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Prevalence of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain

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birds in southern Spain

Jordi Figuerola^{1*}, Miguel Angel Jiménez-Clavero², Gema Rojo², Concepción

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Gómez-Tejedor² & Ramón Soriguer¹¹*Estación Biológica de Doñana-CSIC, Avda. María Luisa s/n, 41013 Sevilla, Spain*

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²*Laboratorio Central de Veterinaria, , Carretera de Algete Km 8, 28110 Algete**(Madrid), Spain.*

*Department of Wetland Ecology, Estación Biológica de Doñana, CSIC, Avda. María

Luisa s/n, 41013 Sevilla, Spain. E-mail: jordi@ebd.csic.es, Tel: 34 954 23 23 40, Fax:

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Prevalence of West Nile virus neutralizing antibodies in colonial aquatic
birds in southern Spain

Jordi Figuerola^{1*}, Miguel Angel Jiménez-Clavero², Gema Rojo², Concepción
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The rapid expansion of West Nile virus (WNV) throughout the New World has raised interest in understanding the population dynamics and patterns of dispersal of emerging infectious diseases by wildlife. WNV affects humans, although its main reservoirs are various species of birds. Here we analyze the prevalence of WNV-neutralizing antibodies in nearly full-grown chicks belonging to seven different species of colonial waterbird at three localities in southern Spain. Chicks with neutralizing antibodies against WNV were detected in three species and at all three localities. However, the low antibody titres suggest the presence of antibodies is probably due to maternal transfer of antibody, presumably from exposure of the adult birds to WNV or a similar flavivirus at some stage of their lives. The analyses of the movements of tagged birds confirmed that all species with antibody visit regions that have had reports of WNV infection over the last decade.

Introduction

West Nile virus (WNV) is a flavivirus associated with wild animals and mainly occurs in birds, although occasional transmission to humans and horses has been documented (Zeller & Schuffenecker, 2004). The virus's usual cycle involves transmission between birds via a number of ornithophilic mosquito species (Work *et al.*, 1955; Hayes *et al.*, 2005). Other modes of infection may include oral, faecal and transovarian transmission (Banet-Noach *et al.*, 2003; Komar *et al.*, 2003; Hayes *et al.*, 2005) and it has also been suggested that the virus can be transmitted to birds that feed on dead infected birds.

West Nile disease was first described in a febrile woman in Uganda in 1937, while in Europe and the Mediterranean basin (Zeller & Schuffenecker, 2004) several outbreaks affecting humans and/or equines have occurred recently after an absence of 30 years in Algeria (1994), Morocco (1996, 2003), Romania (1996), Tunisia (1997), Czech Republic (1997), Italy (1998), Portugal (1998, 2004), Russia (1999-2001), Israel (1999-2000), and France (2000, 2003) (Zeller & Schuffenecker, 2004; Hubalek *et al.*, 2000; Del Giudice *et al.*, 2004; Esteves *et al.*, 2005; Schuffenecker *et al.*, 2005). In Spain, evidence of recent WNV circulation has been demonstrated by detection of the presence of WNV-specific antibodies in humans (Bofill *et al.*, 2006). Unlike the situation in the New World, where WNV was first recorded in 1999 in New York (Ludwig *et al.*, 2002) and then subsequently spread across all of North America and part of central America (CDC, 2005), in Europe WNV has traditionally been considered a rare and minor arbovirolosis (Zeller & Schuffenecker, 2004). In the New World, epidemic relapses occur every summer-autumn, killing thousands of birds and producing severe illness and even fatalities in a small portion of the human population exposed to the virus (Zeller & Schuffenecker, 2004).

In the work described here we examined sera from fledgling birds breeding in southern Spain for WNV-neutralizing antibodies. This area is within 100 km of recent WNV foci in Portugal and Morocco and previous results have shown a significant prevalence of antibodies in some species of wild birds in this area (Jiménez-Clavero *et al.*, 2005). The objectives of this study were to determine the prevalence of WNV-neutralizing antibodies in nearly full-grown chicks of a number of different colonial species of waterbirds living in mosquito-rich habitats (wetlands, streams and marshes) and to then investigate the implications of these results in light of the migratory and dispersive movements of the bird species studied.

