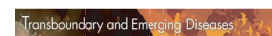


## ORIGINAL ARTICLE



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# The role of different *Culex* mosquito species in the transmission of West Nile virus and avian malaria parasites in Mediterranean areas

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## Abstract

Vector-borne diseases, especially those transmitted by mosquitoes, have severe impacts on public health and economy. West Nile virus (WNV) and avian malaria parasites of the genus *Plasmodium* are mosquito-borne pathogens that may produce severe disease and illness in humans and birds, respectively, and circulate in an endemic form in southern Europe. Here, we used field-collected data to identify the impact of *Culex pipiens*, *Cx. perexiguus* and *Cx. modestus*, on the circulation of both WNV and *Plasmodium* in Andalusia (SW Spain) using mathematical modelling of the basic reproduction number ( $R_0$ ). Models were calibrated with field-collected data on WNV seroprevalence and *Plasmodium* infection in wild house sparrows, presence of WNV and *Plasmodium* in mosquito pools, and mosquito blood-feeding patterns. This approach allowed us to determine the contribution of each vector species to pathogen amplification. Overall, 0.7% and 29.6% of house sparrows were positive to WNV antibodies and *Plasmodium* infection, respectively. In addition, the prevalence of *Plasmodium* was higher in *Cx. pipiens* (2.0%), followed by *Cx. perexiguus* (1.8%) and *Cx. modestus* (0.7%). Three pools of *Cx. perexiguus* were positive to WNV. Models identified *Cx. perexiguus* as the most important species contributing to the amplification of WNV in southern Spain. For *Plasmodium* models,  $R_0$  values were higher when *Cx. pipiens* was present in the population, either alone or in combination with the other mosquito species. These results suggest that the transmission of these vector-borne pathogens depends on different *Culex* species, and consequently, their transmission niches will present different spatial and temporal patterns. For WNV, targeted surveillance and control of

*Cx. perexiguus* populations appear as the most effective measure to reduce WNV amplification. Also, preventing *Culex* populations near human settlements, or reducing the abundance of these species, are potential strategies to reduce WNV spillover into human populations in southern Spain.

#### KEYWORDS

basic reproduction number  $R_0$ , *Culex modestus*, *Culex perexiguus*, *Culex pipiens*, emerging infectious diseases, flavivirus, Haemosporidia, mosquitoes, *Passer domesticus*, vector-borne pathogens, zoonosis

## 1 | INTRODUCTION

The number of infectious diseases has increased in recent decades due in part to factors such as habitat alteration, the introduction of alien species and climate change operating on a global scale (Patz, Graczyk, Geller, & Vittor, 2000). These factors can directly affect the incidence of vector-borne pathogens causing human diseases (Vorou, Papavassiliou, & Tsiodras, 2007). Indeed, diseases transmitted by arthropod vectors, such as mosquitoes, have had a severe impact, throughout history, on public health for human populations, livestock and wildlife and represent an important problem with significant wide-ranging economic and conservation implications (National Academies of Sciences, Engineering, and Medicine, 2016). Consequently, understanding the factors that affect the transmission of pathogens is of great importance for ecological, evolutionary, health and economic reasons. The transmission of vector-borne pathogens involves the interaction of these organisms with their insect vectors and vertebrate hosts. Here, we use epidemiological methods to identify the key vector species responsible for the transmission of two mosquito-borne pathogens – the re-emerging West Nile virus (WNV) and Haemosporidians of the genus *Plasmodium*.

West Nile virus is an emerging zoonotic arbovirus of the genus *Flavivirus* (family *Flaviviridae*) naturally circulating between birds and mosquitoes (Kilpatrick, LaDeau, & Marra, 2007). It is a generalist multi-host multi-vector pathogen with complex eco-epidemiology that can replicate in more than 300 species of birds (<https://www.cdc.gov/westnile/dead-birds/index.html>) and is vectored by a wide range of different species of mosquitoes (Engler et al., 2013). Occasionally, a WNV-infected mosquito bites humans or other mammals (e.g. horses), transmits the virus and can produce a WNV-associated disease. Most of the humans infected by the virus remain asymptomatic (>80% of the total of individuals infected) or present mild flu-like syndrome (Sambri et al., 2013). In only <1% of the cases, infected people develop severe illness including meningitis, encephalitis, acute flaccid paralysis and even death (Sambri et al., 2013). Likewise, most WNV infections are asymptomatic in horses, where the attack rate has been estimated to be below 10%, and the mortality rate can approach 50% in horses with clinical disease (Angenvoort, Brault, Bowen, & Groschup, 2013; Garcia-Bocanegra et al., 2011; Schuler, Khaitsa, Dyer, & Stoltenow, 2004). Naturally circulating in the old world, WNV was introduced to

North America in 1999 with dramatic consequences for humans and wildlife health (Kilpatrick et al., 2007). In Europe, several WNV outbreaks have occurred during the last decades where, in 2018, a significant increase in the incidence of the virus occurred compared to the previous years, resulting in 1,500 human WNV infections and 180 fatalities recorded in Europe (Zannoli & Sambri, 2019). In southern Spain, local circulation of WNV has been observed since at least 2004, as supported by virus isolation from mosquitoes (Engler et al., 2013; Vázquez et al., 2011), seroconversion in resident birds (Ferraguti, Martínez-de la Puente, et al., 2016; Figuerola, Soriguer, Rojo, Tejedor, & Jiménez-Clavero, 2007; Martínez-de la Puente et al., 2018) and outbreaks recorded in horses (García-Bocanegra et al., 2011, 2012). European birds do not usually show any clinical signs or mortality (Malkinson & Banet, 2002), and WNV infection occurs asymptomatically for 5–10 days post-infection (Pérez-Ramírez, Llorente, & Jiménez-Clavero, 2014). Given the short duration of the viraemia, the detection of exposure to WNV is normally based on the detection of antibodies (Pérez-Ramírez et al., 2014).

