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# Size matters: West Nile Virus neutralizing antibodies in resident and migratory birds in Spain

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**Abstract**

The rapid range expansion of West Nile Virus has raised interest in understanding the population dynamics and dispersal patterns of emerging infectious diseases by wildlife. We analyzed different ecological and evolutionary factors related to West Nile Virus neutralizing antibody prevalence in 72 bird species sampled in southern Spain. Prevalence of antibodies reached its maximum during the autumn and winter in comparison to summer months. Prevalence of antibodies was directly related to body mass and migratory behaviour. The greater prevalence of antibodies observed in summer migrants can be explained, among other factors, by the diversity of localities involved in their life cycles or the geographic areas visited during their migrations. Greater prevalence in larger species was explained by their longevity because the relationship was already significant when analyzing only first year birds, and probably also involved a high attraction to vectors by larger hosts. Coloniality and winter gregarism were unrelated to the prevalence of antibodies against this highly host generalist pathogen. Evolutionary relationships between species were unrelated to differences in the prevalence of antibodies. Our results suggest larger species as good candidates for easy, faster and cheaper monitoring of local, seasonal and annual changes in WN virus serology.


*Keywords:* Dispersal; Host ecology; Migratory birds; Monitoring program; Reservoir diversity; Virus dispersal; West Nile Virus ecology

## 1. Introduction

West Nile Virus (WNV) is a member of the *Flavivirus* genus (family Flaviviridae), transmitted by mosquito bites. Humans infected by WNV may develop a variety of signs ranging from mild fever to more severe illnesses such as acute encephalitis,

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poliomyelitis, meningitis, or hepatitis and is fatal in a small percentage (<1%) of cases (Hubálek and Halouzka, 1999). WNV is widely distributed throughout Africa, Asia, Europe, Australia (Kunjin virus). It was first detected in New York in 1999, and in just 6 years has spread throughout all of North America (CDC, 2005). It has been suggested that one of the causes of this rapid expansion is the high mobility of the virus' avian reservoirs (Rappole and Hubálek, 2003) and its wide host range (already detected in more than 285 avian species; CDC, 2005).

In Europe and Africa WNV infection is usually non-fatal for birds (Hubálek and Halouzka, 1999); in the New World, however, the virus has killed many birds (Marra et al., 2004), and reduced populations of more susceptible hosts by up to 45% since WNV arrival (LaDeau et al., 2007). As an example of the different epidemiology in Europe and North America, while experimental infection with WNV of North American birds usually result in high mortalities (i.e. 32.3% of 87 experimentally infected birds of 25 species, Komar et al., 2003), experimental infections done in Europe have reported no apparent mortality due to WNV (9 geese experimentally infected by Malkinson and Banet, 2002). The reasons for this high virulence in North America remain largely unknown; nevertheless, the fact that species from the Nearctic have never been exposed to the virus and the higher pathogenicity observed in the introduced strain (Brault et al., 2004) may explain these differences.

A number of ecological factors can be associated with a higher prevalence or diversity of pathogens in birds: migratory behaviour, coloniality or gregarism, habitat use, mating systems, and immune system capacity (Møller and Erritzoe, 1996; Clayton and Moore, 1997; Figuerola, 1999, 2000; Figuerola and Green, 2000; Tella, 2002). However, to our knowledge, no study has focused on vector-borne generalist pathogens. Despite the thousands of birds that have been tested for WNV or its antibodies in North America, analyses focusing on the relationship between bird ecology and exposure to the virus are still lacking but urgently needed. In this study we take advantage of the differences in the impact of WNV in Europe and in North America to analyze the relationship between bird ecology and phylogeny and prevalence of WNV neutralizing antibodies. The relevance of this study is twofold, on the one hand, the low host specificity of WNV makes this system different from the pathogens used in previous studies (mainly blood parasites and ectoparasites), and may affect the relevance of different ecological factors. On the other hand, given the relevance of WNV for human health and wildlife conservation we also aim to identify the characteristics of the species that can be most useful for monitoring in Europe.

