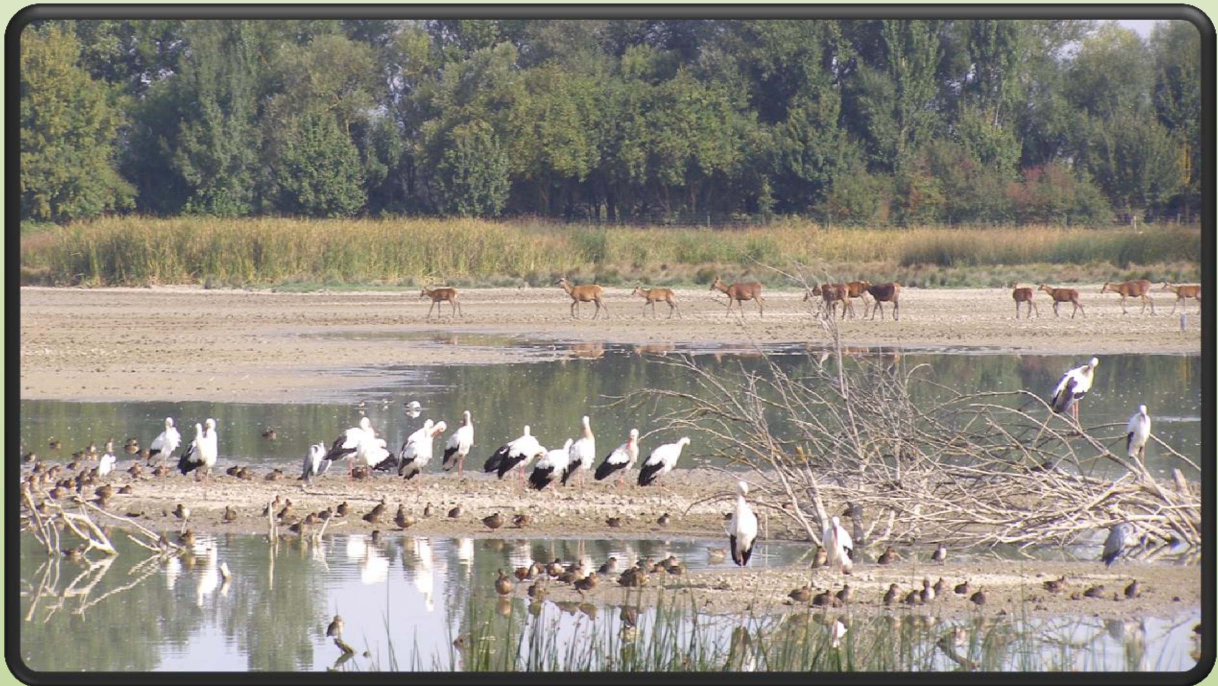
Pathogen dynamics in wild bird species: circulation of avian influenza viruses in natural vs. anthropic ecosystems and concurrent infections with other agents in waterbirds

Olalla Torrontegui Vega



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Pathogen dynamics in wild bird species; circulation of avian influenza viruses in natural vs. anthropic ecosystems and concurrent infections with other agents in waterbirds

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2017

Work performed at the Animal Health Department of NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario for the degree of Doctor of Phylosophy in Biology with international mention at the University of the Basque Country

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Thesis outline

Influenza A virus (genus *Influenzavirus A*) is the taxonomic name of avian influenza virus (AIV). AIVs circulate naturally in waterbirds around the globe and may infect poultry and mammals, humans included. However, infections in the latter hosts are not frequent.

While waterfowl are recognised primary hosts for AIV, information about the epidemiological role of other groups of birds is still lacking. Furthermore, whereas studies on the reservoir hosts are abundant, longitudinal works taking into account AIV and host species diversity within different ecosystems are still scarce.

This thesis aims to shed light on the epidemiology of AIV within its natural maintenance host community (wild birds) in relation to a variety of envionments. Understanding ecosystems' health and changes is essential for gaining knowledge about the complex host-pathogen interactions of AIV with their hosts, reservoirs and vectors in the wild. Increasing knowledge on ecosystem dynamics can therefore provide valuable information following the One Health concept. This is, on why, how and where these interactions take place among humans, domestic animals and wildlife.

<u>Therefore the main objectives of the thesis are</u>:

1. To determine long-term avian influenza virus epidemiology in a natural wetland ecosystem taking into account the circulating viral strains, hosts' ecological traits and some other factors with potential effect on viral detection.
2. To determine the role of Passeriformes on AIV epidemiology.
3. To assess AIV prevalence dynamics in selected waterbird species that use landfills for foraging in South-central Spain.
4. To investigate by means of a comparative study a range of selected agents' prevalence in AIV naturally infected and non-infected birds.

In this regard, this work contains the following studies:

1. **Long-Term Avian Influenza Virus Epidemiology in a Small Spanish Wetland Ecosystem Is Driven by the Breeding Anseriformes Community.**

   A longitudinal non-invasive study based on AIV surveillance in a natural aquatic ecosystem in relation to the ecology of its hosts and environmental conditions. This work delves into understanding AIV epidemiology in the context of the ecology of the wild avian host community.

2. **Avian influenza virus prevalence in Passeriformes hosts in Spain is higher in the Atlantic bioregion.**

   This is a surveillance-study targeting a diverse and ubiquitous bird order such as Passeriformes (song-birds) to evaluate its implication in AIV epidemiology taking into account the diversity of habitats they inhabit.

3. **Avian Influenza Virus in wintering aquatic birds at landfills. Are these places hotspots for surveillance?**

   This work is a non-invasive surveillance approach to assess AIV transmission in anthropic environments such as landfills taking into account the role of sympatric birds mixing. Humanised areas such as landfills, attractive places for many birds due to the predictable constant food supply, generate different patterns of species mingling that may constitute new pathways of AIV circulation and may make them important in AIV epidemiology.

4. ***Mycobacterium* sp. and *Salmonella* sp. are more prevalent when coinfecting with low pathogenic Avian Influenza Virus in wild waterbirds.**

   The last study evaluates concomitant AIV infections with other bacterial and viral agents. The goal of it is verifying whether AIV infection is involved in other microbial infections' prevalence meaningful to animal health.

Literature review

Avian Influenza Virus: morphology, replication and assembly

Classification

Avian Influenza Virus (AIV) pertains to the family *Orthomyxoviridae,* genus *Influenzavirus A,* species *Influenza A virus* (Lefkowitz et al., 2017).

Genus *Influenzavirus A* can be distinguished from *Influenzavirus B*, *C* and *D* genera (types) by the antigenicity of their internal virion nucleoproteins and matrix proteins (Dimmock et al., 2014; Webster et al., 1992).

Furthermore, type A influenza has a distinct epidemiology as compared to other influenza types; it is the only one capable of bearing antigenic shift in addition to the antigenic drift (further described on page 15). It also infects a broad range of hosts, including birds and mammals, and it is capable of triggering pandemics, sometimes with high associated mortality-rates, whereas B and C influenza types are more host-restricted (humans and seals or humans and swine, respectively) and do not cause pandemics (Ohishi et al., 2002; Osterhaus et al., 2000). Indeed, clinical signs of disease are usually mild. As for the recently discovered Influenza D viruses, they are only known to affect cattle and do not infect or cause illness in people (Ducatez et al., 2015).

In 1955 type A influenza virus was shown to be the causative agent of a chickens' disease known as "fowl plague" (Alexander, 1982; Webster et al., 2007). Since then, *Influenza A virus* have also been known to infect mammal orders like Artiodactyla (wild boars and pigs) (Alexander, 1982; Suarez, 2000), Perissodactyla (horses) (Alexander, 1982; Suarez, 2000), Carnivora (cats, dogs and pinnipeds) Chiroptera (bats) and humans to a wide variety of avian species (Keawcharoen et al., 2004; Lee et al., 2009; Leschnik, 2007; Marschall and Hartmann, 2008; Mehle, 2014; Nielsen et al., 2001; Rimmelzwaan et al., 2006; Song et al., 2009, 2008; Thiry et al., 2007; Tong et al., 2013, 2012; Zhu et al., 2013).

Virion structure

AIV particles are 80-120 nm in size, pleomorphic: ranging from spherical to filamentous forms. Virions are enveloped and have a segmented, negative polarity and single stranded ribonucleic acid (RNA) genome (Baltimore classification V) (Figure 1).

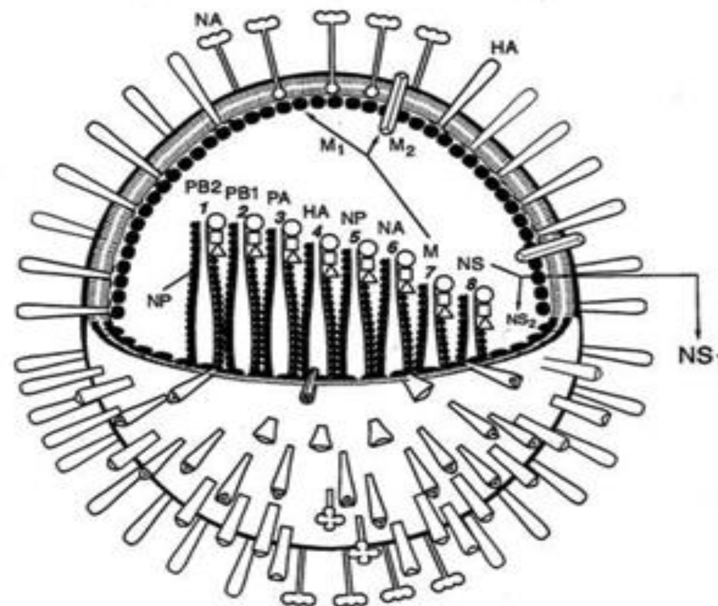

Figure 1. Diagram of an avian influenza virion (Shaw and Palese, 2013).

Number of genome segments (1-8) and encoding proteins are detailed.

Genome structure and organisation

The AIV genome is divided into eight segments that encode at least ten relevant proteins (among which 2 are non-structural) necessary for viral replication and assemblage (Schrauwen et al., 2014) (Figure 2).

The main proteins that constitute the AIV virion are:

Polymerase basic protein 2 (PB2):

PB2 is the cap-binding protein encoded by RNA segment 1. Thus, it is active during the initiation of messenger RNA (mRNA) transcription. It integrates along with polymerase basic protein 1 (PB1) and polymerase acidic protein (PA), the RNA dependent RNA polymerase (RdRp) complex that catalyses RNA replication (Kawaoka, 2006; Webster

et al., 1992).

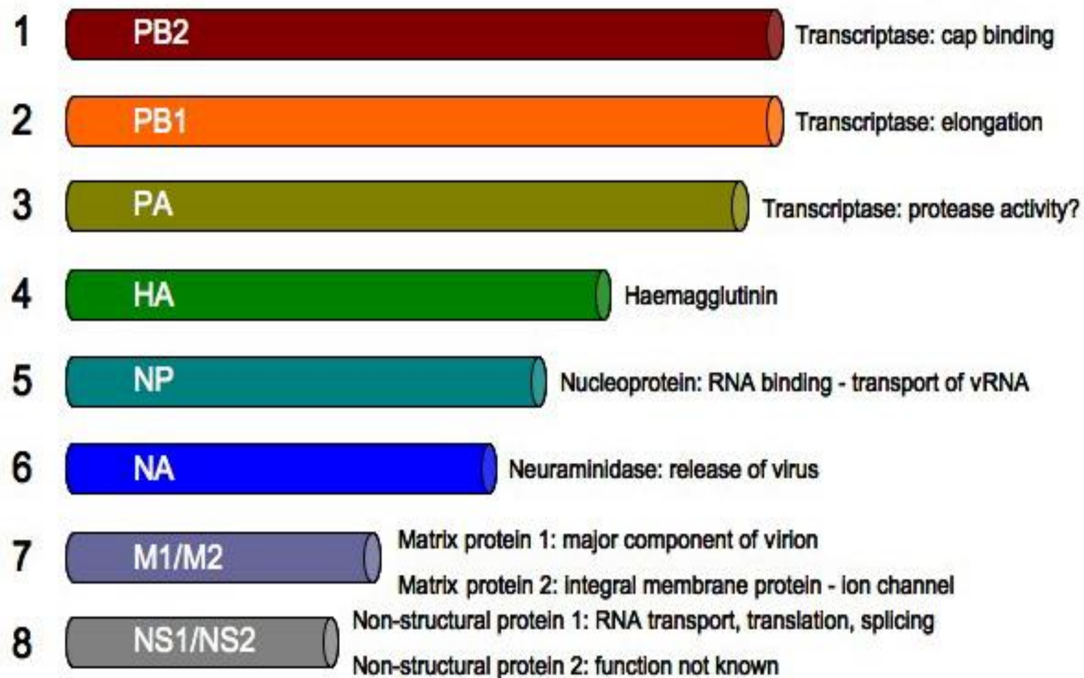

Figure 2. AIV genome segments and associated proteins after transcription.

Each protein's function is also summarised. ("http://www.bios.net/daisy/influenza/4133/3929.html," n.d.)

Polymerase basic protein 1 (PB1):

It is encoded by segment 2 and is responsible for the polymerase activity in the mRNA elongation as well as in RNA and vRNA synthesis (Kawaoka, 2006; Webster et al., 1992).

PB1-F2 is an accessory protein with proapoptotic activity by translation of an overlapping open reading frame (ORF) of the PB1 RNA, that has been found in some strains (Bouvier and Palese, 2008), PB1 N40 is also another protein encoded by the PB1 subunit (Schrauwen et al., 2014).

Polymerase acidic protein (PA):

It is encoded by RNA segment 3 and it is responsible for proteolytic activity. It also contains a second ORF, which encodes the PA-X proteins. PA-155 and PA-N182 proteins are also encoded by the PA (Schrauwen et al., 2014).

Haemagglutinin (HA):

It is responsible for virion attachment and entry. HA is encoded by RNA segment 4, it is an integral membrane glycoprotein and acts as the major surface antigen.

HA is embedded on virion's surface as a homotrimer containing a globular head supported by a stem that is inserted in the viral membrane (Mehle, 2014). Since HA is recognisable by the host immune system, it is considered the main antigenic protein of *Influenza A virus*. This interaction acts as a natural selective force which is enhanced during replication by the non-error-correcting RNA polymerase's action, making the HA a highly mutable protein.

Its implication relies on the capability of the HA to bind the sialic-acids of the host cell membrane. It also plays an important role as a fusion protein in the release of RNA into the host cell cytoplasm by causing membrane merging between the viral particle and the host endosome. HA is a polypeptide that requires activation by host proteases to cleave the non-infectious precursor HA0 form (Runstadler et al., 2013). Cleaved HA0 divides into active HA1 and HA2, which form the active homotrimers. A discrete receptor binding site (RBS) placed in the globular head of HA1, recognises target cells' surface receptors during attachment, whereas HA2 stem takes part primarily during membrane fusion and cell entry with the help of a fusion peptide (Mehle, 2014).

Nucleoprotein (NP):

It is encoded by the RNA segment 5 and it is a structural and functional unit within the RNP. Evidence implicates NP as a key factor in replication (Kawaoka, 2006).

Neuraminidase (NA):

It is encoded by the RNA segment 6 and it is also an integral glycoprotein with antigenic properties. NA is a tetramer involved in viral release from the host by sialic acid-glycoprotein cleavage.

Matrix protein (M):

It is encoded by the RNA segment 7. Matrix protein 1 (M1) lines the inner side of the

nucleocapsid under the virion's envelope and mediates in the transport of the newly synthesized vRNPs to the cytoplasm (Schrauwen et al., 2014). Matrix protein 2 (M2) is an integral protein believed to act as a proton channel during HA synthesis.

Non-structural proteins:

They are encoded by the RNA segment 8. Non-structural protein 1 (NS1) works as an antagonist to block the type 1 interferon (IFN) mediated host antiviral response. More recently it has been discovered that non-structural protein 2 (NS2) mediates the nuclear export of viral ribonucleoproteins (Schrauwen et al., 2014).

M42 protein:

It has recently been discovered. It encodes a novel M2-like protein with a variant extra-cellular domain (Schrauwen et al., 2014).

The active form of the genome for transcription is the viral ribonucleoprotein (RNP), which forms the nucleocapsid. The nucleocapsid shows helical symmetry and it is enclosed within a matrix protein (Alexander, 1982). RNPs encapsulate each viral RNA (vRNA) segment and they are composed of nucleoproteins (NP) and viral RNA-dependent RNA polymerase (RdRp) complex (Kawaoka, 2006). RNPs are transcribed and replicated in the nucleus of the cell (Figure 3).

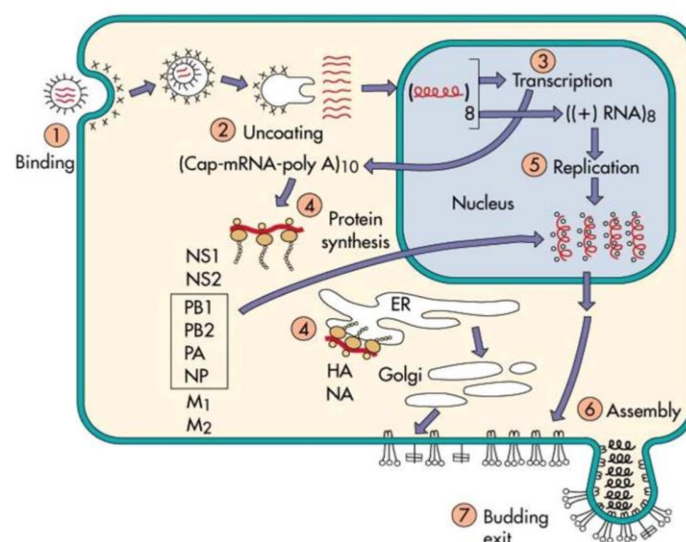

Figure 3. Stages of avian influenza virus replication (1-7).

ER: Endoplasmic Reticulum. Reproduced from (Murray et al., 2005).

Stages of Viral Replication

Unlike most negative-sense RNA viruses, transcription of the influenza virus genome takes place in the nucleus of the infected cells where viral mRNA is synthesized (Dimmock et al., 2014; Elton et al., 2006). New viral particles are formed in a process detailed below (Flint et al., 2015)(Figure 3):

A.– Attachment (1): takes place by a receptor-mediated endocytosis mechanism; the viral particle binds to a sialic acid placed at the cellular membrane.

B.– Absorption: the virion is endocytosed by an endosome and carried into the cytoplasm.

C.– Membrane fusion (2): the low pH inside the endosome triggers a conformational change within the HA which provokes viral and host membrane fusion. As a consequence, the eight viral segments are released into the cytoplasm.

D.– Transcription (3): - viral segments are transported into the nucleus via nuclear pores and copied by the virion's RNA polymerase into viral mRNA using the 5' ends of host mRNAs as primers for synthesis.

E – Assemblage (4-6): the newly synthesised mRNA is transported to the cytoplasm. Those mRNA that encode the viral membrane proteins (HA, NA and M2) will be translated by the endoplasmic reticulum's ribosomes and transported to the cell surface for being incorporated into the host membrane (4). On the other hand, the remaining proteins will be translated by cytoplasmic ribosomes, where most of them will return to the nucleus for participating in the synthesis of new mRNA (5). These proteins will also induce nucleocapsids' export to the cytoplasm. Once the synthesis has been completed, the plasma membrane's proteins will assemble the nucleocapsids.

F.– Release of progeny viral particles (7): occurs by budding from the plasma membrane, cleavage is mediated by the NA.

AIV evolutionary pathways

In AIV the antigenic properties of the two main integral glycoproteins (HA and NA) cause a high selective force in molecular composition. Low fidelity during replication caused by the lack of error-correcting system of the RdRp, also promotes high diversity within subtypes. As a result, excluding the newly discovered bat-influenza subtypes, there are 16 HA and 9 NA different antigenic subtypes that could be assembled in 144 possible combinations (Olsen et al., 2006).

The high variability of these viruses between and within strains makes their subtype-based classification insufficient for providing key information about their evolutionary history, infectivity or pathogenicity (Boyce et al., 2009). In this regard, each AIV nomenclature conveys the following:

An isolate's features (WHO, 1980): 1.) antigenic type (A); 2.) host from which it has been isolated; 3.) geographic origin; 4.) reference identification number; 5.) year of isolation and 6.) the HA and NA subtypes between parentheses.

E.g.: A/Mallard/Netherlands/14/2010(H3N8)
In some cases, the long-lasting and continuous circulation of the same AIV subtype in different geographical areas concurrently, has led to the appearance of distinct genotypes (also named clades) caused by multiple reassortment events. That is the case of highly pathogenic AIV (HPAIV) H5N1 (Dundon et al., 2012).

Because of this genetic diversity, since 2008, highly pathogenic H5N1 (HP H5N1) clades have been classified according to their HA homology (*i.e.* clade 1; clade 2; clade 2.2; clade 2.3.2. *etc*.) with regard to A/goose/Guangdong/1/1996 (H5N1) (first isolate). The *WHO/OIE/FAO H5N1 Evolution Working Group* has formally identified 20 distinct clades of this virus subtype (Dundon et al., 2012).

Antigenic drift

It consists of point mutations in the genome driven by the RdRp's inexistent proofreading mechanism (Figure 4). The mutation rate for a RNA virus is estimated to be in 3x 10^{-4} (*vs.* proofreading DNA-polymerase: 10^{-9}-10^{-10}) (Dimmock et al., 2014). In

other words, the degree of genetic variation achieved in a single RNA genome generation would take a DNA genome between 300,000-3,000,000 genome generations (Dimmock et al., 2014).

Species-specific RdRp activity is an impediment to host switching. Avian influenza virus-derived RdRps, do not work properly in mammalian cells. However, a single mutation in the PB2 subunit, is sufficient to overcome restriction in human cells by conversion of the avian-origin glutamic acid residue at position 627 to the human-origin lysine residue (Mehle, 2014).

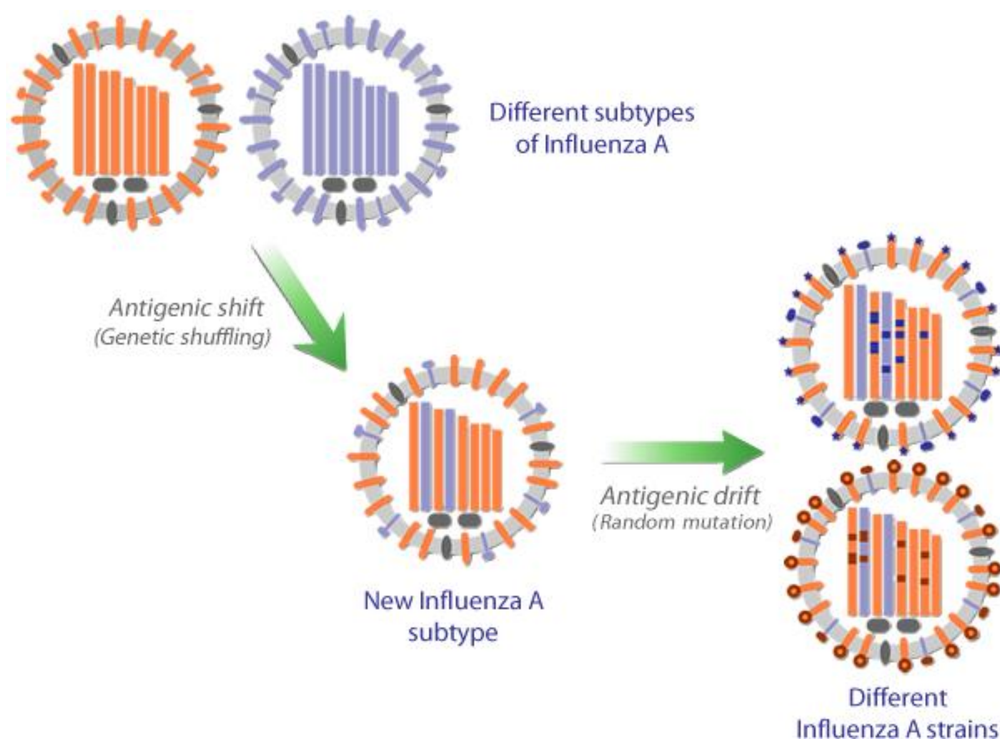

Figure 4. Evolution of AIV: antigenic shift *vs*. antigenic drift.
("Victorian Infectious Disease Reference Laboratory (VIDRL)," n.d.)

Antigenic shift

Because AIV genome is segmented, if a given host cell happens to be concurrently infected by more than one influenza subtype at a time; progeny viruses can have a new rearrangement/reassortment of its genes by combining the segments of the parental strains creating a totally new viral particle (Abolnik et al., 2007; Castrucci et

al., 1993; Hinshaw et al., 1980; Nam et al., 2011; Van Reeth and Nicoll, 2009) (Figure 4).

AIV pathogenesis

Viruses are obligate microparasites which need a host to complete their life cycle. The relation of the parasitised host with the environment in terms of competition for resources, trophic interactions and biodiversity, may be consequently affected (Preston and Johnson, 2010). Microbes usually evolve to reach an equilibrium with their natural hosts without causing disease. Although parasites rarely cause extinctions, in some cases, when pathogens invade naïve host populations dramatic effects may arise (Preston and Johnson, 2010).

AIV receptor specificity

More than 50 molecular species of sialic acids (Sias) are known in nature; among them, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are important in type A and B influenza infections. Viral affinity for host cellular receptors is a crucial step in the process of infection because it will determine viral host range and pathogenicity (Watanabe et al., 2012). In the case of IAV, this affinity it is mediated by (Sias)-galactose association (sialyl sugar structures). The most common sialyl terminal is the N-acetylneuraminic acid and binds the galactose through an α2,3 (NeuAca2,3 Gal) or α2,6 linkage (NeuAca2,6 Gal) (Watanabe et al., 2012). The expression profile of these receptors on the epithelium lining the digestive apparatus of birds is dominated by NeuAca2,3 Gal, whereas along the upper respiratory epithelium of humans long NeuAca2,6 Gal glycans are present. Swine hosts harbour both types. Hence, when an avian adapted strain begins to evolve gaining affinity to a α2,6 Sia bound, it means an early step in adaptation to infecting humans (Watanabe et al., 2012).

Mutations at the cleavage site

Host trypsin-like cellular enzymes will cleave the inactive HA0 to the active forms HA1 and HA2 by proteolysis (Runstadler et al., 2013). Sequence variation at the cleavage

site between HA1 and HA2 will determine tissue tropism and pathogenicity (Watanabe et al., 2012).

Monobasic cleavage sites

Low virulent strains have a single arginine (R) amino acid at the cleavage site and a lysine (K) at position -4 (underlined below).

i.e. PEKQTR/GLF

Trypsin-like enzymes, responsible for this cleavage, are restricted to respiratory and intestinal tracts (Alexander, 2000).

Polybasic cleavage sites

Some strains of the H5 and H7 subtypes are known to evolve into highly pathogenic avian influenza virus forms (HPAIV) through acquiring polybasic amino acids insertions at the HA0 cleavage site (underlined below) (Alexander, 2000; Runstadler et al., 2013).

i.e. PQRESRRKK/GLF

These changes will allow ubiquitous proteases to cleave enabling the infection of multiple tissues and further systemic virus spread.

However, acquisition of a polybasic cleavage site by itself has been proven not to be sufficient for HPAIV conversion in chickens, which suggests that other changes involving additional viral proteins are necessary (Runstadler et al., 2013).

The World Health Organisation (WHO) and the World Organisation for Animal Health (OIE) define a HPIAV when it has an IVPI >1.2 (intravenous pathogenicity index) in 6-week-old chickens or causes at least 75% mortality in 4 to 8 week-old chickens infected intravenously. However, any H5 or H7 subtypes not meeting these criteria are sequenced for comparison to other H5 and H7 subtypes. Among the potential causes for these highly pathogenic variants to thrive in poultry, are the presence in terrestrial birds (chicken, turkey, and quail) of either avian-type (Siaα2-3Gal) or human-type (Siaα2-6Gal) receptors on the epithelial cells. This suggests that these species can

support the replication of both avian and human influenza viruses and act as "adaptation hosts" for receptor switching of avian strains (Watanabe et al., 2012).

Epidemiology of AIVs

It is in the avian class where most AIV subtypes and subtype-combinations have been found, especially within the Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, shorebirds) orders (Webster et al., 1992).

As genetic analyses have further evidenced, these two avian orders are considered the natural reservoirs for *Influenza A virus* and the source of all influenza A viruses in other species, except for bat-endemic strains (H17-H18, N10-N11) (Reed & Medical 2003) (Figure 5).

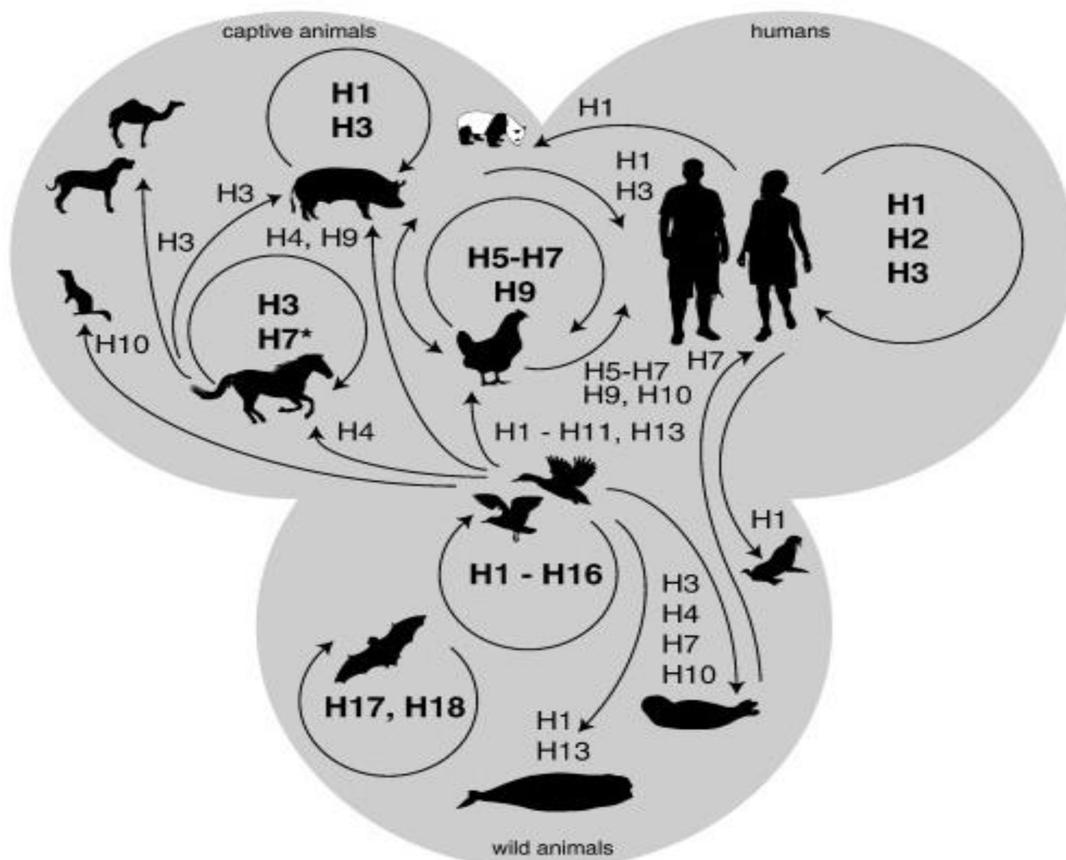

Figure 5. *Influenza A virus* host range and host-associated HA subtypes (H).

