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1 **Detection of Low Pathogenic Avian Influenza Viruses in Wild Birds in Castilla-La
2 Mancha (South Central Spain)**

3

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12

13 **Abstract**

14

15 The Iberian Peninsula is located along the East Atlantic and Black Sea/Mediterranean
16 flyways and is the third ranking European country as wintering quarter for wild
17 migrating birds after Turkey and Rumania. For these reasons, Spanish wetlands are of
18 importance in AIV surveillance, and of great interest for the study of the epidemiology
19 of LPAIV under Mediterranean climate conditions. Nevertheless, information on
20 prevalence of LPAIV viruses in Spain is still scarce and is restricted to two serological
21 surveys carried out in the south of the country during 1990 and 1994 and one virological
22 study performed recently in North East Spain. In the present study we analysed the
23 prevalence of AIV circulating in wild birds in continental wetlands in central Spain and
24 determined temporal, spatial and species variation. Real time RTPCR was performed on
25 1435 faecal samples and cloacal swabs from 54 species. An overall AIV prevalence of

2.6% was detected with a peak during November and December, when thousands of migrating wild birds arrive to Spain for wintering. Highest prevalence rates were detected in *Phoenicopteriformes* and *Anseriformes.* AIV prevalence obtained from cloacal swabs and fresh faeces did not vary significantly, which supports faecal sampling as an appropriate method for large scale LPAIV surveillance programs. Viral culture was achieved in samples obtained from two Mallards and a White stork, in which subtypes H7N9 and H11N9, respectively, were identified. Our results reflect a similar scenario in AIV epidemiology in small continental wetlands as compared to large coastal humid areas in Europe and underline the importance of including species such as flamingos and storks in surveillance programs, since their role in AIV ecology in these areas could be more important than previously considered.

Keywords: Avian influenza, wild birds, faecal samples, ecology, Spain.

Introduction

Avian influenza viruses (AIV) have been isolated from at least 105 wild bird species of 26 different families; however, waterfowl such as *Anseriformes* and *Charadriiformes* are considered the natural reservoir (Webster et al., 1992; Olsen et al., 2006).

The zoonotic potential of the currently circulating H5N1 subtypes and its devastating effect on the health and well-being of avifauna and domestic poultry are worldwide of major concern (Olsen et al., 2006). Migratory waterbirds were included among the main suspects for the long distance transport of highly pathogenic AIV (HPAIV) H5N1 (Normile et al., 2005), especially after the outbreak at Qinghai Lake, China that caused the death of thousands of wild birds (Chen et al., 2005). More recently, experimental

51 studies have shown that some species like the Mallard (*Anas platyrhinchos*) are able to
52 survive H5N1 infections and shed virus over a period of time, thus being a candidate
53 species for long distance transmission of H5N1 HPAIV (Keawcharoen et al., 2008).
54 However, the true role of waterbirds in the spread of H5N1 remains unclear. The
55 attention drawn to H5N1 has also evidenced significant gaps in our knowledge of the
56 ecology of AIV in wild migratory birds. This underlines the need for multidisciplinary
57 research to better understand ecology of AIV in their natural host and environment
58 (Munster et al., 2007).
59 AIV have a global distribution and have been isolated on all continents, except
60 Antarctica. However, most AIV records in wild birds come from North America and
61 Northern Europe, where a large body of evidence of the circulation of low pathogenic
62 AIV (LPAIV) of various subtypes in aquatic birds exists. Although most of these
63 studies have focused on summer/early fall season, some of them involved waterfowl on
64 their wintering grounds as is the case in coastal Louisiana (Stallknecht et al. 2000) and
65 Texas (Hanson et al. 2005; Ferro et al. 2008).
66 Information on AIV prevalence in wild birds in southern Europe is scarce, except from
67 Italy where long-term surveillance has been carried out (De Marco et al., 2003) and
68 France, from where data on AIV prevalence in waterbirds in the Camargue have
69 become available recently (Lebarbenchon et al., 2007).
70 The only information on prevalence of LPAIV in Spain corresponds to two serological
71 surveys carried out in the south of the country during 1990 and 1994, with an average
72 seroprevalence varying from 6 to 40% (Arenas et al., 1990; Astorga et al., 1994), and a
73 recently published study performed on wild birds from Catalonia (North-East Spain), in
74 which 5% of sampled birds were found infected with LPAIV. With view to HPAIV
75 H5N1, only one case has been confirmed in wild birds in the North of Spain in June

76 2006 (Barral et al., 2008). This report also states that 8% of the wild birds examined in
77 the Basque Country were infected with LPAIV.
78 The Iberian Peninsula is located along the East Atlantic and Black Sea/Mediterranean
79 flyways. During spring and autumn migration, thousands of birds stop to rest and feed
80 in wetlands in Spain before undertaking the journey to Africa or Northern Europe. Also,
81 Spain is the third ranking European country in importance as wintering quarter after
82 Turkey and Rumania (Muñoz et al., 2006). The number of birds wintering in Spain has
83 in the recent past increased to 1.500.000 birds (Martí and Del Moral, 2002). For these
84 reasons, Spanish wetlands are of importance in AIV surveillance, and of great interest
85 for the study of the epidemiology of LPAIV under Mediterranean climate conditions.
86 The focus for AIV surveillance is generally on large coastal wetlands; however, the
87 particular conditions of small continental wetlands in the Mediterranean may imply a
88 different epidemiological scenario. The reduced availability of open water in these areas
89 leads to high concentrations of waterfowl and other birds in and around these wetlands,
90 and the development of dense vegetation. Water, vegetation and prey availability also
91 attract mammals such as wild boar or small carnivores. Although part of the species that
92 frequent these wetlands are highly mobile (e.g Flamingos) and can come in close
93 contact with humans in urban areas (e.g White storks), they are rarely included in AIV
94 surveillance schemes. We expected that close continuous monitoring of inland wetlands
95 could yield interesting information on the importance of this type of wetlands for the
96 epidemiology of LPAIV as well as reflect in general terms the dynamics of LPAIV
97 infections in waterfowl and other species in the Iberian Peninsula.
98 The necessity of sampling large numbers of water birds during surveillance programs
99 triggered by the H5N1 epidemic has led to use of fresh faecal samples collected in the
100 field as non-invasive, cost effective alternative (Pannwitz et al., 2009). However, the

