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

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Abstract

The Iberian Peninsula is located along the East Atlantic and Black Sea/Mediterranean flyways and is the third ranking European country as wintering quarter for wild migrating birds after Turkey and Rumania. For these reasons, Spanish wetlands are of importance in AIV surveillance, and of great interest for the study of the epidemiology of LPAIV under Mediterranean climate conditions. Nevertheless, information on prevalence of LPAIV viruses in Spain is still scarce and is restricted to two serological surveys carried out in the south of the country during 1990 and 1994 and one virological study performed recently in North East Spain. In the present study we analysed the prevalence of AIV circulating in wild birds in continental wetlands in central Spain and determined temporal, spatial and species variation. Real time RTPCR was performed on 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 platyrhynchos*) 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

2006 (Barral et al., 2008). This report also states that 8% of the wild birds examined in the Basque Country were infected with LPAIV.

The Iberian Peninsula is located along the East Atlantic and Black Sea/Mediterranean flyways. During spring and autumn migration, thousands of birds stop to rest and feed in wetlands in Spain before undertaking the journey to Africa or Northern Europe. Also, Spain is the third ranking European country in importance as wintering quarter after Turkey and Rumania (Muñoz et al., 2006). The number of birds wintering in Spain has in the recent past increased to 1.500.000 birds (Martí and Del Moral, 2002). For these reasons, Spanish wetlands are of importance in AIV surveillance, and of great interest for the study of the epidemiology of LPAIV under Mediterranean climate conditions.

The focus for AIV surveillance is generally on large coastal wetlands; however, the particular conditions of small continental wetlands in the Mediterranean may imply a different epidemiological scenario. The reduced availability of open water in these areas leads to high concentrations of waterfowl and other birds in and around these wetlands, and the development of dense vegetation. Water, vegetation and prey availability also attract mammals such as wild boar or small carnivores. Although part of the species that frequent these wetlands are highly mobile (e.g. Flamingos) and can come in close contact with humans in urban areas (e.g. White storks), they are rarely included in AIV surveillance schemes. We expected that close continuous monitoring of inland wetlands could yield interesting information on the importance of this type of wetlands for the epidemiology of LPAIV as well as reflect in general terms the dynamics of LPAIV infections in waterfowl and other species in the Iberian Peninsula.

The necessity of sampling large numbers of water birds during surveillance programs triggered by the H5N1 epidemic has led to use of fresh faecal samples collected in the field as non-invasive, cost effective alternative (Pannwitz et al., 2009). However, the

particular climatic conditions in our study area, with very high temperatures in summer, reduced humidity and increased UV radiation may negatively influence survival of AIV viruses in faecal matter.

Thus, the main objectives of this study were to analyse the prevalence of AIV circulating in wild birds in central Spain, determine temporal, spatial and species variation, and with view to the particular climatic conditions in our study area, analyse the efficacy of the use of fresh faeces to assess AIV prevalence.

Materials and Methods

Study area. All samples were collected in Castilla-La Mancha [UTM coordinates: 30S 294,348-681,063 4,208,706-4,575,340 (Figure 1); minimum altitude=244m, maximum altitude=2274m]. The South Central Spanish Plateau is a flat region devoted to agriculture surrounded by medium-high mountainous elevations, crossed east to-west by the Toledo Mountains. The study region has a typical Mediterranean continental climate, with dry periods both in summer and winter, rains concentrated in autumn and spring, and hot summers (above 35°C) and cold winters (below 0°C). Fresh faeces were obtained in different types of wetlands (reservoirs, lakes and rivers).

Specimens. A total of 1435 samples were analyzed. Fresh faeces were collected at resting places in natural lakes, reservoirs and rivers, from large monospecies wild bird flocks, mainly *Anatidae* (n=1063). Cloacal swabs and faeces were obtained from wild birds upon admission to wildlife rehabilitation centres (n=201), from diseased birds collected in different wetlands during a botulism outbreak (n=86) and from birds shot by hunters (n=85).

Sample collection. Sterile cotton swabs/faeces were placed in transport medium (Hank's balanced solution containing 10% glycerol, 200U/ml penicillin, 200µg/ml streptomycin, 100U/ml polymixin B sulphate, 250µg/ml gentamycin and 50U/ml nystatin, Munster et al., 2007). The samples were maintained at 4° C until arrival at the laboratory after a maximum of 4 hours, where they were maintained frozen at -80° C until analysis.

