

1 **Antibodies to West Nile Virus and related Flaviviruses in wild boar, red foxes and**
2 **others mesomammals from Spain**

3
4 Ana-Valeria Gutiérrez-Guzmán^a, Joaquín Vicente^a, Raquel Sobrino^a, Elisa Perez-Ramírez^b,
5 Francisco Llorente^b and Ursula Höfle^{a*}

6
7 ^aInstituto de Investigación en Recursos Cinegéticos IREC, (CSIC-UCLM-JCCM), Ciudad Real, Spain

8 ^bCentro de Investigación en Sanidad Animal del Instituto Nacional de Investigación y Tecnología Agraria y
9 Alimentaria (CISA-INIA), Ctra Algete-El Casar, s/n, 28130, Valdeolmos (Madrid), Spain

10

11 * Corresponding author: Instituto de Investigación en Recursos Cinegéticos IREC, (CSIC-UCLM-JCCM),
12 Ronda de Toledo s/n, 13005 Ciudad Real, Spain. Tel: +34 926 295450; fax: +34 926 295451.

13 *E-mail addresses:* ursula.hofle@uclm.es (U. Höfle).

14

15

16 **Abstract**

17 Red foxes (*Vulpes vulpes*), wild boar (*Sus scrofa*) and Iberian pigs (*Sus scrofa domestica*)

18 that are raised extensively outdoors, as well as other wild mesomammals from south

19 central Spain and wild boar from Doñana National Park (DNP), were tested for antibodies

20 against West Nile Virus (WNV) and related flaviviruses by ELISA and against WNV by

21 VNT. Mean flavivirus seroprevalence according to ELISA was $20.4 \pm 7.8\%$ (21 out of 103)

22 in red foxes, $12.6 \pm 2.8\%$ (69 out of 545) in wild boars, and $3.3 \pm 2.7\%$ (6 out of 177) in

23 Iberian pigs. A stone marten (*Martes foina*) also tested positive. Flavivirus seroprevalence

24 in wild boar was significantly higher in DNP, and increased with age. Haemolysis of the

25 serum samples limited interpretation of VNT to 28 samples, confirming WNV

26 seroprevalence in one red fox, four Iberian pigs and nine wild boars. ELISA positive,

27 microVNT negative samples suggest presence of non-neutralizing antibodies against WNV

28 or antibodies to other antigenically related flaviviruses. Despite the importance of wetlands

29 for flavivirus maintenance and amplification, WNV/flavivirus seroprevalence in wild boar
30 and red foxes was not associated to wetland habitats. This is the first report of exposure of
31 red foxes to WNV. With view to use of the tested species as sentinels for flavivirus
32 activity, limited exposure of Iberian pigs that would be available for regular sampling, low
33 numbers of foxes collected and concentration of wild boar harvest in the winter season are
34 mayor drawbacks.

35

36 *Keywords:* Flavivirus, West Nile virus, Red fox, Wild boar, Iberian pig, Seroprevalence

37

38 **1. Introduction**

39

40 Flaviviruses have a worldwide distribution, and a number of them such as West Nile Virus
41 (WNV) are the cause of zoonoses of considerable importance for public health. The
42 flavivirus (family *Flaviviridae*) members of the Japanese encephalitis antigenic complex
43 (JEV) are maintained in a mosquito vector and bird reservoir cycle where humans, wild
44 and domestic mammals can be implicated as incidental hosts (Weissenböck et al., 2010).
45 Flavivirus activity has been rather limited in Spain until the last decade in which evidence
46 of circulation of different mosquito-borne flaviviruses has been found with increasing
47 frequency. In Spain, flavivirus activity main comprises three mosquito-borne flaviviruses
48 (WNV, USUV and BAGV; Figuerola et al., 2007; Busquets et al., 2008; Vazquez et al.,
49 2010, 2011; Agüero et al., 2011).

50 WNV is a re-emerging zoonotic virus responsible of outbreaks in humans, domestic
51 animals (horses) and wildlife. In Spain, evidence of exposure to WNV has been found in
52 humans (Kaptoul et al., 2007; Anonymous, 2010), birds (Figuerola et al., 2007; Höfle et
53 al., 2008; López et al., 2008; Jiménez-Clavero et al., 2008), mosquitos (Vázquez et al.,
54 2010, Sotelo et al, 2011a), and horses (Jiménez-Clavero et al., 2010; OIE, 2010).

55 USUV has caused disease and mortality in birds in Europe and recent human cases in Italy
56 (Pecorari et al., 2009), but as yet in Spain it has only been detected in mosquitoes
57 (Busquets et al., 2008; Vázquez et al., 2011) and no related disease has been reported in
58 birds or humans. In contrast, Bagaza virus (BAGV), a Flavivirus of the Ntaya serocomplex
59 that had never been detected before in Europe, was recently isolated from an outbreak of
60 lethal disease in free-living game birds in Southern Spain (Agüero et al., 2011).