101 particular climatic conditions in our study area, with very high temperatures in summer,
102 reduced humidity and increased UV radiation may negatively influence survival of AIV
103 viruses in faecal matter.
104 Thus, the main objectives of this study were to analyse the prevalence of AIV
105 circulating in wild birds in central Spain, determine temporal, spatial and species
106 variation, and with view to the particular climatic conditions in our study area, analyse
107 the efficacy of the use of fresh faeces to assess AIV prevalence.
108

109 **Materials and Methods**

110

111 **Study area.** All samples were collected in Castilla-La Mancha [UTM coordinates: 30S
112 294,348-681,063 4,208,706-4,575,340 (Figure 1); minimum altitude=244m, maximum
113 altitude=2274m]. The South Central Spanish Plateau is a flat region devoted to
114 agriculture surrounded by medium-high mountainous elevations, crossed east to-west
115 by the Toledo Mountains. The study region has a typical Mediterranean continental
116 climate, with dry periods both in summer and winter, rains concentrated in autumn and
117 spring, and hot summers (above 35ºC) and cold winters (below 0ºC). Fresh faeces were
118 obtained in different types of wetlands (reservoirs, lakes and rivers).
119 **Specimens.** A total of 1435 samples were analyzed. Fresh faeces were collected at
120 resting places in natural lakes, reservoirs and rivers, from large monospecies wild bird
121 flocks, mainly *Anatidae* (n=1063). Cloacal swabs and faeces were obtained from wild
122 birds upon admission to wildlife rehabilitation centres (n=201), from diseased birds
123 collected in different wetlands during a botulism outbreak (n=86) and from birds shot
124 by hunters (n=85).
125

126 **Sample collection.** Sterile cotton swabs/faeces were placed in transport medium
127 (Hank's balanced solution containing 10%glycerol, 200U/ml penicillin, 200µg/ml
128 streptomycin, 100U/ml polymixin B sulphate, 250µg/ml gentamycine and 50U/ml
129 nystatin, Munster et al., 2007). The samples were maintained at 4º C until arrival at the
130 laboratory after a maximum of 4 hours, where they were maintained frozen at -80º C
131 until analysis.
132 **Sample period.** Samples were collected from July 2005 to July 2007, from 18 different
133 locations. With view to host ecology of the most important group of birds sampled
134 (waterfowl) we defined the period August-October as the period of congregation of
135 birds prior to autumn migration "Autumn migration" (n= 237), November-January as
136 the period of arrival and stay of wintering birds "Wintering" (n=376), February- April
137 as the period of arrival of breeding birds "Spring migration" (n=301) and May-July as
138 the post-breeding period when most of the adult waterbirds moult "Breeding/moult"
139 (n=521) (Table 2). Sampling was carried out during wintering in eleven locations, while
140 ten locations were sampled after "breeding/moult", nine during autumn and six during
141 spring migration. Ten locations were sampled in more than one period. One location
142 (wetland A) was sampled monthly in order to determine variation in prevalence along
143 time. In this site, faeces were collected every month from March 2006 to March 2007,
144 excluding May when no samples could be obtained. Admissions of waterbirds to
145 rehabilitation centres occurred mostly during the period "breeding/moult" and hunter
146 harvested ducks were sampled during the hunting season in winter ("wintering").
147 **Virus detection.** RNA was extracted using commercial kits (High Pure RNA isolation
148 kit, Roche Diagnostics, Germany) according to the manufacturer's instructions.
149 Influenza A virus was detected using a real-time PCR (RTPCR) assay targeting the
150 matrix gene as described by Ward et al., (2004) with modifications in the probe

151 sequence as recommended by Munster et al., (2007). Amplification and detection was
152 performed on an iQ5 real time detection system (BioRad) with the TaqMan EZ RT-PCR
153 Core Reagents kit (Applied Biosystems, New Jersey, USA). Pools of five individual
154 samples were processed and upon identification of any influenza A virus positive pool
155 the RNA isolation and RTPCR procedures were repeated for the individual samples
156 within each positive pool. Individual RTPCR positive samples were subsequently used
157 for virus isolation.
158 **Virus isolation and characterization.** For influenza A virus detection in RTPCR
159 positive samples, 200 µl of the original material were inoculated into the allantoic
160 cavity of 9-11-day-old embryonated specific pathogen free chicken eggs following OIE
161 recommendations (OIE, 2009). The allantoic fluid was harvested as the embryo died or
162 after 7 days if the embryo was still alive. RNA from allantoic fluid was extracted using
163 commercial kit (QIAamp Viral RNA® Mini Kit) and RTPCR to detect influenza A type
164 matrix gene was carried out (Spackman et al., 2002). When no influenza A virus was
165 detected, the allantoic fluid was passaged twice in embryonated chicken eggs.
166 **Sequence analysis.** The haemagglutinin and neuraminidase were sequenced when
167 possible following the protocol described by Hoffmann et al., (2001) with minor
168 modifications. The sequences obtained were compared with those already available in
169 GenBank database by nucleotide sequence homology searches made at the network
170 server of the National Center for Biotechnology Information (NCBI) using BLAST.
171 **Meteorologic data.** Data on mean temperature and humidity for each month of the
172 study period were obtained from the Agencia Estatal de Meteorología (AEMET),
173 Ministerio de Medio Ambiente, Medio Rural y Marino for the stations nearest to the
174 wetland in which monthly sampling was carried out.