In this paper, we first analyze the relationship between host evolutionary and ecological characteristics and the prevalence of WNV neutralizing antibodies in birds. Second, as we report important differences in the prevalence of antibodies according to host characteristics we used a statistical power analysis to discuss the relevance of our results in relation to WNV monitoring in Europe.

## 2. Materials and methods

Between January 2003 and February 2005 we captured 1213 individuals belonging to 72 species (49 genera, 22 families, and 8 orders). Birds were captured without damage using mist-nets and walk-in-traps in the Guadalquivir and Odiel Marshes (SW Spain). Blood samples were taken with syringes from the brachial, femoral, or jugular vein, birds were marked with numbered aluminum rings and released after manipulation. The volume of blood extracted depended on the size of the species and never exceeded 1% of body mass (range 0.080–1 ml). Blood was collected in eppendorf tubes, allowed to clot at ambient temperature, and placed into coolers until centrifugation during the same day. All samples were obtained from adult (full grown) individuals to ensure that the antibodies were not the result of the passive transfer of maternal immunity (Gibbs et al., 2005). When possible age was determined (471 first-year individuals and 540 after first-year individuals) according to Prater et al. (1977), Baker (1993) and Svensson (1996).

WNV strain Eg101 and the E6 clone of Vero cells used for virus propagation were obtained from Hervé Zeller (Institut Pasteur de Lyon). The Usutu virus (SAAR 1776 isolate) was obtained through the Centre for Ecology and Hydrology, Oxford, UK, and propagated in Vero cells (American Type Culture Collection, Manassas, VA). Virus titers were

determined by end-point titration following the method used by Reed and Muench (1938). WNV-Neutralizing antibody titers were determined by a micro-virus-neutralization test (micro-VNT) in 96-well plates, adapted from a previously described method (Jiménez-Clavero et al., 2001). A recent study shows that a micro-VNT assay and the standard $PRNT_{90}$ perform comparably in sensitivity at detecting anti-WNV antibodies in birds (Weingartl et al., 2003). Serum samples were inactivated at 56 °C for 30 min prior to the analysis. Dilutions of test sera (25 ml) were incubated with 100 $TCID_{50}$ of WNV strain Eg101 in the same volume (25 ml) for 1 h at 37 °C in Eagle's medium (EMEM) supplemented with L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, followed by the addition of 50 ml of a suspension ($2 \times 10^5$ cells/ml) of Vero E6 cells in the same medium plus fetal calf serum to a final concentration of 5%. The mixture was further incubated for 6–7 days (37 °C in a 5% $CO_2$ and saturating humidity atmosphere) until cytopathic effect (cpe) was observed in control wells containing 10 $TCID_{50}$ of virus. The screening of samples was performed at 1:10 and 1:20 dilutions of tested sera (dilutions considered before the addition of virus, that is, in a volume of 25 ml). Only samples yielding positive neutralization (complete absence of cpe) at 1:20 were scored as positives and further titrated by analyzing serial serum dilutions from 1:20 to 1:640. Neutralizing serum titer was considered as the highest value of the reciprocal serum dilution giving a complete absence of cpe.

The specificity of the assay was assessed in two ways. First, by analyzing a panel of sera from an external quality assessment, consisting of serum samples containing antibodies from other four flaviviruses, that proved negative for neutralization titers in our WNV assay, while duplicate testing of all WNV antibody-positive serum samples proved positive (>1:20) for neutralization titers (Niedrig et al., 2007). Second, we also compared the neutralizing antibodies titers of 18 samples tested in parallel for WNV and Usutu virus (a closely related JEV group avian virus). In none of the cases showed higher antibody titers more specific to Usutu than to WNV (see Figuerola et al., 2007a, for more details). We cannot discard that the serology to WNV observed in some of the samples, particularly those from birds flying from Central Europe (e.g. *Turdus philomelos, Sylvia atricapilla*) could be attributed to cross-reacting antibodies to other flaviviruses (particularly TBEV) that can be prevalent in Central Europe. However, this seems unlikely since the technique has shown no cross reactivity to TBEV-positive sera.

