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Avian Influenza virus in sympatric wintering aquatic birds: rubbish dumps as hotspots for surveillance?

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1. ABSTRACT

The circulation of Avian Influenza viruses (AIV) in wild birds has been intensively studied, due to their implication in the maintenance and spread of these viruses. Most of these studies have focused on natural aquatic environments, where birds aggregate and in which viral survival is enhanced. Nevertheless, other places such as rubbish dumps also lead to massive aggregation of wild birds, including some known AIV reservoirs. Particularly, during the winter months, Spanish rubbish dumps harbor a great number of wintering wild birds. This is the case in rubbish dumps in the province of Ciudad Real (community of Castilla-La Mancha, Spain), where these animals also visit the surrounding wetlands where AIV circulation had been detected in previous years. In this study we analyzed the prevalence of AIV genome excretion in sympatric species that use two rubbish dumps in Ciudad Real, during the wintering season 2014-2015 and determined temporal, spatial and species variation. We tested 1190 fresh fecal samples, cloacal and oral swabs for AIV genome excretion by real time-RT PCR for the AIV matrix gene. We found an overall prevalence of 0.6%, peaking in October, which coincides with the arrival of migratory wild birds to Spanish territories. A higher prevalence was detected in gulls, which are known AIV reservoirs in natural environments, followed by cattle egrets and white storks. The detection of AIV genome excretion in all studied species indicates that all might play a role in the epidemiology of these viruses.

Our results indicate that AIV circulates in wild birds that visit the studied rubbish dumps, and, at least in gulls, probably continuously during the wintering season. This underlines the potential importance of developing surveillance tasks in these places, namely through collection of fresh feces, a cost-effective sampling method appropriate for large scale LPAIV surveillance in wild birds.

Key words: AIV, cattle egrets, gulls, non-invasive sampling methods, rubbish dumps, white storks, wintering.

2. INTRODUCTION

2.1. Etiology

Avian Influenza viruses (AIV) belong to the family *Orthomyxoviridae*, genus Influenza virus. Their genome consists of eight segments of single-stranded RNA. This characteristic enables viral evolution through exchange of genes between different viruses infecting a single cell at the same time (genetic reassortment/genetic shift) (Macken et al., 2006). The characterization of AIV is based on the antigenic properties of two transmembrane glycoproteins: the hemagglutinin (HA) and neuraminidase (NA) (Webster et al., 1992). There are 16 HA and 9 NA subtypes described in wild birds (Fouchier et al., 2005), although some infrequently (Kam et al., 2004) and others seem to be species-specific such as HA13 and H16 that primarily infect *Laridae* species (Olsen et al., 2006).

2.1.1. Highly pathogenic and low pathogenic AIV

AIV are also classified into highly pathogenic AIV (HPAIV) and low pathogenic AIV (LPAIV) according to their pathogenicity in chickens (Munster & Fouchier 2009). The HA cleavage site is constituted of one or two basic amino acids at distinct positions in the case of LPAIV (Wood et al., 1993), and is recognized by trypsin-like enzymes specific from respiratory and gastrointestinal epithelia. These proteases cause the rupture of the HA, which allows the fusion of the viral envelope and the host cellular membrane (Skehel & Wiley 2000). On the other hand, HPAIV present a multibasic aminoacid motif at the HA cleavage site, which is recognized by subtilisin-like endoproteases that exist in almost every host tissue, leading to systemic infections with high mortality rates in gallinaceous bird species (Horimoto et al., 1994, Rott et al., 1995). Pathogenicity of HPAIV in wild birds can be very different from the chicken and varies with species. Thus, in wild birds the infection with HPAIV can cause death, but may also be asymptomatic. Some individuals have also been described to develop temporal cross-protective immunity due to previous infections with LPAIV (Seo et al., 2002).

2.2. LPAIV in natural environments

The viruses circulating in natural ecosystems are generally LPAIV with tropism for the intestinal tract, leading to excretion in high concentrations in feces. Transmission occurs mainly via the fecal-oral route through direct or indirect contact with contaminated water (Webster et al., 1992). In aquatic environments virus survival is enhanced, which allows viral exchange in bird populations in different time frames (Munster & Fouchier 2009).

Although AIV primarily infect birds, interspecies transmission to mammals, including humans, have been described (Kalthoff et al., 2010) (Figure 1). Wild birds, especially aquatic wild birds from the orders Anseriformes and Charadriiformes, constitute the main reservoir of these LPAIV (Webster et al., 1992) and host a great diversity of subtypes, providing a scenario that allows for a slow (but not negligible) viral evolution (Olsen et al., 2006) and potential transmission to non-avian species. These LPAIV are thought to be adapted to their natural hosts and appear to have little or no effects on their health, although some studies revealed that the infection can potentially reduce body weight, probably due to the reduced intestinal tract function, which can have a negative impact in the survival and reproductive success (Kuiken 2013). There are two recognized lineages of LPAIV: Eurasian and American. Wild waterfowl have been shown to spread LPAIV along their migratory routes (Webster et al., 1992, Munster et al., 2007), and as these migrations connect bird populations of different continents, although rare, exchanges of genes from both lineages have been recorded (Fouchier & Munster 2009).

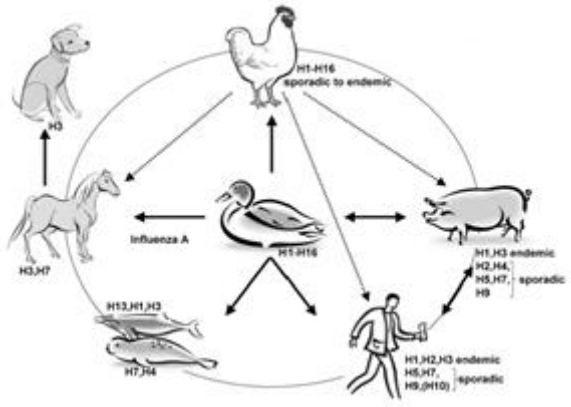

Figure 1 - Schematic illustration of influenza A virus transmission among different species (Kalthoff et al., 2010)

2.2.1. Factors that shape LPAIV circulation

The efficient circulation and transmission of AIV, and thus the prevalence, are modulated by ecological factors related to the host species and to the virus itself. Regarding the host species, these factors are related to host ecology (age, foraging and migratory behavior, habitat selection, bird community composition, frequency of aggregation), but also to intrinsic differences in susceptibility and ability to function as host (Munster et al., 2009). Regarding virus specific ecological factors, the conditions within aquatic habitats, such as temperature, pH or salinity, determine the environmental persistence of AIV (Brown et al., 2009).

2.3. Role of wild birds in HPAIV epidemiology

2.3.1. Genesis of HPAIV

The transmission of LPAIV from wild birds to poultry produces minor effects on their health status, such as a reduction in weight gain and decline in egg production (Capua & Mutinelli 2001). However, LPAIV can evolve into HPAIV after transmission to poultry with subsequent mutation and selection of HPAIV by multiple host passages during recirculation in the flock (Subbarao et al., 2006). This has been described only with subtypes H5 and H7 (Olsen et al., 2006), and for this reason any H5/H7 infection in poultry, both LPAIV and HPAIV, are classified as "notifiable avian influenza" (OIE, EU directive 2005/94/EC).

2.3.2. HPAIV circulation in wild birds

Highly pathogenic H5N1 influenza viruses have been circulating in Asia, causing serious outbreaks on poultry farms since 1997 (Chen et al., 2006). Less frequently, HPAIV have also been isolated from wild birds (Becker 1996, Ellis et al., 2004). Some cases of disease and death in wild birds have been reported (Ellis et al., 2004), but this virus did not seem to seriously affect wild bird populations until April 2005, when an outbreak occurred in a migratory bird population at Qinghai Lake in China (Chen et al., 2005). This episode triggered a westward spread of HPAIV H5N1 into eastern and central Europe, and early in 2006, H5N1 HPAIV had already reached 20 European countries, including Spain, and multiple cases of fatalities in wild birds were reported (Kalthoff et al., 2010).