Sample period. Samples were collected from July 2005 to July 2007, from 18 different locations. With view to host ecology of the most important group of birds sampled (waterfowl) we defined the period August-October as the period of congregation of birds prior to autumn migration "Autumn migration" (n= 237), November-January as the period of arrival and stay of wintering birds "Wintering" (n=376), February- April as the period of arrival of breeding birds "Spring migration" (n=301) and May-July as the post-breeding period when most of the adult waterbirds moult "Breeding/moult" (n=521) (Table 2). Sampling was carried out during wintering in eleven locations, while ten locations were sampled after "breeding/moult", nine during autumn and six during spring migration. Ten locations were sampled in more than one period. One location (wetland A) was sampled monthly in order to determine variation in prevalence along time. In this site, faeces were collected every month from March 2006 to March 2007, excluding May when no samples could be obtained. Admissions of waterbirds to rehabilitation centres occurred mostly during the period "breeding/moult" and hunter harvested ducks were sampled during the hunting season in winter ("wintering").

Virus detection. RNA was extracted using commercial kits (High Pure RNA isolation kit, Roche Diagnostics, Germany) according to the manufacturer's instructions. Influenza A virus was detected using a real-time PCR (RT-PCR) assay targeting the matrix gene as described by Ward et al., (2004) with modifications in the probe

sequence as recommended by Munster et al., (2007). Amplification and detection was performed on an iQ5 real time detection system (BioRad) with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, New Jersey, USA). Pools of five individual samples were processed and upon identification of any influenza A virus positive pool the RNA isolation and RTPCR procedures were repeated for the individual samples within each positive pool. Individual RTPCR positive samples were subsequently used for virus isolation.

Virus isolation and characterization. For influenza A virus detection in RTPCR positive samples, 200 µl of the original material were inoculated into the allantoic cavity of 9-11-day-old embryonated specific pathogen free chicken eggs following OIE recommendations (OIE, 2009). The allantoic fluid was harvested as the embryo died or after 7 days if the embryo was still alive. RNA from allantoic fluid was extracted using commercial kit (QIAamp Viral RNA® Mini Kit) and RTPCR to detect influenza A type matrix gene was carried out (Spackman et al., 2002). When no influenza A virus was detected, the allantoic fluid was passaged twice in embryonated chicken eggs.

Sequence analysis. The haemagglutinin and neuraminidase were sequenced when possible following the protocol described by Hoffmann et al., (2001) with minor modifications. The sequences obtained were compared with those already available in GenBank database by nucleotide sequence homology searches made at the network server of the National Center for Biotechnology Information (NCBI) using BLAST.

Meteorologic data. Data on mean temperature and humidity for each month of the study period were obtained from the Agencia Estatal de Meteorología (AEMET), Ministerio de Medio Ambiente, Medio Rural y Marino for the stations nearest to the wetland in which monthly sampling was carried out.

Statistical analysis. We analysed the complete dataset in order to determine differences between sampling locations, sample type and host species using Chi square tests. All analysis were carried out using STATISTICA 6.0 software.

Results

Between July 2005 and July 2007 a total of 1435 samples (cloacal swabs and fresh faeces) were collected from wild birds from different locations in central Spain (Figure 1). The sample set included birds from 22 families belonging to more than nine orders (Table 1). Our sampling and processing procedures revealed a prevalence of 2.6% (37 out of 1435) of AIV in our sample set.

Overall prevalence for each sampling site varied considerably, with local prevalence of up to 10% and sites with negative results (Figure 1). AIV was detected both in fresh faeces from lakes and reservoirs and in cloacal swabs from birds admitted to rehabilitation centres and hunted ducks. Comparison of results between natural lakes (3.3%, 28 positives out of 829) and reservoirs (1.6%, 3 positives out of 192) revealed no statistically significant differences (χ^2 test, $p=0.19$).

Throughout our study we collected samples from a total of 57 species. 43.8% of the samples were obtained from the order *Anseriformes* (Table 2). The highest AIV prevalence was detected in the order *Phoenicopteriformes* (28.6%, 2 out of 7). However, as all flamingo samples were collected at the same location on the same date, the possibility of several faeces originating from a single animal can not be ruled out. When flamingos are excluded, the highest AIV prevalence was found in *Anseriformes* (29 out of 628, 4.6%). AIV was more often detected in dabbling ducks (28 out of 514, 5.4%) than in the rest of species (χ^2 test, $p<0.001$). On species level (and excluding

flamingos), Mallards had the highest prevalence (25 out of 415, 6%) as compared to other species sampled (χ^2 test, $p < 0.001$).