On the other hand, avian Haemosporidians (Sporozoa: Haemosporidia) are widespread mosquito-borne parasites infecting birds (Valkiūnas, 2005) commonly used as models for the study of the ecology of vector-borne diseases (Fecchio, Chagas, Bell, & Kirchgatter, 2020; Rivero & Gandon, 2018). Most evidence suggests that species of avian *Plasmodium* are generalist parasites transmitted by mosquitoes of different genera (Ferraguti et al., 2013; Kimura, Darbro, & Harrington, 2010) that produce chronic infections with deleterious effects on their vertebrate hosts (Asghar et al., 2015; Marzal et al., 2011; Merino, Moreno, Sanz, & Arriero, 2000) being identified as potential drivers of some avian population declines (Atkinson, Woods, Dusek, Sileo, & Iko, 1995; Van Riper, Van Riper, Goff, & Laird, 1986).

The transmission of mosquito-borne pathogens, including WNV and avian malaria parasites, is commonly driven by the abundance and community composition of insect vectors in the area (Ferraguti et al., 2018; Martínez-de la Puente et al., 2018). With over 3,500 species worldwide, mosquitoes are by far the most common arthropod vectors of pathogens (Tolle, 2009). Factors including the vectorial competence of the mosquito species in the area and the contact rates between reservoirs and susceptible hosts may determine the success of pathogen transmission in a given population. It is important to note that mosquito species vary in their vector competence for the transmission of a

particular pathogen (Beerntsen, James, & Christensen, 2000), and thus, different species may play different roles in the transmission cycles of each pathogen studied (Gutiérrez-López, Martínez-de la Puente, Gangoso, Soriguer, & Figuerola, 2020; Martínez-de la Puente, Martínez, Rivero-de Aguilar, Herrero, & Merino, 2011; Santiago-Alarcon, Palinauskas, & Schaefer, 2012).

West Nile virus and avian *Plasmodium* are both multi-vector mosquito-borne pathogens, with a wide diversity of mosquito species being involved in their transmission where large evidence supports a key role of *Culex* mosquitoes being involved in their transmission (Becker et al., 2010; Engler et al., 2013; Valkiūnas, 2005; Vogels, Göertz, Pijlman, & Koenraadt, 2017). Additional to the capacity of the pathogens to replicate in the mosquitoes, mosquito feeding preferences may play a key role modulating the amplification and transmission success of vector-borne pathogens (Kilpatrick, Daszak, Jones, Marra, & Kramer, 2006; Muñoz et al., 2012), with some mosquito species being mainly involved in the transmission between reservoir vertebrate hosts (i.e. birds), while others act as bridge vectors between infected birds and humans or horses (Kilpatrick et al., 2005). For instance, the diet of *Cx. pipiens* in Europe includes mammals, birds and reptiles (Brugman et al., 2018), with birds and humans representing 69%–97% and 0%–18% of the blood meals, respectively (Gómez-Díaz & Figuerola, 2010). These differential blood-feeding patterns will determine the contact rates between mosquitoes and infected/susceptible individuals and hence affect the risk of pathogen transmission (Kilpatrick et al., 2006; Muñoz et al., 2012).

Quantitative and modelling approaches are essential tools to understand the transmission dynamics of pathogens and to identify and evaluate prevention and control measures (Huppert & Katriel, 2013). Models on WNV transmission involving mosquito and bird populations, or humans, vector and host populations, have yielded valuable insights into WNV epidemiology (Bowman, Gumel, Van den Driessche, Wu, & Zhu, 2005). For example, the occurrence of WNV outbreaks may be decreased by mosquito control strategies, while the control of avian populations may increase WNV risk to humans (Wonham, de-Camino-Beck, & Lewis, 2004). The basic reproduction number ( $R_0$ ) is a measure used in epidemiology to quantify outbreak potential. It can be calculated for models with multiple host species and multiple vector species that can differ in their contributions to transmission potential (Diekmann, Heesterbeek, & Roberts, 2009). We use an epidemiological method (Funk, Nishiura, Heesterbeek, Edmunds, & Checchi, 2013; Nishiura, Hoyer, Klaasen, Bauer, & Heesterbeek, 2009) to estimate the relative contributions of different mosquito species to  $R_0$  of WNV and avian *Plasmodium* in an area with active circulation. In particular, we considered the species *Cx. pipiens*, *Cx. modestus* and *Cx. perexiguus*, which may play an important role in the transmission of avian *Plasmodium* (Gutiérrez-López et al., 2020; Santiago-Alarcon et al., 2012; Ventim et al., 2012) and WNV (Engler et al., 2013) in Mediterranean areas. In addition, experimental infections support the competence of these mosquitoes for WNV (Balenghien et al., 2008; Jupp & McIntosh, 1970). Wild house sparrows (*Passer domesticus*) were used as the focal bird species as they are common hosts for *Culex* mosquitoes (Rizzoli et al., 2015),

are competent for WNV (del Amo et al., 2014) and represent a WNV-host reservoir of major importance in the wild (Komar et al., 2001). In addition, house sparrows usually show a high prevalence of infection by avian malaria parasites worldwide (Ferraguti et al., 2018; Marzal et al., 2011), with these parasites likely contributing to the current population decline of this species (Dadam et al., 2019).

