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Exposure to West Nile Virus in Wild Lagomorphs in Spanish Mediterranean Ecosystems

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ABSTRACT

Background: West Nile virus (WNV) is the most widely distributed mosquito-borne flavivirus. Over the past decade, its spread across Europe has raised significant concerns for both public and animal health. Although WNV exposure has been evidenced in various wild mammal species in Spain, no seroepidemiological studies have been conducted on this flavivirus in wild lagomorphs so far.

Aim: This study aimed to assess WNV exposure in European wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations inhabiting Spanish Mediterranean ecosystems.

Methods: Sera from 540 wild lagomorphs (399 European wild rabbit and 141 Iberian hares), from 106 hunting grounds distributed throughout Andalusia (southern Spain), were collected between the 2018/2019 and 2022/2023 hunting seasons.

Results: Antibodies against flavivirus were detected by blocking enzyme-linked immunosorbent assay (bELISA) in 5.0% (27/540; 95% CI: 3.2–6.8) of the wild lagomorphs. Exposure to WNV was confirmed in 4.8% (19/394; 95% CI: 2.7–6.9) of wild rabbits and 0.7% (1/141; 95% CI: 0.0–2.1) of Iberian hares by virus microneutralisation test. Anti-WNV antibodies were found in wild lagomorphs sampled from three (2.8%) hunting grounds located in western Andalusia during the seasons 2020–2021 and 2021–2022. Remarkably, this spatiotemporal distribution overlaps with the largest outbreak of WNV in Spain. Antibodies against Usutu virus and Bagaza virus were not detected in the wild lagomorph populations analysed.

Conclusions: This study constitutes the first report of WNV exposure in wild rabbit in Spain and in Iberian hare worldwide. While these species seem not play a primary role in the epidemiology of the virus, they could serve as sentinel for monitoring WNV in Iberian Mediterranean ecosystems.

Summary

- First report of WNV exposure in Iberian hare worldwide.
- Seropositivity found in wild lagomorphs overlaps with the largest WNV outbreak in Spain.
- Wild lagomorphs could serve as sentinels for monitoring WNV in Iberian Mediterranean ecosystems.

1 | Introduction

Flaviviruses (genus *Orthoflavivirus*, family *Flaviviridae*) are endemo-epidemic *vector-borne pathogens* in Europe, representing a significant threat for public health. Among them, West Nile virus (WNV) has the broadest geographical spread (Cuervo et al. 2022), being considered as the leading cause of arboviral encephalitis in humans worldwide (Ciota 2017). During the past two decades, the WNV has spread to various regions of eastern and central Europe, while circulating endemically in the Mediterranean basin (ECDC 2024).

In Spain, WNV circulation has been confirmed in bird, mammal and mosquito species since the early 2000s (Figuerola et al. 2007; Jiménez-Clavero et al. 2008). However, cases in humans were sporadic until 2020, when an unprecedented WNV epidemic occurred in south-western Spain, with 77 clinical cases and 7 deceased (Rodríguez-Alarcón et al. 2021). Since then, human cases have been reported annually in this area (ECDC 2024), establishing the virus as endemic in this region.

The natural enzootic cycle of WNV primarily involves birds as primary hosts and mosquitoes, especially those of the *Culex* genus, serving as competent vectors (Vogels et al. 2017), while mammals, mainly horses and humans, are generally considered dead-end hosts due to their low levels of viraemia (Blitvich 2008). Due to the significance of these viruses, a growing number of studies focused on wildlife have identified an increasing number of species susceptible to WNV, thereby expanding the range of potential hosts beyond the primary ones (Jeffrey Root 2013). In Iberian Mediterranean ecosystems, WNV exposure has been evidenced in different wild mammal species, including wild ruminants (García-Bocanegra et al. 2016), red foxes (*Vulpes vulpes*) and wild boar (*Sus scrofa*) (Gutiérrez-Guzmán et al. 2012).

