Pigeons exhibit low susceptibility and poor transmission capacity for H5N1 clade 2.3.4.4b high pathogenicity avian influenza virus

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Abstract

The ongoing panzootic of H5N1 high pathogenicity avian influenza virus (HPAIV) has caused the deaths of over half a billion wild birds and poultry, and has led to spillover events in both wild and domestic mammals, alongside sporadic human infections. A key driver of this panzootic is the apparent high viral fitness across diverse avian species, which facilitates an increased interface between wild and domestic species. *Columbiformes* (pigeons and doves) are commonly found on poultry premises, yet little is known about their potential role in contemporary HPAIV disease ecology. Here we investigated the epidemiological role of pigeons (*Columba livia*) by determining their susceptibility using decreasing doses of clade 2.3.4.4b H5N1 HPAIV (genotype AB). We investigated infection outcomes and transmission potential between pigeons and to chickens. Following direct inoculation, pigeons did not develop clinical signs, and only those inoculated with the highest dose shed viral RNA or seroconverted to H5N1-AB, revealing a MID_{50} of 10^5 EID_{50}. Even in the high-dose group, only low-level shedding and environmental contamination was observed, and low-level viral RNA were present in the tissues of directly inoculated pigeons, with no distinct pathological lesions. Pigeons did not transmit the virus to pigeons or chickens placed in direct contact. We observed distinct differences in sialic acid receptor distribution in the pigeon respiratory tract compared to chickens and ducks. Together, these findings suggest that pigeons have low susceptibility to clade 2.3.4.4b H5N1 HPAIV and are unlikely to contribute significantly to virus maintenance, transmission to poultry, or zoonotic infection.

Word count: 248

43 # Introduction

44 Avian influenza viruses (AIVs) are a diverse group of economically important pathogens, classified
45 according to their subtype determined by their haemagglutinin (HA, H1-H16 and H19) and
46 neuraminidase (NA, N1-N9) genes [1, 2]. High pathogenicity AIV (HPAIV) outbreaks in *gallinaceous*
47 poultry (e.g. chickens and turkeys), are typified by rapid onset of high morbidity and mortality [3].
48 Among HPAIVs, H5Nx viruses of the A/goose/Guangdong/1/1996 (gs/Gd) lineage remain a major
49 global concern and continue to evolve into new clades based on the phylogeny of the HA glycoprotein
50 [4]. In addition, gs/Gd lineage H5Nx viruses have undergone extensive reassortment among the NA and
51 internal genetic segments creating a range of different subtypes and genotypes within each subtype [5].
52 Europe has experienced clade 2.3.4.4 epizootics since 2014 [6], where clade 2.3.4.4b has been
53 particularly prominent since autumn 2020 [5, 7, 8], with significant wild bird mortality and damaging
54 economic impacts upon the poultry industry [9]. During autumn 2021, H5N1 emerged as the dominant
55 subtype among European clade 2.3.4.4b HPAIVs and has been maintained and disseminated in wild
56 bird populations, particularly aquatic bird species, facilitating spread to the Americas (North America
57 and then South America) via bird migration [10], and onward towards the Antarctic region [11]. This is
58 the largest recorded HPAIV panzootic in terms of detections in wild birds and numbers of poultry
59 outbreaks [12]. Alongside high infection in wild bird and poultry, there have been numerous reports of
60 infections in wild and domestic mammals with clade 2.3.4.4b H5N1, including sustained infection in
61 domestic cattle in the United States of America (USA), and sporadic human infections, which underline
62 the zoonotic potential and pandemic risk [13]. Since 2021, a high degree of genetic diversity has been
63 observed within the H5N1 clade 2.3.4.4b HPAIV, particularly within the internal gene segments though
64 reassortment with local indigenous low pathogenicity avian influenza viruses (LPAIVs). As a result,
65 over 12 different genotypes have been detected in wild birds or poultry in the United Kingdom (UK)
66 alone, including genotype AB among the dominant genotypes, which was also the dominant genotype
67 in continental Europe [5, 14].

68 A key hallmark of this H5N1 clade 2.3.4.4b HPAIV panzootic is the broad avian host range
69 which the viruses exhibit, and the apparent increased fitness in some wild bird species compared to

70 previous H5Nx gs/Gd HPAIVs [12, 15, 16]. In the UK, there have been extensive infections in seabird
71 populations including *Laridae* (gulls) and *Sulidae* (e.g. gannets) [17, 18]. Among wild bird species, the
72 order *Columbiformes* (pigeon and doves) have been viewed as potential bridging species to captive
73 birds and humans for several infectious diseases [19, 20]. Pigeon behaviour includes foraging on
74 agricultural land, along with colonisation of urban habitats shared with waterfowl (in municipal parks)
75 and humans [21-23]. *Columbiformes* exist as a single family called *Columbidae*, consisting of 41 groups
76 of pigeons and doves, and representing over 300 species [24], and the terms pigeon and dove are used
77 interchangeably for any of the species. *Columbiformes* are very common worldwide, and five species
78 of *Columbidae* are regularly seen in the UK, including the feral pigeon (*Columba livia*), stock dove (*C.*
79 *oenas*), wood pigeon (*C. palumbus*), collared dove (*Streptopelia decaocto*) and turtle dove (*S. turtur*)
80 [24]. Feral pigeons, common pigeons, racing pigeons, city pigeons and rock doves are all the same
81 species (*C. livia*) which descended from wild rock doves following domestication at least 2000–5000
82 years ago; they are abundant worldwide, and have high connectivity with human settlements [24].
83 Historically, *Columbiformes* have been regarded as a relatively unimportant hosts for AIV
84 infections, including HPAIVs [25-27]. However, multiple different subtypes of AIV have been
85 identified in pigeons, including H1, H2, H3, H5, H6, H7, H9 and H11, suggested at least a limited
86 capacity for pigeons to harbour certain influenza viruses [28]. In addition, the emergence and global
87 spread of clade 2.3.4.4b H5N1 HPAIVs since autumn of 2021 has been attributed in part to the broad
88 species range of these viruses, yet relatively little is known about the role of pigeons during this
89 panzootic. Through recent passive wild bird surveillance programmes (targeting wild birds which are
90 found dead) in the UK and continental Europe, few HPAIV H5Nx positive pigeons have been reported
91 [12, 29], suggesting an absence of high mortality in pigeons, but also reducing surveillance samples,
92 and still leaving questions on their infection status. Similarly, infrequent mortalities have been observed
93 in free-living *Columbiformes* in other regions, such as in the USA [30]. However, clade 2.3.4.4b
94 detections appear to be an overrepresented clade in pigeons compared to other H5 clades or subtypes
95 [28]. Therefore, given these considerations, it remains unclear whether subclinical infection and
96 transmission of these HPAIV can occur in pigeons. Their frequent presence at poultry facilities and
97 close association with humans underscore the need to assess the potential role pigeons may play, not

only in terms of their susceptibility to contemporary clade 2.3.4.4b HPAIV, but also in determining whether they are capable of sustaining inapparent infections within their populations. This is essential to evaluate their capacity to act as a bridging host for virus transmission to commercial poultry species, such as chickens, and to assess any associated zoonotic risk [19].

Here we selected one of the major European H5N1 clade 2.3.4.4b H5N1 HPAIV genotypes, namely AB (AIV09), which became epidemiologically prominent during 2022 [5, 8]. We used this virus to quantify the susceptibility of pigeons (*C. livia*) to H5N1 clade 2.3.4.4b HPAIV, and to investigate the impact in terms of clinical disease, pathology, tissue tropism, seroconversion and viral shedding dynamics following infection. We also explored the ability of pigeons to maintain infection by assessing pigeon-to-pigeon transmission and assessed the ability of pigeons to act as bridging species to poultry by assessing pigeon-to-chicken transmission.