61 Mammals can be naturally exposed to flaviviruses, either by bite from infected vectors, or
62 by ingestion of infected carrion or diseased prey (Austgen et al., 2004; Marra et al., 2004).
63 In fact, serological evidence of exposure to flaviviruses, mainly WNV, has been reported
64 in wild and domestic mammals from America, Africa, Asia and Eastern Europe (Root et
65 al., 2005; Bentler et al., 2007; Halouzka et al., 2008; Ohno et al., 2009, El-Harrak et al.,
66 2011). In Spain, a report from 1980 described the presence of antibodies to flaviviruses in
67 rodents (Chastel et al., 1980). WNV seropositivity has been evidenced in horses (Jiménez-
68 Clavero et al., 2010) and also clinical disease and mortality have been reported in this
69 species (OIE, 2010). Recently, we reported on exposure of wild juvenile ungulates, namely
70 wild boar and Iberian red deer (*Cervus elaphus*) to flaviviruses (Boadella et al., 2011).

71 Flavivirus exposure of free-living mammals with a broad geographic range and high
72 population density, such as wild boar (Acevedo et al., 2007), red fox (*Vulpes vulpes*), and
73 other species could be a useful indicator of viral circulation and expand the knowledge on
74 virus ecology in Mediterranean ecosystems (Platt et al., 2008). Likewise, the extensively
75 reared Iberian pig could come in contact with vectors and thus be a useful source of
76 information on flavivirus activity in the region.

77 The objective of this work is to determine, through antibody detection, the degree of
78 exposure to flaviviruses (especially WNV) of wild boar, red foxes, and other medium sized
79 wild and domestic mammals in South-Central Spain and to further assess whether this

80 information can be useful to monitor flavivirus activity in a given area. Also, the data
81 collected in this study from wild boar will be analyzed to test whether it is correct to
82 assume that the risk of exposure to flaviviruses is higher in wetland habitats, where a high
83 mosquito abundance (Dale and Knight, 2008) and a high concentration of a wide range of
84 wild bird species, either resident or migratory (López et al., 2008) is present, and during
85 specific seasons, in which mosquito activity is more likely. Thus, in this work we compare
86 antibody prevalence data among different geographical regions, habitats and seasons. The
87 effect of host factors such as age, sex and body condition on seroprevalence is also
88 assessed.

89

90 **2. Material and methods**

91

92 *2.1 Study area*

93

94 The study includes 23 sampling sites in south-western Spain (41°15 N and 38°04 N, 5°20 O
95 and 0°59 W, minimum altitude=244m, maximum altitude=2274m, relative humidity=64%,
96 mean temperature=14°C), and one in Doñana National Park (DNP; 36°56 N, 6°21 W, mean
97 altitude=12m, relative humidity=80%, mean temperature=19°C), in the Guadalquivir river
98 marshes in southern Spain (Figure 1). Of the 23 sampling sites in south-western Spain, 2
99 correspond to wetlands with abundance of migratory and resident birds; 9 are devoted to
100 agriculture and 12 are made up of typical Mediterranean forest in small mountain chains.
101 DNP is one of the main wetlands in Western Europe with high density and diversity of
102 resident and migratory birds.

103

104 *2.2 Sampling*

105

106 Blood samples of wild boar (n=545), red fox (n=103) and other wild mammals (European
107 wild cat *Felis silvestris silvestris* n=1, stone marten *Martes foina* n=6, European otter *Lutra*

108 *lutra* n=2, European badger *Meles meles* n=7, common genet *Genetta genetta* n=2) were
109 obtained from hunting drives, trapping programs for National Park management or from
110 animals found dead, mostly road-kills. Blood was collected from the thoracic cavity of
111 freshly dead animals. Blood of Iberian pigs (n=177) was collected from the infraorbital
112 sinus during the annual official active sanitary surveillance procedures (Real Decreto (RD)
113 1186/2006, published October 13th, 2006, now replaced by RD 599/2011, published April
114 29th 2011). Wild boars were sampled in 2007-2010, Iberian pigs in 2009-2010, red foxes in
115 2006-2008 and other small mammals in 2003-2007. Wild boar and red fox samples were
116 grouped by season (spring, summer, autumn, winter) and habitat type (wetland,
117 Mediterranean forest, and agricultural crops) for statistical analysis.
118 The age of wild boars was determined using dentition patterns, classifying them into
119 piglets (< 7 months), weaners (7 to 12 months), juveniles (12 to 24 months) and adults
120 (>2years; Matschke, 1967). When kidney data was available, the kidney fat index (KFI)
121 was obtained as indicator of body condition (Batcheler and Clarke, 1970).
122 Red fox samples were classified according to sex and age (juveniles and adults), and the
123 mean KFI was obtained when possible.
124 All Iberian pigs sampled were adults and body condition information was not available.
125 Upon arrival at the laboratory, blood samples were centrifuged for at least 10 min at
126 2,000g for serum separation and the serum was stored at -20° C until testing.

127 128 *2.3 Serological tests*

129 Presence of antibodies against WNV and closely related flaviviruses was analysed using a
130 commercial competitive enzyme-linked-immunosorbent assay (cELISA; ID Screen®
131 West Nile Competition, ID Vet, Montpellier, France) based on purified whole WNV
132 antigen for detection of antibodies directed against the PrM-E envelope protein common to
133 flaviviruses. The test was performed according to manufacturer's instructions.

134 To confirm ELISA positive samples, neutralizing antibody titers to WNV were determined
135 by a micro virus-neutralization test (micro VNT) previously described by Figuerola et al.
136 (2007), using Vero cells and WNV strain E101.