In bold HA subtypes that are endemic for each of the depicted animals. Arrows indicate the directionality of interspecies transmission. (Short et al., 2015)

AIVs are separated phylogenetically into two main lineages: the Eurasian and the American (Verhagen and Fouchier, 2011). Most of these subtypes are low pathogenic but after transmission into poultry some strains may become highly pathogenic over time.

Table 1. Predominant hosts associated with each HA and NA subtype. Adapted from (Schrauwen et al., 2014; Shaw and Palese, 2013).

Haemagglutinin Subtype	Predominant hosts	Neuraminidase Subtype	Predominant hosts
H1	human, swine, avian	N1	human, swine, avian
H2	human, swine, avian	N2	human, swine, avian
H3	avian, human, swine, equine	N3	avian
H4	avian	N4	avian
H5	avian, (human)	N5	avian
H6	avian, (human)	N6	avian
H7	avian, equine, (human)	N7	equine, avian
H8	avian	N8	equine, avian
H9	avian, (human)	N9	avian
H10	avian		
H11	avian		
H12	avian		
H13	avian		
H14	avian		
H15	avian		
H16	avian		

Those hosts between () represent detections but to which the virus is not endemic yet.

The extraordinary mutation rates of AIVs give them capability of broadening their host range by successful adaptation. H5N1, H7N7, H7N9 viruses have been transmitted from chicken to humans and H9N2 virus from quail to humans, but these viruses have not become endemic in the human population "yet" (Table 1) (Chan et al., 2013).

Phylogenetic studies have shown numerous AIV species-associated lineages (i.e. avian influenza, swine influenza, human influenza). This occurs when the influenza virus accumulates nucleotide sequence changes in different genes at different pace, which leads to evolution of a species-specific virus lineage (Watanabe et al. 2012). Hence, for interspecies transmission, not only the species infected needs to be studied but also interspecies interactions are paramount for understanding the ecology of the circulating AIVs (Webster et al. 1992; Olsen et al. 2006).

Wild birds

The first reported avian influenza outbreak in wild birds was a highly pathogenic H5N3 in common terns (*Sterna hirundo*) detected in 1961 in South Africa. At least 1300 bird deaths were recorded (Alexander, 2000; Stallknecht and Shane, 1988). Since then, AIVs have been isolated in more than 100 wild bird species and 26 families (Fuller et al., 2010; Olsen et al., 2006).

AIVs circulating in wild birds are the progenitors, either directly or indirectly of all pandemic and highly pathogenic influenza viruses (Webster et al., 1992). Prevalence is age-related and varies with time, space and host species, usually showing seasonal peaks and annual variation (Stallknecht and Shane, 1988).

The transmission and maintenance of AIVs in the wild bird population occurs mainly via contaminated water ingestion through the faecal-oral route (Brown and Stallknecht, 2008) (Figure 6).

Although HPAIV cases in wild avian hosts have been reported, circulation of these variants within wild birds is rare (Alexander, 2000; Stallknecht and Shane, 1988). Indeed, the emergence of HPAIV strains in wild birds requires previous infection in poultry (Ferenczi et al., 2016; Stallknecht and Shane, 1988).

Figure 6. AIV replication and transmission in most Anseriformes hosts.

AIV particles are shed within the faeces to the water by infected hosts where new hosts acquire them through the faecal-oral route.

This way, most circulating subtypes affecting wild birds are low pathogenic (LP) and associated infections often develop with unapparent clinical signs and in absence of gross and histological lesions (Kuiken, 2013). However, the impact of low pathogenic strain infections should not be underestimated since they can have subclinical functional effects (reduction of foraging capacity, e.g. (Hoye et al., 2016) and their effect could amplify (or be amplified by other) coinfecting agents (Alexander, 2000).

Anseriformes

The Anseriformes order comprises ducks, geese and swans (commonly named waterfowl). Their members harbour the greatest AIV subtype diversity (number of subtypes and subtype combinations) (Wallensten et al., 2007).

The mallard duck, *Anas platyrhynchos* harbours the highest subtype richness and frequencies of AIV detection (Chan et al. 2013). Indeed, with exception of H16 all HAs/NAs have been detected in this duck species (Fereidouni et al. 2014) (Table 2).

In addition, *Anas* genus members are considered the major reservoir of AIVs where infected hosts are usually asymptomatic, indicating a long-standing equilibrium, possibly owing to virus adaptation over many generations (Bengtsson et al., 2016; Watanabe et al., 2012).

Table 2. AIV subtype combinations found in different wild bird orders.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16
N1	***		***		***	***	***		***		***					
N2	***	***	***	***	***	***	***		***		***					
N3	***	***	***		***	***	***			***	***					
N4						***	***	***								
N5					***	***						***				
N6			***	***		***	***									
N7						***	***		***	***						
N8			***	***	***	***	***		***							
N9		***		***	***	***	***		***		***					

Anseriformes (green), Charadriiformes (purple), Procellariiformes (blue), more than one order (grey), and findings in domestic birds (***) based on GenBank data. (Olson et al., 2014)

This pathogen-host affinity may be mediated by the genetic proximity of mallard ducks with other phylogenetically related interespecifics. Indeed, among birds, low genetic distances are frequent even if they show large morphological differences (Kraus et al., 2012). As concerns the Anatidae family, it shows a remarkable hybridisation in the wild, although each species maintains its distinctive morphological features. In this regard, dabbling ducks (*Anas platyrhynchos*, *A. acuta*, *A. crecca*, *A. penelope* and *A. strepera*) will pertain to a sympatric superspecies complex resulting in fertile offspring (Kraus et al., 2012).

In general, anatids are gregarious outside the breeding season, but solitary while nesting. They build their nests on the ground, close to the water or in shallow submerged areas on floating platforms anchored to vegetation. Nest-parasitism is a common feature within the Anseriformes in which females lay eggs in nests of other conspecifics (Van Dijk et al., 2014b).

In general, the Anseriformes are monogamous, but they present different parental care strategies according to the species they belong to; while swans and geese form family units sharing parental care and create lifetime bonds, duck females are the main responsibles for incubation and young rearing until they are capable of flying.

The chicks are precocial, that is, well-developed, active and alert at hatching and able

to procure food themselves (Whitworth et al., 2007). In *Anas platyrhynchos* hatching of chicks of a clutch is synchronous (Van Dijk et al., 2014b).

All waterfowl undergo a brief post-breeding flightless period where flying feathers are shed simultaneously. This occurs during the breeding season in all females and in those species where males participate in the chick rearing.

The mallard duck is a dabbler, it feeds mainly on surface water allowing effective faecal-oral transmission of AIV (Figure 6)(Wright et al., 1992). Dabbling ducks tend to aggregate in large numbers outside the breeding season and especially during migration. They choose different breeding grounds depending on the mate, even causing a switch in their traditional migration route, a habit called abmigration (Kraus et al., 2011; Olsen et al., 2006).

There is little connectivity between northern and southern hemisphere Anatidae populations; in Afrotropical regions for example, seasons are determined by rainfall rather than by temperature (as it happens in temperate regions) which drives the breeding dynamics and aggregation waterfowl (Gaidet, 2016; Olsen et al., 2006).

The existing epidemiological studies in waterfowl are inconsistent regarding location, sampling periods or number and richness of species sampled. Prevalence values as high as 60% have been recorded for ducks in early autumn in Canadian breeding areas whereas in Europe, AIV detection rate values of 25.7% have also been registered (October 2005) (Olsen et al., 2006; Wallensten et al., 2007). Overall AIV prevalence of the Anseriformes members for all conducted studies in Europe regardless sampled year and season are summarised in Table 3.

A review performed by Kuiken (2013) based on 17 studies about natural and experimental infections with LPAIV in waterbirds, concluded that most of the challenged individuals did not reveal significant signs of disease (reflected on the absence of clinical signs or gross lesions). In a more recent research performed with mallard ducks monitoring the movements of naturally AIV-infected and non-infected individuals, they observed no differences in traits between groups, which comes in

agreement with the idea that natural reservoirs are poorly impacted by AIV infection (Bengtsson et al., 2016).

Charadriiformes

This taxonomic order comprises several families that include shorebirds, gulls, terns and auks. Together with waterfowl, Charadriiformes are the most common hosts of LPAI viruses (Webster et al., 1992).

Like waterfowl, Charadriiformes are quite gregarious outside the breeding season (but in contrast to ducks, also during the breeding season depending on species) when large migrating flocks congregate at wetlands and estuaries for foraging and roosting (Whitworth et al., 2007). Nests are rudimentary (some pebbles or bits of vegetation) and chicks are precocial.

Delaware Bay, USA, is the only site in the world where AIV isolations from shorebirds (Scolopacidae) have consistently been reported (mainly from *Arenaria interpres*) (Gaidet et al., 2012b). Infections are thought to be due to the high concentration of shorebirds that use this site in May (Hanson et al., 2008).

AIV epidemiological studies in gulls reveal the existence of a great diversity of HA subtypes. Multiple factors have been found to affect AIV dynamics in these birds such as HA receptor differences and clusters of gull specific clades (Wille et al., 2011). AIVs of the subtypes H13 and H16 are associated with this bird order (Fouchier and Munster, 2009).

Gulls are larger resourceful birds with a highly-developed social structure. They are very adaptable and many species are frequent in anthropic environments such as human residue landfills and domestic poultry farms (Whitworth et al., 2007). In the wild they are generalistic foragers that prey or scavenge on fish or even eggs and young of seabirds (Svensson et al., 2010). However, several gull species breed inland close to lakes and marshes where they nest on the ground in large colonies (Whitworth et al., 2007).

In a study carried out on black-headed gulls in the Netherlands AIV infections were

more prevalent during the second half of the breeding season, with prevalence of up to 72% per week (Verhagen et al., 2014a). Infected and non-infected sampling gull yearlings had similar body conditions, suggesting that the H13 and the H16 viruses detected in that study had little impact on their health status. An associated study found high percentage of circulating LPAIVs 21.6% (24/111) in nestling black-headed gulls. In addition, based upon the absence of histopathological lesions associated to AIV replication in intestinal epithelium as evidenced by immunohistochemistry the study concluded that in natural LPAIV infections the viruses caused minimal pathogenicity (Hofle et al., 2012).

Closely related terns (Sternidae) are also considered important in AIV epidemiology for their susceptibility to HPAI infections as the one resulted in Common Terns in 1961 (Whitworth et al., 2007).

Additional prevalence values for members of this order in Europe are detailed in Table 3.

Other wild bird species

Surveillance studies on host ecology in relation to the epidemiology of AIV in the wild have usually skewed data. Typically, species that are an easy target to spot (or to be found in accessible areas or in big aggregations) or to capture are the ones selected for studies. On other occasions, the ones in which AIV has been prevalent have been given preference (Verhagen and Fouchier, 2011). Furthermore, sampling studies conducted during the full annual cycle are also scarce which brings some extra difficulties in determining what time of the year AIVs are more prevalent or more persistent (Olson et al., 2014).

Therefore, the role of other bird species not meeting the above mentioned criteria as participants in the AIV maintenance-community is not fully clear (Caron et al., 2015). In this context it is commonly accepted that birds in which AIVs are endemic share habitat with the Anseriformes reservoirs at some point (Verhagen and Fouchier, 2011).

The following summarises some wild birds of interest in AIV epidemiology but with less relevance than the reservoirs (Anseriformes and Charadriiformes):

Gruiformes is a taxonomic order composed of coots, gallinules, rails and cranes. In general, they have generalistic feeding habits and most of them are gregarious except the coot (*Fulica atra*), which can be found in large flocks. Eurasian coots have also been found to be frequent hosts of LPAIV infections, overall prevalence being as high as 2.6% in pooled data (Table 3) (Reperant et al., 2012).

Table 3. A review of wild bird species in which AIV has been detected in Europe.

Order, family	Common name	Scientific name	Country	N	%
Anseriformes, Anatidae	Greater white-fronted goose	*Anser albifrons*	The Netherlands, Sweden	4325	2.2
	Greylag goose	*Anser anser*	Germany, the Netherlands, Sweden	1432	1.5
	Pink-footed goose	*Anser brachyrhynchus*	The Netherlands, Sweden	285	2.1
	Bean goose	*Anser fabalis*	The Netherlands	466	0.6
	Barnacle goose	*Branta leucopsis*	The Netherlands	1257	0.6
	Brent goose	*Branta bernicla*	The Netherlands, Sweden	715	0.6
	Northern pintail	*Anas acuta*	France, the Netherlands, Sweden	920	3.3
	Common teal	*Anas crecca*	France Germany, Iceland, the Netherlands, Sweden	2414	8.3
	Eurasian wigeon	*Anas penelope*	France, the Netherlands, Sweden	3596	2.5
	Mallard	*Anas platyrhynchos*	Czechoslovakia, France, Germany, Hungary, Italy, the Netherlands, Sweden	15962	8.7
	Garganey	*Anas querquedula*	France, Romania		
	Northern shoveler	*Anas clypeata*	France, the Netherlands	284	3.2
	Common shelduck	*Tadorna tadorna*	France, Italy, Sweden	1244	4.7
	Tufted duck	*Aythya fuligula*	The Netherlands	157	1.3
	Long-tailed duck	*Clangula hyemalis*	Germany		
	White-winged scoter	*Melanitta fusca*	Germany		
	Common scoter	*Melanitta nigra*	Norway		
	Common eider	*Somateria mollisima*	The Netherlands, Sweden		
	Bewick's swan	*Cygnus colombianus bewickii*	The Netherlands, Sweden	153	2
	Mute swan	*Gygnus olor*	Germany, the	1362	1.5

Netherlands, Sweden

Cont.

Order, family	**Common name**	**Scientific name**	**Country**	**N**	**%**
Charadriiformes, Charadriidae	Waders		Germany, the Netherlands, Sweden, Italy	3000	1.2
Charadriiformes, Laridae	Black-headed gull	*Chroicocephalus ridibundus*	Germany, the Netherlands, Sweden	2395	1.5
	Common gull	*Larus canus*	The Netherlands	226	0.9
	Herring gull	*Larus argentatus*	The Netherlands	862	0.6
	Greater black-backed gull	*Larus marinus*	The Netherlands, Sweden		
	Mediterranean gull	*Larus melanocephalus*	France		
	Common tern	*Sterna hirundo*	Germany	875	1.5
	Arctic tern	*Sterna paradisea*	Germany		
	Sandwich tern	*Sterna sandwicensis*	Germany	351	0.3
Columbiformes, Columbidae	Eurasian collared dove	*Streptopelia decaocto*	Hungary		
Galliformes, Phasianidae	Common pheasant	*Phasianus colchicus*	Hungary	399	0.5
Gaviiformes, Gavidae	Arctic loon	*Gavia arctica*	Romania		
Gruiformes, Alcidae	Common murre	*Uria aalge*	Sweden	843	0.7
Gruiformes, Rallidae	Eurasian coot	*Fulica atra*	Germany, Hungary, Italy, the Netherlands	2610	2.6
Pelecaniformes, Ardeidae	Grey heron	*Ardea cinerea*	Romania		
Suliformes	Great cormorant	*Phalacrocorax carbo*	Germany, Romania	4500	0.4

AIV prevalence is detailed only in species with more than 150 sampled individuals, adapted from (Reperant et al., 2012). Taxonomic classification according to (Gill and Donsker, 2017)

Storks and Herons were first grouped in the Ciconiiformes taxonomic order. In recent

phylogenetic studies, only storks are included within (herons and egrets now pertain to Pelecaniformes) (Gill and Donsker, 2017). Herons, egrets as well as storks share similar feeding habits and breeding ecology and none are recognised as remarkably prevalent hosts of AIV (Perez-Ramirez et al., 2010; Whitworth et al., 2007). Storks and herons are medium to large wading birds that are associated with wetland ecosystems although human waste landfills also provide a constant food source for their dietary habits (Gilbert et al., 2016). Most of these birds breed in colonies of large stick nests in the upper branches of trees or as in the case of *Ciconia ciconia* on rooftops and other artificial structures (Whitworth et al., 2007). Chicks are altricial at hatching. That is, very little developed and require of parental care.

Grebes (Podicipediformes) are diving and fish eating birds rarely found outside water ecosystems. This group is not considered a common AIV host (Whitworth et al., 2007). However, the only case in which HP H5N1 has been reported in a wild bird in Spain has been from a Great Crested Grebe (*Podiceps cristatus*) (Barral et al., 2008).

Cormorants (Suliformes) are considered occasional hosts of AIVs including the widespread Great Cormorant (*Phalacrocorax carbo*) (Gill and Donsker, 2017; Whitworth et al., 2007). Large breeding colonies can be found either on cliffs in coastal areas or at inland wetlands. Chicks are altricial (Whitworth et al., 2007).

Raptors (Falconiformes) are diurnal birds of prey such as hawks, eagles and falcons and are known to have been fatally affected by HP H5N1 virus likely through consumption of infected birds (Reperant et al., 2012). Although information in natural infections with LPAIV variants is scant (Gunnarsson and Jourdain, 2009).

With over 5000 species Passeriformes (passerines) is one of the most diverse bird orders (Stallknecht and Shane, 1988). Corvids and sparrows have broad habitat preferences, and they can easily be seen close to anthropic ecosystems and thus, in close contact to poultry and livestock. Indeed, both have also been known to become infected with HP H5N1(Whitworth et al., 2007). The presence of many of these species is associated with aquatic habitats, home to the main AIV reservoirs so their role as bridge hosts or as reservoirs still needs structured surveillance (Stallknecht and Shane, 1988). The AIV prevalence in Passeriformes from a recent review determined overall

values in 1% (248/29258) however values as high as 30% were also recorded from birds sampled in Slovakia (Caron et al., 2010; Gronesova et al., 2008). Nevertheless, AIV has rarely been isolated from these hosts. Thus, evidence for birds shedding infective particles remains limited and so it may their potential in virus transmission (Lebarbenchon et al., 2007).

Poultry

Poultry is defined as domesticated birds such as ducks, geese, chickens and turkeys (Boyce et al., 2009). In addition, since 2006 game birds are also regarded as poultry.

As previously mentioned, a LP AIV variant to become HP requires infecting gallinaceous poultry. Clinical symptoms in poultry range in severity depending on the pathogenicity of the strain (OIE, 2015; Swayne and Halvorson, 2008). In this regard, LPAIVs do not cause severe disease outbreaks whereas HP variants can cause up to 100% mortality within 48 hours post-infection (Watanabe et al., 2012). Therefore, infections in poultry are very relevant, especially the H5 and H7 subtypes in terms of the economic impact they may produce and also for the zoonotic character of some subtypes, that can cause disease in persons with a continuous close contact with infected birds (Alexander, 1982). For these reasons, the inclusion of wild birds as targets for early detection of the circulating AIV subtypes is highly recommended within AIV surveillance programs.

Mallard and gamebird restocking

A study conducted by Champagnon and colleagues (2009) described the massive introduction of hand-reared mallards (*Anas platyrhynchos*) within European grounds for restocking purposes (to minimise the impact caused on wild duck population by human hunting activities). In Europe, mallard population restocking by humans has been calculated in a million individuals per year (Champagnon et al., 2009). It is believed that this activity poses demographic impacts on the wild population by means of genetic pollution; this is, by decreasing the average fitness of individuals. As a matter of fact, decrease in bill lamellar density has already been reported which directly affects filtering efficiency at foraging (Champagnon et al., 2009).

Another example are released gallinaceous game bird species, in which high mortalities are recorded in the following weeks after the release (Champagnon et al., 2009). Since they are used to human presence while being fed, the susceptibility to be hunted is increased (searching for food can be more difficult for them and cause weight loss making them more disease prone). Research carried out in the Camargue, (South France), already pointed out the increased likelihood of viral exchange among game bird facilities and wild habitats (Vittecoq et al., 2012). The contemporaneous mallard population is a mixture of farmed birds with wild individuals and mixed hybrids, whose impacts in disease dynamics should be further investigated (Champagnon et al., 2009).

A study based on amplified fragment length polymorphism (AFLP) analysis has revealed that captive-reared birds tend to have lower genetic diversity as compared to their wild counterparts (De Marco et al., 2014) This may also have an impact when it comes to an influenza virus becoming successful in infecting new hosts. If the virus accomplishes adaptation to a new population, the more genetically homogeneous a flock is the lesser restraints it will have to infecting a high number of hosts with genetic similarity.

The role of wild birds in the spread of highly pathogenic variants

After the first report of a HPAIV outbreak in 1959 by a H5 subtype virus, others (including H7) have continuously followed, affecting mainly poultry. Most of these outbreaks have shown very limited spread and self-limitation, sometimes to a single flock of birds (Alexander, 2000). However, during the 80's and 90's several outbreaks of the disease became widespread in the US, Mexico and Pakistan causing serious damages to the poultry industry (Alexander, 2000). One of these cases was the HP H5N1, which was considered an Asian phenomenon until 2005 after the first detection in China in 1996 but it became a cause for concern when it started to expand westward reaching Europe and Africa in 2006 (Alexander, 2000).

Prior to introduction into Europe, H5N1 extensively circulated in Asian poultry with recurrent spillovers to wild birds and humans. Ever since, more than 60 countries (24 European) have been affected and HP H5N1 circulation still continues in the present

days (Adlhoch et al., 2014). The outbreaks in 2006 triggered intensive AIV surveillance programs that also included wild birds. Sampling efforts conducted during 2006 in different wild bird species in Europe are summarised in Table 4.

Table 4. Number of birds sampled for different bird orders during 2006.

Order	Number sampled in 2006	Number sampled February to May	Number (proportion) positive H5N1 HPAI	Number (proportion) positive LPAI
Anseriformes	64 487	30 481	535 (1·8)	1428 (2·2)
Charadriiformes	12 527	5581	5 (0·1)	99 (0·8)
Passeriformes	8961	6278	1 (0·02)	8 (0·1)
Falconiformes	6845	6203	18 (0·3)	8 (0·1)
Ciconiiformes	4550	3825	5 (0·1)	6 (0·1)
Columbiformes	4043	2684	0	6 (0·2)
Gruiformes	3714	2759	2 (0·1)	15 (0·4)
Galliformes	3099	1143	0	0
Pelecaniformes	1504	1055	2 (0·2)	3 (0·2)
Strigiformes	1023	924	4 (0·4)	4 (0·4)
Podicipediformes	310	248	4 (1·6)	3 (1)
Phoenicopteriformes	308	15	0	
Piciformes	72	54	0	0
Procellariiformes	44	37	0	0
Coraciiformes	40	22	0	0
Apodiformes	28	10	0	0
Gaviiformes	23	12	0	0
Threskiornithidae	18	18	0	0
Cuculiformes	12	3	0	0
Psittaciformes	6	2	0	0
Accipitriformes	4	3	0	0
Caprimulgiformes	3	3	0	0
Unknown	9085	5312	15 (0·3)	35 (0·4)
Total	120 706	66 672	591	1615

Values between parentheses express percentage of AIV-positives. HPAI: highly pathogenic avian influenza virus; LPAIV: low pathogenic avian influenza virus (Hesterberg et al., 2009).

The spread of HP H5N1 from Asia to Europe may have occurred as a result of inadequate poultry-production activities or illegal transport of poultry (Adlhoch et al., 2014). However, the same authors sustain that spread in Europe occurred because of cold weather driving substantial numbers of infected birds to migrate further west.

Experimental infections with HP H5N1 carried out in 6 different waterfowl species revealed that mallards were not clinically or pathologically affected although they had

abundant viral shedding (mainly pharyngeal) (Keawcharoen et al., 2008). In that experiment they concluded that mallards could act as effective long-distance vectors of HP H5N1 whereas pochards and tufted ducks (genus *Aythya*) which had developed severe neurological disease (also seen in field observations) were more likely to act as sentinels in the wild.

More recently a HP H5N8 stroke Europe. This was a reassortant virus carrying genes of HP H5N1. In 2010 an ancestral H5N8 strain was notified from China but it was not until 2014 that it reached European territories. In the end of that year Europe had its first detections in a healthy *A. crecca* in Germany and in *A. penelope* in the Netherlands (Adlhoch et al., 2014). In total, nine cases and 4 countries affected were reported. At that time, infection with H5N8 seemed to be mild in wild mallards but to cause severe illness with mass mortalities in the galliforms.

In late 2016, the Food and Agricultural Organization of the United Nations (FAO) released a warning about the spread of HP H5N8 in September after being detected in waterbirds in Russia. Outbreaks in Europe began to be detected in late October. In November 2016 there had been at least 56 outbreaks of HP H5N8 in eight countries in Europe and two in the Middle East (Figure 7). Hundreds of thousands of birds died as a direct result of the virus or through the culling of birds in an attempt to stop the spread of the virus. Both wild birds and poultry were affected including waterfowl, gulls, chickens and turkeys.

Although import of poultry and live captive birds from Asia in 2014 outbreaks should not be excluded, it is unlikely due to the simultaneous nature and geographic spread of the cases. Spread by migratory birds was considered, although at that time outbreaks in wild Asian birds should have been found (Adlhoch et al., 2014).

It is suspected that wild migratory birds could be responsible for the spread of the HP H5N8 strain across distant places. However, this variant affects severely wild birds too, which may impede long distance movements of the birds affected.

Effects of host ecology and environment on AIV epidemiology

A good understanding of host-pathogen interactions is fundamental for wildlife infectious diseases management (Gaidet et al., 2012a).

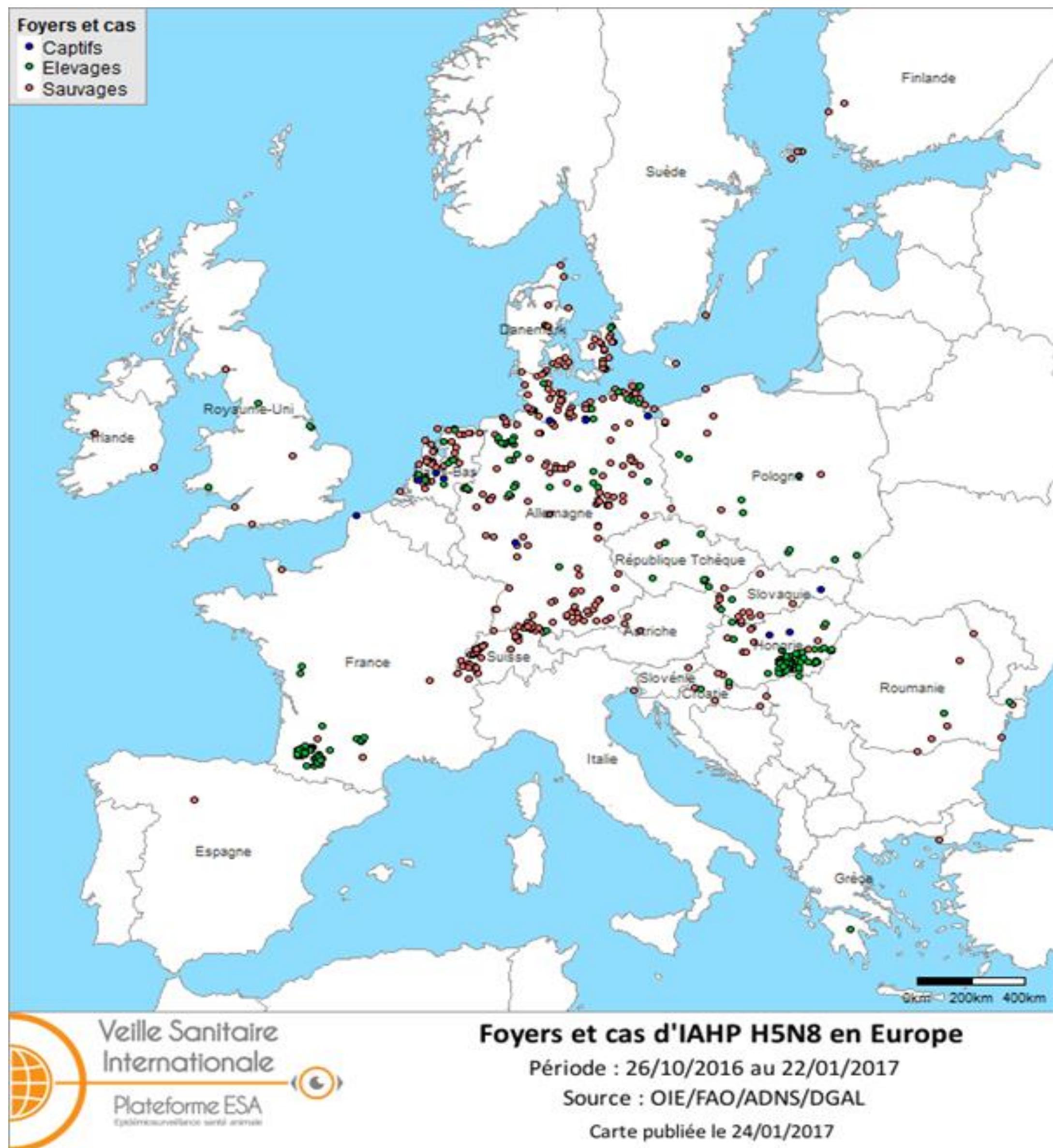

Figure 7. Latest highly pathogenic avian influenza virus outbreak (H5N8) affecting Europe between 26/10/2016 until 22/01/2017.

Coloured dots represent captive birds (blue), poultry production facilities (green) and wild birds (red) respectively.

Hence, as important as studying AIVs from a molecular/virological perspective, it is studying the ecology of their hosts and the environments which these inhabit.