175 **Statistical analysis.** We analysed the complete dataset in order to determine differences
176 between sampling locations, sample type and host species using Chi square tests. All
177 analysis were carried out using STATISTICA 6.0 software.
178
179 **Results**
180
181 Between July 2005 and July 2007 a total of 1435 samples (cloacal swabs and fresh
182 faeces) were collected from wild birds from different locations in central Spain (Figure
183 1). The sample set included birds from 22 families belonging to more than nine orders
184 (Table 1). Our sampling and processing procedures revealed a prevalence of 2.6% (37
185 out of 1435) of AIV in our sample set.
186 Overall prevalence for each sampling site varied considerably, with local prevalence of
187 up to 10% and sites with negative results (Figure 1). AIV was detected both in fresh
188 faeces from lakes and reservoirs and in cloacal swabs from birds admitted to
189 rehabilitation centres and hunted ducks. Comparison of results between natural lakes
190 (3.3%, 28 positives out of 829) and reservoirs (1.6%, 3 positives out of 192) revealed no
191 statistically significant differences (χ^2 test, p=0.19).
192 Throughout our study we collected samples from a total of 57 species. 43.8% of the
193 samples were obtained from the order *Anseriformes* (Table 2). The highest AIV
194 prevalence was detected in the order *Phoenicopteriformes* (28.6%, 2 out of 7).
195 However, as all flamingo samples were collected at the same location on the same date,
196 the possibility of several faeces originating from a single animal can not be ruled out.
197 When flamingos are excluded, the highest AIV prevalence was found in *Anseriformes*
198 (29 out of 628, 4.6%). AIV was more often detected in dabbling ducks (28 out of 514,
199 5.4%) than in the rest of species (χ^2 test, $p<0.001$). On species level (and excluding

200 flamingos), Mallards had the highest prevalence (25 out of 415, 6%) as compared to
201 other species sampled (χ^2 test, $p<0.001$).
202 As reported by Munster et al. (2007), timing relative to migration, instead of the
203 absolute time point, is determinant for virus prevalence. Based on this statement we
204 decided to group the months of the year in 4 categories, corresponding to the main
205 annual biological phenomena affecting most of the wild birds included in this study, as
206 explained in the materials and methods section. Seasons in which a positive result was
207 found for every species are shown in Table 2. Due to differences in sample sizes among
208 species and based on prevalence results, we decided to include only data from anatid
209 species, White storks (*Ciconia ciconia*), Common coots (*Fulica atra*) and Cattle egrets
210 (*Bubulcus ibis*) in temporal prevalence variation analysis. Those species are the best
211 represented in our data set (more than 120 samples each) and AIV have been detected in
212 all of them.
213 As shown in Figure 2, the peak of AIV prevalence was detected during wintering both
214 for the whole dataset (5.7%), and for wetland A (12.2%), reaching maximum prevalence
215 in November and December. However, high AIV prevalence was also evidenced during
216 autumn migration in the combined data (3.6%), although no positives were found in the
217 same period for wetland A. In spring, prevalence was much lower (2% and 1.9%
218 respectively). A slightly higher AIV prevalence was observed for the moult and
219 breeding period (2.2%) in the complete dataset, while AIV was not detected in this
220 period in wetland A. However, ongoing studies (data not shown) have evidenced
221 presence of AIV also in wetland A after breeding and during moult in successive years.
222 AIV prevalence obtained from cloacal swabs (2.5%, 9 positives out of 355) and fresh
223 faeces (2.5%, 27 positives out of 1080) did not vary significantly (χ^2 test, $p=0.98$).

224 Detection of LPAIV in wetland A occurred in seasons with lowest mean temperatures
225 and highest mean humidity in the sampling period (Figure 2).
226 Three influenza A virus isolates were obtained from the 37 positive samples, which
227 means an overall recovery rate of 8.3%. Two of the isolates were obtained from
228 Mallards (*Anas platyrhynchos*) and the other one from a White stork. The two virus
229 isolates obtained from Mallards were identified by sequencing as H7N9 (GenBank
230 accession numbers GU354035 and GU354036), and the isolate obtained from the white
231 stork was identified as H11N9 (Accession numbers GU354037 and GU354038). The
232 pathogenicity of the H7 virus isolate was determined by the study of the sequence at the
233 cleavage site (PETPKGR*GLF) that characterised this strain as of low pathogenicity. A
234 sample of the three isolates was also sent to the Spanish National Reference Laboratory
235 (SNRL) for official confirmation.
236 It was also possible to sequence some of the other positive samples and different LPAI
237 subtypes were identified including H5N2, H3N8 and H5, H12 and N8 genes all of them
238 from samples obtained in Mallards. In the two H5 positive samples the AIV was
239 identified as low pathogenic by RT-PCR (Payungporn et al., 2006).
240
241
242 **Discussion**
243
244 Information on AIV ecology in Mediterranean countries is scarce and most of the
245 existing studies provide data corresponding to only one season (Lebarbenchon et al.,
246 2007; Terregino et al., 2007). Continuous sampling throughout a year enabled us to
247 relate variation of AIV prevalence to seasonal movements and behavioural changes,
248 especially in orders such as *Anseriformes*, *Ciconiformes* and *Gruiformes*, well