In a first model, we investigated the effects of taxonomic relationships on West Nile Virus prevalence by using Generalized Linear Mixed Models (GLMM). GLMM allows a more versatile analysis of correlation than standard regression methods, because the error distribution of the dependent variable and the function linking predictors to it can be adjusted to the characteristics of the data (Littell et al., 1996). Our response variable was the antibody status (1 present, 0 absent), and we used a binomial distributed error and a logistic link function, to ensure linearity, and statistics adjusted to model dispersion. Binomial errors are adequate to analyze binary response variables. Goodness-of-fit of the model was assessed by checking the overdispersion parameter and the Generalized Chi-Square statistic (Littell et al., 1996). Period (a three levels factor, summer: birds captured in June–August, autumn: September–November and winter: December–March) and age (first-year or adult bird, not including unknown age birds in the analyses) were included as fixed factors in the analyses. Species was included as a repeated subject effect (i.e. observations of a same species are correlated) and the interaction between species and period was included as a random factor. The statistical significance of each nested taxonomic level (Genera, Family and Order) was tested using Z-statistics for random effects using the macro GLIMMIX for SAS 8.2 (Littell et al., 1996). As age had no significant effect ($F_{1,27} = 1.88$, $P = 0.18$, $N = 957$), we report the results of analyses excluding this variable to include the full dataset and range of species.

In a second model, we analyzed the relevance of different ecological factors. Species body mass (log transformed mean values to fit a normal distribution as judged by checking the normal quantile plot), migratory behaviour (resident or migratory species), breeding sociality (solitary or colonial breeders), and winter sociality (solitary or gregarious species) were included as fixed factors in the analyses. Values for

Table 1
Model analyzing the relationship between host ecology, period of capture and presence of West Nile Virus (WNV) neutralizing antibodies in the blood of 1213 individuals birds captured in south-west Spain

| | Estimate ± S.E. | $F$ | d.f. | $P$ |
|---|---|---|---|---|
| Body mass | 0.746 ± 0.288 | 6.72 | 1,69 | 0.01 |
| Migratory behaviour | 2.032 ± 1.084 | 0.71 | 1,69 | 0.40 |
| Period | | 3.55 | 2,29 | 0.04 |
| Summer | 0 | | | |
| Autumn | 2.194 ± 1.286 | | | |
| Winter | 2.287 ± 1.133 | | | |
| Coloniality | | 0.67 | 1,68 | 0.42 |
| Winter gregarism | | 2.82 | 1,68 | 0.10 |
| Body mass × Migratory behaviour | | 0.71 | 1,68 | 0.40 |
| Body mass × Period | | 1.21 | 2,1135 | 0.30 |
| Body mass × Coloniality | | 0.65 | 1,68 | 0.42 |
| Body mass × Winter gregarism | | 3.23 | 1,68 | 0.08 |
| Migratory behaviour × Period | | 3.71 | 2,29 | 0.04 |
| Migratory species in autumn | −1.021 ± 1.366 | | | |
| Migratory species in winter | −3.653 ± 1.431 | | | |
| Others | 0 | | | |
| Migratory behaviour × Coloniality | | 1.63 | 2,67 | 0.20 |
| Migratory behaviour × Winter gregarism | | 1.41 | 2,67 | 0.25 |
| Period × Coloniality | | 1.28 | 3,26 | 0.30 |
| Period × Winter gregarism | | 0.99 | 3,26 | 0.41 |
| Coloniality × Winter gregarism | | 1.55 | 2,67 | 0.22 |

Final model was obtained after backwards variable selection. Only variables with $P < 0.05$ are interpreted as statistically significant and parameter estimates are given. For variables not included in the model no parameter estimate is presented and the $F$ and $P$ values correspond to the values when added to the final model.

these variables were taken from literature (Cramp, 1982–1994) and were validated by four independent ornithologists according to the ecology of the species in Spain. For each individual we also included the period of collection to control for seasonal differences in the prevalence of antibodies. To test the relationship between ecological factors and antibody prevalence we followed a stepwise-backward selection procedure starting from an initial model including all the two-way interactions between factors.