Methods

Virus

The genotype of H5N1 clade 2.3.4.4b HPAIV used for this study was A/chicken/England/014330/2022 (H5N1) (GISAID accession number: EPI_ISL_13370704), herewith referred to as H5N1-AB. This isolate is representative of the dominant genotype which circulated in the UK and continental Europe during the 2021-2023 epizootic and is classified as genotype AB or AIV-09 as defined by the European Union Reference Laboratory (EURL) [31] and Byrne, James [5] respectively. H5N1-AB was propagated in 9-day-old specified pathogen free (SPF) embryonated fowls' eggs (EFE) and titrated in EFEs to determine 50% egg infectious dose (EID_{50}), as previously described [15]. The H5N1-AB stock was diluted in 0.1M pH 7.2 phosphate buffered saline (PBS) to prepare the desired dose for all *in vivo* infections. The precise dose was confirmed by titration of the inoculum in EFEs.

Animals and pre-sampling

Forty-eight domestic pigeons (*C. livia*) and twenty-four high-health-status Hy-Line Brown chickens (*Gallus gallus*) were sourced from a commercial UK supplier, and Hy-Line UK Ltd (Studley, UK), respectively. The pigeons were quarantined for five weeks in a separate enclosure by the supplier.

124 Pigeons were approximately three months old at the time of procurement and quarantined for a further
125 two weeks upon arrival in the high containment animal facility. The high-health-status chickens were
126 reared from one-day-old to three-weeks of age in clean environment. Both species were acclimatised
127 for a minimum of seven days prior to infection. The birds were housed under controlled conditions,
128 with temperatures maintained at 21–22°C and relative humidity between 50–60%. Prior to inoculation,
129 approximately 1 ml of blood was collected from the brachial vein of each bird, and oropharyngeal (Op)
130 and cloacal (C) swabs were obtained. All birds tested negative for antibodies against H5N1-AB via
131 haemagglutination inhibition (HI) assay and were also negative for influenza A viral RNA (vRNA) in
132 swab samples.

133 ***In vivo* study design**

134 To assess a response to three different H5N1-AB inoculation doses, three separate identical rooms were
135 used, with pigeons and chickens randomly allocated into experimental groups. For a single given dose
136 (and room), three groups were used, consisting of; (**i**) directly inoculated pigeons (n=8), (**ii**) contact
137 pigeons (n=8), and (**iii**) contact chickens (n=8) (Figure S1), where the 'donor' (D0) and 'recipient' (R1)
138 birds were designated as directly inoculated and contact birds, respectively. For each dose (and room),
139 pigeons were housed in "walk-in chicken runs" (Omlet, UK), consisting of a plastic-coated metal cage
140 measuring 2.2 × 2.2 × 1.5 metres. These enclosures were equipped with wall-hanging and floor-located
141 feeding troughs and drinkers, side-mounted apex, and flat perches and nesting boxes. Chickens were
142 housed on the floor of the same cages on autoclaved straw litter, with access to floor-located feeding
143 troughs, drinkers, scratch mats, and boxed shelters. On the inoculation day, the D0 pigeons were
144 separated for six hours from the R1 birds (pigeons and chickens) and inoculated with 0.1 mL of
145 phosphate-buffered saline (PBS) containing H5N1-AB, via the intra-nasal and intra-ocular routes. The
146 majority of the inoculum was administered intra-nasally, and a single drop (~20 µl) was administered
147 intra-ocularly. The inoculum doses were 10^2 (low), 10^4 (medium), and 10^6 (high) EID_{50}. At 3 days post-
148 infection (dpi), two D0 pigeons from each dose group were culled for post-mortem (PM) analysis. All
149 surviving birds were culled at 14 dpi, with PM analysis also performed at this time on three D0 pigeons
150 from the high-dose room only.

151 **Clinical samples and processing**

152 Op and C swabs were taken daily from all birds from 1 to 14 dpi. Swabs were immersed in 1 mL
153 Leibovitz L-15 Medium (L-15) (Gibco, USA) and stored at -80˚C until required for RNA
154 extraction. Blood was collected at 14 dpi by cardiac puncture, under terminal anaesthesia, from all
155 surviving birds. Sera were separated from clotted blood by centrifugation, heat-inactivated at 56°C for
156 30 minutes and used for downstream serological analyses. At PM a range of tissues were collected
157 including feathers, cervical trachea, pectoralis muscle, heart, liver, pancreas, spleen, kidney, lung, caecal
158 tonsil, bursa, jejunum, caecum, ileum, colon, proventriculus and brain. Tissues were stored in bijoux
159 tubes at -80˚C, and a section of each was transferred into L-15 medium and roughly chopped;
160 supernatants were used for RNA extraction. In addition, environmental samples were collected before
161 inoculation and daily from 1-10 dpi, then at 12 and 14 dpi from the high dose room only as drinking
162 water (wall-hanging and floor located drinkers from pigeons and chickens, respectively) and faeces
163 (pigeon faeces were collected from high perches and/or nesting boxes). Additionally, the chicken
164 scratch-mat was swabbed, and a sample of soiled straw-litter taken. Pigeon feathers were also collected
165 daily in the housing environment, with these being identified by their appearance and colour, to
166 determine the species of origin. RNA was extracted directly from all liquid samples, swabs (scratch
167 mat) were processed as described above, and solid samples (faeces, straw litter and environmental-
168 origin feathers) were prepared as a 10% (volume/volume) suspension in PBS (pH 7.2) as previously
169 described [32].

170 **Clinical scoring and monitoring**

171 Clinical inspections were carried out twice daily from 1 to 3 dpi, then daily until end of study (14 dpi).
172 Birds were monitored against pre-defined clinical score sheets as previously described [15] by
173 experienced animal technicians.

174 **RNA Extraction and real-time reverse transcription-PCR (RT-PCR)**

175 vRNA was extracted from swabs, environmental samples and PM tissue homogenates, using MagMAX
176 CORE extraction chemistry (Applied Biosystems, Warrington, UK) upon a Kingfisher (Thermo Fisher
177 Scientific, Glasgow, UK) as previously described [15]. For H5N1-AB vRNA detection, matrix (M)-

178 gene RT-PCR primers and probes were used [33]. A ten-fold dilution series of H5N1-AB vRNA at a
179 known EID_{50} was used to establish standard curves using MXPro software (Aria) to determine relative
180 equivalent units (REU), as previously described [15]. For M-gene RT-PCR, Cq values <36.00 (>$10^{1.98}$
181 REU) were considered as AIV positive, sub-threshold values in the range Cq 36.01–39.99 and Cq 40.00
182 (No Cq) were discussed, but were interpreted as negative as previously defined [15]. For sample
183 producing a subthreshold Cq value by M-gene RT-PCR, an H5 RT-PCR, which specifically detects
184 clade 2.3.4.4b H5 HPAIV RNA [34] was also performed. An individual bird was considered as
185 productively infected based on at least one positive swab and/or positive seroconversion to H5N1-AB
186 (described below).

187 **Serology**

188 Sera were tested for H5N1-AB HA reactive antibodies by the haemagglutination inhibition (HI) assay
189 using four haemagglutination units of H5N1-AB. Pigeon sera were pre-absorbed with packed chicken
190 red blood cells prior to testing as previously described [35]. Sera with a reciprocal HI titre ≥16 were
191 considered positive, values <16 are discussed but considered negative according to international
192 standards [35].