Migration connects distant populations (Figure 2). At the stopovers/destination, where wild birds frequently aggregate, direct and indirect contact between birds allows for pathogen transmission and posterior spread along the migratory flyways. Despite being controversial, the coincidence between the geographic expansion of HPAIV and the migratory flyways (Tian et al., 2015), as well as the detection of HPAIV in countries without reports of outbreaks in poultry (Olsen et al., 2006) appear to support the theory of HPAIV spread through migratory birds movements. Thus, wild birds can also play an important direct role in the epidemiology of HPAIV.

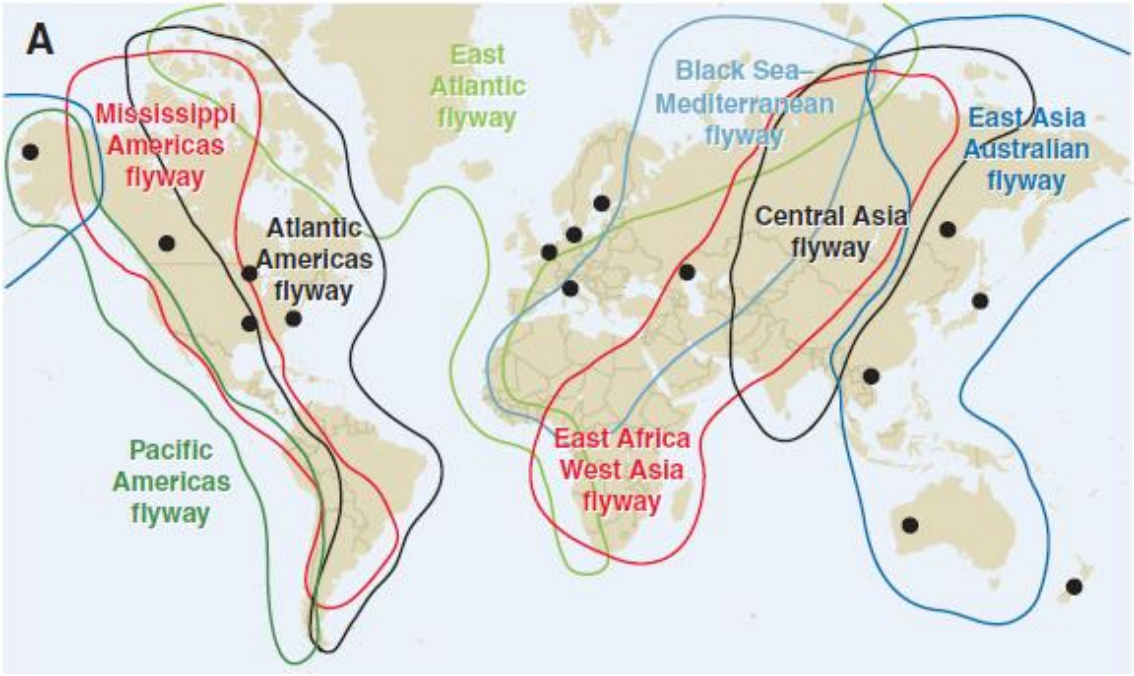

Figure 2- Main general migratory flyways of wild bird populations (Olsen et al., 2006).

2.4. Impact of AIV circulation

The circulation of HPAIV is responsible for important economic losses associated with disease and control measures in the poultry industry. HPAIV can also be implicated in massive deaths in wild birds, as described in South Africa in 1961 (Becker 1966) and China in 2005 (Chen et al., 2005). However, the potential effect on the security of public health is the main concern associated to AIV, as wild bird community represents a reservoir of precursors of potential zoonotic viruses (Kalthoff et al., 2010). Thus, studying the circulating subtypes allows for earlier implementation of measures that prevent future pandemics.

2.5. AIV in Spain

The information about circulation of AIV in Spain is scarce. Spanish wetlands act as resting and stopover feeding points for birds that migrate between Africa and Northern Europe. In fact, Spain is one of the most important wintering areas in Europe (Muñoz et al., 2006). Despite this location along migratory routes that also cross several European countries affected after HPAIV outbreaks in wild birds, there is only one known report of HPAIV H5N1 in a free-living crested grebe (*Podiceps cristatus*) (Barral et al., 2008). This might be an indicator of the unfavorable conditions for the persistence and spread of this HPAIV subtype (Winker et al., 2007). The situation appears to be different in the case of LPAIV. In the 90´s, two serological studies indicated that LPAIV were circulating among wild birds in southern Spain, detecting a seroprevalence of 40% (Arenas et al. 1990) and 6,2% (Astorga et al., 1994) . High prevalence were also found in poultry flocks, mainly in turkeys, which suggest an enzootic form of LPAIV circulation in this region (Arenas et al. 1990). More recent studies have used molecular methods to study LPAIV circulation in wild aquatic birds in Spain (Pérez-Ramírez et al., 2010, Busquets et al., 2010, Pérez-Ramírez et al., 2012).

2.6. Rubbish dumps and demography of wild birds

The availability of abundant and constant feeding resources at rubbish dumps attracts regularly a great number of opportunistic species (Garrido y Sarasa 1999). This has caused changes in the diet, as well as in the migratory behavior of many species. In Spain, this change in the migratory behavior seems to be particularly evident for white storks. For this species, the existence of rubbish dumps has contributed to an increase in the number of resident individuals in the last years (Tortosa *et al.*, 1995), although Spanish rubbish dumps are also important stopovers and wintering areas for white storks of the western European flyway (Hernández 2015). Likewise, the number of gulls has experienced great increases, partially due to the existence of these easy feeding resources. In Salamanca, the black headed gull (*Chroicocephalus ridibundus*) and lesser black backed gull (*Larus fuscus*) have changed their status from “rare or moderately abundant wintering” to species highly common in winter, that feed mostly at the city rubbish dump (Blanco 2007).

2.7. Rubbish dump users as AIV study targets

2.7.1. White storks, cattle egrets and gulls: ecology

White storks (*Ciconia ciconia*, order Ciconiiformes), cattle egrets (*Bubulcus ibis*, order Pelecaniformes) and gulls such as the black headed gull (BHG), yellow legged gull (*Larus michahellis*) (YLG) and lesser black backed gull (LBBG) that belong to the order Charadriiformes and family *Laridae* (Mullarney et al., 1999) are all opportunistic species that often visit rubbish dumps (SEO/Birdlife 2008). In past years the number of white storks that depend on these feeding resources has increased (Blanco 1996). Rubbish dumps also represent one of the most important food sources for YLG that usually use them year round. All these species are also commonly seen in other humanized landscapes, living in close contact with humans. The five species present a colonial breeding behavior and are related to aquatic environments, being frequently seen in wetlands. In fact, wetlands in the province of Castilla-La Mancha present some of the largest breeding colonies in Spain for BHG. White storks, BHG and LBBG are migratory species, wintering in Spain. Nevertheless, there are also some resident non-migrant individuals. In the other hand, both cattle egrets and YBG are mostly resident, although some individuals display some movements of variable range. In fact, cattle egrets from the Iberian Peninsula have been detected in other countries during the wintering season (SEO/Birdlife 2008).