Seasonality; temporal and spatial variation of AIV with host ecology

Seasonality is defined as periodic environmental changes that rule many organisms' life-cycle (Altizer et al., 2006). These changes will determine time for reproduction, migration or other behavioural changes. Since seasonality drives AIV host behaviour, and these behaviours vary depending on host species, factors such as geographical location and time of the year may affect prevalence (Hoye et al., 2010). Furthermore, these differences may fluctuate within a year or interannually (Brown and Stallknecht, 2008).

Related to these factors, one trait that has been linked to AIV prevalence in the wild is bird migration. Migration is a common feature in many AIV avian host species. It ranges from short local movements to intercontinental migrations (Olson et al., 2014) (Figure 8). As a general rule, breeding areas are located in high latitudes where there is less competition for quality food resources, less predators and parasites (Altizer et al., 2011). These grounds are visited during the spring and summer months when weather conditions are mild. When summer is about to finish, breeders and their fully-grown offspring begin their journey southwards to escape from harsh conditions of the winter (*i.e.* scarcity of food caused by frosts) (Altizer et al., 2011). Many waterbirds are long-distance migrants and they congregate in large multispecies flocks during the course of migration and at the staging grounds while migrating.

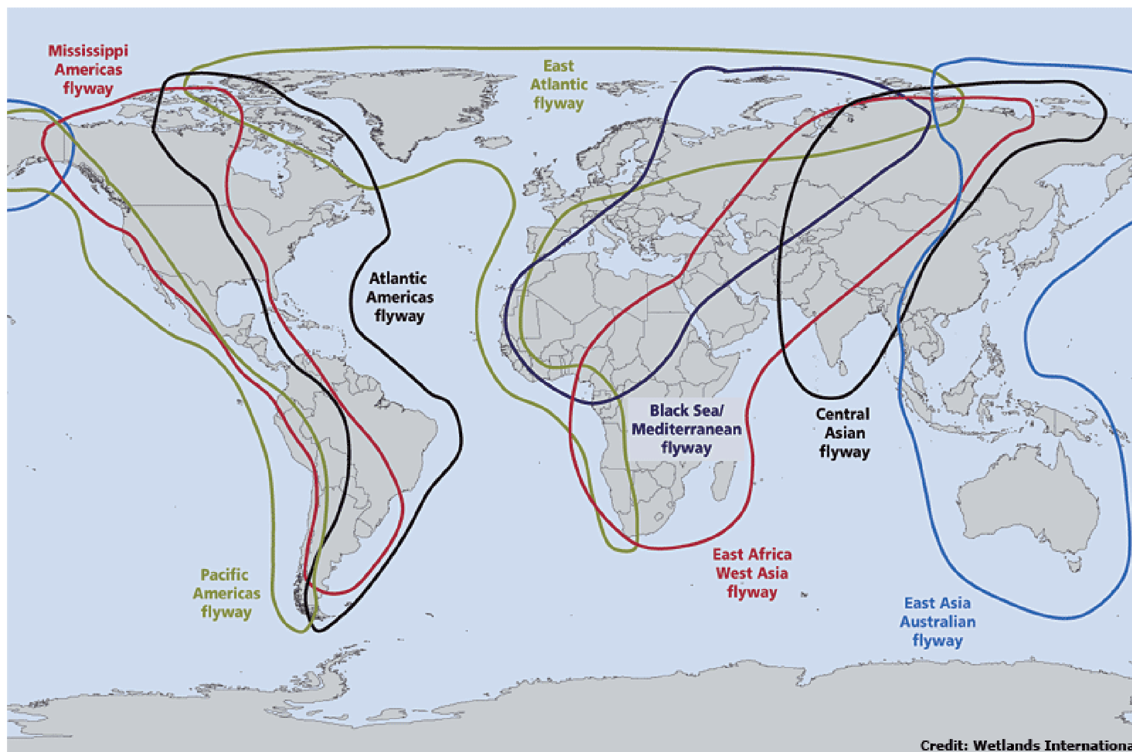

Figure 8. Main global migratory routes used by wild birds ("Wetlands international," n.d.)

This way, AIV can be transmitted between individuals from the same or different species depending on the susceptibility of the host community and the circulating strains' characteristics (Kleijn et al., 2010; Verhagen et al., 2015). Unfortunately, patterns of bird migration tend to be rather complex and variable between species, and they can even be different for various subpopulations within the same species (Bengtsson et al., 2016; Reed and Medical, 2003). Under natural circumstances, bird migration has natural fluctuations in the route and hence the areas migrating birds tend to visit. Some factors such as food abundance or cold temperatures may cause in some species nomadic wandering into new areas beyond their normal range, a trait named vagrant migration. This variation in the ordinary migration route may change the ordinary patterns of AIV epidemiology (Reed and Medical, 2003; Reperant et al., 2010). Furthermore, bird migration is known to cause lowered fitness in part due to physiological stress, which may cause immunosuppression and therefore reactivate latent infections or increased susceptibility towards infectious diseases (Bengtsson et al., 2016; Reed and Medical, 2003).

Mallard migration trends permit influenza viruses to be transmitted between different mallard subpopulations (Olsen et al., 2006; Verhagen and Fouchier, 2011). A feature to take into account is that populations from higher latitudes have been shown to be more prone to migrate rather than the ones inhabiting lower latitudes very likely in an attempt to procure mild environmental conditions and food supplies as previously mentioned (Bengtsson et al., 2016).

AIV epidemiological trends

In the Northern Hemisphere AIV prevalence peak is in late summer/early autumn within the Anseriformes populations in association with premigration staging and high concentrations of juveniles (with up to 30% of infection rates within this age group), presumably due to their naïve immune system (Brown and Stallknecht, 2008; Olsen et al., 2006). Some authors noted that once in wintering areas, prevalence rapidly decreases, probably as a consequence of acquired herd immunity and loss of infected individuals during migration "migratory culling" (Latorre-Margalef et al., 2014). However, other researches sustain that prevalence during the wintering season could be as high as 60% and decline during spring migration (Kleijn et al., 2010). Wintering individuals have also been shown to be able to amplify locally circulating AIVs (Verhagen et al., 2014b). As regards to the breeding season, the influx of susceptible hosts is believed to consist of local recruitment and not migrants (Latorre-Margalef et al., 2014).

An explaination for this temporal variation may be mediated to a big extent by host population immunity (Latorre-Margalef et al., 2014). Heterosubtypic immunity could provide partial protection against other strains (cross-protective immunity) which is believed to last for a month approximately (Latorre-Margalef et al., 2014). Thus, temporal succession in the arrival of mallard subpopulations from different breeding areas to premigratory staging grounds could likely bring different viral richness. These bird stopovers during migration could act as a "cocktail shaker" of both viral and host diversity favouring intra- and interspecies host transmission with AIVs (Latorre-Margalef et al., 2014).

Mixed AIV infections

In AIV epidemiology, mixed infections with more than one influenza virus subtype concomitantly seldom occur, creating an opportunity for genetic mixing by reassortment (Lindsay et al., 2013; Olsen et al., 2006; Sharp et al., 1997).

A study conducted in the late 70's in waterfowl, found that the better adapted an AIV subtype is to its avian host population the greater its ability to prevent mixed infections with other subtypes (Sharp et al., 1997). Thus, poorly adapted subtypes tend to participate significantly in mixed infections. This suggests that some subtypes may be well adapted to a particular avian host but not to others or that certain species of ducks have an increased exposure to them. A given subtype may infect more than one species so there may be species-specific levels of adaptation. In this same study, mallards (*A. platyrhynchos*) and pintails (*A. acuta*) were significantly more likely to be infected by a single subtype than other species of ducks combined but no species was more likely to be infected by more than one AIV subtype (Sharp et al., 1997). Furthermore, single infections within these bird species were 2.5 times higher in juveniles than in adults but only 1.6 times higher as for infections with more than one AIV subtype suggesting a minor role of the immune system during mixed infections. The results also revealed that all subtypes are not maintained by all avian species and that reassortment did not occur randomly in natural populations (Sharp et al., 1997).

On the other hand, a transient, low-level humoral response would allow protection against reinfection (Olsen et al., 2006). Indeed, a study revealed that in free-living breeding mallards, half of the eggs that were collected from different clutches, had maternal antibodies (IgY) however those detections were known to decrease to minimum levels 14 days after chick hatching. It evidences thus, hatchlings' protection against pathogens during their first days of life (Van Dijk et al., 2014b)

Effect of environmental conditions on AIV dynamics

The environment may also act as a selective force in AIV epidemiology; especially it is expected to have an impact in viral fitness between and within the different AIV subtypes.

In the wild, the environmental conditions modulating AIV dynamics are very complex and so they are the interacting factors that may influence detection and perpetuation of these viral particles. Because the environment provides a place for infection in different species that share the same habitats but not necessarily at the same time, the true role of it in AIV epidemics or in sustaining viral tenacity should be further investigated (Stallknecht and Brown, 2009).

A microbe-host dyad is in a delicate balance, as it can easily get disturbed (Mills et al., 2010; Preston and Johnson, 2010). Economic development and inadequate land use policies are leading to unprecedented perturbations with a negative impact in the natural microbial ecology (Crowl et al., 2008). International travel and commerce, together with the exponential trend of human demographic expansion are contributing to an imbalance that favours the emergence of new infectious diseases (Preston and Johnson, 2010).

Human practices are causing habitat fragmentation due to continuous modifications in the natural ecosystems (Mills et al., 2010). In relation to migration, habitat loss is promoting the migrating animals to overcrowd at stopover sites, favouring interspecies transmission of pathogens (Reed and Medical, 2003). Likewise human residue landfills have created artificial environments with aggregations of high numbers of birds of different species due to the constant abundant availability of food. In this regard, gaining knowledge in the role of infectious agents in community ecology is paramount to shed light on disease emergence in wildlife and associated impacts on public health.

Climate change

Wetlands are considered one of the most threatened habitats under the effects of climate change. Ducks, which inhabit them, may also suffer the consequences of such changes but little is known about it.

Climate change comprises a number of alterations in some environmental parameters such as a gradual increase in mean temperature, more precipitation at high latitudes and increased drought areas around the Mediterranean, but also sea-level rise

creating new shallow coastal habitats or changes in wind regimes that could affect the timing of avian migration (Guillemain et al., 2013).

Increasing temperatures may contribute to early spring migration as a result of an improvement of foraging ground conditions. In this regard, positive North Atlantic Oscillation (NAO) index values, characterised by mild and wet winters have been linked to early spring migrants arrival in northern Europe (Guillemain et al., 2013).

However, flexible breeders such as *A. platyrhynchos* and *A. americana* do not seem to be affected by fluctuating environments caused by the NAO index. Nevertheless, foraging habitat loss due to a decrease in wetland extension is deemed to cause a negative impact in the breeding success of mallards over time (Guillemain et al., 2013).

AIV and abiotic factors

Ultra violet (UV) radiation damages the structure of nucleic acids and therefore acts as a biocide. However, no effect has been observed when UV has been irradiated into faecal samples suggesting that UV light does not penetrate the faecal matter (Stallknecht and Brown, 2009).

Detection of AIVs has been linked to the presence of freshwater bodies, especially to wetland environments. For efficient faecal-oral transmission, as it occurs with their main reservoirs (dabbling ducks), it has been proposed that AIV remains floating attached to faecal matter, sediment surface or biofilms (Lacroix-Gueu et al., 2005; Stallknecht and Brown, 2009).

In a study carried out by Brown and colleagues (2007), they tested AIV persistence in water and salinity of wild type strains (low pathogenic) and two different HP H5N1 subtypes. They noted that HP strains had a shorter persistence in remaining infective in the environment than the wild type AIVs in conditions of low salinity. Nevertheless H5 and H7 wild subtypes persisted for long periods in water. They concluded that persistence was highly variable within same virus subtype and between HP H5N1 strains. Salinity and temperature were inversely proportional to H5 and H7 persistence.

Beyond the factors involved in AIV tenacity, ice should have special attention since it may represent a long-term environmental reservoir or a crucial factor for strain re-emergence (Fuller et al., 2010; Stallknecht and Brown, 2009). Besides, it has been documented that influenza viruses remain infectious in lake water up to 4 days at 22ºC and over 30 days at 0ºC (Olsen et al., 2006).

Diagnostic techniques

Nowadays there is a vast number of investigations to develop new techniques for viral detection that not only can be used for human health purposes but which are also widely used in veterinary research. These techniques are focused mainly in viral RNA extraction, isolation and pathotyping as well as in phylogenetic studies among the different AIV strains detected.

Since the clinical forms are highly variable depending on the species affected and the infecting subtype and strain, they can range from asymptomatic (especially in wild reservoirs) to highly morbid with up to 100% mortality (*i.e.* HP H5N1). Hence, despite the complexity of having an accurate diagnosis there is a need for effective laboratory-techniques to allow viral detection.

Because these techniques can be very diverse, numerous authors have pointed out the need of becoming more homogeneous and standardised so as to be comparable among different studies worldwide.

AIV isolation and propagation in specific pathogen free (SPF) chicken eggs

The isolation of AIV is usually performed by inoculating either faeces or tissue samples from infected animals into the chorioallantoic sac of 9-11 days of incubation SPF embryonating chicken eggs where the virus will be propagated (OIE, 2015). However, other inoculation routes have also been used for isolation (*i.e.* the yolk or the amniotic). Chorioallantoic fluid is harvested when the embryo dies or after 7 days post-inoculation and tested for the presence of the hemagglutinating antigen or confirmed directly by a rRT-PCR, an agar gel immunodiffusion (AGID) assay, or a commercial

immunoassay kit specific for type A influenza detection (Woolcock, 2008).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Conventional RT-PCR and after the 2000s, Real-Time RT-PCR (rRT-PCR) have been widely used for AIV detection during routine surveillance, disease outbreaks, and for research purposes. Their main advantages are high sensitivity and specificity, as well as time and cost effectiveness. Moreover, many types of samples can be used for analysis once the virus has been inactivated. As a result, it is a biosafe technique although the viability of the virus cannot be determined (Spackman and Suarez, 2008a)

rRT-PCR, aims at identifying conserved sequences of some AIV genes, such as the M or NP1 genes for a preliminary detection. Semiquantitative analyses such as the one provided by the threshold cycle (C_T), indicates that the target gene amplification has achieved. Low C_T values indicate high number of virus particles in the sample and thus, high values small numbers of virus genome copies (Munster et al., 2009)

Once an AIV-positive is confirmed by these means, another rRT-PCR should be applied in order to identify the circulating subtype; thus, targeting the genetically diverse HA and NA. Because sampling avian specimens for subtype identification is still laborious, the amplification of genes targeting potentially highly pathogenic H5 and H7 subtypes beforehand is a priority.

Serodiagnostic tests

On the other hand, if the factor to be monitored is the exposure of an animal to the virus, then indirect techniques can also be applied (Spackman and Suarez, 2008a)

Antibodies to several viral proteins (HA, NA, NP, M) are produced (during the course of infection with an influenza virus). These antibodies can be detected and cuantified using different techiniques. Widely used serodiagnostic tests are the Hemagglutination Inhibition test (HI) and the Enzime Linked Immunosorbent Assay (ELISA) (OIE, 2015). Since the host population has often antibodies against influenza already a fourfold or greater increase in titre is necessary to indicate recent influenza infection.

Chapter 1. Long-term avian influenza virus epidemiology in a small Spanish wetland ecosystem is driven by the breeding Anseriformes community

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Torrontegui O, Alvarez V, Acevedo P, Gerrikagoitia X, Höfle U, Barral M. Plos One 2017 (In evaluation)

Abstract

During 2007-2009 and 2012-2014, the detection of Avian Influenza Virus (AIV) in fresh faecal samples by non-invasive sampling methods and host identification by *COI* barcoding were studied in a wild avian community of a northern Spanish wetland. Data analysis provided 4.5-year useful information to clarify several aspects within the ecology of AIVs. Global prevalence significantly decreased during the second sampling period (0.3%) as compared to the first one (6.6%). Circulating subtype detections were also distinct between and within periods, with a noteworthy H5 and H7 subtype variety during the first sampling period. *Anas platyrhynchos* was the main host identified in AIV detections although the host from all positive samples could not be ascertained. We modelled AIV prevalence with regard to avian host community ecology and meteorological data from the wetland. Statistical analysis revealed seasonal differences in AIV detection, with a highest prevalence during the breeding season as compared to the rest of the hosts' life-cycle events. The model also showed that the lower AIV prevalence during the second study period was associated with a significant reduction of Anseriformes breeding in the wetland, thus revealing a long-term fluctuation of AIV prevalence, and potentially also subtype diversity, driven by the breeding Anseriformes community.

Introduction

Avian Influenza Viruses (AIV) (family *Orthomyxoviridae*, genus *Influenzavirus A*) are antigenically very diverse. The antigenic properties of two surface glycoproteins, the 16 known haemagglutinin variants (HA) and 9 neuraminidases (NA) act as effective selective forces on these viruses' evolution, leading to at least 144 potential HA/NA subtype combinations. In addition, the segmented fashion of their genome can lead to reassortment by generating new strains during mixed infections (Sharp et al., 1997). Since the affected hosts have little acquired protection against a new reassortant virus, genetic reassortment evades host immunity and enables virulent AIV subtypes to trigger epizootic or panzootic events (Schrauwen and Fouchier, 2014). Although most AIV subtypes are low pathogenic (LPAIV) to wild birds and poultry, H5 and H7 subtypes can become highly pathogenic (HPAIV) after infecting domestic gallinaceous birds (Munster et al., 2005; Pérez-Ramírez et al., 2012). Therefore, also LPAIVs need to be under stringent surveillance as they pose a serious risk to both animal and public health.

The avian orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, shorebirds) are considered the natural reservoirs of AIV (Webster et al., 1992). These species harbour all known subtypes and infections often occur in absence of clinical signs (Bengtsson et al., 2016; Kuiken, 2013; Olson et al., 2014; Webster et al., 2007). The presence of Anseriformes particularly, is considered essential for AIV transmission and environmental persistence (Pérez-Ramírez et al., 2012). However, among all the Anseriformes representatives, the role of the mallard duck (*Anas platyrhynchos*) in AIV epidemiology in natural settings has to be highlighted (Daoust et al., 2011; Gunnarsson et al., 2012). Mallards harbour the highest AIV subtype diversity, prevalence is usually high and most viral isolations are recovered from this taxon (Olson et al., 2014; Tolf et al., 2012). LPAIV replication is more common in the digestive tract of wild bird hosts in which the transmission route is faecal-oral in aquatic environments. However, in some avian host species and with specific AIV subtypes, respiratory shedding can occur (Gaidet et al., 2012a; Kleijn et al., 2010; Van Dijk et al., 2014a).

AIV prevalence in wild ecosystems is very dynamic and dependent on a wide variety of factors *i.e.* time of the year, location, circulating subtype and infected host species (Latorre-Margalef et al., 2014; Pérez-Ramírez et al., 2012). For this reason, long-term studies are of special interest when conducting AIV monitoring in aquatic ecosystems. Yet, the existing studies are scarce and very heterogeneous (Latorre-Margalef et al., 2014); while some tended to aim at a single avian order (or even taxon), others were conducted during certain periods of the year only or focused on different sampling locations sometimes with low representative samplings conducted (Olson et al., 2014). Consequently, comparable epidemiological results from natural environments are difficult to obtain.

The aim of this longitudinal study was to evaluate AIV dynamics in a natural wetland ecosystem taking into account virological aspects and hosts' ecological traits during two different sampling periods. Because we considered important not to alter the avian community's actual distribution, our sampling strategy was based on non-invasive sampling methods (collection of environmental fresh faecal samples and further host identification by *COI* barcoding). This way all roosting water bird species were regarded as potential hosts and interspecies natural mixing was taken into account. Special attention was paid to the circulating viral strains, to the host species harbouring AIVs as well as to ecological factors with a potential effect on viral detection.

Material and Methods

Ethics statement

All procedures were conducted according to the Spanish (RD 53/2013) and the European legislation (Directive 2010/63/EU) for the protection of animals used for scientific purposes.

Study area

Salburua (42°51'N 002°39'W; altitude 500-510 m) is a 217.46 *Ha* wetland area located in Vitoria-Gasteiz, Basque Country (Spain) (Annex I). From an animal health

classification of the Spanish biodiversity, this water ecosystem pertains to the "Northern-Plateau" bioregion (Ministerio de Agricultura Alimentación y Medio Ambiente, 2013; Muñoz et al., 2010). Under the influence of the Atlantic climate, there is a noteworthy thermal oscillation along the year with dry summers. Annual mean temperature is 11.4º C (5.1º C in winter and 17.9º C in summer) and the annual mean precipitation is 823.4 mm. Number of ground frost days is moderate (40.8 days/year)) ("Ficha Informativa de los Humedales de Ramsar," 2006).

Salburua wetland is composed of various lagoons surrounded by meadows and a small oak grove. It received the *Wetland of International Importance* Ramsar designation in 2002 and *Site of Community Importance* in 2004 within the European Natura 2000 Network ("Natura 2000," 2003, "The List of Wetlands of International Importance," n.d.). From an ornithological perspective, the wetland is strategically situated, as many bird species use these lagoons for wintering, breeding or for stopping over along the East Atlantic flyway while migrating. On the other hand, since 2005, when monitoring plans for AIV began in the Basque Country, this wetland has had a frequent LPAIV record and it is where the only known case of HPAIV H5N1 in the Iberian Peninsula has been recorded so far in a wild bird (Barral et al., 2008; Pérez-Ramírez et al., 2012).

Sample collection

Fresh avian faeces were collected at dawn at roosting sites of waterbirds (scattered islets in the wetland area where diverse waterbird species tend to aggregate for resting). Samples were kept refrigerated until analysis within the next 24 hours.

This study was designed with the goal of better understanding AIV ecology and dynamics and it was composed of two sampling periods. Hence, results from a previous research that took place in the same study area were also used for data analysis and they were also submitted to complementary analysis (virus subtyping and host identification). In this first period, samplings were performed once every three months during 2007-2009, with a total of 667 samples analysed during 8 sampling visits, from which 44 were AIV-positive (6.6%) (for details see (Pérez-Ramírez et al., 2012)). As for the second sampling period, 2725 samples were collected monthly from March 2012 until September 2014 consisting of 31 sampling visits.

AIV detection

Four to five individual faecal samples were pooled according to the appearance and location where they were found. Viral RNA extraction was performed with a commercial kit (RNeasy Mini Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. Samples were screened with a TaqMan™ (ThermoFisher Scientific Inc.) real time reverse transcription polymerase chain reaction (rRT-PCR) for AIV matrix-gene detection using a pair of primers previously described (Spackman et al., 2002). Amplification for 40 cycles was carried out using AgPath-ID™ One-Step RT-PCR Reagents (ThermoFisher Scientific Inc.).

At detection of an AIV-positive pool, another extraction from the pool-composing units was performed individually in an attempt to identify the positive sample by AIV matrix gene rRT-PCR. In addition, a H5- and H7- AIV subtype-specific rRT-PCR was performed (Aguero et al., 2007; Monne et al., 2008; Spackman et al., 2002).

The same viral genomic detection procedure and reagents were used for both sampling periods.

AIV isolation

In AIV-positive samples, approximately 25mg of the original faecal samples were homogenised with 500µl of Hank's Balanced Salt Solution (ThermoFisher Scientific Inc.) supplemented with penicillin (2000u/ml) and streptomycin (2mg/ml) (ThermoFisher Scientific Inc.) and sodium bicarbonate 7.5% (ThermoFisher Scientific Inc.), final pH 7.0-7.4. The mixture was inoculated into the allantoic cavity of five embryonated specific pathogen free (SPF) eggs after 9-11 days of incubation (OIE, 2015). Post-inoculation embryo survival was observed by egg candling every 24h. Once the embryo died or 7 days post-inoculation if the embryo was still alive, the allantoic fluid was harvested. Viral RNA extraction was carried out by incubating at 58ºC for 1-3h stirring gently, 180µl of the allantoic fluid, 2.7µl carrier RNA (1µg/µl) and 20µl Proteinase K (20mg/ml) followed by extraction in a Biosprint 96 robot (Qiagen, Hilden, Germany) with a Biosprint 96 DNA Blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Samples were again analysed for the presence of AIV matrix gene by rRT-

PCR (Spackman et al., 2002). In cases where no AIV was isolated, the harvested allantoic fluid was reinoculated into a new set of SPF eggs and the isolation procedure was carried out as described above.

AIV subtype identification and pathogenicity

Haemagglutinin (HA) and neuraminidase (NA) identification were determined either by conventional RT-PCR, rRT-PCR or sequencing (Elizalde et al., 2014; Fereidouni et al., 2009b; Hoffmann et al., 2016; Tsukamoto et al., 2008). Pathogenicity of the H5- and H7-positive samples was also determined by the study of the cleavage site sequence (Hoffmann et al., 2007, 2001; Payungporn et al., 2006).

Host identification

DNA was extracted from AIV-positive faecal samples using MagMAX^{TM} Total Nucleic Acid Isolation Kit (ThermoFisher Scientific Inc.) following the manufacturer's instructions. PCR for the cytochrome c oxidase I (*COI*) gene was performed using a nested PCR method with the AWCF1 and AWCR6 pair of primers for the first round and the pair AWCintF4 and AWCintR6 for the second round (Lijtmaer et al., 2012). Alternatively, first round primers External F1 and External R1 (Cheung et al., 2009) were used combined with the former second round primers. The nested PCR amplicons were purified using IllustraTM ExoProStarTM1-Step (GE Healthcare Europe, Freiburg, Germany) following the manufacturer's instructions. The 277 bp amplified PCR fragment of the *COI* gene was sequenced in an AB3130 Genetic Analyzer (ThermoFisher Scientific Inc.) using BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific Inc.). The obtained sequences were compared with published ones at the network server of the National Centre for Biotechnology Information (NCBI) with BLAST® (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 5. Predictors related to the avian community inhabiting Salburua wetland used for building the model.

FACTOR	**PREDICTOR**	**DEFINITION**
Avian community	Census of wild birds	Total wild bird counts per census session (monthly)
	Species richness	Number of wild bird species
	Order	Counts of waterbird orders per census (Anseriformes, Charadriiformes, Gruiformes, Pelecaniformes, Podicipediformes)
	Species	Counts of waterbird species per census
Implication as AIV reservoir	Anseriformes and Charadriiformes	Counts of Anseriformes and Charadriiformes individuals
	Anatini	Counts of dabbling ducks
	Anatini richness	Number of dabbling duck species
	Aythyini	Counts of diving ducks
	Aythyini richness	Number of diving duck species
	non-Anseriformes and non-Charadriiformes	Counts of non-Anseriformes and non-Charadriiformes individuals
Niche (interspecies feeding associations)	Grazers	Counts of *Anas penelope*, *Anas crecca*, *Anser anser* and *Fulica atra*
	Divers	Counts of *Aythia ferina-Fulica atra* association
Host life-cycle associations	**Phenology**	Waterfowl life-cycle events: SM, BR, AM, W
	Breeding couples	Counts of breeding couples: *Anas clypeata, Anas platyrhynchos, Anas strepera, Anser anser, Aythya ferina, Aythia fuligula, Ardea cinerea, Ardea purpurea, Ciconia ciconia, Circus aeruginosus, Charadrius dubius, Fulica atra,* ***Gallinula chloropus****, Himantopus himantopus, Ixobrychus minutus, Larus michaellis, Nycticorax nycticorax,* ***Podiceps cristatus****, Rallus aquaticus* and ***Tachybaptus ruficollis***
	Breeding Anseriformes	Counts of breeding couples of Anseriformes members
	Breeding non-Anseriformes	Counts of all breeding couples excluding Anseriformes

FACTOR	PREDICTOR	DEFINITION
	Breeding non-Charadriiformes	Counts of all breeding couples excluding Charadriiformes
	Summer visitor species	Counts of bird species recorded during the summer months in consulted bibliography
	Summer visitor species richness	Number of bird species recorded during the summer months in the consulted bibliography
	Wintering birds	Counts of wintering birds
	Wintering species richness	Number of wintering species
	Migratory species	Counts of bird species recorded as migratory in the consulted bibliography
	Migratory species richness	Number of bird species bird species recorded as migratory in the consulted bibliography
	Resident species	Counts of resident bird species
	Resident species richness	Number of resident bird species
Meteorological data	Mean Temperature	Sampling day, 7 days before sampling and 15 days before sampling (Cº)
	Maximum Temperature	Sampling day, 7 days before sampling and 15 days before sampling (Cº)
	Minimum Temperature	Sampling day, 7 days before sampling and 15 days before sampling (Cº)
	Total Precipitation	Sampling day, 7 days before sampling and **15 days before sampling** (l/m^2)
	Mean Humidity	Sampling day, **7 days before sampling** and 15 days before sampling (%)
	Mean Wind	Sampling day, 7 days before sampling and **15 days before sampling** (Km/h)
	Maximum gust of wind	**Sampling day**, 7 days before sampling and 15 days before sampling (Km/h)

VIF<3 in bold. W: wintering season (November-January); SP: northward spring migration (February-April); BR: breeding (May-July) and AM: southward autumn migration (August-October) (Perez-Ramirez et al., 2010). Species distribution was considered according to (*Atlas de las aves en invierno en España 2007-2010*, 2012; Birding Euskadi/Eusko Jaurlaritza, n.d.; Gill and Donsker, 2017; Martí and Del Moral, 2003; SEO/BirdLife, 2008; Svensson et al., 2010), and Luis Lobo's personal communications

Longitudinal epidemiological analysis

Ecological data

Wetland authorities provided monthly data from bird species' abundance all over the wetland area. Data from waterbird counts (Table 5, Annex II) were grouped according to: a) **taxonomic order abundance**; b) **taxonomic implication as AIV reservoir** (a division was created within the Anseriformes taking into account feeding habits; Anatini (dabbling ducks) and Aythyini (diving ducks) subfamilies; c) **habitat-use for foraging** (niche) (grazers, gulls and divers); d) **breeding pair counts** from the most frequent species; and e) **host life-cycle related associations** such as migratory behaviour or breeding period were also registered. Daily meteorological parameters were obtained from the Basque Meteorological Agency (Euskalmet).

Statistical analysis

We used the number of samples positive for AIV detection in each sampling period (35 periods; 8 for 2007-2009 and 27 for 2012-2014) in relation to sample sizes as response variable. Generalized linear model (binomial distribution, logit link function) (Hosmer and Lemeshow, 1989) was used to assess the effects of ecological factors (namely, phenology, bird counts and climate; see below) explaining variations in AIV positivity in this longitudinal study. In addition to the aforementioned variables, predictors described in Table 5 were included as covariables. We avoided multicollinearity derived problems using variance inflation factor (VIF); covariables with VIF>3 were not considered for modelling (Zuur et al., 2010). VIFs were calculated for each variable as the inverse of the coefficient of non-determination of the regression of each predictor against all others by using the R package "HH" (Heiberger R.M, 2012). The variables selected after controlling the VIF were considered in the GLM. The final model was obtained using a forward-backward stepwise procedure based on the corrected Akaike Information Criteria to compare models (Akaike, 1974). Differences were considered significant when $P < 0.05$

Table 6. Percentage of AIV-positive detected and number of analysed samples at Salburua wetland for each year and bird phenological event.

