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High exposure of West Nile virus in equid and wild bird populations in Spain following the epidemic outbreak in 2020

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Abstract

A cross-sectional study was conducted to assess the circulation and risk factors associated with West Nile virus (WNV) exposure in equine and wild bird populations following the largest epidemic outbreak ever reported in Spain. A total of 305 equids and 171 wild birds were sampled between November 2020 and June 2021. IgG antibodies against flaviviruses were detected by blocking enzyme-linked immunosorbent assay (bELISA) in 44.9% (109/243) and 87.1% (54/62) of unvaccinated and vaccinated equids, respectively. The individual seroprevalence in unvaccinated individuals (calculated on animals seropositive by both bELISA and virus microneutralization test [VNT]) was 38.3% (95%CI: 33.1–43.4). No IgM antibodies were detected in animals tested (0/243; 0.0%; 95%CI: 0.0–1.5) by capture-ELISA. The main risk factors associated with WNV exposure in equids were age (adult and geriatric), breed (crossbred) and the absence of a disinsection programme on the facilities. In wild birds, IgG antibodies against flaviviruses were found in 32.7% (56/171; 95%CI: 26.8–38.6) using bELISA, giving an individual WNV seroprevalence of 19.3% (95%CI: 14.3–24.3) after VNT. Seropositivity was found in 37.8% of the 37 species analysed. Species group (raptors), age (>1-year old) and size (large) were the main risk factors related to WNV seropositivity in wild birds. Our results indicate high exposure and widespread distribution of WNV in equid and wild bird populations in Spain after the epidemic outbreak in 2020. The present study highlights the need to continue and improve active surveillance programmes for the detection of WNV in Spain, particularly in those areas at greatest risk of virus circulation.

KEYWORDS
equines, risk factors, Spain, surveillance, West Nile, wild birds

1 | INTRODUCTION

West Nile virus (WNV; genus *Flavivirus*) is a vector-borne virus with worldwide distribution, which is mainly transmitted among vertebrate hosts by blood-feeding ornithophilic mosquito species (David & Abraham, 2016). Birds act as natural reservoirs that contribute to maintaining the natural WNV cycle, whereas other vertebrate hosts such as horses and humans are considered dead-end hosts because viraemia is insufficient to carry on the natural amplification cycle and infect other species (Rossi et al., 2010).

The genome of WNV consists of an enveloped, single-stranded and positive-sense RNA. To date, at least eight genetic lineages have been identified (Pérez-Ramírez et al., 2017). Of these, lineages 1 and 2 are the most widely distributed, and the most important for public and animal health (Christova et al., 2020). Both lineages have circulated in Europe during the last decade, causing hundreds of cases in humans, birds and horses (ECDC, 2022). The spatial spread of WNV during this period clearly shows the emergence and re-emergence of this virus in Europe (ECDC, 2022). The number of outbreaks of WNV reported in horses, wild birds and humans in European Mediterranean countries has increased dramatically in the last few years and constitutes a major concern for European health authorities (ECDC, 2022).

In Spain, antibodies against WNV in humans, horses and wild bird species and clinical infections in humans and raptors had already been detected before 2010 (García-Bocanegra, Busquets, et al., 2011, 2012; Jiménez-Clavero et al., 2008; Kaptoul et al., 2007). WNV lineages 1 and 2 have also been molecularly confirmed in different animal species in this country in the last decade. Lineage 1 is the most widely distributed and is responsible for outbreaks in humans, horses and birds in Spain (García-Bocanegra et al., 2018; Casimiro-Soriguer et al., 2021); to date, lineage 2 has only been found in mosquitoes and wild birds (Busquets, Alba, Allepuz, Aranda, & Núñez, 2008; Aguilera-Sepúlveda et al., 2022). The first clinical case of WNV in horses was reported in summer 2010 and was associated with lineage 1. A total of 36 WNV outbreaks in horses and two cases in humans were reported that year, all of them in Andalusia (southern Spain) (García-Bocanegra, Jaén-Téllez, et al., 2011). Circulation of WNV subsequently became endemic in this region, with periodic but limited detection of human and equine cases in the period 2011–2019 (RASVE, 2022). Of note, an active surveillance study carried out between 2018 and 2019 in provinces bordering on Andalusia highlighted a high circulation of WNV, with seroprevalence rates of 19.7% and 18.2% in horses and wild birds, respectively (Bravo-Barriga et al., 2021; Guerrero-Carvajal et al., 2021). The significantly higher seropositivity values detected in these studies compared to those previously found in the two species (7.1% in horses and 1.1% in birds) (García-Bocanegra et al., 2012; Jurado-Tarifa et al., 2016) suggested that there may have been a change in the epidemiology of WNV in the south of Spain in recent years. Indeed, in 2020, an unprecedented epidemic outbreak of WNV lineage 1 in this country affected hundreds of horses (Figure 1), and 77 humans developed a severe neuroinvasive disease with a fatality rate of 11.3% (Casimiro-Soriguer et al., 2021; Rodríguez-Alarcón et al., 2021). This epidemic outbreak was particularly important in Andalusia, where more than 90% of total cases in humans and horses were reported. The aims of the present study were (1) to evaluate the circulation of WNV in equids and wild birds after the epidemic outbreak in 2020 in southern Spain, and (2) to investigate the potential risk factors associated with WNV exposure in these species.

2 | MATERIALS AND METHODS

2.1 | Study design and sample collection

Between April and July 2021, a cross-sectional study was carried out to evaluate the prevalence of antibodies against WNV in equids in three Andalusian provinces (Cadiz, Huelva and Seville) where WNV cases had been detected in horses and humans during the epidemic outbreak of 2020. Taking into account the number of equids in these provinces ($n > 10{,}000$), an estimated prevalence of 20% (Guerrero-Carvajal et al., 2021), an accepted error of 5% and a 95% confidence interval (95%CI) resulted in 246 animals to be sampled. A total of 305 equids, including 277 horses, 27 mules and 1 donkey, were finally included in the study. Sampling was based on the stratified equid census in each province. Herds were chosen by simple random sampling from the herd census provided by the Regional Government of Andalusia. Based on herd size, between 1 and 17 animals were selected by systematic sampling. None of the sampled equids showed general or nervous clinical signs compatible with WNV infection (García-Bocanegra et al., 2018) at the time of sample collection.

