Investigating Factors Driving Shifts in Subtype Dominance within H5Nx Clade 2.3.4.4b High-Pathogenicity Avian Influenza viruses

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Abstract

H5Nx clade 2.3.4.4b high-pathogenicity avian influenza viruses (HPAIVs) have decimated wild bird and poultry populations globally since the autumn of 2020. In the United Kingdom (UK) and in continental Europe, the H5N8 subtype predominated during the first epizootic wave of 2020/21, with few detections of H5N1. However, during the second (2021/22) and third (2022/23) epizootic waves, H5N1 was the dominant subtype. The rapid shift in dominance from H5N8 to H5N1 was likely driven by a combination of virological, immunological, and/or host-related factors. In this study, we compared viral fitness and immunological responses in ducks, a key reservoir species, using dominant genotypes of H5N1 (genotype AB) and H5N8 (genotype A) from the second wave. While viral shedding dynamics were similar for both viruses, H5N8 was more pathogenic. Antigenic analysis of post-infection duck sera revealed that the haemagglutinin (HA) protein was antigenically similar across clade 2.3.4.4b H5 HPAIVs, but neuraminidase (NA) proteins displayed different patterns of cross-reactivity. We also modelled a scenario where ducks were pre-exposed to H5N1 (genotype C) or H5N8 (genotype A) from the first wave and subsequently challenged with either homologous or heterologous subtypes from the second wave (genotype AB or A). Despite the absence of seroconversion, pre-exposure to different subtypes resulted in varying clinical outcomes following challenge. These findings indicate that both viral and immunological factors likely played significant roles in the emergence and spread of H5Nx HPAIVs in wild bird populations.

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46 # INTRODUCTION

47 Influenza A viruses are subtyped according to the genetic and antigenic relatedness of the two surface
48 glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Currently, seventeen different HA
49 subtypes and 9 different NA subtypes of avian influenza virus (AIV) have been detected in wild birds.
50 The HA protein can be broadly split into five clades, which form two groups; group one (clade 1, H8, H9,
51 H12, H19; clade 2, H1, H2, H5 and H6; clade 3, H11, H13, and H16) and group two (clade 1, H3, H4,
52 and H14; clade 2, H7, H10, and H15) [1, 2]. A similar approach can be applied to the NA protein, which
53 can be divided into two groups (group one, N1, N4, N8 and N5; group two, N6, N9, N7, N2 and N3) [2].
54 Due to the segmented nature of the AIV genome, which consists of eight genomic segments, different HA
55 and NA combinations can arise via reassortment when two or more AIVs co-infect the same cell.
56 Reassortment of the internal gene segments can also generate new genotypes with different biological
57 properties. Wild aquatic birds (mainly Anseriformes [waterfowl; ducks and geese etc.], and
58 Charadriiformes [shorebirds; gulls and waders etc.]) serve as the reservoir for AIV in which nearly every
59 combination of HA and NA subtypes can be found [2].

60 Since 2014, there have been repeated introductions of clade 2.3.4.4 H5Nx high pathogenicity
61 avian influenza virus (HPAIV) of the A/goose/Guangdong/1/96 (gsGd) lineage into the European poultry
62 sector, driven by the movements of wild bird species on migratory pathways [3]. The gsGd lineage
63 epidemiology has been recently dominated by clade 2.3.4.4b HPAIVs, which continue to circulate in wild
64 bird populations, and because of continual reassortment with other circulating AIVs, multiple different
65 NA subtypes have emerged and established dominance in wild birds across Europe with resultant poultry
66 incursions, including H5N8 (2014/15 and 2016/17), H5N6 (2017), H5N8 (2020/21) and H5N1 (2021/22
67 and 2022/23) [4-10].

68 During winter 2020/21 (wave 1), the magnitude of H5Nx epizootics escalated considerably [11].
69 In the United Kingdom (UK) and continental Europe, the first H5Nx epizootic wave (2020/21

70 autumn/winter) was the largest ever at the time. Wave 1 was dominated by a single subtype, H5N8, which
71 was responsible for 96% of detections in UK poultry. A minor population of the H5N1 subtype was also
72 circulating during this period, with H5N1 being responsible for 4% of detections in UK poultry [9, 10].
73 During wave 1, all H5N8 HPAIVs detected in the UK shared >98.1% sequence identity across all gene
74 segments existing as a single genotype (European Reference Laboratory [EURL]: genotype A; H5N8-W1
75 [12]), similarly all H5N1 detections also formed a single genotype (genotype C; H5N1-W1) [10].

76 At the end of the first wave (summer 2021) and at the start of the second wave (W2; 2021/22),
77 H5N1 HPAIV emerged as the dominant subtype in the UK, with less than 1% of detections in wild birds
78 and poultry being of other subtypes (H5N2 and H5N8), with only a single detection of H5N8 from a mute
79 swan (*Cygnus olor*) in November 2021 [10, 13]. This initial H5N1 was genetically highly similar to
80 H5N1-W1 which circulated as a minority subtype in the previous season (both were genotype C).
81 Similarly, the single detection of H5N8 (H5N8-W2) was highly genetically related to the H5N8-W1 virus
82 that had dominated previously (both were genotype A) [10].

83 As the second wave developed (autumn 2022), and transitioned into the third wave (2022/23),
84 reassortment with other circulating AIVs contributed to increased genetic diversity within the H5N1
85 HPAIVs. However, importantly, little variation was observed in the HA gene between all H5N1 and
86 H5N8 genotypes detected since wave 1. Four distinct H5N1 genotypes were detected more than once in
87 poultry in the UK until the end of wave 3 (October 2023): genotypes AB, BB, C, and AL [10, 14-18].
88 These genotypes have had varied detection frequencies, with the AB genotype (H5N1-W2) providing
89 50.9% of detections since it was first detected at the start of the second wave in the UK. This genotype of
90 H5N1-W2 shared the PB1, NP, NA, MP, and NS segments with H5N1-W1 [10, 19], but the PB2 and PA
91 segments were most closely related to H5N3 AIVs from the first wave [10].

92 The rapid transition in dominance from H5N8 to H5N1 between wave 1 and wave 2 suggests (i) a
93 potential phenotypic change in the virus, or (ii) potential differential immune evasion mechanisms
94 between the subtypes. Therefore, in this study, virological and host factors which may influence the

continued evolution of H5Nx clade 2.3.4.4b HPAIVs in Anseriformes were investigated. Specifically, we investigated the relationship between the two epizootic seasons, where H5N8 dominated in wave 1 and H5N1 dominated in wave 2. Using virus isolates representative of those circulating in wave 2 of the epizootic in 2021/22 (H5N1-W2 and H5N8-W2), we compared *in vivo* fitness in ducks and interrogated antigenicity against HA and NA. The changing subtype dominance was modelled by exposing ducks to wave 1 viruses (H5N1-W1 and H5N8-W1) and then challenging with homologous or heterologous wave 2 viruses (H5N1-W2 or H5N8-W2).

METHODS

Viruses and propagation

Six avian influenza virus (AIV) strains were used in this study (Table S1). Two viruses represented the first wave epizootic (Oct 2020–Sept 2021): A/chicken/England/030786/2020 (H5N8, genotype A; H5N8-W1; GISAID: EPI_ISL_17212363) and A/mute swan/England/234255/2020 (H5N1, genotype C; H5N1-W1; GISAID: EPI_ISL_766876). De-engineered versions of both viruses were generated by substituting the multi-basic cleavage site (MBCS) with a single-basic cleavage site (SBCS) via reverse genetics (RG) (described previously [20]), resulting in SB-H5N8-W1 and SB-H5N1-W1.

For in vivo challenge studies, two HPAIVs from the second wave epizootic (Oct 2021–Sept 2022) were used: A/chicken/Scotland/054477/2021 (H5N1, genotype AB; H5N1-W2; GISAID: EPI_ISL_9012696) and A/mute swan/England/298902/2021 (H5N8, genotype A; H5N8-W2; GISAID: EPI_ISL_13369742).

All viruses were propagated in 9-day-old specified pathogen free (SPF) embryonated fowl eggs (EFEs) and titrated in EFEs to determine the 50% egg infectious dose (EID_{50}), as previously described [21]. The full genomes of all viruses used in this study were sequenced, as previously described [10] to confirm that no amino-acid polymorphisms had emerged following passage, when compared to the original sequence.

119 **Animals**

120 High health status Pekin ducks (*Anas platyrhynchos*) (Cherry Valley hybrid; Cherry Valley Farms Ltd.,
121 UK) of mixed sex (52% female; 48% male, total 36) were obtained at one-day old and reared until 3
122 weeks of age at time of infection. Ducks were acclimatised for seven days prior to the first procedures
123 which included swabbing (oropharyngeal (Op) and cloacal (C)) and bleeding from a superficial vein prior
124 to inoculation. These samples were tested using an M-gene real time reverse transcription polymerase
125 chain reaction (RT-PCR) [22] and serological testing [23] using the ID Screen® Influenza A Antibody
126 Competition Multi-species ELISA kit (IDVet, France). For the IVPI test, white Leghorn SPF chickens
127 (*Gallus gallus domesticus*) (Valo, Germany) were reared at APHA until 4 weeks-of-age.

128 **Experimental design**

129 The statutory IVPI test was performed on de-engineered viruses (SB-H5N1-W1 and SB-H5N8-W1) as
130 per WOAH guidelines [23]. Virus isolates were diluted 1:10 in isotonic saline, and 100 µl (10^8
131 EID_{50}/bird) was administered intravenously into the wing vein of 4-week-old chickens (n=10).

132 For infection and prior-immunity studies, two groups of immunologically naïve 3-week-old ducks
133 (n=6/group) were inoculated via the ocular-nasal route with 10^5 EID_{50} of either H5N1-W2 or H5N8-W2
134 in 100 µl of phosphate buffered saline (PBS) (Fig. S1). Oropharyngeal (Op) and cloacal (C) swabs were
135 collected daily from 1–14 days post infection (dpi). Birds were euthanised upon reaching humane
136 endpoints or at 14 dpi, when blood was collected via cardiac puncture under terminal anaesthesia.

137 For prior-exposure studies, two groups of ducks (n=12/group) were inoculated oronasally with
138 10^7 EID_{50} of SB-H5N1-W1 (N1) or SB-H5N8-W1 (N8) (Fig. S1). At 13 dpi, blood was collected from all
139 birds, after which each group was split into two (n=6/group) and challenged oronasally with 10^5 EID_{50} of
140 either H5N1-W2 or H5N8-W2. This formed four groups: N1/N1 and N8/N1 (H5N1-W2 challenge), and
141 N1/N8 and N8/N8 (H5N8-W2 challenge). From 1–14 days post challenge (dpc), Op and C swabs were

142 collected daily. Birds were euthanised at humane endpoints or at 14 dpc (28 dpi total), when terminal
143 blood collection was undertaken.