The European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are two endemic and keystone species in the Iberian Peninsula (Delibes-Mateos et al. 2007) and are among the most abundant and significant species in terms of hunting interest. These species are widespread across the Iberian Peninsula, inhabiting diverse environments, including urban and peri-urban areas. They can reach high densities in certain regions, thereby increasing the risk of zoonotic pathogens transmission (Jiménez et al. 2014). In this regard, these wild lagomorph species have been shown to be natural reservoirs for several zoonotic vector-borne pathogens such as *Coxiella burnetii*, *Francisella tularensis* or *Leishmania infantum* (Jiménez et al. 2014; González-Barrio et al. 2015; Castro-Scholten et al. 2024) and they have been considered to be useful sentinel species for some vector-borne diseases (Carvalho et al. 2014). Furthermore, natural exposure to WNV has been evidenced in European wild rabbits (Arthur et al. 1990) and European brown hares (*Lepus europaeus*) (Juřicová and Hubálek 1999) although upon infection, these species remain asymptomatic (McLean et al. 2002). However, there are no seroepidemiological studies on WNV in European wild rabbit in Spain and Iberian hare worldwide. Thus, the aim of the present study was to assess exposure to WNV in wild lagomorph populations of Spanish Mediterranean ecosystems.

2 | Material and Methods

2.1 | Study Area and Sampling Collection

A cross-sectional epidemiological study was conducted in Andalusia, southern Spain (36° N–38°60′ N, 1°75′ W–7°25′ W), across the hunting seasons 2018/2019–2022/2023. This region accounted for nearly 83% of the total WNV cases detected in human and horses in Spain since the first detection in 2010 (RNVE 2023; RASVE 2024). For the European wild rabbits, sample size was calculated as a minimum of 385 animals, assuming a prevalence of 50%, which provides the highest sample size in studies with unknown prevalence, with a 95% confidence interval (95% CI) and a desired precision of ±5%. Whenever possible, 42 wild rabbits were sampled in each of the eight provinces within the study area to ensure a 95% probability of detecting at least one positive animal, assuming a minimum within-province prevalence of 7% (Thrusfield and Christley 2018). Hunting grounds were randomly selected for sampling in each province. Ultimately, a total of 399 wild rabbits from 53 hunting grounds distributed across all eight provinces were sampled during the study period. A convenience sampling was used for the Iberian hare, as its geographic distribution is restricted to certain areas due to recent population declines, primarily resulting from the impact of myxomatosis on this species (García-Bocanegra et al. 2019, 2021). A total of 141 Iberian hares from 62 hunting grounds were sampled in the same study area and period. Blood samples were collected from the hearth or thoracic cavity of all animals on the field. Samples were kept refrigerated until arrival at the laboratory, and then centrifuged at 400 *g* for 10 min. The resulting serum was stored at −80°C until serological analysis were performed.

During sampling, an epidemiological questionnaire was completed through direct interviews with gamekeepers at each hunting ground. The information collected included the characteristics of the hunting ground, the presence of diseases (myxomatosis and rabbit haemorrhagic disease) and control measures, management practices and the presence of other sympatric species. Additionally, data on each individual animal were recorded, including species, location, year of sampling, age (determined by bodyweight and body length; Morris 1972), kidney fat index and sex.

2.2 | Serological Analysis

Sera from wild lagomorphs were analysed using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (10.WNV.K3 IngezimWest Nile Compac, Gold Standard

Diagnostics, Madrid, Spain) to detect antibodies that block the reaction of a horseradish peroxidase-conjugated monoclonal antibody specifically recognising an epitope within the envelope protein domain III of WNV. The assay was performed according to the manufacturer's instructions. Results were expressed as a percentage of inhibition (PI) and it was calculated as follows: $PI = 100 - [(OD_{sample}/OD_{NC}) \times 100]$, in which OD_{sample} is the optical density of a sample and OD_{NC} is the mean optical density of the negative control (NC). Samples with PI > 40% were considered positive, < 30% negative and between 30% and 40% doubtful.