137

138 *2.4 Molecular tests*

139 To determine WNV and flavivirus genome presence, nucleic acids of available tissue
140 (spleen) of antibody-positive wild boar (n=69) were extracted (*High Pure RNA Tissue Kit*,
141 Roche Diagnostics, Barcelona, Spain), and analysed by real time reverse transcription-
142 polymerase chain reaction (RRT- PCR) for WNV (TaqMan MGB PCR, QuantiTEC
143 Probe® RT-PCR, Qiagen, Madrid, Spain; Jiménez-Clavero et al., 2006), and Flavivirus
144 (QuantiTEC® SYBR®Green RT-PCR, Qiagen, Madrid, Spain) detection (Moureau et al.,
145 2007).

146

147 *2.5 Statistical analysis*

148 A Chi square (χ^2) test for homogeneity was used to compare the mean flavivirus
149 seroprevalence in wild boar between sampling sites and between DNP and the mean
150 seroprevalence from the combined sampling sites from south-western Spain.

151 To study factors that affect exposure to flaviviruses in wild boar we performed a
152 generalized mixed model (GzMM) where flavivirus seropositivity was the response
153 variable and “sex”, “age class”, “season”, “sampling year” and “habitat” were the
154 categorical explanatory variables. Sample origin was included a random variable. For this
155 analysis we used a binomial error and a logit link. Also a generalized mixed model
156 (GzMM) was performed in order to study the relationship between body condition (as
157 log₁₀-transformed KFI, continuous response variable) and WNV antibody presence in
158 wild boar. We included “WNV seropositivity” (as categorical 0=absence, 1=presence),

159 “sex”, “age class”, “season” and “origin” (all of them as categorical) as explanatory
160 variables. “WNV seropositivity” interactions with “sex” and “age” were added to the
161 models. Sampling year was included as random variable. Identity error and a normal link
162 were applied. Finally the Chi square (χ^2) test for homogeneity was used to compare
163 flavivirus seroprevalence in red foxes among age groups and according to sex. All analyses
164 were performed with the SPSS software package, version 19.0 (IBM SPSS Statistics, New
165 York, NY, USA).

166

167 **3. Results**

168

169 Antibodies against flaviviruses were detected by ELISA in $20.4 \pm 7.8\%$ (21 out of 103) of
170 the red foxes, $12.6 \pm 2.8\%$ (69 out of 545) of the wild boars, in $3.3 \pm 2.7\%$ (6 out of 177)
171 of the Iberian pigs and in one stone marten (Table 1, Figure 1). In wild boar, a significantly
172 higher mean seroprevalence was found in DNP ($27 \pm 7.1\%$) as compared to sampling sites
173 in south-western Spain (Figure 1, $\chi^2 = 45.764$, d.f. 6, $p < 0.001$) and the general mean
174 prevalence in south-western Spain ($6.9 \pm 2.5\%$) respectively $\chi^2 = 31.7$, d.f. 1, $p < 0.05$).

175 Habitat, season and sampling year did not affect flavivirus seroprevalence in wild boar.

176 Also, flavivirus seroprevalence in wild boars and red foxes was apparently not affected by
177 sex and body condition. However, adult wild boar had a significantly higher flavivirus
178 antibody prevalence ($19.7 \pm 5.8\%$, 35 out of 178), than juveniles ($7.8 \pm 5.2\%$, 8 out of 103),
179 weaners ($6.5 \pm 4.7\%$, 7 out of 107), and piglets ($5 \pm 9.6\%$, 1 out of 20) (GLZMM, $F = 4.136$,
180 d.f. 4, $p < 0.05$, Figure 2).

181 Due to the strong haemolysis in sera from wild boars and red foxes, only 32% (21 wild
182 boar, 6 Iberian pig and 1 red fox samples) of the total samples tested by microVNT gave a
183 readable result. In wild boar, WNV neutralizing antibodies were found in 9 of the 69
184 ELISA positive samples (13%). More precisely 5 of 41 ELISA positive samples from DNP

185 (12.2%), 2 of 7 ELISA positive samples from wetland habitats in south-western Spain
186 (28.6%), and 2 of 7 samples (28.6%) from Mediterranean forest habitat had WNV
187 neutralizing antibodies (Table 1, Figure 1). In the case of the Iberian pig, none of the
188 samples was haemolytic, and WNV neutralizing antibodies were detected in four (66.6%)
189 of the six ELISA positive samples (Figure 1). The stone marten ELISA positive sample
190 was negative by VNT. One sample of a red fox from a Mediterranean forest habitat
191 presented a high titre of WNV neutralizing antibodies, while neutralization titres for WNV
192 in wild boar and Iberian pigs were relatively low (Table 2).
193 All spleen samples analyzed by RRT-PCR were negative for the presence of WNV and
194 flavivirus genome.

195

196 **4. Discussion**

197 The results of the present study newly confirm the exposure of red foxes, Iberian pigs and
198 a stone marten from south-central Spain to flaviviruses. Mesomammals have previously
199 been confirmed to be exposed to WNV and other flaviviruses. However, to our knowledge,
200 this is the first report of flavivirus exposure in red fox and stone marten. Antibodies to
201 flaviviruses, mainly WNV, have been found in other members of the *Canidae* family, such
202 as gray foxes (*Urocyon cinereoargenteus*) or coyotes (*Canis latrans*; Bischof and Rogers,
203 2005; Bentler et al., 2007), and other mesomammals from North America (Dietrich et al.,
204 2005; Root et al., 2005; Bentler et al., 2007; Gómez et al., 2008; Blitvich et al., 2009), but
205 had not been reported from Europe. Disease due to WNV has been documented in both
206 wolves (*Canis lupus*) and domestic dogs in connection with WN fever outbreaks in horses
207 and humans (Lanthier et al., 2004) and dogs have actually been proposed as potential
208 sentinels for WNV surveillance (Resnick et al., 2008).