144 **Clinical scoring and monitoring**

145 Birds were observed and scored against a defined clinical score sheet (Table S2) twice a day (morning
146 and afternoon). Following the development of clinical disease, birds were additionally observed at a third
147 timepoint (evening). Birds were terminated whenever clinical endpoints were reached (cumulative score
148 >7; Table S2). For ethical reasons, any birds housed singularly, due to euthanasia or death of others
149 within the group, were also euthanised.

150 **Clinical sample collection, processing, and immunohistochemistry**

151 Swabs were individually cut and placed into 1ml of Leibovitz L-15 Medium (Gibco; (LM) [24]), and the
152 supernatant used for RNA extraction or stored at -80°C until further use. During post-mortem
153 examination, approximately 50 milligrams (mg) of tissue from selected organs was collected into 1ml LM
154 and mechanically homogenised, with the clarified homogenate being used for RNA extraction. In
155 addition, tissues were harvested from all major organs and fixed in 10% (v/v) neutral buffered formalin
156 for a minimum period of 5 days before being embedded in paraffin. Four-micron thick serial sections
157 were stained with haematoxylin and eosin (H&E), and for immunohistochemistry (IHC), using a mouse
158 monoclonal anti-influenza A nucleoprotein antibody (Statens Serum Institute, Copenhagen, Denmark) as
159 described previously [25]. The overall distribution of virus-specific staining in each tissue was assessed
160 using a semi-quantitative scoring system (0 = no staining, 1 = minimal, 2 = mild, 3 = moderate and 4 =
161 widespread staining) modified from [26]. Specificity of immunolabelling was assessed in positive control
162 sections by replacing the primary antibody with a matching mouse IgG isotype; no non-specific cross-
163 linking was observed.

164 **Environmental sample collection and processing**

165 Following infection with HPAIV at 14 dpi, environmental samples of drinking water and pond water
166 were collected on alternate days from each duck pen. Air samples were collected, as described previously
167 [24, 27], from the environment of ducks infected with either H5N1-W2 or H5N8-W2. Viral RNA (vRNA)
168 was extracted directly from water samples, and from supernatants of gelatine air filters dissolved in LM
169 as described previously [27].

170 **RNA extraction and AIV reverse transcription Real-Time PCR (RT-PCR)**

171 vRNA was extracted from LM obtained from swabs, tissues, feathers, and environmental samples using
172 the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific™) and the robotic
173 Mechanical Lysis Module (KingFisher Flex system; Life Technologies), according to the manufacturer's
174 instructions. Extracted RNA were tested by the M-gene RT-PCR using the primers and probes as
175 previously described [22]. A ten-fold dilution series of RNA extracted from a stock of H5N1-W2 HPAIV
176 with a known EID_{50} titre was used to construct a standard curve and Ct values converted to relative
177 equivalent units (REU) as previously described [24, 28]. RT-PCR Ct values <36 (>$10^{1.794}$ REU) were
178 considered as AIV positive, Ct values ≥36 (≤$10^{1.794}$ REU) were considered negative.

179 **Serum processing and hemagglutination inhibition assays (HI)**

180 Whole blood samples were centrifuged for 5 minutes at 2000 rpm to separate the serum from the clot.
181 Sera were incubated at 56°C for 30 minutes to inactivate complement. The duck sera were pre-absorbed
182 by adding packed chicken red blood cells and incubated at 4°C overnight to prevent non-specific
183 agglutination [23]. Hemagglutination inhibition assays (HI) were conducted using different antigens
184 initiated at four HA units. Reciprocal HI titres of >1/16 were considered seropositive as defined
185 internationally [23]. All sera were also tested by the ID Screen® Influenza A Antibody Competition
186 Multi-species ELISA kit (IDVET, France) according to the manufacturer's instructions.

187 **Influenza pseudotype virus (PV) generation**

188 Lentiviral pseudotype viruses (PVs) containing H5Nx glycoproteins were generated for H5N1-W2
189 (genotype C) (A/chicken/England/053052/2021), H5N1-W2 (genotype AB)
190 (A/chicken/Scotland/054477/2021) and H5N8-W1 (genotype A) (A/chicken/England/030786/2020)
191 (Table S1). Existing PV were used for H5N8-14 (A/gyrfalcon/Washington/41088-6/2014) [29]. The HA
192 and NA genes were subcloned from pHW2000 into pCAGGS using a directional *NotI-XhoI* restriction
193 digest strategy. PVs were generated as previously described [29] employing p8.91 gag-pol vector as
194 retroviral core. PV titres were assessed by transduction of HEK293T/17 cells as previously described [29]
195 employing the Bright-Glo assay system (Promega) and read as relative luminescence per unit (RLU).

196 **Pseudotype virus neutralisation assay (PVNA)**

197 For PVNA, serum was first prediluted 1:100 and tested in duplicate against H5 PVs. Chicken reference
198 serum raised against for A/chicken/Scotland/59 (H5N1) (product code: RAA7002) and
199 A/duck/England/036254/14 (H5N8) (Product code: RBB6425) (APHA Scientific, UK). Briefly, sera were
200 serially diluted 2-fold in a 96-well white plate and 10^6 RLU of PV was added to each well. The plate was
201 incubated for 1 hour at 37°C to allow binding of the antibody to the antigen. Next, 10^4 HEK293T/17 cells
202 were added to each well. PV-only and cell-only controls were included in the plate to represent the 0%
203 and 100% neutralisation of the PV. The plate was incubated for 48h at 37°C at 5% CO_2 before reading.
204 Data were normalised and plotted on a neutralisation percentage scale and the reciprocal of the serum
205 dilution which induces 50% neutralisation or IC_{50} was calculated as previously described [30].

206 **Pseudotype-based enzyme-linked lectin assay (pELLA)**

207 To investigate NA reactive antibodies HA and NA PVs were employed. H5Nx PVs were generated and
208 titrated via the pELLA method as previously described [31]. Optical density at 450nm (OD_{450}) was
209 determined using the Multiskan™ FC microplate photometer with SkanIt™ Software for data analysis.
210 Readings were normalised to 100% and 0% OD_{450}, and the dilution that resulted in 90% OD_{450} was

211 selected for inhibition assays. The inhibition of NA activity by serum samples was evaluated via pELLA.
212 Serum was tested in duplicate against H5N1 and H5N8 PVs. Sera were initially diluted 1:10 and then
213 serially diluted 2-fold in 50µL SD. 50µL of these dilutions were then transferred to fetuin-coated plates
214 containing 50µL of SD to all wells, excluding SD-only and PV-only controls, representing 100% and 0%
215 inhibition, respectively. 50µL of the H5Nx PV 90% OD_{450} as determined in the titration was then
216 transferred to all wells of the fetuin-coated plate except for SD-only control. All other steps were
217 followed as per NA PV titration via the pELLA. The IC_{50} was calculated as the inverse dilution of serum
218 that resulted in 50% inhibition of NA activity as determined via GraphPad Prism.

219 **Statistical analysis**

220 Area under the curve (AUC) analysis of individual bird shedding profiles was performed using GraphPad
221 Prism v8, with values expressed as REU. Birds that did not shed were assigned an AUC value of 0 REU.
222 Mean AUC values were calculated and compared for statistical significance using one-way ANOVA with
223 multiple comparisons in GraphPad Prism v8. Statistical significance was defined as $p < 0.05$. For pELLA
224 analysis, mean and standard deviation medians were derived for each data point based on two replicates
225 per dilution. Kaplan–Meier survival curves were evaluated using the Gehan–Breslow–Wilcoxon test in
226 GraphPad Prism v8.

227 # RESULTS

228 **Comparative fitness in ducks of 2.3.4.4b H5N1 and H5N8 HPAIV circulating during the second**
229 **epizootic wave.**

230 The *in vivo* fitness of H5N1-W2, a HPAIV representative of the dominant genotype (AB) during the
231 second epizootic wave, was compared with H5N8-W2 (genotype A) from the same time period (Fig. S1).
232 Inoculation of naïve ducks with either virus resulted in productive infection, with high vRNA shedding
233 from the Op cavity (Fig. 1A and B). Both viruses showed similar shedding profiles; however, H5N8-W2
234 exhibited a higher mean peak shedding on 3 dpi, while H5N1-W2 peaked later, at 4 dpi, with a lower

235 mean titre. Notably, H5N1-W2-infected ducks shed vRNA for a longer duration. C shedding was broadly
236 similar, though a higher proportion of ducks shed vRNA after H5N1-W2 infection (H5N1-W2, 100%;
237 H5N8-W2, 50%). However, AUC analysis showed no significant difference between H5N1-W2 and
238 H5N8-W2 in overall Op or C shedding (Op, p-value 0.229; C: p-value 0.477). Environmental
239 contamination was found in the drinking and pond water with vRNA detected from 3 dpi in both groups,
240 but less consistently in the H5N1-W2 group (Fig. 1C and D). vRNA was present in water on 3, 9, and 11
241 dpi for H5N1-W2, and on 3, 5, 7, and 9 dpi for H5N8-W2. Airborne vRNA was only detected at 7 dpi in
242 the H5N1-W2 group.

243 **Clinical outcomes in ducks with H5N1 and H5N8 HPAIV circulating during the second epizootic**
244 **wave.**

245 All ducks infected with H5N1-W2 survived until the end of the study (14 dpi), whereas only 50% of those
246 infected with H5N8-W2 survived (two ducks were euthanised at 5 dpi and one at 7 dpi) (Fig. 1E). H5N1-
247 W2 infection induced fewer clinical signs, with a total clinical score of 8, compared to a score of 90 in the
248 H5N8-W2 group (Table S2). Ducks infected with H5N8-W2 also exhibited more severe neurological
249 signs, including tremors (7 counts), torticollis (1 count), and seizures (1 count) (Table S2, Fig. S2A and
250 B).

