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

Cecilia Di Genova[1*], Caroline J Warren[1*], Simon Johnson[1], Sofia Riccio[1], Kelly Roper[1], Saumya S. Thomas[1], Audra-Lynne Schlachter[2], David Jorge[2], Kajal Ralh[1], Jafar Hassan[1], Elizabeth Billington[1], Ian H. Brown[1,3±], Marek J Slomka[1], Ashley C. Banyard[1,3@], Joe James[1,3@]

[1]Department of Virology, Animal and Plant Health Agency (APHA-Weybridge), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK

[2]Department of Pathology, Animal and Plant Health Agency (APHA-Weybridge), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK

[3]WOAH/FAO International Reference Laboratory for Avian Influenza, Animal and Plant Health Agency (APHA-Weybridge), Woodham Lane, Addlestone, Surrey, KT15 3NB, UK

±Current address: The Pirbright Institute, Pirbright, Woking, United Kingdom

@ Corresponding author Email: Ashley.banyard@apha.gov.uk and Joe.James@APHA.gov.uk

*Authors contributed equally

Key words: Pigeon; high pathogenicity avian influenza virus; H5N1; HPAIV; transmission; pathogenicity

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 and are highly connected to humans in urban settlements, yet relatively 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 for each dose. 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 naive pigeons or chickens placed in direct contact. Overall, these findings suggest that pigeons have a low susceptibility to clade 2.3.4.4b H5N1 HPAIV and are less likely to significantly contribute to disease ecology, incursions into poultry, or pose a significant zoonotic threat.

Word count: 249

Introduction

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

A key hallmark of this H5N1 clade 2.3.4.4b HPAIV panzootic is the broad avian host range 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

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

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

109 Methods

110 Virus

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

120 Animals and pre-sampling

121 Forty-eight domestic pigeons (*C. livia*) and twenty-four high-health-status Hy-Line Brown chickens
122 (*Gallus gallus*) were sourced from Simons Rodents (Cambridge, UK) and Hy-Line UK Ltd (Studley,
123 UK), respectively. The pigeons were approximately three months old at the time of procurement, while

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

131 ***In vivo* study design**

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

149 **Clinical samples and processing**

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

168 **Clinical scoring and monitoring**

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

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

173 vRNA was extracted from swabs, environmental samples and PM tissue homogenates, using MagMAX
174 CORE extraction chemistry (Applied Biosystems, Warrington, UK) upon a Kingfisher (Thermo Fisher
175 Scientific, Glasgow, UK) as previously described [15]. For H5N1-AB vRNA detection, matrix (M)-
176 gene RT-PCR primers and probes were used [33]. A ten-fold dilution series of H5N1-AB vRNA at a

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

185 **Serology**

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

191 **Pathology and immunohistochemistry (IHC)**

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

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

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

201 # Results

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

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

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

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

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

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

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

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

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

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

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

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

254 The tissue tropism of the H5N1-AB was evaluated in pigeons in the high dose group following direct
255 inoculation. vRNA levels were quantified in tissue extracts obtained from various organs of two D0
256 pigeons at 3 dpi (pigeons #41 and #42) and three D0 pigeons at 14 dpi (pigeons #43, #46, and #47) (Fig.
257 4). At 3 dpi, vRNA was only detected in tissues from pigeon #41 while pigeon #42 was negative across
258 all tissues. In pigeon #41, vRNA was detected in the brain, caecal tonsil, caecum, colon, heart, kidney,
259 lung and proventriculus; the highest vRNA levels were found in the brain, caecal tonsil, and lung, with
260 titres of $10^{4.02}$, $10^{3.33}$, and $10^{3.32}$ REU, respectively (Fig. 4). Subthreshold levels of vRNA were identified
261 in the bursa, cervical trachea, jejunum and spleen, vRNA was also detectable in these samples by the
262 H5 RT-PCR. In contrast, only subthreshold levels of vRNA were identified in the lung and spleen of
263 pigeon #42 (Fig. 4), although these were also detected by an H5 RT-PCR. By 14 dpi, at study-end,
264 vRNA was undetectable in tissues from all three pigeons (pigeons #43, #46, and #47) (Fig. 4).

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

274 Discussion

275 The ongoing panzootic which started in 2021 is the largest H5Nx HPAIV outbreak on record for Europe
276 and the Americas in terms of number of infected wild birds and poultry incursions, with the scale and
277 extent of spread attributed to enhanced fitness in wild birds, and the broader avian species range [15,
278 38]. In this study we demonstrated the limited susceptibility, absent inter- and intra- species
279 transmission, and low-level tissue dissemination in pigeons (*C. livia*) following inoculation with a
280 contemporary European H5N1 clade 2.3.4.4b HPAIV (genotype AB). This study clearly showed that

281 pigeons are unlikely to act as a direct bridging species due to productive infection for the transmission
282 of this virus to chickens, as an important commercial poultry species. However, their role as an indirect
283 bridging species, through fomite transmission, was not assessed in this work and requires further study.
284 Our findings indicate that a high infectious dose (10^6 EID_{50}) of H5N1-AB is required to cause
285 detectable infection in the directly inoculated (D0) pigeons. Infection among all eight (100%) high dose
286 D0 pigeons was confirmed by vRNA shedding and/or positive serology. D0 pigeons inoculated with
287 low (10^2 EID_{50}), or medium (10^4 EID_{50}) doses exhibited no detectable vRNA shedding and no
288 seroconversion, demonstrating the limited susceptibility of pigeons to these viral doses. Even when
289 pigeons were infected in the high-dose group, shedding was minimal, was predominantly detected from
290 OP samples, and was short-lived (up to 7 dpi). In some instances, our analyses included subthreshold
291 detections of vRNA by M-gene RT-PCR (vRNA titres >Ct 36 or <$10^{1.98}$ REU). While we have presented
292 these data and attributed REU values, these detections arise late in the RT-PCR amplification. In these
293 instances, we also tested the RNA using an H5 RT-PCR assay which detects vRNA from a different
294 gene segment to further increase our confidence in detection and quantification of vRNA. Indeed,
295 previous H5N1 gs/Gd HPAIV outbreak investigations have shown that such high Ct value samples, as
296 measured by M-gene RT-PCR testing, are unlikely to represent infectious virus [39, 40]. Therefore,
297 these subthreshold detections are of little significance. Indeed, this consideration was reflected in the
298 absence of pigeon-to-pigeon transmission and pigeon-to-chicken transmission with H5N1-AB, which
299 correlated with low viral environmental contamination. Environmental contamination has been
300 consistently shown to be associated with efficient H5Nx clade 2.3.4.4b HPAIV transmission among
301 ducks [15, 37] and pheasants [41], with viral environmental contamination reported during UK H5N1
302 HPAIV poultry farm outbreaks in recent years [32]. These low levels of shedding, environmental vRNA,
303 and absence of transmission to either pigeons or chickens further underscored the limited potential for
304 pigeons to act as significant amplifiers of H5N1 HPAIV in shared habitats with other avian species.
305 Several studies have historically conducted experimental infection studies with AIV in
306 pigeons. A review published in 2014 summarised the findings from 22 experimental pigeon infection
307 studies conducted with a range of LPAIVs and HPAIVs and concluded that minimal mortalities and
308 low-level virus shedding over brief periods were generally observed in pigeons [42]. Specifically,

