ORIGINAL ARTICLE

Association Between Outbreaks of Highly Pathogenic Avian Influenza Subtype H5N1 and Migratory Waterfowl (Family *Anatidae*) Populations

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Impacts

- Highly pathogenic avian influenza (HPAI) virus subtype H5N1 threatens poultry production and human health. Exposure to infected sick or dead poultry is a strong risk factor for human disease.
- A study was conducted to test the hypothesis that HPAI virus subtype H5N1 can be introduced to poultry populations via infected migratory waterfowl. The timing of poultry outbreaks in Romanian villages during the autumn of 2005 were predicted by the distance from migratory waterfowl sites.
- Incorporating information about migratory waterfowl into risk assessments in this region might assist in preventing or reducing the risk of future outbreaks and therefore the risk of pandemic flu.

Keywords:
Avian influenza H5N1; poultry; epidemic; migratory waterfowl; *Anatidae*; Romania

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Summary

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 threatens poultry production and human health. Understanding the role that migratory waterfowl play in introducing and maintaining this infection is critical to control the outbreaks. A study was conducted to determine if the occurrence of HPAI subtype H5N1 outbreaks in village poultry in Romania, 2005–2006, was associated with proximity to populations of migratory waterfowl. Reported outbreaks – which could be grouped into three epidemic phases – and migratory waterfowl sites were mapped. The migratory waterfowl site closest to each outbreak was identified. The distances between outbreaks occurring in phase 1 and 2 of the epidemic and the closest migratory waterfowl site were significantly ($P < 0.001$) less than in phase 3, but these distances were only useful in predicting when outbreaks occurred during phase 1 (October–December, 2005) of the epidemic. A spatial lag ($\rho = 0.408$, $P = 0.041$) model best fit the data, using distance and [distance]*[distance] as predictors ($R^2 = 0.425$). The correlation between when outbreaks were predicted to occur and when they were observed to occur was 0.55 ($P = 0.006$). Results support the hypothesis that HPAI virus subtype H5N1 infections of village poultry in Romania during the autumn of 2005 might have occurred via exposure to migratory populations of waterfowl.

Introduction

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 is a threat to world health: it has caused numerous disease outbreaks in domestic poultry and wild bird populations, and there is a fear that it could become the next pandemic influenza strain (Alexander, 2000). Because exposure to sick or dead poultry is a strong risk factor for human disease caused by HPAI subtype H5N1 (Dinh et al., 2006), the threat of pandemic flu can be

effectively reduced by controlling and preventing the spread of HPAI virus subtype H5N1 through national poultry flocks.

Avian influenza was first described in 1878 (Alexander, 2001). The influenza virus genome consists of eight segments of negative-sense, single-stranded RNA. Influenza viruses are classified – based on antigenic differences in the nucleoprotein and matrix protein – into A, B, and C types. Influenza type A has been isolated from a range of avian and mammalian species. Based on phylogenetic analyses, aquatic birds are believed to be the primordial source of this influenza virus type (Webster et al., 1992; Krauss et al., 2004). Avian influenza virus infection is endemic in a range of free-living bird species world-wide (Alexander, 2000), particularly species associated with water (Anseriformes – ducks, geese, and swans; Charadriiformes – gulls, terns, and shorebirds) (Stallknecht and Shane, 1988). Waterfowl can be infected by all subtypes of type A influenza viruses, with few or no symptoms (Woebser, 1997). Influenza A viruses in waterfowl are able to replicate in the respiratory and intestinal tracts. More virus is shed in the digestive tract than the respiratory tract (Beard et al., 1984), potentially contaminating water sources (Webster et al., 1992; Rene and Bicout, 2007). Factors such as amount of organic material, pH, salinity and water temperature may determine environmental persistence (Stallknecht et al., 1990a,b) and these viruses can probably persist for longer periods in the winter months (Webster et al., 1978; Beard et al., 1984; Fichtner, 1987).

