

Tracking possible ways of transmission of the highly pathogenic avian influenza virus H5N1 to Greece using ringing recoveries of wild birds

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Received: 26 November 2009

Accepted after revision: 3 November 2010

In recent years, a highly pathogenic type of avian influenza virus H5N1 was spread from south-eastern Asia through China, Mongolia and Russia to Europe creating outbreaks and resulting in massive deaths to poultry, and some wild bird species. It has been much debated whether wild birds play an active role in H5N1 geographical spread. We used recoveries of some bird species ringed near outbreak areas to track the possibility of transmission of H5N1 to Greece. Recoveries of great cormorant, grey heron, mute swan, mallard, common pochard, tufted duck, common coot and black-headed gull suggested as a possible way of transmission areas of Russia, Ukraine and eastern Balkans. These areas had the highest number of outbreaks and are situated near the mostly affected areas of Asia. Another possible way of H5N1 transmission to Greece, as suggested by recoveries of great cormorant, white stork, greater flamingo, common pochard and common moorhen is from west and northwest European countries, especially areas of Germany, France and Czech Republic. The role of wild birds in the virus transmission over long distances remains unclear and outbreak incidents in Western Europe and Greece in 2006 rather followed a cold spell in the Black Sea region spreading the virus through infected wild birds travelling short distances. In Greece, only dead waterfowl, mainly mute swans, were detected carrying the virus without any apparent temporal or geographical pattern.

Key words: H5N1, wild birds, virus transmission, Greece.

INTRODUCTION

Bird flu (avian influenza) is a contagious poultry disease that manifests with mild to severe forms of disease (Padhi *et al.*, 2004). Aquatic birds worldwide are a natural reservoir of influenza viruses A, which can be transmitted to other species and humans (Webster *et al.*, 1992). Some subtypes (H5 and H7) can become highly pathogenic when transmitted to poultry. In this case, they can cause outbreaks of highly pathogenic avian influenza (HPAI) (Olsen *et al.*, 2006).

In 1997, an outbreak of HPAI H5N1 caused massive deaths in poultry in Hong Kong also resulting in human infection and deaths (De Jong *et al.*, 1997; Subbarao *et al.*, 1998). Follow-up studies in central China from 1999 showed that the H5N1 virus was endemic in

domestic birds in the region and that multiple evolutionary lines of the virus existed (Li *et al.*, 2004; Chen *et al.*, 2006). HPAI H5N1 reappeared in 2002 in aquatic birds in two Hong Kong parks and other wild and captive birds (Ellis *et al.*, 2004) whereas by 2004 it had devastated the poultry industry in large areas of south Asia forming a great pandemic threat to humans (WHO, 2005). In 2005, virus outbreaks killed numbers of wild migratory birds in Qinghai Lake, China (Liu *et al.*, 2005) further expanding in China and Mongolia (Munster *et al.*, 2006; Olsen *et al.*, 2006). Further, outbreaks concerning domestic and/or wild birds with virus strains genetically related to those of Lake Qinghai were reported in Russia and Kazakhstan (Lvov *et al.*, 2006), in Turkey, Romania, Ukraine and Croatia (October 2005) and in Ukraine (December 2005) (FAO, 2006). The initial outbreaks in Romania, Turkey and Ukraine, occurred close to wetlands used by waterfowl and waders, coinciding with their autumn

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migration movements, far from any known area of the virus occurrence (Gilbert *et al.*, 2006).

In spring of 2006, the virus was transmitted to large numbers of mute swans (*Cygnus olor*) and other wild birds across the Western Europe, in areas where, despite constant surveillance, previous outbreaks in domestic birds had not been reported (Gilbert *et al.*, 2006). These outbreaks took place during unusual movements of waterfowl and waders, associated with a cold spell in the Black Sea where it is believed that the virus H5N1 (HPAI) had been endemic since autumn 2005 (Gilbert *et al.*, 2006; Gauthier-Clerc *et al.*, 2007). After these events, wild birds were suspected of spreading the virus HPAI H5N1 over long distances during migration (Li *et al.*, 2004; Chen *et al.*, 2005).