193 **Pathology and immunohistochemistry (IHC)**

194 Following post-mortem, harvested tissue samples were fixed in 10% (v/v) buffered formalin for a
195 minimum period of five days and routinely processed for histopathology, as previously described [36].
196 Briefly, four-micron thick serial tissue sections were either immunolabelled against influenza A virus
197 NP (Statens Serum Institute, Copenhagen, Denmark), or were stained with haematoxylin and eosin
198 (H&E) as previously described [15]

199 **Sialic acid receptor distribution analysis in pigeon, chicken and duck tissues**

200 To assess the presence and abundance of α2-3-linked sialic acid (2-3Sia) and α2-6-linked sialic acid (2-
201 6Sia) in respiratory tissues of different bird species, formalin-fixed, paraffin-embedded (FFPE) tissues
202 from three uninfected, healthy individuals per species were examined. Pigeon tissues were collected
203 during the present study. As comparator species, FFPE tissues from chickens (Hy-Line UK Ltd) and

204 Pekin ducks (Cherry Valley hybrid) previously prepared in an earlier study [15] were used. Sections of
205 4 µm thickness were dewaxed through xylene then rehydrated through absolute alcohol, quenched for
206 endogenous peroxidase using 3% hydrogen peroxide in methanol (VWR International) for 15 min.
207 Slides were assembled into Shandon cover plates to facilitate IHC using the Sequenza system (Thermo
208 Fischer Scientific) and samples underwent a biotin block using Avidin and Biotin solutions (abcam) for
209 10 mins, followed by a protein block using a 1% BSA (Sigma Aldrich) solution for 20 min. Samples
210 were then incubated for 1 hour with either *Sambucus Nigra* Lectin (SNA) (Vector Labs, B-1305) 1/1500
211 1.33ug/mL to demonstrate a2-6Sia, *Maakia Amurensis* Lectin I (MAL I) (Vector Labs, B-1315) 1/1500
212 1.33ug/mL, or *Maakia Amurensis* Lectin II (MAL II) (Vector Labs, B-1265-1) 1/750 1.33ug/mL to
213 demonstrate a2-3Sia-N-acetylneuraminic acid (NAc) and 2-3Sia respectively. This was followed by
214 incubation with ABC (Vector Labs) for 30 mins at and visualised using 3,3-diaminobenzidine
215 tetrahydrochloride (Sigma Aldrich) for 10 min. "Lectin TBST" (20mM Tris Base, 100mM NaCl, 1mM
216 $CaCl_2$, 1mM $MgCl_2$ in dH_2O, adjusted to pH 7.2 using HCl) was used for rinsing sections between
217 incubations and to dilute the biotinylated lectins, ABC and block. Sections were then counterstained
218 within Mayer's haematoxylin (Pioneer Research Chemicals Ltd.), dehydrated and cleared in absolute
219 alcohol and xylene, and glass coverslips mounted using ClearVue Mounting Medium (Epredia). To
220 confirm the specificity of the lectin binding, matching serial sections were incubated in 100uL of
221 Neuraminidase (Roche) overnight at 37°C following the protein step block and prior to the incubation
222 with the lectins. Presence of histochemical signal in respiratory epithelial cells was evaluated semi-
223 quantitatively on a scale of - (no labelling) to ++++ (abundant labelling).

224 **Statistical analysis and determining minimum infectious dose (MID)**

225 All statistical analyses were performed using Prism version 8.4 software (GraphPad, San Diego, CA,
226 USA), as indicated on the corresponding figure. The minimum infectious dose (MID) and 50% MID
227 (MID_{50}) were calculated as previously described [37].

228 # Results

229 **A high dose of H5N1-AB is required to establish infection and seroconversion in pigeons.**

230 To assess the virulence, pathogenesis, and transmission characteristics of H5N1-AB in pigeons, groups
231 of eight D0 pigeons were inoculated with one of three H5N1-AB doses; low (10^2 EID_{50}), medium (10^4
232 EID_{50}), or high (10^6 EID_{50}).

233 Pigeons inoculated with the low or medium doses did not shed H5N1-AB vRNA from the Op or C
234 cavities (Fig. 1A, B, D & E). A single pigeon in the medium-dose group at 2 dpi exhibited a single
235 subthreshold level of vRNA from in the OP cavity (Fig. 1B), but this was not detectable using an H5
236 RT-PCR. In contrast, vRNA was shed by seven of the eight pigeons (87.5%) in the high-dose group
237 between 1 and 7 dpi (Fig. 1C, F & G). However, OP shedding levels remained low, with mean peak
238 vRNA titres of $10^{3.74}$ REU, and an individual maximum titre reaching $10^{4.12}$ REU. Cloacal shedding was
239 comparatively lower and more sporadic, only a single C swab was positive for vRNA from pigeon #48
240 at 5dpi with a titre of $10^{2.41}$ REU. Four of the eight pigeons exhibited subthreshold cloacal shedding, all
241 these were also detectable by the H5 RT-PCR (Fig. 1F & G). No clinical signs were observed in the D0
242 pigeons throughout the duration of the study (1-14dpi), except for one pigeon in the high-dose group
243 (Pigeon #43) at 1 dpi. This pigeon displayed transient clinical signs which included shivering and a
244 hunched posture, with lowered tail and wings (Table S1).

245 Consistent with the absence of vRNA shedding, pigeons in the low- and medium- dose groups did
246 not seroconvert, as evidenced by HI titres <1/16 against homologous H5N1-AB at 14 dpi (Fig. 2A &
247 B). In contrast, all pigeons in the high-dose group seroconverted, demonstrating detectable antibodies
248 to H5N1-AB by HI (Fig. 2C & D).

249 Together from shedding and seroconversion data for D0 pigeons, a dose of 10^2 or 10^4 EID_{50} resulted
250 in no detectable infection in pigeon (n=0/6), whereas a dose of 10^6 EID_{50} resulted in 100% infection
251 (n=6/6). The estimated MID_{50} for H5N1-AB in pigeons was therefore $>10^4$ but $\leq 10^6$ EID_{50}, and the
252 calculated MID_{50} was 10^5 EID_{50}.

253 **Transmission of H5N1-AB is ineffective between pigeons and from pigeons to chickens.**

254 To investigate the transmission efficiency and dynamics of H5N1-AB within species (pigeon-to-pigeon)
255 and between species (pigeon-to-chicken), we introduced, and cohoused, contact (R1) pigeons and
256 chickens to each dose group (low, medium and high).

257 Pigeon-to-pigeon transmission efficiency was determined to be 0% (0/8) across all three dose
258 groups based on H5N1-AB vRNA shedding from the R1 birds (Fig. 1A–F, teal symbols). One R1
259 contact pigeon (pigeon #39) in the high-dose group exhibited a single subthreshold level of vRNA in
260 an Op sample at 2 dpi ($10^{0.929}$ REU) (Fig. 1C), although no vRNA was detected in this sample using the
261 H5 RT-PCR. Additionally, none of the contact pigeons in any dose group seroconverted to H5N1-AB
262 (Fig. 2A–C). Among the R1 pigeons, negative but subthreshold HI titres (<16 HI) were detected in five
263 of eight (62.5%) and one of eight (12.5%) pigeons cohoused in the medium- and high-dose groups,
264 respectively (Fig. 2B & C). None of the R1 pigeons across all dose groups exhibited clinical signs until
265 study-end at 14 dpi with the exception of one R1 pigeon (#01) in the low-dose group (which exhibited
266 huddling and altered body position) (Table S1).

267 Pigeon-to-chicken transmission efficiency was similarly 0% (0/8). None of the contact R1
268 chickens in any dose group shed detectable H5N1-AB (Fig. 1A–F, pink symbols), nor did they
269 seroconvert to H5N1-AB (Fig. 2B–C). Additionally, no clinical signs were observed among any of the
270 R1 chickens during the study period (up to 14 dpi) (Table S1).

271 **Limited environmental H5N1-AB contamination resulted from pigeon infection.**

272 We also investigated the extent of environmental H5N1-AB contamination in the high-dose group by
273 collecting various samples throughout the study period (1–14 dpi). Only a single sample from floor-
274 level drinking water at 5 dpi was positive for vRNA, albeit at low levels with a titre of $10^{2.32}$ REU. All
275 other environmental samples were negative (either undetectable or subthreshold). Subthreshold vRNA
276 titres were obtained from a single pigeon faecal sample collected at 3 dpi, and a sample collected from
277 the floor-level scratch mat at 7 dpi (Fig. 3). Both these subthreshold detections of vRNA were also
278 detectable by the H5 RT-PCR. No vRNA was detected in the elevated drinking water, pigeon feathers,
279 bedding, or chicken faeces.

280 **H5N1-AB did not exhibit widespread tissue dissemination in infected pigeons.**

281 The tissue tropism of the H5N1-AB was evaluated in pigeons in the high dose group following direct
282 inoculation. vRNA levels were quantified in tissue extracts obtained from various organs of two D0
283 pigeons at 3 dpi (pigeons #41 and #42) and three D0 pigeons at 14 dpi (pigeons #43, #46, and #47) (Fig.

