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Infectivity and pathobiology of H7N1 and H5N8 high pathogenicity avian influenza viruses for pigeons (Columba livia var. domestica)

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Abstract

Avian influenza (AI) is one of the most important viral diseases in poultry, wildlife and humans. Available data indicates that pigeons play a minimum role in the epidemiology

24 of AI. However, a degree of variation exists in the susceptibility of pigeons to highly
25 pathogenic AI viruses (HPAIVs), especially since the emergence of the
26 goose/Guangdong H5 lineage. Here, the pathogenesis of H5N8 HPAIV in comparison
27 with a H7N1 HPAIV and the role of pigeons in the epidemiology of these viruses were
28 evaluated. Local and urban pigeons (*Columba livia var. domestica*) were intranasally
29 inoculated with 10^5 ELD_{50} of A/goose/Spain/IA17CR02699/2017 (H5N8) or
30 A/Chicken/Italy/5093/1999 (H7N1) and monitored during 14 days. Several pigeons
31 inoculated with H5N8 or H7N1 seroconverted. However, clinical signs, mortality,
32 microscopic lesions and viral antigen were only detected in a local pigeon inoculated
33 with H5N8 HPAIV. This pigeon presented prostration and neurological signs that
34 correlated with the presence of large areas of necrosis and widespread AIV antigen in
35 central nervous system, indicating that the fatal outcome was associated with
36 neurological dysfunction. Viral RNA in swabs was detected in few pigeons inoculated
37 with H7N1 and H5N8, but it was inconsistent, short-term and at low titers. The present
38 study demonstrates that the majority of pigeons were resistant to H5N8 and H7N1
39 HPAIVs, despite several pigeons developed asymptomatic infections. The limited viral
40 shedding indicates a minimum role of pigeons as amplifiers of HPAIVs regardless of
41 the viral lineage and suggests that this species may represent a low risk for
42 environmental contamination.
43

44 **Keywords**: highly pathogenic avian influenza; Gs/GD lineage; H7N1; pigeons;
45 pathogenicity; shedding.
46

47 **Highlights**

48

49 • H7N1 and H5N8 HPAIVs can produce subclinical infections in pigeons.

50 • The mortality caused by H5N8 HPAIV in one pigeon was associated with
51 neurological dysfunction.

52 • Pigeons represent a low risk for HPAIVs environmental contamination.

53

54 1. Introduction

55

56 Pigeons are synanthropic birds that congregate in habitats where high amounts of food,
57 water and shelters for roosting and nesting are available. Pigeons preferentially forage
58 on agricultural areas (Rose *et al.,* 2006). A high number of these birds can be present in
59 the vicinities of poultry holdings. Therefore, pigeons can come into direct contact with
60 domestic poultry, particularly with those present in backyards and in free-range
61 husbandry, and also contaminate feed and water with their feces. In these areas, pigeons
62 are also exposed to numerous species of wild birds, including their predators, and they
63 can be the gate to wild birds pathogens go through reaching poultry species. Pigeons are
64 one of the most common birds found in urban areas, where forage is based on spilled
65 food, then living in proximity with humans and sharing habitats with aquatic birds (e.g.
66 with mallards in parks) (Rose *et al.,* 2006). Pigeons are also present in live bird markets
67 (LBMs), backyards and professional lofts, especially the breeds raised for meat or for
68 contests, including the multi-million dollar pigeon racing industry (Hunnam *et al.*,
69 2018).

70

71 The close association with wild birds, poultry and humans suggests that pigeons are
72 likely to be recurrently exposed to Influenza A viruses (IAVs). Therefore, infected
73 pigeons could act as spreaders of avian influenza viruses (AIVs) between farms and be

74 involved in the zoonotic transmission into human population as a “bridging species”.
75 However, available data indicates that pigeons play a minimum role in the
76 epidemiology of AI. Several studies have detected specific antibodies and several
77 subtypes of AIVs by molecular techniques in pigeons but the overall prevalences are
78 very low (Kohls *et al.,* 2011; Teske *et al*., 2013). In most experimental trials, pigeons
79 were resistant or minimally susceptible to highly pathogenic AIV (HPAIV) infection, as
80 demonstrated by the inconsistent presence of clinical signs, lack of mortality, lack of
81 lesions and none or low seroconversion rates (Panigraphy *et al*., 1996; Kaleta &
82 Hönicke, 2004; Liu *et al.,* 2007; Smietanka *et al*., 2011, Yamamoto *et al.,* 2012; Kwon
83 *et al*., 2017; Xiang *et al*., 2017; Albonik *et al*., 2018). Moreover, pigeons are considered
84 poor propagators of HPAIVs by the transient and low viral shedding observed after
85 experimental inoculation and the lack of transmission to co-housed pigeons and/or
86 chickens (Boon *et al.,* 2007; Liu *et al*., 2007; Werner *et al*., 2007; Brown *et al*., 2009,
87 Yamamoto *et al.,* 2012; Kwon *et al*., 2017; Xiang *et al*., 2017). Thus, they are usually
88 referred as dead-end hosts.
89
90 The limited replication of AIVs in pigeons could be in part associated with the
91 expression pattern of sialic acid (SA) molecules in this species: epithelial cell surfaces
92 from the upper respiratory tract organs contain mainly SA in an a2,6 configuration
93 (human-like), whereas a2,3 SA (avian-like) are almost restricted to the rectum (Liu *et*
94 *al.,* 2009). However, a degree of variation exists in the susceptibility of pigeons to
95 HPAIVs, especially evident since the emergence of the Asian-origin goose/Guangdong
96 H5 lineage (Gs/GD) of HPAIVs. Particular HPAIVs belonging to Gs/GD H5 lineage,
97 including H5N1 and H5N8, have been isolated from pigeons that died naturally or
98 presented evident signs of infection (Mansour *et al.,* 2014; Mansour *et al.,* 2017;