Although many wild birds are reservoirs of influenza A virus, aquatic birds such as Anseriformes (ducks, geese and swans) and Charadriiformes (particularly gulls, terns and waders) constitute the largest natural reservoir of low pathogenic influenza viruses (LPAI) (Webster *et al.*, 1992) and have an almost global distribution, except for the more arid regions of the world (Del Hoyo, 1996). Ducks (Anatidae, Anatinae) present the highest risk of transmission to domestic birds (Delogu *et al.*, 2003), because: a) they host avian influenza viruses with the greatest diversity and distribution (Stallknecht & Shane, 1988), b) they have primarily been associated with virus strains in historical outbreaks of HPAI in poultry (Munster *et al.*, 2006), c) they may be excreting large amounts of HPAI H5N1 virus while remaining relatively healthy and capable of carrying the virus over long distances (Hulse-Post *et al.*, 2005) and d) they have more frequent direct contacts with domestic fowls than with other groups of wild birds (Tracey *et al.*, 2004). In Europe, the species mostly affected were mute swan and whooper swan (*Cygnus cygnus*), whereas mortality also occurred in other species of waterfowl, waders and occasionally in raptors, gulls and herons (Olsen *et al.*, 2006). So far, the strain of HPAI H5N1, which originated from Southeast Asia, has been the cause of death for more than 60 species of wild birds (Ellis *et al.*, 2004; Olsen *et al.*, 2006).

Poultry trade and transport of contaminated materials are possible ways of spread of HPAI in general (Alexander, 2000). There is no doubt that for the preservation and spread of H5N1 virus in many countries, particular farming practices and agro-ecologic conditions have played a key role (Gilbert *et al.*, 2006; Martin *et al.*, 2006). Nevertheless, migratory birds can carry pathogens, particularly those that do not signif-

icantly affect their health and therefore do not impede migration (Olsen *et al.*, 2006). Many species of waterfowl and waders make regular long distance trips (Del Hoyo, 1996) which may result in the transfer of LPAI between countries or even continents. Many bird populations within large continents occur along the main migration routes in high densities (Alerstam *et al.*, 1990) being further associated spatially and temporally with favourable stopover, breeding and wintering areas. Therefore, infected birds could transmit viruses to other populations and these in turn carry the viruses into new regions (Olsen *et al.*, 2006).

Due to its particular position and habitat diversity, Greece constitutes a major migration corridor between Europe and Africa and wintering area for numerous waterfowl breeding in the north (Athanasίου, 1987; Handrinos & Akriotis, 1997). Thus, at least some species, having been in contact with the H5N1 virus in outbreak areas of other countries, are expected to transmit it to Greece. In this paper we examined the possibility of transmission of HPAI H5N1 virus based on recoveries of particular bird species ringed near outbreak areas; we also aimed at finding out which species are most likely to transmit the virus and from which areas.

MATERIALS AND METHODS

From a total of 60 species of wild birds found to be infected to date with HPAI H5N1 around the world (Ellis *et al.*, 2004; Olsen *et al.*, 2006), 38 occur in Greece. To understand the mechanisms of the geographical spread of H5N1 by wild birds, it is necessary to track their migratory movements. This research was based on 13 species for which there are available published ringing and recovery data (Akriotis & Handrinos, 2004). The species studied are the following (common names according to Birdlife International, www.birdlife.org):

1. Great cormorant (*Phalacrocorax carbo*)
2. Grey heron (*Ardea cinerea*)
3. Little egret (*Egretta garzetta*)
4. White stork (*Ciconia ciconia*)
5. Greater flamingo (*Phoenicopterus ruber*)
6. Mute swan (*Cygnus olor*)
7. Mallard (*Anas platyrhynchos*)
8. Common pochard (*Aythya ferina*)
9. Tufted duck (*Aythya fuligula*)
10. Common buzzard (*Buteo buteo*)
11. Common coot (*Fulica atra*)
12. Common moorhen (*Gallinula chloropus*)
13. Black-headed gull (*Larus ridibundus*)

Using the coordinates of ringing areas abroad and recovery sites of each species in Greece (Akriotis & Handrinos, 2004), maps were created using the Google Earth software, thus illustrating a rough picture of the origin of birds arriving in Greece. Then, H5N1 outbreaks were located near ringing areas based on data compiled from information held by the Food and Agricultural Organization (F.A.O.) (www.fao.org), the World Organization for Animal Health (O.I.E.) (www.oie.int) and various government sources. These were provided to *Nature* by the U.N. F.A.O. Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases (EMPRES) (www.fao.org/EMPRES) (Butler, 2006). The distances of the birds' ringing areas from the virus outbreaks were measured on the Google Earth maps. Ranges of distances were used to describe distances of the birds' ringing areas from the virus outbreaks. Within a particular range, distances from the virus outbreaks were averaged, if more than one. Information was also compiled on group size, flock density, mixing with other species and contact risk with poultry according to Atkinson *et al.* (2006), Pfeiffer *et al.* (2006) and Veen *et al.* (2007), as an indication of the possibility of the virus transmission by the species studied. The information considered did not concern the transmission possibilities during breeding.