Positive and doubtful sera were further tested by micro virus neutralisation test (micro-VNT) to confirm the presence of specific neutralising antibodies against WNV as well as against Usutu virus (USUV) and Bagaza virus (BAGV), two other flaviviruses which could cross-react in the ELISA tests (Llorente et al. 2019), and whose circulation has been previously evidenced in the study area (García-Bocanegra et al. 2013; Jurado-Tarifa et al. 2016). Micro-VNTs were performed with 60 μL of each serum according to the previously described protocol (Llorente et al. 2019). The immune response was considered specific for a WNV when neutralising antibodies showed titres equal or higher than 1:10 by micro-VNT and at least fourfold higher than titres obtained for any of the other viruses analysed (Calisher et al. 1989). When titre differences did not reach this threshold, the result was considered inconclusive and the specific virus to which the animal was exposed could not be determined.

2.3 | Statistical Analysis

Animals were classified as seropositive for WNV if the sera tested positive/doubtful by ELISA and positive for specific WNV antibodies by micro-VNT. Seroprevalence was calculated by dividing the number of seropositive animals by the total number of animals tested, using two-sided exact binomial 95% confidence intervals (95% CI). Continuous variables were categorised taking the percentiles 33 and 66 as cut points to homogenise the scales of the explanatory variables. Associations between WNV seroprevalence and epidemiological variables were assessed through a Pearson's chi-squared or Fisher's exact tests, as appropriate. Variables with a $p < 0.05$ in the bivariate analysis were selected for further examination. Collinearity between pairs of variables was assessed using Cramer's V test. In cases in which a correlation was detected (Cramer's V coefficient ≥ 0.6), the variable with the highest a priori biological association with flavivirus was retained. Finally, the selected variables were included in a multiple logistic regression model to assess the risk factors potentially associated with WNV exposure in wild lagomorphs. The model was re-run until all remaining variables presented statistically significant values ($p < 0.05$). Statistical analyses were conducted using SPSS v25.0 software (Statistical Package for Social Sciences Inc., Chicago, IL, USA).

3 | Results and Discussion

During the last decade, the number of outbreaks and the spatial distribution of WNV have drastically increased, providing evidence of the emergence and/or re-emergence of the virus across Europe (ECDC 2024). In particular, the Mediterranean Basin has witnessed a significant increase in the number of notified WNV outbreaks caused by both lineages 1 and 2, raising considerable concerns for public and animal health. In Spain, Andalusia stands out as a hotspot for WNV circulation, attributed to favourable climatic conditions, the presence of competent vector species and the influx of susceptible migratory wild birds (Jourdain et al. 2007; Cuervo et al. 2022). In these epidemiological scenarios, different studies have suggested that surveillance in large game species could be a useful complementary tool for national surveillance programs to monitor WNV (Gutiérrez-Guzmán et al. 2012; García-Bocanegra et al. 2016).

In the present study, flavivirus antibodies were detected in 27 (4.8%; 95% CI: 3.0–6.6) of 540 wild lagomorphs tested by bELISA and only one sample was doubtful, specifically one European wild rabbit. Five serum samples positive by ELISA could not be analysed by micro-VNT due to low sample volume. Specific neutralising antibodies against WNV were detected in 20 (90.9% of ELISA positive/doubtful) animals, supposing an overall individual seroprevalence to WNV of 3.7% (95% CI: 2.1–5.3). Also, one positive and the doubtful European wild rabbit by bELISA had micro-VNT titres, but they did not allow to differentiate the presence of WNV-, USUV- or BAGV-specific antibodies (Table 1). The bELISA PI and titres of neutralising antibodies to WNV, USUV and BAGV are shown in Table 1. WNV seroprevalence was determined only from samples that tested positive by both ELISA and micro-VNT, which may have led to a slight underestimation.