Materials and Methods

Nearly full-grown chicks of seven different species of bird were sampled at three different locations (Doñana, Odiel Marshes and Fuente de Piedra) (Table 1). Chicks were trapped by hand at or near the nest before they were old enough to fly. Blood samples were taken with syringes from the brachial or femoral vein and birds were released after manipulation. The volume of blood extracted depended on the size of the species and never exceeded 1% of body mass (range 0.100-1 ml). Blood was collected in microfuge tubes, allowed to clot at ambient temperature and then placed in coolers until centrifuged (always on the same day).

Both the WNV strain E101 and the E6 clone of Vero cells (Vero E6) used for virus propagation were obtained from Hervé Zeller (Institut Pasteur de Lyon). Viral titres were determined by end-point titration (Reed & Muench, 1938). Neutralizing antibody titres to WNV were determined using a micro virus-neutralization test (micro-VNT) in 96-well plates using an adaptation of a previously described method (Weintgartl *et al.*, 2003). Serum samples were inactivated at 56°C for 30 minutes prior

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to analysis. Dilutions of test sera (25 μ l) were mixed with a 25 μ l volume containing 100 TCID₅₀ of WNV strain E101 and incubated for 1 h at 37° C in Eagle's medium (EMEM) supplemented with L-glutamine, 100 U penicillin/ml and 100 μ g streptomycin/ml. A 50 μ l volume of a suspension of Vero E6 cells (2×10^4 cells/ml) was added to the same medium with fetal calf serum to a final concentration of 5% v/v. The plates were incubated for 6-7 days at 37°C until cytopathic effect (cpe) was seen in control wells containing 10 TCID₅₀ of virus. Serum samples were screened at dilutions of 1/10 and 1/20. Samples that neutralized the virus at one or both of the dilutions tested were tested again to confirm the result and then further titrated by testing serial serum dilutions from 1/10 to 1/640. The neutralization titre was reciprocal of the highest dilution completely preventing cpe.

All the available information on the migratory and/or dispersive movements of those species containing neutralizing antibodies were examined. For a number of years Greater Flamingos (*Phoenicopterus ruber*) have been the focus of intensive study using both sightings of individuals marked with polyvinylchloride (PVC) rings and satellite transmitters (Amat *et al.*, 2005). Several hundred Yellow-legged Gulls (*Larus cachinnans*) and thousands of Glossy Ibis (*Plegadis falcinellus*) chicks from the colonies sampled in this study have been individually marked with PVC rings bearing a three-digit code that can be read with the aid of telescopes at a distance of over 100 metres. The worldwide observations and recaptures of these marked birds have been used to characterize the approximate ranges of these species. Good information is also available on the movements of PVC-ringed White Storks (*Ciconia ciconia*), although none of the chicks of this species contained neutralizing antibodies and so data for this species is only presented for comparative purposes and because it is thought that C.

ciconia introduced West Nile from central Europe into Israel in 1998 (Malkinson *et al.*, 2001).

Results

The results are presented in Table 2. Neutralization at tenfold or higher dilutions was detected in three of the seven species examined. The prevalence of neutralizing antibody in *P. ruber* was similar in Doñana and Fuente de Piedra ($\chi^2 = 2.56$, 1 df, $p = 0.11$). The highest prevalence (11.0-17.9%) was seen in this species. Neutralizing antibody was detected in birds at all three localities - in *L. cachinnans* in Odiel and *P. falcinellus* in Doñana. The titres were low in all three species, with maximal titres of 40.

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Discussion

There are three explanations for the presence of neutralizing antibodies in chicks.