The available ringing data used in the analysis originated between 1985 and 2004, partly differing from the years of outbreaks of the virus in many areas. Thus, all analyses in this paper are based on the hypothesis that the migration routes of birds studied have not changed substantially in recent years.

The names and the borders of the countries presented on maps of this paper is responsibility of Google Earth.

RESULTS

Below, the bird species or groups are presented taxonomically.

Pelecaniforms

Great cormorant

Behavioural characteristics of cormorant (Table 1) reflect considerable possibilities of H5N1 transmission. Of 57 ringed great cormorants recovered in Greece, most originated from northern European countries, such as Denmark, Sweden and Germany, but also from Romania and Ukraine (see online supplementary material, Fig. S1). Of them, 50 were located

near H5N1 outbreak areas and a total of 21 ringing sites were situated near 56 outbreaks.

Distances to outbreaks varied from 4 km (Rügen, Germany) to 109 km (Chernomorskiy, Ukraine) (Butler, 2006). Most distances were reported up to 40 km far from the ringing sites most of which were situated in some north and south-eastern European countries (see online supplementary material, Fig. S2).

Ardeids and flamingos

Grey heron

Behavioural characteristics of grey heron (Table 1) reflect medium possibilities of H5N1 transmission. Ringed grey herons recovered in Greece ($n = 19$) mainly originated from northeastern and eastern European countries (see online supplementary material, Fig. S3). Seven birds were ringed near H5N1 outbreak areas whereas four ringing sites were situated near 19 outbreaks. The ringing sites nearest to outbreaks (and a higher number of birds ringed) occurred in Ukraine and Romania (21-80 km) (see online supplementary material, Fig. S2). One ringing place in Bulgaria was close to eight different outbreak sites in Romania, distant less than 80 km.

The Crimean Peninsula in Ukraine may be considered as a critical area for the possible spread of the virus by the grey heron to Greece due to the high density of outbreak sites and its proximity to the mostly affected areas of Asia. Romania may also be a critical area due to the high density of outbreaks.

Little egret

Behavioural characteristics of little egret (Table 1) suggest a rather limited possibility of transmission of H5N1. Seven little egrets were recovered in Greece, originating from Balkan and east European countries but also from Tunisia (see online supplementary material, Fig. S4). Six of the recovered birds were ringed near H5N1 outbreaks whereas five ringing areas were near 18 outbreaks. Most outbreak distances in Romania did not exceed 60 km from ringing areas (see online supplementary material, Fig. S2), but the evidence for the role of the little egret in the possible spread of the virus to Greece is inadequate due to the small number of ringing recoveries.

White stork

Behavioural characteristics of white stork (Table 1) suggest a rather low possibility of H5N1 transmission.

TABLE 1. Behavioural characteristics of bird species studied in relation to their possible transmission potential of H5N1 (according to Veen *et al.*, 2006)

	Group size	Flock density ^a	Mixing with other species ^b	Contact risk with poultry ^c
Great cormorant	Medium ^d	Medium ^e	High	Medium
Grey heron	Small ^f	Low ^h	Medium	Medium
Little egret	Medium	Medium	Medium	Low
White stork	Large ^g	Low	Medium	Low
Greater flamingo	Large	High	Medium	No
Mute swan	Medium	Low	Medium ⁱ	Medium
Mallard	Medium	High ^j	High	High
Common pochard	Medium	High	High ⁱ	Low
Tufted duck	Medium	High	High ⁱ	Low
Common buzzard ^k	Small	Low	Low	Low
Common coot	Large ^g	High	High	Medium
Common moorhen	Small	Low	Low	Medium
Black-headed gull	Large	Medium	High	High

^a During migration and winter

^b Mixed foraging, mixed roosts, mixing at moulting areas

^c As living in proximity to domestic poultry and could be considered as bridge species (Pfeiffer *et al.*, 2006)