Year	**SM**		**BR**		**AM**		**W**		**Total**	
	Prev (%) ± CI	Pos/N	Prev (%) ± CI	Pos/N	Prev (%) ± CI	Pos/N	Prev (%) ± CI	Pos/N	Prev (%) ± CI	Pos/N
2007	/	/	/	/	/	/	0.0	0/95	0.0	0/95
2008	0.0	0/154	31.4 ± 16.2	11/35	2.1 ± 4.3	1/47	0.0	0/136	3.2 ± 1.8	12/372
2009	1.0 ± 2.0	1/98	18.2 ± 27.2	2/11	31.9 ± 9.7	29/91	/	/	16.0 ± 5.1	32/200
Total 2007-09	**0.4 ± 0.8**	**1/252**	**28.3 ± 13.5**	**13/46**	**21.7 ± 7.0**	**30/138**	**0.0**	**0/231**	**6.6 ± 1.9**	**44/667**
2012	0.0	0/214	1.0 ± 1.5	2/192	0.0	0/102	0.0	0/203	0.3 ± 0.4	2/711
2013	0.0	0/305	0.0	0/106	0.9 ± 0.8	4/465	0.3 ± 0.5	1/384	0.4 ± 0.3	5/1260
2014	0.0	0/159	0.3 ± 0.6	1/341	0.0	0/129	0.0	0/125	0.1 ± 0.3	1/754
Total 2012-14	**0.0**	**0/678**	**0.5 ± 0.5**	**3/639**	**0.6 ± 0.5**	**4/696**	**0.1 ± 0.3**	**1/712**	**0.3 ± 0.2**	**8/2725**

(AM): Southward autumn migration; (BR): breeding; (SM) northward spring migration; (W): wintering season. (Prev): prevalence; (CI): 95% confidence interval; (N): number of samples; Pos/N: AIV-positives found for the number of samples analysed in each sampling set.

Results

AIV prevalence and subtype richness

A total of 2725 faecal samples were collected during 2012-2014. Global AIV prevalence was 0.3% (8/2725), which was significantly lower when compared to the 2007-2009 period 6.6% (44/667) (GLMz: Z=-8.04, p<0.001) (Table 6). Sampling effort and AIV prevalence detected at each sampling time are detailed in Figure 9. Viral recovery-rate from both sampling periods was 48% (25 virus isolations out of the 52 AIV positive records). AIV isolations were only achieved from samples taken during autumn migration (Table 7).

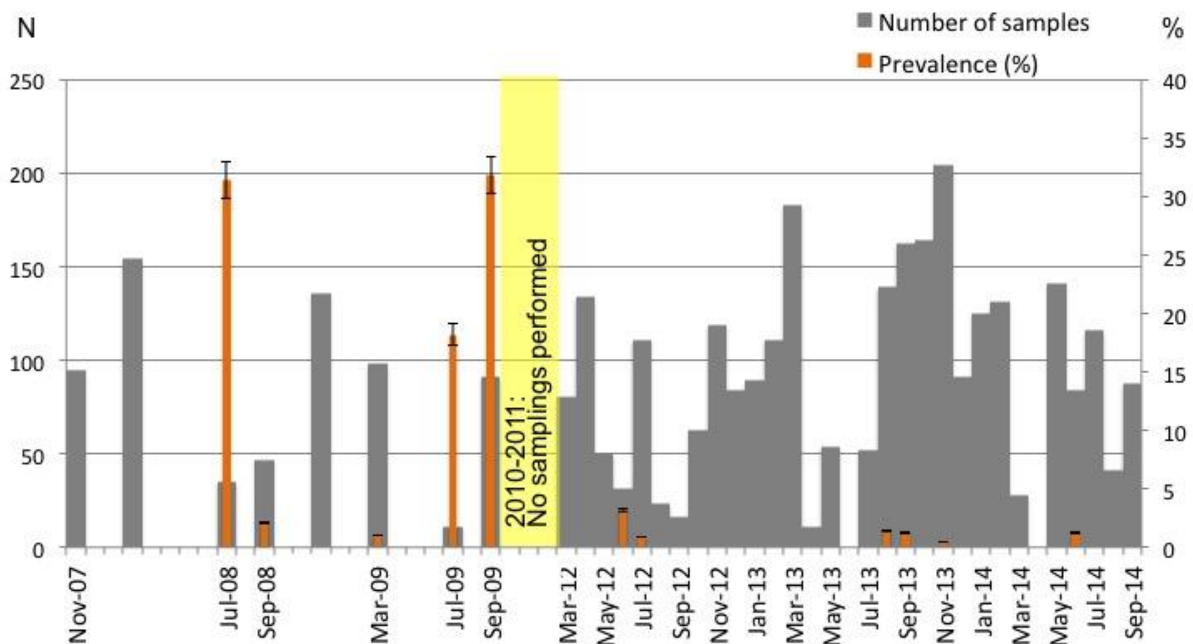

Figure 9. Sampling effort (grey bars) and AIV prevalence (orange bars) at each sampling time (between brackets).

The yellow shaded area indicates that during 2010-2011 no samplings were conducted.

Considering both sampling periods, 2007-2009 and 2012-2014, 11 different viral subtypes were identified (Table 7). H3N8 was the most frequent subtype (25% of all AIV-positive samples) followed by H11N9 (17%). A high diversity of circulating low pathogenic H5 (PQRETR*GLF) and H7 (PEIPKGR*GLF) strains was found (Table 7).

Table 7. AIV subtypes detected in aquatic bird faecal samples in Salburua wetland. Distribution according to sampling year, waterbird phenology, growth in embryonated SPF chicken eggs and identified host species.

Year	**Phenology**	**N**	**Isolation N**	**Subtype**	**Identified Host (N)**
2008	BR	11	8	H3N8	*Anas platyrhynchos* (7)+ND (4)
	AM	1	0	H5N2	*Anas platyrhynchos* (1)
2009	SM	1	0	ND	ND (1)
	BR	2	0	ND	ND (2)
	AM	1	1	H4N?	ND (1)
	AM	1	0	H6N5	ND (1)
	AM	2	2	H7N2	*Anas platyrhynchos* (1)+ND (1)
	AM	1	0	H7N8	*Anas platyrhynchos* (1)
	AM	1	1	H7N9	*Anas platyrhynchos* (1)
	AM	4	1	H7N?	*Anas platyrhynchos* (2)+ND (2)
	AM	4	3	H11N2	ND (4)
	AM	9	6	H11N9	*Anas platyrhynchos* (4)+ND (5)
	AM	3	0	H11N?	*Anas platyrhynchos* (2)+ND (1)
	AM	1	1	H7/H11 N4/N9[i]	ND (1)
	AM	2	0	ND	*Anas platyrhynchos* (1)+ND (1)
Total 2008-09		**44**	**23**		**20**
2012	BR	1	0	H3N8	*Anser anser* (1)
	BR	1	0	ND	ND[ii] (1)
2013	AM	1	1	H3N2	*Anas platyrhynchos* (1)
	AM	1	1	H3N8	ND (1)
	AM	1	0	H12N5	*Anas platyrhynchos* (1)
	AM	1	0	ND	ND (1)
	W	1	0	H5N?	*Anas platyrhynchos* (1)
2014	BR	1	0	ND	*Anser anser* (1)
Total 2012-14		**8**	**2**		**5**

AM: Southward autumn migration; BR: breeding; SM northward spring migration; W: wintering season. ND: not determined; i: a mixed infection from an isolate, it was not possible to elucidate what haemagglutinin type corresponded to its respective neuraminidase; ii: this sample pertains to a pool from which the positive unit was not possible to determine and from which 3 matched with *Fulica atra* and 1 to *Anser anser*.

Host identification was successful in 48% (25/52) of the AIV-positive samples (Table 7). All identified host species were anatids (*Anas platyrhynchos*: 44% (23/52); and *Anser anser*: 3.8% (2/52)).

Longitudinal study

After VIF analysis, the following variables were selected for modelling: migratory species richness, resident species richness, summer visitor species richness, number of: breeding moorhens *(Gallinula chloropus)*, breeding grebes *(Podiceps cristatus)*, breeding little grebes couples *(Tachybaptus ruficollis)*, breeding Anseriformes, breeding non-Anseriformes and breeding non-Charadriiformes (Table 5 and Figure 10).

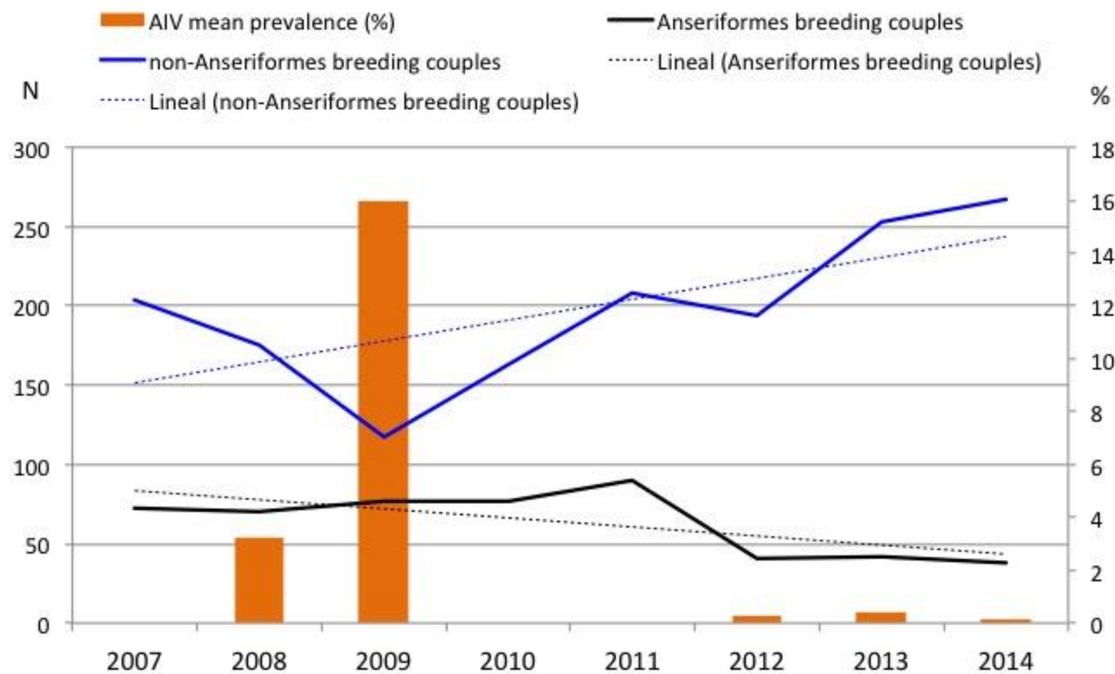

Figure 10. Mean AIV prevalence for each year and counts of Anseriformes and non-Anseriformes breeding pairs in Salburua wetland.

Mean humidity of sampling day, total precipitation of sampling day, daily maximum gust of wind, precipitation total 15 days before sampling (bs), mean wind 15 days bs and humidity 7 days bs, phenology and sampling year were also considered for building our model. The final model explains 95% of the deviance (Table 8). Results indicate a strong positive relation of AIV prevalence with the number of Anseriformes breeding couples (Table 8). There is also a

positive relation with wind during the 15 days before each sampling event, with resident species richness and with the breeding season.

Table 8. Variables retained in the final model for avian influenza virus prevalence in fresh faecal samples of waterbirds at Salburua wetland.

	Estimate	SE	z value	Pr(>|z|)
(Intercept)	-38.053	7.946	-4.789	***
Breeding Anseriformes couples	0.141	0.024	6.005	***
Phenology				
BR	2.701	0.998	2.707	**
SM	-6.930	1.331	-5.208	***
W	-3.715	1.384	-2.684	**
Resident species richness	1.117	0.319	3.502	***
Mean wind 15 days before sampling (Km/h)	0.989	0.282	3.504	***
	-0.179	0.088	-2.024	*

Coefficients (estimates and standard error), statistical test value (z value) and significance (***p<0.001, **p<0.01 and *p<0.05); (SE): standard error; coefficients for phenology are relative to southward autumn migration (August-October); (BR): breeding; (SM): northward spring migration; (W): wintering season.

In addition to these variables, Tukey test evidenced significant differences in AIV prevalence according to waterbird phenology; namely higher prevalence rates during the breeding season followed by the autumn migration and wintering, while AIV was less prevalent during spring migration (with no significant differences between wintering and spring migration periods) (Figure 11).

Discussion

Our study uses a small wetland ecosystem to explore the interaction between LPAIV and avian host ecology as well as the influence of the latter on long-term LPAIV prevalence fluctuations. We found seasonal patterns in LPAIV prevalence that matched results from previous studies in Spain and elsewhere (Latorre-Margalef et al., 2014; Munster et al., 2007; Pérez-Ramírez et al., 2012; Van Dijk et al., 2014a) (Figure 9 and Figure 12) and a strong longitudinal variation in LPAIV prevalence that was linked to the size and composition of the Anseriformes breeding community of the wetland (Figure 11).

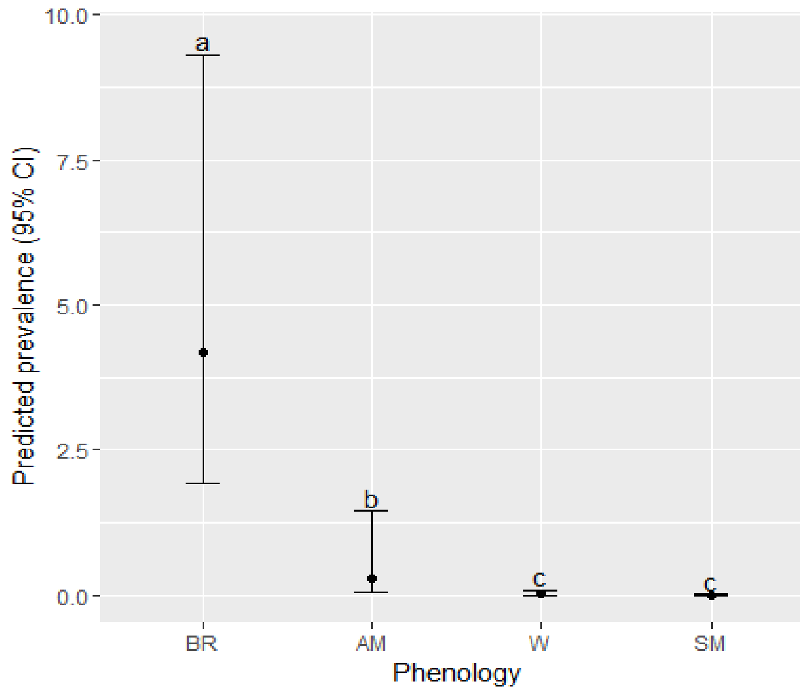

Figure 11. Predicted avian influenza virus prevalence at Salburua wetland in relation to waterbird phenology.

(BR: breeding; AM: southward autumn migration; W: wintering; SM: northward spring migration). Means sharing the same letter did not differ significantly (Tukey tests p>0.05)

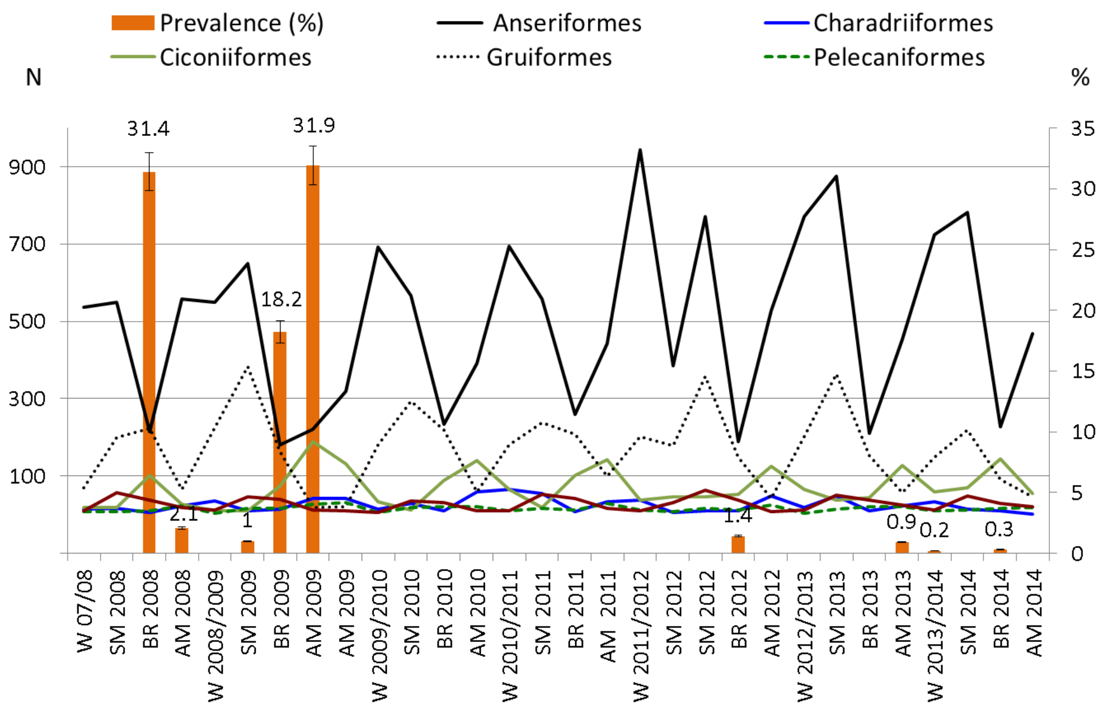

Figure 12. Avian community composition at Salburua wetland.

Mean taxonomic order counts and AIV prevalence according to host phenology. During 2010-2011 birds were counted but no samplings for AIV detection were performed.

Thus, our results prove that the waterfowl breeding community composition drives the long-term fluctuation of AIV prevalence in our study wetland. More precisely, these drivers are the number of Anseriformes breeding couples. Bird counts revealed a drastic decrease within this order during the course of our study, both within the member species altogether and as separate taxa (Figure 10 and 12). The breeding pair decline was most dramatic in the mallard (Figure 13).

Previous studies have pointed out the outstanding role of the Anseriformes within the AIV epidemiology where the mallard is a notorious representative in influenza ecology (De Marco et al., 2014; Munster et al., 2007). The mallard is the most abundant anatid in Western Paleartic (its natural distribution range) (Keawcharoen et al., 2008). This widespread distribution is conferred by its high adaptability towards a wide variety of habitats ranging from natural ecosystems to humanised environments (Keawcharoen et al., 2008).

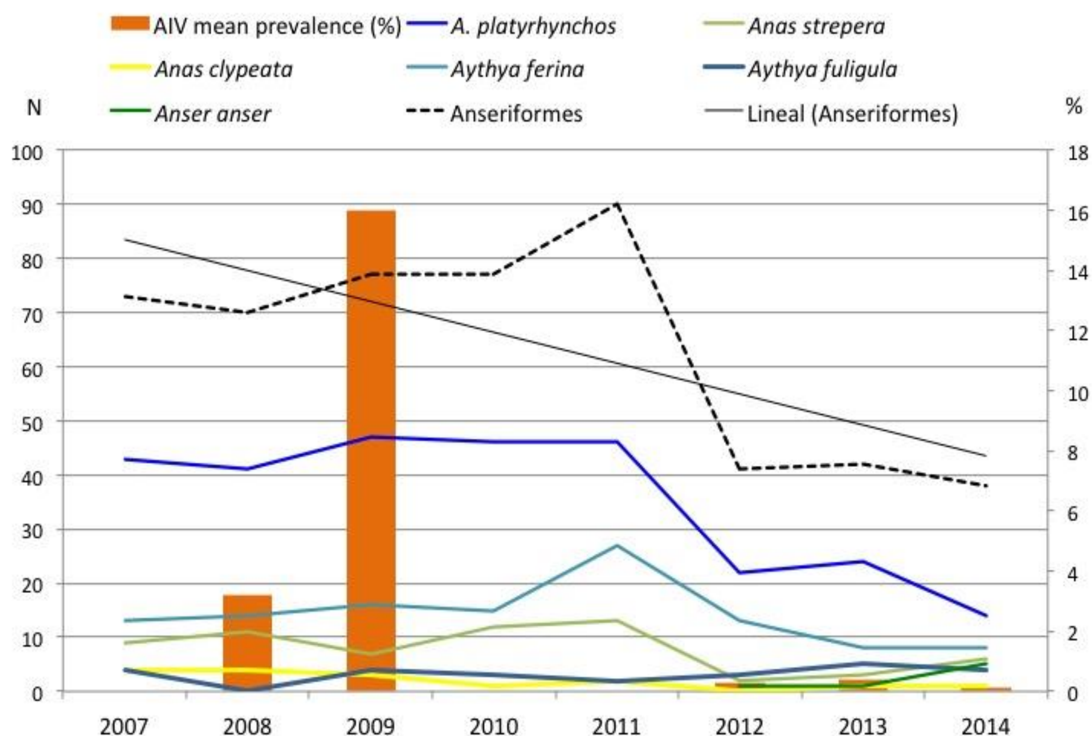

Figure 13. Mean AIV prevalence for each year and counts of Anseriformes breeding pair members in Salburua wetland, Spain.

During 2010 and 2011 breeding pairs were counted but no samples for AIV detection were taken.

Mallards are social birds and capable of moving across long distances during migration, especially subpopulations from high latitudes. However, this bird species is a partial migrant, meaning that a fraction of its wild population is migrant while another is sedentary (Figure 14) (Van Dijk, 2014). This trait may be involved in a different response towards an AIV infection influencing prevalence dynamics (Verhagen et al., 2014b).

Thus, a plausible scenario is that migrants could be bringing new AIVs into the wetland whereas the residents may drive the epizootic of these strains during the breeding season in a similar way to what has been observed during prevalence peaks occurring during the AM elsewhere (Verhagen et al., 2014b; Wallensten et al., 2007).

In fact, significant seasonal differences in AIV prevalence were consistently found in our longitudinal study with regard to host phenology.

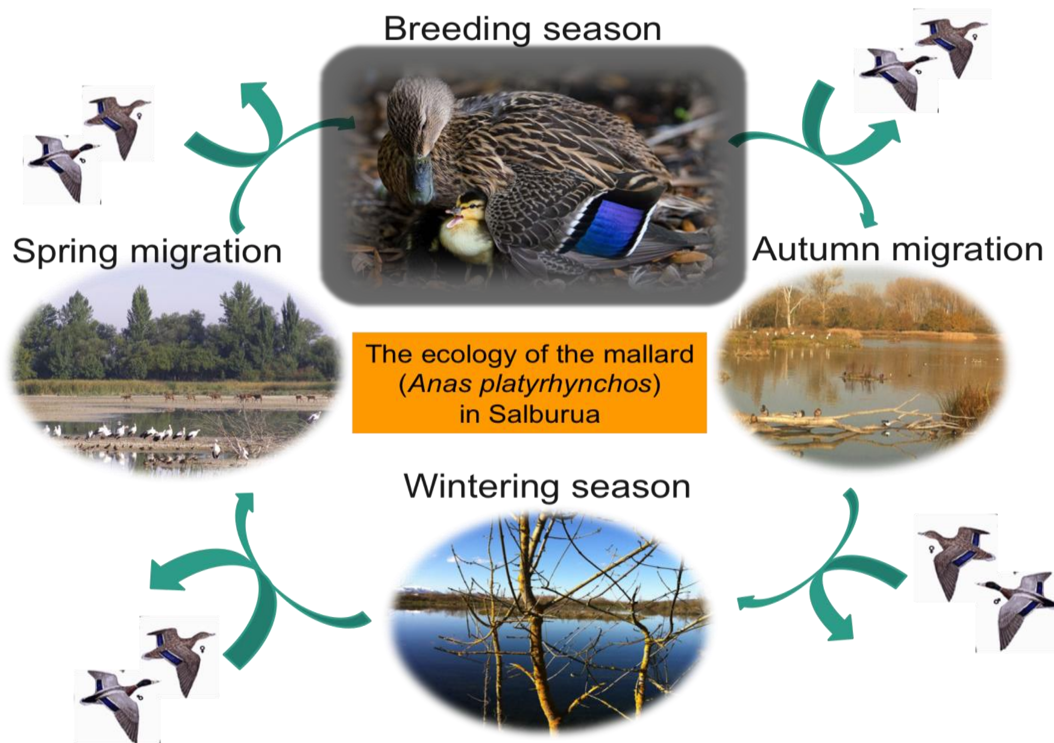

Figure 14. The ecology of the mallard in Salburua wetland.

This bird species is a partial migrant, which means that a fraction of its metapopulation is migratory whereas another fraction is sedentary. In Salburua numbers of migratory flocks join temporarily the local population during all phenological events (Wintering season, Spring migration, Breeding season and Autumn migration) (Personal elaboration).

This finding confirms the existence of seasonal patterns in the epidemiology of these viruses in wild ecosystems as previously stated (Latorre-Margalef et al., 2014; Munster et al., 2007;

Van Dijk et al., 2014a). AIV prevalence always peaked during the same periods in waterfowl phenology both inter- and intra-annually in Salburua wetland (Figure 9 and Table 6); namely during the breeding season (late boreal summer) and/or during the southward autumn migration (early boreal autumn). A temporal trend in AIV prevalence that has also previously been reported (Gunnarsson et al., 2012; Henriques et al., 2011; Munster et al., 2007). However, as our sampling strategy consisted of harvesting environmental fresh faecal samples alone, respiratory-tract affinity by potential circulating viral strains during the other periods should not be excluded (Fereidouni et al., 2010; Van Dijk et al., 2014a).

Autumn southward migration is an ordinary trait in most waterfowl sub-populations (some others are resident). During this period different-origin migrating birds congregate in large numbers within the same area joining the local avian community, which facilitates multiple-strain AIV mixing by host-to-host transmission (within and between species) (Latorre-Margalef et al., 2013) (Figure 4). Like in our study, other research groups did not find any relation between AIV detection rates and host density at species level, however they did when they compared it with the wildfowl community, which suggests aggregation of infection through interspecies mixing (Gaidet et al., 2012a).

The significant relation of 15 days bs wind with prevalence also detected by our model may be related to harsh environmental conditions that made the birds remain at the same place rather than scatter, also contributing to host aggregation and virus environmental enhancement.

AIV detections from wintering populations have been rare, suggesting that prevalence decreases as autumn migration is drawing to an end and more birds have already gained immunity against the circulating viruses (Latorre-Margalef et al., 2014). Heterosubtypic immunity may have been protecting the birds against infection from phylogenetically close strains, causing AIV infections to be both less frequent and less diverse during this season and the following spring northward migration (Latorre-Margalef et al., 2014). Nevertheless, some studies have reported high AIV detections during the wintering season such as in Guatemala, The Netherlands and Iran or during the spring migration in Sweden. AIV circulation during these periods in some geographical regions could also contribute to year-

round virus perpetuation (Fereidouni et al., 2010; González-Reiche et al., 2016; Kleijn et al., 2010; Wallensten et al., 2006).

Thus, integrating the ecology of both host and LPAIV, a likely scenario is that significantly higher AIV detection rates found during the BR and the AM are characterised by an input of hosts that are immunologically naïve in the wetland (chicks).

The fluctuation in number of these effectives will be modulated by the number of breeding couples (as a general rule, the more breeding couples the more offspring) and hence, it will have a direct impact on AIV infections. High AIV circulation may contribute to some subtypes persistence by associating to certain social groups of hosts (which does not exclude new AIV emergence during other phenological periods (Verhagen et al., 2012). Inversely, less progeny will be expected from a reduced number of breeding couples meaning a reduction on virus input to the ecosystem. AIVs environmental persistence and transmission among the avian hosts will also be negatively affected contributing to a lower AIV global prevalence. However, the intrinsic properties of each AIV subtype may modulate these host depending factors.

We observed seasonal and temporal variation in AIV subtype prevalence between sampling periods (Table 7) except for H3N8, which was detected in both sampling periods. In agreement with another research which had also H3N8 and H11N9 among their most abundant detected subtypes, H3N8 was predominant during the breeding season whereas H11 subtypes were only detected during autumn migration (Latorre-Margalef et al., 2014). A considerable proportion of the AIVs detected belonged to LPAIV H5 or H7 subtype. Mallards harboured the greatest number of AIV positive cases and subtype richness for both periods (Table 7). H7 subtypes were frequently detected during 2009, all harboured by mallards, although no H7 subtype was detected in mallards in northern Europe between 2008-2009 (Latorre-Margalef et al., 2014). In contrast, H5 was abundant among findings in the Camargue (France) and Northern European birds during the same period whereas we only detected one sample positive to H5N2 (Latorre-Margalef et al., 2014; Lebarbenchon et al., 2009). Autumn migration appeared to be the period of the highest subtype richness for both sampling periods, very likely due to a variety of strains brought in by different migrating mallard subpopulations (Verhagen et al., 2014b).

For AIV surveillance in wild birds, the use of non-invasive sampling techniques such as fresh faeces collection has been proven to be a cost-effective tool; large sample sizes can easily be removed from the ecosystem and natural avian species distribution is not discriminated (Pannwitz et al., 2009). Capture of birds for swab and blood collection, depending on capture methods, tends to narrow the sample down to specific species and the role of other bird species and potential interspecies transmission in terms of AIV-epidemiology may be missed. Hence, the former sampling strategy gives a more realistic picture of which bird species are being infected and when, at the same time avoiding handling and consequently stressing the animals (Pannwitz et al., 2009). The drawbacks are that individual infections, respiratory viral shedding or previous contact with circulating strains based on antibody detection in sera cannot be monitored. In this regard, although mallard represented at least 44% of the AIV hosts, it may not necessarily be the only host involved in the epidemiology of the AIVs during both sampling periods at this wetland. The low efficiency of the barcoding technique used did not allow the correct host species identification of the remaining samples. Thus, there is a lack of relevant data concerning the role of other potential hosts in this epidemiological study.

During our sampling-periods no aquatic bird mortality was related to the presence of AIV. Besides, we do not know to what extent the different viral subtypes found during our samplings affect the health status or behaviour of the infected birds. Several studies suggest that LPAIV infections are not pathogenic in their natural reservoir (Kleijn et al., 2010; Kuiken, 2013; Vittecoq et al., 2012). In any case, considering the hazardous potential of the high diversity LPAIV H5 and H7 subtypes found at this wetland, it should be regarded as a hotspot for AIV surveillance.