## 2 | MATERIALS AND METHODS

### 2.1 | Study area and sampling collection

This study was conducted in southern Spain, an area characterized by a typical Mediterranean climate with a long dry summer season. During 2013, the mean annual temperature varies between 17 and 21°C, with an annual average rainfall ranging between 500 and 700 L/m<sup>2</sup>, where most of the precipitation occurs in winter. Sampling sites included 49 areas comprising four sampling sites belonging to a constant monitoring programme of the mosquito control service of the Huelva province, and 45 localities at Cadiz, Huelva and Seville provinces (Andalusia; southern Spain) sampled in the context of the study by Ferraguti, Martínez-de La Puente, et al. (2016, see for further detail on the study area characteristics).

From January to December 2013, mosquitoes were trapped using different methods including BG-sentinel traps baited with dry ice as a source of CO<sub>2</sub>, Centers for Disease Control and Prevention (CDC) incandescent light traps also baited with dry ice and a CDC backpack aspirator (model 2,846). For further details of mosquito sampling, identification and quantification procedures (see Ferraguti, Martínez-de La Puente, et al., 2016). House sparrows were sampled using mist-nets at the same 45 localities during capture sessions in July–October, after the bird breeding season, to maximize the capture of juveniles and to better reflect pathogen circulation during the season from hatching until capture. For further details of the bird captures and blood sampling (see Ferraguti et al., 2018).

### 2.2 | Mosquito blood meal identification

Genomic DNA was extracted from the abdomen of the engorged mosquitoes using the DNA Kit Maxwell<sup>®</sup> 16LEV kit (Gutiérrez-López, Martínez-de la Puente, Gangoso, Soriguer, & Figuerola, 2015). The vertebrate origin of the blood meals was assessed following Alcaide et al. (2009) to amplify and sequence a fragment of the vertebrate mitochondrial cytochrome c oxidase 1 gene. Amplicons were sequenced in the MacroGen sequencing service (MacroGen Inc.). Labelled DNA fragments of PCR-positive products were resolved through an ABI 3130xl automated sequencer (Applied Biosystems). Sequences were edited using the software Sequencher<sup>™</sup> v4.9 (Gene Codes Corp, © 1991–2009). Hosts were assigned to vertebrate species when agreement was ≥98% to sequences of known species in GenBank DNA sequence database (National Center for Biotechnology Information Blast)

or the Barcode of Life Data Systems. Information on the feeding patterns of the *Cx. pipiens* mosquitoes included in this study was previously published in Martínez-de la Puente et al. (2016).

### 2.3 | WNV and avian *Plasmodium* detection in mosquitoes and in birds

Unfed mosquito females were grouped in pools generally containing from 1 to 50 females from the same species, sampling site and date of collection. Afterwards, pools were homogenized in a range of 500–700  $\mu$ l of minimal essential medium (MEM solution), supplemented with 200 U/ml of antimicrobial drugs (penicillin/streptomycin) and 10% of foetal bovine serum. The genetic material was extracted from pools of the species *Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus* using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's recommendations and, subsequently, screened for the detection of WNV (with a protocol modified from Sánchez-Seco et al., 2005) and avian *Plasmodium* (following Hellgren, Bensch, & Malmqvist, 2008). Positive pools for flavivirus screening were analysed with the RT-nested PCR method described by Vázquez et al. (2012). A total fragment of 1,000 nt amplified in the NS5 gene was sequenced to characterize the detected flavivirus. The prevalence of WNV and *Plasmodium* in mosquitoes was calculated using *EpiTools* software (Sergeant, 2019) available from AusVet Animal Health Services, <http://epitools.ausvet.com.au/>. This algorithm estimates the prevalence of infection and confidence limits from pooled samples taking account of differences in pool size and assuming 100% sensitivity and specificity (Sacks, Bolin, & Crowder, 1989).

The analysis of WNV antibodies in the serum of house sparrows was done in two steps. First, we used the epitope-blocking ELISA Kit INgezim West Nile Compact (INGENASA). Subsequently, samples that gave positive or doubtful results in the ELISA were tested for the presence of WNV antibodies using the micro-virus-neutralization test (micro-VNT) against WNV strain Eg-101 (GenBank accession n° AF260968). The specificity of the antibodies towards WNV was confirmed by testing in parallel the neutralization against Usutu virus (USUV) strain SAAR-1776 (GenBank accession n° AY453412). This was done because USUV is a mosquito-borne flavivirus circulating in the area and WNV can present cross-reaction with Usutu antibodies (Llorente et al., 2019). For further details on the detection of WNV infections in house sparrows from the studied area (see Martínez-de la Puente et al., 2018). In addition, genomic DNA was extracted from the bird blood cellular fractions and used to molecularly determine the bird's infection status for *Plasmodium* (Hellgren, Waldenström, & Bensch, 2004; see also Ferraguti et al., 2018).