309 investigations with earlier gs/Gd lineage H5Nx HPAIVs have also shown similar results; experimental
310 infection with clade 2.2 and clade 2.3.2.1 H5N1 HPAIVs demonstrated low levels of viral shedding
311 [43]. Studies with H5N2, H5N8 and H5N6 clade 2.3.4.4 HPAIV also found inconsistent, short-term and
312 at low viral shedding viral shedding [44-47]. At most, these studies with earlier H5Nx clade 2.3.4.4
313 HPAVs reported infrequent pigeon mortalities. Recently, and comparably to our study, Root et al (2024)
314 investigated infection and transmission dynamics in common pigeons (*C. livia*) following experimental
315 infection with a contemporary American clade 2.3.4.4b H5N1 HPAIV, representative of the North
316 American epizootic [48]. Root et al (2024) used a dose of $10^{6.6}$ plaque forming units (PFU), in excess,
317 but broadly equivalent to the high dose used in this study (10^6 EID_{50}), to inoculate eight pigeons, some
318 of which failed to shed virus and only a single bird shed infectious levels exceeding 10^3 PFU. Six of
319 eight (75%) inoculated pigeons demonstrated seroconversion [48].

320 The maximal shedding titres observed by Root et al. (2024) are broadly equivalent to those
321 reported in our study (10^3 PFU compared to $10^{4.12}$ REU)[48]. Importantly both of these titres are
322 considerably lower than those shed from the Op cavity of ducks and chickens following experimental
323 infection with a similar clade 2.3.4.4b H5N1 HPAIV from the same epizootic, which typically range
324 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
325 vRNA shedding from infected pigeons in our study, we also observed very low levels of vRNA in the
326 environmental sources, particularly in the drinking water; whereby water sources have previously
327 correlated with detectable levels of vRNA and the potential for transmission [15]. In our study, low
328 levels of vRNA were detected only in a single sample of drinking water. Water contamination from
329 ducks infected with a similar European clade 2.3.4.4b H5N1 HPAIV, from the same epizootic, was
330 consistently positive during the shedding period, with maximal titres of $10^{4.60}$ REU [15]. Therefore,
331 water contamination by pigeons was not only significantly less frequent, but also >2 $\log_{10}$ lower ($10^{2.32}$
332 REU) compared to than from infected ducks. By considering the proportions of D0 pigeons infected by
333 a given dose, the MID_{50} of H5N1-AB was calculated as 10^5 EID_{50}. While there may be some genotype
334 variation, this pigeon MID_{50} is comparatively higher than MID_{50} values calculated from experimental
335 infections of other avian species with similar clade 2.3.4.4b viruses, including ducks (<10^2 to 10^3)[15,

336 37, 49] and chickens ($10^{3.2}$ to 10^{5}) [15, 50-52]. Overall, these studies indicate that pigeons are less
337 susceptible to H5 clade 2.3.4.4b HPAIV infection, compared to ducks and chickens.

338 The low vRNA shedding titres and negligible environmental contamination coupled with the
339 relatively high MID_{50} in pigeons likely explains the lack of transmission we observed between pigeons
340 in this study. Root et al. (2024) also investigated transmission from 8 directly infected pigeons to two
341 contact pigeons, both (100%) of the contact pigeons shed virus on a single day post contact, and
342 seroconverted via HI, suggesting some transmission had occurred. This observed transmission
343 difference from our study may be explained by housing density or experimental design differences,
344 which may have altered the frequency or degree of contact between pigeons. Equally, despite both
345 viruses being clade 2.3.4.4b H5N1 HPAIV representatives of the current panzootic, these viruses differ
346 in their internal genes and so impact of genotype must be considered. We selected an AB genotype
347 representative of the dominant genotype detection in the UK and continental Europe [5, 31], whereas
348 Root et al. (2024) selected an American H5N1 HPAIV genotype. Soon after the start of the European
349 H5N1 HPAIV epizootic in 2021, the virus was translocated into North America via bird migration where
350 it underwent extensive reassortment with indigenous American LPAIVs. Thus, genetic differences
351 within the virus may have different propensities for infection and transmission in pigeons. However,
352 the general observations of a lack of clinical signs and low shedding titres were consistent between the
353 two genotypes in pigeons.

354 Pigeons are frequently observed both on and near poultry farms and have a high connectivity
355 with human activity; with confirmation that pigeons were one of the most frequently observed avian
356 species on poultry farms using camera trap studies [53]. In addition, air sampling from directly inside
357 the poultry houses affirmed presence of genomic DNA from *Columbiformes* [54], indicating strong
358 interactions between pigeons and poultry. Therefore, we investigated the transmission risk from pigeons
359 to chickens, through co-housing. Consistent with the low virus shedding in pigeons, and lack of
360 environmental contamination and pigeon-to-pigeon transmission, we observed 0% transmission to co-
361 housed chickens, based on shedding and seroconversion in the contact chickens. One previous study
362 with H5N6 clade 2.3.4.4 HPAIV similarly showed 0% transmission from infected pigeons to chickens
363 [46]; however, this has not been assessed by any study with contemporary H5N1 HPAIV, but indicates

364 a limited ability of pigeons to act as bridging species for poultry incursions of H5Nx clade 2.3.4.4b
365 HPAIVs. While in this study we investigated the ability of pigeons to directly transmit H5N1 clade
366 2.3.4.4b to chickens and pigeons, we did not assess the role pigeons may play as 'vectors' in indirect
367 transmission, as this was outside of the scope of this study. Rodents have been hypothesised to act in
368 this way though mechanically transferring contaminated material into poultry houses [55]. It is
369 conceivable that pigeons and *Passeriformes* (perching birds) may also act as vectors for fomite
370 transmission, although this requires further study. However, the limited risk of pigeons to act as bridging
371 species is supported by a lack of association being observed in the UK between *Columbiformes*
372 distribution and infected poultry premises during the 2021-2023 panzootic, when spatial generalized
373 additive models were used based on public bird observations reports [56].