Particularly relevant is to understand the influence of host ecology on pathogen transmission for preventing and managing wildlife diseases emergence (Gaidet et al., 2012a). From this perspective, we provide a long-term study on AIV epidemiology in a natural ecosystem where prevalence follows seasonal and annual patterns as previously mentioned but in which long-term prevalence fluctuation is linked to the breeding community composition and size. The use of non-invasive sampling techniques based on environmental

samples has proven effective, although an efficient host-identification tool is still necessary for optimising this sampling strategy.

Acknowledgements

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Chapter 2. Avian influenza virus prevalence is higher in Passeriformes hosts in the Atlantic bioregion in Spain.

Olalla Torrontegui, Vega Alvarez, Diego Villanua, Jose Manuel Hernández, Ursula Höfle, Marta Barral. Veterinary Research 2017 (In evaluation)

Abstract

Avian influenza viruses (AIVs) are known to replicate well in wild Anseriformes and Charadriiformes bird orders. The role of other birds is not fully clear although it may also be important in the epidemiology of the virus. We monitored the prevalence of AIVs within the Passeriformes order, since it is present in a wide variety of habitats and results may help focusing AIV surveillance. Passerines are ubiquitous birds that can be present in natural aquatic ecosystems such as wetlands; home to the AIV reservoirs, and anthropic environments where interspecies spillover events could pose a risk to public health. Our work consisted of monitoring AIV prevalence in Passeriformes from three bioregions of Spain, considering habitat diversity and ecological characteristics of the sampled species.

Our results revealed 1.8% (10/571) global AIV prevalence throughout 2005-2015. Animals obtained from the Atlantic bioregion had significantly higher AIV prevalence (4%; 5/149) than those obtained from the Northern-plateau (1.3%; 4/306) or from the South-Central bioregion (0/116) ($p<0.05$). Species affected by AIV appeared to be highly tolerant towards altitudinal restrictions of the ecosystems they inhabit $p<0.05$. AIVs were detected in 5 out of 22 studied taxonomic families including Fringillidae (7.7%), Turdidae (3.5%), Sylviidae (2.2%), Muscicapidae (1.8%) and Sturnidae (1.8%).

Prevalence patterns did not reveal seasonal trends, which may suggest a possible implication of passerines as maintenance hosts throughout the year in the epidemiology of AIVs.

Introduction

Influenza A viruses, family *Orthomyxoviridae*, genus *Influenzavirus A*, have been responsible for numerous outbreaks worldwide affecting a wide range of hosts (Alexander 2000; Olsen et al. 2006). Avian influenza viruses (AIVs) are known to transmit well via the faecal-oral route in wild waterbirds of the Anseriformes and Charadriiformes taxonomic orders, which harbour most subtypes and in which the virus does not usually cause severe clinical symptoms (Kuiken, 2013; Watanabe et al., 2012). However, AIVs have a segmented RNA genome that is highly mutable and adapts to infecting other animals such as mammals (humans included) and domestic fowl by means of gene reassortment (Webster et al., 1992). Because once they have infected poultry H5 and H7 subtypes may evolve into highly pathogenic (HP) forms and provoke high mortality rates within the avian hosts, they are of concern for the poultry industry. AIVs may become zoonotic as well so they pose a risk to public health (Spackman and Suarez, 2008b). Considering the above, close surveillance of the ecology and distribution of influenza A viruses in the wild is necessary for anticipation of future outbreaks.

Most AIV monitoring programs have targeted conspicuous bird species in which infection with AIVs is more frequently reported (Globig et al., 2009; Verhagen and Fouchier, 2011). Whether some AIV subtypes are endemic on less sampled bird orders as well (from a surveillance-strategy perspective) is not completely clear (Slusher et al., 2014). Indeed, certain avian taxa could also act as bridge hosts or some strains as transient pathogens which adds extra difficulty in identifying the hosts' true role in AIV ecology (Caron et al., 2015). Evidence suggests that birds in which AIVs are endemic, share habitat with the Anseriformes at some point (Verhagen and Fouchier, 2011).

Passeriformes are interesting targets of AIV epidemiological surveillance considering their high taxonomic diversity as well as the wide range of ecosystems they inhabit. Every year thousands of birds fly long distances along migratory flyways in which the passerines outnumber by large the waterbirds. Although AIV epidemics in the former may not be as frequent as in the latter, the heterogeneous ecology of the Passeriformes could contribute to decisive viral input to many ecosystems (Newton, 2008). Indeed, the

common presence of some passerine species in anthropic environments such as urban settlements and poultry farms, could be critical in intra- and interspecies spillover events (Fuller et al., 2010; Pearson et al., 2016). Thus, systematical long-term ecological studies focusing on Passeriformes in different ecosystems with regard to AIV will shed light upon this matter. Research concerning passerines and AIV already exist. However, most of it has been focused on experimental infections to test the susceptibility and reservoir potential of these birds when challenged with highly pathogenic variants. Besides, other studies have been part of multi-order bird samplings for AIV surveillance which are often conducted at specific times of the year (Lebarbenchon et al., 2007; Munster et al., 2007; Slusher et al., 2014). Epidemiological studies aiming at passerines are scarce and let alone the ones focused on both environmental and taxonomic diversity with regard to AIV prevalence throughout the year. With this purpose, our approach was based on a virological survey in a variety of regions and ecosystems throughout Spain for AIV infection evidence in passerine species. We focused on diverse habitats regarding as relevant locations those aquatic environments where AIVs had already been reported.

Material and methods

Sample collection

Samples from passerines were obtained between 2005 and 2015 through active 81.1% (486/571) and passive 14.2% (81/571) AIV surveillance. Samples were taken by swabbing the cloaca (N=403) or the oral cavity (N=58). Additionally, two samples (cloacal and oral) from 110 birds were taken and pooled (N=95) or placed separately (N=15) in Universal Transport Medium (UTM™, COPAN, Italia S.P.A.). Alternatively, 1ml viral transport medium containing cryoprotectants, antibiotics and antifungals was used (Munster et al., 2007). Swabs were kept refrigerated at 4ºC prior to RNA extraction within the first 24 hours after collection or were stored frozen (-80ºC) until analysis.

Virological analysis

Samples were fully homogenised by vortexing. Then, 1ml of the swab kept in 3ml UTM™ was used for viral RNA extraction using RNeasy Mini Kit commercial kit (Qiagen, Hilden, Germany) or instead, 180 µl of the 1ml viral transport medium solution with 20 µl Proteinase K using a Biosprint 96 DNA Blood Kit and then extracted with a Biosprint 96 robot (Qiagen, Hilden, Germany) following the manufacturer's instructions. Samples were screened with a TaqMan™ rRT-PCR targeting the matrix-gene (Spackman et al., 2002). Amplification was performed in an ABI7500 real time detection system with One-Step Ag Path RT-PCR kit (ThermoFisher Scientific).

All positives were tested for the presence of H5- and H7- subtypes with a specific rRT-PCR (Aguero et al., 2007; Monne et al., 2008; Spackman et al., 2002). The remaining haemagglutinin (HA) and neuraminidase (NA) identifications were performed by rRT-PCR (Elizalde et al., 2014; Fereidouni et al., 2009a; Hoffmann et al., 2016; Tsukamoto et al., 2008).

AIV isolation

All rRT-PCR positive samples were inoculated into the allantoic cavity of a 9-11 day-old SPF embryonated chicken egg following OIE recommendations (OIE, 2015). When the embryo died or 7 days post-inoculation, the allantoic fluid was harvested and viral RNA extraction was performed for AIV detection by rRT-PCR (Torrontegui et al., 2017).

If no AIV was detected during isolation; the harvested allantoic fluid was passed into a new set of eggs and proceeded likewise the previous isolation protocol.

Ecological data

Environmental and ecological data from the sampled species individuals were gathered (Table 9). This information included: a.- **taxonomy:** a phylogenetic classification of passerines including family and species (Payevsky, 2014) (Table 10); b.- **sampling year**; c.- **phenology of waterfowl reservoirs** (wintering, northward spring migration, breeding season and, southward autumn migration); d.- **season**; e.- **bioregions**: Atlantic,

Table 9. Prevalence distribution of AIV in passerines according to spatio-temporal variables and host ecological traits.

Factors	**Variables**		**N**	**AIV+**	**AIV %**
Spatio-temporal	Bioregions* (p=0.03)	Atlantic	149	6	4
		Northern-Plateau	306	4	1.3
		South-Central	116	0	
	Sampling year	2005	33	2	6.1
		2006	73	5	6.8
		2007	35	0	
		2008	74	0	
		2009	2	0	
		2010	15	0	
		2011	56	0	
		2012	55	0	
		2013	16	0	
		2014	209	3	1.4
		2015	3	0	
	Season	Winter	99	3	3
		Spring	65	2	3.1
		Summer	184	2	1.1
		Autumn	221	3	1.4
AIV-host ecology	Phenology	Wintering	58	2	3.4
		Spring migration	112	4	3.6
		Breeding season	52	1	1.9
		Autumn migration	347	3	0.8
	Environment	Aquatic	377	7	1.9
		Terrestrial	185	3	1.6
	Relevance for aquatic birds	Yes	374	7	1.9
		No	197	3	1.5
	Diet	Insectivores	520	9	1.7
		Omnivores	32	1	3.1
		Granivores	19	0	

Cont.

Factors	Variables		N	AIV+	AIV %
AIV-host ecology	Habitat	Coastline	34	2	5.9
		Crops-pastures	99	2	2
		Inland forest	18	1	5.6
		Protected wetland	306	4	1.3
		Urban ecosystem	34	1	3
	Age	Juveniles	249	2	0.8
		Adults	181	5	2.8
	Sex	Females	62	0	
		Males	52	2	3.9
	Migration trends	Resident	85	2	2.4
		Migrant	486	8	1.6
	Type of surveillance	Active	486	6	1.2
		Passive	81	3	3.7
	Clinical history	Disease/weakness	7	0	
		Hunting	60	1	1.7
		Ringing campaign	426	5	1.2
		Traumatism	23	1	4.3
		ND	8	0	

N: number of samples analysed; AIV+: number of AIV-positive samples; AIV%: percentage of AIV-positive samples; *: significant with Fisher's test ($p<0.05$).

Northern-Plateau, and South-Central (García-Bocanegra et al., 2016; Muñoz et al., 2010) (Figure 15); f.- **type of environment from which the birds were found or captured**: aquatic (wetland, river or estuary) *vs.* terrestrial (crops, pastures or forests); g.- **habitat from which the birds were found or captured**: coastline (littoral ecosystems where human settlements are less than 10,000 people), urban (more than 10,000 inhabitants regardless of proximity to the ocean), forest, protected wetland (well preserved aquatic ecosystems both inland and coastal), crops and pastures; h.- **relevance for aquatic birds**: areas with frequent waterbird presence regardless of the ecosystem (meaning higher likelihood of exposure to AIVs); i.- **diet**; j.- **sex and age** (when available); k.- **migration trends**: resident *vs*. migratory (because in some cases

Table 10. AIV prevalence distribution with regard to the sampled passerine taxonomic diversity. Families with AIV-positive individuals are highlighted in bold, number of species analysed are between brackets and AIV-positive species specified.

Family	AIV+ species	N	AIV+	AIV%
Acrocephalidae (5)		103	0	
Aegithalidae (1)		3	0	
Cettiidae (1)		11	0	
Cistiocolidae (1)		2	0	
Corvidae (4)		25	0	
Emberizidae (1)		1	0	
Fringillidae (6)		13	1	7.7
	Pyrrhula pyrrhula	1	1	100
Hirundinidae (2)		15	0	
Locustellidae (2)		8	0	
Motacillidae (1)		1	0	
Muscicapidae (9)		56	1	1.8
	Erithacus rubecula	23	1	4.3
Paridae (2)		3	0	
Passeridae (3)		11	0	
Phylloscopidae (4)		38	0	
Prunellidae (1)		2	0	
Regulidae (1)		1	0	
Remizidae (1)		2	0	
Sturnidae (2)		55	1	1.8
	Sturnus vulgaris	53	1	1.9
Sylviidae (3)		46	1	2.2
	Sylvia borin	17	1	14.3
Timaliidae (1)		1	0	
Troglodytidae (1)		3	0	
Turdidae (5)		171	6	3.5
	Turdus merula	29	2	6.9
	Turdus philomelos	119	3	2.5
	Turdus viscivorus	1	1	100
Total (58)		**571**	**10**	**1.8**

N: number of samples analysed; AIV+: number of AIV-positive samples; AIV %: percentage of AIV-positive samples; (): Number of species analysed.

there were multiple migratory variants *i.e.* summer visitors, spring migrants, wintering birds…, species showing both migratory and sedentary behaviours the migratory was

considered more important for AIV ecology and it was included); l. - **altitudinal amplitude of species**; m.- **habitat amplitude of species**; n.- **bird species density**; o.- **climatic- and soil-specialisation gradients of species** (l-o; (Carrascal, 2006))

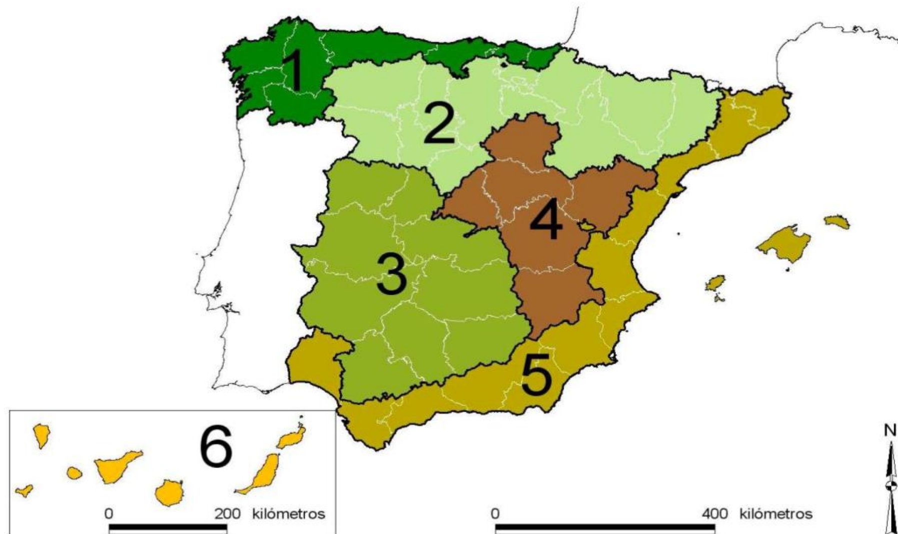

Figure 15. Main bioregions for wildlife sampling (Ministerio de Agricultura Alimentación y Medio Ambiente, 2013). Atlantic (1), Northern-plateau (2) and South-Central Ecosystems (3) have been sampled in this chapter.

Statistical analysis

The relation between AIV prevalence and qualitative variables was determined with Fisher's test in SAS® 9.3 statistical software. As for the quantitative ecological variables, normality was checked with Kolmogrov-Smirnov normality test in SAS®. If data-distribution was not normal Wilcoxon test was employed with SAS® to compare the value of the quantitative ecological variables between AIV-positive and negative birds.

Results

Between 2005 and 2015, 571 passerine birds that belonged to 57 species within 22 families (Table 10, Annex IV) were sampled in 3 different bioregions of Spain (Figure 15 and Table 9).

Global AIV prevalence for the whole sample set was 1.8% (10/571). All samples were negative to H5 and H7 subtypes and detected subtypes were demonstrated to be low pathogenic. No virus was isolated by inoculation of 5 of the positive samples in SPF chicken eggs. The majority of AIV-positive samples (N=9) were detected from cloacal swabs except one, positive from a pool. AIV was detected in 5 taxonomic families and 7 passerine species although prevalence did not differ significantly within taxa (Table 10). Inoculation was performed in 5 out of the 10 AIV-positive cases because for the rest of the cases there was not enough sample material left.

AIV prevalence was significantly higher in samples form the Atlantic bioregion (4% 6 out of 149) than in the other two sampled bioregions (Fisher's test $p=0.03$) (Table 9 and Figure 15). Wilcoxon test revealed significant differences with altitudinal amplitude between AIV-positive ($\chi=0.635556$) and AIV-negative birds ($\chi=0.505567$) ($p<0.05$) (Table 11). No significant influence of any of the studied factors on AIV prevalence could be identified in our sample set (Table 9).

Table 11.- Wilcoxon Two-Sample Test for the above-mentioned quantitative variables in AIV negative (AIV-) and AIV positive (AIV+) number of samples (N).

Variables	**(Mean ± SD) N=AIV-**	**(Mean ± SD) N=AIV+**
Altitudinal amplitude* (p=0.04)	(0.5 ± 0.2) 476	(0.64 ± 0.07) 9
Habitat amplitude	(0.31 ± 0.14) 474	(0.33 ± 0.14) 9
Bird density	(11.85 ± 10.80) 475	(11.28 ± 9.1) 9
Climatic and soil specialisation gradients	(3.978 ± 1.12) 533	(4.08 ± 1.39) 10

Altitudinal amplitude of passerine species: (0: least tolerance, 1: highest tolerance); habitat amplitude of passerine species: (0: least tolerance, 1: highest tolerance); bird density (1: scattered, 10: dense); climatic and soil specialisation gradients of passerine species (1: least specialized, 10: highest specialisation).

Discussion

This study provides valuable information about the frequency of AIV detection in a diverse avian order with few previous similar studies.

By comparative analysis of prevalence according to parameters that group the possibility of AIV exposure in a specific species we tried to identify species or sampling characteristics relevant for the planning of passerine multispecies AIV surveillance monitoring in a variety of ecosystems.

Data analysis revealed significant differences in AIV prevalence attending to the geographical origin of the samples. The highest prevalence was held in the Atlantic bioregion of Spain. Indeed, all AIV-positive species in this study (excluding the ubiquitous *Turdus merula*) showed a marked distribution affinity towards the Northern-half of the Iberian Peninsula and the presence of some taxa is even confined to the Atlantic bioregion (Carrascal, 2006) (Annex I). Previous epidemiological studies conducted in aquatic birds in Spain and northern Europe already observed more AIV detections in the northern regions (Munster et al., 2007; Pérez-Ramírez et al., 2012). The Spanish Atlantic bioregion is characterised by low annual insolation, frequent precipitations, broad altitudinal amplitude with mixed tree cover of coniferous and broadleaf forests (Carrascal, 2006). Apart from a geographical latitude gradient shaping AIV epidemiology (Munster et al., 2007)(linked to the flyways used by the AIV reservoirs) there may be a biogeographical zoning underlying AIV prevalence as well. Considering our results, the aforementioned Atlantic influence in determining the biotic and abiotic factors shaping northern Spain may facilitate, in a similar manner, AIV circulation at the virus-host-environment interface.

The global prevalence found during 2005-2015 in Spain was 1.8%, higher than the overall prevalence reviewed by Slusher and colleagues (2014) in 19 publications about molecular-based surveillance on passerines in natural settings (1% 248/29258). Because of the heterogeneity of the literature sources for building the meta-analysis (differences in sampling time, host species' taxonomy and ecology…), the prevalence value should only be considered as a reference value and not as comparable data.

In our study AIV detections varied in time and space as previously reported in AIV epidemiology (Verhagen and Fouchier, 2011), although temporal variations in prevalence were likely due to the uneven sample size among the sampled years (Table 9). AIV detections in the Passeriformes were found throughout all seasons which may indicate a potential role of these birds as maintenance hosts in viral perpetuation (in contrast to the AIV seasonal peaks found in the Iberian Peninsula during wetland-based multi-order samplings (Busquets et al., 2010; Henriques et al., 2011; Perez-Ramirez et al., 2010; Pérez-Ramírez et al., 2012)). However, because of the lack of standardised samplings in the reservoir species in passerine sampling areas during the most prevalent years (2005-2006) (as well as the scarcity of sample material and thus the absence of virus isolates necessary for conducting phylogenetic studies), it is not possible to assess whether infections in passerines corresponded to spillover events or if these birds are indeed effective hosts in viral circulation. A study carried out from mid-March to late June in France in 2006 in which the passerines represented 59.5% (n=621) of their sample size (n=1044), the authors did not find a single AIV-positive among the 15 different families that were sampled (Lebarbenchon et al., 2007).

In contrast, a study carried out in Slovakia during 2007 found 30% (32/105) prevalence in 12 migrant passerine species sampled in July. Subtype richness in the sampled population was remarkably high and mixed-infections from the respiratory and intestinal tract of some individuals shedding simultaneously different AIV subtypes were also reported (Gronesova et al., 2008). This underpins the importance of taking both oral and cloacal samples from the same specimen during AIV surveillance programs.

A more recent publication from the same area also found high AIV detection-rates during April and June/July 2008 13.6% (72/530) and 17.5% (21/120) respectively, where reed warblers and sedge warblers (*Acrocephalus shoenobaenus*) were not only the most prevalent species but also the ones presenting the highest subtype richness. Furthermore, the authors found mixed infections with different shedding routes as well in *Acrocephalus* spp. (Borovská et al., 2011). Despite the high AIV prevalence detected in passerines in these two studies, they are both restricted to very short periods of time in order to be able to monitor the evolution of prevalence fluctuation.

In the review of Slusher and colleagues (2014) and in two more publications (Pearson et al., 2016; Račnik et al., 2008) it was pointed out that viral detections were reported more frequently from passerines belonging to peridomestic species associated with agricultural environments. Some of these families like Turdidae and Sturnidae are well known for their broad environmental plasticity, meaning they have little restrictions to inhabit diverse habitats, anthropic environments included. Besides, sparrows (*Passer* spp.) which are birds that can often be observed intimately linked to human activities such as farming or urbanised settlements, have been challenged in a number experimental infections where susceptibility to the AIVs has been proven (Han et al., 2012; Nemeth et al., 2013; Yamamoto et al., 2013). In our sample set (N=11) none of the sparrows tested positive but members, both of the Turdidae and Sturnidae families carried AIV (Table 10).

Our analyses also revealed a relation of high altitudinal amplitude tolerance of the AIV-positive taxa (Table 11), which is in agreement with the high altitudinal variation that is found in the Atlantic bioregion (Carrascal, 2006). Thus, AIV dissemination risk to poultry and livestock farms by ubiquitous passerine families during an outbreak should not be neglected. Considering all the aforementioned, efforts should be made in order to improve the true representation of this group of birds in AIV epidemiology. Nevertheless, obtaining similarly large sample sizes among species, sampling locations and consistency of the measured variables is challenging in this taxonomic order.

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Chapter 3. Avian influenza virus in wintering aquatic birds at landfills in south-central Spain. Are these places hotspots for surveillance?

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Abstract

Aquatic wild birds are the undebated reservoir for Avian Influenza Virus (AIV). Therefore, they are intensively studied to better understand their role in AIV maintenance and spread. To date, AIV surveillance focuses on natural aquatic environments where different bird species aggregate and viral survival is enhanced. Nevertheless, artificial habitats such as human waste residue landfills hold also aggregations of wild birds, AIV reservoir species included. The use of landfills as a predictable food source has significantly influenced population size, migratory traits and feeding behaviour of white storks (*Ciconia ciconia*) and black-headed gulls (*Chroicocephalus ridibundus*) among others. Thus, bird sampling at human waste residue landfills could be a useful surveillance tool in addition to what it is performed at wetlands, especially during periods of aggregation of large numbers of birds. During the wintering season 2014-2015 the prevalence of AIV in sympatric avian species at two landfills in Ciudad Real was analysed by rRT PCR and species related temporal variation in AIV prevalence determined. We collected and tested 1186 fresh faecal samples from white storks (N =689), cattle egrets (N =116) and mixed flocks of gulls (N=381) as well as cloacal and oral swabs from five individuals found dead. AIV overall prevalence was 0.6%, peaking in October (1.92%). Prevalence differed significantly between the sampled species, being highest in gulls (1.31%). H16N3 subtype was detected from a cattle egret and H11N9 from a white stork, while gulls harboured both in addition to a H11N3 subtype. Subtype sharing among the sampled species suggests that AIV interspecies transmission may occur between wild birds that mix at human waste residue landfills, albeit phylogenetic analyses are still pending. Our results indicate that AIV circulates in wild birds that forage at human waste residue landfills and probably continuously during the wintering season at least in gulls. Fresh faeces collection at human waste residue landfills offers a cost-effective sampling method for large-scale LPAIV surveillance in these type of environments.

Introduction

Avian Influenza Viruses (AIVs) belong to the *Orthomyxoviridae* family, genus *Influenzavirus A*. AIV characterization is based on the antigenic properties of two transmembrane glycoproteins: the haemagglutinin (HA) and the neuraminidase (NA) (Webster et al., 1992). AIVs are also classified into highly pathogenic (HPAIV) and low pathogenic (LPAIV) forms according to their pathogenicity when infecting chickens (Munster and Fouchier, 2009). Pathogenicity of HPAIV in wild birds can be very different from that of the chicken and varies among species. Cross-protective immunity against HPAIV by previous infections with LPAIV has also been described in some individuals (Seo et al., 2002).

AIVs circulating in natural ecosystems are generally LPAIV. Replication takes place mainly in the intestinal tract (despite some strains have respiratory tract affinity too) and progeny virions are excreted to the environment within the faeces. Transmission occurs mainly via the faecal-oral route, especially in aquatic environments (Webster et al., 1992). Despite being controversial, the coincidence between LPAIV and HPAIV geographical expansion along the migratory flyways, as well as HPAIV detections in countries without previous outbreak reports in poultry, support the theory that HPAIV can spread by means of migratory bird movements (Olsen et al., 2006; Tian and Xu, 2015; Verhagen et al., 2015).

Abundant and constant food resources provided by human waste residue landfills attract a great number of opportunistic species and thus these have become alternative stopover/wintering locations for migratory birds to forage (Garrido and Sarasa, 1999). In Spain, this has induced a change in many species' migratory behaviour, which seems to be particularly evident in the case of white storks and gulls in which human waste residue landfills have significantly contributed to an increase in resident individuals during the last decades (Blanco, 1996; Blanco et al., 2006; SEO/BirdLife, 2008; Tortosa et al., 1995). The Charadriiformes order is considered the second most important LPAIV reservoir after the Anseriformes (Webster et al., 1992). Gulls, members of the former, gather in large numbers at rubbish dumps, and have often been seen mixing with other avian species that benefit from human waste.

Foraging in recently discharged residues in all species feeding at landfills is followed by considerable resting periods in large flocks often around and in small pools or ponds formed by rainfall or runoff from the landfill premises or on surrounding fields. Thus faecal-oral LPAIV transmission by contaminated water could be enhanced. However, the true role of human waste residue landfills as LPAIV host-to-host transmission interface still remains unclear.

The goal of the present work is to study AIV prevalence dynamics through autumn and winter, in selected sympatric species that use human waste residue landfills for foraging in central Spain. We investigated AIV spatio-temporal patterns and host species variation with regard to AIV prevalence.

Material and Methods

Fresh faecal samples were collected at two human waste residue landfills (Alcázar de San Juan 39º25´N 3º13'E and Almagro 38º51'N 3º39'E) in the Autonomous region of Castilla – La Mancha, in South-central Spain from white storks (*Ciconia ciconia*), cattle egrets (*Bubulcus ibis*) and three gull species; black-headed gulls (BHG), lesser black-backed gulls (LBBG) and yellow-legged gulls (YLG) (*Chroicocephalus ridibundus*, *Larus fuscus* and *Larus michaellis* respectively).

Due to the serious decline and local extinction of the white stork in Southern, Central and Northern Europe during the early twentieth century, numerous reintroduction programs and ringing schemes have been in place for decades for this species across Europe that largely employ rings for visual recapture by reading through telescopes. For the sampled landfills white stork ring-readings have been carried out since 1996 and more intensively between 2011 and 2016. Visual recapture data reveals the use of the sampled human waste residue landfills for stopover on southward and northward migration as well as wintering by white storks originating from central Europe as well as by local residents. It is very likely that a similar situation applies to the different gull species that compose the mixed gull flocks at the landfills, but especially for the black headed gull, while in contrast cattle egrets are resident in the area.

Fresh faeces were collected from mono-species flock units at the resting sites after flushing them by approaching (since gull flocks were usually mixed they were considered as a mono-familiar flock unit) at each rubbish dump. Sampling was performed every 15 days during the autumn migration and wintering season of white storks, LBBG and BHG in Spanish territories (from September 2014 to March 2015), in order to study AIV prevalence dynamics (stopover of migrating birds on southward migration (September), wintering (October-December) and stopover on northward migration (January-March)).

Approximately, 30 samples were removed from each flock unit every sampling time. Only fresh samples were individually taken from the environment with a sterile cotton swab and placed inside a small zip-lock bag.

Faecal samples were maintained refrigerated (4-10º C) during transport to the laboratory facilities. Five individual samples each were pooled together (according to the flock species and landfill) 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)). Additional cloacal and oral swabs were obtained from dead BHG (N=2) or sick LBBG (N=1), BHG (N=1) and white stork (N=1) found at the sampling areas. Oral and cloacal swabs from dead or sick animals were processed together and individually.

Viral RNA was extracted using a commercial kit (High PureRNA isolation kit, Roche Diagnostics, Germany), according to the manufacturer's instructions. 200µl transport medium were used for eluting 50 µl RNA. RNA was quantified using NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, DE, USA) and screened following a rRT-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. Amplification and detection was performed using an iQ5 real time detection system (BioRad, Applied Biosystems, New Jersey, USA) for all the rRT-PCR assays.

AIV positives (AIV+) were also analysed for H5 and H7 subtypes by rRT-PCR as described by Munster et al (2009) and Spackman (2002) respectively. All AIV-positive samples were submitted for viral isolation and sequence analysis. 100-200 µl of the

original faecal material was inoculated into the allantoic cavity of 9-11 day-old embryonated SPF chicken eggs following OIE recommendations (OIE, 2009). The allantoic fluid was harvested after death of the embryo or at the 7th day after inoculation. RNA was extracted using a commercial kit (QIAmp Viral RNA Mini kit, Qiagen, Hilden, Germany) and M gene specific rRT-PCR for AIV detection (Spackman et al., 2002). If no AIV was detected, the allantoic fluid was passaged twice in a new set of embryonated chicken eggs.

AIV+ haemagglutinin and neuraminidase subtyping was also performed. (Elizalde et al. 2014; (Hoffmann et al., 2016).

We compared AIV prevalence among species, sampling months and migratory periods using Fisher's exact test. Statistical analysis was performed with SAS® 9.3 statistical software.