251 Post-mortem examination was conducted on three H5N1-W2-infected ducks and six H5N8-W2-
252 infected ducks. As no H5N1-W2 ducks required culling, comparisons between groups are only possible at
253 14 dpi. Among the three H5N8-W2-infected ducks culled between 5–7 dpi (ducks 101, 103, and 104),
254 vRNA was detected in all sampled tissues from ducks 103 and 104 (5 dpi), while duck 101 tested
255 negative in the heart, lung, caecal tonsil, proventriculus, and liver (Fig. 2A). Notably, all three showed
256 high vRNA levels in brain and feather samples, with mean titres of $10^{8.41}$ and $10^{5.48}$ REU, respectively. Of
257 the six surviving ducks culled at 14 dpi, vRNA was detected only sporadically, and at a low level, in
258 brain, muscle, or feathers in one H5N8-W2-infected duck (106) and one H5N1-W2-infected duck (100).
259 Another H5N1-W2-infected duck (99) had detectable vRNA only in the pancreas and kidney.

260 IHC analyses was also performed on tissues from these birds. At 5 dpi, brain samples from
261 H5N8-W2 ducks showed acute non-suppurative encephalitis with extensive viral antigen in neurons (Fig.
262 2B and S3A and B). By 14 dpi, only minimal multifocal gliosis and sparse antigen were observed,
263 indicating viral clearance. In the heart, acute myocardial degeneration with monocytic infiltration and
264 abundant antigen was seen at 4–5 dpi. Pectoral muscle displayed mild non-suppurative myositis with
265 variable antigen labelling. By 14 dpi, lesions in both heart and muscle had resolved to mild chronic
266 inflammation and lymphoid hyperplasia. Minimal antigen remained in macrophages within lymphoid
267 aggregates, suggesting resolution of active viral replication.

268 **Serological responses and antigenic cross-reactivity between H5N1 and H5N8 subtypes.**

269 Serological responses following infection with H5N1-W2 and H5N8-W2 were compared. All surviving
270 infected ducks (n=6 H5N1-W2; n=3 H5N8-W2) were positive by NP ELISA (data not shown) and
271 seroconverted against the homologous antigens in the HI assay, at 14 dpi (Fig. 3A). Lower, but not
272 statistically significant, HI titres were detected when sera from both infections were tested against the
273 H5N8-W2 compared to the H5N1-W2 antigen. No statistically significant differences were observed
274 between reactivity of viruses with sera of the same subtype from wave 2 or wave 1 (H5N1-W2 compared
275 to H5N1-W1 and the reciprocal) (Fig. 3A). To assess HA-specific antibody responses, PVs displaying
276 only the HA proteins of clade 2.3.4.4b H5N1 and H5N8 strains were used, alongside an earlier clade
277 2.3.4.4c H5 (H5N8-14) PV as a reference. Although both sera neutralised H5N8-14 PV, IC_{50} values were
278 significantly lower than those for the more recent H5 PVs (Fig. 3B). No significant differences in
279 neutralisation titres were found between the 2.3.4.4b H5 PVs using either serum (Fig. 3B). To evaluate
280 NA-specific responses, pELLA was performed using PVs containing both HA and NA. H5N1 and H5N8
281 antisera inhibited their homologous PVs with IC_{50} values of $10^{3.68}$ and $10^{4.89}$, respectively (Fig. 3C and D).
282 Pre-infection sera showed no cross-reactivity to either PV. Post-infection sera from both groups (H5N1-
283 W2 and H5N8-W2 sera) robustly inhibited H5N8 PV (IC_{50} $10^{4.28}$ for H5N1-W2 and $10^{4.58}$ for H5N8-W2).

284 However, H5N1-W2 sera only inhibited the H5N1 PV (IC_{50} $10^{3.68}$). H5N8-W2 sera did not inhibit H5N1
285 PV, with inhibition curves failing to reach 50% (Fig. 3C and D).

286 **H5N1 and H5N8 clade 2.3.4.4b H5Nx HPAIV without MBCSs have reduced pathogenicity yet are**
287 **able to productively infect chickens.**

288 To replicate the natural exposure scenario observed between 2020–2022, we next modelled sequential
289 infection in wild birds, with initial exposure to wave 1 viruses (H5N1-W1 or H5N8-W1) followed by re-
290 infection with wave 2 viruses (H5N1-W2 or H5N8-W2). To ensure survival after primary exposure, we
291 attempted to reduce the pathogenicity of the viruses while retaining the antigenicity. Therefore, we used
292 RG to generate low pathogenic variants (SB-H5N1-W1 and SB-H5N8-W1) by replacing the multi-basic
293 cleavage site (MBCS) with a single-basic cleavage site (SBCS) (Fig. S4C). Both SB viruses reached high
294 titres in embryonated fowl eggs (SB-H5N1-W1, $10^{10.50}$ EID_{50}/ml; SB-H5N8-W1, $10^{10.00}$ EID_{50}/ml),
295 comparable to their wildtype HPAIV counterparts (H5N1-W1, $10^{9.85}$; H5N8-W1, $10^{9.48}$). IVPI testing
296 confirmed a low pathogenic phenotype (SB-H5N1-W1, 0.15; SB-H5N8-W1, 0.0), yet all surviving birds
297 seroconverted by 10 dpi, confirmed by homologous HI titres (Fig. S4A). One mortality occurred in the
298 IVPI at 6 dpi in the SB-H5N1-W1 group. vRNA was detected in multiple tissues and swabs from this
299 animal (Fig. S4B) but sequencing confirmed retention of the engineered SBCS (Fig. S4C).

300 **The effect of homo- and hetero- subtypic prior exposure on shedding dynamics of the H5N1 and**
301 **H5N8 clade 2.3.4.4b HPAIV.**

302 We next inoculated ducks, as surrogates for wild aquatic birds, with SB-H5N1-W1 or SB-H5N8-W1
303 viruses, followed by homologous (N1/N1, N8/N8) or heterologous (N1/N8, N8/N1) HPAIV challenge
304 with H5N1-W2 or H5N8-W2 (Fig. S1). Following inoculation with the de-engineered viruses, vRNA was
305 detected in only three of 12 ducks in the SB-H5N1-W1 groups (N1/N1, N1/N8), all below the positive
306 threshold; no vRNA was detected in the SB-H5N8-W1 groups (N8/N8, N8/N1). No clinical signs were
307 observed, and all ducks were seronegative by HI at 13 dpi (HI titres <2).

308 Following challenge with H5N1-W2, ducks previously exposed to SB-H5N1-W1 (N1/N1)
309 exhibited a 4-day delay in vRNA detection in Op swabs compared to naïve controls (Fig. 4A). A similar
310 delay was observed in C swabs (Fig. 4E). Despite this, both groups reached comparable peak vRNA
311 levels, with Op approximately 2 $\log_{10}$ higher than C shedding (Fig. 4A, E). Total shedding (AUC) did not
312 differ significantly between groups (Op, p-value 0.9991; C, p-value 0.9941). vRNA was detected in water
313 from the N1/N1 group from 7–11 dpi; a 4-day delay compared to the naïve H5N1-W2 group (Fig. 5A vs
314 Fig. 1C). In the heterologous challenge (N1/N8), prior SB-H5N1-W1 exposure delayed shedding onset by
315 2 days relative to naïve H5N8-W2-infected ducks, though both groups peaked at 3 dpi (Fig. 4B). Water
316 contamination was detected at 3 dpi in both groups but persisted longer (until 9 dpi) in the naïve (H5N8-
317 W2) group (Figs. 1D, 5D). The delay in shedding onset in the N1/N1 and N1/N8 groups was most
318 apparent early in infection (Fig. 4A–B).

319 In contrast, prior SB-H5N8-W1 exposure had minimal impact on vRNA shedding. Ducks
320 subsequently challenged with H5N1-W2 (N8/N1) or H5N8-W2 (N8/N8) showed similar vRNA shedding
321 profiles to naïve controls (Fig. 4C–D, G–H). Environmental contamination was also similar (Figs. 5B–C).
322 Water from the N8/N1 group showed comparable levels to naïve H5N1-W2-infected ducks, although
323 shedding was more prolonged in the naïve H5N1-W2 ducks (Figs. 1C, 5B). For the N8/N8 group, vRNA
324 was detected in water, but sample collection ceased at 5 dpi due to mortality in all ducks, limiting
325 comparison (Figs. 1D, 5C, 6B).

326 **The effect of homo- and hetero- subtypic prior exposure on disease outcomes and pathogenesis.**

327 In the H5N1-W1 pre-exposed ducks, no mortality was observed following challenge with H5N1-W2
328 (N1/N1), consistent with the survival of naïve H5N1-W2-infected ducks (Fig. 6A). Both groups also
329 exhibited reduced clinical severity compared to ducks exposed to or infected with H5N8 viruses (Fig. S2).
330 In contrast, prior exposure to H5N1-W1 reduced mortality and clinical signs following H5N8-W2
331 challenge (N1/N8) compared to the naïve group, but not statistically significantly (Fig. 6B, Table S2).

332 However, in the N8 pre-exposed group, homologous H5N8 re-challenge (N8/N8) resulted in
333 statistically significantly higher mortality ($p = 0.0139$, Gehan-Breslow-Wilcoxon test) and greater clinical
334 severity compared to the naïve H5N8 group (Fig. 6B and S2B, D), with 100% of ducks culled due to
335 severe disease (two at 4 dpc, four at 5 dpc), compared to 50% in the naïve group (two at 5 dpc, one at 7
336 dpc) (Fig. 6). Prior H5N8-W1 exposure also increased mortality upon H5N1-W2 challenge (N8/N1).

337 Tissue tropism was assessed only in ducks that succumbed to infection. vRNA was detected in
338 multiple tissues from N1/N8, N8/N1, and N8/N8 groups, with the highest titres in brain samples (Fig.
339 7A). High vRNA levels were also found in feathers, cervical trachea, and intestines of N1/N8 and N8/N8
340 ducks. In contrast, no vRNA was detected in tissues from the N8/N1 bird, which also showed lower IHC
341 scores across tissues compared to H5N8-W2-challenged birds (Fig. 7A and B). Histopathological lesions
342 and immunolabelling in N1/N8 and N8/N8 ducks mirrored those in naïve H5N8-W2-infected birds (Figs.
343 S3C, S3D). Moderate multifocal non-suppurative encephalitis with gliosis and perivascular cuffing was
344 observed in the brain, with abundant neuronal antigen. Acute myocardial degeneration and necrosis with
345 co-localised viral antigen were evident in the heart and pectoral muscle (Fig. 7B). In contrast, N8/N1
346 ducks displayed minimal lesions, and no tissue samples were available from the N1/N1 group.