2.7.2. Role in AIV epidemiology

2.7.2.1. White storks

White storks have been considered potential vectors for the introduction of HPAIV H5N1 into the European Union, based on ecological and behavioral features such as gregariousness and habitat use (Veen et al., 2007). AIV shows a strong persistence in water under favorable conditions, and given that this virus is transmitted primarily by fecal-oral route in avian species (Stallknecht et al., 1990), it is probable that white storks can eventually contact with AIV when feeding or resting in wetland habitats shared with important AIV reservoirs (Fouchier & Munster 2009). In fact, different strains of influenza virus, both HPAIV and LPAIV, have been reported in white storks in different European countries, such as Germany, Poland, Slovenia, Spain

(Globig et al., 2009, Müller et al., 2009, Smietanka & Minta 2014, Pérez-Ramírez et al., 2010, Slavec et al., 2012). Despite these occasional findings, other authors were not able to detect evidence of AIV circulation in this species in Germany, France, Czech Republic and Serbia (Müller et al. 2009, Kaleta 2003, Kaleta & Kummerfeld 2012, Lebarbenchon et al., 2007, Nagy et al. 2007, Šekler et al., 2009)

2.7.2.2. Cattle egrets

The habitat use and colonial breeding behavior might allow the circulation of AIV in cattle egrets. Some authors have reported the detection of both LPAIV in Spain (Pérez-Ramírez et al., 2010) and HPAIV (Kayali et al., 2011) in Egypt, in free living individuals, which indicates that this species might act as an AIV host in their natural environment. Besides, Phuong et al., 2011 demonstrated the susceptibility of cattle egrets to the infection with HPAIV H5N1 AIV under experimental conditions. Nevertheless, surveillance activities carried out in France did not detect any AIV positive cattle egret (Lebarbenchon et al., 2007) and more studies are needed in order to better understand the role of cattle egrets in AIV epidemiology.

2.7.2.3. Gulls

Charadriiforms are recognized as one of the main reservoirs of AIV in natural environments (Webster et al., 1992). Their colonial breeding behavior allows for a high contact rate, which might create a good scenario for viral transmission (Lebarbenchon et al., 2007). The aggregation of animals during migration and winter, the assemblage of distinct bird populations and feeding patterns may also pose risk factors for AIV infection (Munster et al., 2007). As expected, many cases of AIV detection in different gull species have been reported worldwide, either in dead animals or live, healthy animals (Anna et al., 2010, Spackman et al., 2009, Sivay et al., 2012 Jurinović et al., 2014, Verhagen et al., 2012, Marco et al., 2005, Busquets et al., 2010, Slavec et al., 2020), including the HPAIV H5N1 (Ellis et al., 2004, Marchenko et al. 2011, Sakoda et al., 2012, Savić et al., 2010) . Though the subtypes H13 and H16 are the most commonly found in gulls[4], different subtypes have been detected (Anna et al., 2010, Spackman et al., 2009, Sivay et al., 2012, Jurinović et al., 2014, Marco et al., 2005, Busquets et al., 2010, Slavec et al., 2012, Ellis et al., 2004, Marchenko et

al., 2011, Sakoda et al., 2012, Savić et al., 2010). Nevertheless, some studies carried out in southern Europe (Marco et al., 2005, Lebarbenchon et al., 2007, Pérez-Ramírez et al., 2010), but also in China (Chen et al., 2006) failed to detect the circulation of AIV in these species.

The described species use the studied rubbish dumps as feeding points and frequently aggregate around small ponds that exist inside and around them. Also, some of them move between these rubbish dumps and surrounding wetlands (Hernández & Höfle 2014), which may contribute to the fecal-oral transmission cycle of AIV characteristic of aquatic environments. Therefore, they represent potentially interesting targets for AIV surveillance studies

2.8. Objectives

The aim of the present work is to study AIV prevalence dynamics during wintering in sympatric species in the use of two Spanish rubbish dumps: the white stork, three species of gulls and the cattle egret. Specifically, this study tried to capture the temporal and spatial patterns, and also host species variation in prevalence in both rubbish dumps, in the period between September and March when changes in the bird community occur continuously due to wintering and migration.

Taking into consideration the recent spread of HPAIV H5N8 in some European countries (Adlhoch et al., 2014), this short term active monitoring study also aimed to obtain information about the possible circulation of HPAIV H5 subtype in wild birds in Spain.

Figure 3 – Aggregation of YLG and LBBG in a small pound inside Alcázar de San Juan rubbish dump. The slope behind the gulls covers a recent rubbish deposit and on top the tubing of the gas from fermentation.

Figure 4 – Aggregation of white storks in a small pond around Almagro rubbish dump.

3. MATERIALS AND METHODS

3.1. Study area

The samples were collected at two active rubbish dumps in the province of Ciudad Real in the Community of Castilla-La Mancha, Spain (UTM coordinates: 30S 294,348–681,063 4,208,706–4,575,340) (Figure 5) one located close to the small town of Alcázar de San Juan and the other close to the town of Almagro . In Almagro, the entry for sampling purposes was temporarily denied for security reasons (explosive gas in a recently sealed part of the dump) and sampling was carried out around two ponds of runoff of the rubbish dump outside the fenced premises.

Figure 5 - Study area. The community of Castilla-La Mancha is represented in grey. Almagro rubbish dump is represented by the symbol in the center and Alcázar de San Juan rubbish dump is represented by the symbol in the upper right of the figure that represents this province.

3.2. Study species

The species included in this study were white storks, cattle egrets and three species of gulls (LBBG, YLG and BHG). Unfortunately, cattle egrets could not be sampled in Almagro rubbish dump as these had changed to the newly sealed area (too dangerous for sampling activities) for resting and did not use the areas around the runoff ponds.

3.3. Study period

Samples were collected from September 2014 to March 2015 (Figure 6). The beginning of the sampling period in Almagro rubbish dump was postponed for 15 days due to the lack of water in the pounds at the end of September and beginning of October. The sampling period was selected in order to include the wintering of white storks, LBBG and BHG in Spanish territories and because in previous studies LPAIV prevalence was highest in winter (Pérez-Ramirez et al., 2010, Pérez-Ramírez et al., 2012). The high number of wintering individuals allowed for an acceptable sample size, which would have been difficult for all the studied species throughout the rest of the year. In each of the two sampling areas, samples were taken every 15 days approximately. This periodicity was chosen to obtain data that reflect the changes in the

dynamics of the studied species (arrival from the breeding countries, stopover during migration to wintering quarters further south, wintering, and stopover on the way to and departure to the breeding grounds). Also, the LPAIV infection in waterbirds has been described as having a short duration (up to one to two weeks) (Latorre-Margalef et al., 2009, Hénaux & Samuel 2011), and with this periodicity we reduce the possibilities of detecting the same positive individual twice.

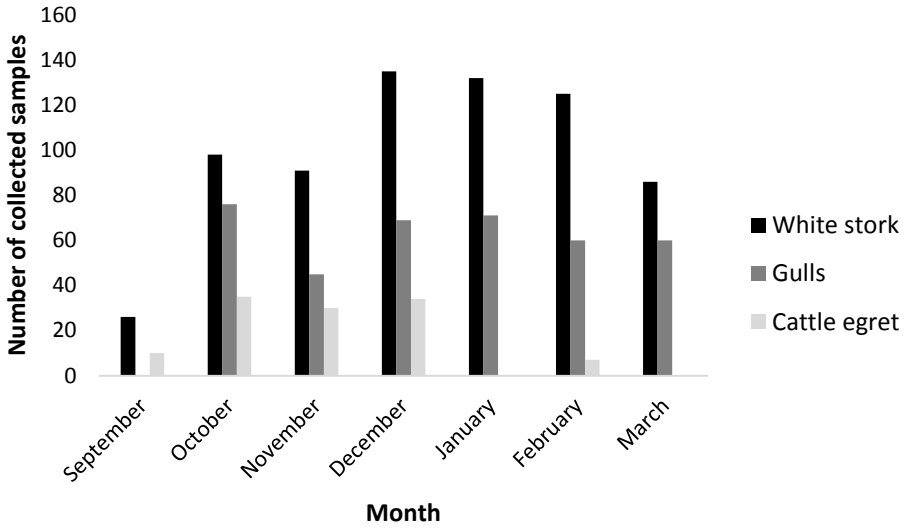

Figure 6 – Number of fresh fecal samples collected by month at Alcázar de San Juan and Almagro rubbish dumps from white storks (black), gulls (dark grey) and cattle egrets (light grey) between September 2014 and March 2015.