Migratory waterfowl might be responsible for the spread of influenza A viruses between regions (Krauss et al., 2004; Oyana et al., 2006), and HPAI and low pathogenic avian influenza (LPAI) in poultry are often assumed to occur from exposure to wild avian species (Morgan and Kelly, 1990; Khawaja et al., 2005; Velkers et al., 2006). For example, a number of separate outbreaks of the same virus subtype (suggesting a common link) occurred in California during 2000–2001 where no clear connection could be determined epidemiologically. These outbreaks coincided with migratory bird patterns, suggesting that the virus was introduced by the migratory birds (Hanson et al., 2005). In several European outbreaks of HPAI in poultry, LPAI viruses of the same subtype have also been identified in an aquatic bird species (for example, mallards) from the same locations (Munster et al., 2005). However, the relationship between outbreaks of HPAI and LPAI in poultry remains mostly speculative, with the transport of infected poultry and contaminated poultry products being blamed in some cases for spreading the disease within regions.

Highly pathogenic avian influenza subtype H5N1 (A/Hong Kong/156/97) was first identified during an outbreak of disease in poultry and humans in Hong Kong during 1996 and 1997 (Subbarao et al., 1998). This virus strain was eradicated from poultry, but it continued to circulate in geese (Sims et al., 2005). Wild birds are suspected of spreading HPAI subtype H5N1 via their migration routes westward across Asia and into Europe, the Middle East and Africa during 2005–2006 (Gilbert et al., 2006). Evidence to support this hypothesis include: (1) a major disease outbreak discovered in wild birds in Lake Qinghai (western China) in May 2005; (2) outbreaks in Turkey, Romania, and Ukraine during October, November and December, 2005; and (3) the infection of large numbers of mute swans and other wild bird species across Western Europe in the spring of 2006 (Gilbert et al., 2006). It is plausible that some migratory waterfowl species inhabiting wetlands in the West Siberian lowlands could have been infected during the spring and summer of 2005, and then spread HPAI subtype H5N1 to domestic poultry in the Black Sea region (directly, or indirectly via non-migratory wild birds) during their period of overwintering. Previous research on this Romania epidemic has shown that spatially and temporally it consisted of two parts – disease introduction, local spread and sporadic outbreaks, and long-distance disease spread with rapid epidemic propagation (Ward et al., 2008a). In particular, the spatiotemporal pattern of the first part of the epidemic is consistent with the hypothesis that the environment and landscape (specifically the Danube River Delta) played a critical role in the introduction and initial spread of HPAI subtype H5N1 during the autumn and winter of 2005. Furthermore, the risk of an outbreak in the first part of this epidemic was strongly associated (odds ratio 5.08, 95% CI 1.08–23.9) with a village being <5 km of a regularly flooded land area (Ward et al., 2008a), a habitat suitable for migratory waterfowl.

To plan the most effective control programmes, the sources of influenza A virus infecting poultry populations, and therefore potentially human populations, must be known. Specifically, understanding the role that migratory waterfowl play in introducing and maintaining these viruses within the environment is critical to control the outbreaks of avian influenza in poultry. The aim of this study was to determine if the occurrence of HPAI subtype H5N1 outbreaks in village poultry in Romania, 2005–2006, was associated with proximity to populations of migratory waterfowl.

Materials and Methods

Data source

Highly pathogenic avian influenza subtype H5N1 was first reported as a cause of an outbreak of disease in village poultry in Romania on 7 October 2005. Highly pathogenic avian influenza subtype H5N1 had first been

reported from this region (Turkey) on 1 October 2005 (Food and Agriculture Organisation, 2008). Following identification of HPAI subtype H5N1 in Romania, it was subsequently reported from Ukraine on 2 December. During early 2006, outbreaks in poultry or wild birds were also reported for the first time from Greece (30 January), Bulgaria (31 January), Slovakia (17 February), Serbia (28 February) and Hungary (4 February) (http://www.fao.org/docs/eims/upload//241290/AIDEnews_mar08_no51.pdf).

Between October 2005 and June 2006, 165 outbreaks of HPAI subtype H5N1 were reported in 161 village poultry populations in Romania. Outbreaks were reported from 23 of the 41 counties and municipalities in Romania, covering about half (128 000 km^2) the area of Romania. Data available included the outbreak location (*X*, *Y* coordinates) and the reported date of occurrence. Outbreak locations represented the village in which HPAI subtype H5N1 was identified. As this is a backyard production system, the entire village flock was assumed to be infected (separate backyards were not differentiated). The coordinate information represents the official geographic location of an affected village.