^d Several tens to few hundred individuals

^e Often 2-5 m between individuals

^f Often up to a few tens of birds

^g Several hundreds to thousands of individuals

^h Often > 5 m between individuals

ⁱ Atkinson *et al.* (2006)

^j Often < 2 m between individuals

^k Behavioural characteristics (except for contact risk with poultry) were evaluated by the authors

Most of the 37 ringed white storks recovered in Greece originated from the north and northwest, mainly from Germany ($n = 23$, 62%) (see online supplementary material, Fig. S5). The sites where 31 storks were ringed were situated near H5N1 outbreaks whereas 27 ringing sites were situated near a total of 60 H5N1 outbreak areas in a considerable number of countries, mostly along the eastern migration corridor of the species. Most outbreak areas were up to 60 km distant from ringing sites, with Germany being the most critical for the spread of the virus due to both high number of outbreaks and ringing sites in their vicinity (see online supplementary material, Fig. S2).

Greater flamingo

Behavioural characteristics of greater flamingo (Table 1) reflect low possibilities of H5N1 transmission. Most of the 26 ringed flamingos recovered in Greece came from west European countries (France and Spain, totally $n = 20$, 77%), with some coming from Turkey and one from Kazakhstan (see online supplementary material, Fig. S6). In total, five ringing sites occurred near

11 outbreaks. Nineteen birds were recovered from places close to outbreak areas in France, Turkey and Iraq. France (Ain, Saint-Mitre-les-Remparts) seems to be the most critical country for the spread of the virus to Greece due to the highest numbers of flamingos ($n = 14$) ringed in less than 21-40 km away from outbreaks (see online supplementary material, Fig. S7).

Swans and ducks

Mute swan

Behavioural characteristics of mute swan (Table 1) suggest a medium possibility of H5N1 transmission. It is one of the 26 species which pose a relatively high risk of spreading H5N1 along their migration routes from outbreak sites outside the European Community to within EC borders (Atkinson *et al.*, 2006). Mute swans are more likely to act as sentinels for HPAI H5N1 in wild bird populations (Teifke *et al.*, 2007).

Seven (58%) of the 12 ringed mute swans recovered in Greece originated from Ukraine and the rest from north European countries (see online supplementary material, Fig. S8). Ten of the recoveries con-

cerned birds ringed near H5N1 outbreaks whereas in total, six ringing sites were situated near 19 outbreak areas. Most mute swans' ringing sites were up to 60 km away from outbreaks detected especially in Ukraine (where also a highest number birds were ringed) (see online supplementary material, Fig. S9). Therefore, the Crimean Peninsula in Ukraine can be considered as the most critical area for the possible spread of the virus by mute swan to Greece.

Mallard

Behavioural characteristics of mallard (Table 1) suggest a high possibility of H5N1 transmission. It is one of the two species posing a relatively higher risk of spreading H5N1 along their migration routes from outbreak areas outside the European Community to within EC borders (Atkinson *et al.*, 2006).

Of 35 ringed mallards recovered to Greece, most ($n = 32$, 91%) originated from Russia (district of Astrakhan region) (see online supplementary material, Fig. S10). Of them, 24 were ringed near H5N1 outbreaks whereas totally 13 ringing sites were situated near 26 outbreak areas. Most distances from outbreaks were from 60 to over 100 km away from ringing sites being highest in number in Russia, mainly near the Volga Delta area (see online supplementary material, Fig. S9). The latter constitutes one of the most critical areas for virus transmission to Greece, considering its geographical proximity to heavily affected areas in Asia.

Common pochard

Behavioural characteristics of common pochard (Table 1) suggest some possibilities of H5N1 transmission. Pochard is one of the 26 species which pose a relatively high risk of spreading H5N1 along their migration routes from outbreak sites outside the European Community to within EC borders (Atkinson *et al.*, 2006).

Common pochards recovered in Greece ($n = 15$) were ringed mainly in Russia ($n = 8$, 53%) and most of the rest in Western Europe (see online supplementary material, Fig. S11). Of birds recovered, 14 were ringed near H5N1 outbreak areas, while totally 11 ringing sites were near 33 outbreaks. The distances from outbreaks were in most cases less than 100 km away from ringing sites, being highest in number in Russia but, within the framework of the available sample, also in Germany and Switzerland (see online supplementary material, Fig. S9). The regions of No-

vosibirsk, Omsk, Kurgan, Tiumen, and Chelyabinsk in Russia, where all outbreaks were located, can be considered as the most critical for the possible spread of the virus by the common pochard to Greece due to the high density of outbreak sites and their proximity to the mostly affected areas of Asia.