Firstly, these chicks may have been infected by mosquitoes, suggesting local circulation of WNV. However, if this was the case, at least some individuals would be expected to have high titres (80 or greater), as has been reported in experimentally (Komar *et al.*, 2003) or naturally infected birds (i.e. individuals repeatedly captured and sero-converted in the field; authors' unpublished data). In addition there would probably be differences in the prevalence between localities because the number of mosquitoes and prevalence of the virus would not necessarily be the same in each locality.

The second possibility is that the antibodies were passively transferred. Maternal antibodies transferred via egg yolk may survive for over a month (Komar, 2001; Gibbs *et al.*, 2005). Greater Flamingos, the species in which we found the highest prevalence of antibodies, feed their chicks with a secretion that is produced by glands in the upper

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digestive tract and that is very rich in blood, protein, carbohydrate and fat (Lang, 1963).

This is a similar mechanism to that used by pigeons to feed their chicks.

Immunoglobulin A and immunoglobulin G are present in the crop milk produced by pigeons (Engberg *et al.*, 1992) and consequently chicks may receive maternal and paternal antibodies, even after hatching. Parental transmission of antibodies, although not yet demonstrated in flamingos, would explain the similar prevalences of WNV-neutralizing antibodies in Fuente de Piedra and Doñana. The habitat in Doñana is only suitable for breeding by Greater Flamingos some years, and birds breeding there originate mainly from the Fuente de Piedra population and, to a lesser extent, La Camargue (Nager *et al.*, 1997; Rendón-Martos *et al.*, 2001). The transfer of antibodies by this secretion could also explain the higher prevalence of antibodies in this species as both males and females would be able to transfer antibodies to the chicks.

The third possibility is that the antibody was not induced by WNV infections, but by a cross-reacting virus antigenically related to WNV. Neutralizing antibody titres are often determined in parallel against different flaviviruses to establish the specificity of the immune response. Unfortunately, this analysis could not be carried out during our study due to the small volumes of sera available from most birds. However, WNV foci have been identified in two places within 100 km of the sampling areas, in Kenitra (Morocco, Schuffenecker *et al.*, 2005) and the Algarve (Portugal, Esteves *et al.*, 2005), and IgM specific for WNV has been detected recently in a human in southern Spain (Bofill *et al.*, 2006), confirming WNV activity close to the areas under study. In addition, a previous survey of adult wild birds from these areas detected WNV neutralizing antibody titres in a number of bird species, and in all cases the titres against another related flavivirus (Usutu virus) were consistently lower than those against WNV (Figuerola *et al.*, unpublished data). The specificity of the virus-neutralization test

employed in our study has been analyzed previously as part of an external quality assurance test (Niedrig *et al.*, 2006) using a panel of sera with specificity for different flaviviruses (WNV, yellow fever virus, dengue virus and tick-borne encephalitis virus), and titres of 10 or higher were only detected in sera from WNV-infected birds. We cannot rule out the possibility of a cross-reaction with a related flavivirus, particularly in birds with lower titres. Nevertheless, even if these low titres were discounted, the main conclusions of this study would remain unchanged.

Although we were unable to determine conclusively whether the antibodies had a parental origin or were produced by the chicks, the differences between the species and similarities between localities support the importance of parental transmission as an explanation for our results. Results from a previous study indicate that some birds breeding in Spain have been exposed to WNV and that the prevalence of antibodies is higher in migratory species (Figuerola *et al.*, unpublished data). All the species included in our study were migratory. In recent years the movements of *Phoenicopterus ruber* have been studied with satellite transmitters (Amat *et al.*, 2005). During the breeding season flamingos very frequently move distances of up to 200 km between wetlands in southern Spain to collect food for their chicks. During the non-breeding season movements are less frequent but occur over greater distances (up to 1,189 km) and individuals visit localities in northern and north-western Africa (Algeria, Tunisia, Morocco, Western Sahara, and Mauritania) that overlap with areas affected by recent outbreaks of WNV (see Figure 1A).

Many Glossy Ibises stay in the Doñana area until work ends in the rice fields and then move to North Africa or, as was the case during the extreme drought of the winter 2004-2005, to western Europe (Figure 1B). Most sightings of Yellow-legged Gulls from

the colonies studied have occurred in Spain, although some individuals have been observed along the Atlantic coast of France, Portugal and Morocco (see Figure 1C).