99 Elgendy *et al*., 2016; Abolnik *et al.,* 2018). Moreover, particular Gs/GD H5N1 HPAIVs
100 isolates acquired virulence to pigeons under experimental conditions: some birds
101 presented clear nervous signs before death or died in the absence of clinical signs, in
102 association to viral replication in internal organs (Klopfleish *et al*., 2006; Werner *et al.,*
103 2007; Yu *et al.,* 2007; Brown *et al.,* 2009; Phonaknguen *et al*., 2013). Presence of
104 pigeons near poultry holdings has been considered a risk factor of introduction of
105 Gs/GD H5 HPAIVs in backyard chickens (Biswas *et al.,* 2009), indicating the possible
106 involvement of pigeons in Gs/GD H5 HPAIVs outbreaks in poultry. In addition, Gs/GD
107 H5N1 HPAIV of clade 1 was efficiently transmitted to co-housed chickens
108 (Phonaknguen *et al.,* 2013). Since particular HPAIVs may have acquired unusual
109 avidity towards pigeons, it is important to characterize the pathobiology of different
110 HPAIVs in this species.
111
112 The outcome after infection with HPAIVs is influenced by the virus isolate, but also
113 largely by numerous host factors. Several experiments in chickens demonstrated that the
114 susceptibility to infection is highly dependent of the genetic background of the
115 breed/line (Sironi *et al*., 2008; Hunt *et al.,* 2010; Lee *et al*., 2016; Matsuu *et al.,* 2016;
116 Park *et al*., 2019). However, the existence of type-specific differences in peri-domestic
117 avian species, including pigeons, has not been evaluated to date. Moreover, the different
118 types of pigeons are present in diverse environments, and some of them are raised as
119 domestic birds; thus, they could play a different role in the epidemiology of AI in case
120 of infection.
121
122 Considering the differences in outcome and shedding after infection with different
123 HPAIVs in pigeons and the potential breed-specific variations in susceptibility between

124 breeds, herein we evaluated the differential pathobiology of two HPAIVs in two types
125 of pigeons. A l H7N1 isolated in Italy during the 1999-2000 epidemics and a H5N8
126 belonging to Gs/Gd H5 lineage isolated in Spain during the 2016/2017 European
127 epidemics were inoculated in local and urban pigeons. The infectivity, the pathogenesis
128 and the viral shedding were assessed.

129

130

131
2. Materials and methods

132

133
2.1. Viruses

134 The viruses used in this study were: A/Chicken/Italy/5093/1999 (H7N1), isolated in
135 1999-2000 during an Italian epidemic that mainly affected Veneto and Lombardia
136 regions (kindly provided by Dr. Ana Moreno from the *Instituto Zooprofilattico*
137 *Sperimentale della Lombardia e dell'Emilia Romagna*) and
138 A/Goose/Spain/IA17CR02699/2017 (H5N8 clade 2.3.4.4. group B), isolated in
139 Catalonia (Northern Spain) during the 2016/2017 European epizootics. Both viruses are
140 highly pathogenic based on the aminoacid sequences at the HA0 cleavage site:
141 PEIPKGSRVRR↓GLF (H7N1) and PLREKRRKR↓GLF (H5N8). Virus stocks were
142 produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 24-
143 48 hpi, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered
144 viruses in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean
145 egg lethal doses (ELD_{50}) were determined by Reed and Muench method (Reed &
146 Muench, 1938). The consensus full genome sequences corresponding to the eight
147 segments of H5N8 are available in Genbank under accession numbers: MK494920 to
148 MK494927 (H5N8).

2.2. Animals and facilities

In total, 70 pigeons (*Columba livia domestica*) of approximately 6 months of age were used in the present study. Two different types of pigeons were included: *Colom del vol català* (35 birds) and urban pigeons (35 birds). Colom del vol català is a local breed of Catalonia (northern Spain) generally present as a domestic species in backyard flocks that has been selected for flight in flock and plumage colours. Urban pigeons gather in large numbers and are extensively present in urban,peri-domestic and agricultural areas, usually foraging on spilled food. Local and urban pigeons were obtained from an accredited local supplier.

At arrival, the animals were individually identified and placed in separated negative-pressured HEPA-filtered boxes present in BSL-3 facilities in *Centre de Recerca en Sanitat Animal* (*Programa de Sanitat Animal*, IRTA). In order to ensure animal welfare the installations were enriched with perches, as described in the Spanish Royal Decree 53/2013 that lays down the basic obligations and general principles concerning the animal protection in experimentation. Prior to infection, serum samples were obtained from all animals to ensure that they were seronegative to IAV and Newcastle disease virus (NDV) by cELISA (ID-VET, Montpellier, France). In addition, oral swabs (OS) and cloacal swabs (CS) were collected from 5 random pigeons of each group (10 animals/breed) and confirmed to be negative to IAV by one-step qRT-PCR.