3.4. Sample collection

Fresh feces (n=1185) were collected at resting places, in each rubbish dump, from monospecies flocks after flushing the flocks by approaching them. The three species of gulls that belong to the *Laridae* family were usually mixed in the same flock (being the LBBG the less frequent). Thus, samples from gulls were obtained from monofamiliar flocks. With this method of non-invasive sampling, we ensure the use of only fresh material and a high probability that the collected feces belong to individuals of a determined species/family (Pérez-Ramírez et al., 2010). Approximately 30 samples were collected for each species/family in each sampling moment, with the goal of obtaining a representative number, adapted to the available logistics. Although we defined a maximum number of samples to be collected in each visit (30 samples by species), unfortunately we could not always achieve this number due to the small size of the sampled flocks. Thus, differences in the sample size were obtained between

different species and study months at both rubbish dumps. We collected a total of 692 fecal samples from white storks, 377 from gulls and 166 from cattle egrets. When the samples were collected near ponds, feces closer than 5 cm to the shoreline or inside the water were avoided. Each sample was collected using a sterile cotton swab and placed inside a small zip-lock bag. Also, cloacal and oral cotton swabs were obtained from dead BHG (n=2) or sick LBBG (n=1), BHG (n=1) and white stork (n=1) found in the sampling areas. The collection of these individual samples was done at the IREC facilities and thus they were processed immediately afterwards.

Fecal samples were maintained refrigerated (4 to 10º C) and transported within 2-3 hours to the laboratory facilities at IREC. The samples were either processed immediately or stored at (4º C) and processed the next day.

3.5. Processing

Fecal samples collected at both rubbish dumps were pooled into transport medium (Hank's balanced solution containing 10% glycerol, 200 U/ml penicillin, 200mg/ml streptomycin, 100 U/ml polymixin B sulphate, 250mg/ml gentamycine and 50 U/ ml nystatin (Munster et al., 2007). Each pool contained feces from 5 individuals of the same species/family, collected in the same rubbish dump and on the same day. The samples collected from dead or diseased animals were processed the same way but were not pooled. Nevertheless, the oral and cloacal swab of each animal were put together, as this appears to allow a higher detection rate of LPAIV (Ip et al., 2012). The processed samples were frozen at -80º C until further analysis. The portion of feces that was not included in the pools was also frozen at -80º C.

3.6. RNA Extraction

Viral RNA was extracted using a commercial kit (High PureRNA isolation kit, Roche Diagnostics, Germany), according to the manufacturer's instructions. Briefly, 200µl of transport medium were directly used to extract the RNA, and the nucleic acid was eluted in a final volume of 50 µl elution buffer. A negative control of extraction was used in each extraction assay. The quantity of extracted RNA was determined using Nanodrop (NanoDrop 1000 Spectrophotometer V3.7, Thermo Fisher Scientific, Wilmington, DE, USA). The extracted RNA was frozen at -80º C until further analysis.

3.7. Real time RT-PCR

The extracted RNA was screened following the real-time RT-PCR (RRT-PCR) protocol targeting the influenza A virus matrix gene as described by Ward et al. 2004 and modified by Munster et al. 2007. Briefly the reaction mix for each sample contained 13.5 µl of ultrapure water, 2.5 pmol (0,5 µl) of a Taqman FAM-labeled probe 1293 (5´–6-carboxyfluorescein–TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC–6 carboxytetramethylrhodamine–3´), 20 pmol (0,5 µl) of primer 1074 (5´-CAA-AGC-GTC-TAC-GCT-GCA-GTC-C-3´), 15 pmol (0,5 µl) of primer 1073 (5´-AAG-ACC-AAT-CCT-GTC-ACC-TCT-GA-3´), 5 µl of Taqmanfast virus master mix (Applied Biosystems) and 5 µl of the extracted RNA. The reaction was performed under the following conditions: 50ºC for 2 minutes, 60ºC for 30 minutes, followed by 45 cycles of 95ºC for 3 minutes, then 94ºC for 20 seconds and finally 60ºC for one minute. Samples with a CT ≤40 were considered positive. For the pools that yielded positive results, the RNA extraction was repeated for the individual samples within each pool and analyzed in the same way. A positive and negative control of amplification were used in each RRT-PCR assay. Amplification and detection was performed using an iQ5 real time detection system (BioRad, Applied Biosystems, New Jersey, USA) for all the RRT-PCR assays.

3.8. AIV isolation and typing

Samples that yielded positive results in RRT-PCR abovementioned were then analyzed for H5 by real-time RT-PCR as described by Munster et al 2009. The reaction mixfor each sample contained 13.5 µl of ultrapure water, 5pmol (0,5 µl) of 1150 probe, 10 pmol (0,5 µl) of primer 1148, 10 pmol (0,5 µl) of primer 1149, 5 µl of Taqmanfast virus master mix (Applied Biosystems) and 5 µl of the extracted RNA. The reaction was performed under the following conditions: 50ºC for 2 minutes, 60ºC for 30 minutes, followed by 50 cycles of 95ºC for 7 minutes, then 94ºC for 20 seconds and finally 60ºC for one minute. A positive and negative control of amplification were used in the RRT-PCR assay. Amplification and detection were performed using an iQ5 real time detection system (BioRad, Applied Biosystems, New Jersey, USA). All the samples that yielded positive results in RRT-PCR protocol targeting the influenza A virus matrix were also submitted for viral isolation and sequence analysis. Because these procedures have to be carried out in BSL3 facilities, they were done at the Basque institute for Agricultral Research (NEIKER). Briefly, for AIV isolation, 100-200 µl of the original

fecal 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 when death of the embryo was detected, or, in the case it survived, at the day seven after inoculation. RNA from this fluid was extracted using a commercial kit (QIAmp Viral RNA Mini kit, Qiagen, Hilden, Germany) and RRT-PCR to detect AIV matrix gene was done (Spackman et al., 2002). If no AIV was detected, the allantoic fluid was passaged two times in embryonated chicken eggs. The sequences obtained from isolated virus were compared with published sequences by sequence homology searches at the network server of the National Centre for Biotechnology Information (NCBI) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

3.9. Census and ring lecture data

Monthly mean numbers and lectures from long distance identification rings were available for white storks and allowed an approximate idea of the number of commensals and the countries of origin of a proportion of these (Hernández & Höfle 2014, Hernández 2015, personal comment). We compared these monthly mean numbers with the mean number of samples collected from white storks at the same month.

3.10. Statistics

Due to the low AIV prevalence found in this study, we could not apply any statistical model in order to comprehensively analyze factors that could influence AIV prevalence in the sampled species, between sample sites and throughout time. We used Fisher's exact test to compare AIV prevalence between the two rubbish dumps, excluding the months of September (no samples at Almagro) and cattle egrets (no samples at Almagro) and Chi square test to compare AIV prevalence between species and sampling months. We compared sample size between rubbish dumps using a U Mann Whitney test. We also used this test to compare the numbers of samples obtained from gulls and white storks respectively in the two rubbish dumps and between white storks and gulls in Almagro. We also used the U Mann-Whitney test to compare sample size between months with and without AIV positive samples within each rubbish dump. Finally we compared sample size between species (cattle egrets, gulls and white storks) in Alcázar de San Juan using a Kruskal Wallis test. For all tests p was set at $p=0.05$. SPSS statistics software (IBM armonk, NY, USA) version 19.0 was used in all analysis.