We estimated the sample size necessary to detect increases of 10, 20, 30 and 40% in WNV seroprevalence with the program G-Power (Buchner et al., 1997). Effect sizes were calculated for prevalences between 1 and 55%, and sample size necessary to obtain a power of 0.80 when using a Chi-Square test was estimated. A power of 0.80 indicates that a significant result ($P < 0.05$) will be obtained in 80% of the analyses of datasets with statistical differences of that magnitude, and is the threshold value usually used in ecology (Bausell and Li, 2002).

## 3. Results

Of the 1213 individuals tested, 126 (10.4% of individuals from 24 out of 72 species) had WNV neutralizing antibodies, with titers ranging from 1:20 to over 1:640 (see Electronic Appendix A). Important interspecific differences in the presence of WNV neutralizing antibodies were found, with prevalences ranging from 0 to 42.9%. However, taxonomic levels were unrelated to these differences in prevalence (Genera, $Z = 0.80$, $P = 0.21$; Family, $Z = 1.11$, $P = 0.14$; Order, $Z = 0.58$, $P = 0.28$).

Multivariate analyses indicate that antibody prevalence was unrelated to host sociality (Table 1). Prevalence of antibodies changed seasonally (Table 1) with significantly higher prevalences in autumn (mean ± S.E.: 10.29% ± 12.71) than in summer (a test of Least-Square means difference, 2.09 ± 7.72, $t_{29} = 2.46$, $P = 0.02$), and intermediate prevalences in winter (3.27% ± 9.12, contrast with autumn, $t_{29} = 1.85$, $P = 0.07$; contrast with summer, $t_{29} = 0.61$, $P = 0.55$).

Although migratory behaviour was not directly related to antibody prevalence (migrants, 5.23% ± 7.41; residents, 3.32% ± 8.13), a significant interaction with season was found (Table 1). Prevalence did not change with season in resident species ($F_{2,29} = 2.11$, $P = 0.14$) but only in migratory species ($F_{2,29} = 6.15$, $P = 0.006$). When comparing migrant and resident species within each period, in summer migrants (i.e. species wintering in Africa) tended to have higher prevalences of antibodies that residents (5.56% ± 8.58 vs. 0.77% ± 8.85; $t_{29} = 1.88$, $P = 0.07$). Winter migrants (i.e. coming from central and northern Europe) tended to have lower prevalences than resident species (1.48% ± 10.49 vs. 7.06 ± 12.16; $t_{29} = 1.70$, $P = 0.09$). Large species (as estimated from their body size) had higher prevalences of antibodies (Table 1, Fig. 1).

As age may affect the relationship between antibody prevalence and body mass, the analyses were repeated using only less than 1 year old birds (417 individuals), confirming that the relationship between prevalence and body mass was significant also when considering only birds of the same age ($F_{1,43} = 5.61$, $P = 0.02$).

Power analyses indicate that the sample size necessary to detect significant changes in seroprevalence depends dramatically on initial seroprevalence (Fig. 2). For example, 4857 individuals are necessary to detect a 40% increase in seroprevalence when initial seroprevalence is 1% (i.e. in our study *Passer domesticus* had a prevalence of 0%), but only 74 individuals are necessary when focusing in species with 40% prevalence (i.e. *Fulica atra*, with 42.6% prevalence or *Larus ridibundus*, with 42.9%).