209 The high flavivirus antibody prevalence rate evidenced in red foxes could be due to higher
210 exposure to frequent infected mosquito bites or to consumption of infected prey, although
211 this route of infection has only been reported in experimentally infected domestic cats and
212 naturally infected raptorial birds (Garmendia et al., 2000; Austgen et al., 2004).
213 Iberian pigs that could be more easily accessible than wild boar and red foxes for sampling
214 as sentinels were apparently less exposed to flavivirus. In a study including juvenile
215 individuals we showed that wild boar could be an interesting sentinel species for
216 flaviviruses surveillance (Boadella et al., 2011). As the frequently flavivirus positive red
217 foxes are generally available only in low numbers and as wild boar is mostly harvested in
218 winter, Iberian pigs that are farmed extensively and thus exposed to mosquitoes could be a
219 valuable alternative, not the least because they are accessible for sampling around the year,
220 and as blood samples are of better quality, than samples obtained from wild boar carcasses.
221 Our study shows however that exposure to flaviviruses in this species is much lower than
222 in wild boar in the same period. Previously, antibodies against WNV have been found in
223 feral swine in North America by ELISA with a mean prevalence in 2001-2004 (22.5%)
224 similar to the one encountered in DNP, and by VNT in 6.5% wild boar in the Czech
225 Republic (Gibbs et al., 2006; Halouzka et al., 2008).
226 In this study results for flavivirus seroprevalence in mesomammals by ELISA and WNV
227 seroprevalence by VNT differed. Currently, VNT is considered the gold standard to
228 confirm exposure to WNV (Dauphin and Zientara, 2006). Here, the high degree of
229 haemolysis present in the serum samples of wild boar and red foxes has only allowed
230 correct interpretation in a reduced number of samples (n=28). ELISA, on the other hand, is
231 less prone to haemolysis interference, but detects antibodies of cross related flaviviruses,
232 particularly viruses of the Japanese encephalitis serocomplex. Thus, in this study we
233 discuss flavivirus seroprevalence in general, as ELISA positive, microVNT negative

234 samples could contain either non-neutralizing antibodies against WNV or antibodies to
235 other antigenically related flaviviruses. However, to date only the mosquito borne USUV
236 and BAGV, and the tick borne Spanish sheep encephalomyelitis virus (SSEV) have been
237 reported in the country (Marin et al., 1995; Busquets et al., 2008; Agüero et al., 2011).
238 The substantially higher flavivirus seroprevalence found in DNP as compared to south-
239 central Spain (CLM) suggests a potential link to a habitat that favours vector abundance
240 and reservoir host (wild bird) presence. Nevertheless, no association of flavivirus
241 seroprevalence to wetland habitats could be established in wild boar in south-central Spain.
242 Flavivirus, namely WNV activity has been shown to vary with time due to climatic factors
243 that affect vector abundance (e.g. Platonov et al., 2008). In this study, flavivirus exposure
244 was detected between 2005 and 2010. For the years 2003 and 2004 only data for three
245 badgers, two genet, a wild cat and a stone marten were available, which is insufficient to
246 conclude about flavivirus activity in the study area. We were unable to detect a relation of
247 seroprevalence to year or season, however we also do not know about the duration of
248 antibody persistence in our test species, and most of our samples were from the winter (low
249 mosquito density) season which is when hunting drives take place.

250 The significantly higher seroprevalence found in adult wild boars in comparison to
251 juveniles, weaners and piglets (Figure 2), coincides with previous results in North
252 American feral swine (Gibbs et al., 2006) and in camels (El Harrak et al., 2011), and could
253 be explained by a longer time span of possible exposure or due to antibody persistence. In
254 adult pigs, antibodies to JEV have been found to persist for more than three years possibly
255 due to frequent re-inoculation by mosquito bites (Geevarghese et al., 1994), while other
256 experimental studies detected persistent antibodies in absence of re-infections only until 28
257 days post infection (Blitvich et al., 2003; Teehee et al., 2005).

258 Finally, in this work, we found antibodies against WNV and/or other flaviviruses but viral
259 genome was not detected, suggesting absence of active infection or low possibilities of
260 viral genome detection due to transitory viraemia or other host characteristics. Material
261 other than spleen that might have been more suitable for flavivirus genome detection was
262 not available for this study.

263 The results obtained in this study document the exposure of widely distributed wild and
264 domestic mammals in Spain to flaviviruses, specifically in areas where flavivirus activity
265 has been previously reported, but do not reveal Iberian pigs as good sentinel species for
266 flavivirus surveillance. With the samples available for this study we could neither
267 demonstrate increased exposure to flavivirus in wetlands nor a relation of flavivirus
268 exposure to season and thus mosquito abundance. The association of Flavivirus prevalence
269 with age suggests that juvenile individuals may be of more interest for surveillance.
270 Additional studies aimed at evaluating flavivirus circulation in other regions and the degree
271 of exposure of other widely distributed mammals would be of interest.