249 represented by a large sample size and of which samples from all seasons were
250 available.
251 Overall, we confirmed a low average LPAIV prevalence (2.6%, 37 out of 1435), similar
252 to what has been stated in previous studies carried out in Northern Europe (Munster et
253 al., 2007), Africa (Gaidet et al., 2007), Italy (Cattoli et al., 2007) and France
254 (Lebarbenchon et al., 2007), but lower than what has been reported from the north of
255 Spain (Barral et al., 2008; Busquets et al. 2010). However, comparison among studies
256 should be done cautiously due to differences related to sampling design, species
257 targeted and laboratory methods (Olsen et al. 2006).
258 AIV prevalence in *Anseriformes* was slightly lower than described by Munster et al.,
259 *(*2007) (4.5% versus 6.9% in Sweden and The Netherlands), but similar to data obtained
260 from other Mediterranean countries such as Italy (Cattoli et al., 2007). In the Mallard
261 (*Anas platyrhynchos*), the most represented species in this study, prevalence was lower
262 than described in preceding studies (Munster et al., 2007; Terregino et al., 2007;
263 Busquets et al., 2010).
264 The higher prevalence of AIV observed in dabbling ducks is consistent with findings in
265 previous studies (Olsen et al., 2006; Munster et al., 2007) and has been attributed to the
266 feeding habits of these species, as virus shed by faeces may remain infectious for
267 prolonged periods in surface waters as long as temperature, salinity and pH are
268 favourable (Brown et al., 2009).
269 Information on LPAIV in *Phoenicopteridae* is scarce. Reasons for this may be that
270 these birds are not very abundant in Europe and North America, their capture is costly
271 and time-consuming and collection of faeces is not always possible as they usually
272 remain in the water. In many LPAIV studies in wild birds, samples from
273 *Phoenicopteriformes* are not included (Munster et al., 2007; Gaidet et al., 2007). The

274 high AIV prevalence we obtained for flamingos in our study resulted from a low
275 number of samples (n=7), collected on one single day from the same location, and thus
276 must be considered with caution. Cross contamination of the positive samples is
277 unlikely, because different persons collected the individual samples, however we cannot
278 completely rule out that both positive samples belonged to the same individual. If
279 feeding behaviour is considered as an important factor for the exposure to AIV, the
280 flamingo, that filters surface and profound water, is a species that may be exposed
281 frequently. Their breeding behaviour in large colonies could also favour AIV
282 transmission among adults and juveniles. Lebarbenchon et al. (2007) did not find AIV
283 in samples from 113 greater flamingo chicks in the Camargue, France, while a
284 prevalence of 25.3% (19 positives out of 75) was detected in Greater Flamingo in
285 Northern Italy during winter 2004-2006 (Terregino et al., 2007), achieving successful
286 isolation of an H6N2 virus. Likewise, high seroprevalences (43%) were evidenced in
287 Flamingos from South Spain by Arenas et al. 1990, and AIV was detected by RTPCR in
288 2.5% of 154 Flamingos sampled in Catalonia, Spain (Busquets et al.2010). All these
289 data are in accordance with our findings. Flamingos are actually considered a semi-
290 resident, fairly mobile species, thus AIV prevalence in this species, their movements
291 and their presence in areas where infected waterfowl have been detected may have
292 important implications for AIV ecology and surveillance (Terregino et al., 2007).
293 In North America, some species in the order *Charadriiformes* are considered to play an
294 important role in LPAIV epidemiology (Stallknecht and Shane 1988; Krauss et al.,
295 2004), while in Europe its role remains unclear, with prevalences that are mostly low
296 (Fouchier et al., 2003; Olsen et al., 2006; Cattoli et al., 2007; Busquets et al., 2010). We
297 did not detect AIV in any species of this order sampled in our study, possibly because
298 only a reduced number of samples from the genus *Larus* (n=65) was included and no

299 samples from *Calidris*, *Sterna* and *Uria* genuses in which LPAIV infection has been
300 detected in preceding studies (Kaleta et al., 2005; Munster et al., 2007; Fouchier et al.,
301 2003) were available.
302 Few studies include samples from *Ciconiformes,* despite behavioural traits that might
303 favour AIV transmission and recirculation. While Lebarbenchon et al. (2007) did not
304 detect AIV in samples of 185 *Ciconiformes*, Muller et al. (2009) found LPAIV in 3 out
305 of 103 faeces of adult storks sampled in Germany during 2006 by means of RTPCR. In
306 fact, HPAI H5N1 has been previously recorded in several species of *Ciconiformes* such
307 as Little Egrets (*Egretta garzeta*) and Grey herons (*Ardea cinerea*) in Hong Kong (Ellis
308 et al., 2002) and White storks in Germany (Globig et al., 2009).We found a low
309 prevalence in this order (1%, 3 positives out of 308 samples), but achieved virus
310 isolation from a cloacal swab of a White stork admitted to a rehabilitation centre due to
311 trauma. Virus from the two positive Cattle egrets could not be isolated. Given the
312 behaviour of White storks as colony breeders, as an at least partially migratory species,
313 their increased census in eastern and central Europe and specifically in Spain and their
314 usual association to human activity, they could be an interesting species to include when
315 planning AIV surveillance programs in wild birds.
316 In the case of *Gruiformes*, prevalence (1.9%) is higher than reported in previous studies
317 (0.7% in Gaidet et al., 2006; 0.4% in Munster et al., 2007), but similar to data obtained
318 in Italy (De Marco et al., 2004) and North East Spain (Busquets et al., 2010). The
319 detection of AIV in three Common coots (*Fulica atra*) out of 160 in our study may
320 reflect interspecific transmission from Mallards, as coots in the studied wetlands are
321 closely associated with other waterfowl, especially Mallards, which would also be
322 consistent with the findings of De Marco et al. (2004).