Blood samples were collected by jugular vein puncture using sterile 10-ml blood tubes without anticoagulants (BD Vacutainer, USA). Samples were transported to the laboratory under refrigerated conditions (4°C) within 24–48 h of collection, centrifuged at 400 g for 15 min to obtain serum and stored at −20°C until analysis. An epidemiological questionnaire comprising an on-farm interview with the owners was conducted during sampling. A total of 29 explanatory variables were included to provide information on levels of exposure to potential risk factors. The variables were grouped by the following topics: (1) individual data, (2) herd data and (3) health and biosecurity data (Table S1).

In addition, between November 2020 and June 2021, 171 blood samples from wild birds admitted to wildlife rehabilitation centres (WRC) were collected by official veterinarians of the Regional Government of Andalusia in the provinces of Cadiz ($n = 60$), Huelva ($n = 54$) and Seville ($n = 57$). The number of birds to be sampled ($n = 174$) was calculated assuming an estimated seroprevalence of 13% (Jurado-Tarifa et al., 2016), a 95%CI and an accepted error of 5%. Samples were obtained by cubital, jugular and/or medial metatarsal veins puncture using sterile tubes without anticoagulants. Handling procedures were designed to reduce stress and minimize suffering in subjects. Blood samples were processed and stored as described for equids. Individual data related to wild birds were provided by the WRC (Table S2). No neurological clinical signs compatible with flavivirus infection were observed in the sampled birds.

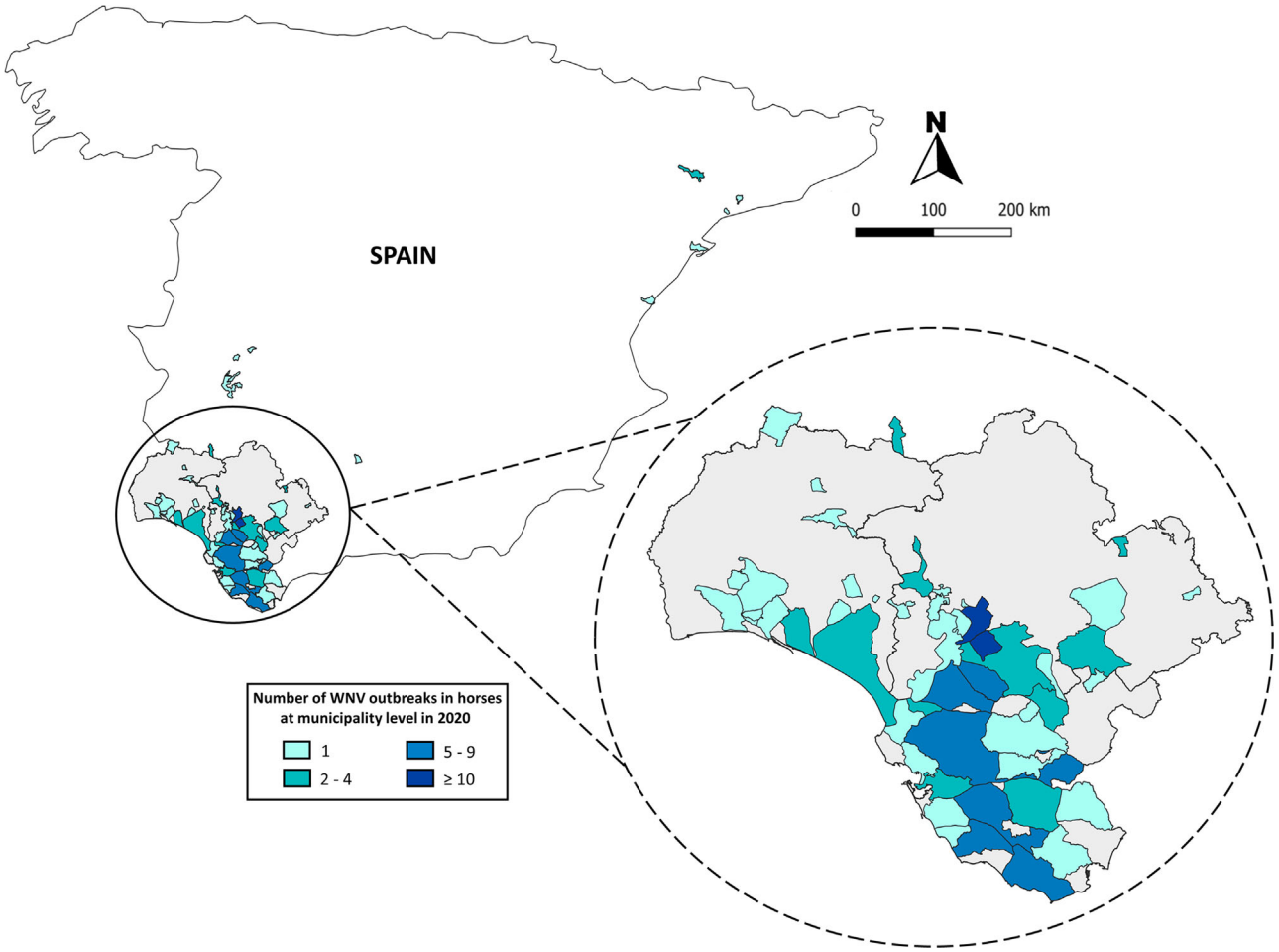

FIGURE 1 Distribution of West Nile virus (WNV) outbreaks in horses officially reported in Spain during the 2020 epidemic at the municipal level

2.2 | Serological analysis

Sera from equids and wild birds were analysed using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (Ingezim West Nile Compac R.10.WNV.K3, Eurofins Ingenasa, Madrid, Spain), which detects antibodies targeting epitopes on domain III of the envelope protein of flaviviruses belonging to the Japanese encephalitis antigenic complex. In addition, samples from horses were tested for the detection of IgM antibodies against WNV by a commercial capture ELISA (cELISA; Ingezim West Nile IgM R.14.WNV.K2, Eurofins Ingenasa, Madrid, Spain) using a monoclonal antibody specific to equine IgM and a monoclonal antibody specific to WNV E glycoprotein (domain III). ELISAs were performed according to the manufacturer's recommendations.

