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1 Size matters: West Nile virus neutralizing antibodies in resident and migratory birds in
2 Spain

3

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15

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 not only explained by their longevity because the relationship was already significant when analyzing only first year birds, and probably also involves 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.

Key-words: 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, 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 six 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

63 use, mating systems, and immune system capacity (Møller and Erritzoe, 1996; Clayton
64 and Moore, 1997; Figuerola, 1999, 2000; Figuerola and Green, 2000; Tella, 2002).
65 However, to our knowledge, no study has focused on vector-borne generalist pathogens.
66 Despite the thousands of birds that have been tested for WNV or its antibodies in North
67 America, analyses focusing on the relationship between bird ecology and exposure to
68 the virus are still lacking but urgently needed. In this study we take advantage of the
69 differences in the impact of WNV in Europe and in North America to analyze the
70 relationship between bird ecology and phylogeny and prevalence of WNV neutralizing
71 antibodies. The relevance of this study is two fold, on the one hand, the low host
72 specificity of WNV makes this system different to the pathogens used in previous
73 studies (mainly blood parasites and ectoparasites), and may affect the relevance of
74 different ecological factors. On the other hand, given the relevance of WNV for human
75 health and wildlife conservation we also aim to identify the characteristics of the species
76 that can be most useful for monitoring in Europe.

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

82

83 **2. Materials and methods**

84 Between January 2003 and February 2005 we captured 1,213 individuals
85 belonging to 72 species (49 genera, 22 families, and 8 orders). Birds were captured
86 without damage using mist-nets and walk-in-traps in the Guadalquivir and Odiel
87 Marshes (SW Spain). Blood samples were taken with syringes from the brachial,

88 femoral, or jugular vein, birds were marked with numbered aluminum rings and
89 released after manipulation. The volume of blood extracted depended on the size of the
90 species and never exceeded 1% of body mass (range 0.080-1 ml). Blood was collected
91 in eppendorf tubes, allowed to clot at ambient temperature, and placed into coolers until
92 centrifugation during the same day. All samples were obtained from adult (full grown)
93 individuals to ensure that the antibodies were not the result of the passive transfer of
94 maternal immunity (Gibbs et al., 2005). When possible age was determined (471 first-
95 year individuals and 540 after first-year individuals) according to Prater, Marchant and
96 Vourinen (1977), Baker (1993) and Svensson (1996).

97 WNV strain Eg101 and the E6 clone of Vero cells used for virus propagation
98 were obtained from Hervé Zeller (Institut Pasteur de Lyon). The Usutu virus (SAAR
99 1776 isolate) was obtained through the Centre for Ecology and Hydrology, Oxford, UK,
100 and propagated in Vero cells (American Type Culture Collection, Manassas, Va). Virus
101 titers were determined by end-point titration following the method used by Reed and
102 Muench (1938). WNV-Neutralizing antibody titers were determined by a micro virus-
103 neutralization test (micro-VNT) in 96-well plates, adapted from a previously described
104 method (Jiménez-Clavero et al., 2001). A recent study shows that a micro-VNT assay
105 and the standard PNRT₉₀ perform comparably in sensitivity at detecting anti-WNV
106 antibodies in birds (Weingartl et al., 2003). Serum samples were inactivated at 56 °C for
107 30 minutes prior to the analysis. Dilutions of test sera (25 •l) were incubated with 100
108 TCID₅₀ of WNV strain Eg101 in the same volume (25 •l) for 1 h at 37 °C in Eagle's
109 medium (EMEM) supplemented with L-glutamine, 100 U/ml penicillin, 100 •g/ml
110 streptomycin, followed by the addition of 50 •l of a suspension (2x10⁴ cells/ml) of Vero
111 E6 cells in the same medium plus fetal calf serum to a final concentration of 5%. The
112 mixture was further incubated for 6-7 days (37 °C in a 5% CO₂ and saturating humidity

113 atmosphere) until cytopathic effect (cpe) was observed in control wells containing 10
114 TCID₅₀ of virus. The screening of samples was performed at 1:10 and 1:20 dilutions of
115 tested sera (dilutions considered before the addition of virus, that is, in a volume of 25
116 μ l). Only samples yielding positive neutralization (complete absence of cpe) at 1:20
117 were scored as positives and further titrated by analyzing serial serum dilutions from
118 1:20 to 1:640. Neutralizing serum titer was considered as the highest value of the
119 reciprocal serum dilution giving a complete absence of cpe.