347 **DISCUSSION**

348 During the H5Nx clade 2.3.4.4b panzootic, a notable shift in NA subtype dominance occurred in the UK
349 and continental Europe. In the 2020–2021 season (wave 1), H5N8 predominated, with 26 infected poultry
350 premises (IPs) and 285 wild bird cases reported in the UK, compared to only 2 IPs and 18 wild bird
351 detections of H5N1. However, by 2021–2022 (wave 2), H5N1 became dominant, with 157 IPs and 1,629
352 wild bird detections, while H5N8 was detected only once, in a single mute swan (*C. olor*), with no poultry
353 cases [9, 10]. This rapid shift may reflect (**i**) changes in viral virulence or infectivity, (**ii**) differential
354 immune evasion, or (**iii**) a combination of both.

355 To assess virological fitness, representative H5N1 and H5N8 viruses from wave 2 were compared
356 *in vivo*. Both H5N1-W2 and H5N8-W2 established productive infections, with all ducks shedding virus
357 and seroconverting. Shedding kinetics were similar, with higher viral loads from Op swabs than C swabs.
358 H5N1-W2 showed a more prolonged Op shedding period, though total viral load did not differ
359 significantly between groups. Notably, H5N8-W2 caused 50% mortality, while H5N1-W2 caused none.
360 Previous studies have shown that an earlier H5N1-W2 HPAIV (genotype C) had comparable infectivity
361 and transmissibility to other clade 2.3.4.4 HPAIVs, with enhanced fitness in Anseriformes [32-34].
362 Recently, Bordes, Germeraad [35] compared two H5N1 genotypes of different dominance (genotype C
363 [wave 1 – low] and genotype AB [wave 2 – high]) in three species of Anseriformes (Pekin ducks [*A.*
364 *platyrhynchos*], Eurasian wigeon [*Mareca Penelope*] and barnacle geese [*Branta leucopsis*]) [35]. In all
365 three species the dominant genotype AB was less pathogenic than less dominant genotype C; in pekin
366 ducks no mortalities were seen for genotype AB compared to 40% mortalities with genotype C. The
367 observation of high mortalities with early wave 1 H5N1 (genotype C) parallels with our findings of high
368 mortalities for early wave 1 H5N8. Furthermore, consistent with our observations here, viral shedding
369 kinetics were comparable between genotype AB and genotype C [35]. Bordes, Germeraad [35] did not
370 assess transmission dynamics, and likewise transmission was not assessed here in our study. However,
371 several studies highlighted a correlation between transmission efficiency and the extent of environmental
372 contamination, particularly in water sources, for H5Nx 2.3.4.4b viruses [24, 33]. In our study,
373 environmental contamination, particularly of pond and drinking water, was detected more frequently with
374 H5N1-W2. This, combined with reduced vRNA shedding duration and higher mortality for H5N8-W2,
375 suggests that H5N1-W2 may transmit more efficiently in wild waterfowl. The infrequent contact between
376 wild birds implies that premature host death could limit transmission opportunities, as previously
377 proposed [36]. Together, these data indicate phenotypic differences between H5N8 and H5N1 that may
378 explain the ecological shift in subtype dominance from H5N8 in wave 1 to H5N1 in wave 2 in the UK
379 and Europe.

380 Following initial characterisation of infection dynamics and pathogenicity, we investigated the
381 potential role of antibody-mediated immunity in the observed shift in subtype prevalence between the first
382 two outbreak waves. Some avian species are susceptible to HPAIV yet can survive infection. Ringing
383 data suggest wild Anseriformes have life expectancies ranging from 3 to 20 years, depending on species
384 [37], implying that individuals tolerant to infection may persist across multiple epizootics and encounter
385 diverse AIV subtypes and genotypes [24, 38]. Antigenic relatedness and associated cross-reactivity
386 between similar HA and/or NA proteins can complicate interpretation of serological data in wild birds.
387 Nevertheless, HA- and NA-specific antibodies have been detected in several wild waterfowl species [33,
388 39, 40]. To explore this further, we first assessed serological responses in naïve ducks surviving infection
389 with H5N1-W2 or H5N8-W2, finding that all birds seroconverted by HI. Although not statistically
390 significant, titres were lower when sera from both groups were evaluated against H5N8-W2 antigen than
391 H5N1-W2. To better resolve HA-specific responses, we evaluated antibody titres using HA-only PVNAs.
392 No significant differences in IC_{50} values were observed between sera from H5N1 and H5N8-infected
393 birds. This similarity in HA antigenicity among clade 2.3.4.4b H5Nx viruses aligns with previous
394 findings [41, 42] and likely reflects the high amino acid identity across their HA proteins (Table S3).

395 Although most of the antibody response in birds exposed to AIV targets HA [43], antibodies
396 against NA also contribute to protective immunity [44, 45]. However, the composition and function of the
397 antibody repertoire in wild bird species remain poorly characterised, particularly regarding the relative
398 roles of HA- and NA-specific responses in protection. To investigate this, we assessed NA-specific
399 antibodies using the pELLA assay [31]. While pELLA does not directly quantify neutralisation, it
400 correlates well with NA inhibition activity [46]. Sera from ducks surviving infection showed detectable
401 NA-specific antibodies, indicating a reactive immune response to NA [44, 45]. Previous studies have
402 demonstrated a protective role for NA antibodies in Canada geese (*Branta canadensis*) and mallards (*A.*
403 *platyrhynchos*), where prior LPAIV exposure reduced disease severity upon NA-homologous H5Nx
404 HPAIV challenge [47, 48]. Similarly, NA antibodies have provided protection against H5N1 HPAIV in

405 chickens [49]. These findings support the concept of "permissive immunity", whereby NA antibodies
406 limit viral release from infected cells, thereby mitigating disease without fully blocking infection [50, 51],
407 in line with our observations. Additionally, neutralising NA antibodies against N1 and N8 have been
408 detected in wild ducks in North America [52]. Interestingly, H5N1-W2-specific antisera in our study
409 exhibited broader cross-subtype reactivity, inhibiting both H5N1 and H5N8 PVs, while H5N8-W2
410 antisera reacted only with homologous PVs. This raises the hypothesis that NA-reactive antibodies may
411 persist following H5Nx infection, with H5N1-induced antibodies conferring broader cross-reactivity.
412 Notably, this pattern mirrors the epidemiological shift from H5N8 to H5N1 dominance between waves 1
413 and 2, raising the possibility that immunity to H5N8 may have facilitated the emergence of H5N1 by
414 allowing subsequent infection in previously exposed hosts.

415 To further examine the impact of prior subtype exposure on infection outcome, we de-engineered
416 the MBCS of H5N1-W1 and H5N8-W1 HPAIVs to contain a SBCS, minimising potential severe
417 outcomes ducks. The low pathogenicity phenotype of both mutants was confirmed by IVPI, as per
418 international guidelines [21]. Unexpectedly, for ducks inoculated with 10^7 EID_{50} of these de-engineered
419 viruses no viral shedding or seroconversion was detected. This contrasted with chickens in the IVPI,
420 which developed robust serological responses, including one mortality with vRNA detected in multiple
421 tissues, while retaining the SBCS in HA. Additionally, *in ovo* amplification produced similar viral titres
422 for both wildtype and de-engineered viruses, together suggesting the modified viruses remained
423 replication competent in animal systems. Collectively, these findings indicate that while the viruses are
424 viable, the MBCS is essential for productive infection of ducks with clade 2.3.4.4b H5Nx viruses.

425 Whilst the lack of seroconversion to the de-engineered viruses was unexpected, other host
426 responses outside of antibody generation are also likely important for infection outcome following AIV
427 infection, and so we investigated the effect of homosubtypic and heterosubtypic re-infection. Prior
428 exposure had limited impact on vRNA shedding following HPAIV challenge. However, in the N1/N1
429 group, shedding and environmental contamination were delayed compared to naïve H5N1-W2-infected

430 ducks, with fewer positive cloacal swabs. This delay was more pronounced in homologous (N1/N1) than
431 heterologous (N1/N8) challenge (4 vs. 2 days, respectively). Disease severity also varied depending on
432 the priming subtype. Prior SB-H5N1-W1 exposure reduced clinical scores and mortality following
433 challenge with either H5N1-W2 or H5N8-W2. Conversely, prior SB-H5N8-W1 exposure increased
434 disease severity and mortality for both challenge viruses. This may reflect differences in (**i**) infection
435 efficiency of the challenge virus, (**ii**) replication of the SB-H5N8-W1 virus, or (**iii**) host immune
436 modulation and the clonal nature of the priming vs. challenge viruses. Earlier studies found that pre-
437 exposure to LPAIV, especially with seroconversion, conferred protection from HPAIV-induced disease
438 [11, 53, 54], though these studies used older birds (13 weeks–18 months) compared to the 3-week-old
439 ducks used here. Age-related immune differences, particularly within the first six weeks post-hatch [55-
440 57], therefore may explain the differences observed. In the N8/N8 group, we obtained inverse results
441 following challenge, with the pre-exposed birds having higher mortality than the naïve/N8 group. Unlike
442 previous prior exposure studies that used genetically distinct LPAIVs [11, 54, 58], in the current study we
443 used RG to de-engineer the MBCS and retain close genetic relationships between de-engineered AIV W1
444 exposure and HPAIV W2 challenge. A recent study exposing mallards to a H4N6 found minimal
445 antibody responses, as we saw here, but detected evidence of complement activation early in infection
446 [59]. We did not investigate complement activation, but this could be a factor to be considered in future
447 studies of this type. While the exact mechanism remains uncertain, prior exposure does not greatly impact
448 viral shedding but does appear to alter disease severity. While cross-subtype immunisation and challenge
449 has been reported to cause vaccine-associated enhanced respiratory disease (VAERD) in pigs and ferrets
450 [60, 61], there have been no reports of VAERD in avian hosts, but subtype mismatch during our prior
451 exposure and challenge has shown an increase in clinical scores. When considering the epidemiological
452 switch in dominance from H5N8 to H5N1, birds would potentially be exposed to H5N8 and subsequently
453 encounter H5N1. In this scenario, our data suggest that prior exposure to H5N8 may not protect against
454 subsequent exposure to H5N8, but could allow ducks to tolerate H5N1 infection, although allowing

455 infection and transmission of H5N1 among Anseriformes. All N8/N1 ducks shed H5N1-W2 to similar
456 titres as the H5N1-W2 naïve birds, but 80% survived infection.