During the experimental procedures, food and water were provided *ad libitum*. The experimental design was approved by the ethical commission of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) and the Government of Catalonia (*Departament*

174 *de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*)
175 under reference code CEEA 92/2018-10253.
176

177 **2.3. Experimental design and sampling**

178 64 pigeons (32 local and 32 urban) were randomly separated into 4 infected groups of
179 16 birds each. After 5 days of acclimation, for each virus (H7N1 and H5N8) 16 local
180 and 16 urban pigeons were inoculated with the corresponding virus diluted in PBS in
181 order to inoculate 10^5 ELD_{50} in a final volume of 0.05 mL (0.025 mL inoculated in each
182 nostril). 6 pigeons that were previously demonstrated seronegative to IAV and NDV by
183 cELISA test were euthanized prior to infection in order to collect tissue samples as
184 negative control birds.
185

186 All birds were monitored daily for clinical signs until 14 day post-inoculation (dpi). An
187 OIE scoring for AIV infection was used (OIE, 2017). Moribund pigeons were
188 anesthetized using ketamine/xylazine (20 mg/kg body weight, Imalgene 100 and 5
189 mg/kg body weight, Rompun 20 mg/ml) via the intramuscular route, euthanized with
190 intravenous pentobarbital (140 mg/kg body weight, Euthasol 400 mg/ml) and scored as
191 dead. The mortalities and MDT were recorded in each group. In order to evaluate viral
192 shedding, OS and CS were obtained from the first 9 birds of each group (selected
193 previously to the inoculation) at 1, 3, 6, 10 and 14 dpi. Pulps from immature feathers
194 (FP) were collected from the ventral area in the same birds at 3 and 6 dpi. 2 birds of
195 each group were sacrificed, using the combination of sedation and euthanasia described
196 above, at 3, 6, 10 and 14 dpi (at 14 dpi only one pigeon was euthanized) to evaluate
197 gross lesions, and tissues were collected and formalin-fixed from the necropsies
198 performed for pathological studies. The selection of birds was biased towards those

found dead or presenting evident clinical signs of disease. At the end of the study, serum samples were obtained from all survivor pigeons in order to evaluate seroconversion.

2.4. Pathological examination and immunohistochemical testing

A standardized necropsy was performed in order to detect gross lesions and collect tissues for pathological studies. Tissue samples were collected, immersed in 10% formalin for fixation during 72 hours and embedded in paraffin wax. Samples included skin, thymus, ocular conjunctiva, pectoral muscle, nasal cavity, trachea, lung, central nervous system, heart, spleen, liver, kidney, proventriculum, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius. Tissue samples collected at 3, 6 and 10 dpi were subjected to microscopic examination. Microtome sections of 3 µm of thickness (Leica RM2255, Nussloch, Germany) from formalin-fixed, paraffin-embedded (FFPE) tissues were processed, stained with H/E and then examined under light microscopy. An IHC technique was performed as described previously in serial sections of the tissues (Sánchez-González *et al*., 2020). Sections were counterstained with Mayer's haematoxylin and examined under light microscopy. The positivity in the tissues was semi-quantitatively assessed taking into consideration the percentage of NP-positive and negative cells. The samples were classified as follows: no positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells (+++) in a tissue section. Positive and negative controls were used. The positive control was a central nervous system from a chicken experimentally infected with H7N1 HPAIV (Chaves *et al*., 2011), and the negative controls consisted in the same tissue incubated with PBS instead of the primary antibody and the tissues collected from seronegative pigeons that were euthanized prior to infection.

224

225 ## 2.5. Viral RNA quantitation in swabs and feather pulps

226 Swabs were placed in 0.5 ml of sterile PBS enriched with Penicillin-Streptomycin
227 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Nystatin (Sigma-
228 Aldrich, Missouri, USA) at a final concentration of 6% The pulps were separated from
229 the feathers and homogenized in 0.5 ml of sterile PBS with 6% antibiotics. All samples
230 described above were conserved at -75°C until further use. Viral RNA was extracted
231 from OS, CS and FP using Magattract 96 cador pathogen kit and BioSprint 96
232 equipment (Qiagen, Valencia, CA, USA), following manufacturer's instructions. A
233 highly conserved region of AIV M1 gene was detected by one-step Taqman RT-PCR in
234 Fast7500 equipment (Applied Biosystems, Foster City, CA, USA), using the primers
235 and probe as well as conditions of amplification previously described (Spackman *et al.*,
236 2002; Busquets *et al.,* 2010). Samples presenting a Ct value lower than 40 were
237 considered positive to IAV RNA. To extrapolate the genome equivalent copies (GEC)
238 present in the samples, a standard curve obtained by amplification of M1 gene fragment
239 (99 bp) was used. The limit of detection of the technique was 2,07 log GEC.

240

241 ## 2.6. Seroconversion

242 To evaluate seroconversion, sera of all survivor animals were tested by a cELISA test
243 (ID Screen® Influenza A Antibody Competition Multi-species, ID-VET, Montpellier,
244 France), following manufacturer's instructions.

245

246 # 3. Results

247

248 ## 3.1. Clinical signs and mortality

Clinical signs were only observed in a local pigeon inoculated with H5N8 HPAIV. At 5 dpi, this pigeon presented reluctance to movement, severe apathy and nervous signs, including tremor and ataxia. At 6 dpi, the bird presented similar nervous signs and was prostrated, and was consequently euthanized for ethical reasons. The remaining local and urban pigeons inoculated with H5N8 HPAIV did not present any evident clinical sign. Local and urban pigeons inoculated with H7N1 HPAIV did not exhibit any evident clinical sign through the study.

3.2. Gross lesions

Consistent gross lesions were only observed in the pigeon inoculated with H5N8 HPAIV that was euthanized at 6 dpi. The lesions were restricted to the pancreas, which presented a generalized mild discoloration with small petechial haemorrhages. The remaining birds inoculated with H5N8 and H7N1 HPAIVs serially necropsied at 3, 6, 10 and 14 dpi did not present any evident macroscopic lesion, neither in negative control animals.

