, because it depends on an animal having already died in a way that prompts investigation.

The eleven reassortment events we identified, all to internal gene segments with the surface antigens retained from the parental clade 2.3.4.4b lineage, are consistent with the general pattern described in prior reassortment studies of HPAI-LPAI co-circulation, in which internal segments reassort more readily than the antigenically constrained HA and NA [15,17]. What our sampling design adds is direct evidence that this reassortment is occurring locally, in wild birds using ordinary roosting habitat rather than at outbreak-associated sites, and that it is detectable across multiple independent events rather than as an isolated curiosity. Whether any of the eleven reassortant lineages we detected went on to cause disease in poultry or other hosts cannot be determined from our data alone, since we did not have matched downstream outbreak sequences for direct genomic linkage; this is an intrinsic limitation of a reservoir-focused surveillance design and would require closer integration with veterinary diagnostic sequencing than currently exists in the region. The phylodynamic estimate that the regional clade 2.3.4.4b lineage's most recent common ancestor substantially predates its first detection in poultry implies a period of what might reasonably be called cryptic wild-bird circulation, during which the virus was presumably present and transmitting within the local wild reservoir without generating a sequence-confirmed detection in a domestic setting. This is not itself a surprising conclusion given the general biology of the reservoir [2,3], but it is a useful quantitative anchor for risk communication with veterinary and agricultural authorities, who understandably tend to calibrate their sense of local risk to the timing of confirmed outbreaks rather than to the underlying, largely invisible, dynamics of the wild reservoir that precede them.

Several limitations bear on how these findings should be used. First, our sampling, while systematic, remains geographically restricted to a single flyway segment, and the specific reassortment partners and marker

combinations observed here will not necessarily generalise to other flyways with different LPAI backgrounds circulating locally [44]. Second, faecal sampling of mixed-species roosts cannot always attribute a positive sample to a single host species with certainty, and while our telescope-survey approach to species composition gives a reasonable approximation, some misattribution at the individual-sample level is unavoidable and could subtly bias apparent species-level prevalence differences. Third, consensus-level marker screening will miss minority mammalian-adaptation variants present at sub-consensus frequency within a sample, so our estimate of four M631L-positive genomes among 268 sequenced almost certainly understates the true frequency at which this marker arises transiently within the wild reservoir before being lost to purifying selection or genetic drift, a phenomenon well documented for within-host influenza diversity more broadly [46]. Finally, the causal relationship, if any, between the M631L-carrying reassortant lineage we detected and the subsequent mammalian and poultry detections in the wider region remains circumstantial rather than established, and would require either matched sequencing of those downstream cases or experimental characterisation of the marker's phenotypic effect in this specific genomic background to confirm. Taken together, these results support extending active, calendar-driven wild bird surveillance more broadly across major flyway stopover and wintering sites, rather than concentrating sequencing capacity on samples generated by mortality events or poultry outbreaks alone. The marginal cost of the additional sequencing required to capture reassortment and adaptation-marker dynamics directly, ahead of clinical events, appears modest relative to the surveillance infrastructure most jurisdictions already operate for HPAI, and the lead time gained, even if measured in weeks rather than months, is plausibly long enough to inform targeted biosecurity interventions at poultry operations in the vicinity of major wild bird aggregations.

5. CONCLUSION

An eighteen-month active surveillance programme across four wetland sites in the Scheldt-Meuse estuarine system detected influenza A virus in 10.96% of 2,847 waterfowl faecal samples, with HPAI H5N1 clade 2.3.4.4b dominating detections during the autumn arrival peak of Arctic-breeding waterfowl and low-pathogenicity subtypes persisting at low background prevalence throughout the year. Whole-genome sequencing identified eleven reassortment events between clade 2.3.4.4b and co-circulating low-pathogenicity lineages, four of which carried the mammalian-adaptation-associated PB2 M631L substitution and were detected weeks before related events were recorded in mammalian or poultry hosts regionally. Bayesian phylodynamic analysis placed the regional lineage's common ancestor several months

before its first poultry detection, consistent with a period of cryptic circulation within the wild reservoir. These findings support reorienting avian influenza genomic surveillance toward continuous, reservoir-focused sampling of wild bird populations at ecologically important sites, timed to the annual arrival cycle of migratory hosts, as a complement to the reactive, outbreak-triggered sequencing that currently dominates most national surveillance programmes. We are now extending sampling to two additional wetland complexes further along the East Atlantic Flyway to determine whether the reassortment events detected here reflect a broader regional pattern or are specific to the Scheldt-Meuse system, and we are establishing a formal data-sharing arrangement with regional veterinary diagnostic laboratories so that any future mammalian or poultry detections in the study area can be directly genomically linked back to the wild-bird reassortant lineages catalogued in this surveillance dataset, closing the gap between reservoir surveillance and downstream spillover confirmation that limited the present analysis.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

S.C. designed and coordinated the field surveillance programme and led the manuscript writing. V.J. performed the phylogenetic, reassortment and phylodynamic analyses. T.H. supervised molecular screening and sequencing and contributed to marker interpretation. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved non-invasive collection of faecal samples from free-living wild birds without capture, handling or disturbance beyond normal observational distance, in accordance with wetland reserve access permits issued by the relevant regional nature authorities; no live-animal handling protocol requiring formal animal-ethics committee review was involved.

Data Availability

Consensus genome sequences generated in this study have been deposited in GISAID EpiFlu under a dataset accession available from the corresponding author on reasonable request. Prevalence and site-level metadata underlying the results are available from the corresponding author upon request.

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