All bELISA-positive sera samples categorized as flavivirus-seropositive were subsequently confirmed by virus microneutralization test (VNT) against WNV (strain Morocco 03) at the National WNV Reference Laboratory in Algete (Madrid, Spain), as previously described (Schuffenecker et al., 2005). A serum sample was considered WNV-positive if both bELISA and VNT tests were positive. Given the possibility of cross-reactivity with antigenically related flaviviruses, bELISA-positive and doubtful samples from wild birds were also tested by VNT against Bagaza virus (BAGV; Spain/RLP–Hcc1/2010), which is a flavivirus that has been detected in wild bird species in the study area in the last decade (García-Bocanegra et al., 2013). Samples that showed neutralization and the absence of cytopathic effect at dilutions ≥10 were considered positive. The neutralizing immune response observed was considered specific when VNT titres for a given virus were ≥4-fold higher than those obtained for the other virus. Two-to-fourfold differences in VNT titres were considered positive for WNV and/or BAGV. Samples showing a ≤2-fold difference in VNT titres between the viruses examined were considered positive for flaviviruses but inconclusive for any specific virus.

2.3 | Statistical analysis

The prevalence of WNV antibodies was calculated as the ratio of the number of positive samples to the total number of samples tested, with 95%CI, using an exact calculation based on the binomial distribution (Thrusfield & Christley, 2018). The association between results

and epidemiological variables was initially assessed by bivariate analysis using Pearson's chi-squared test or Fisher's test, as necessary. Variables with *p*-values <.10 were selected for multivariate analysis. Collinearity between pairs of variables was estimated using Cramer's *V*, selecting the variable with the highest biological plausibility if a correlation coefficient between variables >.6 and a *p*-value <.05 was obtained. Finally, the effect of the previously selected independent variables of the response variable (WNV seropositivity) was analysed using generalized estimating equations (GEE). For statistical analysis, it was assumed that seropositive animals followed a binomial distribution, and the variable 'municipality' was included as a random effect. To select the most accurate model, the quasi-likelihood information criterion was considered. Statistical analysis was performed using RStudio (R Core Team, 2022).

3 | RESULTS

The epidemiological questionnaire revealed that 62 of the 305 equines sampled had previously been vaccinated against WNV and so were excluded from the seroprevalence calculations and risk factor analysis. Information on the WNV vaccination protocol was obtained for 48 of the 62 vaccinated horses. Most of these (89.6%; 43/48) had been vaccinated within the last 12 months (89.6%; 43/48), whereas the remaining 5 individuals (10.4%) had been vaccinated more than 12 months previously. In vaccinated equids, no statistically significant differences were found between time of last WNV vaccination and seropositivity ($p = .111$).

Of the vaccinated individuals, 87.1% (54/62) showed IgG antibodies to flavivirus by bELISA. In the unvaccinated equids, seropositivity to flavivirus was 44.9% (109/243). Significantly higher seropositivity was found in vaccinated compared to unvaccinated individuals ($p < .001$). IgM antibodies against WNV were not detected in any of the 243 unvaccinated animals (0.0%; 95%CI: 0.0–1.5). Specific anti-WNV antibodies were confirmed in 93 (85.3%) of the 109 bELISA-positive animals using the VNT. The individual seroprevalence in unvaccinated equids was therefore 38.3% (95%CI: 33.1–43.4). Titres of 1:10, 1:20, 1:40, 1:60, 1:80, 1:160 and 1:320 were found in 14.0%, 18.3%, 24.7%, 1.1%, 20.4%, 10.8% and 10.8% of seropositive equids, respectively.

WNV seroprevalence was 40.3% (29/72) in the province of Cadiz, 38.0% (35/92) in Seville and 36.7% (29/79) in Huelva (Table 1, Figure 2). No statistically significant differences between provinces were found ($p = .902$). At least one WNV seropositive individual was detected in 89.3% (25/28) of the herds analysed. Within-herd seropositivity ranged between 12.5% and 100% (mean: 42.01%; median: 33.3%). The frequency of seropositivity at municipal level is shown in Figure 2.

A total of 17 of 29 explanatory variables were initially selected from the bivariate analysis ($p < .10$) (Table 2). Explanatory variables 'cleanliness of facilities' and 'internal deworming programme' were excluded from the statistical analysis due to the low response rate (<5%) in at least one of the categories. The variables 'distance to urban area', 'rodent control programme' and 'water sources' were excluded from the multivariate analysis due to collinearity. The final model showed that the main risk factors potentially associated with WNV exposure in equids were age (adult and geriatric animals), breed (crossbred individuals) and the absence of a disinsection programme on the facilities (Table 3).

With respect to the wild birds, a total of 56 (32.7%) of 171 wild birds tested had IgG antibodies to flavivirus. Of these, 32 were confirmed by VNT, and 5 bELISA-positive sera could not be tested by VNT due to cytotoxicity or low sample volume. Consequently, the individual seroprevalence to WNV in wild birds was 19.3% (95%CI: 14.3–24.3). Titres of 1:10 (18.8%), 1:20 (21.9%), 1:40 (28.1%), 1:80 (12.5%), 1:120 (3.1%), 1:160 (9.4%) and 1:320 (6.3%) were obtained. No antibodies against BAGV were detected by VNT in bELISA-positive birds (Table 4).