323 A peak of prevalence is observed in south central Spain during winter, which is
324 consistent with results obtained by Munster et al. (2007) in Northern Europe. More
325 precisely, the results from Munster et al. (2007) reflect a peak in AIV prevalence early
326 in fall migration during the months of October and November, with a subsequent
327 decline, and a North-South gradient, being virus prevalence in Mallards in The
328 Netherlands 3-fold lower as compared with Sweden. Considering that these birds
329 continue southward migration they would presumably arrive in Spanish wetlands by
330 November- December, which is when we detected the highest prevalence in our study
331 area. Also, prevalence was lower than in Northern Europe, which would support the
332 hypothesis of a North-South gradient of virus prevalence due to a progressive decrease
333 of virus shedding, development of immunity or loss of infected individuals during
334 southbound migration (Muzzafar et al., 2006; Terregino et al., 2007). Nevertheless,
335 although mean prevalence was lower both than in Sweden and in the Netherlands,
336 locally (as in the case of wetland A during wintering) we found high prevalences. This
337 could be explained by recirculation of AIV due to the high concentration of wintering
338 waterbirds in Spanish wetlands.
339 Both, our results and data from previous studies on wild birds wintering in wetlands in
340 Northern Italy in which considerable prevalence (5-8%) and seroprevalence of AIV was
341 detected, confirm the important role that Mediterranean wintering areas play in AIV
342 epidemiology (Terregino et al., 2007; Cattoli et al., 2007; De Marco et al., 2003). As in
343 our case, in studies from the US, medium to high prevalences were detected during
344 winter (2-10%), mainly from dabbling ducks and especially from teals (Hanson et al.,
345 2005; Ferro et al., 2008). Mallards, Northern shovelers (*Anas clypeata*) and Gadwalls
346 (*Anas strepera*) which represent most of AIV carriers in our study area were also
347 frequently found infected in those studies.

348 For our whole dataset, relatively high prevalences (although lower than during winter),
349 were also detected during autumn migration (3.6%). In contrast, in wetland A no AIV
350 was found in the same period, probably due to small sample size. Other studies carried
351 out in South Europe also detected high prevalences in early fall although, contrary to
352 our results, a marked decrease was evidenced afterwards, during November, December
353 and January (Lebarbenchon et al., 2010).
354 Wild birds returning northward in spring generally have lower viral titres than
355 southward migrants, but in high enough loads to re-establish the infection in their
356 northern breeding grounds (Krauss et al., 2004; Webster et al., 1992). We detected a
357 low AIV prevalence (2%, 5 out of 246) during spring migration (February to April) in
358 our study area. However, it is difficult to elucidate whether these carriages belong to
359 resident individuals or to waterfowl flying northward to their reproductive areas.
360 Post-breeding is considered an important period for AIV transmission and perpetuation
361 due to the presence of high numbers of juvenile birds susceptible to infection. AIV
362 prevalence in this period was 2.2% (9 out of 406), much lower than during wintering. In
363 the case of the wetland used for annual variation studies, wetland A, we did not find
364 AIV positive samples during June and July 2006, although it should be taken into
365 account that only 64 samples were analysed for this period in 2006. Data from ongoing
366 studies reveal LPAIV prevalences of 2.3% in the same area in 2007 and 2.8% in 2008
367 (Pérez-Ramírez et al., unpublished data).These results underline the importance of long-
368 term studies due to the considerable interannual variations in AIV prevalence (Krauss et
369 al., 2004). In fact, in our study, prevalence variation has been observed when
370 individually comparing seasons from both years. Nevertheless, high infection rates have
371 been consistently found during wintering.

372 Climatic conditions in winter are favourable for AIV persistence in faeces and the
373 environment and recirculation in the waterbird community (Webster et al., 1992; Brown
374 et al., 2007). Thus LPAIV detection in wetland A during the period with higher
375 humidity and lower temperatues may reflect both, increase in persistence of LPAIV in
376 the environment, and higher numbers of suitable hosts (increase in waterbird
377 populations during wintering).
378 Isolation rate in our study was low (8.3% of real time RTPCR positives), but preceding
379 studies obtained similar or lower isolation rates (3.14% in Gaidet et al., 2007; 8.1% in
380 Cattoli et al., 2007; 8.3% in Pereda et al., 2008; 0% in Pannwitz et al., 2009). Low
381 numbers of viral copies and inability of some AIV to grow to high enough titers to be
382 detected in embrionated chicken eggs could be responsible for this (Ip et al., 2008,
383 Runstadler et al., 2007).
384 Some authors have considered cloacal swabs more suitable for AIV detection by means
385 of RTPCR techniques, since the virus replicates mainly in epithelial cells (Slemons and
386 Easterday, 1978) and this kind of sample is supposed to be less prone to contamination.
387 However, fresh faeces collection is a convenient, non invasive and cost effective
388 method and has frequently been used for monitoring LPAIV in wild birds (Pannwitz et
389 al., 2009; Lebarbenchon et al., 2007; Gaidet et al., 2007). Drawbacks include negative
390 effects of external agents (UV light, temperature and humidity) or the fact that it is not
391 always possible to establish an accurate association between droppings and individual
392 birds (Yasué et al., 2006).
393 Our results are in concordance with reports from other authors (Gaidet et al., 2007;
394 Lebarbenchon et al., 2007; Pannwitz et al., 2009) which support faecal sampling as an
395 appropriate method for large scale LPAIV surveillance programs, since capture of large
396 numbers of birds is extremely labour-intensive, costly, time-consuming and causes

more disturbances to wild waterbird species and their habitats. This may be different for HPAIV as for example in the case of HPAI H5N1, in which excretion of the virus primarily via the upper respiratory tract makes the combination of cloacal and tracheal swabs mandatory for virus detection (Ellström et al., 2008).

Conclusion

Our results reflect a similar scenario in AIV epidemiology in small continental wetlands as compared to large coastal humid areas. Our data support the hypothesis of Mediterranean wintering areas as key points in AIV epidemiology. Fresh faecal samples and cloacal swabs proved equally effective as tools for active surveillance of AIV in wild birds. Finally, our results reveal the importance of including species such as flamingos and storks in surveillance programs, since their role in AIV ecology in these areas could be more important than previously considered.