374 Following direct infection of pigeons, we observed no clinical signs and no mortalities; this
375 finding is broadly consistent with the outcomes of other similar experimental pigeon infection studies
376 [42, 44, 45, 47, 48]. We detected overall low vRNA tissue titres, but viral dissemination occurred in one
377 of the two pigeons at 3dpi, with the highest vRNA titres in the brain, lung, and caecal tonsil. Importantly,
378 no nucleoprotein was detected in any tissue by IHC. We also did not observe any histopathological
379 lesions associated with viral associated tissue damage. Together, these results indicated that while vRNA
380 was present at low levels, H5N1-AB was not replicating to high levels in these organs, suggesting that,
381 although systemic dissemination of the virus can potentially occur in pigeons, it is highly variable
382 between pigeons and does not result in efficient replication producing high virus titres. By 14 dpi, no
383 vRNA was detected in any tissues, suggesting efficient viral clearance in pigeons [57]. A similarly low
384 level of virus dissemination in pigeon tissues has been previously identified following infection with
385 earlier gs/Gd clades [43]. However, there do appear to be virus strain-specific differences in terms of
386 infectivity and infection outcome in pigeons. Lui et al (2022) performed pigeon experimental infection
387 studies with two clade 2.3.4.4 H5N6 HPAIVs [46]. One strain caused no clinical disease with minimal
388 viral shedding, whereas the other strain caused 25% mortality, with 58% of pigeons displaying
389 neurological signs and wide dissemination in tissues with this strain [46]. In addition, there have been
390 several documented reports describing infection and mortalities of *Columbiformes* with H5Nx HPAIV,
391 including common pigeons [42, 58], wood pigeons [59] and African mourning doves (*S. decipiens*)

392 [60]. A recent case of natural HPAI H5N1 clade 2.3.4.4b infection was recorded in a wood pigeon (*C.*
393 *palumbus*) found deceased in a wildlife centre in Germany [59]. Gross examination revealed mild
394 splenomegaly and discolouration of the pancreas, while histopathological analysis identified neuronal
395 necrosis within the grey matter of the cerebral hemispheres and brainstem, demonstrating pronounced
396 neurotropism of the virus [59]. This wood pigeon mortality may have been due to a greater individual
397 susceptibility to systemic H5N1 HPAIV infection, with resultant death or the ability of different
398 genotypes to infect. Similarly, natural infection of pigeons with an older H5N1 clade 2.2.1 HPAIV in
399 Egypt was associated with 50% mortalities, with congestion of the internal organs, particularly the lungs
400 and brain being observed [58]. Interestingly, when an isolate from this outbreak was assessed in
401 experimental pigeon infections, some clinical signs did ensue, but with reduced mortality (10%)
402 compared to the field observations [58]. Passive surveillance of found dead wild birds in the UK since
403 the start of the clade 2.3.4.4b H5N1 HPAIV panzootic in October 2021 has shown H5 HPAIV infection
404 in only 23 *Columbidae* out of 3465 (0.66%) [16].

405 All these above observations suggest that while in some instances H5 HPAIV infection can be
406 fatal in pigeons, mortality is more likely to occur in a field environment. This outcome is possibly due
407 to different exposure doses, routes or repeated exposure being achieved in some instances under field
408 conditions, with co-morbidities and environmental stressors rendering the pigeons potentially more
409 susceptible to HPAIV infection or with more severe disease outcomes. This may also explain differences
410 in experimental studies, since pigeons are not routinely raised as specified pathogen free and often
411 naturally sourced, consequently the exact health status is often unknown and likely not uniform. Pigeons
412 in the wild have a high propensity for infection with a range of bacterial and viral pathogens [20, 61].
413 One of the most significant is avian paramyxovirus type 1 (APMV-1), also referred to Newcastle disease
414 virus (NDV) when virulent forms are seen in poultry [62, 63]. APMV-1 have specific lineages referred
415 to as pigeon paramyxovirus type 1 (PPMV-1) in pigeons, and while prevalence is difficult to assess,
416 between 5 - 17% common pigeons have been found as actively infected, dependent on season [64]. The
417 pigeons used in our study were confirmed negative for APMV-1 infection. However, interestingly co-
418 morbidities between HPAIV and PPMV-1 have been hypothesised for exacerbated mortality in natural

419 infected pigeons in South Africa [65], although the interaction between co-infections and impact on
420 disease outcomes require further study.

421 Despite these sporadic instances of mortality, most surveillance and experimental infection
422 data, including from this study, have shown that pigeons have a low susceptibility to H5Nx clade
423 2.3.4.4b HPAIVs, and indeed many other AIVs. The underlying reason behind this is likely multi-
424 factorial. For a species to be susceptible to AIV, the host cell must be susceptible (possess the right
425 receptor for the virus) and permissive (possess host factors to allow viral replication). Influenza viruses
426 bind to sialic acid (Sia) and the majority of AIVs, including the H5N1-AB used in this study, have a
427 preference for α2-3-linked sialic acid (2-3Sia), instead of α2-6-linked sialic acid (2-6Sia) [66]. Previous
428 analyses of pigeon tissues demonstrated that the epithelial surfaces of the larynx, trachea, bronchus, and
429 bronchiole of pigeons contained abundant 2-6Sia, with little or no 2-3Sia present [67]. In contrast,
430 chicken, turkey and duck tissues demonstrated mainly 2-3Sia being present in respiratory tissues [67,
431 68]. While more detailed investigation of the pigeon glycome is required, these observations suggest a
432 potential mechanism for their low susceptibility. Due to the paucity of pigeon host-specific reagents,
433 AIV replication dynamics and related host-responses during AIV infection in pigeon cells remain
434 largely unexplored. However, analysis of the pigeon immune response to HPAIV has found relatively
435 little change in either cytokines production [57] or proinflammatory factors [43] following infection
436 with H5 HPAIV. Interestingly, it has been noted that pigeons appear to have a higher basal expression
437 of interferon stimulated genes (ISG) compared to other avian species [43]. This observation may be
438 linked to different efficiencies of pigeon MDA5 to sense PAMPs, and eliciting an interferon response,
439 compared to other birds [69]. Therefore, it is also possible that higher basal ISG expression may
440 contribute to the low pigeon susceptibility to infection.

441 Under the prevailing circumstances of the ongoing H5Nx clade 2.3.4.4b panzootic [70], the
442 negligible role of pigeons in active disease ecology including direct transmission of these viruses is at
443 least reassuring, particularly given their widespread presence in urban environments and agricultural
444 landscapes. While the virus can infect pigeons at high doses, their limited shedding, lack of efficient
445 transmission to co-housed birds, and minimal environmental contamination suggest they are unlikely
446 to contribute significantly directly to the epidemiology of this virus in wild bird populations or

commercial poultry. However, the indirect role pigeons may play though fomite transmission (i.e. bringing contaminated material into a poultry premises) requires further investigation. These findings 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.