The results of seropositivity to flavivirus and WNV in wild birds are shown in Table 4. Seropositivity to flavivirus and specific anti-WNV antibodies was found in 17 (45.9%; 95%CI: 32.4–59.4) and 14 (37.8%; 95%CI: 24.7–50.9) of the 37 avian species, respectively. Wild birds belonging to the order Accipitriformes showed the highest frequency of seropositivity to WNV (46.3%; 19/41), followed by the Ciconiiformes (22.2%; 2/9), Strigiformes (16.1%; 9/56) and Falconiformes (11.8%; 2/17).

TABLE 1 Seroprevalence of West Nile virus (WNV) in equines and wild birds in western Andalusia, southern Spain

Species group	Province	No. sampled animals (vaccinated/unvaccinated)	% bELISA positive (vaccinated/unvaccinated)	% VNT positive to WNV
Equines	Seville	107 (15/92)	93.3/43.5	38.0
	Cadiz	102 (30/72)	90.0/48.6	40.3
	Huelva	96 (17/79)	76.5/43.0	36.7
	Total	305 (62/243)	87.1/44.9	38.3
Wild birds	Seville	57	35.1	18.2
	Cadiz	60	43.3	27.1
	Huelva	54	18.5	11.5
	Total	171	32.7	19.3

Abbreviations: bELISA, blocking enzyme-linked immunosorbent assay; VNT, virus microneutralization test.

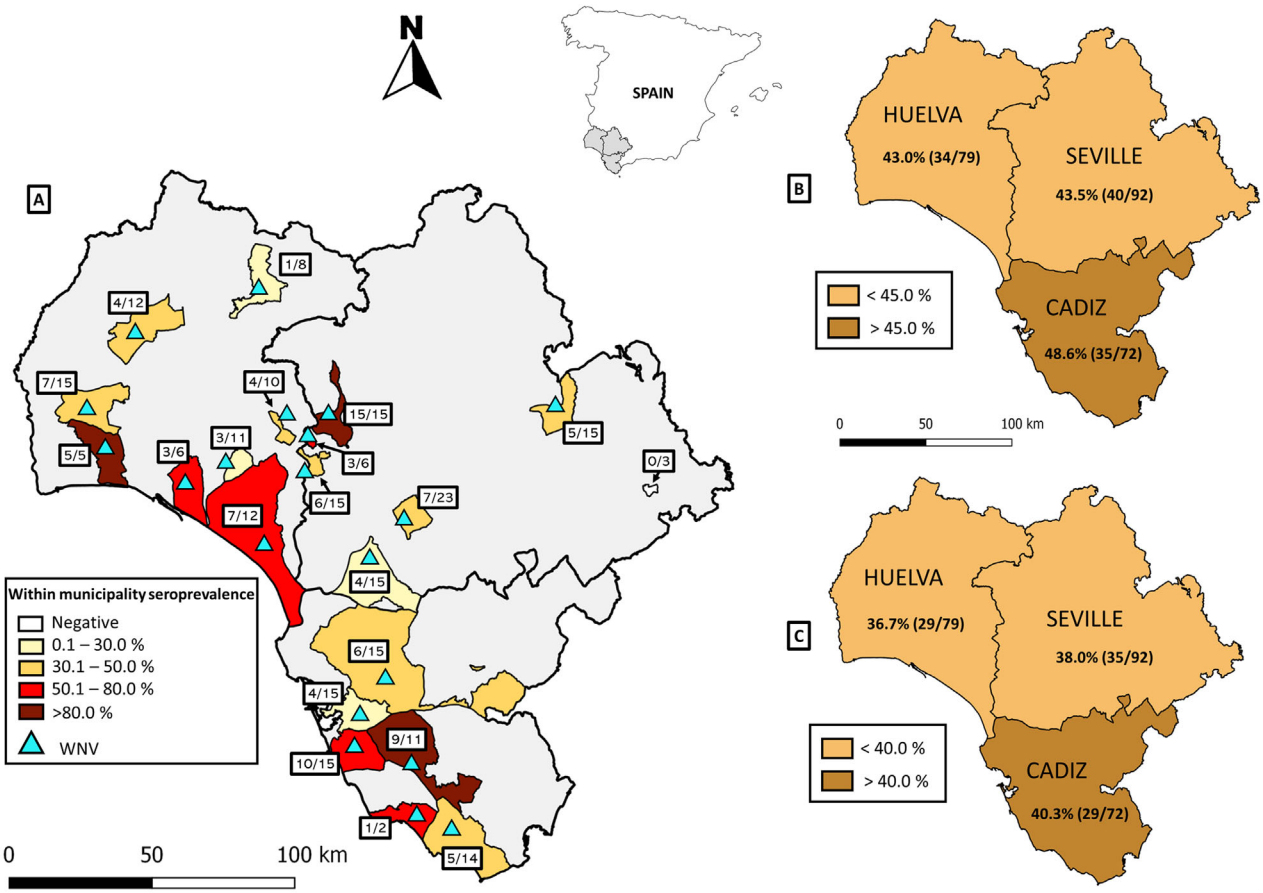

FIGURE 2 Map of the study area showing the locations of the sampled equines; colour gradations show flavivirus seropositivity by municipality and blue triangles indicate municipalities where West Nile virus (WNV) was confirmed by virus microneutralization test (VNT) (a); overall seroprevalence of flavivirus (b) and WNV (c) at the province level in the study area

At least 1 WNV seropositive bird was detected in 26 (36.7%) of 71 municipalities sampled. WNV seroprevalence was 27.1% (16/59) in Cadiz, 18.2% (10/55) in Seville and 11.5% (6/52) in Huelva (Table 1) (Figure 3). No statistically significant differences between provinces were found (p = .112), although marginally statistically differences between Huelva and Cadiz were reported (p = .055).