Nineteen (4.8%; 95% CI: 2.7–6.9) of 394 European wild rabbits were positive for anti-WNV antibodies. Although our results were higher, they align with the only previous serosurvey conducted on this species globally. In that study, one (0.4%) of the 269 animals sampled in southeast France reacted positively to WNV antigen in a haemagglutination inhibition (HI) test (Arthur et al. 1990). These findings confirm that European wild rabbits are naturally exposed to this virus. Only one (0.7%; 95% CI: 0.0–2.1) Iberian hare showed specific antibodies against WNV. To our knowledge, this is the first report of WNV exposure in this hare species, thus increasing the range of susceptible mammals to this zoonotic pathogen. Previous surveys detected positive reaction to WNV antigen in 18 (9.3%) of 193 European brown hares in Czech Republic by HI test (Juřicová and Hubálek 1999) and in the five (100%) hares of this species sampled in Israel using the same diagnostic method (Akov and Goldwasser 1966). Although accurate comparisons are difficult due to variations in the number of animals tested, epidemiological contexts and serological methods used, our results suggest a limited but relevant exposure of the European wild rabbit and, to a lesser extent, Iberian hare populations, to WNV in the Mediterranean ecosystems of southern Spain. However, additional studies on these lagomorph species, including a larger number of animals and broader geographic coverage, are warranted to determine their role in the epidemiology of WNV in the Iberian Peninsula.

The distribution of WNV seroprevalence according to species, age, sex, hunting season and province is shown in Table 2. Although species, hunting season and province showed

TABLE 1 | Values obtained in the serological analysis.

Animal identification	Species	Province	bELISA PI (%)	WNV micro-VNT titre	USUV micro-VNT titre	BAGV micro-VNT titre	Result
1	Wild rabbit	Almería	42.67	1:20	1:20	< 1:10	Undetermined *Orthoflavivirus*
3	Wild rabbit	Cádiz	94.04	1:320	1:10	< 1:10	WNV
4	Wild rabbit	Cádiz	92.26	> 1:1280	1:20	< 1:10	WNV
5	Wild rabbit	Cádiz	73.22	> 1:1280	1:160	< 1:10	WNV
6	Wild rabbit	Cádiz	94.93	> 1:1280	1:20	< 1:10	WNV
7	Wild rabbit	Cádiz	91.46	> 1:1280	1:40	1:10	WNV
8	Wild rabbit	Cádiz	94.40	1:640	1:10	< 1:10	WNV
9	Wild rabbit	Cádiz	94.13	1:640	1:20	< 1:10	WNV
10	Wild rabbit	Cádiz	94.13	> 1:1280	1:20	1:10	WNV
11	Wild rabbit	Cádiz	94.13	1:640	1:80	< 1:10	WNV
12	Wild rabbit	Cádiz	92.79	1:640	1:20	1:10	WNV
14	Wild rabbit	Cádiz	94.31	> 1:1280	1:20	< 1:10	WNV
15	Wild rabbit	Cádiz	92.88	1:320	1:10	< 1:10	WNV
16	Wild rabbit	Cádiz	93.42	1:640	1:20	< 1:10	WNV
17	Wild rabbit	Cádiz	90.92	1:640	1:10	< 1:10	WNV
18	Wild rabbit	Cádiz	92.35	1:640	1:20	< 1:10	WNV
19	Wild rabbit	Cádiz	88.97	> 1:640	1:10	< 1:5	WNV
20	Wild rabbit	Cádiz	92.08	> 1:1280	1:20	1:10	WNV
21	Wild rabbit	Cádiz	94.31	1:320	1:40	—	WNV
25	Wild rabbit	Granada	30.56	1:10	1:10	< 1:10	Undetermined *Orthoflavivirus*
27	Wild rabbit	Huelva	92.44	1:320	1:40	1:10	WNV
28	Iberian hare	Sevilla	78.65	1:320	1:80	< 1:10	WNV

Abbreviations: BAGV, Bagaza virus; bELISA, blocking enzyme-linked immunosorbent assay; Micro-VNT, micro virus neutralisation test; PI, percentage of inhibition; USUV, Usutu virus; WNV, West Nile virus.