Results

Between September 2014 and March 2015 a total of 1186 fresh faecal samples were collected, from white storks, gulls and cattle egrets (Table 12). Global AIV prevalence was 0.6% (7/1186). None of the samples taken from dead or diseased animals (N =5) was AIV-positive.

There were significant differences in AIV prevalence among the sampled species (p=0.04); gulls (1.31%; N= 5/381), cattle egrets (0.86%; N= 1/116) and white storks (0.15%; N= 1/689) (Figure 16).

As for the migratory periods, AIV prevalence was 0.82 (5/613) and 0.37 (2/537) during the wintering season and northward migration respectively. Despite AIV prevalence peaked in October (1.92%, 4/208) differences in prevalence were not significant neither monthly nor seasonally (Figures 16 and 17).

All AIV-positives were LPAIV. Viral recovery rate was 66.6% (4/6). 6 out of the 7 positive cases belonged to either H11 or H16 subtypes. Gulls harboured H11N3, H11N9 and H16N3 (x2) subtypes.

Table 12. AIV prevalence (%) in white storks, cattle egrets and gulls sampled in two human waste residue landfills in south-central Spain during September 2014-March 2015. Number of collected samples, number of AIV-positives in rRT-PCR and AIV subtypes are also detailed.

Taxa	N	RT-PCR positive	Prevalence (%)	AIV subtype
Ciconia ciconia	689	1	0.15	H11N9
Bubulcus ibis	116	1	0.86	H16N3
Laridae	381	5	1.31	H16N3(x2);H11N3;H11N9;ND(x1)
Total	**1186**	**7**	**0.6**	

The positive sample from a cattle egret was a H16N3 subtype and the white stork carried H11N9 (Table 12).

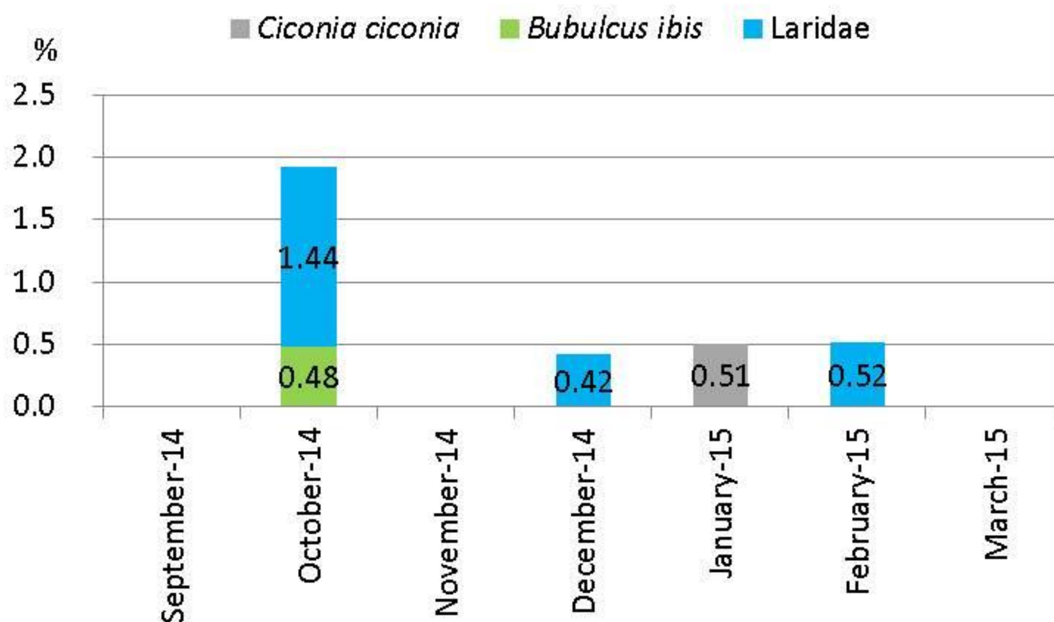

Figure 16. AIV monthly prevalence (%) in the sampled species from September 2014 to March 2015.

Prevalence for each bird species is detailed within the colour bars: white storks (grey), gulls (blue) and cattle egrets (green).

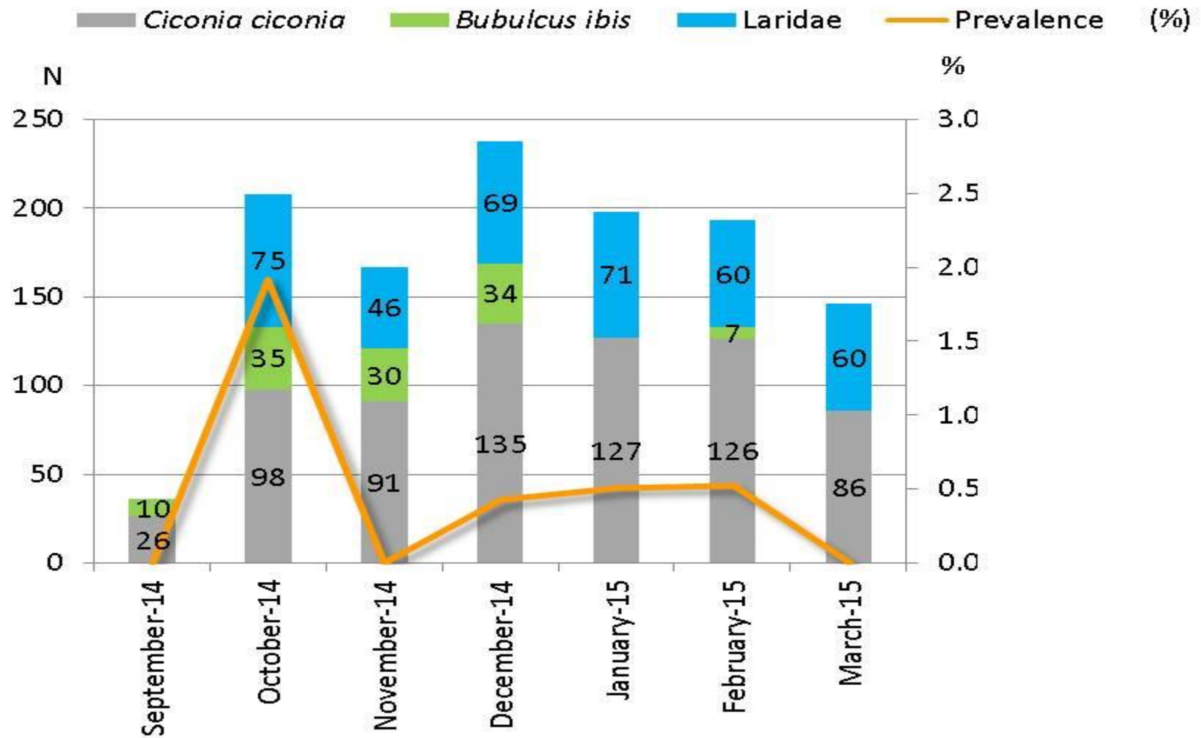

Figure 17. Temporal AIV prevalence fluctuation for the sampled period and species sampled.

Monthly AIV prevalence (%) is represented with an orange line and sample size for each bird species is detailed within the colour bars: white storks (grey), gulls (blue) and cattle egrets (green).

Discussion

The present study focuses on AIV epidemiology in aquatic birds visiting Spanish human waste residue landfills using a non-invasive sampling strategy.

AIV prevalence in water bird epidemiology is highly variable in natural ecosystems depending on location, time of the year and host species targeted (Busquets et al., 2010; Gronesova et al., 2008; Lopez-Martinez et al., 2013; Pérez-Ramírez et al., 2012). In previous studies conducted in natural environments in Spain LPAIV prevalence varied between 2.6% (2005-2007) and 5% (2006-2009) in Castilla-La Mancha (south-central Spain) and Catalonia (northeastern Spain) respectively (Busquets et al., 2010; Perez-Ramirez et al., 2010). In addition, a third study performed between 2007 and 2009 from wetlands in Castilla-La Mancha, Catalonia and Basque Country, found a 1.7% overall AIV prevalence (Pérez-Ramírez et al., 2012). Albeit, in the latter authors found important fluctuations in prevalence between years, varying from 0.82% to 7.7%.

The low AIV prevalence detected in our study may be due to the lack of samples from the Anatidae family, which usually have the highest AIV prevalence (Webster et al., 1992). As herbivores, these are usually not present at human waste residue landfills, which may also influence the AIV subtypes that circulate in the landfill environment. However, in a recent long-term study carried out in Spain with notorious Anatidae presence, the overall prevalence detected during 2012-2014 (0.3%) was below the overall prevalence detected in this study (Torrontegui et al., 2017).

Sampling during a full annual cycle might have allowed for differentiation between the importance of resident and migratory individuals in the maintenance of AIV in the landfill habitat. Census data for the study period is available only for white storks at both study sites, showing a significant tenfold decrease in white stork numbers between April and July as compared to the migratory and wintering season, with also a significantly lower proportion of storks originating from central or Northern Europe (data not shown) and this is likely to be also the case for the three gull species included in the study.

A trend for gulls to be more frequently found to excrete AIV as compared to the other species, is in agreement with other studies pointing at Charadriiformes as AIV reservoirs in natural environments (Webster et al., 1992) (Figure 17, Table 12).

Previous studies have shown that H16 AIV subtype, is endemic to gulls (Hanson et al., 2008; Jourdain et al., 2011; Verhagen et al., 2014a; Wahlgren, 2011). This suggests a possible spillover of these strains to other species through shared habitat. In this regard, interspecies variation in AIV prevalence can be explained by intrinsic differences in host susceptibility and ecology (Munster and Fouchier, 2009). An explanation for higher detection-rates in gulls may be due to the use of aquatic bodies more frequently than the other studied species (in this study in scattered ponds at the landfills), and thus higher exposure to the virus in the medium in which it is known to persist for longer periods (Munster and Fouchier, 2009).

The low prevalence found among white storks may indicate that this species may have a limited role in AIV epidemiology, as observed in previous studies in Germany

suggesting that infections are more likely related to spillover events from reservoir species such as gulls (Kaleta and Kummerfeld, 2012).

Infection with the AIVs has been shown in mallards to be bidirectional between migratory and resident birds (Verhagen et al., 2014b). Thus also here migrating birds arriving from breeding grounds may allow the introduction and circulation of new viral strains into the studied human waste residue landfills, while they could also become infected by endemic AIV strains present in the resident bird community.

However, our sampling technique does not allow identifying the individual host. Hence, whether a migrant or a resident individual shed the virus detected could not be determined.

Furthermore, taking into account the proximity of the studied landfills to wetlands and the mobility of studied birds, wetlands as the source of AIV infection in our individuals should not be neglected.

Even if non-invasive techniques do not detect oral viral shedding fresh faecal sample collection has been described as an appropriate method for large-scale LPAIV surveillance programs in wild birds as it is cost-effective and causes little impact in the wild bird community (Perez-Ramirez et al., 2010).

In summary, the present study identified circulation of AIV in sympatric species foraging at the studied urban waste landfills evidencing that these places may also act as appropriate environments for AIV transmission. AIV was detected in all studied species, demonstrating they were all susceptible towards an AIV infection. Higher AIV detection rates in gulls are in agreement with their epidemiological role as AIV reservoirs in natural ecosystems. As described in other studies, the arrival of migrating wild birds during the wintering season to Spain could be associated with a peak in AIV detection within wild bird populations foraging at urban waste landfills.

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Chapter 4. *Mycobacterium* sp. and *Salmonella* sp. are more prevalent when coinfecting with low pathogenic avian influenza virus in wild waterbirds

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Abstract

Circulation of infectious agents in wild birds is common. Some of them may be a risk to domestic animals by means of spill over events and some others are zoonotic agents, posing a risk to public health. Infections by some agents have been shown to increase susceptibility of the host to others leading to coinfections and altered host-to-host transmission patterns, however little is known about the frequency and impact of this feature in wild bird disease epidemiology.

In order to verify whether Avian Influenza Virus (AIV) excretion in wild waterbirds was related to the presence of other coinfecting microorganisms, 73 AIV-positive and 73 AIV-negative samples (80 faeces and 66 swabs), were tested towards the following selected agents presence: West Nile Virus, Avian Paramyxovirustype-1, *Salmonella* sp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Mycobacterium avium* species, *Mycobacterium tuberculosis* complex and *Mycobacerium* sp. by Real Time PCR.

AIV-positive samples (all of them low pathogenic) were coupled with negative replicates of the same sampling characteristics (bird species, sampling-time and location) for coinfection evaluation.

Mycobacerium sp. and *Salmonella* sp. were found to be more prevalent among the AIV-positive samples than among the negatives (42.9% vs. 22.8%; p=0.02 and 15.2% vs. 0%; p=0.0005 respectively).

Coinfections with other agents occurred in 48% (35/73) of the AIV-positives whereas among the negatives, detections of any of the selected agents occurred in 23.3% (17/73)(p=0.003). Multiple coinfections occurred in 9.6% (7/73) of all the AIV-positive samples that corresponded to 20% (7/35) of the coinfection cases.

The prevalence of coinfections differed significantly between sampling years (p=0.001), host families (p=0.002), host species (p=0.003) as well as AIV subtypes (p=0.03).

In this work we observed that AIV detection in faecal/swab samples of wild aquatic birds increases the possibility of detection of other microbial agents. Further studies

on a larger field sample set or under experimental conditions are necessary to infer causality and further ecological and epidemiological trends of coinfections.

Introduction

Microbial agents circulation, some of them with zoonotic potential, in wild birds is common (Tsiodras et al., 2008). Infective particles are often replicated in the digestive tract and released to the environment within the faeces. Faecal contamination of feed by birds poses therefore, a risk of pathogen transmission to domestic animals (Pearson et al., 2016). In addition, the low pathogenicity of these microorganisms in the avian host, may make migratory bird species act either as long-distance vectors for a wide range of microbes or as mechanical carriers when they transport infected ectoparasites (Diego et al., 2009; Dundon et al., 2012; Hubálek, 2004).

Horizontal transmission of pathogens is more likely to happen where frequent intra- and interspecies contacts take place, *i.e.* in stopover areas during migration (Hubálek, 2004). There, the transmission of pathogens between hosts is also modulated by biotic (*i.e.* tenacity of the microorganism, number of hosts and vectors) and abiotic factors (*i.e.* temperature, humidity) (Acevedo-Whitehouse and Duffus, 2009; Breban et al., 2009; Diego et al., 2009; Drosten, 2013; Hubálek, 2004; Rohani et al., 2009).

Another less well-known factor is the interaction of different microorganisms when they infect the same host. These types of co-infections may have an impact on host health by, modifying the type, degree and duration of shedding patterns thus, affecting transmission (Pantin-Jackwood et al., 2015).

Avian Influenza Virus (AIV), family *Orthomyxoviridae*, genus *Influenzavirus A*, is a –ss RNA virus which is frequently hosted by wild aquatic birds that belong to the Anseriformes and Charadriiformes taxonomic orders, where the majority of infections (the low pathogenic forms) undergo with inapparent disease signs (Keawcharoen et al., 2008; Olsen et al., 2006; Webster et al., 1992).

In humans, concomitant infections of *Influenzavirus A* with bacterial pathogens are associated with an increase in mortality as well as morbidity and they have also shown

to alter the gut microbiota and favour *Salmonella* infection in the mouse model (Klein et al., 2016).

Results from experimental coinfections of two domestic duck pathogenic forms of AIV with a virulent avian paramyxovirus strain (APMV-1) and low or highly pathogenic forms of AIV, suggested that one virus could interfere with the replication of the other by competition on cell receptor attachment or by hindering replication; a phenomenon called viral interference (Pantin-Jackwood et al., 2015; Pedersen et al., 2014).

Yet, the processes underlaying in co-infection susceptibility and prevalence between low pathogenic avian influenza virus (LPAIV) infections with other agents is not fully clear.

The objective of this study was to determine in naturally LPAIV infected and non-infected birds the prevalence of a range of selected agents, some of which with zoonotic potential, including APMV-1, West Nile Virus (WNV), *Salmonella* sp., *Mycobacterium avium* subspecies, *Mycobacterium tuberculosis* complex, *Mycobacerium* sp., *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Factors associated with these agent's coinfection with LPAIV were also investigated.

Material and methods

Sampling design

AIV-positive faecal and cloacal swab samples from wetland-related avian species of known origin and sampling time were selected with an equal number of AIV-negative samples of similar characteristics.

Faecal samples

Between 2007-2009 and 2012-2014, fresh faecal samples from waterbirds were gathered at Salburua wetland (2º 38' 10, 74'' W / 42º 51' 31, 33'' N), located in the Basque Country (Spain), during AIV epidemiological surveillance studies (Pérez-Ramírez

et al., 2012; Torrontegui et al., 2017). 40 AIV-positive samples were selected and matched with 40 AIV-negative samples from the same month and year of the positive outcomes. Host identification by *COI barcoding* was only performed in AIV-positive samples due to the low success in species determination. As there were many negative samples available for coupling a random selection procedure with the SAS® 9.3 statistical software was performed.

Swabs

Cloacal swab samples were taken between 2005-2015 by the local Administrations during official AIV-surveillance programs which included birds found dead, hunted, admitted to rehabilitation centres or captured during ringing events. Sample size consisted of 33 AIV-positive birds of known species, origin and collection time and coupled for comparison with an equal number of AIV-negative birds of the same characteristics. For the cases with more than one negative coupling-sample possibility, a random selection procedure with SAS® was also applied.

Because seasonality may act as a potential interacting factor altering microbial community composition, the month and year of the sample collection was given preference when coupling AIV-positive and AIV-negative samples. Since intra- and interspecies contact between birds may favour infection with the circulating agents, geographical proximity was also given priority at the time of AIV-positives and negatives matching.

Total sample size consisted of 146 samples (N_{AIV+}= 73; N_{AIV-}=73). All AIV-positive samples were low pathogenic, but included H5/H7 subtypes.

Molecular genomic AIV-detection and host-identification from faeces

RNA samples were screened with a TaqMan™ (Thermo Fisher Scientific) rRT-PCR for matrix-gene detection of AIV (Spackman et al., 2002), with previously reported modifications (Torrontegui et al., 2017). AIV positive samples were subtyped with

specific rRT-PCR (Elizalde et al., 2014; Fereidouni et al., 2009b; Hoffmann et al., 2016, 2001; Tsukamoto et al., 2008).

Host identification was performed by means of *COI barcoding* using previously described primers and protocols (Lijtmaer et al., 2012; Torrontegui et al., 2017)

Reverse transcription real-time polymerase chain reaction for the detection of selected viruses

35mg faecal material or alternatively 1ml from a swab embedded in 3ml Universal Transport Medium, UTM™ (COPAN Italia S.P.A) was used for viral RNA extraction using a commercial kit (RNeasy Mini Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions.

A multiplex rRT-PCR was performed for polymerase and matrix gene sequences amplification of APMV-1 (Kim et al., 2008).

A rRT-PCR was directed to a highly conserved sequence within the 3' non-coding region of the WNV genome (Jimenez-Clavero et al., 2006)

Real-time polymerase chain reaction for the selected bacteria detection

DNA was extracted from 30mg faecal samples using MagMAX™ Total Nucleic Acid Isolation Kit (Ambion, Applied Biosystems) following the manufacturer's instructions.

Yersinia enterocolitica (YE) and *Y. pseudotuberculosis* (YP) were detected by the *ail* gene amplification in two independent TaqMan real-time polymerase chain reactions (rPCR) (Arrausi-Subiza et al., 2015; Thisted Lambertz et al., 2008a, 2008b).

A rPCR amplifying the *ttr*BCA locus was used for *Salmonella* detection (Malorny et al., 2004).

A tetraplex rPCR, that included an internal amplification control, was performed for the genus *Mycobacterium*, the *M. avium* subspecies and the *M. tuberculosis* complex detection (Sevilla et al., 2015).

Data analysis

We used Fisher's tests in SAS® 9.3 statistical software to compare the prevalence of each selected agent in AIV-positive *vs*. AIV-negative samples.

We considered the existence of coinfections, when at least one of the selected agents appeared in AIV-positive samples, or multiple coinfections if more than one agent was detected in AIV-positive samples.

We also evaluated the factors associated with the presence of coinfections or multiple coinfections cases including AIV virological characteristics (HA and NA subtype, H5/H7-positive or not), host biological features (taxonomic family, species and bird phenology), sampling year and type of surveillance (Faeces/Swab samples) (Fisher's test).

All statistical analyses were performed with SAS® software. Differences were considered significant when P < 0.05.

Results

Prevalence of each selected agent in AIV-positive *vs*. AIV-negative samples

Mycobacterium sp. and *Salmonella* sp. prevalence was significantly higher in AIV-positive than AIV-negative samples (p=0.02; and p=0.0005, respectively) (Table 13). *Mycobacterium* sp. was the most frequently detected microorganism (Table 13). APMV-1 was found in both AIV-positive and AIV-negative samples with no significant differences between both groups (Table 13). A single *Y. enterocolitica*-positive was detected from an AIV-negative sample. WNV, *M. avium, M. tuberculosis* complex and *Y. pseudotuberculosis* were not detected in any sample.

Coinfections with other agents occurred in 48% (35/73) of the AIV-positives significantly more frequently than in AIV-negative samples, in which detections of any

of the selected agents occurred in 23.3% (17/73) (p=0.003). *Mycobacterium* sp. was identified in 80.7% (30/35) of the coinfection cases followed by *Salmonella* sp. in 29% (10/35) and APMV-1 in 5.71% (2/35) (Table 14, coinfection combinations with regard to host species are also detailed).

Table 13. Prevalence of the selected agents in AIV-positive and AIV-negative samples from wild birds from Northern Spain.

		Faeces		**Swabbing**		**TOTAL**	
		Prev(%)	Pos/N	Prev(%)	Pos/N	Prev(%)	Pos/N
Mycobacterium sp.*	AIV+	54.1	20/37	30.3	10/33	42.9	30/70
	AIV-	24.1	7/29	21.4	6/28	22.8	13/57
Salmonella sp.*	AIV+	27.3	9/33	3.0	1/33	15.2	10/66
	AIV-	0.0	0/38	0.0	0/33	0.0	0/71
YE	AIV+	0.0	0/37	0.0	0/33	0.0	0/70
	AIV-	2.5	1/40	0.0	0/33	1.4	1/73
APMV-1	AIV+	3.2	1/31	3.0	1/33	3.1	2/64
	AIV-	0.0	0/35	9.1	3/33	4.4	3/68

YE: *Yersinia enterocolitica*; APMV-1: Avian Paramyxovirus Serotype-1; Prev: prevalence; Pos: number of positive samples; N: number of samples tested; AIV+: avian influenza virus-positive samples; AIV-: avian influenza virus-negative samples; (*): Statistically significant (p<0.05) between AIV+ and AIV.

Multiple coinfections occurred in 9.6% (7/73) of all the AIV-positive samples that corresponded to 20% (7/35) of the coinfection cases. In these concomitant infections, *Mycobacterium* sp. was always present and appeared coinfecting either with *Salmonella* sp. in 71.4% (5/7) or APMV-1 in 28.6% (2/7). Only infections by single agents were detected among the AIV-negatives (Table 14).

Table 14. Summary of coinfection cases combinations with the studied agents and avian hosts harbouring them.

AIV	N	APMV-1	Myc.	Salm.	YE	Host
Positive	23	- / ND	Positive	- / ND	-	A.p. (x19); A.a. (x1); L.m. (x2); ND (x1)
	5	-	Positive	Positive	-	A.p. (x3); C.r. (x1); ND (x1)
	5	-	-	Positive	-	A.p. (x1); ND (x4)
	2	Positive	Positive	-	-	A.p. (x2);
	38	-	- / ND	- / ND	-	A.p. (x12); A.s. (x1); A.c. (x4); C.r. (x1); C. a. (x2); L.m.(x7); ND (x11)
Total Positive	73	2	30	10	0	
Negative	13	-	Positive	-	-	A.p. (x5); L.m. (x1); ND (x7)
	3	Positive	-	-	-	A.p. (x2); L.m. (x1)
	1	-	-	ND	Positive	ND
	56	- / ND	- / ND	- / ND	-	A.p. (x8); A.c. (x5); L.m. (x7); C.r. (x2); C.a. (x2); ND (x32)
Total Negative	73	3	13	0	1	

N: number of samples analysed; AIV: Avian Influenza Virus; APMV-1: Avian Paramyxovirus Serotype-1; Myc: *Mycobacterium* sp.; Salm: *Salmonella* sp.; YE: *Yersinia enterocolitica*; +: positive; -: negative ; ND: not determined. A.p.; *Anas platyrhynchos*; A.a.: *Anser anser*; L.m.: *Larus michaellis*; C.r.: *Chroicocephalus ridibundus*; A.s.: *Anas strepera*; A.c.: *Ardea cinerea*; C.a.: *Circus aeruginosus*

Potential interacting factors affecting coinfection cases

The prevalence of coinfections differed significantly between sampling years (p=0.001), host families (p=0.002), host species (p=0.003) as well as AIV subtypes (p=0.03) (Table 15). No significant relations were found between the tested variables and multiple coinfection cases (Table 15).

Table 15. Coinfection or multiple-coinfection prevalence of AIV and selected agents according to the temporal, host and pathogen specific variables.

Variables		Coinf (%)	N_Coinf/N	MCoinf (%)	N_MCoinf/N
Sampling year*	2006	29.4	5/17	5.9	1/17
	2007	21.4	3/14	0	0/14
	2008	100	8/8	25	2/8
	2009	50.0	14/28	10.7	3/28
	2013	66.7	2/3	0	0/3
	2014	100	1/1	0	0/1
	2015	100	2/2	50	1/2
Phenology	W	60.0	3/5	20	1/5
	SM	30.0	3/10	0	0/10
	BR	35.3	6/17	5.9	1/17
	AM	56.1	23/41	12.2	5/41
Host family*	Anseriformes	66.7	26/39	12.8	5/39
	Charadriiformes	27.3	3/11	9.1	1/11
	Falconiformes	0	0/2	0	0/2
	Pelecaniformes	0	0/4	0	0/4
Host species*	*A. platyrhynchos*	67.6	25/37	13.5	5/37
	A. strepera	0	0/1	0	0/1
	A.anser	100	1/1	0	0/1
	A. cinerea	0	0/4	0	0/4
	C. ridibundus	50	1/2	50	1/2
	C. aeruginosus	0	0/2	0	0/2
	L. michaellis	22.2	2/9	0	0/9
AIV high risk	H5/H7	72.7	8/11	27.3	3/11
	Other	43.5	27/62	6.5	4/62
AIV Subtype	H3N8	100	8/8	12.5	1/8
	H4N?	100	1/1	100	1/1
	H5N2	100	1/1	100	1/1
	H5N3	100	1/1	0	0/1
	H6N5	0	0/1	0	0/1
	H7H11/N4N9	0	0/1	0	0/1
	H7N?	75	3/4	25	1/4
	H7N2	50	1/2	0	0/2
	H7N8	100	1/1	100	1/1
	H7N9	100	1/1	0	0/1
	H11N?	33.3	1/3	0	0/3
	H11N2	0	0/2	0	0/2
	H11N9	44.4	4/9	0	0/9
	H12N5	0	0/1	0	0/1
Surveillance	Active	56.5	26/46	10.9	5/46
	Passive	33.3	8/24	8.3	2/24

Coinf: a single selected agent detection in AIV-positive samples; MCoinf: multiple coinfections (more than one of the selected agents coinfecting with AIV); (*): Statistically significant ($p<0.05$) with coinfection (Coinf). (**): Statistically significant ($p<0.005$) with coinfection (Coinf). No significant relations were found between the tested variables and multiple coinfection cases.

Discussion:

The ecology and composition of gastrointestinal tract microbiota in wild birds is rather complex; factors such as type of diet or bird phenology may modulate some agents' prevalence or the appearance of some others (Friend and Franson, 1999).

However, interactions such as competition or facilitation between agents may also have an impact albeit there is still limited knowledge upon these relations (Deriu et al., 2016; Pantin-Jackwood et al., 2015). The present study, comparing selected microorganisms in AIV-positive and AIV-negative wild birds, found differences in prevalence between these two groups where AIV-positive samples held significantly higher detection rates. This uneven distribution of the tested agents, suggests a connection between the presence of AIV and some microorganisms' detection frequencies (Verhagen and Fouchier, 2011).

Infections with mycobacteria have been reported from a wide range of avian species, especially in aged individuals due to the long incubation periods required (Diego et al., 2009). Mycobacteria detected in this study did not pertain to the pathogenic forms *Mycobacterium avium* subspecies or to the *Mycobacterium tuberculosis* complex but we could not further identify the species despite attempts made to sequence them (data not shown). Hence, it was not possible to elucidate whether these were infectious or free-living environmental bacteria, although *Mycobacterium* sp was observed both in fresh fecal samples and cloacal swabs without significant differences. Another plausible hypothesis is that they were acquired through ingestion not subjected to infectious processes. Neither should be neglected the possibility of hosts acting as asymptomatic carriers accompanied by intermittent excretion as part of a chronic weakening process.

In this regard, environmental persistence of *Salmonella* bacteria is determined by prolonged excretion rates via faecal/oral route of the species affected, and some birds may become lifelong carriers and excretors (Tizard, 2004). Bird aggregation also contributes to increasing the probability of infection. Pathogenicity is highly strain, host stress- and age- dependent; chicks being the most vulnerable (Friend and

Franson, 1999). Although *Salmonella* species was not identified, the fact of only having found *Salmonella* in the AIV-positive samples may be related to altered protective gut microbiota induced by influenza, increasing host susceptibility to secondary *Salmonella*-induced infection, as previously reported in a mouse model (Deriu et al., 2016).

The small number of APMV-1 positives detected in our study and the fact of being found in both groups (AIV-positives and negatives) in very similar proportions does not allow concluding on any interactions between the two viruses. Other studies observed that previous infection with APMV-1 could prevent highly pathogenic AIV replication and mortality in chickens or reduce LPAIV shedding (Costa-Hurtado et al. 2015; Pantin-Jackwood et al., 2015).

The fact of not having found the WNV Arbovirus it is very likely linked to the absence of infected transmission vectors (*Culex* spp.) and not to inherent factors of AIV infection. Attending to the rest of the studied agents it seems that they are not circulating in waterbirds in the area.

Factors potentially involved in concomitant infections with AIV

Previous studies address changes in habitat use, year-long diet, and interspecies contacts as factors that may influence the seasonal exposure to most contagious diseases (Friend and Franson, 1999; Verhagen and Fouchier, 2011).

In our work however, we detected significant interactions with some factors that may play a role in coinfection processes including; sampling year, host taxonomy and the AIV-subtype (Table 15) but a more robust sample size is needed to verify the strength of these trends.