Funding statement

Funding was provided by the Defra and the Devolved Administrations of Scotland and Wales, through SE2227 'FluFocus' and SV3400. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) and Department for Environment, Food and Rural Affairs (Defra, UK) research initiative 'FluTrailmap' [grant number BB/Y007271/1]. Funded by the European Union under grant agreement (101084171) - (Kappa-Flu). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or REA. Neither the European Union nor the granting authority can be held responsible for them.

Acknowledgements

The authors would like to thank Dr. Craig Ross for technical advice and assistance inoculating the pigeons, and colleagues in the Animal Sciences Department at APHA for animal husbandry, clinical assessment and sample collection.

Author contribution

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

Figure Legends

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

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

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

496 Haemagglutinin inhibition (HI) titres from sera collected at 14 days post infection (dpi) from pigeons
497 which were directly inoculated (D0, blue) with low (10^2 EID_{50}) (**A**), medium (10^4 EID_{50}) (**B**) or high
498 (10^6 EID_{50}) (**C**) doses of H5N1-AB, or contact (R1) pigeons (teal) and chickens (pink) cohoused with
499 each group. HI titres were derived using homologous H5N1 HPAIV antigen. Graphs show geometric
500 mean ± geometric SD. **D.** Individual HI titres plotted for pigeons directly infected with the high
501 (10^6 EID_{50}) H5N1-AB. Dotted horizontal lines indicate the positive threshold at $10^{1.98}$ REU.

502 **Fig. 3. Detection of H5N1-AB vRNA in the environment of pigeons and chickens.**
503 Indicated samples were collected from the environment of pigeons directly inoculated with a high dose
504 (10^6 EID_{50}) of H5N1-AB, which also contained direct contact pigeons and chickens. Environmental
505 samples associated with either chickens or pigeons were collected on indicated days post infection (dpi).
506 H5N1-AB vRNA titres were determined by M-gene RT-PCR, with Ct values converted into relative
507 equivalency units (REUs) and displayed graphically as a heat map with high values represented with
508 more intense colouring. Values below the threshold are shown in shades of grey, values above the
509 positive threshold are shown in shades of blue; dotted line in the heatmap indicates the positive
510 threshold at $10^{1.98}$ REU. Cells with crosses indicate no sample obtained. Mean Op and C shedding from
511 the D0 pigeons is also indicated.

512 **Fig. 4. H5N1-AB vRNA distribution in tissues from inoculated pigeons.**
513 Tissues were dissected from two pigeons at 3 days post infection (dpi) and three pigeons at 14 dpi
514 following infection with a high (10^6 EID_{50}) dose of H5N1-AB. Viral RNA titres were determined by M-
515 gene RT-PCR, converted into relative equivalent units (REUs) and displayed graphically as a heat map,
516 with higher titres represented by more intense colouring. Subthreshold viral RNA levels are shown in
517 shades of grey, levels above the positive threshold are shown in shades of blue; dotted line in the
518 heatmap indicates the positive threshold at $10^{1.98}$ REU.
519

520 # References

1. **Alexander DJ**. An overview of the epidemiology of avian influenza. *Vaccine* 2007;25(30):5637-5644.
2. **Fereidouni S, Starick E, Karamendin K, Genova CD, Scott SD et al.** Genetic characterization of a new candidate hemagglutinin subtype of influenza A viruses. *Emerg Microbes Infect* 2023;12(2):2225645.
3. **Alexander DJ, Brown IH**. History of highly pathogenic avian influenza. *Rev Sci Tech* 2009;28(1):19-38.
4. **The World Health Organization Global Influenza Program Surveillance Network**. Evolution of H5N1 avian influenza viruses in Asia. *Emerg Infect Dis* 2005;11(10):1515-1521.
5. **Byrne AMP, James J, Mollett BC, Meyer SM, Lewis T et al.** Investigating the Genetic Diversity of H5 Avian Influenza Viruses in the United Kingdom from 2020-2022. *Microbiol Spectr* 2023;11(4):e0477622.
6. **Alarcon P, Brouwer A, Venkatesh D, Duncan D, Dovas CI et al.** Comparison of 2016-17 and Previous Epizootics of Highly Pathogenic Avian Influenza H5 Guangdong Lineage in Europe. *Emerg Infect Dis* 2018;24(12):2270-2283.
7. **Lewis NS, Banyard AC, Whittard E, Karibayev T, Al Kafagi T et al.** Emergence and spread of novel H5N8, H5N5 and H5N1 clade 2.3.4.4 highly pathogenic avian influenza in 2020. *Emerging Microbes & Infections* 2021;10(1):148-151.
8. **Pohlmann A, King J, Fusaro A, Zecchin B, Banyard Ashley C et al.** Has Epizootic Become Enzootic? Evidence for a Fundamental Change in the Infection Dynamics of Highly Pathogenic Avian Influenza in Europe, 2021. *mBio* 2022;13(4):e00609-00622.
9. **Chatsuwan MZ, E.; Taylor, E.; Horton, D; Wright, H**. 2023. Industry Reinfected: Spotlight on the Economic and Public Health Impacts of Avian Flu. https://www.fairr.org/resources/reports/industry-reinfected-avian-flu [accessed December 2024].
10. **Caliendo V, Lewis NS, Pohlmann A, Baillie SR, Banyard AC et al.** Transatlantic spread of highly pathogenic avian influenza H5N1 by wild birds from Europe to North America in 2021. *Scientific Reports* 2022;12(1):11729.
11. **Banyard AC, Bennison A, Byrne AMP, Reid SM, Lynton-Jenkins JG et al.** Detection and spread of high pathogenicity avian influenza virus H5N1 in the Antarctic Region. *Nature Communications* 2024;15(1):7433.
12. **European Food Safety Authority (EFSA)**. Annual report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2021. *EFSA Journal* 2022;20(9):e07554.
13. **Peacock TP, Moncla L, Dudas G, VanInsberghe D, Sukhova K et al.** The global H5N1 influenza panzootic in mammals. *Nature* 2025;637(8045):304-313.
14. **European Union Reference Laboratory (EURL)**. 2025. EURL | Avian Flu Data Portal. https://eurlaidata.izsvenezie.it/epidemio.php [accessed April 2025].
15. **James J, Billington E, Warren CJ, De Sliva D, Di Genova C et al.** Clade 2.3.4.4b H5N1 high pathogenicity avian influenza virus (HPAIV) from the 2021/22 epizootic is highly duck adapted and poorly adapted to chickens. *J Gen Virol* 2023;104(5).
16. **The Animal and Plant Health Agency (APHA)**. 2025. Research and analysis: Bird flu (avian influenza): cases in wild birds. https://www.gov.uk/government/publications/avian-influenza-in-wild-birds [accessed Jan 2025].
17. **Falchieri M, Reid SM, Ross CS, James J, Byrne AMP et al.** Shift in HPAI infection dynamics causes significant losses in seabird populations across Great Britain. *Vet Rec* 2022;191(7):294-296.
18. **Lane JV, Jeglinski JWE, Avery-Gomm S, Ballstaedt E, Banyard AC et al.** High pathogenicity avian influenza (H5N1) in Northern Gannets (Morus bassanus): Global spread, clinical signs and demographic consequences. *Ibis* 2024;166(2):633-650.
19. **Abolnik C**. A current review of avian influenza in pigeons and doves (Columbidae). *Vet Microbiol* 2014;170(3-4):181-196.
20. **Marlier D, Vindevogel H**. Viral infections in pigeons. *The Veterinary Journal* 2006;172(1):40-51.
21. **Rose E, Nagel P, Haag-Wackernagel D**. Spatio-temporal use of the urban habitat by feral pigeons (Columba livia). *Behavioral Ecology and Sociobiology* 2006;60(2):242-254.