The epidemic curve of reported outbreaks has been analysed previously by Ward et al. (2008a). Based on visual inspection of the epidemic, three epidemic phases were apparent: 7 October–29 December 2005 (days 1–84; 23 outbreaks), 30 December 2005–16 April 2006 (days 85–192; 28 outbreaks), and 17 April–6 June 2006 (days 193–243; 110 outbreaks). Half of all outbreaks were reported during an 85-day period, between 27 February and 23 May (epidemic days 144–229). Previous analysis of this epidemic, using a range of statistical and geostatistical methods, provided insights into how HPAI subtype H5N1 might spread and allowed the generation of disease dispersion hypotheses (Ward et al., 2008a). Risk mapping and the application of cluster statistics showed that outbreaks first appeared in Eastern and Southern Romania, particularly within an area that forms part of the Danube River Delta, and then spread in an East to West direction to central Romania. It was suggested that the evolution of the epidemic could be characterized into two parts: disease introduction, local spread and sporadic outbreaks, and long-distance disease spread with rapid epidemic propagation (Ward et al., 2008a).

Most *Anatidae* (swans, geese and ducks) depend on wetlands throughout much of their life-cycle. These wetlands habitats are often discrete locations, separated in some cases by vast areas of non-wetland habitat (Scott and Rose, 1996). The *Anatidae* includes many migratory populations which utilize a network of sites throughout their range of distribution. Each site in the network plays a critical role, enabling the individuals that use it to move on to the next site in the network (Scott and Rose, 1996). Information on the presence and location of migratory *Anatidae* was sourced from an Atlas of *Anatidae* Populations in Africa and Western Eurasia (Scott and Rose, 1996). This publication combines data from several sources to describe the networks of key sites used by the *Anatidae* in the African/Eurasian region. All the Romanian sites selected in this study are classified by Scott and Rose (1996) as key sites, that is, sites identified as being of international importance for a particular population either because of the average peak counts for an *Anatidae* species in at least three of the most recent 5 years exceeds 1% of the individuals in that species population; or that it regularly contains >1% of the species population during periods of unusually harsh weather; or that it regularly supports >20 000 individuals from the species population; or that the site regularly supports >50 individuals (or 15 breeding pairs) of a globally threatened species. The data generally represent the period 1984–1993 (Scott and Rose, 1996). Available information from this data source included country, site name, location (latitude, longitude), population protection status, species for which the site is important, season and counts. Data were copied from the relevant document table (Annex 2: *Key Sites for Anatidae in Africa and Western Eurasia*), entered into a spreadsheet program (Microsoft® Office Excel 2003, Microsoft Corporation, Redmond WA), processed, saved as a data base file and imported into a GIS program (ArcGIS™ 9.0, ESRI Inc., Redland, CA, USA). Key *Anatidae* sites from all countries neighbouring Romania (Bulgaria, Serbia, Hungary, Moldova and Ukraine) were initially also included, but all reported Romanian outbreaks were found to be closest to sites in Romania and thus these additional sites were excluded from data analysis.

Data analysis

A Romanian national shapefile (WGS 1984 Datum) was created from a shapefile of Romanian counties (Administrative Areas of Europe. ESRI Inc. http://www.esri.com/data/data-maps/overview.html), by dissolving county boundaries (Spatial Analyst. ArcGIS™ 9.0; ESRI Inc.). This national shapefile was projected using Stereographic 70. Village outbreak locations and *Anatidae* sites were mapped by importing *X* and *Y* coordinates, within separate database files, into the GIS program. Village outbreak locations and *Anatidae* sites were mapped (ArcGIS™ 9.0. ESRI Inc.) by importing *X* and *Y* coordinates, within separate database files, into the GIS program.

To measure the distance between outbreaks and breeding sites, a spatial join (which involves matching rows from the join layer to the target layer, based on a spatial

relationship and creation of an output feature class) was created. The target layer was village outbreak locations, and the join layer was *Anatidae* sites. The output included the distance (km) each outbreak location was to the nearest *Anatidae* site (ArcGIS™ 9.0; ESRI Inc.). The distances (km) between each outbreak and the nearest *Anatidae* migratory site were used as a predictor of epidemic day. The epidemic day variable was created from outbreak onset date information by designating the date of the first reported outbreak (7 October 2005) as epidemic day 1 and the date of the last reported outbreak (6 June 2006) as epidemic day 243.