Anseriformes held the majority of the coinfections where its integrating species showed diverse coinfection proportions (Table 15). The mallard duck, known to harbour most AIV-subtypes, was also the species in which we detected more coinfections and it harboured all of the agents included in this study, although these data may also be biased due to the high number of individuals sampled in relation to

the other species. As for the host harbouring APMV-1, wild ducks are regarded by some authors to be among their primary hosts (Hanson et al., 2005).

It is not possible to elucidate whether these infections were already present before exposure to AIV and thus predisposing the host for AIV infection (*i.e.* behavioural changes that enhance contact with AIV) or if by contrast, AIV infected hosts are more prone to acquire additional pathogens. However it is noteworthy that in absence of AIV infection none of the tested agents was detected in combination with any of the other.

In this work we observed that AIV detection in faecal/swab samples of wild aquatic birds increases the possibility of detection of other microbial agents. Our results add circumstantial evidence to previous experimental studies which suggested that AIV infection increased susceptibility to other pathogens (Olsen et al., 2006). Further studies on a larger field sample set or under experimental conditions are necessary to infer causality and further ecological and epidemiological trends of coinfections.

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General Discussion

Studying infectious diseases patterns in spatially complex environments is challenging since there may be multiple transmission processes acting simultaneously depending on the nature of the hosts (i.e. wild or human-associated species) (Biek and Real, 2010).

Microparasites like viruses and bacteria have the potential to alter the environment they inhabit; they are capable of producing immunity or to cause the death of susceptible hosts (Biek and Real, 2010). Host-parasite dynamics are subjected to many selection pressures given in part from high mutation rates and short generation times of pathogens (Altizer et al., 2006). Selective pressures in pathogen evolution, range from within-host to between-host and beyond scales (Metcalf et al., 2015).

Persistence of diseases maintained in wild host reservoirs is deemed to be dependent on particular habitat features or landscape types (Biek and Real, 2010). In the same lines, infectious processes may vary within the wild hosts depending on whether they are reservoirs, bridge-species or maintenance hosts because they may have a distinct contribution to the overall disease patterns according to their ecological traits (Biek and Real, 2010). Gaining knowledge on host-pathogen interactions within the environment is necessary because the diversity of habitat characteristics at various spatial scales may have a direct impact on many infectious disease patterns (Biek and Real, 2010). Thus, in epidemiology it is essential to ascertain how these variables affect the emergence and distribution of infectious diseases.

Long-term monitoring to track changes in host population dynamics and infection prevalence is paramount for accurate prediction on wildlife diseases (Mills et al., 2010). However these longitudinal studies are scarce and perhaps of insufficient duration to reflect changes in environmental factors and address the variables triggering changes in host and pathogen dynamics for a given site (Mills et al., 2010).

The longitudinal study carried out in Salburua (Chapter 1) adds knowledge to AIV epidemiology. The remarkable role of the Anseriformes in AIV dynamics has been confirmed (both by fluctuations evidenced by breeding bird counts and by the important number of anseriform hosts identified by *COI* barcoding) as noted in previous studies (Pérez-Ramírez et al., 2012; Van Dijk, 2014; van Riel et al., 2007).

Seasonal changes are also a source of external variation influencing human and natural systems that may play a role in pathogen evolution by means of generating oscillations in prevalence (Altizer et al., 2006). As observed elsewhere AIV prevalence peaks were detected in the end of the breeding season and in the middle of southward migration (Chapter 1) very likely as a consequence of high numbers of juveniles that amplified viral excretion during a period of great aggregation of individuals (Pérez-Ramírez et al., 2012; Van Dijk, 2014; van Riel et al., 2007). Factors such as number of juveniles and their susceptibility to the virus due to their naïve immune system can decline herd immunity leading to higher risk of infection among susceptible adult hosts as well (Altizer et al., 2006). Thus, reproduction is a periodic phenomenon with potential strong effects on host-parasite dynamics across many host species (Altizer et al., 2006).

In addition to the seasonal variation our results also evidenced a longitudinal fluctuation (reduction) in AIV prevalence that was clearly and directly significantly related to the number of anseriform breeding couples suggesting that the number of breeding Anseriformes may be key as prevalence indicator (chapter 1).

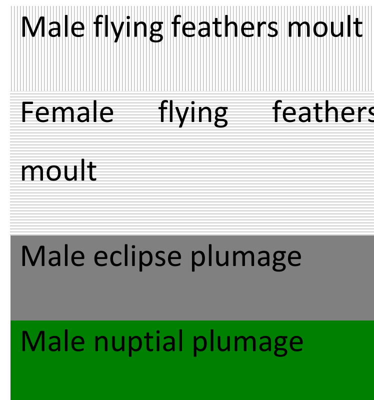
Nevertheless, there is another less studied phenomenon closely related to the breeding season such as moult that should need further research.

Waterfowl moult is a drastic phenomenon since the birds lose all flight feathers simultaneously and thus, flying capability for a month approximately. Because little is known about the true impact of Anseriformes moult in AIV epidemiology, it should need directed research and different sampling strategies (such as capture) from those used in Chapter 1. In this regard, moulting individuals undergoing physiological stress (Cornelius et al., 2011) may be playing a similar role as viral infection amplifiers in the same way immunologically naïve juveniles do, however our sampling design did not allow us to further investigate such hypothesis. In hantavirus hosts, viral load and increased rates of transmission have been suggested as a direct consequence of stress-related immunosuppression (Mills et al., 2010). In Salburua, the mallard, the main host involved in the AIV-positive detections in this work (at least 44% of the AIV-positives), moults during June-August in the case of males and during July-September in the females, soon after the juveniles start to fly (Table 16). Hence, there are moulting

males and late female breeders moulting that share breeding grounds with chicks at some point (Luis Lobo, personal comment).

Table 16 Phenology of *A. platyrhynchos.*

Phenology code	**SM**			**BR**			**AM**			**WI**		
Month	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**I**



Moult of the flying feathers in males takes place after the females start to lay eggs. Females moult their flying feathers when juveniles start to fly. Male moulting periods towards an eclipse or nuptial plumage are highlighted in grey and green respectively. SM: spring migration; BR: breeding season; AM: autumn migration; WI: wintering; I-XII: months of a year.

Stress can facilitate host susceptibility towards infections through activation of glucocorticoid hormones preceding and during the breeding season (Altizer et al., 2006). Recent studies in mammalian and avian hosts suggest that factors weakening the immune system could include harsh weather conditions and lack of food experienced during winter else the cost related to reproduction (Altizer et al., 2006). Behavioural and physiological processes associated to mating activity during the breeding season such as offspring care, testosterone increase in males and laying eggs in females can also trigger reduced immune capacity (Altizer et al., 2006).

Also, during moult the birds are not capable of flying thus they have to stay at the same water body to avoid predation which for shedding birds means more virus input into the same area. This suggests that prevalence during the breeding season may be

linked to numbers of susceptible hosts but also to aggregation patterns due to lack of host dispersal.

Long-term sampling also provides an important record of data in hosts' distribution across latitudinal, elevational, and environmental gradients (Mills et al., 2010). With the aim of broadening the existing information on AIV and host ecology, we decided to delve into studying a ubiquitous order like Passeriformes (Chapter 2). While some passerine species share habitat with waterbird reservoirs and hence, are likely to have a higher exposure to the virus, others are present in anthropic ecosystems where they could play an important role in interspecies transmission acting as bridge hosts. So we tried to evaluate the frequency of detection of AIV in different species and environments. Results revealed that AIV prevalence showed variation with regard to host species' altitudinal gradients and bioregions.

This affinity of AIV-positive species for certain bioregions suggests there may be climatic factors underlying dispersion of hosts and environmental persistence of AIV. Altitude tolerance of the hosts is another of the traits of influence meaning that environment will pose little restrictions on these hosts. Animals highly tolerant to habitat heterogeneity may disperse infectious agents to new and diverse territories like human-made ones, with the additional risk of exposure at the wildlife-domestic interface (Caraco et al., 2016). In addition, high environmental plasticity may make these hosts more prone to contacts with locations of high reservoir abundance, hence increasing the risk of getting infected with AIVs by direct or indirect transmission. Unfortunately sample size and low consistency of the measured variables did not permit to further relate other trends of AIV infection with passerine ecology and environment.

An example of how geographical boundaries may have an influence on host-pathogen genetics is the rare intercontinental exchange of avian influenza virus (AIV) despite the broad host range capable of executing such a transfer (Biek and Real, 2010; Krauss et al., 2007). The vast range of wild bird species capable of harbouring AIV poses one of the main problems for monitoring the virus at both local and global scales (Biek and Real, 2010).

Intrinsic factors (of pathogens and hosts) such as type of communities in wildlife, behaviour, life history, rapid evolutionary changes, transmission mechanisms and pathogen's host range combined with extrinsic factors contribute to disease risk in humans (Han et al., 2016).

Finally, extrinsic factors such as anthropic practices (*i.e.* agriculture or extractive industries like oil drilling, deforestation and mining) may not only affect directly the human-animal and wildlife-livestock interfaces in pathogen transmission but also damage ecosystem health, provoking changes of unpredictable consequences in exposure to infectious agents, influenza included (Han et al., 2016). Increased understanding of human practices' impact on ecosystems can become a useful tool for disease emergence modelling and prediction (Han et al., 2016).

This scenario led us to evaluate AIV transmission in a human-made environment such as landfills (Chapter 3). The enormous quantity of organic waste accumulating at landfills provides a predictable abundant food supply to many wild species, especially birds. Foraging at landfills has potentially changed the migratory patterns of some species such as the white stork (Flack et al., 2016; Gilbert et al., 2016). These behavioural changes may offer new transmission pathways for AIV as well as other infectious agents'. Our results revealed that although prevalence was low there was subtype sharing within the sampled species. Although this result indicates likely interspecies transmission, viral phylogeny would have to be studied to confirm it.

Results from Chapter 4 prove the existence of differences in prevalence of bacterial agents such as *Mycobacterium* sp. and *Salmonella* sp. between AIV-positive and AIV-negative individuals. Despite works evaluating LPAIV infection impact on host health status concluded that these infections undergo with mild clinical signs (Kuiken, 2013), they may have an impact in intestinal flora composition sufficient as to provoke other agents proliferation. Here, the inability of sequencing the species of *Mycobacterium* and *Salmonella* in our work did not allow elucidating to what extent these infections contribute in compromising birds' health status nor if there are several species involved. In the same manner it was not possible to ascertain whether mycobacteria in AIV-positive samples and in AIV-negative samples were the same species or not. As for

Salmonella, the fact of not being found among the AIV-negatives suggests that these bacteria may proliferate in situations of higher vulnerability to secondary infections as is the case of experimental infections in mice with influenza (Deriu et al. 2016).

Pathogen-host interactions with the environment, are embedded in complex and multiple interacting processes, long-term and spatially heterogeneous but of sufficient replicability to draw generalizable conclusions (Mills et al., 2010). Collaboration among governmental and nongovernmental institutions and universities will provide the best opportunity of achieving these intentions (NGO) (Mills et al., 2010). The present thesis represents such a joint effort as each of the chapters is based upon a collaborative effort of ornithological groups and NGO's (ringing and bird counts), researchers and the administration (protected wetlands and urban waste landfills).

For all the above mentioned, we can conclude that avian influenza virus dynamics in wild birds is a complex phenomenon subject to many types of variations. On the one hand, spatial and temporal variations produce a fluctuation in prevalence while dynamics are also influenced on the other hand, by factors inherent to the circulating viral strain and its affinity to the avian host community. An important finding to highlight is the higher detection probability for *Mycobacterium* sp. and *Salmonella* sp. found in AIV-positive birds as compared to AIV-negative ones. This finding underscores that inflections with AIV could be a weakening factor or a facilitating mechanism for secondary infections with other agents that needs further research.

Conclusions

1. Avian influenza virus prevalence in aquatic birds such as the mallard is higher during the breeding season as determined in the longitudinal study undertaken in Salburua wetland.

2. Long-term fluctuation of avian influenza virus prevalence in Salburua wetland is driven by the number of Anseriformes breeding couples.

3. The Atlantic bioregion of the Iberian Peninsula is an adequate climatic unit for conducting avian influenza virus epidemiological studies in Passeriformes.

4. Higher avian influenza virus genome prevalence in passerine species was associated with a broad altitudinal range and hence less restrictions to overcome environment switching.

5. Avian influenza virus circulates in white storks, gulls and cattle egrets feeding at human residue landfills during wintering.

6. Shared avian influenza virus subtypes suggest that human residue landfills may be appropriate scenarios for avian influenza virus subtype intermixing among different wintering hosts.

7. Fresh faecal sample collection from natural aquatic ecosystems as well as from monphyletic flocks resting around human residue landfills is an appropriate method for avian influenza virus surveillance.

8. Avian influenza virus infection in wild waterbirds, increases *Mycobacterium* sp. and *Salmonella* sp. detection-probability thus, coinfections.

9. Multiple infections with more than one bacteria or virus were only found in avian influenza virus positive animals, suggesting that avian influenza virus may predispose other agents' detection probability.

Summary

Influenza A virus (*Influenzavirus A*) is the aetiological agent of avian influenza virus (AIV). AIVs circulate naturally in waterbirds around the globe and may infect poultry and mammals, humans included. However infections in the latter are not frequent.

Wild aquatic birds are considered the natural reservoir of the virus where infections undergo with unapparent signs of disease. Viral replication takes place in the digestive and respiratory tracts. AIVs are well maintained in water ecosystems where they are known to persist for long periods.

AIVs are however highly contagious in the avian hosts. Indeed, some variants have the capability of causing disease or even killing them, whith poultry like chicken, quails or turkeys being especially susceptible. In this regard, AIVs are classified in two pathotypes; low pathogenic avian influenza viruses (LPAIVs) or highly pathogenic avian influenza viruses (HPAIVs) depending on their ability to cause disease or mortality in chickens.

This PhD thesis is composed of 4 chapters in which AIV epidemiology is studied with different approaches. In order to have updated knowledge in AIV epidemiology in the wild avian hosts a literature review has also been undertaken.

Because AIV epidemiology is complex and affects birds of many kinds, we conducted a long-term study, by means of non-invasive sampling, based on AIV surveillance in a natural aquatic ecosystem in relation to the ecology of its hosts, the avian community natural composition and environmental conditions, in a small wetland ecosystem. Statistical analysis revealed seasonal differences in AIV detection, with a highest prevalence during the breeding season as compared to the rest of the hosts' life-cycle events. Mathematical modelling indicated that long-term fluctuation of AIV prevalence, and potentially subtype diversity is driven by the breeding Anseriformes community.

In the second study, the epidemiology within the Passeriformes was studied because of the ubiquitous nature of this taxonomic order as well as for the ecological characteristics of its integrating species. This work allowed us to ascertain that species belonging to the Atlantic bioregion and with less altitudinal restraints were the most

frequently infected with AIV. Hence, this work highlights the importance of environment and host intrinsic characteristics in AIV detection.

On the other hand, AIV transmission in ecosystems such as human residue landfills has also been monitored. The third study aimed at an anthropic environment like landfills for monitoring, by non-invasive techniques, AIV transmission likelihood among sympatric birds. Results revealed that aquatic birds feeding at landfills during winter are exposed to AIV and inter- and intraspecies transmission may occur due to the aggregation in the landfill environment.

Thus, the aforementioned chapters reflect three key scenarios in AIV epidemiology, a longitudinal study within a natural ecosystem with important numbers of reservoir hosts, a study in a less studied taxonomic order from the epidemiological point of view but which inhabit both, natural and anthropic ecosystems and finally a study in an artificial ecosystem such as human residue landfills with new and possibly different transmission pathways.

The Thesis' core concludes with a fourth study that aims at verifying whether AIV excretion is related to the presence of other coinfecting microorganisms in wild waterbirds. Coinfection with other agents occurred more frequently in AIV-positives than in AIV-negatives and concurrent infections with more than one of the selected agents occurred within the AIV-positives only, suggesting that AIV may pose a facilitating effect to other agents' infection. Specifically *Mycobacterium* sp. and *Salmonella* sp. are more prevalent when coinfecting with AIV.

For all above mentioned, this Thesis provides new and relevant information in AIV epidemiology as regards to the ecology of its wild hosts.

Laburpena

Influentza A birusa (*Influenzavirus A*) hegazti-gripea-ren (HG) agente etiologikoa da. HG-ren subtipoen gehiengoa mundu osoko basa-hegazti urtarretan topa daiteke eta etxeko-hegazti zein beste animalia espezie batzuk infektatzeko gai da, gizakiak barne, nahiz eta hauengan ez oso ohikoa izan.

Basa-hegazti urtarrak birusaren ostalari natural direla kontsideratzen da eta birusaren erreplikazioa liseri aparatuan eta arnasbideetan ematen da, askotan gaixotasunaren seinale-kliniko hautemangarriak antzematen ez direlarik. Hala ere, hegaztien artean HG-a kutsakorra da eta birus honen subtipo jakin batzuk zenbait etxeko-hegazti espezie, hala nola, oilasko, ahate, galeper eta indioilar, gaixotuarazteko edota akatzeko gai dira. HG-a bi kategoriatan banatzen da (patogenizitate baxuko (PBHG) eta patogenizitate altuko (PAHG) HG-an hain zuzen), euren ezaugarri molekularren eta txitengan gaixotasuna edo heriotza eragiteko duten ahalmenaren arabera.

HG-ren epidemiologiaren ezagutzan sakontzeko, hegaztiak ostalari direnetan bereziki, bibliografiaren berrikuspena burutu da. Gainera, Doktorego Tesi hau lau kapituluk osatzen dute, non HG-aren birusaren dinamika aztertzen da ikerketa longitudinal baten bidez, ostalarien eta ingurumen naturalaren artean, hala nola Passeriformeen Ordena Taxonomikoa osatzen dutenen artean. Zabortegietan maiz ibiltzen diren hegazti espezie ezberdinen artean, HG-aren transmisioa ebaluatzen da eta bestalde, hegazti urtarretan, animalia-osasun ikuspegitik interesa duten hainbat agente patogenoen prebalentzia ikertu da, HG-ri bai positibo eta bai negatibo izandako laginak aztertuz.

HG-ren epidemiologia konplexua denez eta ostalari moduan diharduten espezie ezberdinetako hegaztiei eragiten dienez, epe luzera burututako ikerketa burutu zen. Laginketa ez-inbaditzaileak eginaz, dimentsio txikiko hezegune batetako hegaztien distribuzio naturala aztertu zen, birusaren ingurugiroan subtipo ezberdinen ekologiari buruz informazioa lortzea ahalbidetzeko. Lortutako emaitzek ezberdintasun esanguratsuak erakutsi zituzten HG-ren prebalentziari dagokionez, ikertutako denboraldien artean eta barnean. Gainera, urtaroen arteko aldaketez gain, HG-aren prebalentziaren beherakada adierazgarria ematen zela ikusi zen ikerketa guztian zehar. Erabilitako modelo matematikoak, prebalentzia hori, erroldatutako Anseriforme bikote ugaltzaileen eta bertako espezieen araberakoa zela ikustarazten zuen. Dena den, HG-ri

positibo emandako gorotz laginetatik, espezie ostalaria identifikatzeko teknikaren efektibitate baxua ikusita, ostalarien ekologiari lotutako beste hainbat faktore kontutan hartu ahal izatea mugatu zuen.

Bigarren azterketan, HG-ren epidemiologia ikertu da Passeriformeetan. Ordena honen nonahiko izaera kontutan izanik, maiz egoten diren ingurunearen dibertsitatea eta ezaugarri ekologikoak kontenplatu ziren. Lan honen arabera, ikertutako espezieak kontutan hartuta, HG-rekin gehien infektatzen zirenak, Atlantiko isurialdetik eta euren habitatean, altitute murrizketa gutxiago zituztenak zirela ikusi zen. Aurkikuntza honek ingurumenaren eta ostalarien ezaugarri intrintsekoen garrantzia azpimarratzen du HG-a detektatzerakoan.

Hirugarren azterketan, gizkakien hondakinak kudeatzen diren zabortegi batean burutu zen lana, leku hauetan maiz ibiltzen diren hainbat basa-hegazti urtarren artean HG-arekin kutsatzeko posibilitatea monitorizatzeko asmoz. Emaitzek prebalentzia baxua erakutsi zuten lagindutako espezieei dagokienez. Suptipo berdinak topatu ziren hainbat espezieetan, zabortegiak HG-ren transmisio leku izan daitezkeela iradokitzen duelarik.

Hiru lan hauek, HG-ren epidemiologian hiru eszenatoki nagusi daudela islatzen dute; ostalarien presentzia garrantzitsua duen ingurumen naturalean eginiko azterketa, HG-ren epidmiologiaren ikuspuntutik begiratuta, ingurune natural eta gizatiartuetan maiz ibiltzen den eta hain ezaguna ez den Ordena baten azterketa eta azkenik, HG-ren epidemiologian garrantzia izan dezakeen, ingurugiro ez natural batean, kutsatzeko bide berri eta ezberdinak izan ditzakeen, hegaztien azterketa.

Tesiaren muina laugarren azterketa batekin burutzen da, hegazti urtarretan HG-ren infekzioarekiko izan dezakeen inpaktua ebaluatzeko asmoz, non HG-ri positibo eta negatibo emandako laginak beste agente batzuekiko infekzioa izateko suszeptibilitatea alderatzen dan. Azterketa honetan, HG-ri positibo ziren laginetan, *Mycobacterium* sp. eta *Salmonella* sp. agenteen prebalentzia altuagoa zela ikusi zen. Gainera, aukeratutako hainbat agenteren aldi-bereko zirkulazioa, HG-ri positibo ziren laginetan soilik gertatzen zen, honek birusak afinitate edo erraztasuna izan dezakeela iradokitzen duelarik beste agente batzuekin infekzioak izaterako orduan.

Resumen

El virus influenza A (*Influenzavirus* A) es el agente etiológico de la influenza aviar. Los virus de la influenza aviar (VIA) circulan de forma natural entre las aves acuáticas de todo el mundo y pueden infectar a las aves de domésticas y a los mamíferos, incluido el ser humano, aunque en este último las infecciones no son frecuentes.

Las aves acuáticas silvestres se consideran los reservorios naturales del virus aunque la infección rara vez transcurre con signos clínicos. El virus se replica tanto en el tracto intestinal como en el respiratorio de las aves infectadas y se puede mantener en los ecosistemas acuáticos durante largos periodos de tiempo. Los VIA se transmiten fácilmente entre las aves y algunos subtipos pueden hacer enfermar o incluso matar a ciertas especies, siendo especialmente susceptibles algunas especies de aves domésticas, como los pollos, los pavos o las codornices. Los VIAs se clasifican en VIA de baja patogenicidad y VIA altamente patógenos en función de sus características moleculares y de la capacidad que tienen de causar enfermedad o mortalidad en pollos.

Esta Tesis Doctoral la componen cuatro capítulos en los que se estudia la epidemiologia de los VIA desde diferentes abordajes. Además, para profundizar en el conocimiento de la epidemiología de los VIA se ha llevado a cabo una revisión de la bibliografía existente.

En primer lugar y debido a que la epidemiología de los VIA es compleja y afecta a hospedadores de diversas especies, se ha llevado a cabo un estudio de larga duración en un humedal de pequeñas dimensiones. En este humedal se ha profundizado, mediante el uso de técnicas no invasivas, en la epidemiologia del VIA en relación con la ecología y la composición de la comunidad de aves presentes en el humedal y las condiciones meteorológicas. Los resultados revelaron diferencias estacionales en la prevalencia de VIA, observándose las prevalencias más altas en la época de cría. El modelo matemático realizado indicó que las fluctuaciones a largo plazo en la prevalencia de VIA y potencialmente la diversidad de subtipos, se encontraban moduladas por el número de parejas Anseriformes reproductoras en el humedal.

En el segundo estudio se abordó la epidemiología de VIA en los paseriformes, debido a la ubicuidad de este orden y a la diversidad de características ecológicas de las

especies que lo integran. Gracias a este trabajo se pudo comprobar que las especies más frecuentemente infectadas con VIA procedían de la vertiente Atlántica y mostraban una mayor tolerancia a cambios en la altitud geográfica. Este trabajo refuerza la importancia del entorno y de las características intrínsecas de cada especie en la detección de los VIA.

En el tercer estudio se monitorizó, mediante técnicas no invasivas, la prevalencia de los VIA en un ambiente humanizado, como son los vertederos de residuos urbanos, estudiando las especies de aves acuáticas que se agregan en ellos durante la invernada. Las aves estudiadas mostraron una prevalencia baja de VIA, pero el hallazgo de los mismos subtipos en especies diferentes sugiere que los vertederos podrían ser importantes lugares de transmisión de los VIA.

Estos tres capítulos ponen en evidencia tres escenarios clave en la epidemiología de los VIA; un estudio longitudinal en un ecosistema natural con una presencia importante de reservorios, un estudio en un orden menos conocido desde el punto de la epidemiología de los VIA, pero que frecuentan una gran diversidad de ambientes naturales y humanizados y por último un estudio en un ecosistema artificial, como son los vertederos de residuos urbanos, con agregación temporal de aves acuáticas.

El núcleo de la tesis culmina con un cuarto estudio cuyo objetivo es verificar si la excreción de los VIA está relacionada con la presencia de otros microorganismos que coinfectan a las aves acuáticas. En este estudio se observó que la infección con VIA incrementaba la posibilidad de detectar otras agentes en las muestras estudiadas. Además, la circulación concomitante de varios de los agentes seleccionados se produjo únicamente en las muestras positivas a VIA, lo que sugiere que la infección con VIA podría facilitar la infección con otros agentes. De forma más concreta, se comprobó que la prevalencia de *Mycobacterium* sp. y *Salmonella* sp. era más alta en los animales infectados con VIA, frente a los que no lo estaban.

Por todo lo anteriormente mencionado esta Tesis aporta información nueva y relevante en relación con la epidemiología de los virus influenza aviar en relación con la ecología de los hospedadores silvestres y de los ecosistemas naturales o artificiales en los que viven.

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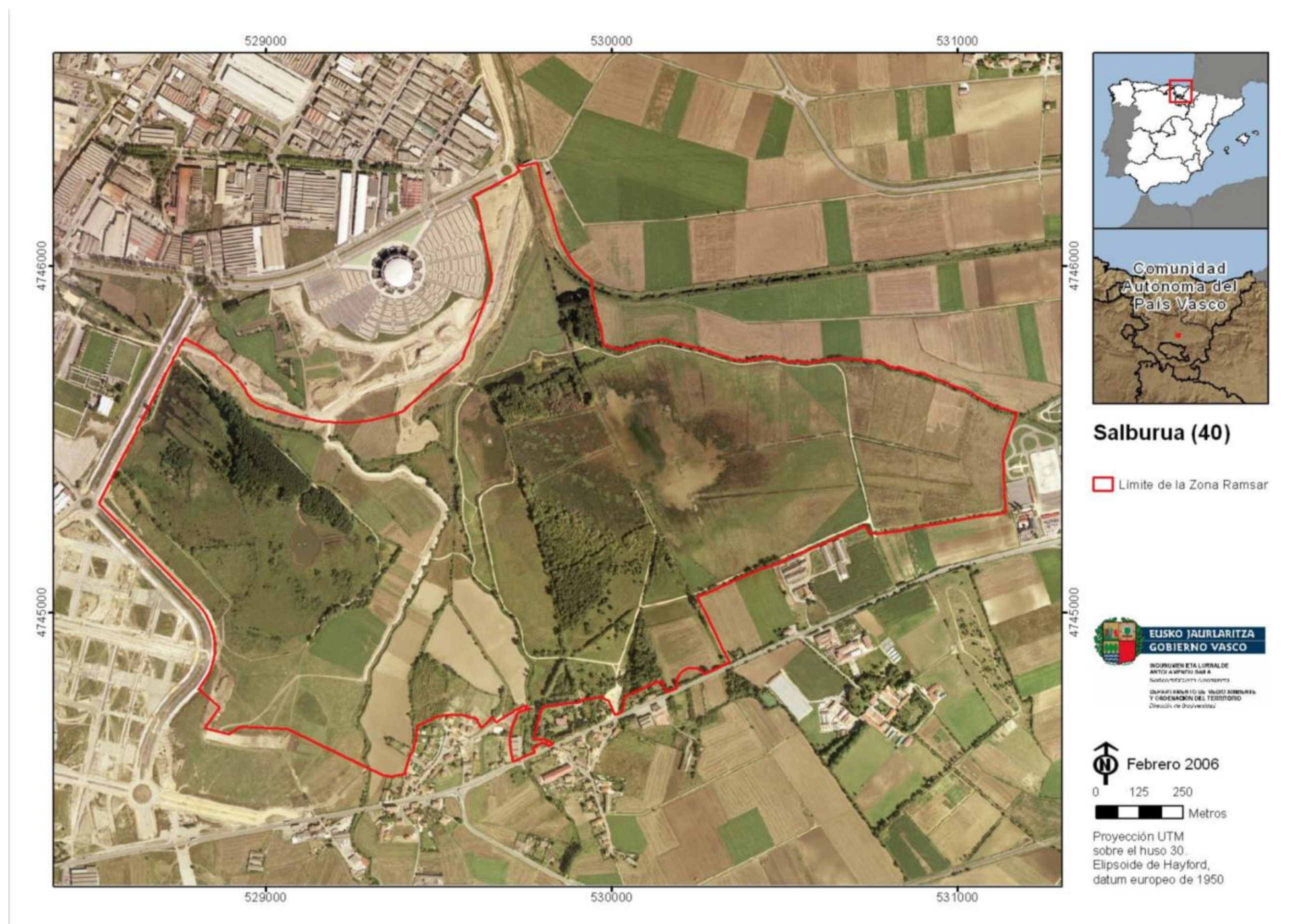
Annexes

Annex I. Aerial view of Salburua wetland and its geographical location (map on the right side).



The red line marks the limits of the Ramsar designation area.

Annex II. Raw data of the main predictors used, namely the avian community inhabiting Salburua wetland, AIV prevalence and meteorological parameters.