Tufted duck

Behavioural characteristics of tufted duck (Table 1) suggest possibilities of H5N1 transmission. It is one of the 26 species which pose a relatively high risk of spreading H5N1 along their migration routes from outbreak sites outside the European Community to within EC borders (Atkinson *et al.*, 2006). Tufted ducks were victims of the outbreaks in Zurich (Switzerland) (Butler, 2006).

Ringed tufted ducks recovered in Greece ($n = 8$) came mainly from Russia ($n = 5$, 62.5%) and some European countries (see online supplementary material, Fig. S12). The distances from outbreak areas varied, being generally shorter in Russia than in other areas (see online supplementary material, Fig. S9).

The regions of Novosibirsk and Chelyabinsk in Russia can be considered as the most critical for the possible spread of the virus by the tufted duck to Greece due to the high density of outbreak sites and their proximity to the mostly affected areas of Asia.

Birds of prey

Common buzzard

Behavioural characteristics of common buzzard (Table 1) suggest low possibilities of H5N1 transmission. Of 27 ringed common buzzards, 21 (78%) were recovered in Greece and originated from Finland (see online supplementary material, Fig. S13). No H5N1 outbreaks occurred in Finland and only three ringing sites of common buzzards were close to outbreaks. In Czech Republic, an outbreak area was 4 km distant from a ringing site at Breclav, being also 80 km away from another two outbreaks in Slovakia. The ringing site in Crimea, Ukraine was 118 km away from an outbreak. Finally, a ringing site in Michalovce, Slovakia was 138 km away from an outbreak area of Romania.

Gruiforms

Common coot

Behavioural characteristics of common coot (Table 1) suggest considerable possibilities of H5N1 trans-

mission. Ringed common coots recovered in Greece ($n = 11$) mainly originated from Lithuania ($n = 4$, 36%), Russia ($n = 2$, 18%) and to a lesser degree from south European countries (see online supplementary material, Fig. S14). Only five of these birds were ringed near H5N1 outbreaks. These ringing sites were situated near 12 outbreak areas. The distances from the outbreaks in Ukraine, Slovakia, Romania and Italy varied considerably (see online supplementary material, Fig. S7).

A critical area for the possible spread of the virus by the common coot to Greece can possibly be the Crimean Peninsula due to the high density of outbreak sites. It is also worth mentioning, that at Feodosiya district in Crimea, 146175 poultry died due to H5N1 in 2006 (Butler, 2006).

Common moorhen

Behavioural characteristics of common moorhen (Table 1) suggest low possibilities of H5N1 transmission. Two (28%) of the seven common moorhens recovered in Greece originated from Germany and the rest from central and eastern European countries (see online supplementary material, Fig. S15). Six of the recovered birds were ringed near outbreak areas whereas the respective ringing sites were situated near 17 outbreaks. In most countries involved, these distances were less than 80 km away (see online supplementary material, Fig. S7) being shortest in Czech Republic; the ringing site in České Budějovice was less than 17 km away from three outbreak areas.

Judging from the high number of outbreak sites, Bavaria in Germany might be the most critical area for the possible transportation of the virus by the common moorhen to Greece.

Charariiforms

Black-headed gull

Behavioural characteristics of black-headed gull (Table 1) suggest high possibilities of H5N1 transmission. It is one of the two species which pose a relatively higher risk of spreading H5N1 along their migration routes from outbreak sites outside the European Union to within EU borders (Atkinson *et al.*, 2006).

Of 86 ringed black-headed gulls recovered in Greece, most originated from Russia ($n = 45$, 52%), Finland ($n = 14$, 16%) and Hungary ($n = 12$, 14%) while the rest from some central and southeastern European countries (see online supplementary material,

Fig. S16). Fifty-four of the recovered birds were ringed near outbreaks while 12 ringing sites were situated near 34 outbreaks. The distances of the gulls' ringing sites from outbreaks varied but, in the countries with the highest number of ringing sites, particularly Russia (Moskovskaya), Hungary and Germany (Bavaria) and also in Ukraine (Crimea), most were no farther from 60 km (see online supplementary material, Fig. S7).

The most critical area for the possible spread of the virus by the black-headed gull to Greece may be Moskovskaya due to the high risk of the virus transmission from this area.