272

273 **Conflict of interest statement**

274 The authors have no conflict of interest.

275

276 **Acknowledgements**

277 We acknowledge the help and excellent comments that greatly improved the manuscript by
278 M.A. Jiménez-Clavero, and the inestimable help with sample collection of fellow students
279 in hunting drives and of the veterinary officers of the JCCM at Iberian pig farms. We are
280 also thankful to P. Acevedo for his help with Figure 1. This study has been supported by
281 projects PAC08-0296-7771 (JCCM), and AG2008-02504GAN. A.V. Gutierrez –Guzman
282 is a JCCM fellow (PAC08-029).

283

284 **References**

285 Acevedo, P., Vicente, J., Höfle, U., Casinello, J., Ruiz-Fons, F., Gortazar, C., 2007.

286 Estimation of European wild boar relative abundance and aggregation: a novel method
287 in epidemiological risk assessment. *Epidemiol. Infect.* 1, 1-

288 Anonymous, 2010. ProMED-mail. VNO, nuevo caso humano - España (Andalucía).

289 ProMED-mail 2010; 6 de octubre; Access no: 20101006.3626. Available
290 at:<http://www.promedmail.org>.

291 Agüero, M., Fernández-Pinero, J., Buitrago, D., Sánchez, A., Elizalde, M., San Miguel, E.,
292 Villalba, R., Llorente, F., Jiménez-Clavero, M.A., 2011. Bagaza virus in partridges and
293 pheasants, Spain, 2010. *Emerg. Infect. Dis.* 17, 1498-1501.

294 Austgen, L., Bowen, R.A., Bunning, M.L., Davis, B.S., Mitchell, C.J., Chang, J.J., 2004.
295 Experimental infection of cats and dogs with West Nile virus. *Emerg. Infect. Dis.* 10,
296 82-86.

297 Batcheler, C.L., Clarke, C.M.H., 1970. Note on kidney weights and the kidney fat index.
298 *New Zeal. J. Sci.* 13, 663-668.

299 Bentler, K.T., Hall, J.S., Root, J.J., Klenk, K., Schmit, B., Blackwell, B.F., Ramey, P.C.,
300 Clark, L., 2007. Serologic evidence of West Nile Virus exposure in North American
301 mesopredators. *Am. J. Trop. Med. Hyg.* 76, 173-79.

302 Blitvich, B.J., Bowen, R.A., Marlenee, N.L., Hall, R.A., Bunning, M.L., Beaty, B.J., 2003.
303 Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile
304 Virus antibodies in domestic mammals. *J. Clin. Microbiol.* 41, 2676-2679.

305 Blitvich, B.J., Juarez, L.I., Tucker, B.J., Rowley, W.A., Platt, B., 2009. Antibodies to West
306 Nile virus in raccoons and other wild peridomestic mammals in Iowa. *J. Wildl. Dis.* 45,
307 1163-1168.

308 Bischof, R., Rogers, D.G., 2005. Serologic survey of select infectious diseases in coyotes
309 and raccoons in Nebraska. *J. Wildl. Dis.* 41, 787-791.

310 Boadella, M., Díez-Delgado, I., Gutiérrez-Guzmán, A.V., Höfle, U., Gortázar, C., 2012.
311 Do Wild Ungulates Allow Improved Monitoring of Flavivirus Circulation in Spain?
312 *Vector Borne Zoonotic Dis.* In press.

313 Busquets, N., Alba, A., Allepuz, A., Aranda, C., Nunez, J.I., 2008. Usutu virus sequences
314 in *Culex pipiens (Diptera : Culicidae)*, Spain. *Emerg. Infect. Dis.* 14, 861-863.

315 Chastel, C., Launay, H., Rogues, G., Beaucornu, J.C., 1980. - Infections à arbovirus en
316 Espagne ; enquête sérologique chez les petits mammifères. *Bull. Soc. Path. Exot.* 73,
317 384-390.

318 Dale, P.E.R., Knight, J.M., 2008. Wetlands and mosquitoes: a review. *Wetlands Ecol.*
319 *Manag.* 16, 255-276.

320 Dauphin, G., Zientara, S., 2007. West Nile virus: Recent trends in diagnosis and vaccine
321 development. *Vaccine* 25, 5563-5576.

322 Dietrich, G., Montenieri, J. A., Panella, N. A., Langevin, S., Lasater, S. E., Klenk, K., Kile,
323 J. C., Komar, N., 2005. Serologic evidence of West Nile virus infection in free-ranging
324 mammals, Slidell, Louisiana, 2002. *Vector-Borne and Zoonotic Diseases.* 5, 288-292.

325 El-Harrak, M., Martín-Folgar, R., Llorente, F., Fernández-Pacheco, P., Brun, A., Figuerola,
326 J., Jiménez-Clavero, M.A., 2011. Rift Valley and West Nile virus antibodies in camels,
327 north Africa. *Emerg. Infect. Dis.* 17, 2372-2374.

328 Figuerola, J., Jiménez-Clavero, M.A., Rojo, G., Gómez, C., Soriguer, R., 2007. Prevalence
329 of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain.
330 *Avian Pathol.* 36, 209-212.

331 Garmendia, A., Herbert, J., Kruijning, V., French, R., Anderson, J., Andreadis, T.,
332 Kumar, A., West, B., 2000. Recovery and identification of West Nile virus from a
333 Hawk in winter. *J. Clin. Microbiol.* 38, 3110-3111.