TABLE 2 | Distribution of seroprevalence against West Nile virus in wild lagomorphs in Andalusia (southern Spain) by animal categories and results of bivariate analysis.

Variable	Categories	No. positives/Overall[a]	Seroprevalence (%)	*p*
Species	Wild rabbit	19/394	4.8	**0.016**
	Iberian hare	1/141	0.7	
Age	Adult	19/384	4.9	0.051
	Subadult	0/112	0.0	
	Young	1/35	2.9	
Sex	Male	9/263	3.4	0.427
	Female	11/268	4.1	
Hunting season	2018/2019	0/42	0.0	**0.010**
	2019/2020	0/43	0.0	
	2020/2021	19/296	6.4	
	2021/2022	1/102	1.0	
	2022/2023	0/51	0.0	
Province	Almería	0/45	0.0	**<0.001**
	Cádiz	18/54	33.3	
	Córdoba	0/92	0.0	
	Granada	0/51	0.0	
	Huelva	1/53	1.9	
	Jaén	0/81	0.0	
	Málaga	0/93	0.0	
	Sevilla	1/66	1.5	

[a]Missing values omitted. Variables with *p* values lower than 0.05 are in bold.

$p < 0.05$ in bivariate analysis, neither of these variables were retained in the final multiple logistic regression model. Seropositive animals were detected in hunting seasons 2020–2021 and 2021–2022 and anti-WNV antibodies were found in a seropositive yearling Iberian hare sampled in 2020. In addition, seropositivity to WNV was confirmed in three (2.8%) of the 106 sampled hunting grounds, these belonging to three different provinces located in south-western Spain (Huelva, Cádiz and Sevilla) (Figure 1). All these findings denote WNV circulation in wild lagomorphs during the last years as well as heterogeneous spatiotemporal distribution of the virus in these mammal species in the study region. This spatial pattern is in line with the outbreaks of WNV reported in human and horses in Spain. Since the first case notified in 2010, a total of 131 human cases and 364 outbreaks in horse herds have been reported in Andalusia, with 98.4% detected in western region and 43.6% between 2020 and 2022 (RNVE 2023; SAS 2024; RASVE 2024). Interestingly, 18 of the 20 WNV-seropositive wild lagomorphs were sampled in September 2020 in a single hunting ground located in the municipality of Vejer de la Frontera (province of Cádiz; south-western Spain). It should be noted that August and September were the highest risk period for WNV outbreaks during the epidemic of 2020 and, notably, both outbreaks in horses and human cases were reported in this municipality (Gonzálvez et al. 2023).

Specific antibodies against USUV and/or BAG were not detected in the bELISA-positive lagomorphs. Nevertheless, exposure to more than one of these viruses cannot be ruled out in these animals, particularly in those two individuals which showed similar titres against both WNV and USUV. Our results denote that positivity found by the bELISA is essentially due to WNV exposure and not to these other flaviviruses in the wild lagomorphs analysed. This finding is in line with the absence of BAGV RNA or antibodies in any of the mammal species studied so far (Llorente et al. 2015; Magallanes et al. 2023). In contrast, anti-USUV antibodies have been detected in equids (Guerrero-Carvajal et al. 2021), red deer (*Cervus elaphus*) (García-Bocanegra et al. 2016) and zoo animals (Caballero-Gómez et al. 2020) from south-western Spain. Although active circulation of USUV has been evidenced in the study area (Bravo-Barriga et al. 2021, 2023; Figuerola et al. 2022), our results suggest a limited exposure to USUV of wild lagomorphs in southern Spain. Nevertheless, future studies are needed to assess the circulation of other flaviviruses in wild lagomorph populations from this region.