### 2.4 | The $R_0$ calculation

The  $R_0$  was calculated based on the approach described by Funk et al. (2013) adapted from Nishiura et al. (2009). The analysis assumes

that the system has been observed in an equilibrium state, allowing us to calculate the forces of infection in all species from measured prevalence. The  $R_0$  is the average number of new cases of infection caused by one infected individual when introduced into a population of susceptible hosts. If  $R_0$  lies below 1, the number of cases will decline and eventually be eliminated, and if  $R_0$  is above 1, the number of cases will increase at least initially and cause an outbreak (and the infection can become endemic, which is what we assume in our analysis). We started by defining and analysing separately a system for the spread of WNV and *Plasmodium* in the resident and common host species (the house sparrow) and one mosquito species. We then extended this to include up to the three target *Culex* species and all the pairwise combinations of them. We compared  $R_0$  for all systems and analysed the relative importance of each vector species for WNV and *Plasmodium* spread. We assumed pure host-vector transmission and therefore ignore any direct transmission from vector to vector and from host to host. For more detail on the mathematical model calculation (see Appendix S1).

### 2.5 | Applied models for data analysis

Values estimated from field data included in the mathematical models are shown in Table 1. First,  $R_0$  was calculated using fixed values for  $f_{hv}$ ,  $i_v$ ,  $n_v$  and  $i_h$  measured from field-collected data, and  $\tau_v$  estimated from Pawelek, Niehaus, Salmeron, Hager, and Hunt (2014). Second, a sensitivity analysis was performed by using first 100, and then 1,000 random values from  $\beta$ -distributions around the mean value of the field data of each of these parameters ( $\alpha$  = field-collected data,  $\beta$  = 100 – field-collected data). In all calculations, the parameters were varied randomly in this way one at a time, while the other parameters were maintained as mean values. Finally, one set of simulations was performed with all the parameters randomized, following a  $\beta$ -distribution, as described above. The  $\beta$ -distribution allowed to maintain random values within the 0–1 range in contrast to using normal distributions. Simulations were performed using 100 and 1,000 randomized values to ensure the consistency of the method. For comparison, all statistical analyses were repeated using also a normal ( $SD$  =  $mean/3$ ) instead of a  $\beta$ -distribution and the conclusions did not change (results not shown). Independent analyses were performed for each habitat category and pathogen by using both specific and randomly simulated values for  $i_v$ ,  $n_v$  and  $i_h$  (see Table S1 for vector information, specific  $i_h$  for each habitat are detailed below,  $f_{hv}$  was a constant parameter in each model). In urban areas, WNV seroprevalence was zero in all cases (Table S2).

Finally, for the case of WNV, due to the absence of positive pools for *Cx. pipiens* and *Cx. modestus*, three different scenarios were simulated with different sets of simulations for *Culex* mosquitoes  $i_v$  based on a hypothetical *Cx. pipiens* and *Cx. modestus* WNV infection prevalence measured as 1/10 (high), 1/100 (medium) and 1/1,000 (low) of the observed prevalence of *Cx. perexiguus*. These scenarios allowed to compare the role of the former two *Culex* species in the transmission of WNV.

**TABLE 1** Parameter estimates of the three *Culex* mosquito species based on the field-collected data. Information of the biting rate of mosquitoes per day ( $\tau_v$ ) was obtained from Pawelek et al. (2014)

Variable	Definition	<i>Cx. pipiens</i>	<i>Cx. perexiguus</i>	<i>Cx. modestus</i>
$N_{\text{tot}}$	Number of mosquito females captured	21,487	4,079	1,100
$N_v$	Number of mosquito pools analysed	1,101	252	138
$n_v$	Fraction of all vectors that are of species $v$	0.729	0.225	0.047
$\tau_v$	Mosquito biting rate per day of vector species $v$	0.333	0.333	0.333
$i_{v\_wnv}^*$	Endemic equilibrium prevalence of WNV infection in vector species $v$	0.000	0.001	0.000
$i_{v\_Plasm}^*$	Overall prevalence of <i>Plasmodium</i> infection in vector species $v$	0.020	0.018	0.007
$f_{hv}$	Fraction of mosquito bites by vector species $v$ that is on house sparrows	0.124	0.015	0.006

Abbreviation: WNV, West Nile virus.

\*Endemic Equilibrium Status.