4. RESULTS

Between September 2014 and March 2015 we collected a total of 1190 samples (fresh feces, cloacal and oral swabs) from sympatric wild birds (white storks, cattle egrets, and gull species) at two rubbish dumps in the province of Ciudad Real, Spain (Table 1). Due to the aforementioned difficulties sample size varied considerably between months and species. Sample size was not significantly different between Alcázar de San Juan and Almagro (Statistic= 149196, p= 0.310). Also, the sample size for white storks between Alcázar de San Juan and Almagro was not significantly different (Statistic= 61706, p= 0.319), while significantly more samples from gulls (Statistic= 3251, p= 0.000) were obtained in Alcázar de San Juan as compared to Almagro. In Alcázar de San Juan significantly less samples were collected from cattle egrets than from white storks and gulls (Statistic= 95447, p= 0.000), but also from gulls than from white storks (Figure 7), while in Almagro significantly more fecal samples were obtained from white storks than from gulls (Statistic= 4753, p=0.000) (Figure 8). Both in Alcázar de San Juan and Almagro, the sample size was not significantly different in months with and without detection of positive samples (Statistic=2 392, p=0.977 and Statistic=38 500, p=0.201, respectively).

We detected seven AIV positive samples by means of a generic RRT-PCR against the AIV matrix protein, and thus a mean prevalence of 0.6% of AIV genome excretion in our sample set. None of the samples collected from dead or diseased animals (n=5) yielded positive results. Because it was impossible to assign the species of origin to each individual sample collected from groups of gulls, these results are presented jointly for the three primary gull species that composed the groups (BHG, YLG and LBBG).

We detected a higher prevalence in Alcázar de San Juan (0.75%, 6 out of 797 samples) as compared to Almagro (0.25%, 1 out of 393) (Figure 9) that was however not significant (χ^2=1.086, d.f=1, p=0.297).

In the whole sample set, the highest AIV prevalence was found in gulls (1.31%, 5 out of 381 samples), followed by cattle egrets (0.86%, 1 out of 116 samples) and white storks (0.14%, 1 out of 693 samples). This interspecies variation in prevalence was not significant (χ^2=5.858, d.f=2, p=0.053). In Alcázar de San Juan, we found positive samples in all studied species. A higher prevalence was found for gulls

(1.27%, 4 out of 316 samples). Lower prevalence were obtained for white storks (0.27%, 1 out of 365 samples) and cattle egrets (0.86%, 1 out of 116 samples), although these differences were not significant (χ^2=1.264, d.f=2, p=0.532) (Figure 7). In Almagro we detected a single positive sample in gulls, and thus, we obtained a prevalence of 1.54% AIV genome excretion (1 out of 65 samples) in this species. Nevertheless, the difference in AIV prevalence in gulls and white storks was not significant (χ^2=0.813, d.f=1, p=0.367).

In Alcázar de San Juan and Almagro, AIV prevalence peaked in October (2.31% in Alcázar de San Juan corresponding to 3 out of 130 samples (Figures 7), and 1.27% in Almagro, corresponding to 1 out of 79 samples (Figure 8), respectively) This variation in prevalence was not significant neither in Alcázar de San Juan (χ^2=8.377, d.f=6, p=0.212) nor in Almagro (χ^2=4.444, d.f=5, p=0.487).

We were able to isolate three viruses out of seven RRT-PCR positive samples, obtaining a recovery rate of 42.86%.

In Alcázar de San Juan, it seems that the variation in the monthly mean number of collected samples matches the variation in monthly mean number of white storks that visit this rubbish dump. We have however not tested this apparent correlation statistically (Figure 10).

Table 1
Number of collected samples, number of AIV positives in real time RT-PCR and AIV prevalence in white storks, cattle egrets and gulls in Alcázar de San Juan and Almagro rubbish dumps between September 2014 and March 2015. Sampling location in bold type represents at least one positive sample in the location (ASJ= Alcázar de San Juan, AL= Almagro). Sampling months in bold type represent at least one positive sample in the month (S= September, O= October, N= November, D= December, J= January, F= February, M= March).

Family	N	RT-PCR positive	Family prevalence (%)	Samplig location	Sampling months
Ciconiidae	693	1	0.14	**ASJ,** AL	S, O, N, D, **J**, F, M
Ardeidae	116	1	0.86	**ASJ**	S, **O**, N, D, F
Laridae	381	5	1.31	**ASJ, AL**	**O**, N, **D**, J, **F**, M
Total	1190	7	0.6	ASJ, AL	S, O, N, D, J, F, M

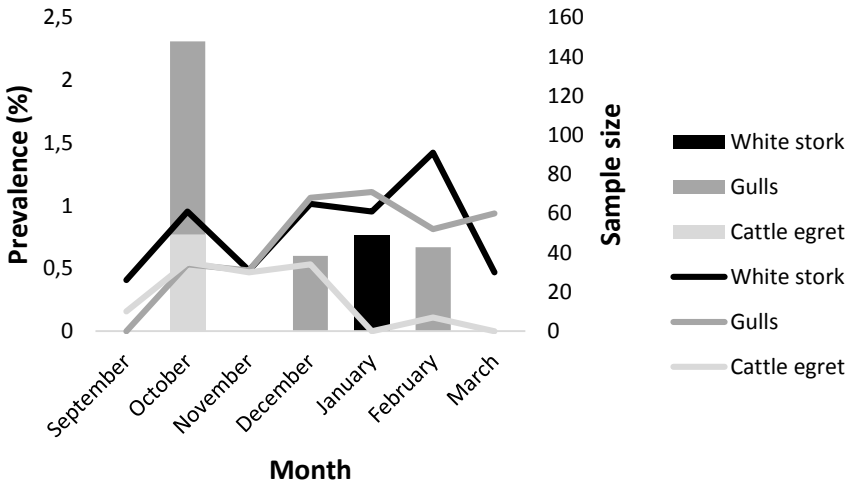

Figure 7 – Prevalence (%) of AIV genome excretion by month in white storks (black), gulls (dark grey) and cattle egrets (light grey) (bars) and sample size (number of fresh fecal samples collected) by month from white storks (black), gulls (dark grey) and cattle egrets (light grey) (lines) at the Alcázar de San Juan rubbish dump between September 2014 and March 2015.

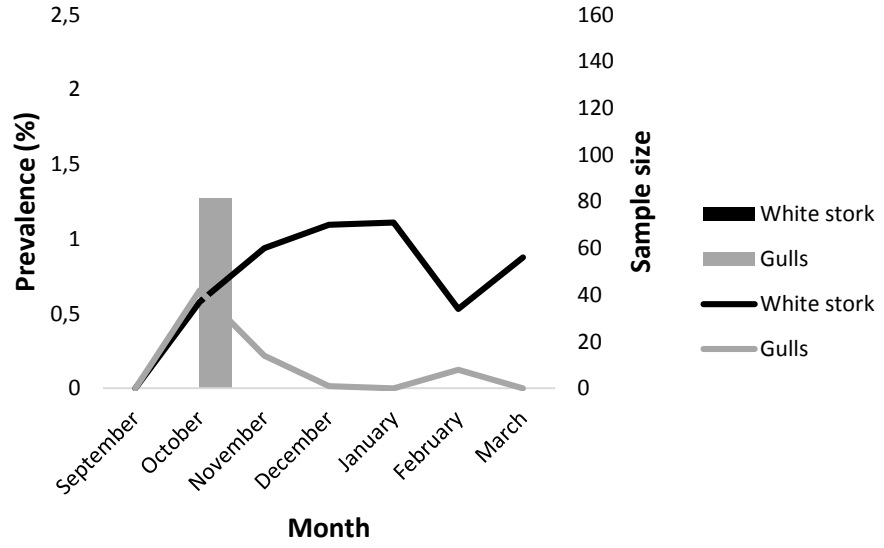

Figure 8 – Prevalence (%) of AIV genome excretion by month in white storks (black) and gulls (dark grey) (bars) and sample size (number of fresh fecal samples collected) by month from white storks (black) and gulls (dark grey) (lines) at the Almagro rubbish dump between September 2014 and March 2015. Cattle egrets were not included because they were not sampled at the Almagro rubbish dump.