120 The specificity of the assay was assessed in two ways. First, by analyzing a
121 panel of sera from an external quality assessment, consisting of serum samples
122 containing antibodies from other four flaviviruses, that proved negative for
123 neutralization titers in our WNV assay, while duplicate testing of all WNV antibody-
124 positive serum samples proved positive (>1:20) for neutralization titers (Niedrig et al.,
125 2006). Second, we also compared the neutralizing antibodies titers of 18 samples tested
126 in parallel for WNV and Usutu virus (a closely related JEV group avian virus). In none
127 of the cases showed higher antibody titers more specific to Usutu than to WNV (see
128 Figuerola et al., 2007a, for more details). We cannot discard that the serology to WNV
129 observed in some of the samples, particularly those from birds flying from Central
130 Europe (e.g. *Turdus philomelos*, *Sylvia atricapilla*) could be attributed to cross-reacting
131 antibodies to other flaviviruses (particularly TBEV) that can be prevalent in Central
132 Europe. However, this seems unlikely since the technique has shown no cross reactivity
133 to TBEV-positive sera.

134 In a first model, we investigated the effects of taxonomic relationships on West
135 Nile virus prevalence by using Generalized Linear Mixed Models (GLMM). GLMM
136 allows a more versatile analysis of correlation than standard regression methods,
137 because the error distribution of the dependent variable and the function linking

138 predictors to it can be adjusted to the characteristics of the data (Littell et al., 1996). Our
139 response variable was the antibody status (1 present, 0 absent), and we used a binomial
140 distributed error and a logistic link function, to ensure linearity, and statistics adjusted
141 to model dispersion. Binomial errors are adequate to analyze binary response variables.
142 Goodness-of-fit of the model was assessed by checking the overdispersion parameter
143 and the Generalized Chi-Square statistic (Littell et al., 1996). Period (a three levels
144 factor, summer: birds captured in June-August, autumn: September-November and
145 winter: December-March) and age (first-year or adult bird, not including unknown age
146 birds in the analyses) were included as fixed factors in the analyses. Species was
147 included as a repeated subject effect (i.e. observations of a same species are correlated)
148 and the interaction between species and period was included as a random factor. The
149 statistical significance of each nested taxonomic level (Genera, Family and Order) was
150 tested using Z-statistics for random effects using the macro GLIMMIX for SAS 8.2
151 (Littell et al., 1996). As age had no significant effect ($F_{1,27} = 1.88$, $P = 0.18$, $N = 957$),
152 we report the results of analyses excluding this variable to include the full dataset and
153 range of species.

154 In a second model, we analyzed the relevance of different ecological factors. Species
155 body mass (log transformed mean values to fit a normal distribution as judged by
156 checking the normal quantile plot), migratory behaviour (resident or migratory species),
157 breeding sociality (solitary or colonial breeders), and winter sociality (solitary or
158 gregarious species) were included as fixed factors in the analyses. Values for these
159 variables were taken from literature (Cramp, 1982-1994) and were validated by four
160 independent ornithologists according to the ecology of the species in Spain. For each
161 individual we also included the period of collection to control for seasonal differences
162 in the prevalence of antibodies. To test the relationship between ecological factors and

163 antibody prevalence we followed a stepwise-backward selection procedure starting from
164 an initial model including all the two-way interactions between factors.

165 We estimated the sample size necessary to detect increases of 10, 20, 30 and
166 40% in WNV seroprevalence with the program G-Power (Buchner et al., 1997). Effect
167 sizes were calculated for prevalences between 1 and 55%, and sample size necessary to
168 obtain a power of 0.80 when using a Chi-Square test was estimated. A power of 0.80
169 indicates that a significant result ($p < 0.05$) will be obtained in 80% of the analyses of
170 datasets with statistical differences of that magnitude, and is the threshold value usually
171 used in ecology (Bausell and Li, 2002).