457 In conclusion, this study identifies distinct virological and serological differences between H5N1-
458 W2 and H5N8-W2 that may explain the rapid shift in subtype dominance during the 2021–2022 epizootic
459 in the UK and continental Europe. While both viruses demonstrated comparable shedding kinetics and
460 environmental contamination in infected ducks as surrogates for wild Anseriformes, H5N8-W2 exhibited
461 higher pathogenicity, leading to greater mortality. However, the delayed onset of shedding and the
462 increased survival associated with H5N1-W2 suggests that H5N1 may be more adept at sustaining
463 transmission, particularly in wild bird populations. Furthermore, NA-reactive antibodies were detected
464 following infection with both subtypes. Notably, N1 antisera cross-reacted with N8, but the reverse was
465 not observed, suggesting that prior H5N8 exposure may not impede subsequent H5N1 infection at the
466 wild waterfowl population scale. In addition, prior exposure may enhance the severity of disease outcome
467 outside of humoral responses, though mechanisms are unknown. Together these data suggest that in
468 ducks genotype A H5N8 from the second epizootic wave is more pathogenic than genotype AB H5N1
469 from the same period, and that H5N1 pathogenicity has decreased between epizootic waves, whereas
470 H5N8 pathogenicity has been maintained or increased. These phenotypic characteristics could favour
471 selection of H5N1 in a partially immune H5N8 population, but the ability of H5N1 viruses to at least in
472 part evade host antibody responses to H5N8 could create an environment in the natural reservoir
473 favouring their selection and persistence. These factors may have a direct correlation with the emergence
474 and complete shift to H5N1 HPAIVs where previously H5N8 was dominant.

475 Following initial selection and shift, H5N1 rapidly underwent reassortment to produce a highly
476 variable number of genotypes that also has likely contributed to the rapid dissemination and spread of this
477 subtype [10]. H5N1 has continued to emerge, reassort and genetically evolve with LPAIVs present in
478 wild birds, co-existing as multiple genotypes that through increasing opportunity for infection and fitness
479 in multiple avian species, remain in dynamic circulation on a global scale. These insights underscore the

480 complex interplay of viral fitness, host immunity, and environmental factors in shaping the dynamics of
481 gsGD-lineage H5Nx HPAIV epizootics. Certainly, a more robust assessment of different HPAIV
482 genotypes is required to understand the impact of these viruses on different avian populations.

483 # Author statements

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491 **Ethics and Safety Statement**

492 The *in vivo* study was reviewed and approved by the local Animal and Plant Health Agency (APHA)
493 Animal Welfare and Ethical Review Body (AWERB) to comply with the relevant UK legislation, in
494 accord with the UK Home Office (HO) Project License PP7633638. Material utilised for all
495 experimentation was initially isolated in EFEs under HO license P5275AD31. According to the UK's
496 Advisory Committee on Dangerous Pathogens (ACDP) and the Specified Animal Pathogens Order
497 (SAPO); the gsGd lineage H5Nx HPAIVs are classified as ACDP hazard group level three pathogens and
498 SAPO level four, hence all animal and laboratory work involving infectious material was conducted
499 within licensed containment level 3 facilities at APHA. Welfare monitoring of up to three times daily was
500 undertaken to assess the birds for humane endpoints following the onset of clinical disease, enabling any
501 decisions to be made concerning the need for euthanasia. All birds had access to food and water *ad*
502 *libitum*.

503 **Conflict of interest**

504 The authors declare no conflicts of interest.

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509 **Author contribution**

510 EB, CW, ST, SJ, SR, AS, AN, JJ and MJS conducted the *in vivo* experimental work. CDG, EB and ST
511 conducted serological work. JY and MI produced the viruses by reverse genetics. NT and KdC provided
512 logistical support and training for PVNA and pELLA. EB, CW, AMPB, JJ, MJS and CDG analysed the
513 data. EB, CW, CDG, MJS, IHB, ACB and JJ wrote the manuscript. JJ, ACB and IHB secured funding.

514 Figure Legends

515 **Fig. 1. Virus shedding, environmental contamination, and survival from ducks infected with H5N1-**
516 **W2 and H5N8-W2 HPAIVs.**

517 **A and B**. Viral shedding measured from swab samples collected from the oropharyngeal (Op; A) and
518 cloacal (C; B) cavities of naïve ducks following infection with H5N1-W2 (blue) or H5N8-W2 (orange)
519 HPAIV. Viral RNA (vRNA) titres were quantified by M-gene RT-PCR and expressed as $\log_{10}$ relative
520 expression units (REU). Individual data points are shown, with coloured lines indicating the group mean.
521 The dotted horizontal line represents the diagnostic Ct threshold (Ct 36.00; $10^{1.794}$ REU) for M-gene RT-
522 PCR positivity. **C and D**. Heatmaps representing quantification of vRNA ($\log_{10}$ REU) for H5N1-W2 (**C**)
523 and H5N8-W2 (**D**). The top two rows of each panel correspond to the mean shedding from OP and
524 cloacal C swabs, as shown in panels A and B. Environmental samples (drinker water, pond water, and air)
525 were collected on alternate days. Heatmap shading indicates vRNA detection: white denotes no Ct value,

526 grey represents samples below diagnostic threshold ($10^{1.794}$ REU), and blue indicates positive samples. **E.**
527 Percentage survival of ducks over a 14-day period post-infection (dpi) with H5N1-W2 (blue) or H5N8-
528 W2 (orange) HPAIV.

529 **Fig. 2. Virus distribution in tissues of ducks infected with H5N1-W2 or H5N8-W2 HPAIV.**

530 Quantification of viral RNA (vRNA) in tissue samples collected from nine ducks infected with H5N1-W2
531 (n = 3) or H5N8-W2 (n = 6) HPAIV. Ducks were euthanised due to severe clinical signs or culled at the
532 study endpoint (14 days post-infection, dpi). vRNA levels were measured by M-gene RT-PCR and
533 expressed as $\log_{10}$ relative expression units (REU), represented as a heatmap. Samples with Ct values
534 below the diagnostic threshold ($10^{1.794}$ REU) are shown in grey, positive samples in blue, and samples
535 with no detectable Ct value in white. **B.** Semi-quantitative scoring of anti-NP staining intensities and
536 distribution in immunohistochemistry (IHC) analysis of tissues (lower to higher intensities, 0-4), while
537 the "X" indicates that no sample was available for IHC.

538 **Fig. 3. Serological responses and antigenic characterization of H5N1-W2 and H5N8-W2.**

539 Serological responses using sera collected from ducks at 14 dpi infected with H5N1-W2 AB (blue) or
540 H5N8-W2 A (orange); sera assessed using HI (**A**) or lentiviral pseudotype viruses (PVs) containing
541 H5Nx influenza virus HA or HA and NA glycoproteins (**B-D**). Coloured horizontal bars indicate
542 geometric mean titres, while the dotted line indicates the diagnostic threshold for positivity. **A**. HI using
543 sera from ducks infected with H5N1-W2 or H5N8-W2, against antigens with homologous or
544 heterologous NAs from 2021 or 2020. **B.** PV neutralisation titres (inhibition concentration 50%; IC_{50})
545 using PVs containing H5 HA only evaluated against individual sera from H5N1-W2 or H5N8-W2
546 infected ducks. (**C-D**) Sera assessed by pELLA using PVs containing HA and NA glycoproteins tested
547 against pre-infection (green), homologous subtype positive control (red), H5N1-W2 (blue) or H5N8-W2
548 (orange) tested against post-infection sera from H5N1-W2 or H5N8-W2 infected ducks.

549 **Fig. 4. Virus shedding from ducks infected with H5N1-W2 and H5N8-W2 HPAIV with prior**
550 **exposure to de-engineered H5N1-W1 and H5N8-W1 AIVs.**

551 Groups of ducks were initially inoculated with either H5N1-W1 (N1) or H5N8-W1 (N8) de-engineered
552 AIVs. After 14 days post-inoculation (dpi) the groups were divided, and ducks were challenged with
553 either HPAIV H5N1-W2 or H5N8-W2. This resulted in homologous (N1/N1 **(A and E)** and N8/N8 **(D**
554 **and H)**, or heterologous (N1/N8 **(B and F)** and N8/N1 **(C and G)** subtype combinations. Shedding was
555 measured from oropharyngeal (OP) (**A-D**) and cloacal (C) (**E-H**) swabs following challenge. Shedding
556 from naïve birds also shown for reference. vRNA titres were determined by M-gene RT-PCR and
557 expressed as $\log_{10}$ REU. Individual shedding values are shown by coloured symbols, with group mean
558 shedding shown by continuous coloured lines. Dotted horizontal lines indicate the M-gene RT-PCR
559 positive cut-off ($10^{1.794}$ REU).

560 **Fig. 5. Viral environmental contamination from ducks challenged with H5N1-W2 and H5N8-W2**
561 **HPAIV, following prior exposure to de-engineered H5N1-W1 and H5N8-W1 AIVs.**

562 Environmental specimens were collected between 1-13 dpc from drinker and pond waters of ducks. The
563 ducks had been challenged with HPAIV H5N1-W2 or H5N8-W2 but previously been exposed to
564 homologous (N1/N1 **(A)** and N8/N8 **(C)**) or heterologous (N1/N8 **(D)** and N8/N1 **(B)**) subtype of de-
565 engineered H5N1-W1 or H5N8-W1 AIVs. vRNA titres were determined by the M-gene RT-PCR.
566 Samples shown as a heatmap with no Ct in white, below the diagnostic cut-off ($10^{1.794}$ REU) shown in
567 grey, positive samples in blue. 'X' indicates that no sample was collected at the specific time point. The
568 top two rows on all graphs represent the mean oropharyngeal (OP) and cloacal (C) shedding titres from
569 all birds.

570 **Fig. 6. Survival of ducks challenged with H5N1-W2 and H5N8-W2 HPAIV, with or without prior**
571 **exposure to de-engineered H5N1-W1 and H5N8-W1 AIVs.**

572 Percentage survival of ducks until 14 dpc following challenge with (A) HPAIV H5N1-W2 or (B) H5N8-
573 W2 with no prior exposure (hashed lines), or ducks which had previously been exposed to (solid lines)
574 homologous (N1/N1 and N8/N8) or heterologous (N1/N8 and N8/N1) subtypes of de-engineered H5N1-
575 W1 or H5N8-W1 AIVs.

576 **Fig. 7. Organ tropism in ducks with prior exposure to de-engineered AIVs before H5N1-W2 and**
577 **H5N8-W2 HPAIV challenge.**

578 Tissue samples were collected from six ducks following H5N1-W2 or H5N8-W2 HPAIV challenge,
579 which had previously been exposed to a homologous (N1/N1 and N8/N8) or heterologous (N1/N8 and
580 N8/N1) subtype of de-engineered H5N1-W1 or H5N8-W1 AIVs. All ducks were euthanised at indicated
581 times (dpc) as the result of severe clinical signs. **A.** vRNA levels, measured by M-gene RT-PCR,
582 expressed as $\log_{10}$ REU and represented as a heatmap. Samples below the diagnostic cut-off ($10^{1.794}$ REU)
583 shown in grey, positive samples in blue, and no Ct in white. Individual bird identifiers allow for
584 comparisons to serological response graphs and IHC-staining results. **B.** Semi-quantitative scoring of
585 anti-NP staining intensities and distribution in IHC analysis of tissues (lower to higher intensities, 0-4),
586 demonstrating abundant viral antigen in the brain, heart and muscle following N1/N8 and N8/N8
587 challenge.