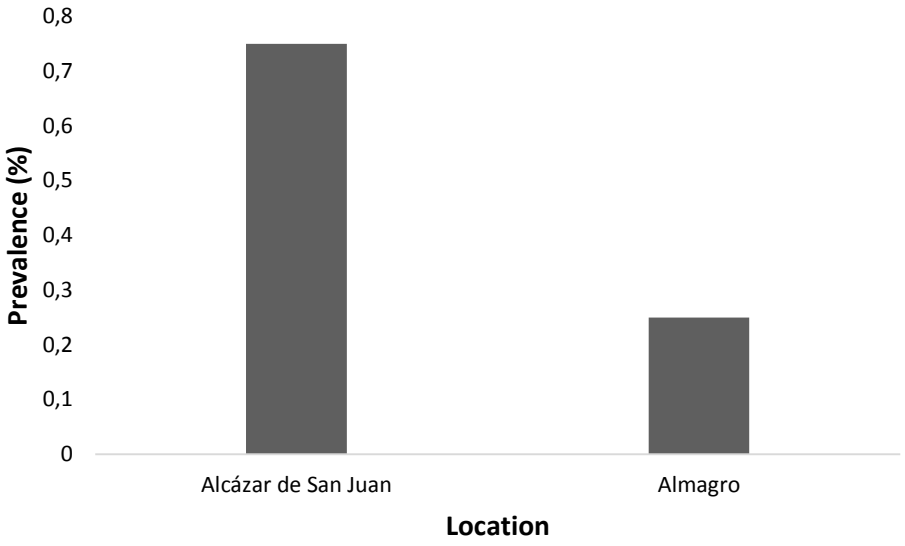

Figure 9– Prevalence (%) of AIV genome excretion in fresh feces collected from aquatic wild birds between September 2014 and March 2015 in Alcázar de San Juan and Almagro rubbish dumps.

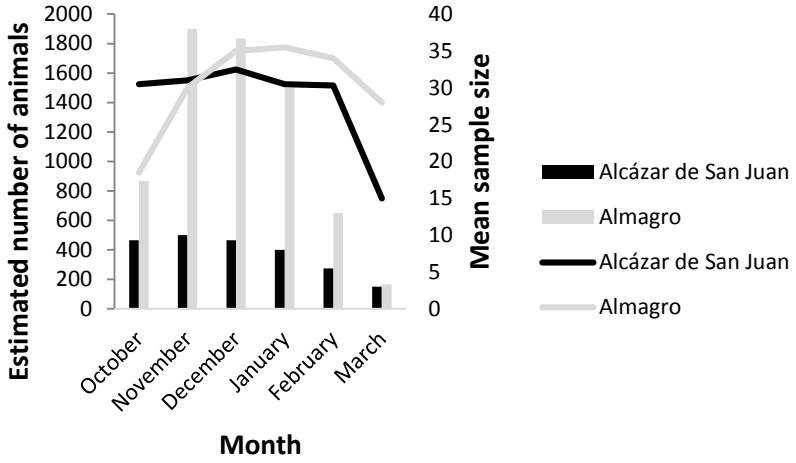

Figure 10- Estimated monthly numbers of white storks at the Alcázar de San Juan rubbish dump (black) and Almagro rubbish dump (grey) between October 2014 and March 2015 (Hernández 2015, in preparation) (bars) and mean sample size (mean number of collected samples) from white storks per month during the same period in Alcázar de San Juan rubbish dump (black) and Almagro rubbish dump (grey) (lines).

5. DISCUSSION

The present study describes the detection of AIV genome in the feces of wild birds sympatric in the use of two rubbish dumps. To the best of my knowledge, this is the first study that explores the dynamics of AIV infections in wild birds in Spanish rubbish dumps. All studied species had already been targeted in previous studies, including a work on AIV circulation in BHG on a rubbish dump in Croatia (Jurinovic et al., 2014). Nevertheless, although it is tempting to compare results of different studies in order to explain variations in AIV prevalence, different surveillance approaches across studies can lead to variations in prevalence (Olsen et al., 2006). Thus, temporal and geographical data, and also number and type of sampled species should be taken in account when drawing conclusions (Busquets et al., 2010).

5.1. AIV genome excretion prevalence

The prevalence of AIV in wild birds tends to be low (Hoye et al., 2010), as evidenced by some studies based on molecular detection method (Pérez-Ramírez et al., 2010, Spackman et al., 2009, Sivay et al., 2012, Slavec et al., 2012, Busquets et al., 2010). Nevertheless, studies on LPAIV prevalence in wild birds in Spain found considerably higher prevalence than in the present study. Precisely in Castilla-La Mancha, one of these studies determined an AIV prevalence of 2.6% between the years 2005 and 2007 (Pérez-Ramírez et al., 2010). A higher prevalence (5%) was found in the study carried out in Catalonia between the years 2006 and 2009 (Busquets et al., 2010). In the cited studies, *Anatidae* represented 50% (686 out of 1374) (Pérez-Ramírez et al., 2010) and 44% (628 out of 1435) (Busquets et al., 2010) of the total of sampled aquatic birds. Birds from the *Anatidae* family are well known AIV reservoirs in nature (Webster et al., 1992), and in fact presented the highest AIV prevalence in both studies, which could explain the difference in mean prevalence as our study did not include ducks because these do not normally forage at rubbish dumps. A third study, that combined data collected from wetlands in Castilla-La Mancha, Catalonia and Basque Country between the years 2007 and 2009 found and overall LPAIV prevalence of 1.7% (Pérez-Ramírez et al., 2012). However, specifically for the autumn migration and wintering (targeted in the present study) these authors found important fluctuations in prevalence between years, varying from 7.7% to as low as 0.82%. Thus, the temporal context can also explain the differences in prevalence between this and

other studies. Another factor could be real time RT-PCR inhibition that has been identified when using feces from YLG for the diagnosis of AIV infection, and was suggested to be due to components of the diet (Busquets et al., 2010). Thus, some positive samples might have been missed in the present study, at least in gulls, contributing to the low prevalence detected. Internal positive controls (IPC) can be used to identify false negatives associated to PCR inhibitors in fecal samples (Busquets et al., 2010). Previous experiments in IREC using IPC did not provide consistent results and thus were not applied in the present study

5.2. Spatial variation in AIV genome excretion prevalence

A higher AIV prevalence was found in Alcázar de San juan (6 out of 797 samples), although the difference in prevalence between the two rubbish dumps was not significant. However, we suspect that the low number of positive samples negatively affects statistical power. Due to the low prevalence of AIV encountered, we would probably have to increase sample size to detect significant differences. In gulls, despite the significantly higher sample size in Alcázar de San Juan (Statistics= 3251, p= 0.000), the prevalence was apparently higher in Almagro. As we could not sample individuals inside the premises of the rubbish dump, our sample size was low in most months and restricted to a fraction of the population leading most probably to an underestimation of the true prevalence in Almagro. However, despite these differences, both rubbish dumps showed the same trend of a higher prevalence of AIV in gulls than in the other two species. An apparently higher prevalence was obtained in white storks in Alcázar de San Juan as compared with Almagro. Sample size in storks was more consistent throughout the study period both in Alcázar de San Juan and Almagro, and, in Alcázar de San Juan, apparently matched the number of storks present (Figure 10). In Almagro this correlation does not seem to be so clear, which can be due to the fact that we did not sample inside the rubbish dump, where the ring lectures were carried out.