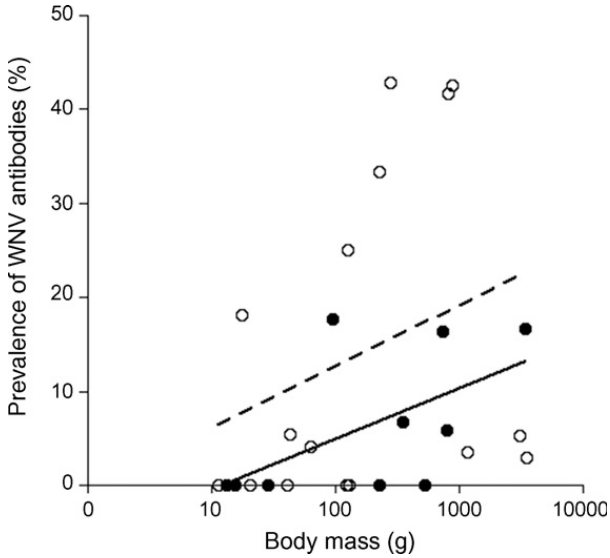


Fig. 1. Prevalence of West Nile Virus (WNV) neutralizing antibodies in relation to body size (grams) in resident (418 individuals) and migratory (795 individuals) birds sampled in south-west Spain. For illustration purposes a regression line has been plotted for migratory and resident species. Open symbols and dotted line correspond to migratory species and filled symbols and continuous line to resident species. Only species with at least ten individuals sampled have been included in the plot.

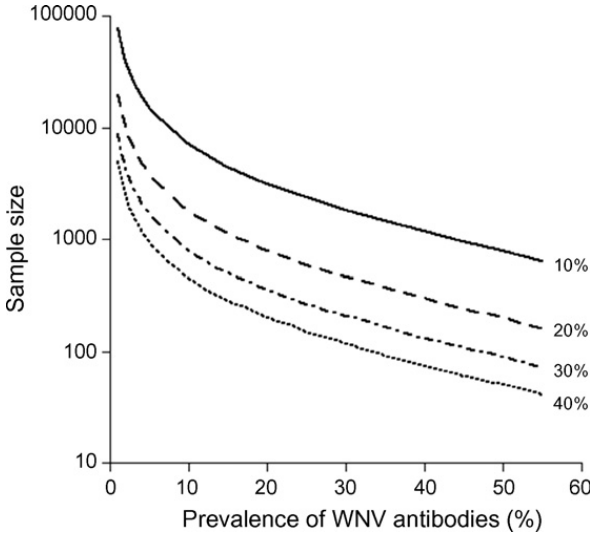


Fig. 2. Sample size necessary to detect with a Chi-square test and a power of 0.80 increases by 10, 20, 30 and 40% in the prevalence of West Nile Virus (WNV) antibodies.

## 4. Discussion

In Spain, clinical signs of WNV disease in birds has only been reported recently (Höfle et al., 2008). WNV neutralizing antibodies had been reported in horses (Jiménez-Clavero et al., 2007), chicks of different colonial breeding waterbirds (Figuerola et al., 2007a), and the rapid seroconversion of common coots during a capture–recapture study has also confirmed the local circulation of WNV in the study area (Figuerola et al., 2007b). Infections with clinical symptoms in humans were reported in 2004 in Badajoz (Spain) and Algarve (Portugal) (Esteves et al., 2005; Kaptoul et al., 2007), overlapping with the collection of samples for this study. Previous records give a seroprevalence of up to 30% in humans in some towns in the Ebro Delta (Lozano and Filipe, 1998) and of 16.5% in northwest Spain (González and Filipe, 1977), presumably with maximum epidemic activity during the 1970s.

However, these results were obtained by haemagglutination-inhibition, a technique burdened by its cross-reactivity with a range of flaviviruses. Recent studies with highly specific neutralization assays show that the prevalence of WNV antibodies in humans living around wetlands in Spain is currently very low (Bofill et al., 2006).

The presence of serum antibodies neutralizing WNV in adult birds indicates previous contact (infection) with WNV or a closely antigenically related flavivirus, and survival to the initial infection. Consequently, for a wild bird population with low pathogenicity WNV infection (such as those usually found in the Old World, Zeller and Schuffenecker, 2004), the higher the prevalence of WNV neutralizing antibodies, the higher the exposure to the virus. This scenario is not applicable when WNV infection results in high mortality, as observed in North America.