The final GEE model showed that group of species (raptors), age (>1-year old) and size (large) were the main risk factors potentially related to WNV exposure in wild birds (Table 3).

4 | DISCUSSION

Spain is the European country with the third highest number of WNV cases reported in animals so far (OIE, 2022), with Andalusia being the most important risk region for WNV circulation. This situation is influenced by both abiotic factors, such as favourable climatic conditions and the presence of a considerable number of wetlands, as well as biotic factors, notably the occurrence of competent vector species and susceptible migratory birds (Cuervo et al., 2021; García-Bocanegra et al., 2018; Jourdain et al., 2007). Our results indicate high viral circulation in both species groups in western Andalusia following the largest WNV epidemic outbreak detected in horses and humans in Spain.

Due to the random sampling method used in the present study to select equids, 62 horses previously vaccinated against WNV were tested but subsequently excluded from seroprevalence estimation and risk factor analysis. Even so, the results obtained provide valuable information to evaluate immunity against this virus in vaccinated equid populations. The significantly higher seropositivity found in vaccinated (87.1%) versus unvaccinated horses (44.9%) shows that vaccination is an effective prophylactic measure to prevent WNV infection and is in accordance with previous evidence (Khatibzadeh et al., 2015).

Despite the fact that the WNV seroprevalence found in the present study could be slightly underestimated due to the sample size of unvaccinated equids, the value obtained (38.3%) is the highest detected in Spain to date. The first study conducted in this country to assess WNV circulation in horses was carried out in Doñana National Park (southeastern Spain) in 2005 and showed a seroprevalence of 8.3% (Jiménez-Clavero et al., 2007). A similar seropositivity (9.8%) was found in horses sampled in Catalonia (northeastern Spain) during the period 2010–2019 (Napp et al., 2021). Another epidemiological survey with a similar design to our study detected a seropositivity of

TABLE 2 Distribution of explanatory variables identified as significant ($p < .10$) in the bivariate analysis and included in the multivariate analysis to determine the risk factors associated with West Nile virus (WNV) exposure in unvaccinated-WNV equids and wild birds in western Andalusia, southern Spain

Species group	Variable	Categories	% Positive	Number/Overall[a]	*p*-Value
Equines	Breed	Purebred	32.7	51/156	0.012
		Crossbred	48.3	42/87	
	Sex	Female	44.7	42/94	0.060
		Male	33.8	49/145	
	Age	Juvenile	25.6	11/43	0.020
		Adult	35.6	48/135	
		Geriatric	51.7	30/58	
	Vaccine rhinopneumonitis (individual)	No	40.7	88/216	0.018
		Yes	18.5	5/27	
	Census	Low (1–5)	55.6	20/36	0.020
		Medium (6–12)	25.5	12/47	
		High (>12)	38.1	61/160	
	Activity	Farming	37.1	43/116	0.008
		Leisure	64.3	18/28	
		Sport	32.3	32/99	
	Housing type	Outside	63.1	41/65	<0.001
		Inside	24.4	19/78	
		Mix	30.6	26/85	
	Animals per housing	Individual	31.0	31/100	0.043
		Collective	43.0	55/128	
	Domestic birds (presence)	No	41.4	72/174	0.075
		Yes	30.4	21/69	
	Wild birds (presence)	No	32.3	50/155	0.008
		Yes	48.9	43/88	
	Distance to urban area	<1 km	29.2	45/154	<0.001
		>1 km	53.9	48/89	
	Disinsection programme on facilities	No	67.8	40/59	<0.001
		Yes	28.8	53/184	
	Rodent control programme	No	57.1	40/70	<0.001
		Yes	30.6	53/173	
	Water sources (presence)	No	28.7	31/108	0.004
		Yes	45.9	62/135	
	River (presence)	No	32.4	58/179	0.001
		Yes	54.7	35/64	
Wild birds	Age	>1 year	29.7	27/91	<0.001
		<1 year	6.7	5/75	
	Size	Large	47.6	10/21	0.002
		Medium	17.6	13/74	
		Small	12.7	9/71	
	Raptor	No	3.8	2/52	<0.001
		Yes	26.3	30/114	

[a]Missing values omitted.

TABLE 3 Potential risk factors associated with West Nile virus (WNV) seropositivity in unvaccinated-WNV equids and wild birds in western Andalusia, southern Spain

Species group	Variable	Categories	β	*p*-Value	OR	95%CI
Equines	Age	Geriatric	1.057	0.014	2.878	1.243–6.665
		Adult	0.663	0.035	1.940	1.048–3.590
		Juvenile	[a]	[a]	[a]	[a]
	Breed	Crossbred	0.627	0.039	1.872	1.033–3.392
		Purebred	[a]	[a]	[a]	[a]
	Disinsection programme on facilities	Yes	−2.079	0.005	0.125	0.029–0.538
		No	[a]	[a]	[a]	[a]
Wild birds	Age	>1 year	1.497	0.001	4.466	1.840–10.840
		<1 year	[a]	[a]	[a]	[a]
	Size	Large	1.298	0.019	3.663	1.234–10.872
		Medium	0.441	0.400	1.555	0.557–4.342
		Small	[a]	[a]	[a]	[a]
	Raptor	Yes	2.061	0.001	7.860	2.402–25.694
		No	[a]	[a]	[a]	[a]

[a]Reference category.

7.1% in horses just before the first WNV outbreaks in this species in Spain in 2010 (García-Bocanegra et al., 2012). A slightly lower seroprevalence (6.4%) was found in a later study conducted in the Balearic Islands (eastern Spain) (Vanhomwegen et al., 2017), whereas a much lower antibody prevalence (1.4%) was found in animals from central Spain between 2011 and 2013 (Abad-Cobo et al., 2017). More recently, Guerrero-Carvajal et al. (2021) found a WNV seroprevalence of 19.7% in horses in Extremadura (which borders our study area to the north). Nevertheless, comparisons between studies should be made with care due to differences in study design, diagnostic technique and epidemic period, among other factors.