3.3. Histopathological findings

Pigeons serially necropsied at 3, 6 and 10 dpi were subjected to microscopic examination. Only the pigeon inoculated with H5N8 HPAIV euthanized at 6 dpi presented microscopic lesions and NP+ cells, which were restricted to the central nervous system and myocardium. The most severe microscopic lesions and the higher percentage (>40%) of cells expressing the presence of viral antigen by means of IHC techniques were observed in the brain. The cerebral hemispheres presented extensive areas of non-suppurative encephalitis consisting in severe spongiosis, gliosis and neuronal chromatolysis, and lymphocytic cuffing; the IHC technique revealed

274 widespread AIV antigen in neurons and glial cells (Figure 1A/B). The myocardium of
275 the same bird presented focal areas of moderate necrosis and degeneration of
276 myocardiocytes with mixed inflammatory infiltrate (lymphoplasmacytic and
277 heterophils); in these areas IHC techniques revealed a low percentage (<10%) of
278 myocardiocytes and inflammatory cells expressing the presence of viral antigen
279 (Figure 1C/D). No microscopic lesions neither NP-positive cells were detected in the
280 pancreas. The remaining tissues of the same bird and the other examined pigeons
281 inoculated with H7N1 or H5N8 HPAIVs did not present evident microscopic lesions
282 nor NP-cells positivity. Negative control animals showed neither microscopic lesions
283 nor viral antigen in tissues.

284

285 3.4. Viral shedding

286 In H7N1 HPAIV-inoculated birds, three OS collected from urban pigeons were positive
287 at 1 dpi, and two urban pigeons different from those at 1 dpi tested positive at 3 dpi. In
288 CS, one urban pigeon maintained detectable levels from 3 to 6 dpi. The levels of viral
289 RNA in OS and CS remained low through the experiment (ranging from 2,93 to 3,86
290 log GEC and from 3,16 to 4,23, respectively) (Figure 2A/B).

291

292 In H5N8 HPAIV-inoculated birds, the OS collected from one local and one urban
293 pigeon tested positive to AIV RNA at 1 dpi; at 3 dpi, one pigeon of each type different
294 from those that were positive at 1 dpi presented detectable levels of viral RNA.
295 Similarly, one pigeon of each type different from those positive at 1 and 3 dpi were
296 positive at 6 dpi. In the case of the CS, viral RNA was detected in two urban pigeons at
297 1 dpi, and only a local pigeon tested positive at 3 dpi. The levels of AIV RNA in OS
298 and CS were generally low; only one CS collected at 1 dpi (5,16 log GEC) and one OS

299 collected at 6 dpi (5,02 log GEC) presented moderate amounts of viral RNA (Figure
300 2C/D).
301
302 No viral RNA was detected in the OS and CS collected at 10 and 14 dpi in any
303 experimental group, or in the swabs collected in the local pigeons inoculated with H7N1
304 HPAIV. No viral RNA was detected in the feather pulps collected at 3 and 6 dpi in any
305 bird.
306
307 In summary, viral RNA from OS and CS was only tested positive in 4 local pigeons
308 inoculated with H7N1 HPAIV, and in two local and three urban pigeons inoculated with
309 H5N8 HPAIV. Oral and cloacal shedding in the same bird was only observed in a local
310 pigeon and in an urban pigeon inoculated with H5N8 HPAIV at 3 and 1 dpi,
311 respectively. The detection in the birds was transient, inconsistent, and the viral
312 shedding restricted from 1 to 6 dpi.
313

314 3.5. Seroconversion

315 All inoculated pigeons were seronegative to AIV prior to infection. At the end of the
316 study (14 dpi), 60% (6/10) of the urban pigeons inoculated with H7N1 HPAIV
317 seroconverted, and no local pigeons tested positive. Regarding H5N8 HPAIV-
318 inoculated birds, 30% (3/10) of the local and 30% (3/10) of the urban pigeons
319 seroconverted.
320
321

322 4. Discussion

323

324 Available data indicate that the susceptibility of pigeons to HPAIVs is dependent of the
325 virus isolate, and may also be influenced by the genetic background of the line as in
326 chickens. In the present study, the differential pathobiology of H7N1 HPAIV and a
327 Gs/GD H5N8 HPAIV in pigeons and the existence of type-specific variations in
328 susceptibility between local and urban pigeons were evaluated.
329
330 Pigeons can be infected with HPAIVs, but birds usually lack evident signs of disease
331 and if so, recover entirely within a short period of time (Panigraphy *et al*., 1996; Kaleta
332 & Hönicke, 2004; Liu *et al.,* 2007; Jia *et al*., 2008; Smietanka *et al*., 2011, Yamamoto *et*
333 *al.,* 2012; Kwon *et al.,* 2017; Xiang *et al*., 2017; Albonik *et al*., 2018). However, some
334 HPAIVs have produced severe clinical signs and mortality in this species. Despite in the
335 majority of cases the mortality ratios have been minimal, infection with particular
336 Gs/GD H5N1 HPAIVs resulted in mortalities up to 37.5% (Klopfleish *et al*., 2006;
337 Werner *et al.,* 2007; Yu *et al.,* 2007; Brown *et al.,* 2009; Phonaknguen *et al*., 2013),
338 demonstrating important differences in virulence between HPAIV strains for pigeons. In
339 the present study, the experimental inoculation of a H7N1 HPAIV and a Gs/GD H5N8
340 HPAIV in pigeons resulted in the lack of evident clinical signs, mortality, gross lesions,
341 microscopic lesions and viral replication in tissues by IHC techniques in all pigeons,
342 except in one local pigeon inoculated with H5N8 HPAIV. However, the infection was
343 established in some birds, as demonstrated by the viral shedding and seroconversion.
344 Thus, the results of the present study support the existing literature that pigeons,
345 although considered resistant, can be naturally and experimentally infected in a
346 subclinical way by some diverse HPAIVs.
347