As reported by Munster et al. (2007), timing relative to migration, instead of the absolute time point, is determinant for virus prevalence. Based on this statement we decided to group the months of the year in 4 categories, corresponding to the main annual biological phenomena affecting most of the wild birds included in this study, as explained in the materials and methods section. Seasons in which a positive result was found for every species are shown in Table 2. Due to differences in sample sizes among species and based on prevalence results, we decided to include only data from anatid species, White storks (*Ciconia ciconia*), Common coots (*Fulica atra*) and Cattle egrets (*Bubulcus ibis*) in temporal prevalence variation analysis. Those species are the best represented in our data set (more than 120 samples each) and AIV have been detected in all of them.

As shown in Figure 2, the peak of AIV prevalence was detected during wintering both for the whole dataset (5.7%), and for wetland A (12.2%), reaching maximum prevalence in November and December. However, high AIV prevalence was also evidenced during autumn migration in the combined data (3.6%), although no positives were found in the same period for wetland A. In spring, prevalence was much lower (2% and 1.9% respectively). A slightly higher AIV prevalence was observed for the moult and breeding period (2.2%) in the complete dataset, while AIV was not detected in this period in wetland A. However, ongoing studies (data not shown) have evidenced presence of AIV also in wetland A after breeding and during moult in successive years. AIV prevalence obtained from cloacal swabs (2.5%, 9 positives out of 355) and fresh faeces (2.5%, 27 positives out of 1080) did not vary significantly (χ^2 test, $p = 0.98$).

Detection of LPAIV in wetland A occurred in seasons with lowest mean temperatures and highest mean humidity in the sampling period (Figure 2).

Three influenza A virus isolates were obtained from the 37 positive samples, which means an overall recovery rate of 8.3%. Two of the isolates were obtained from Mallards (*Anas platyrhynchos*) and the other one from a White stork. The two virus isolates obtained from Mallards were identified by sequencing as H7N9 (GenBank accession numbers GU354035 and GU354036), and the isolate obtained from the white stork was identified as H11N9 (Accession numbers GU354037 and GU354038). The pathogenicity of the H7 virus isolate was determined by the study of the sequence at the cleavage site (PETPKGR*GLF) that characterised this strain as of low pathogenicity. A sample of the three isolates was also sent to the Spanish National Reference Laboratory (SNRL) for official confirmation.

It was also possible to sequence some of the other positive samples and different LPAI subtypes were identified including H5N2, H3N8 and H5, H12 and N8 genes all of them from samples obtained in Mallards. In the two H5 positive samples the AIV was identified as low pathogenic by RT-PCR (Payungporn et al., 2006).

Discussion

Information on AIV ecology in Mediterranean countries is scarce and most of the existing studies provide data corresponding to only one season (Lebarbenchon et al., 2007; Terregino et al., 2007). Continuous sampling throughout a year enabled us to relate variation of AIV prevalence to seasonal movements and behavioural changes, especially in orders such as *Anseriformes*, *Ciconiformes* and *Gruiformes*, well

represented by a large sample size and of which samples from all seasons were available.

Overall, we confirmed a low average LPAIV prevalence (2.6%, 37 out of 1435), similar to what has been stated in previous studies carried out in Northern Europe (Munster et al., 2007), Africa (Gaidet et al., 2007), Italy (Cattoli et al., 2007) and France (Lebarbenchon et al., 2007), but lower than what has been reported from the north of Spain (Barral et al., 2008; Busquets et al. 2010). However, comparison among studies should be done cautiously due to differences related to sampling design, species targeted and laboratory methods (Olsen et al. 2006).

AIV prevalence in *Anseriformes* was slightly lower than described by Munster et al., (2007) (4.5% versus 6.9% in Sweden and The Netherlands), but similar to data obtained from other Mediterranean countries such as Italy (Cattoli et al., 2007). In the Mallard (*Anas platyrhynchos*), the most represented species in this study, prevalence was lower than described in preceding studies (Munster et al., 2007; Terregino et al., 2007; Busquets et al., 2010).

The higher prevalence of AIV observed in dabbling ducks is consistent with findings in previous studies (Olsen et al., 2006; Munster et al., 2007) and has been attributed to the feeding habits of these species, as virus shed by faeces may remain infectious for prolonged periods in surface waters as long as temperature, salinity and pH are favourable (Brown et al., 2009).