5.3. Interspecies variation in AIV genome excretion prevalence

Differences in AIV genome excretion prevalence among the studied species were observed, and gulls presented the highest value as compared to the other species (1.31% versus 0.86% and 0.14% for cattle egrets and white storks, respectively). This differences were not significant (χ^2=5.858, d.f=2, p=0.053), possibly due to the overall

low prevalence, but the marginally significant difference suggests a trend. These differences were also observed in Alcázar de San Juan among the studied species, presenting gulls the highest value (1.27% versus 0.86% and 0.27% for cattle egrets and white storks, respectively). In fact, a higher prevalence in gulls was expected, as aquatic birds from the order Charadriiformes are known AIV reservoirs in natural environments (Webster et al., 1992). Interspecies variation in AIV prevalence can be explained by intrinsic differences in host susceptibility, but also in host ecology (Munster & Fouchier 2009). In the study area, gulls were frequently seen resting in large groups floating inside the ponds (personal observation). Thus, it is possible that gulls use these aquatic environments, known to enhance AIV survival (Munster & Fouchier 2009), more often than the other studied species. Some studies have reported high AIV prevalence in gulls. In the Netherlands a prevalence of 7.4% was reported for BHG in rural environments between the years 2006 and 2009 (Verhagen et al., 2012) and a prevalence of 21.1% was found in this species in Norway in the year 2006 (Anna et al., 2010). However, lower prevalence, more similar to what we found, were reported in BHG sampled in a rubbish dump in Croatia in the year 2009 (0.7%) (Jurinović et al., 2014) and in Catalonia in YLG sampled between the years 2006 and 2009 (1.32%) (Busquets et al., 2010). In Castilla- La Mancha, Pérez-Ramirez et al., 2010 were not able to detect AIV in any of the 36 BHG and 6 LBBG sampled between the year 2005 and 2007. Unfortunately, in the present study it was impossible to identify the species for samples belonging to the *Laridae* family, which complicates comparisons with other studies. In future studies using the same sampling methodology it would be interesting to estimate the proportion of each of the three species in the sample set, using recently developed molecular techniques such as DNA barcoding (Cheung et al., 2009).

We obtained a significantly higher number of samples from white storks (Statistics= 95447, p= 0.000). The low prevalence in white storks (we found a single positive sample) despite the high sampling size, might indicate that in white storks AIV circulates with a low prevalence, and that they thus have a limited role in this viruses' epidemiology, similarly to the results obtained in white storks in Germany (Kaleta & Kummerfeld 2012). However, other studies obtained higher prevalence in this species. For instance, Müller et al., 2009 found a prevalence of 2.9% (3 out of 103) of AIV in white storks sampled in the year 2006 in Germany. One possible explanation is that

these individuals were sampled right after the return to their breeding grounds in Germany, when a high contact rate among individuals with different ages and immune status occurs due to colonial breeding behavior, which may enhance viral transmission. The work of Pérez-Ramirez et al., (2010) carried out in the years 2005 and 2007 in Castilla- La Mancha wetlands, also found a higher prevalence (0.8%, corresponding to 1 out of 128 samples) in white storks. In this case, the only positive sample was found between February and April, which also coincides with the beginning of the breeding season (SEO/Birdlife 2008).

The recurrent low sample size in cattle egrets (we collected less than 30 samples in all sampling moments) might lead us to underestimate the circulation of AIV in this species, and thus their role in the epidemiology of AIV. In fact, higher prevalence were found in the study of Pérez-Ramirez et al., 2010 for cattle egrets (1.4%, corresponding to 2 out of 147 samples, versus the prevalence of 0.86% of the present study). Nevertheless, despite the higher sample size, the 2 positive samples from cattle egrets found in their study were obtained in late May, which coincides with the breeding period in which these animals aggregate in breeding colonies (SEO/Birdlife 2008).

We detected a single positive sample both in white storks and cattle egrets, and thus we cannot exclude a low level of AIV circulation in both host species. Nevertheless both species seem to be of reduced importance for AIV maintenance. In fact, the single positive finding in cattle egrets was detected in October, and might be the result of a spillover from gulls (which presented a higher prevalence in this month). Unfortunately virus typing results are still pending. Although we found AIV genome excretion in all studied species/families, subtyping and sequencing of AIV found in this study would give us more hints about the potential transmission of the virus among them in the studied rubbish dumps.

5.4. Temporal variation in AIV genome excretion prevalence

A higher AIV prevalence was found in October, in Alcázar de San Juan (2.31%) and Almagro (1.27%). Although this temporal variation in prevalence was not significant, probably due to the reasons mentioned above, a higher prevalence was expected during the autumn migration, as was found for many other European countries and specifically, Spain, due to the arrival of migrant birds (Pérez-Ramírez et al., 2012). In the present study, as gulls were the main contributors to the peak in prevalence (two

positive samples in Alcázar de San Juan and one positive sample in Almagro). A higher prevalence in gulls has been reported in late summer-early autumn, which is presumably due to the aggregation of individuals that belong to different age groups (and thus, immune status) in crowded breeding colonies (Olsen et al., 2006). However, a peak in prevalence of AIV in gulls was also described at the beginning of the autumn migration, shortly after they have left their breeding grounds (Munster et al., 2007). AIVs can be transmitted over long distances during migration (Olsen et al., 2006, Lam et al., 2012). The higher number of positive samples in October might thus be due to the arrival of birds that leave their breeding colonies carrying LPAIV and reach Spanish territories. The aggregation of migrant species at the stopover or wintering sites allows the transmission of LPAIV in these crowded sites (Olsen et al., 2006). This transmission may occur from migrant to resident birds and vice-versa. The influx of migrant mallards in Netherlands was recently associated with amplification of endemic LPAIV (Verhagen et al., 2014). Birds that arrive to their wintering territories have carried out a long trip. After this effort they are exhausted and the low immune efficacy might not be sufficient for prevention of AIV infection (Li et al., 2010). Also, migratory individuals may be immunologically naïve to endemic pathogens and thus more susceptible than resident individuals (Leighton 2002). Thus, the arrival of migrating birds from their breeding grounds may allow for the introduction and circulation of viruses into the studied rubbish dumps, or allow for increase of transmission of local endemic AIV. The prevalence decreased after October. LPAIV infections in mallards provided protection against re-infection with homologous and heterologous LPAIV subtypes (Jourdain et al., 2010). In BHG previous infection with a homologous LPAIV virus was associated with a strong, long-lasting protective effect (Verhagen et al., in preparation). The epizootics is gulls appear to occur in late summer-early autumn, as commented above. Thus, a decrease in susceptible/resistant ratio can explain a decrease in AIV prevalence over the study period.

Climatic conditions, such as humidity and temperature, determine environmental viral survival and thus, AIV prevalence (Pérez-Ramirez et al., 2012). Nevertheless, in the present study we were not able to associate these data with AIV prevalence over time due to difficulties in obtaining accurate meteorological data.