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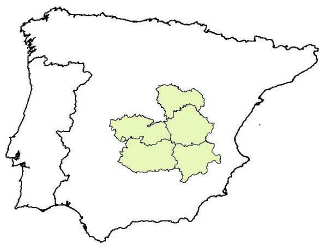
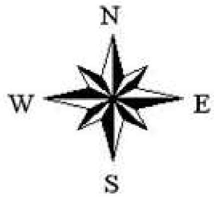
607 *Figure 1*

608 Study area, sample size and AIV prevalence by sampling sites. Number of samples
609 collected is reflected by circle size. Prevalence rates (%) are shown in numbers
610 connected to the respective circles. Wetland A is represented by a striped circle.
611 Dotted circles represent locations were hunted birds were sampled. White circles
612 represent wildlife rehabilitation centres and grey circles represent wetlands (lakes,
613 reservoirs and rivers).

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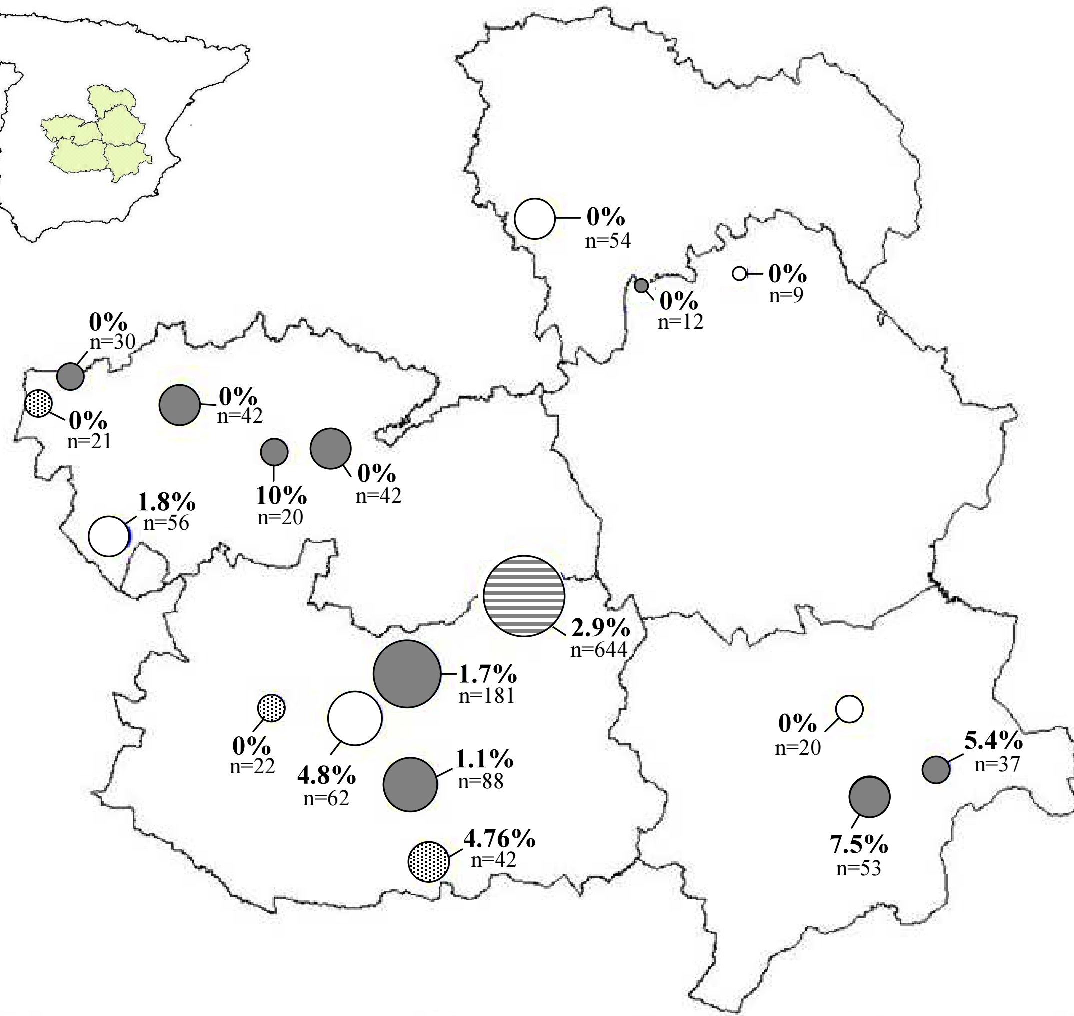
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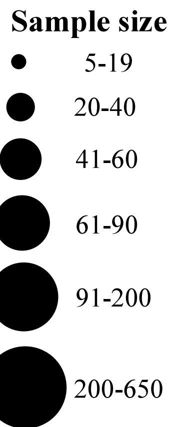
Sample size
5-19
20-40
41-60
61-90
91-200
200-650

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210
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Km

615 *Figure 2*

616 *A*. LPAIV prevalence variation throughout the year in the whole study area (grey) and
617 in wetland A (black), where monthly sampling was carried out. Bars indicate confidence
618 intervals. AM: autumn migration; W: wintering; SM: spring migration; B/M:
619 breeding/moult.

620 *B*. Mean seasonal temperature (MSTemp, grey) in ºC and mean seasonal humidity
621 (MSHum, black) as % of relative humidity (taken from monthly means, obtained from
622 AEMET) in relation to prevalence variation throughout a year in Wetland A.