Information on LPAIV in *Phoenicopteridae* is scarce. Reasons for this may be that these birds are not very abundant in Europe and North America, their capture is costly and time-consuming and collection of faeces is not always possible as they usually remain in the water. In many LPAIV studies in wild birds, samples from *Phoenicopteriformes* are not included (Munster et al., 2007; Gaidet et al., 2007). The

high AIV prevalence we obtained for flamingos in our study resulted from a low number of samples (n=7), collected on one single day from the same location, and thus must be considered with caution. Cross contamination of the positive samples is unlikely, because different persons collected the individual samples, however we cannot completely rule out that both positive samples belonged to the same individual. If feeding behaviour is considered as an important factor for the exposure to AIV, the flamingo, that filters surface and profound water, is a species that may be exposed frequently. Their breeding behaviour in large colonies could also favour AIV transmission among adults and juveniles. Lebarbenchon et al. (2007) did not find AIV in samples from 113 greater flamingo chicks in the Camargue, France, while a prevalence of 25.3% (19 positives out of 75) was detected in Greater Flamingo in Northern Italy during winter 2004-2006 (Terregino et al., 2007), achieving successful isolation of an H6N2 virus. Likewise, high seroprevalences (43%) were evidenced in Flamingos from South Spain by Arenas et al. 1990, and AIV was detected by RTPCR in 2.5% of 154 Flamingos sampled in Catalonia, Spain (Busquets et al.2010). All these data are in accordance with our findings. Flamingos are actually considered a semi-resident, fairly mobile species, thus AIV prevalence in this species, their movements and their presence in areas where infected waterfowl have been detected may have important implications for AIV ecology and surveillance (Terregino et al., 2007).

In North America, some species in the order *Charadriiformes* are considered to play an important role in LPAIV epidemiology (Stallknecht and Shane 1988; Krauss et al., 2004), while in Europe its role remains unclear, with prevalences that are mostly low (Fouchier et al., 2003; Olsen et al., 2006; Cattoli et al., 2007; Busquets et al., 2010). We did not detect AIV in any species of this order sampled in our study, possibly because only a reduced number of samples from the genus *Larus* (n=65) was included and no

samples from *Calidris*, *Sterna* and *Uria* genuses in which LPAIV infection has been detected in preceding studies (Kaleta et al., 2005; Munster et al., 2007; Fouchier et al., 2003) were available.

Few studies include samples from *Ciconiformes*, despite behavioural traits that might favour AIV transmission and recirculation. While Lebarbenchon et al. (2007) did not detect AIV in samples of 185 *Ciconiformes*, Muller et al. (2009) found LPAIV in 3 out of 103 faeces of adult storks sampled in Germany during 2006 by means of RTPCR. In fact, HPAI H5N1 has been previously recorded in several species of *Ciconiformes* such as Little Egrets (*Egretta garzeta*) and Grey herons (*Ardea cinerea*) in Hong Kong (Ellis et al., 2002) and White storks in Germany (Globig et al., 2009). We found a low prevalence in this order (1%, 3 positives out of 308 samples), but achieved virus isolation from a cloacal swab of a White stork admitted to a rehabilitation centre due to trauma. Virus from the two positive Cattle egrets could not be isolated. Given the behaviour of White storks as colony breeders, as an at least partially migratory species, their increased census in eastern and central Europe and specifically in Spain and their usual association to human activity, they could be an interesting species to include when planning AIV surveillance programs in wild birds.

In the case of *Gruiformes*, prevalence (1.9%) is higher than reported in previous studies (0.7% in Gaidet et al., 2006; 0.4% in Munster et al., 2007), but similar to data obtained in Italy (De Marco et al., 2004) and North East Spain (Busquets et al., 2010). The detection of AIV in three Common coots (*Fulica atra*) out of 160 in our study may reflect interspecific transmission from Mallards, as coots in the studied wetlands are closely associated with other waterfowl, especially Mallards, which would also be consistent with the findings of De Marco et al. (2004).

A peak of prevalence is observed in south central Spain during winter, which is consistent with results obtained by Munster et al. (2007) in Northern Europe. More precisely, the results from Munster et al. (2007) reflect a peak in AIV prevalence early in fall migration during the months of October and November, with a subsequent decline, and a North-South gradient, being virus prevalence in Mallards in The Netherlands 3-fold lower as compared with Sweden. Considering that these birds continue southward migration they would presumably arrive in Spanish wetlands by November- December, which is when we detected the highest prevalence in our study area. Also, prevalence was lower than in Northern Europe, which would support the hypothesis of a North-South gradient of virus prevalence due to a progressive decrease of virus shedding, development of immunity or loss of infected individuals during southbound migration (Muzzafar et al., 2006; Terregino et al., 2007). Nevertheless, although mean prevalence was lower both than in Sweden and in the Netherlands, locally (as in the case of wetland A during wintering) we found high prevalences. This could be explained by recirculation of AIV due to the high concentration of wintering waterbirds in Spanish wetlands.