334 Geevarghese, B.H., Shaikh, P., Jacob, G., Bhat, H.R., 1994. Persistence of the
335 haemagglutination-inhibition antibodies to JE and WNV in naturally infected domestic
336 pigs in Karnataka State, India. *National Institute of Virology.* 38, 235-237.

337 Gibbs, S., Marlenee, N.L., Romines, J., Kavanaugh, D., Corn, J.L., Stallknecht D.E., 2006.
338 Antibodies to West Nile virus in feral swine from Florida, Georgia, and Texas, USA.
339 *Vector-Borne and Zoonotic Diseases.* 6, 261-265.

340 Gómez, A., Kilpatrick, A.M., Kramer, L.D., Dupuis, A.P., Maffei, J.G., Goetz, S.J., Marra,
341 P.P., Daszak, P., Aguirre, A., 2008. Land use and West Nile virus seroprevalence in wild
342 mammals. *Emerg. Infect. Dis.* 14, 962-964.

343 Halouzka, J., Juricova, Z., Jankova, J., Hubalek, Z., 2008. Serologic survey of wild boars
344 for mosquito-borne viruses in South Moravia (Czech Republic). *Veterinarni Medicina,*
345 53: 266–271.

346 Höfle, U., Blanco, J.M., Crespo, E., Naranjo, V., Jiménez-Clavero, M.A., Sánchez, A., De la
347 Fuente, J., Gortazar, C., 2008. West Nile virus in the endangered Spanish imperial eagle.
348 *Vet. Microbiol.* 14, 171-178.

349 Jiménez-Clavero, M.A., Agüero, M., Rojo, G., Gómez-Tejedor, C., 2006. A new
350 florogenic real-time RT-PCR assay for detection of lineage 1 and lineage 2 West Nile
351 viruses. *J. Vet. Diagn. Invest.* 18, 459-462.

352 Jiménez-Clavero, M.A., Llorente, F., Sotelo, E., Soriguer, R., Gómez-Tejedor, C.,
353 Figuerola, J., 2010. West Nile virus serosurveillance in horses in Donana, Spain, 2005
354 to 2008. *Vet. Rec.* 167, 379-380.

355 Kaptoul, D., Viladrich, P. F., Domingo, C., Niubo, J., Martínez-Yelamos, S., De Ory, F.,
356 Tenorio, A., 2007. West Nile virus in Spain: Report of the first diagnosed case (in
357 Spain) in a human with aseptic meningitis. *Scan. J. Infect. Dis.* 39, 70-71.

358 Lanthier, I., Hébert, M., Tremblay, D., Harel, J., Dallaire, A.D., Girard, C., 2004. Natural
359 West Nile virus infection in a captive juvenile Arctic wolf (*Canis lupus*). *J. Vet. Diagn.*
360 *Invest.* 16, 326-329.

361 López, G., Jiménez-Clavero, A., Tejedor, C. G., Soriguer, R., Figuerola, J., 2008.
362 Prevalence of West Nile Virus neutralizing antibodies in Spain is related to the
363 behaviour of migratory birds. *Vector-Borne and Zoonotic Diseases.* 8, 615-621.

364 Marin, M. S, McKenzie, J., Gao, G. F., Reid, H. W., Antoniadis, A., Gould, E. A., 1995.
365 The virus causing encephalomyelitis in sheep in Spain- a new member of the tick-
366 borne encephalitis group. *Res.Vet. Sci.* 58, 11-13.

367 Marra, P.P., Griffing, S., Cafrey, C., Kilpatrick, A.M., McLean, R., Brand, C., Saito, E.,
368 Dupuis, A.P., Kramer, L., Novak, R. 2004. West Nile virus and wildlife. *Bioscience.*
369 54, 393-402.

370 Matschke, G.H., 1967. Aging European wild hogs by dentition. *J. Wildlife. Manage.* 31,
371 109.

372 Moureau, G., Temmam, S., Gonzalez, J.P., Charrel, R.N., Grard, G., De Lamballerie, X.,
373 2007. A real-time RT-PCR method for the universal detection and identification of
374 Flaviviruses. *Vector-Borne and Zoonotic Diseases.* 7, 467-76.

375 Ohno, Y., Sato, H., Suzuki, K., Yokoyama, M., Uni, S., Shibasaki, T., Sashika, M.,
376 Inokuma, H., Kai, K., Maeda, K., 2009. Detection of antibodies against Japanese
377 Encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. *J. Vet. Med.*
378 *Scien.* 71, 1035-1039.

379 OIE. West Nile Fever, Spain [consulted 8/11/2010]. Immediate notification, 10/09/2010:
380 World Organisation for Animal Health; 2010. Available at:
381 <http://web.oie.int/wahis/public.php?page=home>.

382 Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabattini, A.M., Tagliazucchi, S.,
383 Savini, G., Monaco, F., Simnoe, M.L., Lelli, R., Rumpianesi, F., 2009. First human
384 case of Usutu virus neuroinvasive infection, Italy, August-September 2009.
385 *Eurosurveillance*. 14, 50.

386 Platonov, A., Fedorova, M.V., Karan, L.S., Shopenskaya, T.A., Platonova, O.V.,
387 Zhuravlev, V.I., 2008. Epidemiology of West Nile infection in Volgograd, Russia, in
388 relation to climate change and mosquito (Diptera: *Culicidae*) bionomics. *Parasitol. Res.*
389 103, 45-53.