At least one seropositive equid was found in 25 of the 28 (89.3%) sampled herds. This, as well as the similar seroprevalence at province level (ranging from 36.7% to 40.3%) and the absence of significant differences between them, indicates a high spatial distribution and homogeneous circulation of WNV in the equine populations of western Andalusia. Our result contrasts with the study carried out by García-Bocanegra et al. (2012) in 2010 in the same region, in which the provinces of Cadiz and Huelva showed significantly higher WNV seroprevalences (14.2% and 13.3%, respectively) when compared to Seville (5.8%). Differences in seroprevalence values between regions and countries have also been observed in other European countries (Metz et al., 2021). Furthermore, previous studies have shown differences in WNV seropositivity in the same study region or country at different times. Petrović et al. (2014) obtained a significantly higher seroprevalence (49.2%) in horses from Vojvodina province (northern Serbia) in 2012 compared to the same region in 2009–2010 (12.0%) (Lupulović et al., 2011). Bażanów et al. (2018) observed a higher seroprevalence in horses from Poland (15.1%), which contrasted with earlier findings in the same country that yielded either negative results (Hubálek et al., 2008) or a very low level of seropositivity (below 1.0%) (Golnik et al., 2008; Niczyporuk et al., 2015). These results highlight the importance of long-term active surveillance to improve our understanding of the epidemiology of WNV in endemic areas.

IgM antibodies were not detected in any of the 243 equines tested, which is consistent with the fact that the sampling period took place between April and July 2021, whereas the WNV outbreaks that occurred in 2020 were detected during the autumn and early winter months (RASVE, 2022). Considering that IgM antibodies are detectable from day 7 to 3 months after infection (Bunning et al., 2002; Ostlund et al., 2000), the absence of IgM antibodies in the tested equids suggests that WNV did not circulate in the study area at least during the first half of 2021.

Risk factor analysis showed that the prevalence of anti-WNV antibodies was significantly higher in adult and geriatric animals compared to juvenile individuals, which is in accordance with previous observations (Cardinale et al., 2017; Guerrero-Carvajal et al., 2021). The higher seropositivity in these age groups probably reflects a cumulative probability of exposure to the virus as well as the continued occurrence of IgG antibodies over time. Seroprevalence was also significantly higher in crossbred animals (48.3%) versus purebreds (32.7%). A possible explanation for this finding is that purebred equids may benefit from better management practices and biosecurity measures associated with more adequate facilities (e.g., presence of mosquito nets, disinsection protocols and installations designed to prevent wild birds from entering), which may reduce the risk of WNV exposure (Petersen et al., 2013). Finally, the GEE model showed that herds where there were disinsection programmes had a significantly lower WNV seroprevalence (28.8%) than herds where this vector control measure was not applied (67.8%). The identification of on-farm disinsection programmes as a protective factor against WNV exposure is in-line with the current scientific literature (ECDC, 2020; Saiz et al.,

TABLE 4 Detection of antibodies against flavivirus and West Nile virus (WNV) in wild bird species from western Andalusia, southern Spain, by blocking enzyme-linked immunosorbent assay (bELISA) and virus microneutralization test (VNT), respectively

		Flavivirus			WNV			
Order	Genus and species	NP	TI	%	NP	TI	%	VNT titre
Accipitriformes	*Aegypius monachus*	1	1	100	0	1	0.0	
	Accipiter gentilis	1	3	33.3	1	3	33.3	1/40[a]
	Accipiter nisus	0	1	0.0	0	1	0.0	
	Aquila adalberti	1	2	50.0	1	2	50.0	1/10
	Aquila fasciata	2	2	100	1	2	50.0	1/20
	Buteo buteo	4	11	36.4	3	11	27.3	1/160(2); 1/320
	Circus aeruginosus	1	2	50.0	1	2	50.0	1/10
	Circus pygargus	5	5	100	3	5	60.0	1/20; 1/40(2)[a]
	Gyps fulvus	8	10	80.0	6	9	66.7	1/10; 1/20; 1/40(2); 1/80; 1/120
	Hieraaetus pennatus	4	5	80.0	3	4	75.0	1/10(2); 1/160
	Parabuteo unicinctus	0	1	0.0	0	1	0.0	
Anseriformes	*Anas platyrhynchos*	0	3	0.0	0	3	0.0	
	Cairina moschata	0	1	0.0	0	1	0.0	
Apodiformes	*Apus pallidus*	0	3	0.0	0	3	0.0	
Bucerotiformes	*Upupa epops*	0	1	0.0	0	1	0.0	
Caprimulgiformes	*Caprimulgus europaeus*	0	2	0.0	0	2	0.0	
Charadriiformes	*Calidris alpina*	0	1	0.0	0	1	0.0	
	Larus audouinii	0	1	0.0	0	1	0.0	
	Larus fuscus	1	22	4.5	0	22	0.0	
	Larus michahellis	0	4	0.0	0	4	0.0	
	Stercorarius skua	0	1	0.0	0	1	0.0	
Ciconiiformes	*Bubulcus ibis*	0	1	0.0	0	1	0.0	
	Ciconia ciconia	2	6	33.3	2	6	33.3	1/20(2)[a]
	Platalea leucorodia	0	2	0.0	0	2	0.0	
Falconiformes	*Falco naumanni*	0	4	0.0	0	4	0.0	
	Falco peregrinus	0	1	0.0	0	1	0.0	
	Falco tinnunculus	3	8	37.5	2	7	28.6	1/20(2)
	Milvus migrans	2	5	40.0	0	5	0.0	
Passeriformes	*Corvus corax*	0	1	0.0	0	1	0.0	
	Corvus monedula	0	1	0.0	0	1	0.0	
	Sturnus unicolor	0	1	0.0	0	1	0.0	
Strigiformes	*Asio otus*	1	4	25.0	1	4	25.0	1/40
	Athene noctua	0	7	0.0	0	7	0.0	
	Bubo bubo	4	8	50.0	2	8	25.0	1/40; 1/80
	Strix aluco	13	35	37.1	3	33	9.1	1/10; 1/40; 1/80[a]
	Tyto alba	3	4	75.0	3	4	75.0	1/40; 1/80[a]; 1/320
Suliformes	*Morus bassanus*	0	1	0.0	0	1	0.0	

Abbreviations: NP, number of positives; TI, total individuals analysed; %, NP/TI.
[a]Detection of BAGV positive individual (titre 1/10).