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760

Fig. 1

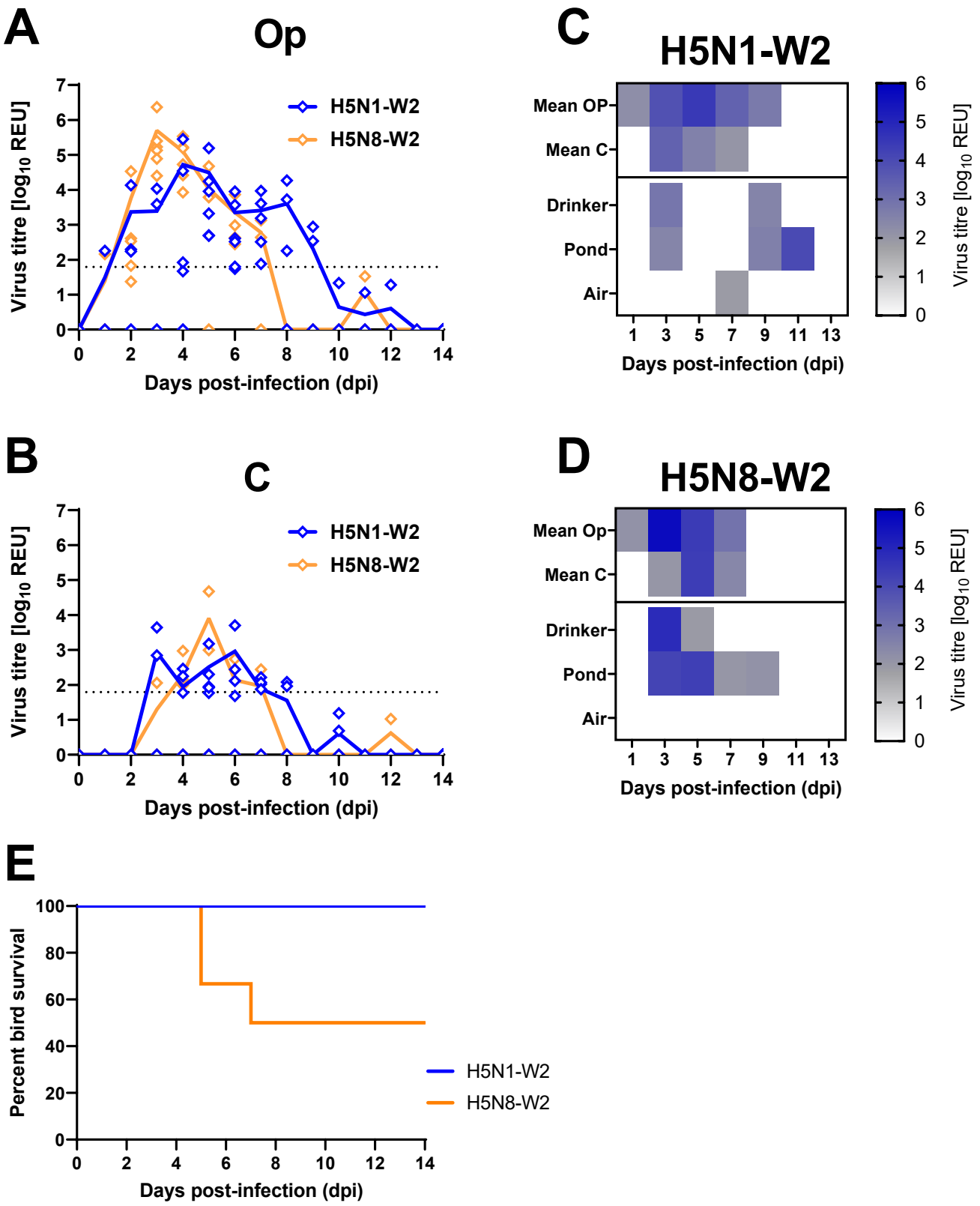

A
Op
H5N1-W2
H5N8-W2
Virus titre [log10 REU]
Days post-infection (dpi)
C
H5N1-W2
Mean OP
Mean C
Drinker
Pond
Air
Days post-infection (dpi)
Virus titre [log10 REU]
B
C
H5N1-W2
H5N8-W2
Virus titre [log10 REU]
Days post-infection (dpi)
D
H5N8-W2
Mean Op
Mean C
Drinker
Pond
Air
Days post-infection (dpi)
Virus titre [log10 REU]
E
Percent bird survival
Days post-infection (dpi)
H5N1-W2
H5N8-W2

Fig. 2

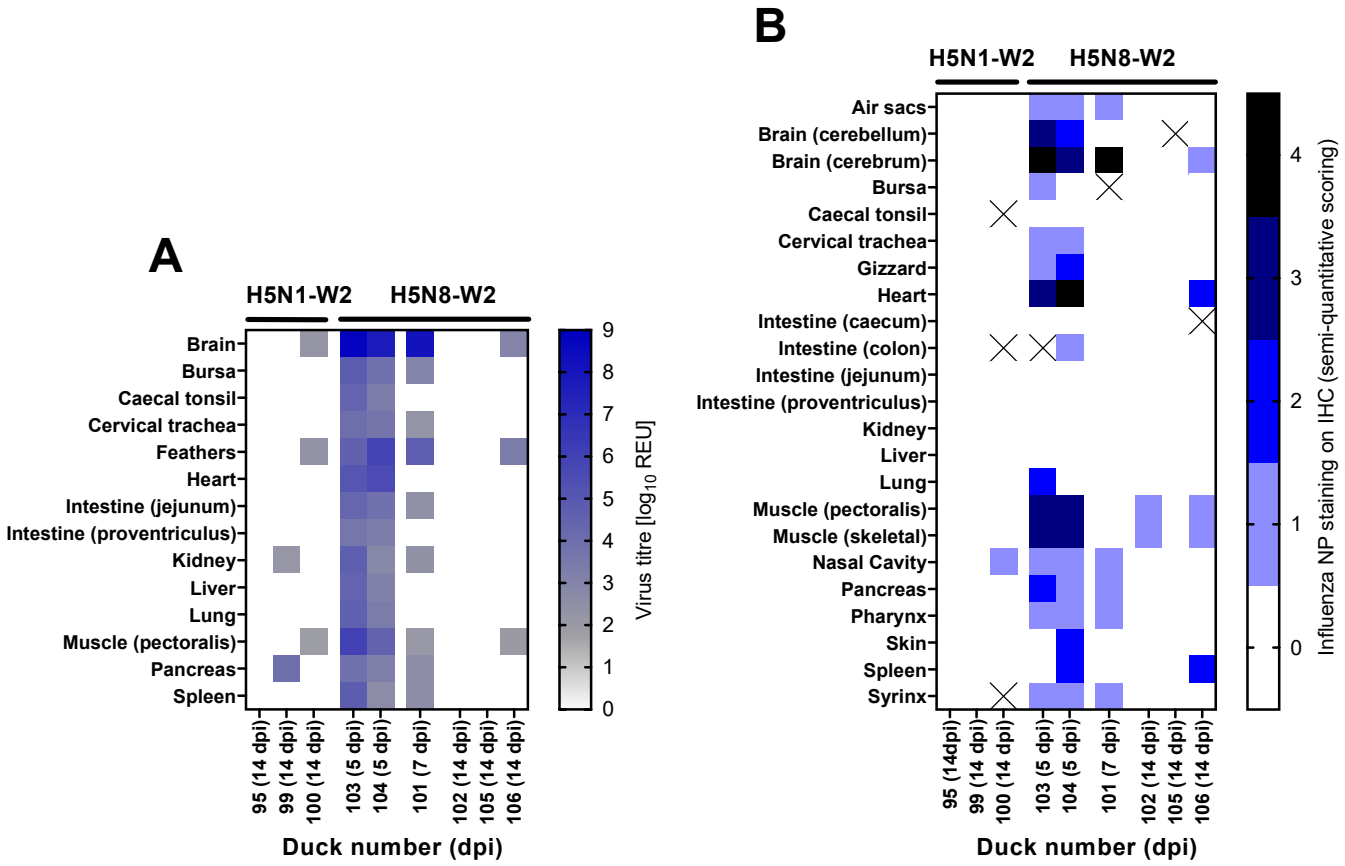

A
H5N1-W2
H5N8-W2
Brain
Bursa
Caecal tonsil
Cervical trachea
Feathers
Heart
Intestine (jejunum)
Intestine (proventriculus)
Kidney
Liver
Lung
Muscle (pectoralis)
Pancreas
Spleen
95 (14 dpi)
99 (14 dpi)
100 (14 dpi)
103 (5 dpi)
104 (5 dpi)
101 (7 dpi)
102 (14 dpi)
105 (14 dpi)
106 (14 dpi)
Duck number (dpi)
Virus titre [log10 REU]
0
1
2
3
4
5
6
7
8
9
B
H5N1-W2
H5N8-W2
Air sacs
Brain (cerebellum)
Brain (cerebrum)
Bursa
Caecal tonsil
Cervical trachea
Gizzard
Heart
Intestine (caecum)
Intestine (colon)
Intestine (jejunum)
Intestine (proventriculus)
Kidney
Liver
Lung
Muscle (pectoralis)
Muscle (skeletal)
Nasal Cavity
Pancreas
Pharynx
Skin
Spleen
Syrinx
95 (14dpi)
99 (14 dpi)
100 (14 dpi)
103 (5 dpi)
104 (5 dpi)
101 (7 dpi)
102 (14 dpi)
105 (14 dpi)
106 (14 dpi)
Duck number (dpi)
Influenza NP staining on IHC (semi-quantitative scoring)
0
1
2
3
4