348 One local pigeon inoculated with H5N8 HPAIV exhibited severe apathy and evident
349 nervous signs, including tremor and ataxia, and had to be euthanized for ethical reasons.
350 Despite the bird was euthanized, it is likely that the infection would have resulted in
351 death within a short period of time. The clinical presentation observed was similar to
352 that previously reported in pigeons inoculated with particular Gs/GD H5N1 HPAIVs
353 (Klopfleish *et al*., 2006; Werner *et al*., 2007; Brown *et al.,* 2009; Phonaknguen *et al*.,
354 2013), but to our knowledge, this is the first experimental study reporting severe clinical
355 signs in pigeons after inoculation with Gs/GD H5N8 HPAIV of clade 2.3.4.4 Group B.
356 The present study demonstrates that despite marginal, Gs/GD H5 HPAIVs harboring
357 distinct NA subtypes other than N1 can produce deadly infections in pigeons. However,
358 the existence of significant differences in virulence between H5N8 and H7N1 HPAIVs
359 in pigeons was not demonstrated due to the low mortalities observed.
360
361
362 The severe clinical signs in the local pigeon inoculated with H5N8 HPAIV correlated
363 well with presence of microscopic lesions and viral antigen in particular tissues. This
364 bird presented large areas of necrosis in the central nervous system associated to
365 widespread presence of viral antigen, indicating that the fatal outcome was likely
366 associated with neurological dysfunction. Several studies have demonstrated the
367 presence of viral antigen and microscopic lesions in the central nervous system in
368 pigeons infected with Gs/GD H5 HPAIVsdemonstrating the strong neurotropism of this
369 lineage (Klopfleish *et al*., 2006; Jia *et al*., 2008; Brown *et al.,* 2009). Despite the
370 mortalities described in pigeons inoculated with HPAIVs are low, the central nervous
371 system generally presents the highest viral loads and more severe microscopic lesions
372 (Klopfleish *et al.,* 2006; Brown *et al.,* 2009). This particular pigeon also presented mild

373 multifocal necrotic and inflammatory lesions in the myocardium associated to moderate
374 amounts of viral antigen in myocardiocytes. This indicates that the virus was circulating
375 in the bloodstream and that the more likely source of spread into the CNS was by the
376 hematogenous route. However, no microscopic lesions and viral antigen were observed
377 in any other organ in that pigeon. This, together with the absence of viral antigen in the
378 remaining pigeons and the lack of detection of viral RNA in feather pulps in all birds
379 indicate that despite H5N8 HPAIV can replicate at high titers in the central nervous
380 system, the virus did not produce a robust systemic infection in pigeons. Therefore, the
381 high detection of viral antigen in the CNS in the severely-affected pigeon could be in
382 part associated to the dissemination of the virus via the olfactory nerve, a route of
383 spread of HPAIVs previously described in mammal species (Yamada *et al*., 2012).
384 The outcome after infection with HPAIVs is dependent on numerous viral but also host
385 factors. Previous reports have demonstrated that the genetic background of the breed
386 largely influences the infection outcome (Sironi *et al*., 2008; Hunt *et al.,* 2010; Lee *et*
387 *al*., 2016; Matsuu *et al.,* 2016; Park *et al*., 2019). To our knowledge, the existence of
388 type-specific differences in susceptibility to AIVs in peri-domestic avian species,
389 including pigeons, has not been tested to date. Herein, several urbans pigeons infected
390 with H7N1 HPAIV shed virus and serocoverted but none of the local. However, we did
391 not detect any evidence of differential susceptibility between the local and urban
392 pigeons regarding clinical presentation andpathogenesis . Taken into account the low
393 mortality rate present in this study, we cannot discard that the highly virulent infection
394 caused by H5N8 HPAIV in one pigeon could be related to the possible existence of
395 other infectious agents and non-infectious factors, such as nutritional deficiencies, stress
396 and immune status that aggravated the infection in that particular pigeon, rather than to
397 type-specific factors. However, why only a particular bird presented higher

398 susceptibility could not be unraveled. All inoculated pigeons lacked antibodies against
399 NDV, and a qRT-PCR targeting a highly conserved region of NDV was performed in
400 brain and pancreas of the pigeon that succumbed to infection in order to detect an early
401 infection, with negative results (data not shown).
402
403 Pigeons are naturally present in a wide range of habitats driven by the availability of
404 resources, and could play a role in the transmission of HPAIVs by direct contact with
405 susceptible species and/or indirectly by contamination of the environment with infective
406 secretions. Most experimental studies demonstrate that the viral shedding after HPAIV
407 infection in pigeons is brief and viral titers are under the minimum threshold required to
408 infect other species, even in sick individuals (Boon *et al.,* 2007; Liu *et al.*, 2007; Werner
409 *et al*., 2007; Brown *et al*., 2009, Yamamoto *et al.,* 2012; Kwon *et al*., 2017; Xiang *et al*.,
410 2017). Therefore, the transmission of HPAIVs between poultry holdings by pigeons is
411 more likely to occur mechanically (e.g. carrying the virus on their feet and feathers).
412 However, one study demonstrated effective transmission of Gs/GD H5N1 HPAIV to
413 co-housed chickens (Phonaknguen *et al.,* 2013), suggesting that pigeons could actively
414 act as a biological vector of particular HPAIVs. Therefore, the shedding pattern of
415 pigeons infected with different HPAIVs should be further assessed to determine if they
416 represent a risk for HPAIV interspecies transmission. In the present study, viral RNA
417 was detected in OS and CS in few pigeons inoculated with the H7N1 HPAIV and the
418 Gs/GD H5N8 HPAIV, but the detection was inconsistent, short and generally at low
419 titers. These results suggest a minimum role of pigeons as amplifiers of HPAIVs
420 regardless of the viral lineage. However, moderate levels of viral RNA were detected in
421 some OS and CS in H5N8 HPAIV-inoculated groups (up to 5,16 log GEC), including in
422 the pigeon that succumbed to infection. Since pigeons often gather in large numbers, we

423 speculate that even a small percentage of pigeons shedding moderate levels of virus
424 could represent a low but potential risk of H5N8 HPAIV environmental contamination
425 and spill over into avian species that are more susceptible. However, transmission
426 studies are required to test this hypothesis.
427