Initially, an ordinary linear regression model (GeoDa™ 0.9.5-i5, Anselin, 2005) was fit to the data (distance the predictor variable and epidemic day the response variable). Moran's *I* statistic was used to identify residual autocorrelation. This statistic measures spatial autocorrelation based on both feature locations (*X*, *Y* coordinates) and feature values (epidemic day) simultaneously. It evaluates whether the pattern is clustered, dispersed, or random. A Moran's *I* value near 1 is indicative of clustering and a value near –1 is indicative of dispersion. A value close to zero indicates that the pattern of features is spatially random (Ward and Carpenter, 2000).

Presence of spatial autocorrelation in the residuals of an ordinary linear regression model can result in model misspecification (for example, identifying significant predictors that might be associated with the outcome simply because of their spatial pattern). To take into account these spatial relationships, spatial regression models are needed. Spatial error and spatial lag regression models are two options for achieving this outcome. These spatial regression models directly include spatial dependency within the regression analysis by either including a spatially lagged dependent variable, or including the spatial correlation within the model error term (Anselin, 2005). Either a spatial lag or spatial error regression model was fit to the data, based on diagnostic tests for spatial dependence, to identify the model that best explained (coefficient of determination, R^2) the variability in epidemic day (GeoDa™ 0.9.5-i5, Anselin, 2005). Observed epidemic day versus epidemic day predicted by the model were assessed for outliers and systematic over- or under-prediction. Several methods of defining spatial relationships (spatial weights) between observations were investigated, including Euclidean (straight-line) distance and nearest neighbours (GeoDa™ 0.9.5-i5, Anselin, 2005).

Results

The spatial relationship between HPAI subtype H5N1 outbreaks and *Anatidae* population sites is shown in Fig. 1. The median distance between outbreaks and the nearest *Anatidae* population was 55 km (interquartile range, 29.9–74.6 km). This distance was more strongly correlated with longitude (r_{SP} −0.73) than with latitude (0.26). The relationship between outbreaks within each of the three identified phases of the epidemic and *Anatidae* population sites is summarized in Table 1. The median distances in phase 1 (34.8 km) and phase 2 (26.2 km) were both significantly ($P < 0.001$) less than in phase 3 (66.5 km), but were not significantly ($P = 0.156$) different from each other. The first two outbreaks reported in Romania, on 7 October 2005, were 49 and 12 km from the nearest *Anatidae* sites. The remaining 21 outbreaks during this phase of the epidemic were located between 11 and 50 km from the nearest *Anatidae* sites. The distance between outbreaks and populations of *Anatidae* were moderately correlated ($r_{SP} > 0.5$) with latitude in phase 2 of the epidemic and with longitude in phase 3 of the epidemic ($P < 0.05$).

Overall, the correlation between epidemic day and distance from the nearest *Anatidae* population was 0.36 ($P < 0.001$). Within phase 1 and phase 2 of the epidemic, epidemic day and distance were not significantly correlated (0.30, $P = 0.161$ and 0.01, $P = 0.939$, respectively). Within phase 3, epidemic day and distance were negatively corre-