Fig. 3

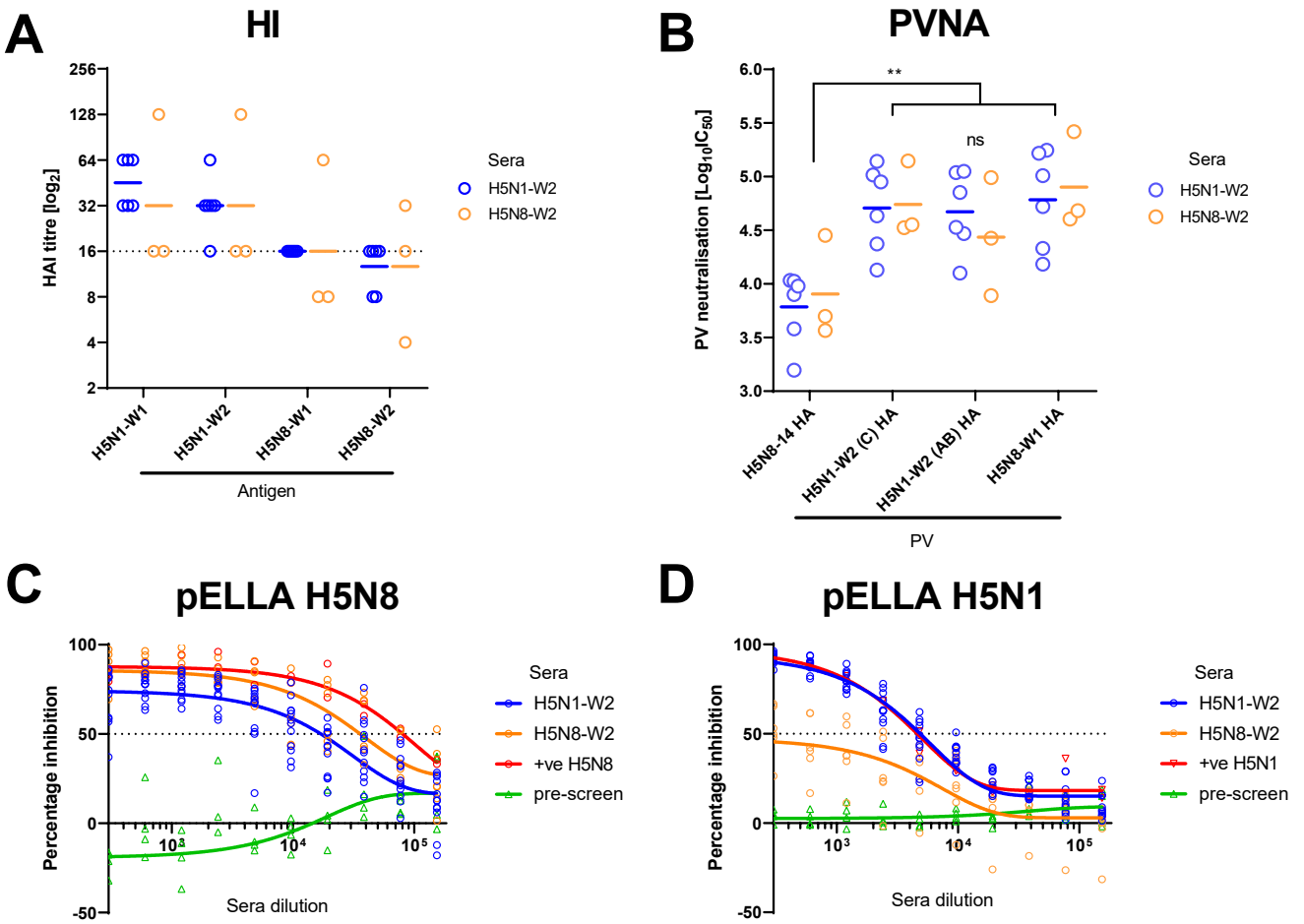

A
HI
HAI titre [log2]
256
128
64
32
16
8
4
2
H5N1-W1
H5N1-W2
H5N8-W1
H5N8-W2
Antigen
Sera
H5N1-W2
H5N8-W2
B
PVNA
PV neutralisation [Log10IC50]
6.0
5.5
5.0
4.5
4.0
3.5
3.0
**
ns
H5N8-14 HA
H5N1-W2 (C) HA
H5N1-W2 (AB) HA
H5N8-W1 HA
PV
Sera
H5N1-W2
H5N8-W2
C
pELLA H5N8
Percentage inhibition
100
50
0
-50
10³
10⁴
10⁵
Sera dilution
Sera
H5N1-W2
H5N8-W2
+ve H5N8
pre-screen
D
pELLA H5N1
Percentage inhibition
100
50
0
-50
10³
10⁴
10⁵
Sera dilution
Sera
H5N1-W2
H5N8-W2
+ve H5N1
pre-screen

Fig. 4

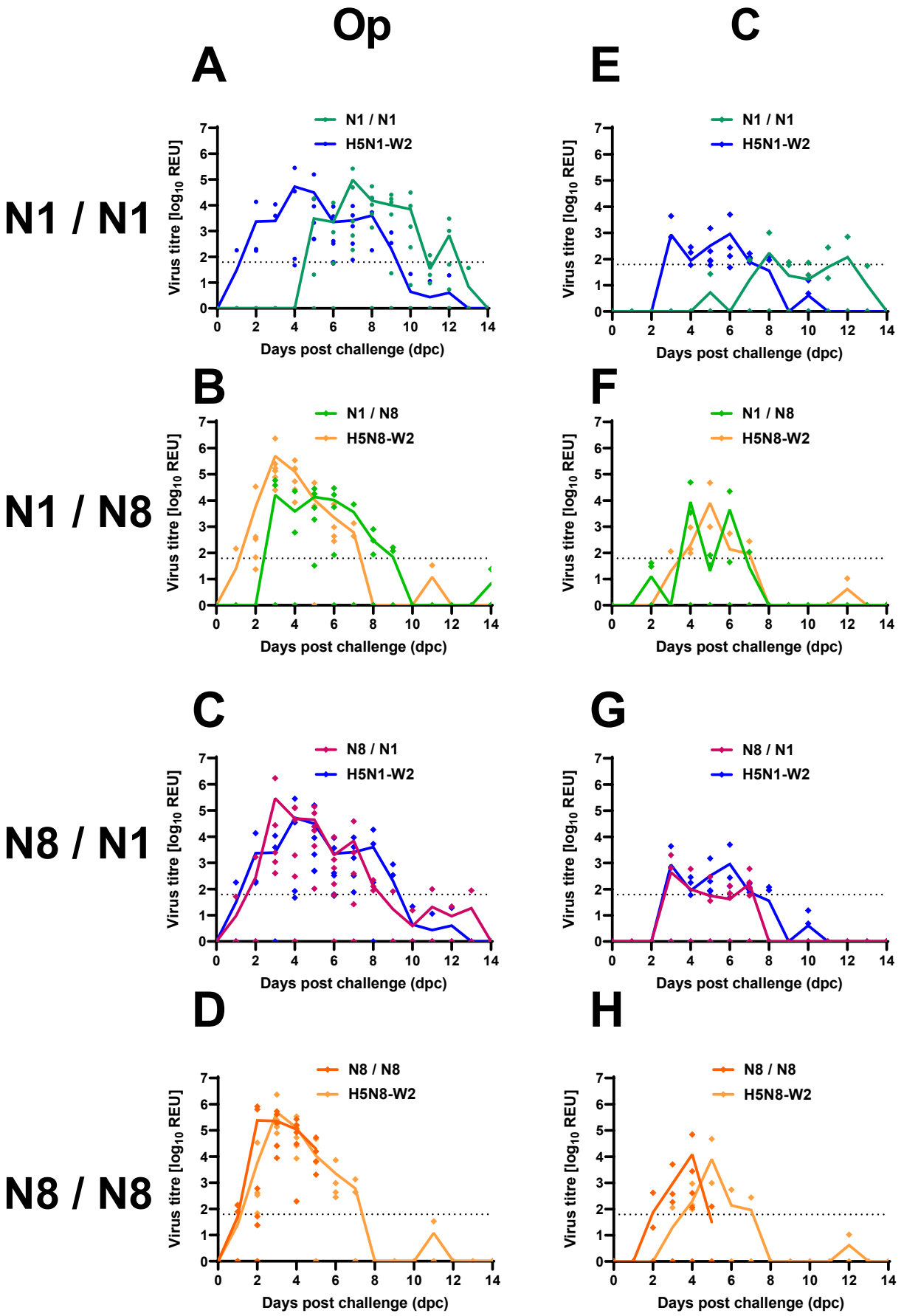

Op
C
A
E
N1 / N1
N1 / N1
H5N1-W2
Virus titre [log10 REU]
Days post challenge (dpc)
B
F
N1 / N8
N1 / N8
H5N8-W2
C
G
N8 / N1
N8 / N1
H5N1-W2
D
H
N8 / N8
N8 / N8
H5N8-W2