172

173 **3. Results**

174 Of the 1,213 individuals tested, 126 (10.4% of individuals from 24 out of 72
175 species) had WNV neutralizing antibodies, with titers ranging from 1:20 to over 1:640
176 (see Electronic Appendix). Important interspecific differences in the presence of WNV
177 neutralizing antibodies were found, with prevalences ranging from 0 to 42.9%.
178 However, taxonomic levels were unrelated to these differences in prevalence (Genera, Z
179 = 0.80, $P = 0.21$; Family, $Z = 1.11$, $P = 0.14$; Order, $Z = 0.58$, $P = 0.28$).

180 Multivariate analyses indicate that antibody prevalence was unrelated to host
181 sociality (Table 1). Prevalence of antibodies changed seasonally (Table 1) with
182 significantly higher prevalences in autumn (mean \pm se: 10.29% \pm 12.71) than in summer
183 (a test of Least-Square means difference, 2.09 ± 7.72 , $t_{29} = 2.46$, $P = 0.02$), and
184 intermediate prevalences in winter (3.27% \pm 9.12, contrast with autumn, $t_{29} = 1.85$, $P =$
185 0.07; contrast with summer, $t_{29} = 0.61$, $P = 0.55$). Although migratory behaviour was
186 not directly related to antibody prevalence (migrants, 5.23% \pm 7.41; residents, 3.32% \pm
187 8.13), a significant interaction with season was found (Table 1). Prevalence did not

188 change with season in resident species ($F_{2,29} = 2.11$, $P = 0.14$) but only in migratory
189 species ($F_{2,29} = 6.15$, $P = 0.006$). When comparing migrant and resident species within
190 each period, in summer migrants (i.e. species wintering in Africa) tended to have higher
191 prevalences of antibodies than residents ($5.56\% \pm 8.58$ vs. $0.77\% \pm 8.85$; $t_{29} = 1.88$, $P =$
192 0.07). In winter migrants (i.e. coming from central and northern Europe) tended to have
193 lower prevalences than resident species ($1.48\% \pm 10.49$ vs. 7.06 ± 12.16 ; $t_{29} = 1.70$, $P =$
194 0.09). Large species (as estimated from their body size) had higher prevalences of
195 antibodies (Table 1, Fig. 1).

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

200 Power analyses indicate that the sample size necessary to detect significant
201 changes in seroprevalence depends dramatically of initial seroprevalence (Fig. 2). For
202 example, 4857 individuals are necessary to detect a 40% increase in seroprevalence
203 when initial seroprevalence is 1% (i.e. in our study *Passer domesticus* had a prevalence
204 of 0%), but only 74 individuals are necessary when focusing in species with 40%
205 prevalence (i.e. *Fulica atra*, with 42.6% prevalence or *Larus ridibundus*, with 42.9%).
206

207 4. Discussion

208 In Spain, clinical signs of WNV disease in birds has only been reported recently
209 (Höfle et al., in press). WNV neutralizing antibodies had been reported in horses
210 (Jiménez-Clavero et al., 2007), chicks of different colonial breeding waterbirds
211 (Figuerola et al., 2007a), and the rapid seroconversion of common coots during a
212 capture-recapture study has also confirmed the local circulation of WNV in the study

213 area (Figuerola et al., 2007b). Infections with clinical symptoms in humans were
214 reported in 2004 in Badajoz (Spain) and Algarve (Portugal) (Esteves et al., 2005;
215 Kaptoul et al., 2007), overlapping with the collection of samples for this study. Previous
216 records give a seroprevalence of up to 30% in humans in some towns in the Ebro Delta
217 (Lozano and Filipe, 1998) and of 16.5% in northwest Spain (González and Filipe,
218 1977), presumably with maximum epidemic activity during the 1970s. However, these
219 results were obtained by haemagglutination-inhibition, a technique burdened by its
220 cross-reactivity with a range of flaviviruses. Recent studies with highly specific
221 neutralization assays show that the prevalence of WNV antibodies in humans living
222 around wetlands in Spain is currently very low (Bofill et al., 2006).

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

230 The results suggest that evolutionary relationships are of little importance in
231 explaining variations in exposure to WNV. This contrasts with the initial studies that
232 identified Corvidae (McLean et al., 2001), Mimidae, and Cardinalidae (Ringia et al.,
233 2004) as bird families that are particularly exposed to WNV infection. In our study both
234 Rallidae (6.7 to 42.6%) and Laridae (25.0 to 42.9%) presented very high antibody
235 prevalence, although our results suggests that these high prevalences were related to the
236 ecology of the species sampled (migratory species of medium and large size), rather
237 than to the birds' taxonomy.