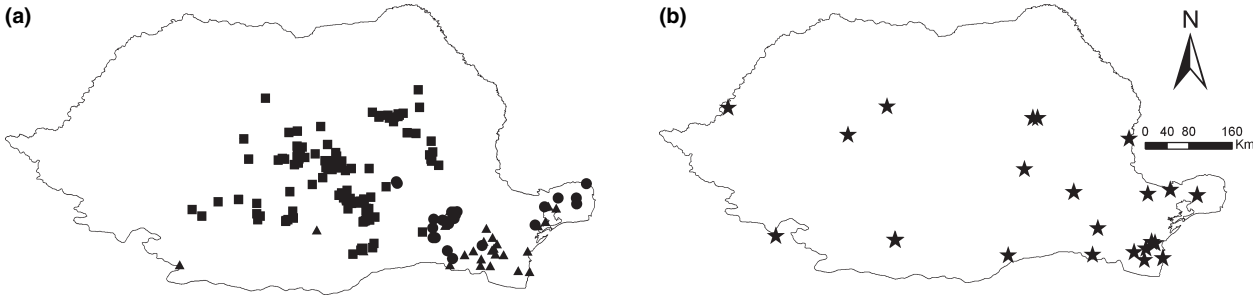

Fig. 1. The spatial relationship between (a) outbreaks [epidemic phase 1 (days 1–84),•; epidemic phase 2 (days 85–192), ▲; epidemic phase 3 (days 193–243),■] of highly pathogenic avian influenza virus subtype H5N1 in poultry located in 161 villages in Romania during a 2005–2006 epidemic, and (b) migratory populations (★) of waterfowl (family *Anatidae*).

Table 1. The relationship between outbreaks of highly pathogenic avian influenza subtype H5N1 in village poultry in Romania and nearby migratory waterfowl (family *Anatidae*) populations

	Epidemic phase[a]		
Statistic	1	2	3
Number of outbreaks	23	28	110
Length (days)	84	108	51
Median distance (km)[b]	34.8	26.2	66.5
Interquartile range (km)[b]	21.1–42.1	14.9–39.3	51.8–78.7
Correlation, distance versus latitude (*P*-value)[c]	−0.33 (0.122)	0.52 (0.005)	−0.09 (0.344)
Correlation, distance versus longitude (*P*-value)[c]	0.21 (0.334)	−0.35 (0.073)	−0.56 (<0.001)

[a]7 October–29 December 2005; 30 December 2005–16 April 2006; 17 April–6 June 2006.
[b]distance between each outbreak and the closest population of migratory *Anatidae*.
[c]Spearman's rank correlation statistic.

lated (−0.22, $P = 0.022$). However, the best fitting ordinary linear regression model (adjusted R^2 0.201) was found for outbreaks reported in phase 1 of the epidemic (Fig. 2), and included distance ($P = 0.016$) and [distance]*[distance] ($P = 0.024$) as predictors: epidemic day = −13.265 + 4.627 [distance] − 0.069 [distance]*[distance]. In no other models fit to the data were these predictor variables significantly ($P < 0.05$) associated with epidemic day, nor was there a substantial amount of variability explained (adjusted $R^2 < 0.05$). The model errors of the best fitting model were normally distributed (Jarque–Bera test statistic 3.294, $P = 0.193$). The Jarque–Bera test is a test for non-normality (Anselin, 2005). It is a goodness-of-fit measure of departure from normality, based on the sample kurtosis and skewness (Bera and Jarque, 1980).

The best-fitting model of phase 1 epidemic day – using distance and [distance]*[distance] as predictors – was further investigated. For this ordinary linear regression model, significant ($P < 0.0001$) spatial autocorrelation (Moran's *I*, 0.30) was detected. Although Moran's *I* statistic is powerful for detecting model misspecifications, for example when residual spatial autocorrelation remains in the model, it is less helpful in suggesting which alternative model specification should be used (Anselin, 2005). Two alternative model specifications to better describe the data, spatial lag and spatial error, were investigated and selected by calculating the Lagrange multiplier test statistics. The standard and robust forms of these statistics were considered. Both the standard Lagrange multiplier statistics for spatial lag (6.068, $P = 0.014$) and error (5.159, $P = 0.023$) models were significant. Since the robust form of the Lagrange multiplier spatial lag model (0.983, $P = 0.321$) was more significant than the robust form of the Lagrange multiplier spatial error model (0.075, $P = 0.785$), a spatial lag model – using distance and [distance]*[distance] as predictors – was fit to the epidemic day (phase 1) data, as recommended by Anselin (2005). The spatial relationship between outbreaks was defined using Euclidean distance (km). The model fit explained 31.3% of the variability in epidemic day. However, defining the spatial relationship by the five nearest neighbouring outbreaks resulted in 42.5% of the variation in epidemic day being explained by the distance and [distance]*[distance] from the nearest *Anatidae* population (Table 2). Defining the spatial relationship by the 10 nearest neighbouring outbreaks explained only 37.1% of the variation in epidemic day. Of the three models fit, the spatial lag parameter (ρ) was only significant (likelihood