Sampling	Month	Year	Phenology	Total waterbirds counts	Number of waterbird species	Counts of Anseriformes	Counts of Charadriformes	Counts of Gruiformes	Counts of Pelecaniformes	Counts of Podicipediformes	Counts of resident birds	Counts of dabbling ducks
1	11	2007	W	598	19	501	14	39	8	8	532	484
.	12	2007	W	567	24	453	16	62	9	9	501	437
.	1	2008	W	838	20	657	13	105	4	16	726	578
2	2	2008	SM	895	20	674	12	162	9	21	810	614
.	3	2008	SM	1110	22	689	25	302	5	73	1005	618
.	4	2008	SM	548	19	283	9	133	12	75	540	218
.	5	2008	BR	427	23	228	7	129	16	29	418	192
.	6	2008	BR	546	16	205	1	240	3	39	540	167
3	7	2008	BR	816	18	214	11	303	11	43	808	194
.	8	2008	AM	615	20	435	5	91	18	23	603	429
4	9	2008	AM	686	23	527	25	52	33	19	504	499
.	10	2008	AM	863	30	711	39	51	18	13	630	673
.	11	2008	W	663	22	478	42	109	5	11	590	433
5	12	2008	W	934	20	512	58	328	2	15	888	455
.	1	2009	W	920	20	656	6	240	1	10	809	600
.	2	2009	SM	1545	19	898	4	596	11	26	1366	841
6	3	2009	SM	1164	26	710	14	357	16	48	1033	623
.	4	2009	SM	650	27	341	13	199	19	62	631	297
.	5	2009	BR	411	30	180	34	124	18	41	383	162
.	6	2009	BR	444	17	151	4	209	20	35	443	139
7	7	2009	BR	614	14	208	4	159	9	42	610	199
.	8	2009	AM	552	28	53	74	23	36	16	521	49
8	9	2009	AM	474	22	390	9	15	18	8	346	383
9	3	2012	SM	1268	22	772	11	357	17	63	1095	632
10	4	2012	SM	.	.	.	.	.	.	.	.	.
11	5	2012	BR	390	17	191	2	132	9	35	386	165
12	6	2012	BR	378	20	126	23	152	15	37	365	96

Sampling	Month	Year	Phenology	Total waterbirds counts	Number of waterbird species	Counts of Anseriformes	Counts of Charadriformes	Counts of Gruiformes	Counts of Pelecaniformes	Counts of Podicipediformes	Counts of resident birds	Counts of dabbling ducks
13	7	2012	BR	588	18	252	3	164	12	41	582	237
14	8	2012	AM	668	24	392	54	64	36	13	547	366
15	9	2012	AM	776	25	526	57	39	26	9	487	518
16	10	2012	AM	903	21	669	31	26	15	4	677	662
17	11	2012	W	866	26	605	12	177	6	4	754	532
18	12	2012	W	1050	24	740	16	187	2	11	831	629
19	1	2013	W	1326	21	969	29	241	5	24	942	859
20	2	2013	SM	1771	24	1162	128	391	10	36	1576	1059
21	3	2013	SM	1579	21	1047	8	424	17	60	1470	927
22	4	2013	SM	842	22	419	10	275	17	58	815	321
23	5	2013	BR	390	22	186	9	128	13	27	379	152
24	6	2013	BR	447	27	162	15	174	29	40	426	146
25	7	2013	BR	600	23	281	8	155	20	44	592	254
26	8	2013	AM	580	26	234	19	73	17	52	560	205
27	9	2013	AM	685	28	479	35	48	25	13	460	464
28	10	2013	AM	861	21	646	12	48	18	12	784	612
29	11	2013	W	897	24	674	45	132	7	7	784	599
30	12	2013	W	1043	24	734	29	158	13	12	899	583
31	1	2014	W	1110	25	765	28	153	12	19	959	572
32	2	2014	SM	1164	24	851	19	159	10	19	1046	645
33	3	2014	SM	1683	25	1147	17	352	12	80	1553	907
34	4	2014	SM	630	20	349	8	148	12	45	605	221
35	5	2014	BR	516	18	261	15	116	9	30	496	129
36	6	2014	BR	498	23	236	5	80	26	30	470	183
37	7	2014	BR	559	22	188	11	80	15	27	547	149
38	8	2014	AM	.	.	.	.	.	.	.	.	.
39	9	2014	AM	620	25	469	5	47	19	21	582	456

Sampling	Number of dabbling duck species	Counts of diving ducks	Number of diving duck species	Counts of Anseriformes and Charadriiformes	Counts of non-Anseriformes and non-Charadriiformes	Counts of grazers	Counts of divers	Counts of breeding couples of Anseriformes	Counts of all breeding couples excluding Charadriiformes	Counts of all breeding couples excluding Anseriformes	Counts of winter visitor birds	Counts of summer visitor birds
1	6	17	2	515	55	42	42	73	277	204	586	15
.	6	15	2	468	80	72	64	73	277	204	557	9
.	6	78	2	669	125	135	168	70	245	175	775	31
2	6	60	2	686	192	167	195	70	245	175	852	7
.	6	71	2	714	380	286	351	70	245	175	1052	13
.	4	65	2	292	220	114	179	70	245	175	485	40
.	4	36	3	235	174	110	144	70	245	175	386	23
.	3	38	3	206	282	217	252	70	245	175	514	58
3	3	20	2	225	357	285	303	70	245	175	794	234
.	4	6	1	440	132	67	73	70	245	175	606	40
4	5	28	1	552	104	25	53	70	245	175	642	42
.	6	36	2	748	82	44	68	70	245	175	824	22
.	6	36	2	511	125	96	124	70	245	175	629	7
5	6	56	2	569	345	329	366	70	245	175	887	14
.	6	48	2	654	251	254	267	77	191	117	874	6
.	6	53	2	898	633	611	621	77	191	117	1498	4
6	7	86	2	723	421	325	401	77	191	117	1080	17
.	6	43	2	353	280	175	215	77	191	117	606	17
.	5	18	2	214	183	109	125	77	191	117	388	21
.	3	12	1	155	264	190	202	77	191	117	428	28
7	3	9	1	212	210	137	146	77	191	117	599	188
.	3	4	2	127	75	12	16	77	191	117	539	360
8	4	6	2	398	41	5	11	77	191	117	465	34
9	5	140	2	783	437	329	469	41	234	194	1138	46
10	.	.	.	.	.	.	.	41	234	194	.	.
11	3	24	2	191	176	115	137	41	234	194	366	18
12	3	28	1	147	204	141	167	41	234	194	346	29

Sampling	Number of dabbling duck species	Counts of diving ducks	Number of diving duck species	Counts of Anseriformes and Charadriiformes	Counts of non-Anseriformes and non-Charadriiformes	Counts of grazers	Counts of divers	Counts of breeding couples of Anseriformes	Counts of all breeding couples excluding Charadriiformes	Counts of all breeding couples excluding Anseriformes	Counts of winter visitor birds	Counts of summer visitor birds
13	3	13	2	253	217	147	158	41	234	194	577	114
14	5	26	2	446	113	37	63	41	234	194	645	121
15	4	8	3	583	74	18	22	41	234	194	771	117
16	4	0	0	693	45	15	8	41	234	194	897	153
17	6	63	2	607	187	172	220	41	234	194	800	57
18	6	98	2	743	200	200	279	41	234	194	962	89
19	6	103	2	991	270	247	328	42	293	253	1264	53
20	7	97	2	1284	437	379	453	42	293	253	1673	38
21	5	113	2	1048	501	390	496	42	293	253	1482	20
22	4	91	2	422	350	244	328	42	293	253	760	61
23	4	32	2	193	168	112	142	42	293	253	357	25
24	4	14	2	175	243	147	159	42	293	253	423	30
25	3	25	2	287	219	131	154	42	293	253	571	93
26	4	26	2	250	142	47	73	42	293	253	553	184
27	4	7	2	506	86	30	29	42	293	253	671	85
28	6	26	1	650	78	43	57	42	293	253	827	129
29	6	68	2	712	146	116	173	42	293	253	825	25
30	6	137	2	749	183	162	276	42	293	253	915	70
31	6	177	2	777	184	180	314	38	303	267	946	84
32	6	199	2	863	188	158	338	38	303	267	972	79
33	5	230	2	1154	444	123	546	38	303	267	1468	67
34	4	124	2	353	205	128	248	38	303	267	518	65
35	3	132	2	276	155	90	222	38	303	267	388	86
36	3	42	3	230	136	72	91	38	303	267	462	129
37	3	35	2	195	122	63	94	38	303	267	519	241
38	.	.	.	.	.	.	.	38	303	267	.	.
39	5	3	1	464	87	36	29	38	303	267	615	57

Sampling	Counts of migratory birds	Number of resident species	Number of winter visitor species	Number of summer visitor species	Number of migratory species	Sampling day. Mean Tª (ºC)	Sampling day. Maximum Tª (ºC)	Sampling day. Minimum Tª (ºC)	Sampling day. Mean Humidity (%)	Sampling day. Total precipitation (l/m2)
1	36	15	18	1	5	6.40	7.00	5.60	83.00	0.00
.	30	19	22	1	7	4.70	9.10	-2.00	84.00	1.00
.	48	16	19	1	5	6.00	12.30	1.60	70.00	0.00
2	28	16	19	1	6	9.70	15.50	1.80	80.00	0.00
.	24	17	21	2	7	11.30	18.10	8.40	78.00	0.20
.	38	16	17	2	5	10.30	15.10	3.90	77.00	0.10
.	24	17	16	5	9	12.50	17.40	9.80	83.00	6.10
.	63	14	13	2	4	17.60	22.20	11.60	79.00	11.00
3	239	16	15	2	6	15.70	25.50	5.50	71.00	0.00
.	50	17	17	3	8	16.30	20.80	11.80	84.00	0.00
4	81	19	21	2	10	11.10	17.80	6.60	77.00	0.00
.	57	22	27	2	12	13.90	20.70	8.90	86.00	0.00
.	54	17	20	1	6	10.60	13.50	9.00	92.00	0.30
5	31	16	19	1	5	4.00	7.80	1.10	94.00	0.00
.	13	16	18	1	5	4.50	12.70	-2.60	85.00	0.00
.	14	15	17	1	4	3.90	11.20	-3.00	82.00	0.00
6	32	20	22	3	8	6.20	18.30	-1.30	85.00	0.00
.	25	20	24	4	10	11.40	18.40	4.90	80.00	0.00
.	31	20	25	6	16	15.00	27.10	9.70	85.00	0.00
.	32	16	13	5	6	21.40	27.70	16.10	83.00	3.20
7	192	14	12	2	3	24.10	34.70	14.50	66.00	0.00
.	421	22	23	6	14	15.40	19.90	13.60	90.00	0.00
8	38	18	19	2	7	13.60	21.20	7.30	82.00	0.80
9	65	19	19	1	8	12.70	18.90	5.30	62.00	0.50
10	.	.	.	.	.	6.60	16.40	-0.10	90.00	1.90
11	28	15	15	1	5	21.20	30.70	9.10	74.00	0.00
12	47	18	18	3	7	13.10	18.60	8.50	91.00	1.00

Sampling	Counts of migratory birds	Number of resident species	Number of winter visitor species	Number of summer visitor species	Number of migratory species	Sampling day. Mean Tª (ºC)	Sampling day. Maximum Tª. (ºC)	Sampling day. Minimum Tª. (ºC)	Sampling day. Mean Humidity (%)	Sampling day. Total precipitation (l/m2)
13	123	16	16	2	5	20.70	33.80	5.50	65.00	0.00
14	178	18	22	2	10	23.10	35.90	19.20	85.00	0.00
15	194	19	23	2	11	18.40	28.00	10.30	84.00	0.00
16	193	18	19	1	8	31.80	.	9.70	.	2.70
17	78	20	23	1	8	10.70	19.00	4.30	90.00	0.00
18	97	19	22	1	6	11.70	15.00	9.10	88.00	6.70
19	65	16	20	1	5	4.60	8.30	0.90	80.00	0.40
20	159	17	21	2	9	8.30	14.30	4.20	85.00	1.70
21	28	18	19	2	5	13.60	19.10	10.20	49.00	1.70
22	69	17	19	3	5	15.40	25.00	10.80	74.00	0.00
23	40	16	19	2	9	10.70	22.20	4.70	85.00	3.30
24	51	21	21	5	10	14.10	20.20	7.80	76.00	0.00
25	109	18	19	5	9	19.30	27.10	12.10	75.00	0.00
26	220	20	22	3	10	19.90	33.80	7.70	69.00	0.00
27	137	22	23	6	11	17.70	29.40	7.30	73.00	0.00
28	154	17	19	2	7	16.70	19.60	14.50	82.00	0.90
29	74	19	22	1	7	9.10	11.80	7.30	81.00	1.50
30	104	19	22	1	7	6.10	10.70	2.10	85.00	0.00
31	117	20	23	1	7	8.70	11.30	4.50	87.00	0.50
32	102	19	22	1	7	5.70	13.90	-3.00	65.00	0.00
33	91	20	21	1	9	8.00	10.80	4.80	93.00	0.20
34	73	16	18	2	5	13.20	22.90	4.30	79.00	0.00
35	95	16	15	3	6	13.70	22.90	5.20	82.00	0.00
36	139	18	19	4	8	15.80	22.20	8.80	76.00	0.00
37	258	18	19	3	9	19.70	29.30	10.60	78.00	0.00
38	.	.	.	.	.	17.00	22.70	13.30	.	0.00
39	75	19	22	2	10	14.30	17.60	9.10	89.00	0.10

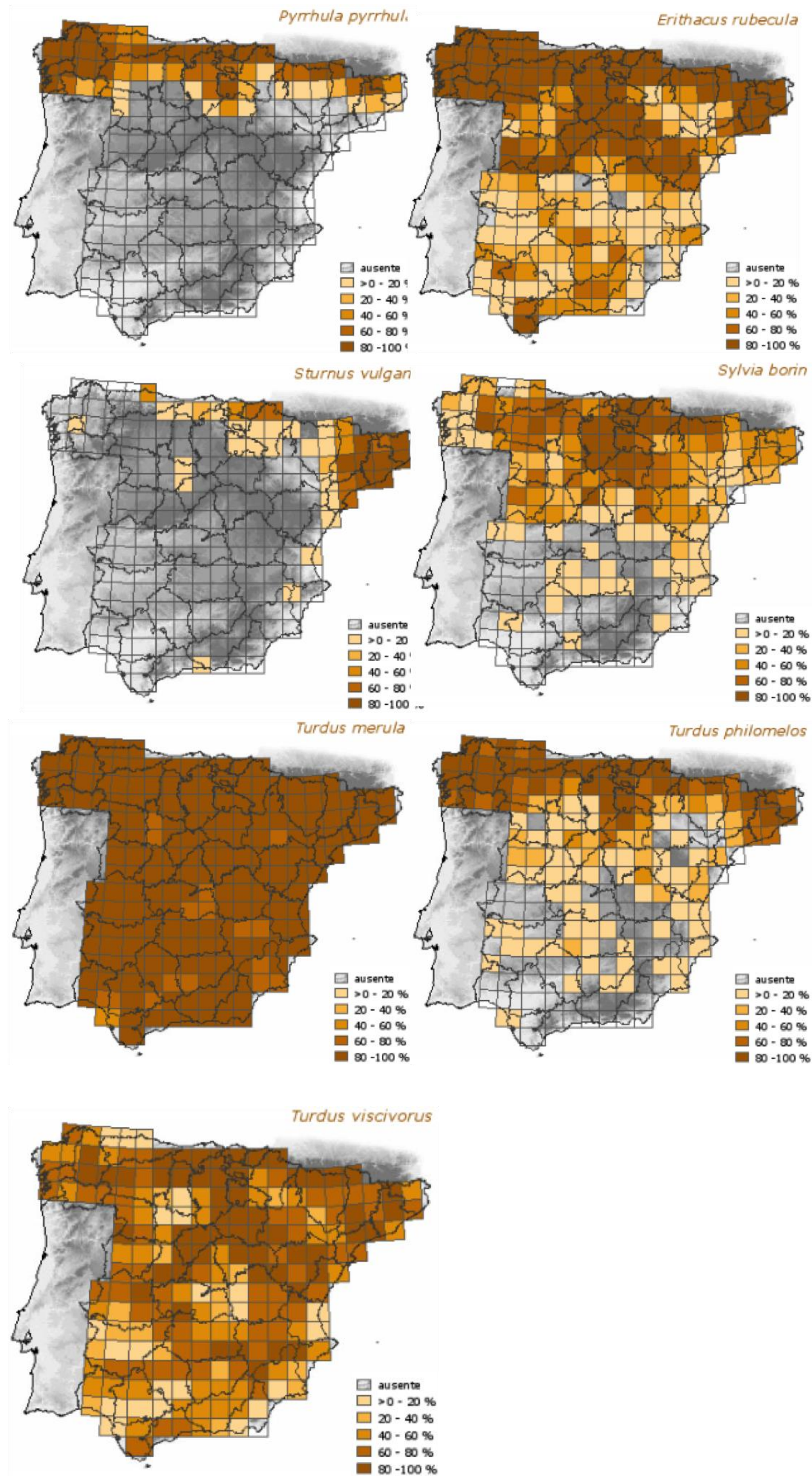
Sampling	Sampling day. Mean wind (Km/h)	Sampling day. Maximum gust of wind (Km/h)	15 days before sampling. Mean Tª (ºC)	15 days before sampling. Maximum Tª (ºC)	15 days before sampling. Minimum Tª (ºC)	15 days before sampling. Mean Humidity (%)	15 days before sampling. Total precipitation (l/m2)	15 days before sampling. Mean wind (Km/h)	15 days before sampling. Maximum gust of wind (Km/h)
1	11.60	38.90	5.75	14.80	-8.50	77.27	17.10	7.45	42.80
.	6.10	28.80	2.87	12.00	-8.10	81.13	3.80	4.91	56.50
.	8.20	46.40	5.97	16.00	-5.50	82.80	21.90	7.76	53.30
2	2.80	19.40	8.02	20.00	-2.90	71.93	0.30	3.93	49.00
.	4.80	50.40	7.01	22.80	-0.50	78.00	97.10	4.57	74.50
.	3.70	32.40	8.85	19.40	-1.40	77.93	68.60	6.04	71.30
.	6.20	50.40	13.67	26.50	0.30	76.60	66.00	3.14	45.40
.	7.90	34.20	17.06	32.20	4.90	78.93	73.50	6.96	51.80
3	5.70	31.00	17.55	31.10	6.80	77.20	26.30	8.47	51.80
.	8.10	32.00	16.69	30.10	6.30	75.86	0.70	7.36	45.40
4	5.80	32.00	14.65	30.40	4.90	80.47	13.40	5.26	46.40
.	3.10	17.30	12.78	24.90	1.10	79.60	25.70	5.10	42.10
.	10.20	44.50	3.73	13.90	-2.10	84.20	39.10	5.39	45.40
5	2.70	31.80	6.38	15.10	1.20	92.18	17.20	9.09	56.40
.	6.30	25.70	2.41	15.20	-7.80	91.47	3.10	5.92	42.00
.	7.60	28.90	5.49	13.70	-2.50	87.07	8.10	9.52	67.40
6	4.30	31.40	6.69	19.80	-2.20	87.80	45.50	7.85	84.70
.	6.90	28.90	9.51	20.30	1.10	84.13	34.30	8.86	51.90
.	6.80	38.40	10.40	28.20	0.90	84.17	18.90	8.81	51.90
.	4.10	35.60	16.51	32.60	5.60	79.53	12.70	8.81	66.70
7	4.50	42.70	17.72	34.70	7.00	77.87	1.50	8.35	60.00
.	9.10	29.30	18.35	35.10	6.70	82.00	3.40	8.35	65.60
8	3.00	20.10	16.54	32.10	4.10	82.73	6.60	7.07	42.70
9	6.30	42.00	7.60	26.10	-3.40	83.60	4.70	7.15	47.60
10	6.40	39.50	8.07	21.50	1.00	90.00	21.10	10.28	55.40
11	3.10	21.50	11.51	26.20	0.50	86.00	44.20	9.04	77.60
12	6.00	35.30	17.18	33.50	4.50	82.47	14.50	7.25	60.00

Sampling	Sampling day. Mean wind (Km/h)	Sampling day. Maximum gust of wind (Km/h)	15 days before sampling. Mean Tª (ºC)	15 days before sampling. Maximum Tª. (ºC)	15 days before sampling. Minimum Tª. (ºC)	15 days before sampling. Mean Humidity (%)	15 days before sampling. Total precipitation (l/m^2)	15 days before sampling. Mean wind (Km/h)	15 days before sampling. Maximum gust of wind (Km/h)
13	3.80	26.10	16.55	32.70	3.00	80.07	1.30	8.18	61.40
14	9.60	48.30	22.75	41.40	7.50	69.33	0.00	5.96	56.40
15	5.60	28.20	17.75	33.10	4.80	82.00	1.20	6.61	43.10
16	6.00	35.30	14.47	30.40	2.80	84.93	13.50	4.68	51.20
17	1.90	13.80	8.58	21.40	-1.00	89.00	27.90	6.54	78.00
18	14.80	73.70	4.47	13.20	-2.30	91.33	35.00	7.15	54.30
19	10.90	44.80	5.04	13.90	-4.60	92.27	119.50	7.55	60.70
20	2.30	11.30	4.35	14.80	-1.40	90.00	107.70	8.20	66.00
21	18.70	74.80	4.93	19.20	-5.70	76.93	36.50	7.98	48.30
22	4.20	27.20	10.09	24.60	0.40	52.00	49.20	11.51	73.40
23	5.80	45.50	10.89	24.30	2.10	80.20	35.50	7.88	45.50
24	10.00	36.70	15.25	31.40	7.00	81.13	41.10	6.98	42.70
25	5.20	27.90	20.89	32.90	9.90	76.00	17.30	6.27	50.10
26	1.70	15.20	18.04	29.90	8.20	80.27	16.30	6.92	44.10
27	2.10	22.90	14.87	28.90	5.00	83.47	2.20	5.62	34.90
28	16.10	48.00	14.29	27.60	-0.20	82.00	17.20	6.71	52.90
29	11.60	36.70	11.59	20.20	1.10	86.87	44.00	9.00	60.30
30	3.40	26.50	2.62	14.10	-4.90	89.87	6.50	3.39	35.30
31	7.90	44.50	8.67	18.20	-1.70	79.13	19.00	12.46	90.00
32	5.20	38.10	7.04	21.30	-1.70	75.67	31.00	15.63	95.60
33	8.40	36.70	8.47	25.90	-1.30	74.00	44.30	6.43	63.90
34	4.70	28.60	12.33	25.90	4.20	76.80	6.20	8.61	55.40
35	6.00	39.90	11.79	25.90	1.10	79.40	15.10	8.11	46.60
36	8.60	37.80	17.09	30.10	4.50	74.67	1.80	7.65	59.30
37	4.60	25.40	16.43	31.20	6.00	80.60	21.80	7.79	44.50
38	7.50	34.20	18.27	32.30	7.90	74.07	7.70	6.88	49.40
39	6.40	28.90	18.75	30.30	9.40	79.93	37.70	4.43	66.70

Sampling	7 days before sampling. Mean Tª (ºC)	7 days before sampling. Maximum Tª. (ºC)	7 days before sampling. Minimum Tª. (ºC)	7 days before sampling. Mean Humidity (%)	7 days before sampling. Total precipitation (l/m2)	7 days before sampling. Mean wind (Km/h)	7 days before sampling. Maximum gust of wind (Km/h)	Number of samples analysed	Number of AIV positive samples	AIV prevalence (%)
1	7.30	14.80	2.40	81.29	12.60	9.27	42.80	95	0	0.00
.	5.76	12.00	-3.60	78.43	0.00	6.27	56.50	.	.	.
.	8.51	16.00	0.60	82.00	17.00	10.03	53.30	.	.	.
2	9.34	20.00	0.00	75.43	0.10	3.30	38.50	154	0	0.00
.	5.17	12.40	-0.40	84.57	79.30	2.23	50.40	.	.	.
.	9.36	19.40	-1.40	75.29	24.60	7.31	71.30	.	.	.
.	12.50	21.20	4.80	80.43	55.00	4.63	45.40	.	.	.
.	19.59	32.20	4.90	74.86	11.50	6.04	51.80	.	.	.
3	16.54	30.70	6.80	74.14	0.00	8.69	41.00	35	11	31.40
.	16.69	30.10	6.30	75.86	0.70	7.36	45.40	.	.	.
4	15.23	27.60	8.10	79.71	0.00	5.36	35.30	47	1	2.10
.	15.43	24.90	4.20	80.00	0.00	4.97	40.70	.	.	.
.	4.07	7.40	0.60	86.29	16.90	5.89	42.10	.	.	.
5	7.16	15.10	1.30	92.43	12.70	6.41	44.50	136	0	0.00
.	2.26	11.00	-5.10	90.29	0.60	6.19	42.00	.	.	.
.	5.14	11.40	-2.50	86.86	2.30	7.66	50.40	.	.	.
6	6.43	12.60	1.20	90.00	35.70	10.00	84.70	98	1	1.02
.	9.81	16.00	2.60	84.57	10.50	10.09	51.90	.	.	.
.	10.69	28.20	2.50	85.57	0.90	9.83	41.30	.	.	.
.	17.09	32.60	9.90	79.86	1.60	10.50	66.70	.	.	.
7	19.03	34.70	9.70	74.29	0.00	8.31	60.00	11	2	18.20
.	17.91	32.40	9.70	85.00	0.80	8.81	40.90	.	.	.
8	15.41	27.80	9.30	83.71	6.40	8.64	42.70	91	29	31.90
9	9.10	26.10	-2.70	80.86	0.00	6.44	39.90	80	0	0.00
10	7.09	13.10	1.00	89.71	15.80	12.46	55.40	134	0	0.00
11	13.44	26.20	2.80	86.43	16.60	9.60	66.70	50	0	0.00
12	16.16	29.40	4.50	82.29	13.20	8.51	60.00	31	1	3.23

Sampling	7 days before sampling. Mean Tª (ºC)	7 days before sampling. Maximum Tª. (ºC)	7 days before sampling. Minimum Tª. (ºC)	7 days before sampling. Mean Humidity (%)	7 days before sampling. Total precipitation (l/m2)	7 days before sampling. Mean wind (Km/h)	7 days before sampling. Maximum gust of wind (Km/h)	Number of samples analysed	Number of AIV positive samples	AIV prevalence (%)
13	16.31	32.10	4.70	82.00	1.30	9.00	61.40	111	1	0.90
14	24.17	40.00	8.20	64.43	0.00	6.31	56.40	23	0	0.00
15	17.47	32.60	4.80	82.86	0.80	6.31	43.10	16	0	0.00
16	12.66	23.70	3.60	88.43	13.50	5.76	48.70	63	0	0.00
17	8.47	21.40	0.70	91.00	14.30	4.31	48.30	119	0	0.00
18	4.16	11.80	-2.30	93.00	7.60	5.29	54.30	84	0	0.00
19	6.43	13.90	1.70	92.86	112.20	11.07	60.70	89	0	0.00
20	4.21	12.80	-1.40	90.00	48.00	5.69	46.90	111	0	0.00
21	7.16	19.20	-5.00	66.71	1.30	8.89	48.30	183	0	0.00
22	13.49	24.60	3.60	38.43	5.10	14.23	72.30	11	0	0.00
23	12.06	21.20	2.10	80.00	9.20	8.77	45.50	54	0	0.00
24	12.94	23.90	7.40	86.00	37.40	7.47	40.90	.	.	.
25	21.09	32.90	12.50	76.29	17.30	6.04	50.10	52	0	0.00
26	18.41	29.90	8.30	77.29	0.00	6.61	43.10	139	2	1.44
27	15.37	28.90	5.00	82.00	0.30	5.67	34.90	162	2	1.23
28	10.94	21.10	-0.20	82.57	1.70	5.67	37.00	164	0	0.00
29	11.49	18.70	1.10	87.57	13.20	10.03	55.40	204	1	0.49
30	1.76	14.10	-4.90	91.71	6.10	2.37	20.40	91	0	0.00
31	6.64	18.20	-1.70	83.57	10.60	6.34	51.20	125	0	0.00
32	7.44	21.30	-1.50	77.29	14.30	14.81	70.60	131	0	0.00
33	8.57	25.90	-1.30	71.43	0.20	5.63	42.70	28	0	0.00
34	12.59	25.90	4.20	79.00	3.70	8.73	41.30	.	.	.
35	12.31	25.90	1.10	78.00	1.30	7.39	38.80	141	0	0.00
36	17.81	30.00	10.20	77.43	1.40	9.43	43.70	84	1	1.19
37	15.67	25.20	7.70	81.00	4.70	8.19	41.30	116	0	0.00
38	16.06	27.00	7.90	67.00	7.60	6.44	39.20	41	0	0.00
39	18.13	27.60	10.20	79.14	28.80	4.81	66.70	88	0	0.00

Annex III. Geographical distribution of the AIV -positive passerine species in Spain. Maps reproduced from (Carrascal, 2006)



Annex IV. List and number of individuals of sampled passerine species.

Sampled species	AIV+	N	Sampled species cont.	AIV+	N
Acrocephalus arundinaceus		10	*Parus major*		1
Acrocephalus paludicola		2	*Passer domesticus*		4
Acrocephalus schoenobaenus		13	*Passer hispaniolensis*		2
Acrocephalus scirpaceus		67	*Passer montanus*		5
Aegithalos caudatus		3	*Phoenicurus phoenicurus*		2
Carduelis carduelis		5	*Phylloscopus collybita*		25
Carduelis chloris		2	*Phylloscopus ibericus*		2
Carduelis spinus		1	*Phylloscopus trochiloides*		1
Cettia cetti		11	*Phylloscopus trochilus*		10
Cisticola juncidis		2	*Pica pica*		11
Coccothraustes coccothraustes		1	*Prunella modularis*		2
Corvus corax		3	*Pyrrhula pyrrhula*	1	1
Corvus corone		9	*Regulus ignicapilla*		1
Emberiza schoeniclus		1	*Remiz pendulinus*		2
Erithacus rubecula	1	24	*Riparia riparia*		8
Ficedula hypoleuca		9	*Saxicola rubetra*		1
Fringilla coelebs		3	*Saxicola torquata*		1
Garrulus glandarius		2	*Sturnus unicolor*		1
Hippolais polyglotta		11	*Sturnus vulgaris*	1	54
Hirundo rustica		7	*Sylvia atricapilla*		22
Locustella luscinioides		1	*Sylvia borin*	1	18
Locustella naevia		7	*Sylvia communis*		6
Luscinia megarhynchos		6	*Troglodytes troglodytes*		3
Luscinia svecica		9	*Turdus iliacus*		10
Motacilla alba		1	*Turdus merula*	2	31
Muscicapa striata		3	*Turdus philomelos*	3	126
Oenanthe oenanthe		1	*Turdus pilaris*		2
Panurus biarmicus		1	*Turdus viscivorus*	1	2
Parus caeruleus		2	**Total**	**10**	**571**

Number of individuals sampled (N) and number of AIV-positive individuals (AIV+) is specified.