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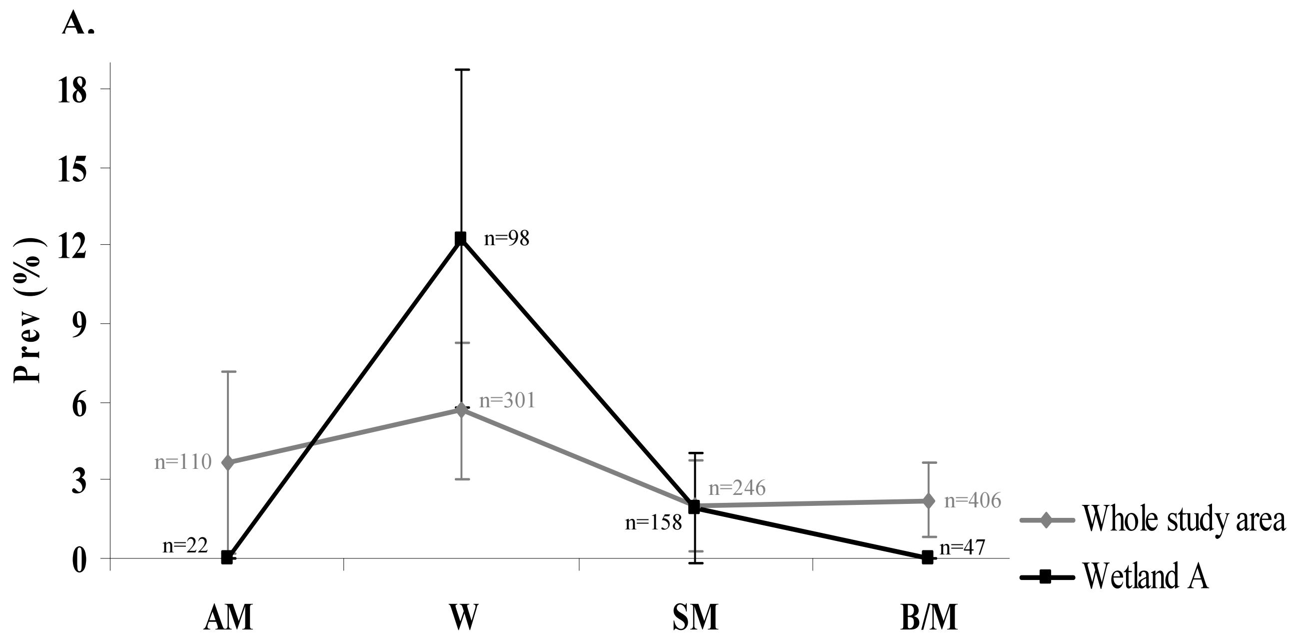
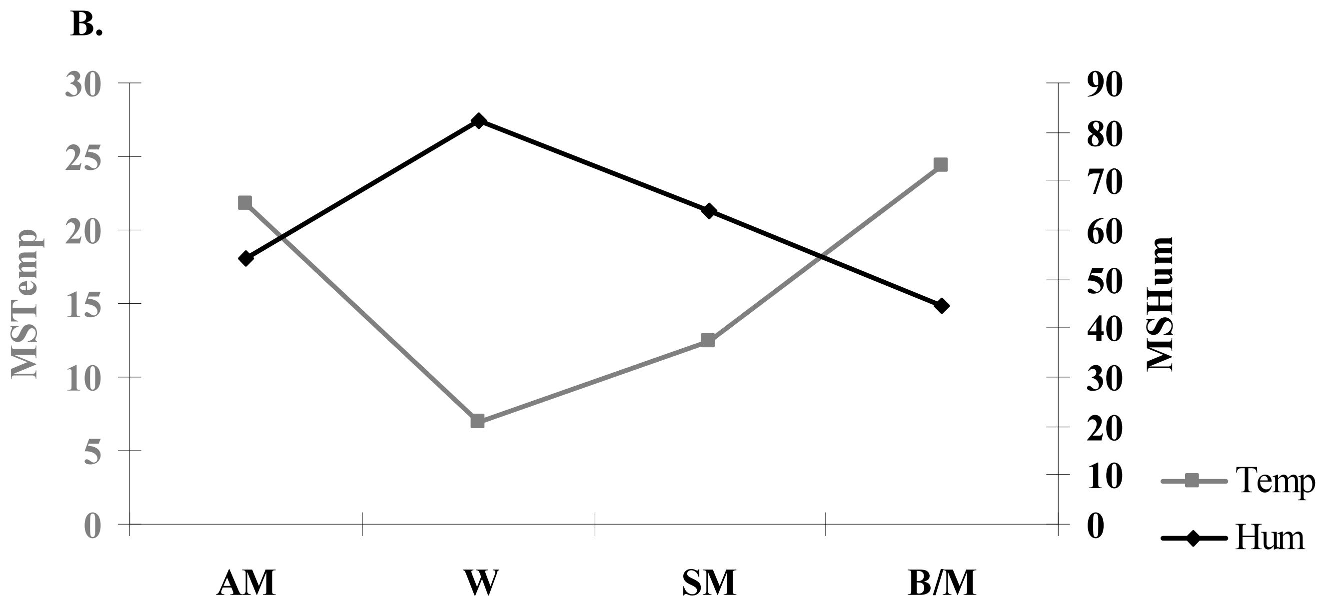
624



625 626 627 628 629 630 631 632 633 634



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648 *Table 1.* Orders and families sampled and AIV prevalences detected.

ORDER	FAMILY	N	INFECTED	FAM PREV (%)	ORDER PREV (%)
Anseriformes (N= 628)	*Anatidae*	628	29	4.6	4.6
Charadriiformes (N=217)	*Charadriidae*	35	0	0	0
	Recuvirostridae	115	0	0	
	Laridae	66	0	0	
	Buhrnidae	1	0	0	
Gruiformes (N=180)	*Rallidae*	163	3	1.8	1.7
	Gruidae	17	0	0	
Pelecaniformes (N=29)	*Phalacrocoracidae*	29	0	0	0
Columbiformes (N=31)	*Columbidae*	31	0	0	0
Ciconiformes (N=308)	*Ciconidae*	129	1	0.8	1
	Ardeidae	179	2	1.1	
Passeriformes (N=24)	*Corvidae*	18	0	0	0
	Other	6	0	0	
Phoenicopteriformes (N=7)	*Phoenicopteridae*	7	2	28.6	28.6
Other (N=11)	*Other*	11	0	0	0

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Table 2. Number of samples, number of LPAIV positives and LPAIV prevalences in different waterbird species in Castilla–La Mancha between 2005-2007. Sampling period in bold type represents at least one positive sample in the season (AM = autumn migration, W = wintering, B/M = Postbreeding/moult, SM = spring migration). Scientific names after The Collins Bird Guide to the birds of Spain and Europe (Mullarney et al., 1999).