428 Several studies detected high titers of HPAIVs within the feather of HPAIV-infected
429 chickens and ducks, and the transmission following feather consumption have been
430 already demonstrated (Yamamoto *et al*., 2007). In this study, the lack of viral RNA and
431 antigen in all FP despite some animals became subclinically infected indicate that
432 feathers from pigeons do not likely play an important role in the interspecies
433 transmission of HPAIVs. In contrast, the large quantities of viral antigen detected in the
434 brain of one pigeon inoculated with H5N8 HPAIV indicate that a high viral load may be
435 present in this organ. It was previously reported that a cat succumbed to Gs/GD H5N1
436 HPAIV after consuming a pigeon carcass infected with the virus (Songserm *et al*.,
437 2006). This fact and the one lethal infection of a pigeon in our study support a low but
438 potential risk of exposure and infection to HPAIV by predators (e.g. cats, crows, and
439 hawks).
440

441 In summary, we found that pigeons can be subclinically infected by diverse HPAIVs...
442 Despite the viral excretion detected in the present study indicate the potential risk of
443 environmental contamination is low, increased surveillance in synanthropic avian
444 species during active outbreaks in poultry are needed in order to avoid the spread
445 between farms and the potential introduction into human population.
446

447 Disclosure statement

448 The authors declare that they have no competing interests.

449

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456 References

457

458 Abolnik, C., Stutchbury, S. & Jacobus Hartman, M. (2018). Experimental infection of 459 racing pigeons (*Columba livia domestica*) with highly pathogenic Clade 2.3.4.4 460 sub-group B H5N8 avian influenza virus. *Veterinary Microbiology*, 227, 127–132.

461 Biswas, P.K., Christensen, J.P., Ahmed, S.S.U., Das, A., Rahman, M.H., Barua, H., 462 Giasuddin, M., Hannan, A.S.M.A., Habib, M.A. & Debnath, N.C. (2009). Risk for 463 Infection with Highly Pathogenic Avian Influenza Virus (H5N1) in Backyard 464 Chickens, Bangladesh. *Emerging Infectious Diseases,* 15, 1931–1936.

465 Boon, A.C.M., Sandbulte, M.R., Seiler, P., Webby, R.J., Songserm, T., Guan, Y. & 466 Webster, R.G. (2007). Role of Terrestrial Wild Birds in Ecology of Influenza A 467 Virus (H5N1). *Emerging Infectious Diseases*, 13, 1720–4.

468 Brown, J.D., Stallknecht, D.E., Berghaus, R.D. & Swayne, D.E. (2009). Infectious and 469 lethal doses of H5N1 highly pathogenic Avian influenza virus for house sparrows 470 (*Passer domesticus*) and rock pigeons (*Columbia livia*). *Journal of Veterinary* 471 *Diagnostic Investigation*, 21, 437–45.

472 Busquets, N., Alba, A., Napp, S., Sánchez, A., Serrano, E., Rivas, R., Núñez, J.I. &

473 Majó, N. (2010). Influenza A virus subtypes in wild birds in North-Eastern Spain
474 (Catalonia). *Virus Research,* 149, 10–8.
475 Chaves, A.J., Busquets, N., Campos, N., Ramis, A., Rivas, R., Valle, R., Abad, F.X.,
476 Darji, A. & Majó, N. (2011). Pathogenesis of highly pathogenic avian influenza A
477 virus (H7N1) infection in chickens inoculated with three different doses. *Avian*
478 *Pathology,* 40, 163–72.
479 Elgendy, E.M., Watanabe, Y., Daidoji, T., Arai, Y., Ikuta, K., Ibrahim, M.S. & Nakaya,
480 T. (2016). Genetic characterization of highly pathogenic avian influenza H5N1
481 viruses isolated from naturally infected pigeons in Egypt. *Virus Genes*, 52, 867–
482 871.
483 Hunnam, J.C., Sloan, S., McCowan, C.I., Glass, E. & Walker, C. (2018). The racing
484 pigeon (*Columba livia domestica*) industry in Victoria, Australia, and
485 epidemiology of a novel Group A rotavirus outbreak. *Transboundary and*
486 *Emerging Diseases*, 66, 2058–2066.
487 Hunt, H.D., Jadhao, S. & Swayne, D.E. (2010). Major Histocompatibility Complex and
488 Background Genes in Chickens Influence Susceptibility to High Pathogenicity
489 Avian Influenza Virus. *Avian diseases*, 54, 572–5.
490 Jia, B., Shi, J., Li, Y., Shinya, K., Muramoto, Y., Zeng, X., Tian, G., Kawaoka, Y. &
491 Chen, H. (2008). Pathogenicity of Chinese H5N1 highly pathogenic avian
492 influenza viruses in pigeons. *Archives of Virology*, 153, 1821–6.
493 Kaleta, E.F. & Hönicke, A. (2004). Review of the literature on avian influenza A
494 viruses in pigeons and experimental studies on the susceptibility of domestic
495 pigeons to influenza A viruses of the haemagglutinin subtype H7. *Dtsch Tierarztl*
496 *Wochenschr.*, 111, 467–72.
497 Klopfleish, R., Werner, O., Mundt, E., Harder, T. & Teifke, J.P. (2006). Neurotropism