No antibodies were found in the chicks of the other four species studied: the Slender-billed Gull (*Larus genei*), the Gull-billed Tern (*Sterna nilotica*), the Common Pochard (*Aythya ferina*) and the White Stork (*Ciconia ciconia*). While *L. genei* and *A. ferina* remain in Europe or the Mediterranean Basin during the winter, *S. nilotica* winters farther south in West and East Africa (Delany & Scott, 2002). *C. ciconia* is a migratory species in Europe and many individuals migrate to Africa (see Figure 1D). Spanish populations, however, have become all but sedentary due to their ability to exploit the permanent food resources provided by rubbish tips (Tortosa *et al.*, 1995) and most sightings of marked *C. ciconia* were in Spain (5733 out of 5760). In previous analyses of adult birds we found that only one of 12 adult *C. ciconia* contained neutralizing antibodies (Figuerola *et al.*, unpublished data). There are no data on the prevalence of antibodies in adults of the other species.

Our results are consistent with the hypothesis that adult birds have been exposed to WNV (or a highly related flavivirus). This may have occurred in Spain or in other areas visited during their migrations. During some of these movements birds visit areas where WNV has been reported over the last decade. However, our results should be interpreted with caution. The presence of antibodies does not indicate that an individual will disperse the virus; rather, it only means that the individual has been exposed to the virus and has as a result developed immunity against it. For successful dispersal of the virus between migratory stopovers, birds would have to accumulate fat reserves, fly for a long time over large distances and then be bitten by a suitable vector during the viraemic phase of the infection, which usually lasts much less than seven days (Komar *et al.*, 2003) after arrival in a new area. Sub-clinical effects of virus infection on the

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activity of birds and their capacity to migrate would further reduce the likelihood of dispersal, and the importance of these processes in long-distance dispersal of pathogens merits further attention, given the growing interest in the role of wildlife in the epidemiology of emerging zoonotic diseases.

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Table 1. Species, locality, and date of blood samples.

Species	Locality	Date	Number Sampled
<i>Aythya ferina</i> Common Pochard	Doñana	8-10 July 2003	21
<i>Ciconia ciconia</i> European White Stork	Doñana	1 May-26 June 2003	78
<i>Larus cachinnans</i> Yellow-legged Gull	Odiel	21 June 2003	18
<i>Larus genei</i> Slender-billed Gull	Doñana	2 July 2003	45
<i>Phoenicopterus ruber</i> Greater Flamingo	Doñana	8 August 2003	106
<i>Phoenicopterus ruber</i>	Fuente de Piedra	12 July 2003	163
<i>Plegadis falcinellus</i> Glossy Ibis	Doñana	23 May-4 July 2003	52
<i>Sterna nilotica</i> Gull-billed Tern	Doñana	2 July 2003	51

Table 2. Summary of the analyses of antibody titres in nearly full-grown chicks of seven different waterbird species in southwest Spain.

Species	0	10	20	40	80	160	% ≥ 10
<i>Aythya ferina</i>	21	-	-	-	-	-	0
<i>Ciconia ciconia</i>	78	-	-	-	-	-	0
<i>Larus cachinnans</i>	17	-	-	1	-	-	5.6
<i>Larus genei</i>	45	-	-	-	-	-	0
<i>Phoenicopterus ruber</i> (Doñana)	87	6	4	9	-	-	17.9
<i>Phoenicopterus ruber</i> (Fuente de Piedra)	145	7	10	1	-	-	11.0
<i>Plegadis falcinellus</i>	51	1	-	-	-	-	1.9
<i>Sterna nilotica</i>	51	-	-	-	-	-	0

Figure 1. Movements of *Phoenicopterus ruber* marked with satellite trackers, redrawn from Amat *et al.*, 2005 (A), *Plegadis falcinellus* (B), *Larus cachinnans* (C), and *Ciconia ciconia* (D).

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