22. **Blechman AD**. *Pigeons: the fascinating saga of the world's most revered and reviled bird*: Open Road+ Grove/Atlantic; 2007.
23. **Jerolmack C**. How pigeons became rats: The cultural-spatial logic of problem animals. *Social problems* 2008;55(1):72-94.
24. **Gibbs D, Barnes E, Cox J**. *Pigeons and doves: a guide to the pigeons and doves of the world*: A&C Black; 2001.
25. **Tudor DC**. Pigeon health and disease. 1991.
26. **Panigrahy B, Senne DA, Pedersen JC, Shafer AL, Pearson JE**. Susceptibility of pigeons to avian influenza. *Avian Dis* 1996;40(3):600-604.
27. **Kohls A, Lüschow D, Lierz M, Hafez HM**. Influenza A virus monitoring in urban and free-ranging pigeon populations in Germany, 2006-2008. *Avian Dis* 2011;55(3):447-450.
28. **Cui N, Wang P, Huang Q, Yuan Z, Su S et al.** Detection of Avian Influenza Virus in Pigeons. *Viruses* 2025;17(4).
29. **European Food Safety Authority (EFSA)**. Annual Report on surveillance for avian influenza in poultry and wild birds in Member States of the European Union in 2020. *EFSA Journal* 2021;19(12):e06953.
30. **APHIS (Animal and Plant health Inspection Service) USDA (United States Department of Agriculture)**. 2024. Detections of Highly Pathogenic Avian Influenza in Wild Birds. https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/wild-birds [accessed December 2024].
31. **European Union Reference Laboratory (EURL)**. 2025. EURL: Avian Flu Data Portal. https://eurlaidata.izsvenezie.it/epidemio.php#:~:text=wild%20birds%20cases.-,Genotypes,genotypes%20present%20in%20each%20country. [accessed 6th January 2025].
32. **James J, Warren CJ, De Silva D, Lewis T, Grace K et al.** The Role of Airborne Particles in the Epidemiology of Clade 2.3.4.4b H5N1 High Pathogenicity Avian Influenza Virus in Commercial Poultry Production Units. *Viruses* 2023;15(4).
33. **Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP et al.** Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 2002;40(9):3256-3260.
34. **James J, Seekings AH, Skinner P, Purchase K, Mahmood S et al.** Rapid and sensitive detection of high pathogenicity Eurasian clade 2.3.4.4b avian influenza viruses in wild birds and poultry. *J Virol Methods* 2022;301:114454.
35. **World Organisation for Animal Health (WOAH)**. Chapter 3.3.4. Avian influenza (including infection with high pathogenicity avian influenza viruses). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* 2021.
36. **Núñez A, Brookes SM, Reid SM, Garcia-Rueda C, Hicks DJ et al.** Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4 Virus: Equivocal Pathogenicity and Implications for Surveillance Following Natural Infection in Breeder Ducks in the United Kingdom. *Transbound Emerg Dis* 2016;63(1):5-9.
37. **Seekings AH, Warren CJ, Thomas SS, Mahmood S, James J et al.** Highly pathogenic avian influenza virus H5N6 (clade 2.3.4.4b) has a preferable host tropism for waterfowl reflected in its inefficient transmission to terrestrial poultry. *Virology* 2021;559:74-85.
38. **Graziosi G, Lupini C, Catelli E, Carnaccini S**. Highly Pathogenic Avian Influenza (HPAI) H5 Clade 2.3.4.4b Virus Infection in Birds and Mammals. *Animals (Basel)* 2024;14(9).
39. **Slomka MJ, Irvine RM, Pavlidis T, Banks J, Brown IH**. Role of real-time RT-PCR platform technology in the diagnosis and management of notifiable avian influenza outbreaks: experiences in Great Britain. *Avian Dis* 2010;54(1 Suppl):591-596.
40. **Slomka MJ, To TL, Tong HH, Coward VJ, Hanna A et al.** Challenges for accurate and prompt molecular diagnosis of clades of highly pathogenic avian influenza H5N1 viruses emerging in Vietnam. *Avian Pathol* 2012;41(2):177-193.
41. **Seekings AH, Liang Y, Warren CJ, Hjulsager CK, Thomas SS et al.** Transmission dynamics and pathogenesis differ between pheasants and partridges infected with clade 2.3.4.4b H5N8 and H5N1 high-pathogenicity avian influenza viruses. *Journal of General Virology* 2024;105(1).
42. **Abolnik C**. A current review of avian influenza in pigeons and doves (Columbidae). *Veterinary Microbiology* 2014;170(3-4):181-196.