In conclusion, our findings confirm that the European wild rabbit and the Iberian hare populations are naturally exposed to WNV, increasing the range of susceptible species to this zoonotic virus. Even though these species do not appear to play a relevant

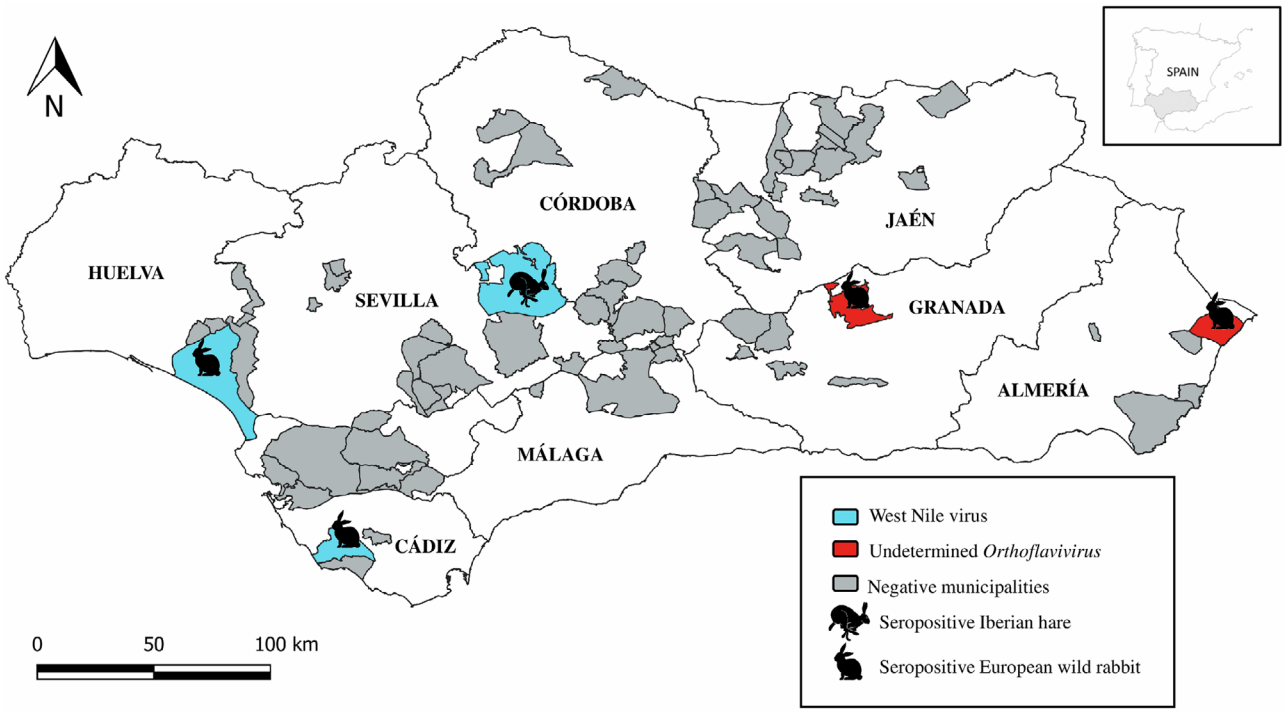

FIGURE 1 | Map of the autonomous region of Andalusia (southern Spain) showing the sampling areas and the geographical distribution of the samples from wild lagomorphs that tested positive for WNV and undetermined *Orthoflavivirus*.

role in the epidemiology of WNV, they could serve as sentinel species for monitoring this mosquito-borne virus. This study underscores the importance of continued surveillance efforts in wildlife populations to better understand the dynamics of flaviviruses transmission and inform public health interventions.

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Ethics Statement

This study did not involve purposeful killing of animals. All samples were collected from legally hunted animals during the hunting seasons or by passive surveillance under Spanish and Andalusian legislation. Consequently, ethical approval was not required.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request.

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