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

251 No effect of winter or breeding sociality on antibody prevalence was found.
252 Although a high prevalence of blood parasites had been reported among social living
253 species (Tella, 2002), the low host-specificity of WNV may make the density of birds the
254 relevant parameter affecting risk of exposition, regardless whether or not it consists of
255 conspecifics. Interestingly, migratory species showed higher antibody prevalence than
256 resident species, but only when comparing summer migrants with residents. Although
257 local circulation of the virus is taking place (since resident species also have
258 antibodies), this higher prevalence observed in migratory birds suggest that these birds
259 spend part of their lives in areas in Africa where the circulation of the virus may be
260 higher than in the surveyed area in Spain. For example, a recent serosurvey in horses
261 detected extremely high prevalences of antibodies (up to 97%) in some sub-Saharan
262 countries (Cabre et al., 2006), areas visited by many European long distance migratory

263 species. Our analyses support the view that species of larger body mass may have
264 increased opportunity for exposure to WNV. Given that we analyzed antibody
265 prevalence in free-living and apparently healthy individuals, this conclusion is not
266 merely a bias caused by the difficulties in finding carcasses of smaller species (Marra et
267 al., 2004), a problem associated with studies based only on dead birds. The direct
268 relationship between prevalence of WNV antibodies and body mass can be explained by
269 several non-exclusive factors. Larger species live longer (Calder, 1984), however we
270 have demonstrated that the relationship between seroprevalence and body mass is also
271 significant when analyzing only first-year birds. We suggest that the larger prevalence
272 of antibodies in larger species is the result of their larger surface area and higher CO₂
273 production (Nagy, 1987), and can host and attract a higher number of ectoparasites
274 (Soliman et al., 2001), mosquitoes, and other biting arthropods that transmit the virus.

275 In conclusion, we suggest that migratory birds of large body mass may provide a
276 means for monitoring WNV prevalence on a large geographical scale (e.g. migratory
277 flyways). Additionally, resident species of large body mass may provide a better
278 description of local WNV prevalence. Further, the past allegations regarding a greater
279 probability of infection by WNV by particular taxonomic groups should be more
280 carefully explored given our findings and the fact that many of those studies were based
281 on the examination of only a few species, and on dead birds, making difficult to
282 separate the effects of exposure, susceptibility and carcass detection probability. From a
283 conservation standpoint, it perhaps would be more beneficial to focus our attention on
284 the effects of WNV on species of greater body mass and on migratory species.

285

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410 Table 1

411 Model analyzing the relationship between host ecology, period of capture and presence
 412 of West Nile Virus (WNV) neutralizing antibodies in the blood of 1,213 individuals
 413 birds captured in South-West Spain. Final model was obtained after backwards variable
 414 selection. Only variables with $p < 0.05$ are interpreted as statistically significant and
 415 parameter estimates are given. For variables not included in the model no parameter
 416 estimate is presented and the F and P values correspond to the values when added to the
 417 final model.

418		Estimate \pm s.e.	F	df	P
419	Body mass	0.746 \pm 0.288	6.72	1,69	0.01
420	Migratory behaviour	2.032 \pm 1.084	0.71	1,69	0.40
421	Period		3.55	2,29	0.04
422	Summer	0			
423	Autumn	2.194 \pm 1.286			
424	Winter	2.287 \pm 1.133			
425	Coloniality		0.67	1,68	0.42
426	Winter gregarism		2.82	1,68	0.10
427	Body mass*Migratory behaviour		0.71	1,68	0.40
428	Body mass*Period		1.21	2,1135	0.30
429	Body mass*Coloniality		0.65	1,68	0.42
430	Body mass*Winter gregarism		3.23	1,68	0.08
431	Migratory behaviour*Period		3.71	2,29	0.04
432	Migratory species in autumn	-1.021 \pm 1.366			
433	Migratory species in winter	-3.653 \pm 1.431			