43. **Morris KM, Mishra A, Raut AA, Gaunt ER, Borowska D et al.** The molecular basis of differential host responses to avian influenza viruses in avian species with differing susceptibility. *Frontiers in Cellular and Infection Microbiology*, Original Research 2023;13.
44. **Bosco-Lauth AM, Marlenee NL, Hartwig AE, Bowen RA, Root JJ**. Shedding of clade 2.3. 4.4 H5N8 and H5N2 highly pathogenic avian influenza viruses in peridomestic wild birds in the US. *Transboundary and emerging diseases* 2019;66(3):1301-1305.
45. **Sánchez-González R, Ramis A, Nofrarías M, Wali N, Valle R et al.** Infectivity and pathobiology of H7N1 and H5N8 high pathogenicity avian influenza viruses for pigeons (Columba livia var. domestica). *Avian Pathol* 2021;50(1):98-106.
46. **Liu K, Gao R, Wang X, Han W, Ji Z et al.** Pathogenicity and transmissibility of clade 2.3.4.4 highly pathogenic avian influenza virus subtype H5N6 in pigeons. *Veterinary Microbiology* 2020;247:108776.
47. **Jeong S, Kwon JH, Lee SH, Kim YJ, Jeong JH et al.** Subclinical Infection and Transmission of Clade 2.3.4.4 H5N6 Highly Pathogenic Avian Influenza Virus in Mandarin Duck (Aix galericulata) and Domestic Pigeon (Columbia livia domestica). *Viruses* 2021;13(6).
48. **Root JJ, Porter SM, Lenoch JB, Ellis JW, Bosco-Lauth AM**. Susceptibilities and viral shedding of peridomestic wildlife infected with clade 2.3.4.4b highly pathogenic avian influenza virus (H5N1). *Virology* 2024;600:110231.
49. **Slomka MJ, Puranik A, Mahmood S, Thomas SS, Seekings AH et al.** Ducks Are Susceptible to Infection with a Range of Doses of H5N8 Highly Pathogenic Avian Influenza Virus (2016, Clade 2.3.4.4b) and Are Largely Resistant to Virus-Specific Mortality, but Efficiently Transmit Infection to Contact Turkeys. *Avian Dis* 2019;63(sp1):172-180.
50. **Leyson C, Youk S-s, Smith D, Dimitrov K, Lee D-H et al.** Pathogenicity and genomic changes of a 2016 European H5N8 highly pathogenic avian influenza virus (clade 2.3. 4.4) in experimentally infected mallards and chickens. *Virology* 2019;537:172-185.
51. **DeJesus E, Costa-Hurtado M, Smith D, Lee D-H, Spackman E et al.** Changes in adaptation of H5N2 highly pathogenic avian influenza H5 clade 2.3. 4.4 viruses in chickens and mallards. *Virology* 2016;499:52-64.
52. **Seekings AH, Warren CJ, Thomas SS, Lean FZX, Selden D et al.** Different Outcomes of Chicken Infection with UK-Origin H5N1-2020 and H5N8-2020 High-Pathogenicity Avian Influenza Viruses (Clade 2.3.4.4b). *Viruses* 2023;15(9).
53. **Graziosi G, Lupini C, Favera FD, Martini G, Dosa G et al.** Characterizing the domestic-wild bird interface through camera traps in an area at risk for avian influenza introduction in Northern Italy. *Poultry Science* 2024;103(8):103892.
54. **Bossers A, de Rooij MM, van Schothorst I, Velkers FC, Smit LA**. Detection of airborne wild waterbird-derived DNA demonstrates potential for transmission of avian influenza virus via air inlets into poultry houses, the Netherlands, 2021 to 2022. *Euro Surveill* 2024;29(40).
55. **Velkers FC, Blokhuis SJ, Veldhuis Kroeze EJB, Burt SA**. The role of rodents in avian influenza outbreaks in poultry farms: a review. *Veterinary Quarterly* 2017;37(1):182-194.
56. **Vickers SH, Raghwani J, Banyard AC, Brown IH, Fournie G et al.** Utilizing citizen science data to rapidly assess changing associations between wild birds and avian influenza outbreaks in poultry. *Proc Biol Sci* 2024;291(2031):20241713.
57. **Hayashi T, Hiromoto Y, Chaichoune K, Patchimasiri T, Chakritbudsabong W et al.** Host cytokine responses of pigeons infected with highly pathogenic Thai avian influenza viruses of subtype H5N1 isolated from wild birds. *PLoS One* 2011;6(8):e23103.
58. **Mansour SM, ElBakrey RM, Ali H, Knudsen DE, Eid AA**. Natural infection with highly pathogenic avian influenza virus H5N1 in domestic pigeons (Columba livia) in Egypt. *Avian Pathol* 2014;43(4):319-324.
59. **Peters M, King J, Wohlsein P, Grund C, Harder T**. Genuine lethal infection of a wood pigeon (Columba palumbus) with high pathogenicity avian influenza H5N1, clade 2.3.4.4b, in Germany, 2022. *Vet Microbiol* 2022;270:109461.
60. **Letsholo SL, James J, Meyer SM, Byrne AMP, Reid SM et al.** Emergence of High Pathogenicity Avian Influenza Virus H5N1 Clade 2.3.4.4b in Wild Birds and Poultry in Botswana. *Viruses* 2022;14(12).

61. **Santos HM, Tsai C-Y, Catulin GEM, Trangia KCG, Tayo LL et al.** Common bacterial, viral, and parasitic diseases in pigeons (Columba livia): A review of diagnostic and treatment strategies. *Veterinary Microbiology* 2020;247:108779.
62. **World Organisation for Animal Health (WOAH)**. Chapter 3.3.14. Newcastle disease (infection with Newcastle disease virus). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* 2021.
63. **Dortmans JC, Koch G, Rottier PJ, Peeters BP**. Virulence of Newcastle disease virus: what is known so far? *Vet Res* 2011;42(1):122.
64. **Annaheim D, Vogler BR, Sigrist B, Vogtlin A, Hussy D et al.** Screening of Healthy Feral Pigeons (Columba livia domestica) in the City of Zurich Reveals Continuous Circulation of Pigeon Paramyxovirus-1 and a Serious Threat of Transmission to Domestic Poultry. *Microorganisms* 2022;10(8).
65. **Abolnik C, Pieterse R, Peyrot BM, Choma P, Phiri TP et al.** The Incursion and Spread of Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4 Within South Africa. *Avian Dis* 2019;63(sp1):149-156.
66. **Yang J, Daines R, Chang P, Karunarathna TK, Qureshi M et al.** The Haemagglutinin Genes of the UK Clade 2.3.4.4b H5N1 Avian Influenza Viruses from 2020 to 2022 Retain Strong Avian Phenotype. *bioRxiv* 2024:2024.2007.2009.602706.
67. **Liu Y, Han C, Wang X, Lin J, Ma M et al.** Influenza A virus receptors in the respiratory and intestinal tracts of pigeons. *Avian Pathology* 2009;38(4):263-266.
68. **James J, Thomas SS, Seekings AH, Mahmood S, Kelly M et al.** Evaluating the epizootic and zoonotic threat of an H7N9 low-pathogenicity avian influenza virus (LPAIV) variant associated with enhanced pathogenicity in turkeys. *Journal of General Virology* 2024;105(7).
69. **Shao Q, Fu F, Zhu P, Yu X, Wang J et al.** Pigeon MDA5 inhibits viral replication by triggering antiviral innate immunity. *Poultry Science* 2023;102(10):102954.
70. **Food and Agriculture Organization of the United Nations (FAO)**. 2025. Global Avian Influenza Viruses with Zoonotic Potential situation update. https://www.fao.org/animal-health/situation-updates/global-aiv-with-zoonotic-potential/en [accessed Jan 2025].