### 3 | RESULTS

A total of 26,666 mosquito females belonging to *Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus* were collected and grouped in 1,491 pools (Table 1). WNV was identified in three pools of *Cx. perexiguus* (two pools collected in August and one in September) from one natural (two positive pools) and one rural area (one positive pool) from the Seville province. Also, 382 (25.62%) pools of unfed mosquitoes were positive for the presence of *Plasmodium* parasites, with a higher prevalence of infection found in *Cx. pipiens*, followed by *Cx. perexiguus* and *Cx. modestus* (Table 2). One mosquito pool of three that were positive for WNV harboured *Plasmodium* parasites. The vertebrate hosts of mosquitoes were identified for 194 *Cx. pipiens*, 34 *Cx. perexiguus* and two *Cx. modestus*. For the case of *Plasmodium* parasites in birds, 765 out of 2,588 individuals analysed were infected ( $i_{h\_Plasm}^* = 29.6\%$ ). The infection prevalence varied, although not significantly, between habitat categories ( $i_{h\_Plasm\_Natural}^* = 29.1\%$ ,  $i_{h\_Plasm\_Rural}^* = 34.2\%$ ,  $i_{h\_Plasm\_Urban}^* = 25.7\%$ ). Unfortunately, not enough serum was obtained for 44 out of the 2,588 birds sampled, so these individuals were excluded from WNV analyses. Here, 17 out of the 2,544 individuals were confirmed for WNV-specific antibodies using VNT ( $i_{h\_wnv}^* = 0.007$ ); these birds were captured in natural and rural areas from Huelva province. Overall, the infection prevalence varied, although not significantly, between habitat categories ( $i_{h\_wnv\_Natural}^* = 0.009$ ,  $i_{h\_wnv\_Rural}^* = 0.012$ ) and no differences were found in any of the simulations (Tables S2 and S3). Usutu-specific antibodies were detected in only 0.04% of birds, and we did not consider this pathogen in further analyses. Six birds of 17 that were positive for WNV antibodies had *Plasmodium* infections.

The  $R_0$  calculations for WNV based on fixed values for the parameters estimated from field data support that the higher  $R_0$  values occur when *Cx. perexiguus* is present in the system, either alone or in combination with the other *Culex* species (Figure 1). Similar conclusions were obtained when values for all parameters were randomized, either varying them one at a time (Tables S3–S6) or

simultaneously (Tables S7). These results did not depend on the number of simulations done in each calculation (100 or 1,000). For the case of *Plasmodium*, the higher  $R_0$  values were obtained when *Cx. pipiens* was included in the system, either alone or in combination with one or both the other mosquito species (Table 3). As in the case of WNV, conclusions were maintained when using simulations with randomized values of other parameters of equation 10, either varying them one at a time (Tables S8–S11) or simultaneously (Figure 2; Table S12).

The three scenarios simulating different WNV prevalence in *Cx. pipiens* and *Cx. modestus* mosquitoes showed different outcomes depending on the estimated prevalence of WNV infection (Figure 3a–c). However,  $R_0 > 1$  was only found in presence of both *Cx. pipiens* and *Cx. perexiguus* and in presence of the three mosquito species. The relative contribution of *Cx. pipiens* to the transmission of WNV decreased as the estimated prevalence of WNV in this species decreased, while the relevance of *Cx. perexiguus* increased. In all three scenarios, *Cx. modestus* was, by far, the least relevant mosquito species for the maintenance of WNV circulation in the area.

Finally, no differences between habitat categories were found in any of the calculations, neither with fixed values for both pathogens (Table S2) nor with randomized parameters in each of the natural, rural or urban habitats (Tables S13–S14). The results were consistent with models based on field-data calculation in all three habitat categories for both WNV and *Plasmodium*.

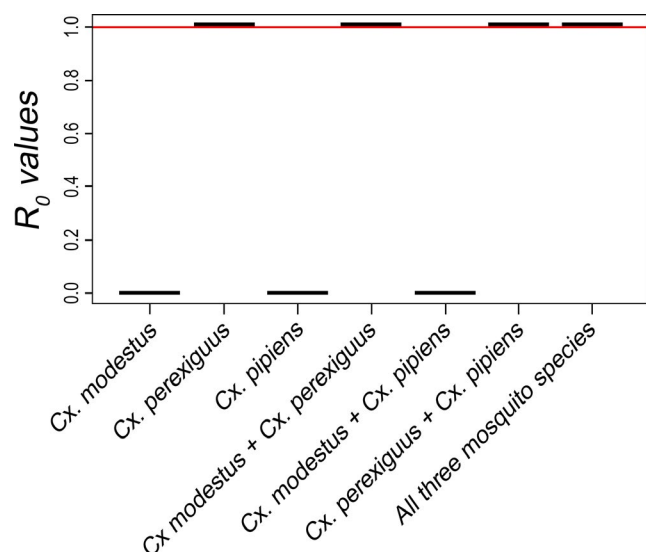
### 4 | DISCUSSION

Most of the applied and theoretical studies of pathogen dynamics derived from model pathogens that affect a single or a limited number of species, a fact that may simplify and facilitate their study. However, many vector-borne zoonotic pathogens are generalists frequently interacting with a wide range of hosts and vector species. Unfortunately, current knowledge of the transmission dynamics of



**TABLE 2** Overall avian malaria parasite prevalence in unfed mosquitoes. For each mosquito species, the total number of females, pools, positive infected samples, prevalence and confidence limits are shown

Mosquito species	No. of females	No. of Pools	Positive pools	Prevalence	Lower 95% CL	Upper 95% CL
<i>Cx. pipiens</i>	21,487	1,101	318	0.020	0.018	0.023
<i>Cx. perexiguus</i>	4,079	252	57	0.018	0.014	0.003
<i>Cx. modestus</i>	1,100	138	7	0.007	0.023	0.013



**FIGURE 1**  $R_0$  results from West Nile virus model based on  $f_{hv}$ ,  $i_v$ ,  $n_v$ ,  $i_h$  and  $\tau_v$  parameters estimated from field data (Table 1). The red line indicates  $R_0 = 1$