498 of Highly Pathogenic Avian Influenza Virus A/Chicken/Indonesia/2003 (H5N1) in
499 Experimentally Infected Pigeons (*Columbia livia f. domestica*). *Veterinary*
500 *Pathology*, 43, 463–70.
501 Kohls, A., Lüschow, D., Lierz, M. & Hafez, H.M. (2011). Influenza A Virus
502 Monitoring in Urban and Free-Ranging Pigeon Populations in Germany, 2006 –
503 2008. *Avian Diseases,* 55, 447–50.
504 Kwon, J.H., Noh, Y.K., Lee, D.H., Yuk, S.S., Erdene-Ochir, T.O., Noh, J.Y., Hong,
505 W.T., Jeong, J.H., Jeong, S., Gwon, G.B., Song, C.S. & Nahm, S.S. (2017).
506 Experimental infection with highly pathogenic H5N8 avian influenza viruses in the
507 Mandarin duck (*Aix galericulata*) and domestic pigeon (*Columba livia domestica*).
508 Vet Microbiol., 203, 95-102.
509 Lee, D-H., Kwon, J-H., Noh, J-Y., Park, J-K., Yuk, S-S., Erdene-Ochir, T-O., Lee, J-B.,
510 Park, S-Y., Choi, I-S., Lee, S-W. & Song, C-S. (2016). Pathogenicity of the
511 Korean H5N8 highly pathogenic avian influenza virus in commercial domestic
512 poultry species. *Avian Pathology*, 45, 208–11.
513 Liu, Y., Han, C., Wang, X., Lin, J., Ma, M., Shu, Y., Zhou, J., Yang, H., Liang, Q.,
514 Guo, C., Zhu, J., Wei, H., Zhao, J., Ma, Z. & Pan, J. (2009). Influenza A virus
515 receptors in the respiratory and intestinal tracts of pigeons. *Avian Pathology*, 38,
516 263–6.
517 Liu, Y., Zhou, J., Yang, H., Yao, W., Bu, W., Yang, B., Song, W., Meng, Y., Lin, J.,
518 Han, C., Zhu, J., Ma, Z., Zhao, J. & Wang, X. (2007). Susceptibility and
519 transmissibility of pigeons to Asian lineage highly pathogenic avian influenza
520 virus subtype H5N1. *Avian Pathology*, 36, 461–5.
521 Mansour, S.M.G., ElBakrey, R.M., Ali, H., Knudsen, D.E.B. & Eid, A.A.M. (2014).
522 Natural infection with highly pathogenic avian influenza virus H5N1 in domestic

523 pigeons (Columba livia) in Egypt. *Avian Pathology*, 43, 319–34.

524 Mansour, S.M.G., Mohamed, F.F., Eid, A.A.. M., Mor, S.K. & Goyal, S.M. (2017). Co-
525 circulation of paramyxo- and influenza viruses in pigeons in Egypt. *Avian*
526 *Pathology*, 46, 367–375.

527 Matsuu, A., Kobayashi, T., Patchimasiri, T., Shiina, T., Suzuki, S., Chaichoune, K.,
528 Ratanakorn, P., Hiromoto, Y., Abe, H., Parchariyanon, S. & Saito, T. (2016).
529 Pathogenicity of genetically similar, H5N1 highly pathogenic avian influenza virus
530 strains in chicken and the differences in sensitivity among different chicken breeds.
531 *PLoS ONE*, 11, e0153649.

532 Panigraphy, B., Senne, D.A., Pederson, J.C., Shafer, A.L. & Pearson, J.E. (1996).
533 Susceptibility of pigeons to avian influenza. *Avian diseases*, 40, 600–4.

534 Park, S.C., Song, B.M., Lee, Y.N., Lee, E.K., Heo, G.B., Kye, S.J., Lee, K.H., Bae,
535 Y.C., Lee, Y.J. & Kim, B. (2019). Pathogenicity of clade 2.3.4.4 H5N6 highly
536 pathogenic avian influenza virus in three chicken breeds from South Korea in
537 2016/2017. *J Vet Sci*., 20, e27.

538 Phonaknguen, R., Chaichoun, K., Wiriyarat, W., Sariya, L., Prayoowong, N., Chaisilp,
539 N., Moonjit, P., Puthavathana, P., Auewarakul, P., Ratanakorn, P. & Songserm, T.
540 (2013). Minimal susceptibility to highly pathogenic avian influenza H5N1 viral
541 infection of pigeons (*Columba Livia*) and potential transmission of the virus to
542 comingled domestic chickens. *Kasetsart J. (Nat. Sci.),* 47, 720–732.

543 Reed, L.J. & Muench, H. (1938). A simple method of estimating fifty per cent
544 endpoints. *Am J Epidemiol,* 27, 493–497.

545 Rose, E., Nagel, P. & Haag-Wackernagel, D. (2006). Spatio-Temporal Use of the Urban
546 Habitat by Feral Pigeons (*Columba livia*). *Behavioral Ecology and Sociobiology*,
547 60, 242–254.

548 Sánchez-González, R., Ramis, A., Nofrarías, M., Wali, N., Valle, R., Pérez, M., Perlas,
549 A & Majó, N. (2020) Experimental infection of domestic geese (Anser anser var.
550 domesticus) with H5N8 Gs/GD and H7N1 highly pathogenic avian influenza
551 viruses. Avian Pathology. DOI: 10.1080/03079457.2020.1809635
552 Sironi, L., Williams, J.L., Moreno-Martin, A.M., Ramelli, P., Stella, A., Jianlin, H.,
553 Weigend, S., Lombardi, G., Cordioli, P. & Mariani, P. (2008). Susceptibility of
554 different chicken lines to H7N1 highly pathogenic avian influenza virus and the
555 role of Mx gene polymorphism coding amino acid position 631. *Virology*, 380,
556 152–6.
557 Smietanka, K., Minta, Z., Wyrostek, K., Jóźwiak, M., Olszewska, M., Domanska-
558 Blicharz, K., Reichert, M., Pikula, A., Habyarimana, A. & van den Berg, T. (2011).
559 Susceptibility of Pigeons to Clade 1 and 2.2 High Pathogenicity Avian Influenza
560 H5N1. *Avian Diseases*, 55, 106–12.
561 Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N.,
562 Payungporn, S., Theamboonlers, A. & Poovorawan, Y. (2006). Avian Influenza
563 H5N1 in naturally infected domestic cat. *Emerging Infectious Diseases*, 12, 681–
564 683.
565 Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L.,
566 Lohman, K., Daum, L.T. & Suarez, D.L. (2002). Development of a real-time
567 reverse transcriptase PCR assay for type A influenza virus and the avian H5 and
568 H7 hemagglutinin subtypes. *J Clin Microbiol.*, 40, 3256–60.
569 Teske, L., Ryll, M. & Rautenschlein, S. (2013). Epidemiological investigations on the
570 role of clinically healthy racing pigeons as a reservoir for avian paramyxovirus-1
571 and avian influenza virus. *Avian Pathology,* 42, 557–65.
572 Werner, O., Starick, E., Teifke, J., Klopfleisch, R., Prajitno, T.Y., Beer, M., Hoffmann,