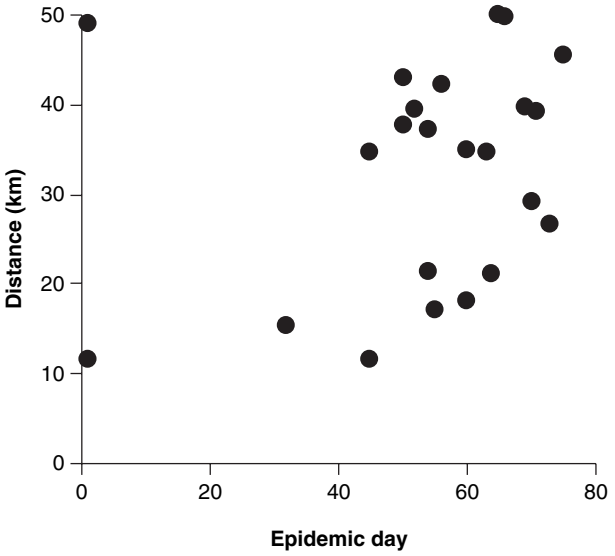

Fig. 2. Epidemic day of reporting (day 1 – October 7, day 80 – 25 December, 2005) of outbreaks of highly pathogenic avian influenza virus subtype H5N1 in village poultry populations in Romania, versus distance of the village from the nearest population of migratory waterfowl (family *Anatidae*), during the first phase of the epidemic in Eastern Romania. The best fitting ordinary (aspatial) linear regression model (adjusted R^2 0.201) of epidemic day included [distance] and [distance]*[distance] from the nearest population of migratory waterfowl. A spatial lag model fit to this data using the same predictors explained 42.5% of the variation in epidemic day.

Table 2. A spatial lag model of epidemic day of occurrence of outbreaks of highly pathogenic avian influenza subtype H5N1, Romania 2005, using distance (km) between outbreaks and the nearest population of migratory waterfowl (family *Anatidae*)

Variable	Coefficient	SE	*z*-value	*P*-value
Spatial lag	0.408	0.199	2.047	0.041
Constant	−27.043	20.967	−1.290	0.197
Distance	4.253	1.530	2.780	0.005
[Distance]*[Distance]	−0.066	0.024	−2.704	0.007

ratio test statistic 4.40, $P = 0.036$) in the model using the nearest five neighbouring outbreaks as the spatial weight.

The correlation (Fig. 3) between epidemic day predicted by the best fitting model – using distance and [distance]*[distance] from the nearest *Anatidae* population and observed epidemic day was 0.55 ($P = 0.006$). The first two outbreaks reported on 7 October 2005 (epidemic day 1) were outliers: the model predicted their occurrence on epidemic days 32 and 41. The closest populations of *Anatidae* to these two outbreaks were a distance of 11.6 and 48.9 km, respectively. In addition, another outbreak (located 34.7 km from the nearest *Anatidae* population) was reported on epidemic day 45 but was predicted to occur on epidemic day 67. The epidemic days of four outbreaks were predicted to be earlier than observed: days 44 versus 55, 65, 66 and day 53 versus 75. The closest populations of *Anatidae* to these four outbreaks were 17.0, 51.1, 49.8 and 45.5 km, respectively.