434	Others	0		
435	Migratory behaviour*Coloniality	1.63	2,67	0.20
436	Migratory behaviour*Winter gregarism	1.41	2,67	0.25
437	Period*Coloniality	1.28	3,26	0.30
438	Period*Winter gregarism	0.99	3,26	0.41
439	Coloniality*Winter gregarism	1.55	2,67	0.22

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

446

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

Figure 1

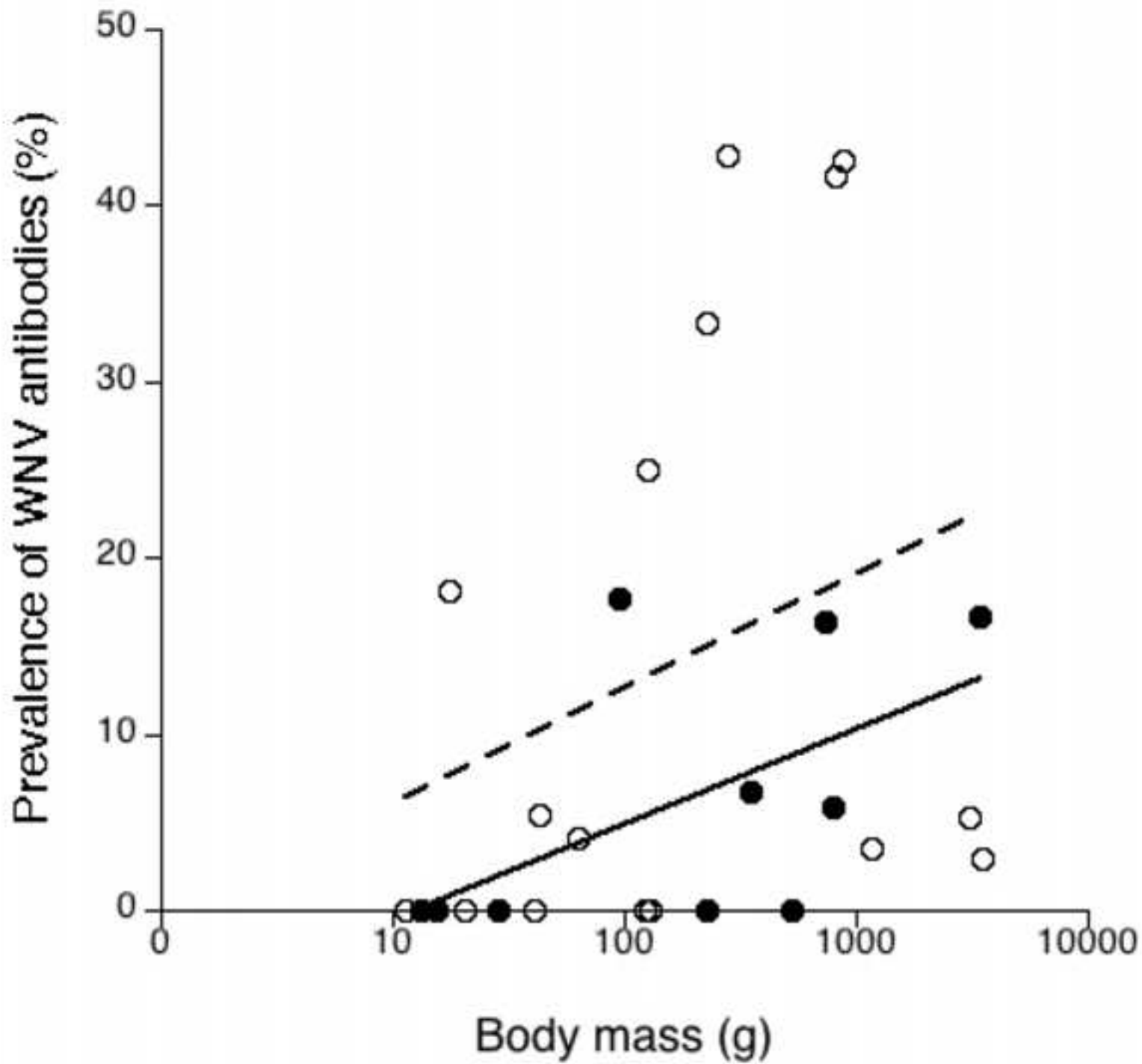


Figure 2