ORDER	FAMILY	SPECIES	N	RTPCR POSIT	PREV (%)	SAMPLING PERIOD
Anseriformes	*Anatidae*	Mallard *(Anas platyrhynchos)*	415	25	6	**AM, W, SM, B/M**
		Greylag goose *(Anser anser)*	50	0	0	W, SM, B/M
		Northern shoveler *(Anas clypeata)*	46	2	4.3	AM, **W, SM,** B/M
		Gadwall *(Anas strepera)*	36	1	2.8	AM, W, SM, **B/M**
		Domestic goose *(Anser anser f. domesticus)*	21	0	0	AM
		Common pochard *(Aythia ferina)*	19	1	5.3	W, SM**, B/M**
		Hybrid mallard (*Anas sp*.)	11	0	0	AM, SM, B/M
		Red-crested pochard *(Netta rufina)*	7	0	0	AM, SM, B/M
		Marbled teal *(Marmaronetta angustirostris)*	6	0	0	SM, B/M
		Common teal *(Anas crecca)*	4	0	0	SM, B/M
		Tufted duck *(Aythia fuligula)*	4	0	0	W, B/M
		Common shelduck *(Tadorna tadorna)*	3	0	0	B/M
		White-headed duck *(Oxyura leucocephala)*	2	0	0	AM, B/M
		Egyptian goose *(Alopochen aegyptiacus)*	2	0	0	AM
		Northern pintail *(Anas acuta)*	2	0	0	SM, B/M
Charadriiformes	*Recurvirostridae*	Black winged stilt (*Himantopus himantopus*)	94	0	0	AM, SM, B/M
		Pied avocet (*Recurvirostra avosetta*)	21	0	0	SM, B/M
	Laridae	Black headed gull (*Larus ridibundus)*	36	0	0	AM, SM, B/M
		Lesser Black headed gull (*Larus fuscus)*	6	0	0	W
		Herring gull *(Larus argentatus)*	1	0	0	AM
		Larus sp.	23	0	0	AM, SM
	Charadriidae	Northern lapwing *(Vanellus vanellus)*	30	0	0	W, B/M
		Unidentified *Charadriidae*	5	0	0	SM
	Buhrnidae	Eurasian stone-curlew *(Burhinus oedicnemus)*	1	0	0	W
Gruiformes	*Rallidae*	Eurasian coot (*Fulica atra*)	160	3	1.9	**W, SM,** B/M
		Common Moorhen (*Gallinula chloropus*)	1	0	0	W
		Purple swamphen (*Porphyrio porphyrio)*	2	0	0	SM, B/M
	Gruidae	Common crane (*Grus grus*)	17	0	0	AM, W, SM

Ciconiformes	*Ardeidae*	Grey heron (*Ardea cinerea*)	13	0	0	AM, W, SM
		Cattle egret (*Bubulcus ibis*)	147	2	1.4	AM, **B/M**
		Little egret (*Egretta garceta*)	2	0	0	AM
		Black-crowned night Heron (*Nycticorax nycticorax*)	17	0	0	AM
	Ciconidae	White stork (*Ciconia ciconia*)	128	1	0.8	AM, W, **SM**, B/M
		Black stork (*Ciconia nigra*)	1	0	0	AM
Columbiformes	*Columbidae*	Pigeon (*Columba livia*)	8	0	0	B/M
		Wood pigeon (*Columba palumbus*)	8	0	0	AM, W, SM
		Eurasian collared dove (*Streptopelia decaocto*)	15	0	0	AM
Pelecaniformes	*Phalacrocoracidae*	Great cormorant (*Phalacrocorax carbo*)	29	0	0	W, SM
Passeriformes	*Motacillidae*	Water pipit *(Anthus spinoletta)*	1	0	0	W
	Corvidae	Common raven *(Corvus corax)*	13	0	0	W, SM
		Eurasian jackdaw *(Corvus monedula)*	1	0	0	AM
		Red-billed chough *(Pyrrhocorax pyrrhocorax)*	1	0	0	B/M
		European magpie *(Pica pica)*	3	0	0	B/M
	Muscicapidae	European robin *(Eritachus rubecula)*	1	0	0	W
		Nightingale *(Luscinia megarhynchos)*	1	0	0	SM
	Sylviidae	Common chiffchaff *(Phylloscopus collybita)*	1	0	0	W
	Passeridae	House sparrow *(Passer domesticus)*	2	0	0	SM
Piciformes	*Picidae*	Great spotted woodpecker *(Picoides major)*	1	0	0	B/M
Falconiformes	*Accipitridae*	Eurasian sparrowhawk *(Accipiter nissus)*	1	0	0	B/M
		Griffon vulture *(Gyps fulvus)*	2	0	0	AM, B/M
		Booted eagle *(Hieraaetus pennatus)*	1	0	0	AM
		Black kite *(Milvus migrans)*	1	0	0	SM
	Falconidae	Peregrine falcon *(Falco peregrinus)*	2	0	0	SM, B/M
		Honey buzzard (*Pernis aviporus*)	1	0	0	AM
Strigiformes	*Strigidae*	Eurasian eagle-owl *(Bubo bubo)*	1	0	0	SM
	Tytonidae	Barn owl *(Tyto alba)*	1	0	0	B/M
Phoenicopteriformes	*Phoenicopteridae*	Greater flamingo *(Phoenicopterus ruber)*	7	2	28.6	**B/M**