5.5. Consequences of LPAIV carriage in rubbish dump guests: potential for local and regional spread

The detection of birds shedding AIV virus at both rubbish dumps is of major importance. Particularly, despite the low prevalence the detection of AIV genome excretion in white storks should not be neglected. In the province of Castilla-La Mancha, the proximity between wetlands (and also the local National Park "Tablas de Daimiel") and rubbish dumps might create an interesting scenario for viral exchange between avian users of these wetlands, as they are used, for instance, by the same white storks for feeding and resting. This situation occurs with the Alcázar de San Juan rubbish dump and a close-by small shallow lake (Hernández & Höfle 2014). At this lake, the peak of LPAIV prevalence was detected in winter in previous years (Pérez-Ramírez et al., 2010).Thus, white stork movements could be a risk factor for the introduction of AIV acquired at the rubbish dump into the wetland especially during the wintering season. Most of the storks observed at Almagro use another wetland for resting at night thus the same scenario applies. Likewise vice-versa white storks foraging at the rubbish dumps could during foraging or resting transmit wetland acquired AIV to other species. As cattle egrets and gulls also use aquatic environments, and AIV genome excretion was detected in both, they can also have a role in the viral circulation between rubbish dumps and wetlands. Foreign European wintering white storks constitute the biggest part of the wintering population at Alcázar de San Juan and Almagro (Figure 11). Thus, there is a potential for viral dispersion from and into different European countries, especially Germany, France and Switzerland (Figure 12) or influx of AIV from these regions through incoming migrants. Lastly, regarding public health, all the studied species present a synanthropic behavior and may thus function as "bridge species" for zoonotic AIV, spreading these viruses into domestic animals and/or different human communities.

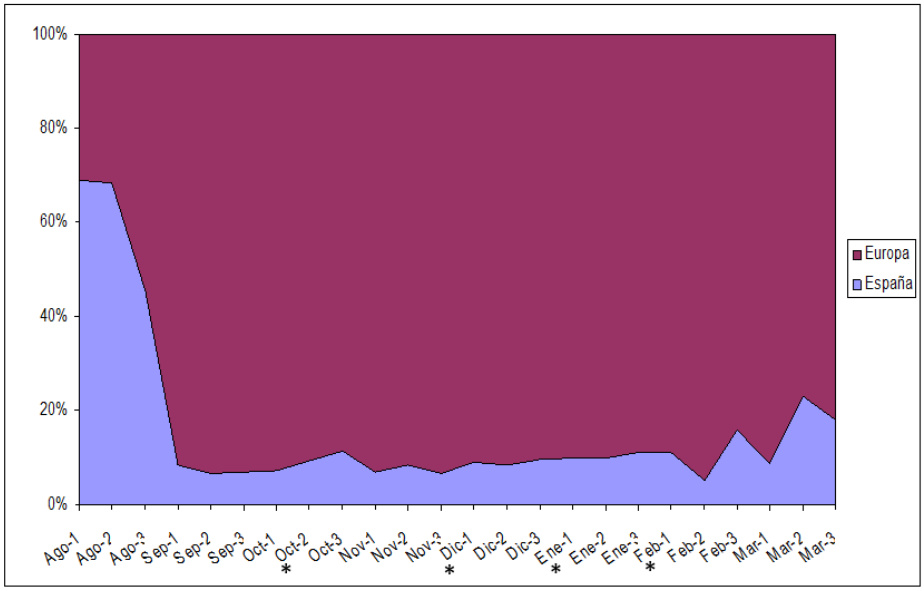

Figure 11 - Proportion between centraleuropean white storks and iberian white storks in Alcázar de San Juan and Almagro rubbish dumps ((*) represents the detection of AIV positive samples in Alcázar de San Juan and Almagro rubbish dumps between September 2014 and March 2015) (graph courtesy of J.M. Hernández)

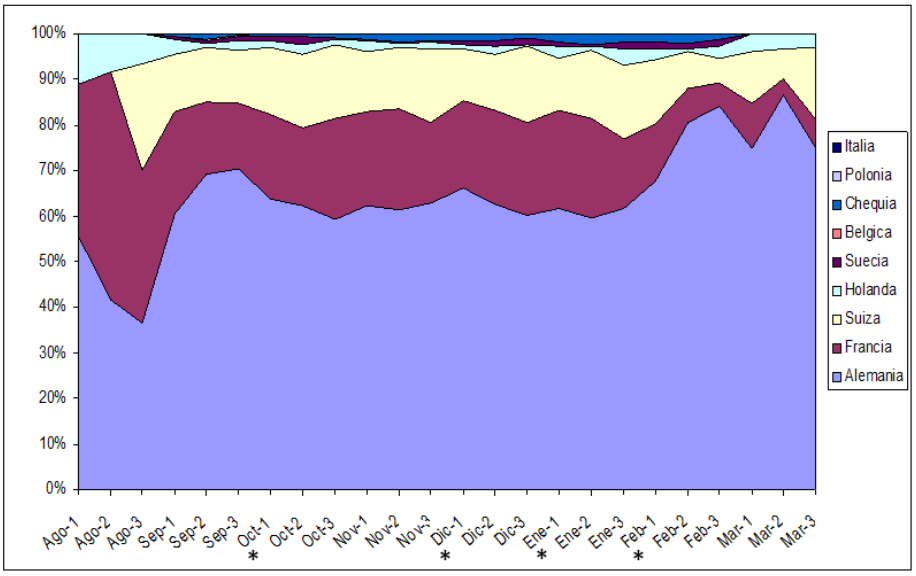

Figure 12 - Proportion of european white storks in Alcázar de San Juan and Almagro rubbish dumps by provenience ((*) represents the detection of AIV positive samples in the Alcázar de San Juan and Almagro rubbish dumps between September 2014 and March 2015) (Graph courtesy of J.M. Hernández).

5.6. Role in HPAIV epidemiology

Taking into accont the recent outbreaks of H5N8 in Europe and the case of H5N1 detected in 2006 (Adlhoch et al., 2014), there is a possibility of introduction of H5 HPAIV in Spain. In fact H5 HPAIV has been detected in white storks (Globig et al., 2009, Śmietanka & Minta 2014), gulls (Ellis et al., 2004, Marchenko et al. 2011, Sakoda et al., 2012, Savić et al., 2010) and cattle egrets (Kayali et al., 2011), but according to our data, during our study period the subtype H5 has not circulated in the wild bird community of the studied rubbish dumps. Nevertheless, HPAIV H5N1, for instance, appears to present more affinity for the respiratory tract (Sturm-Ramirez et al., 2005). Thus, the sampling method applied in the present study may have missed this virus if present.

5.7. Non-invasive sampling methods: pros and cons

The collection of fresh fecal samples has been described as an appropriate method for large scale LPAIV surveillance programs in wild birds (Pérez-Ramírez et al., 2005). In the present study, this non-invasive approach allowed for the collection of a high number of samples from wild birds, which otherwise would be difficult in such a short period of time. It is also cost-effective and causes less impact in the wild bird community, as it does not require capture of the birds. On the other hand, the collection of fresh feces in cases of mixed species flocks does not allow a completely accurate identification of the species-origin of the samples. This handicap can be overcome by DNA barcoding through amplification and sequencing of the mitochondrial cytochrome oxidase I gene from fecal and cloacal samples (Cheung et al., 2009). However, this technique is not yet available at IREC and is pending at the collaborative institution (NEIKER) that currently carries out viral culture and AIV subtyping of the positive samples.

6. CONCLUSIONS

1- The present study identifies a low prevalence of AIV genome excretion in aquatic bird species that forage at the studied rubbish dumps. This indicates that these rubbish dumps where birds aggregate are sites where AIV excretion may occur and where cost-effective sampling for surveillance activities can provide relevant information on AIV circulation in wild birds.

2- AIV genome excretion was detected in all studied species, showing that all of them could to some extent participate in AIV maintenance in the studied rubbish dumps or at least have a role as sentinels.

3- A higher prevalence was obtained in gulls, which agrees with their definition as reservoirs of AIV in natural ecosystems.

4- Despite the high sample size in white storks, this species showed the lowest prevalence. Thus, although white storks can be targeted in surveillance studies (as AIV genome excretion was detected), they do not seem to be a good reservoir for AIV.

5- As described in other studies, the arrival of migrating wild birds to Spain can be associated with a peak in AIV prevalence in wild birds at these territories.

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