284 4). At 3 dpi, vRNA was only detected in tissues from pigeon #41 while pigeon #42 was negative across
285 all tissues. In pigeon #41, vRNA was detected in the brain, caecal tonsil, caecum, colon, heart, kidney,
286 lung and proventriculus; the highest vRNA levels were found in the brain, caecal tonsil, and lung, with
287 titres of $10^{4.02}$, $10^{3.33}$, and $10^{3.32}$ REU, respectively (Fig. 4). Subthreshold levels of vRNA were identified
288 in the bursa, cervical trachea, jejunum and spleen, vRNA was also detectable in these samples by the
289 H5 RT-PCR. In contrast, only subthreshold levels of vRNA were identified in the lung and spleen of
290 pigeon #42 (Fig. 4), although these were also detected by an H5 RT-PCR. By 14 dpi, at study-end,
291 vRNA was undetectable in tissues from all three pigeons (pigeons #43, #46, and #47) (Fig. 4).

292 To further characterise tissue tropism, IHC analysis and histopathological examinations were
293 performed on all the tissues obtained from the same five D0 pigeons in the high-dose group, including
294 the two pigeons at 3 dpi (#41 and #42) and three pigeons at 14 dpi (#43, #46, and #47). Histopathological
295 investigations revealed mild multifocal acute airsacculitis in pigeons #41 (3 dpi), #43, and #47 (both 14
296 dpi). Pigeon #41 additionally exhibited mild multifocal acute dermatitis, moderate multifocal folliculitis
297 of the feather pulp, and moderate multifocal pale irregular areas in the pancreas, suggestive of post-
298 insult regeneration of the parenchyma. No other pathological lesions were identified in any of the
299 examined tissues. IHC analysis did not reveal detectable viral nucleoprotein staining in any of the
300 tissues assessed (Fig. S2).

301 **Pigeons have lower 2-3Sia and higher 2-6Sia abundance in their respiratory tract compared to**
302 **chickens and ducks.**

303 H5N1-AB has a receptor preference for 2-3Sia compared to 2-6Sia [38], therefore we compared 2-3Sia
304 and 2-6Sia receptor distribution across respiratory tissues (nasal turbinates, trachea and lungs) of
305 pigeons, chickens and ducks and using lectin histochemistry, using the lectins SNA (binds 2-6Sia), and
306 the lectins MAL I and MAL II (binds 2-3Sia) (Fig. S3). In the nasal turbinates and trachea, pigeons
307 showed weaker MAL I and MAL II (2-3Sia) staining, whereas chickens and ducks display much
308 stronger MAL II (2-3Sia) labelling (Table 1). In contrast, pigeons exhibit very strong SNA (2-6Sia)
309 labelling in the nasal cavity, while ducks show no detectable staining (Table 1). In the pulmonary
310 airways, pigeons exhibit strong SNA (2-6Sia) and MAL I (2-3Sia) binding, but ducks and chickens

311 often show even higher or comparable levels of staining, particularly ducks with robust MAL II (2-
312 3Sia) and SNA (2-6Sia) labelling (Table 1). A large difference was seen in the pulmonary air capillaries
313 where pigeons lack MAL I (2-3Sia) staining entirely and show only moderate MAL II (2-3Sia) with
314 strong SNA (2-6Sia) labelling. In contrast, ducks show robust MAL II (2-3Sia) and moderate SNA (2-
315 6Sia) staining, while chickens have weaker overall staining (Table 1). Together, these comparisons
316 suggest that pigeons have a relatively lower presence of 2-3Sia but higher 2-6Sia abundance in upper
317 respiratory tissues compared to chickens and ducks.
318

319 **Discussion**

320 The ongoing panzootic which started in 2021 is the largest H5Nx HPAIV outbreak on record for Europe
321 and the Americas in terms of number of infected wild birds and poultry incursions, with the scale and
322 extent of spread attributed to enhanced fitness in wild birds, and the broader avian species range [15,
323 39]. In this study we demonstrated the limited susceptibility, absent inter- and intra- species
324 transmission, and low-level tissue dissemination in pigeons (*C. livia*) following inoculation with a
325 contemporary European H5N1 clade 2.3.4.4b HPAIV (genotype AB). This study clearly showed that
326 pigeons are unlikely to act as a direct bridging species due to productive infection for the transmission
327 of this virus to chickens, as an important commercial poultry species. However, their role as an indirect
328 bridging species, through fomite transmission, was not assessed in this work and requires further study.
329 Our findings indicate that a high infectious dose (10^6 EID_{50}) of H5N1-AB is required to cause
330 detectable infection in the directly inoculated (D0) pigeons. Infection among all eight (100%) high dose
331 D0 pigeons was confirmed by vRNA shedding and/or positive serology. D0 pigeons inoculated with
332 low (10^2 EID_{50}), or medium (10^4 EID_{50}) doses exhibited no detectable vRNA shedding and no
333 seroconversion, demonstrating the limited susceptibility of pigeons to these viral doses. Even when
334 pigeons were infected in the high-dose group, shedding was minimal, was predominantly detected from
335 OP samples, and was short-lived (up to 7 dpi). In some instances, our analyses included subthreshold
336 detections of vRNA by M-gene RT-PCR (vRNA titres >Cq 36 or <$10^{1.98}$ REU). While we have presented
337 these data and attributed REU values, these detections arise late in the RT-PCR amplification. In these
338 instances, we also tested the RNA using an H5 RT-PCR assay which detects vRNA from a different

339 gene segment to further increase our confidence in detection and quantification of vRNA. Indeed,
340 previous H5N1 gs/Gd HPAIV outbreak investigations have shown that such high Cq value samples, as
341 measured by M-gene RT-PCR testing, are unlikely to represent infectious virus [40, 41]. Therefore,
342 these subthreshold detections are of little significance. Indeed, this consideration was reflected in the
343 absence of pigeon-to-pigeon transmission and pigeon-to-chicken transmission with H5N1-AB, which
344 correlated with low viral environmental contamination. Environmental contamination has been
345 consistently shown to be associated with efficient H5Nx clade 2.3.4.4b HPAIV transmission among
346 ducks [15, 37] and pheasants [42], with viral environmental contamination reported during UK H5N1
347 HPAIV poultry farm outbreaks in recent years [32]. These low levels of shedding, environmental vRNA,
348 and absence of transmission to either pigeons or chickens further underscored the limited potential for
349 pigeons to act as significant amplifiers of H5N1 HPAIV in shared habitats with other avian species.

350 Several studies have historically conducted experimental infection studies with AIV in
351 pigeons. A review published in 2014 summarised the findings from 22 experimental pigeon infection
352 studies conducted with a range of LPAIVs and HPAIVs and concluded that minimal mortalities and
353 low-level virus shedding over brief periods were generally observed in pigeons [19]. Specifically,
354 investigations with earlier gs/Gd lineage H5Nx HPAIVs have also shown similar results; experimental
355 infection with clade 2.2 and clade 2.3.2.1 H5N1 HPAIVs demonstrated low levels of viral shedding
356 [43]. Studies with H5N2, H5N8 and H5N6 clade 2.3.4.4 HPAIV also found inconsistent, short-term and
357 at low viral shedding viral shedding [44-47]. At most, these studies with earlier H5Nx clade 2.3.4.4
358 HPAVs reported infrequent pigeon mortalities. Recently, and comparably to our study, Root et al (2024)
359 investigated infection and transmission dynamics in common pigeons (*C. livia*) following experimental
360 infection with a contemporary American clade 2.3.4.4b H5N1 HPAIV, representative of the North
361 American epizootic [48]. Root et al (2024) used a dose of $10^{6.6}$ plaque forming units (PFU), in excess,
362 but broadly equivalent to the high dose used in this study (10^6 EID_{50}), to inoculate eight pigeons, some
363 of which failed to shed virus and only a single bird shed infectious levels exceeding 10^3 PFU. Six of
364 eight (75%) inoculated pigeons demonstrated seroconversion [48].