Fig. 1

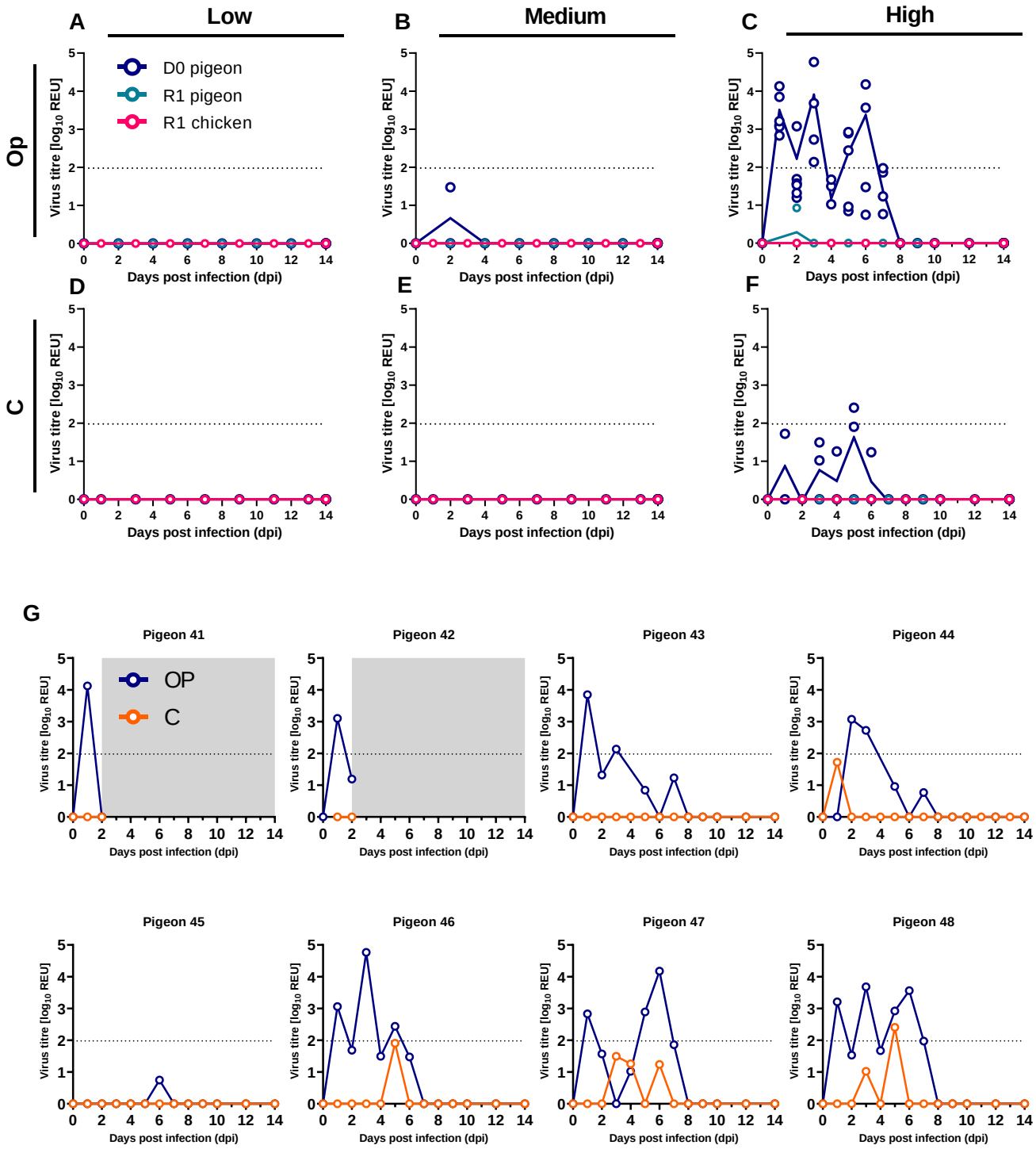

A
Low
B
Medium
C
High
Op
C
D0 pigeon
R1 pigeon
R1 chicken
Virus titre [log10 REU]
Days post infection (dpi)
D
E
F
G
Pigeon 41
Pigeon 42
Pigeon 43
Pigeon 44
Pigeon 45
Pigeon 46
Pigeon 47
Pigeon 48
OP
C

Fig. 2

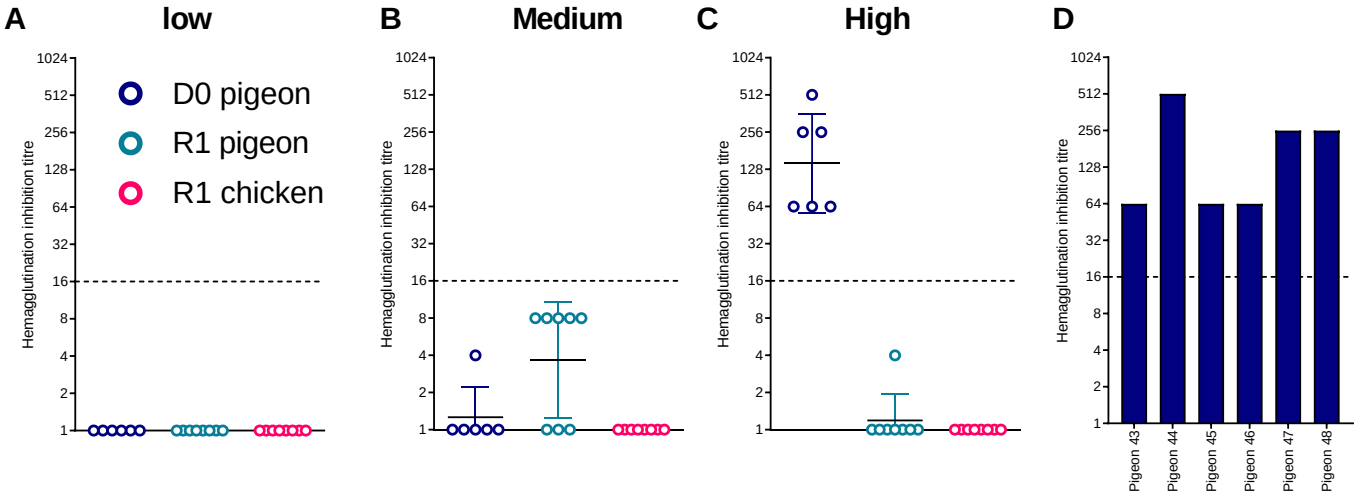

A
low
B
Medium
C
High
D
D0 pigeon
R1 pigeon
R1 chicken
Hemagglutination inhibition titre
1024
512
256
128
64
32
16
8
4
2
1
Pigeon 43
Pigeon 44
Pigeon 45
Pigeon 46
Pigeon 47
Pigeon 48