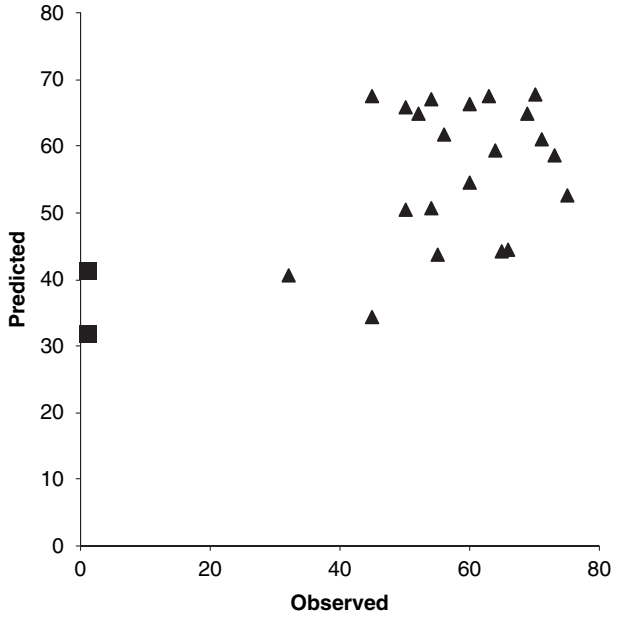

Fig. 3. The correlation (0.55) between when outbreaks of highly pathogenic avian influenza virus subtype H5N1 reported between October and December 2005 in Romania occurred and the day predicted, using a spatial lag model and distance between outbreaks and the nearest population of migratory waterfowl (family *Anatidae*). Two outliers (epidemic day 1), in which the model predicted occurrence on epidemic days 32 and 41, are indicated (■).

Discussion

Infected migratory waterfowl have been suspected of spreading HPAI virus subtype H5N1 from central Asia to Eastern Europe during the second half of 2005, based on migratory flyways and climate (Gilbert et al., 2006). The present study was conducted at a finer spatial scale than previous studies, and results support the hypothesis that migratory waterfowl acted as a potential source of infection during the initial phase of the Romanian epidemic in the autumn of 2005.

Despite results of this and other studies, the links between infected migratory waterfowl and infection of domestic poultry with HPAI virus subtype H5N1 remain circumstantial. Evidence against the hypothesis that HPAI subtype H5N1 is spread by wild birds includes observations that infected wild birds are usually found dead or moribund, thus limiting their opportunity to spread the virus over long distances (Olsen et al., 2006). However, it has been shown experimentally that some species can survive infection and shed the H5N1 virus without apparent disease signs, and many wild birds may be partially immune owing to previous exposures to LPAI influenza viruses (Olsen et al., 2006). In these cases, HPAI viruses may become less pathogenic to ducks, whilst retaining high pathogenicity for chickens. In some parts of Europe, infected wild birds have been found without corresponding reports of outbreaks in poultry (Olsen et al., 2006), and H5 virus circulation has been demonstrated in populations of ducks wintering in Mediterranean areas (Marco et al., 2005).

The actual role played by migratory waterfowl in spreading HPAI virus subtypes in a given situation will depend on the infection status of the population and the opportunity for contact with poultry populations. In this respect, the initial epidemic in Romania in 2005 was unique in that HPAI virus subtype H5N1 infected migratory waterfowl might have been present during autumn in large numbers in the Danube Delta region, where poultry production is village-based and the opportunity for direct or indirect (environmental) contact between wild birds and poultry is high. Studies to estimate the prevalence of infection of wild birds have produced variable results. In Egypt during the 2005–2006 migratory bird season, 203 of 1304 (16%) cloacal swabs were positive for influenza A virus via RT-PCR (Saad et al., 2007). However, H5 was detected in only one sample. In early 2006, a total of 2101 samples representing 61 bird species

in the Czech Republic were examined (Holko et al., 2006; Nagy et al., 2007). Highly pathogenic avian influenza virus subtype H5N1 was detected in 12 Mute swans (*Cygnus olor*). However, following the first HPAI outbreak in poultry, about 300 wild birds representing 33 species were sampled from the outbreak region and paradoxically, all were negative. The Eastern part of Romania, and in particular the Danube River Delta, is an important location on the Siberian-West Africa migratory flyway. At least 23 species of *Anatidae* overwinter in the Black Sea region. Of these, at least six species have a breeding range that extends to West Siberia, including *Cygnus cygnus* (Whooper Swan), *Anas penelope* (Eurasian Wigeon), *Anas crecca* (Common Teal), *Anas acuta* (Northern Pintail), *Anas clypeata* (Northern Shoveler) and *Aythya marila* (Greater Scaup). Highly pathogenic avian influenza subtype H5N1 virus has reportedly been isolated from the first four of these species in Romania (Ontanu et al., 2007). In this region of Romania, poultry production is almost entirely based at the village level. It is common for birds to have free range during the daylight hours, and contact with wild birds is likely. Thus, although the mechanism of introduction of HPAI subtype H5N1 to Romania in 2005 remains speculative, there is some strong evidence supporting migratory birds on the Siberian-West Africa migratory flyway as the source.