2021; van den Berg et al., 2021; Wilson et al., 2020) and highlights the importance of implementing this kind of measure in facilities advantageous for vector breeding. In this respect, vector control using environmental and/or animal insecticides (larvicides or adulticides) is considered one of the main tools for control of WNV (ECDC, 2020).

The seroprevalence of flavivirus detected in wild bird populations in the present study was 32.7% using bELISA. In contrast to what

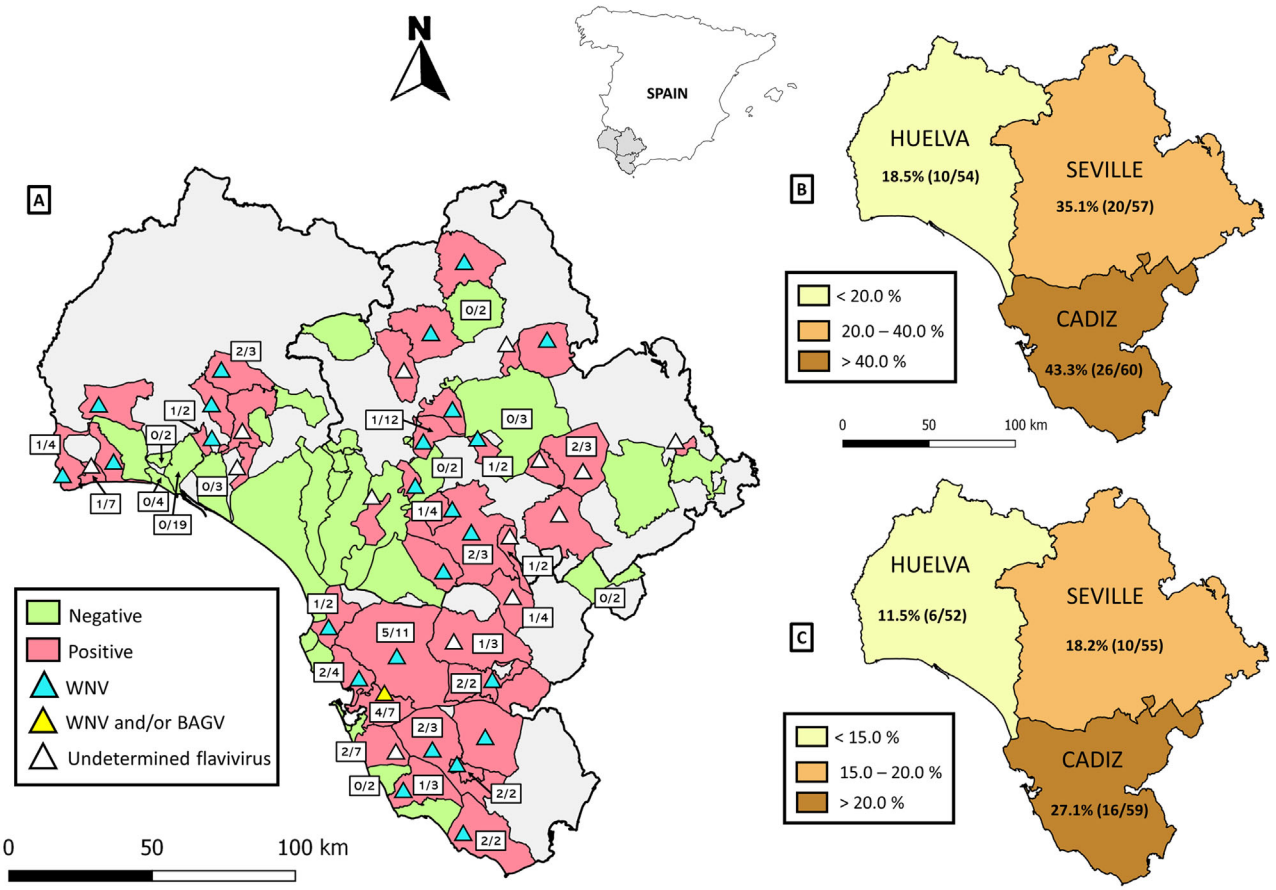

FIGURE 3 Map of the study area showing the locations of the sampled wild birds. Blue, yellow and white triangles indicate the presence of West Nile virus (WNV), WNV and/or Bagaza virus (BAGV) and undetermined flaviviruses at the municipal level, respectively (a); overall seroprevalence of flavivirus (b) and WNV (c) at the provincial level in the study area. The seropositive and the total number of tested birds (*n*) are shown in labels per municipality when $n > 1$.

was observed in unvaccinated equids, where 85.3% of bELISA-positive animals were confirmed by VNT, only 57.1% (19.3% prevalence of anti-WNV antibodies) of birds that presented antibodies against flavivirus by bELISA were confirmed by VNT. Specific antibodies against BAGV were not found in the bELISA-positive wild birds sampled, so that the observed differences between diagnostic techniques could be explained by cross-reactions with other antigenically related flaviviruses, such as Usutu virus, Meaban virus or tick-borne encephalitis virus, which have been detected in wild birds in the Iberian Peninsula (Gamino et al., 2012; Jurado-Tarifa et al., 2016; Napp et al., 2021). Unfortunately, the limited volume of samples as well as the fact that it was not possible to conduct these analyses at the National WNV Reference Laboratory meant that VNT for the detection of neutralizing antibodies against these other flaviviruses could not be performed. Because co-exposure to other antigenically related flaviviruses may affect the outcomes of WNV serological results (Llorente et al., 2019), further VNT analyses, including other cross-reacting flaviviruses not assessed in the sera tested, should be carried out for a better interpretation of the serological results obtained in the present study. The additional analyses may also be useful for obtaining complementary epidemiological information about antigenically related flaviviruses circulating in the study area.