The results suggest that evolutionary relationships are of little importance in explaining variations in exposure to WNV. This contrasts with the initial studies that identified Corvidae (McLean et al., 2001), Mimidae, and Cardinalidae (Ringia et al., 2004) as bird families that are particularly exposed to WNV infection. In our study both Rallidae (6.7–42.6%) and Laridae (25.0–42.9%) presented very high antibody prevalence, although our results suggest that these high prevalences were related to the ecology of the species sampled (migratory species of medium and large size), rather than to the birds' taxonomy.

In North America, the American Crow appears to be particularly susceptible to mortality by WNV (Komar et al., 2003). This has led some researchers to suggest that Corvidae in general might be very at a risk for exposure to the virus. Interestingly, none of the 35 individuals of *Corvus monedula* (the only Corvidae included in our study) had WNV antibodies, even when captured together with individuals of other species with high prevalences. It is important to note that this low (zero) prevalence of antibodies in *Corvus monedula* is not likely to result from the rapid death of infected individuals, given that all attempts we have done to the moment to detect the virus in several hundreds dead water-birds had failed (data not shown). We suggest that the high incidence of West Nile in American Crow can result not only from the transmission by mosquitoes but also from the consumption of corpses of birds dying during the viraemic phase of the infection. In this case the utility of Corvids for monitoring WNV circulation in the wild could be reduced in Europe.

No effect of winter or breeding sociality on antibody prevalence was found. Although a high prevalence of blood parasites had been reported among social living species (Tella, 2002), the low host-specifity of WNV may make the density of birds the relevant parameter affecting risk of exposition, regardless whether or not it consists of conspecifics. Interestingly, migratory species showed higher antibody prevalence than resident species, but only when comparing summer migrants with residents. Although local circulation of the virus is taking place (since resident species also have antibodies), this higher prevalence observed in migratory birds suggest that these birds spend part of their lives in areas in Africa where the circulation of the virus may be higher than in the surveyed area in Spain. For example, a recent serosurvey in horses detected extremely high prevalences of antibodies (up to 97%) in some sub-Saharan countries (Cabre et al., 2006), areas visited by many European long distance migratory species. Our analyses support the view that species of larger body mass may have increased opportunity for exposure to WNV. Given that we analyzed antibody prevalence in free-living and apparently healthy individuals, this conclusion is not merely a bias caused by the difficulties in finding carcasses of smaller species (Marra et al., 2004), a problem associated with studies based only on dead birds. The direct relationship between prevalence of WNV antibodies and body mass can be explained by several non-exclusive factors. Larger species live longer (Calder, 1984), however we have demonstrated that the relationship between seroprevalence and body mass is also significant when analyzing only first-year birds. We suggest that the larger prevalence of antibodies in larger species is the result of their larger surface area and higher $CO_2$ production (Nagy, 1987), and can host and attract a higher number of ectoparasites (Soliman et al., 2001), mosquitoes, and other biting arthropods that transmit the virus.

In conclusion, we suggest that migratory birds of large body mass may provide a means for monitoring WNV prevalence on a large geographical scale (e.g. migratory flyways). Additionally, resident species of large body mass may provide a better description of

local WNV prevalence. Further, the past allegations regarding a greater probability of infection by WNV by particular taxonomic groups should be more carefully explored given our findings and the fact that many of those studies were based on the examination of only a few species, and on dead birds, making difficult to separate the effects of exposure, susceptibility and carcass detection probability. From a conservation standpoint, it perhaps would be more beneficial to focus our attention on the effects of WNV on species of greater body mass and on migratory species.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2008.04.023.

## References

Baker, B., 1993. Identification guide to European Non-Passerines. BTO Guide 24.

Bausell, R.B., Li, Y.-F., 2002. Power Analysis for Experimental Research. Cambridge University Press.