573 B. & Harder, T.C. (2007). Minute excretion of highly pathogenic avian influenza
574 virus A/chicken /Indonesia/2003 (H5N1) from experimentally infected domestic
575 pigeons (*Columbia livia*) and lack of transmission to sentinel chickens. *Journal of*
576 *General Virology,* 88, 3089–93.

577 World Organisation for Animal Health (OIE). (2017). Avian Influenza. *Terrestrial*
578 *Animal Health Code*, Chapter 10.

579 Xiang, B., Liang, J., You, R., Han, L., Mei, K., Chen, L., Chen, R., Zhang, Y., Dai, X.,
580 Gao, P., Liao, M., Xiao, C. & Ren, T. (2017). Pathogenicity and transmissibility of
581 a highly pathogenic avian influenza virus H5N6 isolated from a domestic goose in
582 Southern China. *Vet Microbiol.,* 212, 16-21.

583 Yamada, M., Bingham, J., Payne, J., Rookes, J., Lowther, S., Haining, J., Robinson, R.,
584 Johnson, D., & Middleton, D. (2012). Multiple routes of invasion of wild-type
585 Clade 1 highly pathogenic avian influenza H5N1 virus into the central nervous
586 system (CNS) after intranasal exposure in ferrets. *Acta neuropathologica*, 124,
587 505–516.Yamamoto, Y., Nakamura, K., Kitagawa, K., Ikenaga, N., Yamada, M.,
588 Mase, M. & Narita, M.(2007). Pathogenesis in call ducks inoculated intranasally
589 with H5N1 highly pathogenic avian influenza virus and transmission by oral
590 inoculation of infective feathers from an infected call duck. *Avian Diseases,* 51,
591 744–9.

592 Yamamoto, Y., Nakamura, K., Yamada, M. & Mase, M. (2012). Limited Susceptibility
593 of Pigeons Experimentally Inoculated with H5N1 Highly Pathogenic Avian
594 Influenza Viruses. *The Journal of Veterinary Medical Science,* 74, 205–8.

595 Yu, Z., Song, Y., Zhou, H., Xu, X., Hu, Q., Wu, H., Zhang, A., Zhoy, Y., Chen, J., Dan,
596 H., Luo, Q., Li, X., Chen, H. & Jin, M. (2007). Avian Influenza H5N1 virus in
597 waterowl and chickens, Central China. *Emerging Infectious Diseases,* 13, 772–

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Figures

Figure 1. Serial sections of the central nervous system and heart of a local pigeon experimentally inoculated with H5N8 HPAIV stained with conventional HE staining and IHC techniques against NP nucleoprotein, respectively**.** CNS A/B, diffuse areas of necrosis, gliosis and perivascular cuffing (A) and NP-positive neurons and glial cells (B) Myocardium C/D., multifocal necrosis of myocardiocytes with mild inflammatory infiltrate (C) and NP-positive myocardiocytes (D).

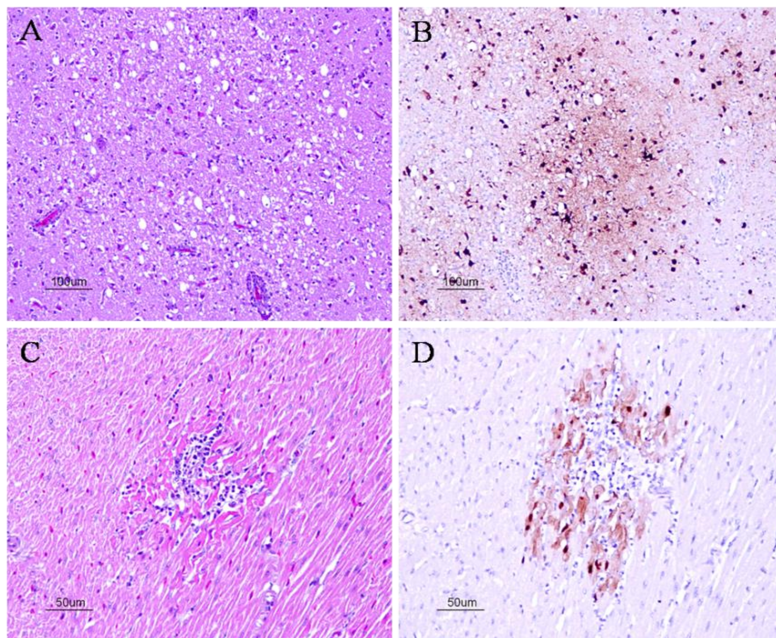

Figure 2. Viral titers expressed as log GEC in OS (A, C) and CS (B, D) obtained from local and urban pigeons inoculated with H7N1 (A,B) or H5N8 (C,D) HPAIVs at different times post-inoculation. The ratios above the columns represent the number of birds shedding virus out of the total sampled. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.