The individual seroprevalence for WNV obtained in wild birds in our study (19.3%) was higher than that previously found in previous serosurveys in the same region. Jurado-Tarifa et al. (2016) detected a seroprevalence of 1.1% in 1052 waterfowl sampled in 2011 in the same provinces. García-Bocanegra, Busquets et al. (2011) found 1.0% seropositivity in 201 wild birds sampled during 2006–2009, and Ferraguti et al. (2016) reported 0.7% seropositivity in 149 wild birds sampled in 2013 in Andalusia. Our results suggest a change in the epidemiology of WNV in wild bird populations in southern Spain over the last few years. The high seropositivity (18.2%) detected in wild birds sampled in Extremadura during 2017–2019 (Bravo-Barriga et al., 2021) supports this hypothesis.

At least one WNV seropositive bird was detected in 36.7% of the municipalities included in the study, which indicates a wide distribution of this virus in the study region. The highest prevalence of WNV antibodies was found in the province of Cadiz (27.1%), followed by Seville

(18.2%) and Huelva (11.5%), which is consistent with the pattern of WNV outbreaks so far reported in equids in these provinces (Figure 1) (RASVE, 2022).

WNV seropositivity occurred in 14 (37.8%; 95%CI: 24.7–50.9) of the avian species analysed. Raptor species had a 7.9 times higher risk of being exposed to the virus than other species. Birds of prey have been shown to be particularly susceptible to WNV infection (Nemeth et al., 2007; Ziegler et al., 2013) and were also among the most frequently infected bird species during the WNV outbreaks (Vidaña et al., 2020). It should be noted that raptors are at the top of the food chain and could have a higher probability of WNV exposure as some species feed on WNV-susceptible birds, thus increasing the risk of virus infection (Komar et al., 2003; Vidaña et al., 2020). In Spain, mortality due to this viral infection has been reported in endangered raptor species such as the Iberian imperial eagle (*Aquila adalberti*) (Höfle et al., 2008) and the golden eagle (*Aquila chrysaetos*) (Jiménez-Clavero et al., 2008), which points to the importance of WNV for conservation. In addition, WNV lineage 2 circulation has been detected in this country in northern goshawks (*Accipiter gentilis*) in recent years (Busquets et al., 2019). The high seroprevalence observed in raptors in our study (26.3%) highlights the role of these species as WNV reservoirs and sentinels.

Seroprevalence for WNV in wild birds was age-related. Significantly higher seropositivity was found in adult birds (>1-year old; 29.7%) than in juveniles (<1-year old; 6.7%), which coincides with findings observed by other authors (Bravo-Barriga et al., 2021; Jurado-Tarifa et al., 2016) and points to a higher probability of WNV exposure in birds over time and/or continued occurrence of antibodies after infection. Although passive transfer of maternal immunity cannot be ruled out, the seropositivity detected in five juvenile individuals, including two resident bird species, suggests recent viral circulation in the study region.

The GEE model also showed significantly higher seropositivity in large species (47.6%) compared to medium (17.6%) and small (12.7%) species, which is in-line with previous studies and could be associated with a greater preference for larger birds in competent mosquitoes (mainly *Culex* spp.) (Figuerola et al., 2008; Victoriano Llopis et al., 2016). In this respect, mosquitoes are attracted to carbon dioxide, which larger birds exhale in greater amounts (Spanoudis et al., 2020). In addition, parts of the body with bald spots in certain large species, such as the neck in vultures or the long legs of waterfowl, could favour increased mosquito bites (García-Bocanegra, Busquets, et al., 2011).

5 | CONCLUSIONS

The present study contributes to a better understanding of WNV transmission. Seroprevalence values obtained in equids (38.3%) and wild birds (19.3%) indicate widespread circulation of WNV in western Andalusia following the largest WNV epidemic outbreak recorded to date in humans and horses in Spain. Surveillance programmes in wild birds and equids should be maintained and reinforced to better prevent future WNV outbreaks in humans and horses in this country, particularly in those areas with the highest risk of virus circulation.

AUTHOR CONTRIBUTIONS

Ignacio García-Bocanegra, María B. Gómez and Juan J. Franco wrote the original draft of the manuscript. Ignacio García-Bocanegra, David Cano-Terriza and Moisés Gonzálvez identified the research question and selected the methodology to be used. Clara I. León, María V. García-Miña and Vicente Fernández-Molera conducted the field sampling. Juan J. Franco, Jesús Barbero-Moyano, María B. Gómez and David Cano-Terriza performed experimental work. All authors contributed to the critical review of the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

DATA AVAILABILITY STATEMENT

All data supporting the findings of the present study are available upon reasonable request from the corresponding author.

ETHICS STATEMENT

Horse samples were obtained within the official Epidemiological Surveillance System of Livestock for the Improvement of the Competitiveness of the Agricultural Sector of the Regional Government of Andalusia, Spain (REF: BOJA no 55 of March 20). Sera from wild birds came from specimens admitted to wildlife rehabilitation centres and subjected to medical check-ups or surgical interventions during the study period. Animal handling and sampling were performed by qualified and trained veterinarians following European (Directive 86/609/CEE) and Spanish (RD 53/2013) legislations. Therefore, no ethical approval was necessary.

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SUPPORTING INFORMATION

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