390 Platt, K.B., Tiawsirisup, B.J., Tucker, B.J., Blitvich, L.C., Bartholomay, L.C., Rowley,
391 W.A., 2008. The potencial of small mammals to contribute to the ecology and
392 epidemiology of West Nile virus. FAVA-OIE Joint Symposium on Emerging Diseases.

393 Resnick, M.P., Grunenwald, P., Blackmar, D., Hailey, C., Bueno, R., Murray, K.O., 2008.
394 Juvenile dogs as potential sentinels for West Nile virus surveillance. *Zoonoses Public*
395 *Health*. 55, 443-447.

396 Root, J.J., Hall, J.S., Mclean, R.G., Marlenee, N.L., Beaty, B.J., Gansowski, J., Clark, L.,
397 2005. Serologic evidence of exposure of wild mammals to Flaviviruses in the Central
398 and Eastern United States. *Am. J. Trop. Med. Hyg.* 72, 622-630.

399 Sotelo, E., Fernández-Pinero, J., Llorente, F., Vázquez, A., Moreno, A., Agüero, M.,
400 Cordioli, P., Tenorio, A., Jiménez-Clavero, MA., 2011. Phylogenetic relationships of
401 Western Mediterranean West Nile virus strains (1996-2010) using full-length genome
402 sequences: single or multiple introductions? *J. Gen. Virol.* 92, 2512–2522.

403 Teehee, M.L., Bunning, M.L., Stevens, S., Bowen, R.A., 2005. Experimental infection of pigs
404 with West Nile virus. *Archives of Virology*. 150, 1249-1256.

405 Vázquez, A., Sánchez, M.P., Ruiz, S., Molero, F., Hernández, L., Moreno, J., Magallanes, A.,
406 Tejedor, C., Tenorio, A., 2010. Putative new lineage of West Nile virus, Spain. *Emerg.*
407 *Infect. Dis.* 16, 549-552.

408 Vázquez, A., Jiménez-Clavero, M. A., Franco, L., Donoso-Mantke, O., Sambri, V.,
409 Niedrig, M., Zeller, H., Tenorio, A., 2011. Usutu virus - potential risk of human disease
410 in Europe. *Eurosurveillance*. 16, 22-26.

411 Weissenböck, H., Hubálek, Z., Bakonyi, T., Nowotny, N., 2010. Zoonotic mosquito-borne
412 flaviviruses: Worldwide presence of agents with proven pathogenicity and potencial
413 candidates of future emerging diseases. *Vet. Microbiol.* 140, 271-280.

414

415

416

417

418

419

420

421

422

423

424

425

426

427

428

429

430
 431 Table 1. Sampling effort by year and habitat type, and flavivirus and WNV seroprevalence by blocking
 432 ELISA and WNV neutralization test in wild boar, red fox, and Iberian pigs.

Species	Habitat	Wet-land location	2006			2007			2008			2009			2010		
			n	ELIS A pos.	VN T rd/+	n	ELIS A pos.	VN T rd/+	n	ELIS A pos.	VN T rd/+	n	ELIS A pos.	VN T rd/+	n	ELIS A pos.	VN T rd/+
Wild boar	Mediterranean forest	DNP SW				30	3	0/0	34	3	0/0	48	1	1/1	19	0	0/0
	Wetland		-----	46	13	3/3	40	12	4/0	40	6	3/1	26	10	4/1		
	Agriculture			30	1	1/1	55	2	2/2	76	2	2/0	2	0	0/0		
				18	0	0/0	62	10	1/0	15	1	0/0	4	0	0/0		
Total wild boar		-----	124	17	4/4	191	32	7/2	179	10	6/2	51	10	4/1			
Red fox	Mediterranean forest		11	0	0/0	17	7	1/1	45	10	0/0						
	Wetland		11	1	0/0	--	--	--	--	--	--						
	Agriculture		1	0	0/0	16	3	0/0	2	0	0/0						
	Total red fox		23	1	0/0	33	10	1/1	47	10	0/0						
Iberian pig	Mediterranean forest											--	--	--	20	0	--
	Wetland											--	--	--	--	--	--
	Agriculture											86	3	3/3	71	3	3/1
	Total Iberian pig											86	3	3/3	91	3	3/1
Total samples			23	1	0/0	157	27	5/5	238	42	7/2	265	13	9/5	142	13	7/2