Bofill, D., Domingo, C., Cardeñoso, N., Zaragoza, J., de Ory, F., Minguell, S., Sánchez-Seco, M.P., Dominguez, A., Tenorio, A., 2006. Human West Nile virus infection, Catalonia, Spain. Emerg. Infect. Dis. 12, 1307.

Brault, A.C., Langevin, S.A., Bowen, R.A., Panella, N.A., Biggerstaff, B.J., Miller, B.R., Komar, N., 2004. Differential virulence of West Nile strains for American crows. Emerg. Infect. Dis. 10, 2161–2168.

Buchner, A., Faul, F., Erdfelder, E., 1997. G·Power: A Priori, Post-hoc, and Compromise Power Analyses for the Macintosh (Version 2.1.2) [Computer Program] University of Trier, Trier, Germany.

Cabre, O., Grandadam, M., Marié, J.-L., Gravier, P., Prangé, A., Santinelli, Y., Rous, V., Bourry, O., Durand, J.-P., Tolou, H., Davoust, B., 2006. West Nile Virus in horses, sub-Saharan Africa. Emerg. Infect. Dis. 12, 1958–1960.

Calder, W.A., 1984. Size, Function and Life History. Harvard University Press, Cambridge, MA.

Centers for Disease Control and Prevention, 2005. Available from http://www.cdc.gov/ncidod/dvbid/westnile/birdspecies.htm.

Clayton, D.H., Moore, J., 1997. Host–Parasite Evolution: General Principles and Avian Models. Oxford University Press, Oxford.

Cramp, S., 1982–1994. The Birds of the Western Palearctic, vols. 1–9. Oxford University Press, Oxford.

Esteves, A., Almeida, A.P.G., Galao, R.P., Parreira, R., Piedade, J., Rodrigues, J.C., Sousa, C.A., Novo, M.T., 2005. West Nile Virus in southern Portugal. Vector Borne Zoonotic. Dis. 5, 410–413.

Figuerola, J., 1999. Effects of salinity on rates of infestation of waterbirds by haematozoa. Ecography 22, 681–685.

Figuerola, J., 2000. Ecological correlates of feather mite prevalence in passerines. J. Avian Biol. 31, 489–494.

Figuerola, J., Green, A.J., 2000. Haematozoan parasites and migratory behaviour in waterfowl. Evol. Ecol. 14, 143–153.

Figuerola, J., Jiménez-Clavero, M.A., Rojo, G., Gómez-Tejedor, C., Soriguer, R., 2007a. Prevalence of West Nile Virus neutralizing antibodies in colonial aquatic birds in southern Spain. Avian Pathol. 36, 209–212.

Figuerola, J., Soriguer, R., Rojo, G., Gómez-Tejedor, C., Jímenez-Clavero, M.A., 2007b. Seroconversion in wild birds and local circulation of West Nile Virus. Spain. Emerg. Infect. Dis. 13, 1915–1917.

Gibbs, S.E.J., Hoffman, D.M., Stark, L.M., Marlenee, N.L., Blitvich, B.J., Beaty, B.J., Stallknecht, D.E., 2005. Persistence of antibodies to West Nile Virus in naturally infected Rock Pigeons (*Columba livia*). Clin. Diagn. Lab. Immunol. 12, 665–667.

González, M.T.G., Filipe, A.R., 1977. Antibodies to arboviruses in Northwestern Spain. Am. J. Trop. Med. Hyg. 26, 792–797.

Höfle, U., Blanco, J.M., Crespo, E., Naranjo, V., Jiménez-Clavero, M.A., Sanchez, A., de la Fuente, J., Gortazar, C., 2008. West Nile virus in the endangered Spanish imperial eagle. Vet. Microbiol. 129, 171–178.

Hubálek, Z., Halouzka, J., 1999. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg. Infect. Dis. 5, 643–650.

Jiménez-Clavero, M.A., Escribano-Romero, E., Douglas, A., Ley, V., 2001. The VP1 N-terminal region of swine vesicular disease virus contains a neutralization site that arises upon cell attachment and is involved in viral entry. J. Virol. 75, 1044–1047.