In some cases, the spread of HPAI virus subtype H5N1 has been more consistent with trade routes than with migration flyways (Gilbert et al., 2006; Kilpatrick et al., 2006). The spread of avian influenza virus via smuggled poultry carcasses and wildlife has been documented (Van den Berg et al., 2005; Beato et al., 2006). By its very nature, the role played by smuggling and the illegal movement of poultry and poultry products is very difficult to define. Although the mechanism of introduction of HPAI subtype H5N1 to Romania in 2005 remains speculative, introduction via migrating waterfowl during the early autumn of 2005 is likely.

While this study focused on proximity to *Anatidae* sites, other studies on this same data set have examined the role of anthropogenic (road networks) and environmental (vegetation type) factors in the spread of the epidemic (Ward et al., 2008b). Proximity to a major road, river or stream, or a regularly flooded land area was found to increase the risk of an outbreak. Results not only supported the role of wild bird populations in the epidemic, but also suggest that the spread of HPAI virus subtype H5N1 might depend on the movement of poultry and poultry products via transportation networks, such as roads. However, the ill-defined nature of such movements (particular during epidemics, when such movements are generally illegal) prevents an accurate assessment of their contribution to spread being made. Previous research (Ward et al., 2008a) supports our assumption that this epidemic can be divided into a predominantly migratory wild vectored phase (phase 1) and a predominantly human-driven phase (phase 3). Thus, separate analyses based on the hypothesized mechanism of spread are appropriate. Fitting models that can describe the entire epidemic progression would be parsimonious. However, the apparently different mechanisms of spread within distinct regions of the country pose analytical challenges. Further investigations of how and why HPAI subtype H5N1 spread is some situations, and not in others, is needed. To accomplish this, additional methods of analysing spatio-temporal outbreak data need to be developed that can provide clues about the likely pathways of disease spread.

It is important to consider scale when interpreting the results of spatial analyses of disease occurrence. While no significant association between migratory *Anatidae* sites and outbreaks were detected at the national scale, a significant and predictive association was detected during phase 1 of the epidemic, an area <25% (28 000 km^2) of that of the national epidemic. This scale effect suggests that interactions between wild migratory birds and village poultry, if responsible for H5N1 infection, occurs at a local scale of probably <50 km (the maximum distance observed during phase 1 of the epidemic). If analysis only focuses on national data, it is possible that such associations can be missed. It is likely that even during phase 1 of the Romanian epidemic, more than one disease transmission process occurred. For example, during the first 60 days of the epidemic (7 October to 5 December 2005), outbreaks <25 km from the nearest *Anatidae* site were reported on epidemic days 1, 32, 45, 54, 55 and 60, whereas outbreaks ≥25 km from the nearest *Anatidae* site were reported on epidemic days 1, 45, 50, 52, 54, 56 and 60. It is plausible that some village poultry populations were infected from local interactions with wild *Anatidae*, whereas other villages might have been secondarily infected from these primary outbreaks.

Active surveillance programmes based solely on the blanket or opportunistic sampling of wild birds are unlikely to provide early warning of HPAI virus subtype H5N1 incursions, as the species responsible for such introductions are usually unknown, prevalence of infection might be very low, and the timing of sampling is critical. One method of increasing the efficiency of detection is to target localities during periods of the year when species suspected of being infected are present. Extensive surveillance studies of wild ducks in the Northern Hemisphere have revealed high LPAI virus prevalences primarily in juveniles (presumably immunologically naiëve), peaking in early fall before southbound migration (Olsen et al., 2006). Although certain broad

ecoregions have experienced a greater number of HPAI subtype H5N1 outbreaks than would be expected simply based on migratory patterns, vegetation or climate alone (Sengupta et al., 2007), such an approach should be refined based on knowledge of the local environmental conditions and the population at-risk. In the Danube Delta region of Romanian, sampling of juvenile, migratory waterfowl, beginning August–September, might increase the likelihood of detection. To design effective surveillance and control programmes for HPAI subtype H5N1, the potential risk posed within an ecoregion, the nature of the landscape, and the potential for interaction between wild and domestic bird populations all need to be considered.

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