DISCUSSION

In this study, ringing recoveries of some wild bird species were used to draw possible ways of transmission of H5N1 to Greece. It would have been more informative if recoveries for these species from the particular ringing sites had been exclusively acquired during the virus outbreaks. Even so, the information provided on the real bird routes between the ringing and the recovery sites is rather poor and may not be represented by a straight line connecting two sites on a map. Additionally, the numbers of birds of one or more species recovered may not indicate how critical a species is for the transmission of the virus and may merely represent a particular ringing effort on these species. Similarly, the importance of ringing sites as virus transmission indicators of the H5N1 may be limited. These areas are usually selected because they offer compensating-for-the-effort benefits, related to the number and diversity of bird species caught. Within this framework, lack of recoveries from neighbouring Turkey, where so many outbreaks have been reported, may not preclude this country as a possible way of the virus transmission to Greece but may indicate reduced ringing effort related to the species selected for this study.

Despite these drawbacks, there is a quite clear association between the origination of the bird species arriving to Greece and a possible way of the virus transmission. Regarding the possibility of the H5N1 transmission from the species considered, most have a tendency of medium or high mixing with flocks of other bird species and some medium to high contact risk with poultry (see Table 1). Of them, mallard and black-headed gull seem as the strongest transmission candidates followed by common coot, cormorant and mute swan, being of the most important ones. Keaw-

charoen *et al.* (2008) tested six *Anas* species and concluded that mallard is the prime candidate for being a long-distance vector of HPAI H5N1 because it was the only species that showed abundant virus excretion without clinical or pathologic evidence of debilitating disease. Pochard and tufted duck seem less likely candidates as long-distance virus vectors because those individuals that excreted the most virus also developed severe neurologic disease (Keawcharoen *et al.*, 2008). Therefore they would not have been able to fly far before succumbing. Instead, they are more likely to act as sentinels for HPAI H5N1 in wild bird populations. Regarding the possible transmission of the virus by the cormorant to Greece, there are two possible “fronts”: one from the western Europe, determined by a higher number of ringing areas near outbreak sites, and another through south-eastern Europe. The latter, may be more critical not only due to the high density of outbreak sites but also due to its geographical position, located near the mostly affected areas of Asia. It is also worth mentioning, that cormorants were victims of the H5N1 outbreaks in the Ukrainian areas of Kherson and Odessa (Butler, 2006).

Generally, regarding the transmission ways, the most possible route seems to be eastern-northeastern, from Russia through Ukraine and eastern Balkans (mainly Romania), as supported by the information presented on great cormorant, grey heron, mute swan, mallard, common pochard, tufted duck, common coot and black-headed gull. Within this region, some areas, such as the Crimean Peninsula in Ukraine, the areas of Volga Delta, Novosibirsk, Omsk, Kurgan, Tiumen, Chelyabinsk and Moskovskaya in Russia, and Romania, seem to be of greater risk. These areas had the highest number of outbreaks and, particularly Ukraine and Russia, are near the mostly affected areas of Asia from where the virus was transmitted. On the other hand, other possible routes of transmission are associated with west and northwest European countries, as supported by the information presented on great cormorant, white stork, flamingo, common pochard and common moorhen. In this region, high density of outbreaks and relatively short distances to ringing sites seem to be more critical. The areas of Bavaria (Germany), Ain (Saint-Mitre-les-Remparts) (France) and south Bohemia (Czech Republic) seemed to pose the highest risk.

The information available on common buzzard makes this species unclear as candidate for the H5N1 transmission to Greece. The common buzzard has been considered as a potential transmitter because it

preys upon waterbirds which may carry the virus (Veen *et al.*, 2006). Virus-carrier birds of prey have also been found through smuggling, as in the case of two infected mountain hawk-eagles seized at the customs office in Brussels airport at a flight from Bangkok (Van Borm *et al.*, 2005).