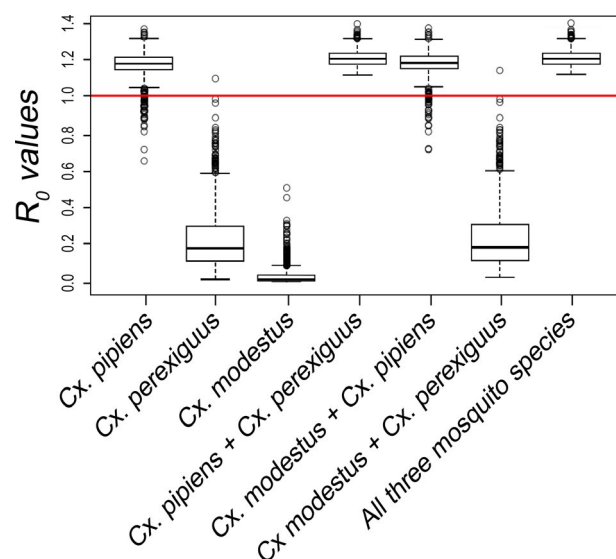
**TABLE 3**  $R_0$  results from *Plasmodium* model based on  $f_{hv}$ ,  $i_v$ ,  $n_v$ ,  $i_h$  and  $\tau_v$  parameters estimated from field data (Table 1)

Mosquito species involved	$R_0$
<i>Cx. pipiens</i>	1.181
<i>Cx. perexiguus</i>	0.216
<i>Cx. modestus</i>	0.038
<i>Cx. pipiens</i> , <i>Cx. perexiguus</i>	1.201
<i>Cx. pipiens</i> , <i>Cx. modestus</i>	1.182
<i>Cx. perexiguus</i> , <i>Cx. modestus</i>	0.219
<i>Cx. pipiens</i> , <i>Cx. perexiguus</i> , <i>Cx. modestus</i>	1.201

Note: In both cases (100 or 1,000 repetition), the results are qualitatively the same.

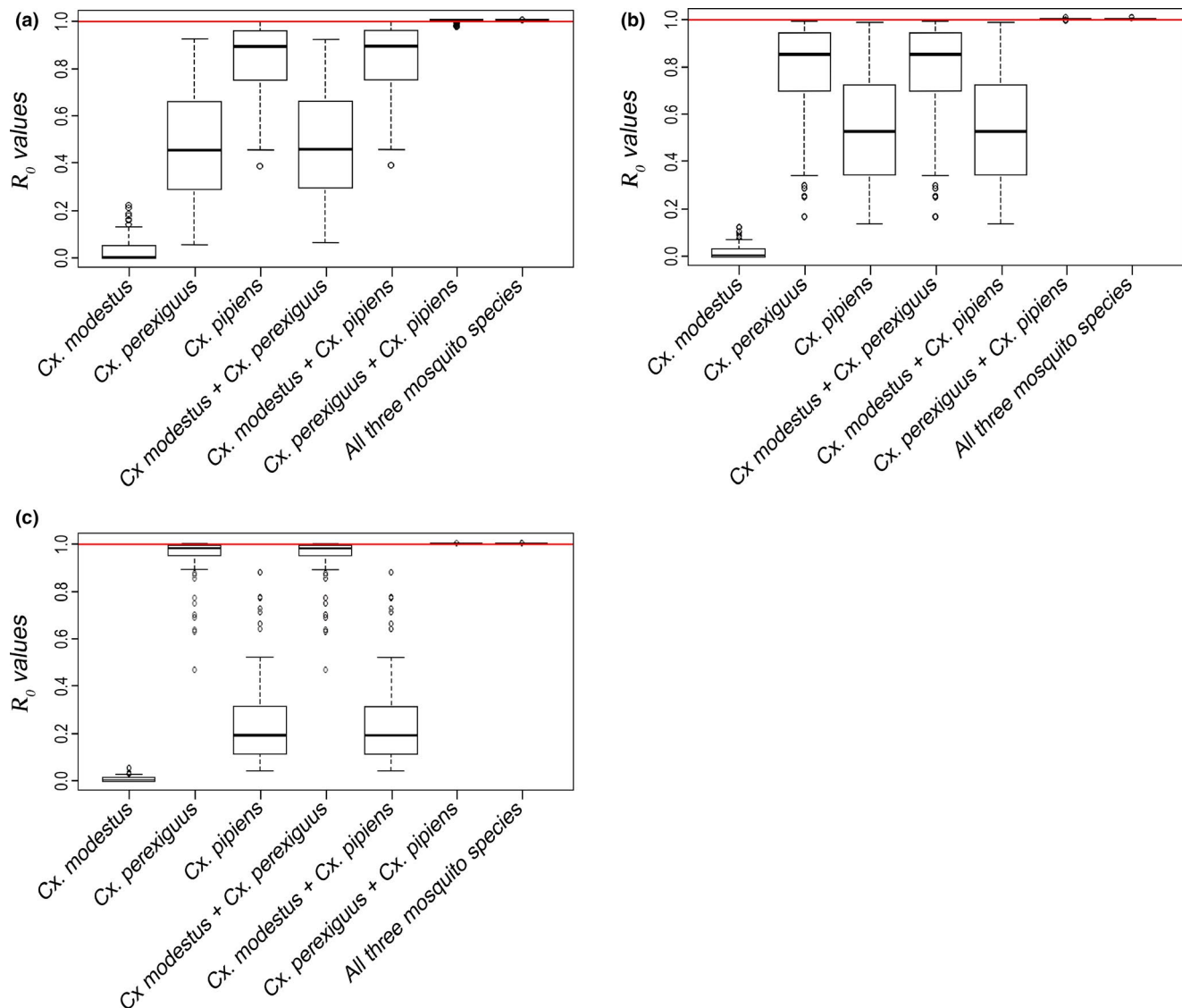
pathogens transmitted simultaneously by several vector species is scarce and the study on the potential role of the vector community on pathogen amplification has rarely been explored. Here, we used mathematical modelling to compare the role of different vector species in the spread of two mosquito-borne pathogens showing contrasting results. One of the main conclusions is that while *Cx. perexiguus* may play a key role in the transmission of WNV, the transmission of avian *Plasmodium* may be mostly influenced by *Cx. pipiens*.