365 The maximal shedding titres observed by Root et al. (2024) are broadly equivalent to those
366 reported in our study (10^3 PFU compared to $10^{4.12}$ REU)[48]. Importantly both of these titres are

367 considerably lower than those shed from the Op cavity of ducks and chickens following experimental
368 infection with a similar clade 2.3.4.4b H5N1 HPAIV from the same epizootic, which typically range
369 from 10^4 to 10^7 REU in ducks and 10^4 to 10^6 REU in chickens [15]. Consistent with the low levels of
370 vRNA shedding from infected pigeons in our study, we also observed very low levels of vRNA in the
371 environmental sources, particularly in the drinking water; whereby water sources have previously
372 correlated with detectable levels of vRNA and the potential for transmission [15]. In our study, low
373 levels of vRNA were detected only in a single sample of drinking water. Water contamination from
374 ducks infected with a similar European clade 2.3.4.4b H5N1 HPAIV, from the same epizootic, was
375 consistently positive during the shedding period, with maximal titres of $10^{4.60}$ REU [15]. Therefore,
376 water contamination by pigeons was not only significantly less frequent, but also >2 $\log_{10}$ lower ($10^{2.32}$
377 REU) compared to than from infected ducks. By considering the proportions of D0 pigeons infected by
378 a given dose, the MID_{50} of H5N1-AB was calculated as 10^5 EID_{50}. While there may be some genotype
379 variation, this pigeon MID_{50} is comparatively higher than MID_{50} values calculated from experimental
380 infections of other avian species with similar clade 2.3.4.4b viruses, including ducks ($<10^2$ to 10^3)[15,
381 37, 49] and chickens ($10^{3.2}$ to 10^5) [15, 50-52]. Overall, these studies indicate that pigeons are less
382 susceptible to H5 clade 2.3.4.4b HPAIV infection, compared to ducks and chickens.

383 The low vRNA shedding titres and negligible environmental contamination coupled with the
384 relatively high MID_{50} in pigeons likely explains the lack of transmission we observed between pigeons
385 in this study. Root et al. (2024) also investigated transmission from 8 directly infected pigeons to two
386 contact pigeons, both (100%) of the contact pigeons shed virus on a single day post contact, and
387 seroconverted via HI, suggesting some transmission had occurred. This observed transmission
388 difference from our study may be explained by housing density or experimental design differences,
389 which may have altered the frequency or degree of contact between pigeons. Equally, despite both
390 viruses being clade 2.3.4.4b H5N1 HPAIV representatives of the current panzootic, these viruses differ
391 in their internal genes and so impact of genotype must be considered. We selected an AB genotype
392 representative of the dominant genotype detection in the UK and continental Europe [5, 31], whereas
393 Root et al. (2024) selected an American H5N1 HPAIV genotype. Soon after the start of the European
394 H5N1 HPAIV epizootic in 2021, the virus was translocated into North America via bird migration where

395 it underwent extensive reassortment with indigenous American LPAIVs. Thus, genetic differences
396 within the virus may have different propensities for infection and transmission in pigeons. However,
397 the general observations of a lack of clinical signs and low shedding titres were consistent between the
398 two genotypes in pigeons.

399 Pigeons are frequently observed both on and near poultry farms and have a high connectivity
400 with human activity; with confirmation that pigeons were one of the most frequently observed avian
401 species on poultry farms using camera trap studies [53]. In addition, air sampling from directly inside
402 the poultry houses affirmed presence of genomic DNA from *Columbiformes* [54], indicating strong
403 interactions between pigeons and poultry. Therefore, we investigated the transmission risk from pigeons
404 to chickens, through co-housing. Consistent with the low virus shedding in pigeons, and lack of
405 environmental contamination and pigeon-to-pigeon transmission, we observed 0% transmission to co-
406 housed chickens, based on shedding and seroconversion in the contact chickens. One previous study
407 with H5N6 clade 2.3.4.4 HPAIV similarly showed 0% transmission from infected pigeons to chickens
408 [46]; however, this has not been assessed by any study with contemporary H5N1 HPAIV, but indicates
409 a limited ability of pigeons to act as bridging species for poultry incursions of H5Nx clade 2.3.4.4b
410 HPAIVs. While in this study we investigated the ability of pigeons to directly transmit H5N1 clade
411 2.3.4.4b to chickens and pigeons, we did not assess the role pigeons may play as 'vectors' in indirect
412 transmission, as this was outside of the scope of this study. Rodents have been hypothesised to act in
413 this way though mechanically transferring contaminated material into poultry houses [55]. It is
414 conceivable that pigeons and *Passeriformes* (perching birds) may also act as vectors for fomite
415 transmission, although this requires further study. However, the limited risk of pigeons to act as bridging
416 species is supported by a lack of association being observed in the UK between *Columbiformes*
417 distribution and infected poultry premises during the 2021-2023 panzootic, when spatial generalized
418 additive models were used based on public bird observations reports [56].

419 Following direct infection of pigeons, we observed no clinical signs and no mortalities; this
420 finding is broadly consistent with the outcomes of other similar experimental pigeon infection studies
421 [44, 45, 47, 48]. We detected overall low vRNA tissue titres, but viral dissemination occurred in one of
422 the two pigeons at 3dpi, with the highest vRNA titres in the brain, lung, and caecal tonsil. Importantly,

423 no nucleoprotein was detected in any tissue by IHC. We also did not observe any histopathological
424 lesions associated with viral associated tissue damage. Together, these results indicated that while vRNA
425 was present at low levels, H5N1-AB was not replicating to high levels in these organs, suggesting that,
426 although systemic dissemination of the virus can potentially occur in pigeons, it is highly variable
427 between pigeons and does not result in efficient replication producing high virus titres. By 14 dpi, no
428 vRNA was detected in any tissues, suggesting efficient viral clearance in pigeons [57]. A similarly low
429 level of virus dissemination in pigeon tissues has been previously identified following infection with
430 earlier gs/Gd clades [43]. However, there do appear to be virus strain-specific differences in terms of
431 infectivity and infection outcome in pigeons. Lui et al (2022) performed pigeon experimental infection
432 studies with two clade 2.3.4.4 H5N6 HPAIVs [46]. One strain caused no clinical disease with minimal
433 viral shedding, whereas the other strain caused 25% mortality, with 58% of pigeons displaying
434 neurological signs and wide dissemination in tissues with this strain [46]. In addition, there have been
435 several documented reports describing infection and mortalities of *Columbiformes* with H5Nx HPAIV,
436 including common pigeons [58], wood pigeons [59] and African mourning doves (*S. decipiens*) [60]. A
437 recent case of natural HPAI H5N1 clade 2.3.4.4b infection was recorded in a wood pigeon (*C.*
438 *palumbus*) found deceased in a wildlife centre in Germany [59]. Gross examination revealed mild
439 splenomegaly and discolouration of the pancreas, while histopathological analysis identified neuronal
440 necrosis within the grey matter of the cerebral hemispheres and brainstem, demonstrating pronounced
441 neurotropism of the virus [59]. This wood pigeon mortality may have been due to a greater individual
442 susceptibility to systemic H5N1 HPAIV infection, with resultant death or the ability of different
443 genotypes to infect. Similarly, natural infection of pigeons with an older H5N1 clade 2.2.1 HPAIV in
444 Egypt was associated with 50% mortalities, with congestion of the internal organs, particularly the lungs
445 and brain being observed [58]. Interestingly, when an isolate from this outbreak was assessed in
446 experimental pigeon infections, some clinical signs did ensue, but with reduced mortality (10%)
447 compared to the field observations [58]. Passive surveillance of found dead wild birds in the UK since
448 the start of the clade 2.3.4.4b H5N1 HPAIV panzootic in October 2021 has shown H5 HPAIV infection
449 in only 23 *Columbidae* out of 3465 (0.66%) [16].