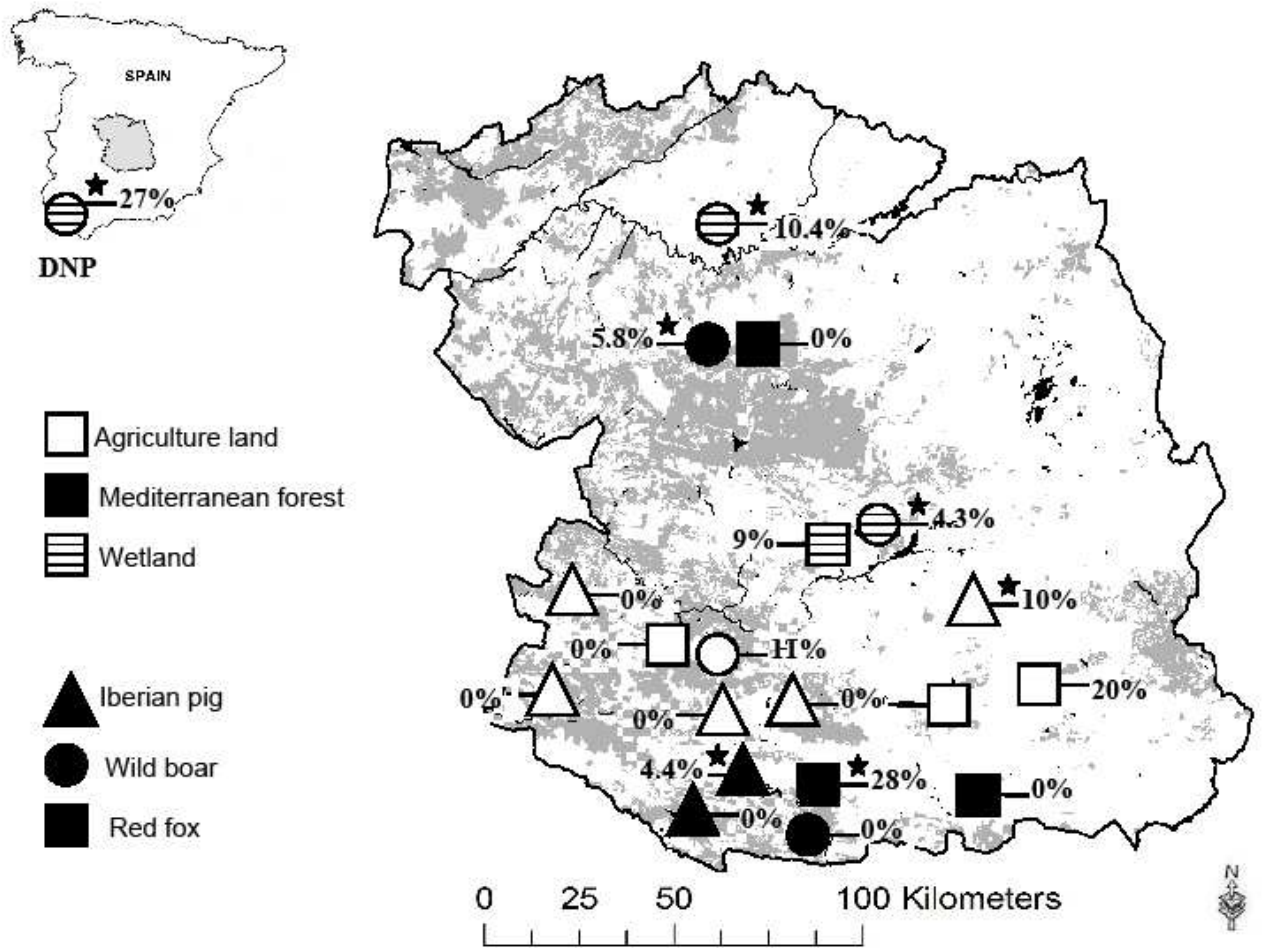
433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456
 457
 458
 459
 460
 461
 462
 463

464 Table 2. Summary of antibody titres against WNV detected in free-living wild
 465 boars, red foxes and Iberian pigs

Species	5	10	20	40	80	160	240	480
Wild boar	1	3	2	1	0	2	--	--
Red fox	--	--	--	--	1	--	--	1
Iberian pig	--	--	1	1	1	1	--	--

467
 468
 469
 470
 471
 472
 473
 474
 475
 476
 477
 478
 479
 480
 481
 482
 483
 484
 485
 486
 487
 488
 489
 490
 491
 492
 493
 494
 495
 496
 497
 498
 499
 500
 501
 502
 503
 504
 505
 506
 507
 508
 509
 510
 511
 512
 513

514 Figure 1. Flaviviruses seroprevalence in wild boar, red fox and Iberian pig from south-
 515 central, Spain. Stars indicate locations with VNT positive samples.



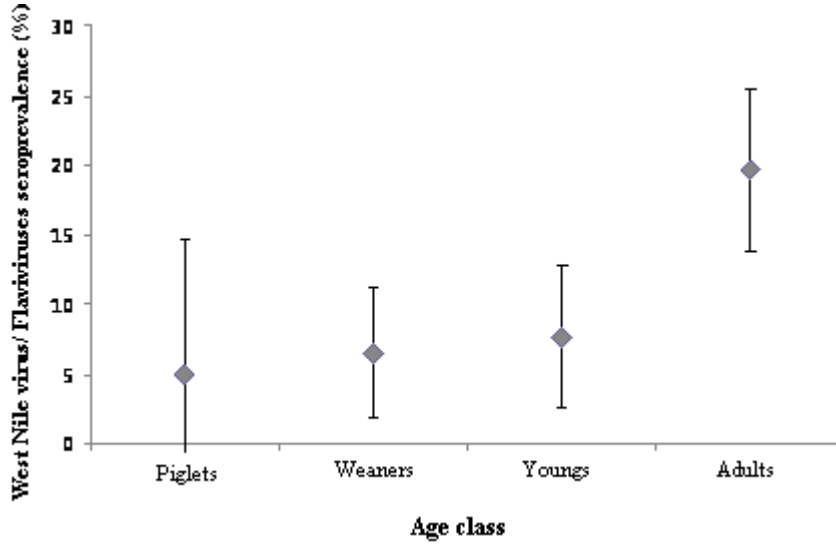
516

517

518 Figure 2. WNV/Flavivirus seroprevalence in wild boars of south central Spain increases

519 with age.

520



521

522