Our  $R_0$  calculations further supported that *Cx. pipiens* and *Cx. modestus* play a secondary role in the amplification of WNV



**FIGURE 2**  $R_0$  results from *Plasmodium* model based on  $f_{hv}$ ,  $i_v$ ,  $n_v$ ,  $i_h$  and  $\tau_v$  random parameters with a  $\beta$ -distribution and 1,000 replicates. The red line indicates  $R_0 = 1$

in southern Spain. However, due to its host feeding patterns and habitat selection *Cx. pipiens* may be important for the transmission of the virus to humans. WNV-positive pools were already detected in *Cx. pipiens* in 2006 and *Cx. perexiguus* in 2008 (Vázquez et al., 2011). Previously, Muñoz et al. (2012) suggested that *Cx. perexiguus* was the most important mosquito species for WNV amplification in the area based on its abundance, vector competence and the proportion of blood meals with an avian origin. Martínez-de la Puente et al. (2018) already reported a positive relationship between the prevalence of WNV infection in house sparrows and the abundance of *Cx. perexiguus*. This species is commonly found during July and August in natural areas such as rice fields, scrubland and sand dunes (Ferraguti, Martínez-de La Puente, et al., 2016) where the density of human populations is negligible (Martínez-de la Puente et al., 2018). This may explain the high incidence of WNV in birds, the relative high incidence in horses, but the very small number of cases reported in humans in the study area. Finally, our results strongly support the need to use VNTs to confirm WNV in all positive and doubtful samples detected by ELISA kits to increase the accuracy of estimates of pathogen seroprevalence in wild birds. USUV was also tested in bird blood samples that gave positive or doubtful results in ELISA test, because this flavivirus circulates between birds and mosquitoes in the area and can be detected by this technique. Thus, it was



**FIGURE 3** Results from the three sets of West Nile virus (WNV) simulations, with 100 randomized values for *Culex* mosquito's  $i_v$  assuming infection prevalence for *Cx. pipiens* and *Cx. modestus* of 1/10 (a), 1/100 (b) and 1/1,000 (c) of the observed WNV prevalence in *Cx. perexiguus*. The red line indicates  $R_0 = 1$

necessary to compare the VNT titres obtained when testing the two flaviviruses in parallel to prevent overestimate the presence of WNV antibodies (Beck et al., 2013).

According to the  $R_0$  models, *Cx. pipiens* is the main vector of avian *Plasmodium* in southern Spain, with the other two mosquito species playing less important roles. Different factors may explain the different importance of these mosquito species for the pathogens studied, including their differential capacities for the transmission of this parasite genus. Unfortunately, limited information is available on the vectorial competence of these mosquito species for avian *Plasmodium*, but molecular xenomonitoring, defined as the molecular screening of pathogens in wild mosquitoes, has identified *Culex* as key vectors of avian *Plasmodium* (Ferraguti et al., 2013; Santiago-Alarcon et al., 2012; Valkiūnas, 2005). Among them, *Cx. pipiens* has repeatedly been incriminated in the transmission of avian *Plasmodium* in different

European countries (Brugman et al., 2018) and different experimental studies support its competence for the transmission of different avian *Plasmodium* lineages, including those infecting house sparrows in the area (Gutiérrez-López et al., 2019, 2020; Valkiūnas et al., 2015). Contrary to the case of *Cx. pipiens*, little is known on the potential role of *Cx. modestus* and *Cx. perexiguus* in the transmission of avian malaria parasites. In Portugal, one out of 11 pools of *Cx. perexiguus* was positive for the presence of avian *Plasmodium* (Ventim et al., 2012). In a single locality from southern Spain, a lower prevalence of infection was found in *Cx. perexiguus* and *Cx. modestus* than in *Cx. pipiens* when mosquito females in pools were molecularly tested for the presence of these parasites (Ferraguti et al., 2013). However, it is important to highlight that molecular detection of parasite DNA in mosquito pools does not necessarily imply ability of parasite transmission (Valkiūnas, 2011).