450 These observations suggest that while H5 HPAIV infection can be fatal in pigeons under certain
451 conditions, mortality is more likely to occur in field settings. This may be attributed to differences in
452 exposure dose, route of infection, or repeated exposure, which may be different in natural environments.
453 Additionally, co-morbidities and environmental stressors likely increase pigeon susceptibility to
454 infection and contribute to more severe disease outcomes. Such factors may also account for the
455 variability observed across experimental studies. Since pigeons are not routinely raised under specific
456 pathogen-free (SPF) conditions and are often sourced from natural or commercial populations, their
457 baseline health status and infection history are typically unknown and heterogeneous. Furthermore, due
458 to the wide range of possible subclinical infections and practical limitations, this information is rarely
459 comprehensively reported in the published literature. Indeed, pigeons in the wild have a high propensity
460 for infection with a range of bacterial and viral pathogens [20, 61]. One of the most significant is avian
461 paramyxovirus type 1 (APMV-1), also referred to Newcastle disease virus (NDV) when virulent forms
462 are seen in poultry [62, 63]. APMV-1 have specific lineages referred to as pigeon paramyxovirus type
463 1 (PPMV-1) in pigeons, and while prevalence is difficult to assess, between 5 - 17% common pigeons
464 have been found as actively infected, dependent on season [64]. The pigeons used in our study were
465 confirmed negative for APMV-1 infection. However, interestingly co-morbidities between HPAIV and
466 PPMV-1 have been hypothesised for exacerbated mortality in natural infected pigeons in South Africa
467 [65], although the interaction between co-infections and impact on disease outcomes require further
468 study.

469 Despite these sporadic instances of mortality, most surveillance and experimental infection
470 data, including from this study, have shown that pigeons have a low susceptibility to H5Nx clade
471 2.3.4.4b HPAIVs, and indeed many other AIVs. The underlying reason behind this is likely multi-
472 factorial. For a species to be susceptible to AIV, the target host cell must be 'susceptible' (possess the
473 corresponding receptor for the virus) and 'permissive' (possess the corresponding host factors to allow
474 viral replication). Influenza viruses bind to sialic acid and the majority of AIV have a preference for 2-
475 3Sia, instead of 2-6Sia [38]. Therefore, to investigate susceptibility, we assessed the 2-3Sia and 2-6Sia
476 receptor abundance on pigeons using chickens and ducks as a comparator susceptible species. We
477 observed a lower relative 2-3Sia, and higher 2-6Sia abundance in proximal respiratory tissues of

478 pigeons when compared with chickens and ducks. This observation aligns with previous analyses of
479 pigeon tissues which demonstrated that the epithelial surfaces of the larynx, trachea, bronchus, and
480 bronchiole of pigeons contained little or no 2-3Sia, but abundant 2-6Sia distribution [66]. In contrast,
481 chicken, turkey and duck respiratory tissues demonstrated a predominance for 2-3Sia [66, 67]. The vast
482 majority of avian origin clade 2.3.4.4b H5N1, including the virus used here (H5N1-AB), have been
483 shown to exclusively bind 2-3Sia in biophysical analysis, with no detectable binding to 2-6Sia [38].
484 While more detailed investigations of the pigeon glycome are required, these observations offer a
485 potential mechanism for their low susceptibility to H5N1 clade 2.3.4.4b HPAIVs, and potentially many
486 other AIVs.

487 With respect to the permissiveness of pigeon cells to clade 2.3.4.4b H5N1 HPAIV, the paucity
488 of pigeon-specific reagents hampers detailed investigation of AIV replication dynamics and associated
489 host responses in these cells. Consequently, this aspect remains largely unexplored. However, analysis
490 of the pigeon immune response to HPAIV has found relatively little change in either cytokine production
491 [57] or proinflammatory factors [43] following infection with H5 HPAIV. Interestingly, it has been
492 noted that pigeons appear to have a higher basal expression of interferon stimulated genes (ISG)
493 compared to other avian species [43]. This observation may be linked to different efficiencies of pigeon
494 MDA5 to sense PAMPs, and eliciting an interferon response, compared to other birds [68]. Therefore,
495 it is also possible that higher basal ISG expression may also contribute to the low pigeon susceptibility
496 to infection.

497 Under the prevailing circumstances of the ongoing H5Nx clade 2.3.4.4b panzootic [69], the
498 negligible role of pigeons in active disease ecology including direct transmission of these viruses is at
499 least reassuring, particularly given their widespread presence in urban environments and agricultural
500 landscapes. While the virus can infect pigeons at high doses, their limited shedding, lack of efficient
501 transmission to co-housed birds, and minimal environmental contamination suggest they are unlikely
502 to contribute significantly directly to the epidemiology of this virus in wild bird populations or
503 commercial poultry. However, the indirect role pigeons may play though fomite transmission (i.e.
504 bringing contaminated material into a poultry premises) requires further investigation. These findings
505 have important implications for surveillance and control strategies, as they suggest that targeted

interventions should focus on species with higher susceptibility and transmission potential, such as wild waterfowl, seabirds, and poultry. Further research into other *Columbiformes* species and their interactions with high-risk avian hosts could provide additional insights into their role, if any, in the spread of H5N1 HPAIV.

Author statements

Ethics statements

The experiments were approved by the Animal and Plant Health Agency (APHA) Animal Welfare and Ethical Review Body (AWERB), ensuring compliance with UK legislation and alignment with UK Home Office project licence PP7633638 and PP9307748. All work involving infectious HPAIV was performed in licensed Containment Level 3 (CL-3) laboratories and animal housing facilities at APHA, meeting Advisory Committee on Dangerous Pathogens level 3 (ACDP-3) and Specified Animal Pathogens Order level 4 (SAPO-4) requirements. All birds had access to food and water *ad libitum.*

Conflict of interest

The authors declare no conflicts of interest.

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Author contribution

Conceptualisation: MJS, AB, JJ; formal analysis: CDG, CJW, MJS, JJ; investigation: CDG, CJW, SJ, SR, KR, ST, ALS, AN, DJ, KR, JH, EB; resources: AN, MJS, AB, JJ; writing—original draft: CDG, CJW, DJ, MJS, JJ; writing—review and editing: CDG, AN, MJS, JJ, AB, IHB, AN, ALS. All authors have read and agreed to the final version of the manuscript.

538 # Figure Legends

539 **Fig. 1. H5N1-AB vRNA shedding from directly inoculated pigeons and contact pigeons and**
540 **chickens.**

541 **A-F.** H5N1-AB vRNA shedding from pigeons which were directly inoculated (D0, blue) with low
542 (10^2 EID_{50}) (**A & D**), medium (10^4 EID_{50}) (**B & E**) or high (10^6 EID_{50}) (**C & F**) doses of H5N1-AB.
543 Shedding is also shown from pigeons (teal) and chickens (pink) placed in direct contact (R1) with D0
544 pigeons. Shedding is shown from swab samples collected from the oropharyngeal (Op) (**A-C**) and
545 cloacal (C) (**D-F**) cavities. **G.** H5N1-AB shedding from each individual D0 pigeon infected with the
546 high dose (10^6 EID_{50}) is presented as separate graphs for Op (blue) and C (orange). Grey shading
547 indicates no data generated as these pigeons were culled for post-mortem analysis at 3dpi. **A-G**. Viral
548 RNA titres were determined by M-gene RT-PCR and converted into relative equivalency units (REU).
549 Dotted horizontal lines indicate the positive threshold at $10^{1.98}$ REU.

550 **Fig. 2. Seroconversion of pigeons directly inoculated with H5N1-AB and from pigeons and**
551 **chickens placed in direct contact.**

552 Haemagglutinin inhibition (HI) titres from sera collected at 14 days post infection (dpi) from pigeons
553 which were directly inoculated (D0, blue) with low (10^2 EID_{50}) (**A**), medium (10^4 EID_{50}) (**B**) or high
554 (10^6 EID_{50}) (**C**) doses of H5N1-AB, or contact (R1) pigeons (teal) and chickens (pink) cohoused with
555 each group. HI titres were derived using homologous H5N1 HPAIV antigen. Graphs show geometric
556 mean ± geometric SD. **D.** Individual HI titres plotted for pigeons directly infected with the high
557 (10^6 EID_{50}) H5N1-AB. Dotted horizontal lines indicate the positive threshold at $10^{1.98}$ REU.