Fig. 3

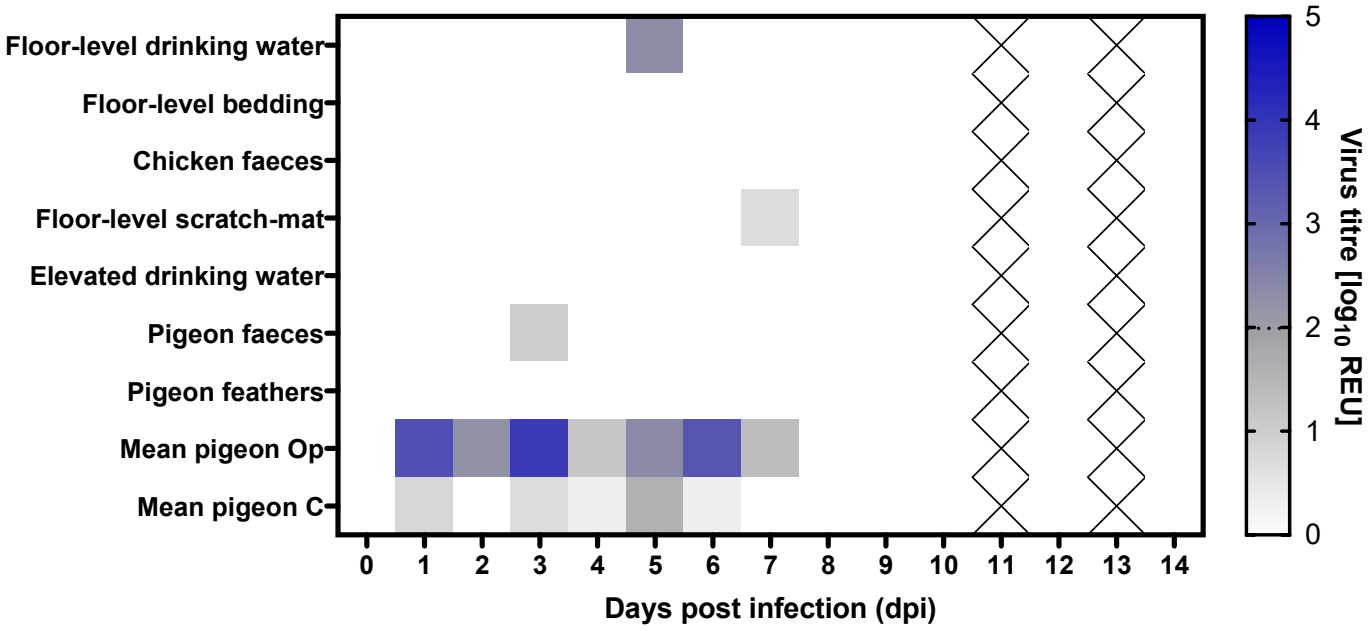
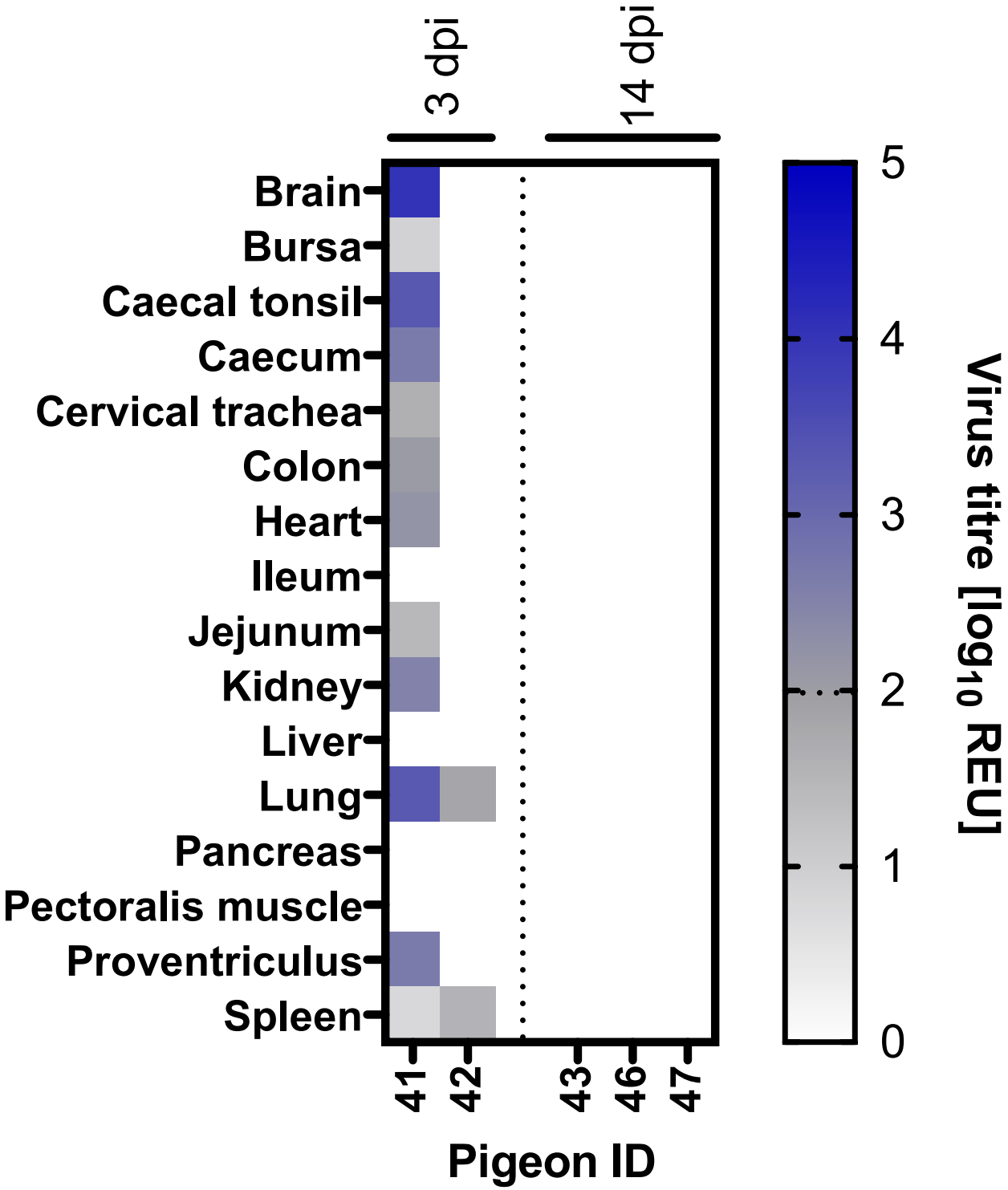

Floor-level drinking water
Floor-level bedding
Chicken faeces
Floor-level scratch-mat
Elevated drinking water
Pigeon faeces
Pigeon feathers
Mean pigeon Op
Mean pigeon C
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days post infection (dpi)
5
4
3
2
1
0
Virus titre [log10 REU]

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]