Fig. 5

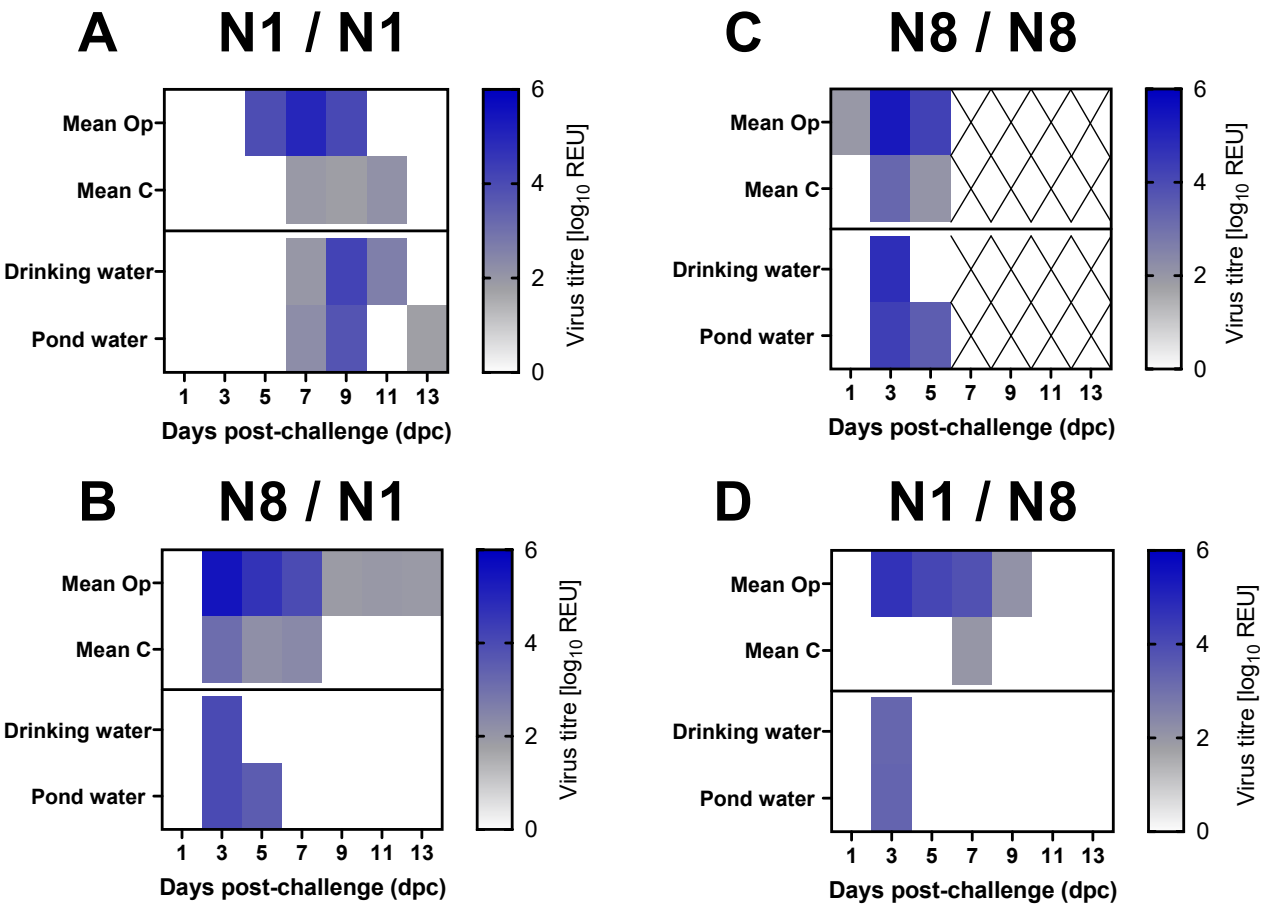

A
N1 / N1
Mean Op
Mean C
Drinking water
Pond water
1 3 5 7 9 11 13
Days post-challenge (dpc)
6
4
2
0
Virus titre [log10 REU]
C
N8 / N8
Mean Op
Mean C
Drinking water
Pond water
1 3 5 7 9 11 13
Days post-challenge (dpc)
6
4
2
0
Virus titre [log10 REU]
B
N8 / N1
Mean Op
Mean C
Drinking water
Pond water
1 3 5 7 9 11 13
Days post-challenge (dpc)
6
4
2
0
Virus titre [log10 REU]
D
N1 / N8
Mean Op
Mean C
Drinking water
Pond water
1 3 5 7 9 11 13
Days post-challenge (dpc)
6
4
2
0
Virus titre [log10 REU]

Fig. 6

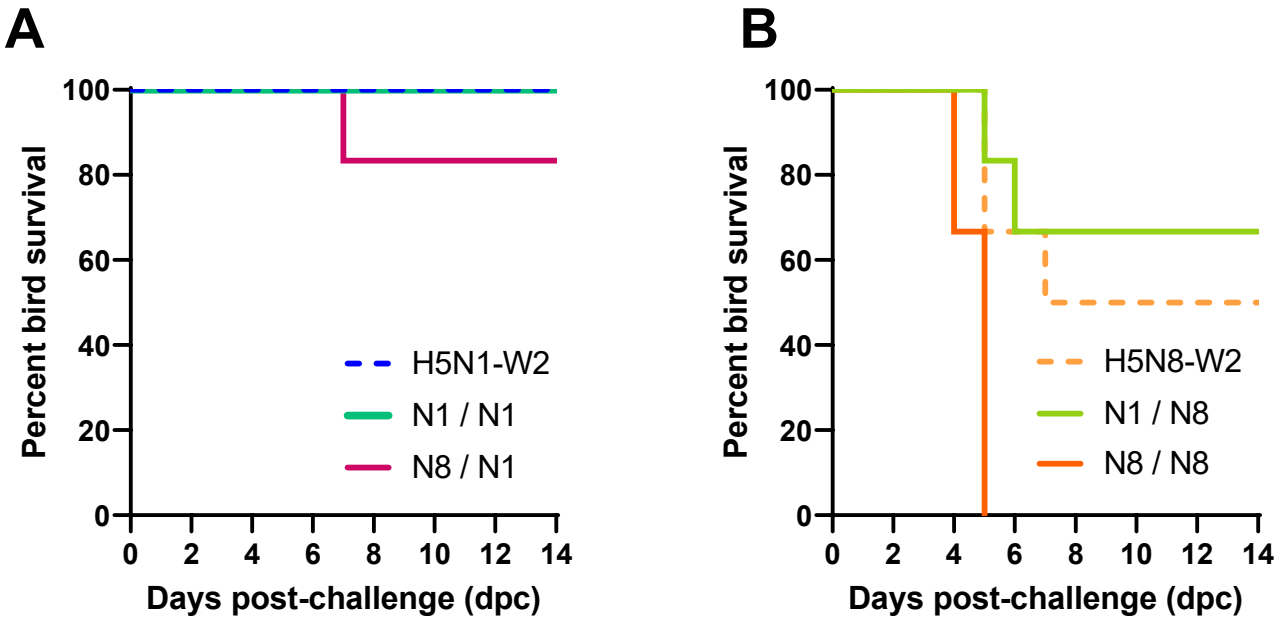
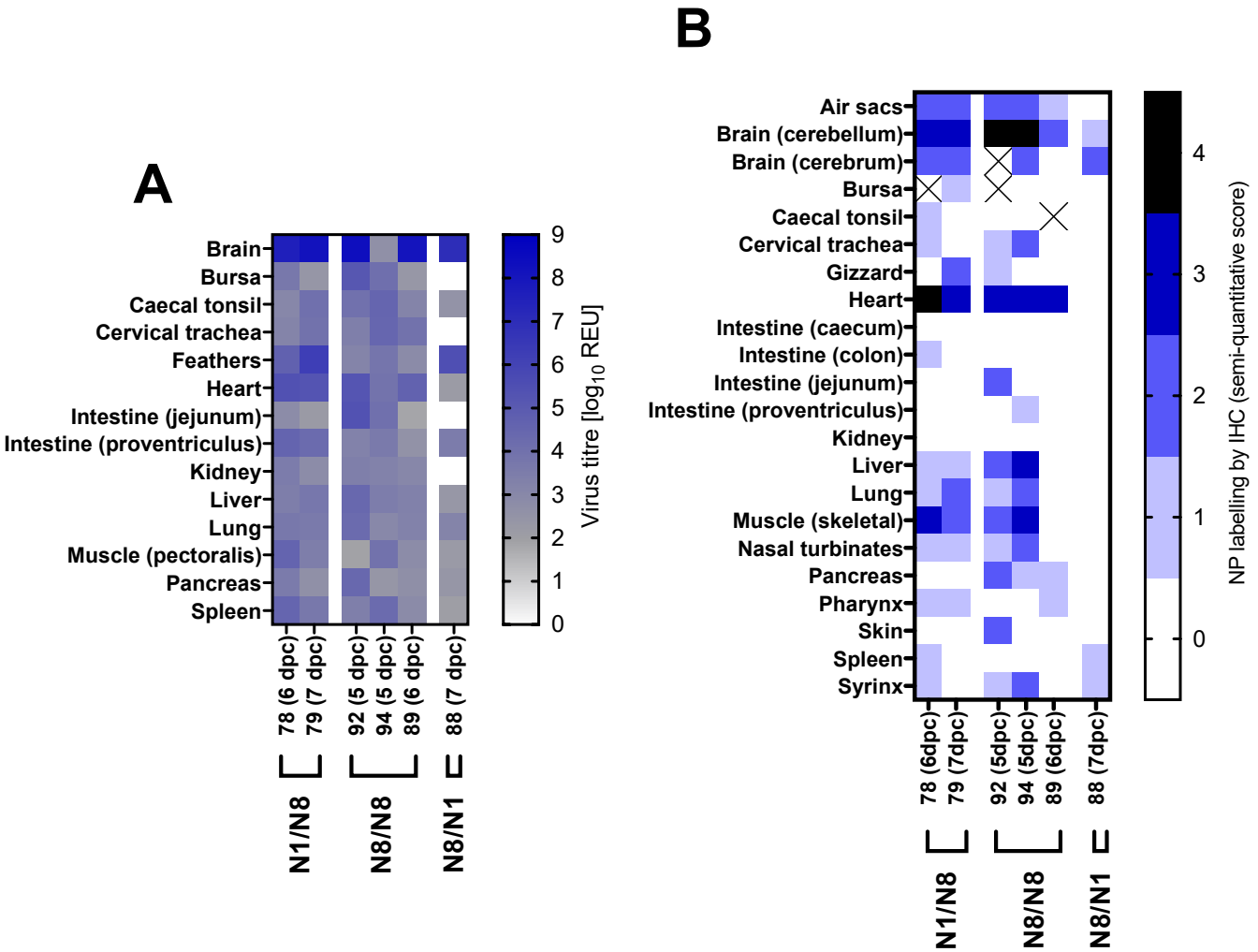

A
100
80
60
40
20
0
Percent bird survival
0
2
4
6
8
10
12
14
Days post-challenge (dpc)
H5N1-W2
N1 / N1
N8 / N1
B
100
80
60
40
20
0
Percent bird survival
0
2
4
6
8
10
12
14
Days post-challenge (dpc)
H5N8-W2
N1 / N8
N8 / N8

Fig. 7


A
Brain
Bursa
Caecal tonsil
Cervical trachea
Feathers
Heart
Intestine (jejunum)
Intestine (proventriculus)
Kidney
Liver
Lung
Muscle (pectoralis)
Pancreas
Spleen
78 (6 dpc)
79 (7 dpc)
92 (5 dpc)
94 (5 dpc)
89 (6 dpc)
88 (7 dpc)
N1/N8
N8/N8
N8/N1
Virus titre [log10 REU]
0
1
2
3
4
5
6
7
8
9
B
Air sacs
Brain (cerebellum)
Brain (cerebrum)
Bursa
Caecal tonsil
Cervical trachea
Gizzard
Heart
Intestine (caecum)
Intestine (colon)
Intestine (jejunum)
Intestine (proventriculus)
Kidney
Liver
Lung
Muscle (skeletal)
Nasal turbinates
Pancreas
Pharynx
Skin
Spleen
Syrinx
78 (6dpc)
79 (7dpc)
92 (5dpc)
94 (5dpc)
89 (6dpc)
88 (7dpc)
N1/N8
N8/N8
N8/N1
NP labelling by IHC (semi-quantitative score)
0
1
2
3
4