Both, our results and data from previous studies on wild birds wintering in wetlands in Northern Italy in which considerable prevalence (5-8%) and seroprevalence of AIV was detected, confirm the important role that Mediterranean wintering areas play in AIV epidemiology (Terregino et al., 2007; Cattoli et al., 2007; De Marco et al., 2003). As in our case, in studies from the US, medium to high prevalences were detected during winter (2-10%), mainly from dabbling ducks and especially from teals (Hanson et al., 2005; Ferro et al., 2008). Mallards, Northern shovelers (*Anas clypeata*) and Gadwalls (*Anas strepera*) which represent most of AIV carriers in our study area were also frequently found infected in those studies.

For our whole dataset, relatively high prevalences (although lower than during winter), were also detected during autumn migration (3.6%). In contrast, in wetland A no AIV was found in the same period, probably due to small sample size. Other studies carried out in South Europe also detected high prevalences in early fall although, contrary to our results, a marked decrease was evidenced afterwards, during November, December and January (Lebarbenchon et al., 2010).

Wild birds returning northward in spring generally have lower viral titres than southward migrants, but in high enough loads to re-establish the infection in their northern breeding grounds (Krauss et al., 2004; Webster et al., 1992). We detected a low AIV prevalence (2%, 5 out of 246) during spring migration (February to April) in our study area. However, it is difficult to elucidate whether these carriages belong to resident individuals or to waterfowl flying northward to their reproductive areas.

Post-breeding is considered an important period for AIV transmission and perpetuation due to the presence of high numbers of juvenile birds susceptible to infection. AIV prevalence in this period was 2.2% (9 out of 406), much lower than during wintering. In the case of the wetland used for annual variation studies, wetland A, we did not find AIV positive samples during June and July 2006, although it should be taken into account that only 64 samples were analysed for this period in 2006. Data from ongoing studies reveal LPAIV prevalences of 2.3% in the same area in 2007 and 2.8% in 2008 (Pérez-Ramírez et al., unpublished data). These results underline the importance of long-term studies due to the considerable interannual variations in AIV prevalence (Krauss et al., 2004). In fact, in our study, prevalence variation has been observed when individually comparing seasons from both years. Nevertheless, high infection rates have been consistently found during wintering.

Climatic conditions in winter are favourable for AIV persistence in faeces and the environment and recirculation in the waterbird community (Webster et al., 1992; Brown et al., 2007). Thus LPAIV detection in wetland A during the period with higher humidity and lower temperatures may reflect both, increase in persistence of LPAIV in the environment, and higher numbers of suitable hosts (increase in waterbird populations during wintering).

Isolation rate in our study was low (8.3% of real time RTPCR positives), but preceding studies obtained similar or lower isolation rates (3.14% in Gaidet et al., 2007; 8.1% in Cattoli et al., 2007; 8.3% in Pereda et al., 2008; 0% in Pannwitz et al., 2009). Low numbers of viral copies and inability of some AIV to grow to high enough titers to be detected in embrionated chicken eggs could be responsible for this (Ip et al., 2008, Runstadler et al., 2007).

Some authors have considered cloacal swabs more suitable for AIV detection by means of RTPCR techniques, since the virus replicates mainly in epithelial cells (Slemons and Easterday, 1978) and this kind of sample is supposed to be less prone to contamination. However, fresh faeces collection is a convenient, non invasive and cost effective method and has frequently been used for monitoring LPAIV in wild birds (Pannwitz et al., 2009; Lebarbenchon et al., 2007; Gaidet et al., 2007). Drawbacks include negative effects of external agents (UV light, temperature and humidity) or the fact that it is not always possible to establish an accurate association between droppings and individual birds (Yasué et al., 2006).

Our results are in concordance with reports from other authors (Gaidet et al., 2007; Lebarbenchon et al., 2007; Pannwitz et al., 2009) which support faecal sampling as an appropriate method for large scale LPAIV surveillance programs, since capture of large 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.

Acknowledgements

We are grateful to all the personnel of the Wildlife Rehabilitation Centres in Castilla-La Mancha for their collaboration and to all the people that helped in the field work. We also thank JCCM for support, Tomás Velasco for sharing his knowledge of wetlands and waterbirds in Central Spain and Vega Álvarez (NEIKER) for technical support. We wish to thank the “Spanish National Reference Laboratory” for official confirmation of isolates.

This work is a contribution to grant INIA RTA2005-111-00, grant JCCM PA298-05 and grant MEC/INIA FAU2006-019-C03. E. Pérez-Ramírez currently has an I3p fellowship from the Spanish Science Council (CSIC).

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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 where hunted birds were sampled. White circles
612 represent wildlife rehabilitation centres and grey circles represent wetlands (lakes,
613 reservoirs and rivers).

Sample size

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 °C 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.

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

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

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

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