558 **Fig. 3. Detection of H5N1-AB vRNA in the environment of pigeons and chickens.**

559 Indicated samples were collected from the environment of pigeons directly inoculated with a high dose
560 (10^6 EID_{50}) of H5N1-AB, which also contained direct contact pigeons and chickens. Environmental
561 samples associated with either chickens or pigeons were collected on indicated days post infection (dpi).
562 H5N1-AB vRNA titres were determined by M-gene RT-PCR, with Cq values converted into relative
563 equivalency units (REUs) and displayed graphically as a heat map with high values represented with

564 more intense colouring. Values below the threshold are shown in shades of grey, values above the
565 positive threshold are shown in shades of blue; dotted line in the heatmap indicates the positive
566 threshold at $10^{1.98}$ REU. Cells with crosses indicate no sample obtained. Mean Op and C shedding from
567 the D0 pigeons is also indicated.

568 **Fig. 4. H5N1-AB vRNA distribution in tissues from inoculated pigeons.**

569 Tissues were dissected from two pigeons at 3 days post infection (dpi) and three pigeons at 14 dpi
570 following infection with a high (10^6 EID_{50}) dose of H5N1-AB. Viral RNA titres were determined by M-
571 gene RT-PCR, converted into relative equivalent units (REUs) and displayed graphically as a heat map,
572 with higher titres represented by more intense colouring. Subthreshold viral RNA levels are shown in
573 shades of grey, levels above the positive threshold are shown in shades of blue; dotted line in the
574 heatmap indicates the positive threshold at $10^{1.98}$ REU.

575

Table 1. Distribution of sialic acid receptors by lectin histochemistry in respiratory tissues of pigeons, chicken and ducks

Tissue	MAL I (2-3Sia-NAc)			MAL II (2-3Sia)			SNA (2-6Sia)		
	Pigeon	Chicken	Duck	Pigeon	Chicken	Duck	Pigeon	Chicken	Duck
Nasal turbinates	+	++	+	+	+++	+++	++++	+++	-
Trachea	++	+++	+++	++	++++	+++	++++	++++	+++
Pulmonary airways	+++	++++	++	++	++	+++	++++	+++	+++
Pulmonary air capillaries	-	-	+	+++	+	++++	++++	++	++

* -, no labelling; +, rare labelling (focal/multifocal, <25% epithelial surface); ++, mild labelling (multifocal, 25-50%); +++, moderate labelling (multifocal, 50-75%); ++++, profuse labelling (diffuse, >75% of epithelial surface).

576

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Fig. 1

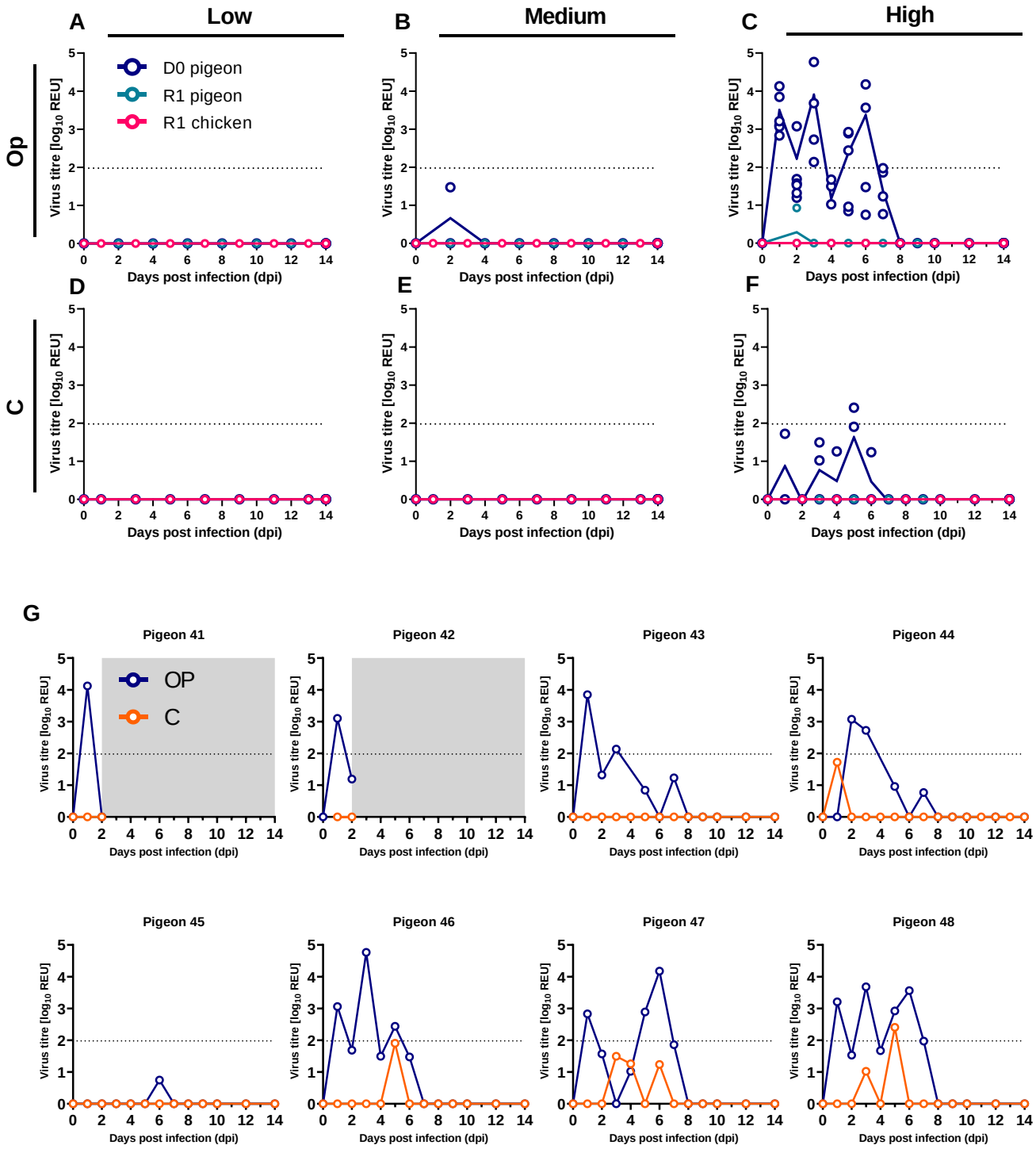

A
Low
B
Medium
C
High
Op
C
D0 pigeon
R1 pigeon
R1 chicken
Virus titre [log10 REU]
Days post infection (dpi)
D
E
F
G
Pigeon 41
Pigeon 42
Pigeon 43
Pigeon 44
Pigeon 45
Pigeon 46
Pigeon 47
Pigeon 48
OP
C

Fig. 2

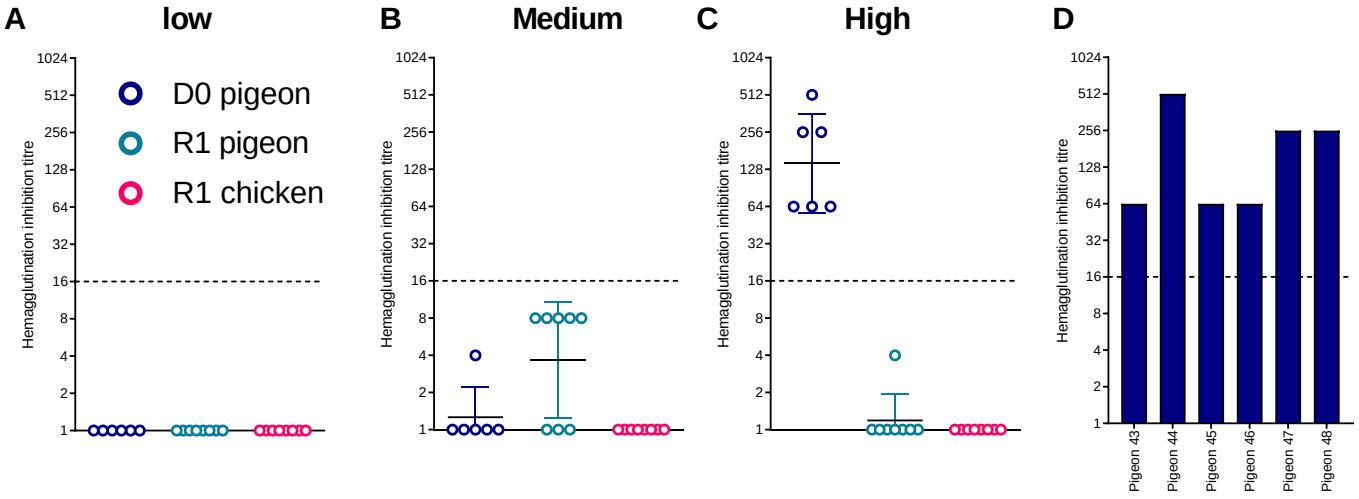

A
low
B
Medium
C
High
D
D0 pigeon
R1 pigeon
R1 chicken
Hemagglutination inhibition titre
1024
512
256
128
64
32
16
8
4
2
1
Pigeon 43
Pigeon 44
Pigeon 45
Pigeon 46
Pigeon 47
Pigeon 48