The probability of spread through the aforementioned migration routes was based on the hypothesis that wild birds can indeed transmit the virus. Despite the fact that around the world more than 60 species of wild birds have been infected with H5N1 virus so far (Ellis *et al.*, 2004; Olsen *et al.*, 2006), it has been much debated whether they play an active role in its geographical spread. Many species of birds experimentally infected in the laboratory, survived the infection and continued transmitting the H5N1 virus without exhibiting symptoms of the disease (Sturm-Ramirez *et al.*, 2004; Hulse-Post *et al.*, 2005; Chen *et al.*, 2006). In two studies (Chen *et al.*, 2006; Lvov *et al.*, 2006) the virus (HPAI) H5N1 was isolated from healthy wild birds, leading to the conclusion that they may be agents of the virus spreading it over long distances during migration. In parallel, it is considered that, as most migratory birds move in flocks, some individuals may die on the way, while others are infected and continue to transmit the virus farther away (Richardson, 2006). Feare & Yasué (2006) consider that there are problems in the methodology of these studies and express reservations about their results.

In contrast to the above, other researchers have the opinion that data are still inadequate to support that the virus is transmitted over long distances during seasonal migration (Feare, 2007). If migratory species were the main agents of the HPAI H5N1 virus spread, outbreaks would have been expected until early January 2006 in regions such as India, Pakistan, Bangladesh, the Middle East, Africa, America and Australia. Nevertheless, none of these countries or regions ever reported outbreaks of the virus since then, while U.S.A. and Australia have never reported any case (OIE, 2009). In contrast, outbreaks in domestic birds occurring in Russia and Kazakhstan in the summer of 2005 were mostly distributed along major trade routes, linking the western China with Russia, rather than along a specific migratory corridor (Gilbert *et al.*, 2006; Gauthier-Clerc *et al.*, 2007). Analyzing 52 cases of virus introduction in several countries, Kilpatrick *et al.* (2006) concluded that poultry and wild birds' trade were a greater risk than migratory species for the introduction of H5N1 in America.

In any case, the spread of H5N1 in Europe showed that wild birds should play a role in the transfer of the virus: by the end of April 2006, outbreaks concerning wild birds had been reported in 13 European countries with no simultaneous outbreaks in poultry except for three cases (Atkinson *et al.*, 2006). The outbreak incidents in Western Europe following the cold spell in the Black Sea region showed that the virus could spread through infected wild birds travelling short distances (Feare, 2007). In some European countries, including Greece, wild birds were found infected with influenza virus H5N1, with no simultaneous reports of cases in poultry. This suggests that wild birds can indeed carry the virus to non-infected areas (Boyce *et al.*, 2008). Although dead swans were the first indicators of the presence of H5N1 in various European countries, their role as prevailing hosts is not self-evident. They may have simply acted as final hosts infected by other migratory bird species (Olsen *et al.*, 2006). The situation in Europe during 2006 and 2007 suggests that the probability of transmission is extremely low (Artois *et al.*, 2009).

In Greece, of 33 dead waterfowl found to be infected with H5N1 between 30/1/2006 and 7/3/2006, 30 were mute swans, one was a cormorant, one a whooper swan (*Cygnus cygnus*) and one a red-breasted goose (*Branta ruficollis*) (OIE, 2009). All were found in northeastern Greece, except for one in the island of Skyros (see online supplementary material, Fig. S17). The survival of the last mentioned species, being strictly protected in the European Community, might be threatened by the H5N1 virus influenza. It is noteworthy that during the period these infected birds were found, no outbreaks took place in wetlands of international importance of this region such as Lake Kerkini, Axios-Loudias-Aliakmon Deltas or in wetlands of western Greece (e.g. Amvrakikos Gulf, Mesolonghi Lagoon). It seemed that all the cases mentioned above were single and randomly scattered. They were mostly connected with unusual movements of aquatic birds, probably due to a cold spell in the Black Sea (Gilbert *et al.*, 2006; Gauthier-Clerc *et al.*, 2007).

The Ministry of Rural Development and Food runs a program of epizootic investigation of avian influenza, collecting samples of wild birds. From 2004 to 2006, wild birds of several species were collected. None of them was positive for the virus, nor any poultry. In addition, within this framework, a considerable number of passerine species and Mediterranean gull chicks have been examined to date, none of which

carried the virus (Kazantzidis, personal communication).

Surveillance of live wild birds would be useful in finding reservoirs for HPAI and obtaining more detailed information on how it spreads because recoveries of dead birds rather provide information on species susceptibility than their transmitting capabilities (Yee *et al.*, 2008). In conclusion, there are areas of some countries which, compared with others, constitute more likely places of H5N1 spread through wild migratory birds. However, convincing evidence is needed on the role of wild birds in the transfer of the virus over long distances. To understand better the spread of H5N1 virus, more and most recent recovery data are needed as well as information on the ecology of the virus in the field.

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