#### 4.1 | Limitation of the epidemiological model

One of the goals of this study was to identify the main vectors involved in the transmission of two different avian pathogens and their relative importance for this transmission. We have used information on vector abundance, host feeding patterns and pathogen prevalence for three different mosquito species, but the information on pathogen incidence on the vertebrate host was restricted to a single host species. Our goal was not to provide an absolute estimate of  $R_0$  for these two pathogens which require to consider a much broader range of species. Although the approach we have used can be generalized to multiple host species and additional vector species (see Funk et al., 2013; Nishiura et al., 2009), this requires information on the prevalence of infection for a broader range of host and vector species that is extremely difficult reliably obtain in the wide range of localities studied. Our approach allowed us to use a simplified system consisting of three vector species and a single host species. We assume that the biting frequencies of the three mosquito species on the abundant house sparrow are typical for their feeding patterns in relation to its abundance and that this pattern extends to other hosts (Molaei, Andreadis, Armstrong, Anderson, & Vossbrinck, 2006; Rizzoli et al., 2015). In other words, any other host species that the vector species use for feeding are assumed to be bitten in the same relative frequencies. In that case, the biting rate on house sparrows can be seen as representative for the biting rate by the three mosquito species and, therefore, as representative for assessing the contribution of each vector relative to the others to the overall WNV transmission. Thus, our results represent the contribution of house sparrows to pathogen amplification, and consequently, the values of  $R_0$  should not be interpreted as absolute estimates but only as a useful tool to compare the contribution of the different mosquito species. In addition, coinfections by different pathogens are commonly found in wild birds, including those composed by WNV and avian *Plasmodium* which could interfere with each other. Although the mechanisms explaining these associations are unknown, a previous study by Medeiros et al. (2014) reported a negative association between *Plasmodium* infections and the presence of WNV antibodies in birds. Here, the proportion of *Plasmodium* infection was similar to that found in house sparrows without WNV antibodies ( $\chi^2 = 0.27$ , 1 df,  $p = .60$ ), and thus, no significant association was found between WNV seroprevalence and *Plasmodium* infection in our study.

Furthermore, several assumptions were made in this study that may affect the  $R_0$  estimates. For instance, we assumed the vectorial transmission of WNV, but transmission from bird to bird may occur through consumption of infected bird preys or contaminated water (Banet-Noach, Simanov, & Malkinson, 2003) and in confined laboratory experiments (Komar et al., 2001; U.S. Geological Survey, 2000). However, these effects are likely to be limited in the case of house sparrows, which are the focus of our study, as this is a granivorous species that may feed on insects but not on other birds. Also, the approaches used here hinge on the assumption of the endemic equilibrium and ignoring spatial heterogeneity in environmental

conditions. The endemic equilibrium allowed us to estimate the force of infection from observed seroprevalence (WNV), prevalence (*Plasmodium*) and abundance of both mosquitoes and house sparrows. This is a strong assumption because under natural conditions these parameters may show a temporal variation. The spatial heterogeneity will lead to spatial variation in conditions and hence in local estimates of outbreak potential. For this reason, we performed several sensitivity analyses using simulations with randomized parameters, which addressed the variability in estimates and showed consistency with our conclusions. These simulations also allowed us to identify potential differences due to the low prevalence of WNV found in mosquitoes.

In summary, based on the analyses reported here, our results suggest that WNV and *Plasmodium* are mainly transmitted by different mosquito species. We suggest that *Cx. perexiguus* may play a central role in the transmission of WNV in southern Spain, while for avian malaria parasites *Cx. pipiens* may be considered the main vector species. These dissimilarities between the niche of transmission of both pathogens in relation to the mosquito vector species highlight the importance of explicitly consider field-collected data on the vector community composition in studies on pathogen transmission. In addition, the role of mosquito species in the transmission of these pathogens may differ between areas, considering the vertebrate and vector communities and the environmental characteristics where these organisms interact. In addition, the estimated  $R_0$  in this study reached values slightly above 1, suggesting that the WNV should be controllable in the area with control programmes focus on *Cx. perexiguus*. Interventions on the populations of this mosquito species near human inhabited areas should be a priority to reduce the risk of WNV spillover into people. In addition to reducing mosquito populations,  $R_0$  may drop by increasing the proportion of blood meals on non-competent hosts such as wild and domestic mammals. However, it is still unclear how increasing vertebrate density may affect vector population abundance (Randolph & Dobson, 2012), and no information is yet available on how the densities of preferred and non-preferred vertebrate hosts determine host selection by the different species of mosquitoes.

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## CONFLICT OF INTEREST

The authors of this manuscript declare that there are no conflict of interests.

## AUTHOR CONTRIBUTION

MF, HH, JMP, MÁJ-C, RS and JF participated in the design of the research; MF, JMP, FL, AV, JF, DR and SR collected field samples and performed the experiments; MF, HH and HV carried out the statistical analyses; MF, JMP, MÁJ-C, FL and AV carried out the molecular and serological laboratory work; and RS, MÁJ-C and JF contributed to the reagents/materials/analysis tools. MF wrote the first draft of the manuscript under the supervision of HH, JMP and JF. All authors read, contributed and approved the final version of the manuscript.

## ETHICAL STATEMENT

Mosquito trapping and bird sampling were carried out with all the necessary permits issued by the regional Department of the Environment (Consejería de Medio Ambiente, Junta de Andalucía) and in accordance with relevant guidelines and regulations for animal research. Procedures were approved by the Ethical Committee of CSIC (resolution from 9th March 2012) and complied with the current Spanish laws. Surveys and sampling on private lands and in private residential areas were conducted with all the necessary permits and oral consent, and in the presence of owners. This study did not affect any endangered species.

## DATA AVAILABILITY STATEMENT

Data have not been archived because this article does not contain new data.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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