Jiménez-Clavero, M.A., Gómez-Tejedor, C., Rojo, G., Soriguer, R., Figuerola, J., 2007. Serosurvey of West Nile virus in equids and bovids in Spain. Vet. Rec. 161, 212.

Kaptoul, D., Viladrich, P.F., Domingo, C., Niubó, J., Martínez-Yélamos, S., de Ory, F., Tenorio, A., 2007. West Nile virus in Spain: reports of the first diagnosed case (in Spain) in a human with aseptic meningitis. Scand. J. Infect. Dis. 39, 70–93.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R., Bunning, M., 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis. 9, 311–322.

LaDeau, S.L., Kilpatrick, A.M., Marra, P.P., 2007. West Nile virus emergence and large-scale declines of North American bird populations. Nature 447, 710–713.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., 1996. SAS System for Mixed Models. SAS Institute, Cary, USA.

Lozano, A., Filipe, A.R., 1998. Anticuerpos frente a virus West Nile y otros virus transmitidos por Artropodos en la población del Delta del Ebro. Rev. Esp. Salud Publ. 72, 245–250.

Malkinson, M., Banet, C., 2002. The role of birds in the ecology of West Nile virus in Europe and Africa. Curr. Top. Microbiol. 267, 309–322.

Marra, P.P., Griffing, S., Caffrey, C., Kilpatrick, A.M., McLean, R., Brand, C., Saito, E., Dupuis, A.P., Kramer, L., Novak, R., 2004. West Nile virus and wildlife. Bioscience 54, 393–402.

McLean, R.G., Ubico, S.R., Docherty, D.E., Hansen, W.R., Sileo, L., McNamara, T.S., 2001. West Nile virus transmission and ecology in birds. Ann. N.Y. Acad. Sci. 951, 54–57.

Møller, A.P., Erritzoe, J., 1996. Parasite virulence and host immune defense: host immune response is related to nest reuse in birds. Evolution 50, 2066–2072.

Nagy, K.A., 1987. Field metabolic rates and food requirement scaling in mammals and birds. Ecol. Monogr. 57, 111–128.

Niedrig, M., Donoso Mantke, O., Altmann, D., Zeller, H., 2007. First international diagnostic accuracy study for the serological detection of West Nile virus infection. BMC Infect. Dis. 7, 72.

Prater, A.J., Marchant, J., Vuorinen, J., 1977. Guide to the identification and ageing of Holartic waders. Br. Trust Ornithol..

Rappole, J.H., Hubálek, Z., 2003. Migratory birds and West Nile virus. J. Appl. Microbiol. 94, 47S–58S.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Ringia, A.M., Bradley, J.B., Koo, H.-Y., van de Wyngaerde, M., Brawn, J.D., Novak, R.J., 2004. Antibody prevalence of West Nile Virus in birds, Illinois, 2002. Emerg. Infect. Dis. 10, 1120–1124.

Soliman, S., Marzouk, A.S., Main, A.J., Montasser, A.A., 2001. Effect of sex, size, and age of commensal rat hosts on the infestation parameters of their ectoparasites in a rural area of Egypt. J. Parasitol. 87, 1308–1316.

Svensson, L., 1996. Guia para la Identificación de los Passeriformes Europeos. Sociedad Española de Ornitología, Madrid.

Tella, J.L., 2002. The evolutionary transition to coloniality promotes higher blood parasitism in birds. J. Evol. Biol. 15, 32–41.

Weingartl, H.M., Drebot, M.A., Hubálek, Z., Halouzka, J., Andonova, M., Dibernardo, A., Cottam-Birt, C., Larence, J., Marszal, P., 2003. Comparison of assays for the detection of West Nile virus antibodies in chicken serum. Can. J. Vet. Res. 67, 128–132.

Zeller, H.G., Schuffenecker, I., 2004. West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. Eur. J. Clin. Microbiol. Infect. Dis. 23, 147–156.