Fig. 3

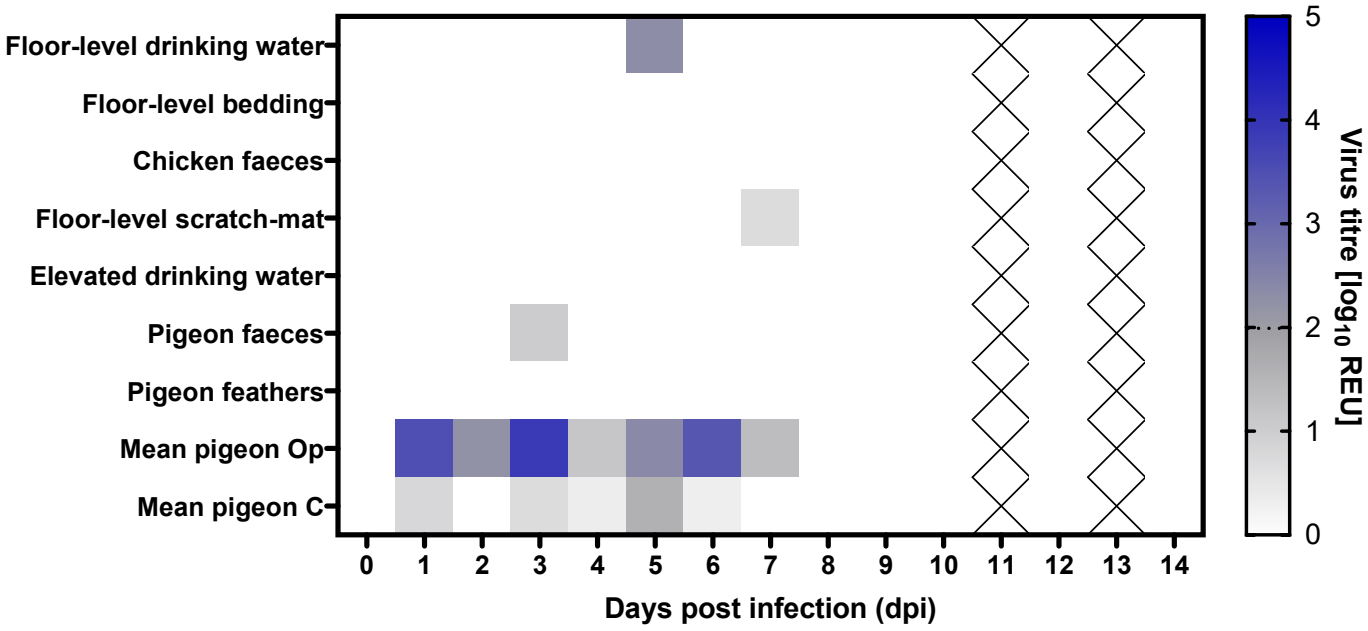
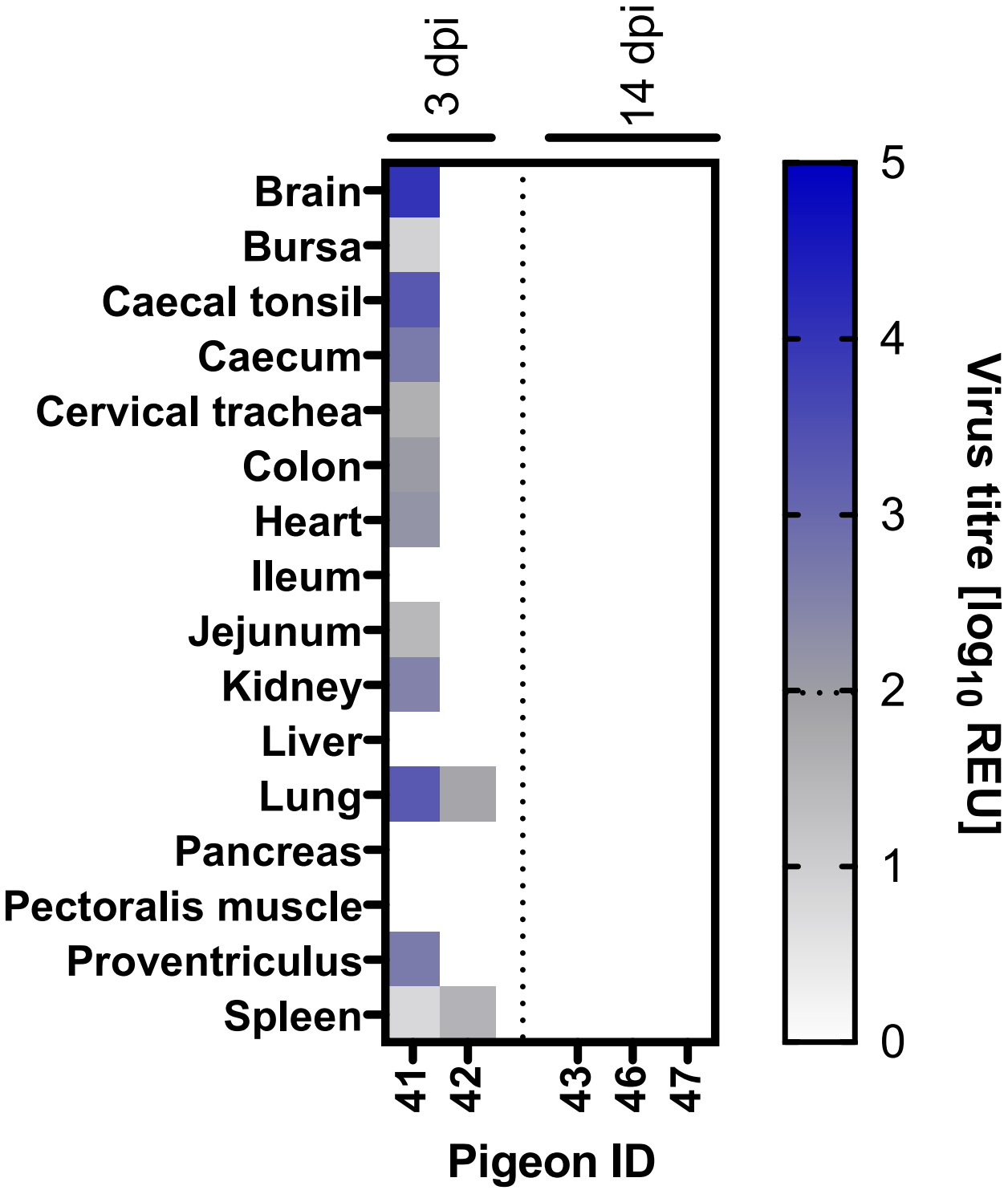

Floor-level drinking water
Floor-level bedding
Chicken faeces
Floor-level scratch-mat
Elevated drinking water
Pigeon faeces
Pigeon feathers
Mean pigeon Op
Mean pigeon C
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days post infection (dpi)
5
4
3
2
1
0
Virus titre [log10 REU]

Fig. 4


3 dpi
14 dpi
Brain
Bursa
Caecal tonsil
Caecum
Cervical trachea
Colon
Heart
Ileum
Jejunum
Kidney
Liver
Lung
Pancreas
Pectoralis muscle
Proventriculus
Spleen
41
42
43
46
47
Pigeon ID
5
4
3
2
1
0
Virus titre [log10 REU]