The mosquito fauna of the western region of Spain with emphasis on ecological factors and the characterization of *Culex pipiens* forms

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ABSTRACT: This study updates the diversity, distribution, and seasonal trends of mosquitoes in a western region of Spain, assesses ecological determinants of *Culex pipiens* s.l., and determines form composition of *Cx. pipiens* s.s. populations. A total of 1,495 mosquitoes of 16 species was collected during 2012-2013, of which *Cx. pipiens* s.l. and *Cx. theileri* were the most abundant. Five new records for *An. maculipennis* s.s., *Orthopodomyia pulcripalpis*, *Aedes (Ochlerotatus) punctor*, *Cx. europaeus*, and *Cx. modestus* were found for this region. *Cx. pipiens* density varied across weather and habitat patterns, correlating positively with high temperatures and with a preference for urbanized areas and rural areas within a proximity of ovine farms. Moreover, molecular identification by CQ11FL was performed in 467 *Cx. pipiens* s.s., detecting both pipiens (66%) and molestus (8.4%) forms coexisting in different habitats (urban, peri-urban and rural) aboveground with a high degree of hybridization (25.7%). The abundance of *Cx. pipiens* in urban areas and farms, with the presence of hybrids, may increase their capacity to act as bridge vectors for the transmission of arboviral infections. These data will be helpful for further implementation of entomological programs focused on risk assessment for arboviruses or other mosquito-borne pathogens. ***Journal of Vector Ecology* 42 (1): 136-147. 2017.**

Keyword Index: *Culex pipiens*, molestus form, hybrids, CQ11FL, ecological factor, Spain.

INTRODUCTION

In Spain, a total of 64 mosquito species from seven different genera have been described (*Aedes*: 27 species; *Anopheles*: 14; *Culex*: 13; *Culiseta*: 6; *Coquillettidia*: 2; *Orthopodomyia*: 1, and *Uranotaenia*: 1) (Bueno-Marí et al. 2012), including *Aedes, Anopheles,* and *Culex,* the main vectors of parasites in Spain. The *Culex pipiens* complex is a monophyletic mosquito group with several species, subspecies, and infraspecific forms with similar morphological features (Harbach 2011, Vinogradova 2000). The main member is the northern house mosquito *Cx. pipiens* s.s., which has a worldwide distribution and is also considered the main vector of arboviruses such as West Nile virus (WNV) in Europe and the U.S.A., Usutu virus (USUV) in Europe, and Rift Valley Fever Virus (RVFV) in Egypt. This mosquito also plays an important role in the transmission of parasites such as *Dirofilaria immitis* (Capelli et al. 2013).

Due to the importance of *Cx. pipiens* in Europe, knowledge of the influence of climatic and ecological factors for its abundance is crucial for an efficient application of control methods based on the areas where they are more abundant and with the highest risk of vector-borne diseases (Pemola Devi and Jauhari 2007). A change in temperature may increase the potential transmission of WNV because it is related to the life cycle dynamics of both vector species and pathogenic organisms (Vogels et al. 2016). WNV and USUV have been detected in *Cx. pipiens* in the Spanish areas of Catalonia and Huelva, respectively (Busquets et al. 2008, Vázquez et al. 2010). In recent years, the virus has been detected in Extremadura and border areas in horses. As Extremadura is classified as an "Important Bird Area of Spain," this region is an important stop for large flocks of migratory birds to Europe, especially during the transitional seasons. Since wild birds are the reservoir of WNV, this geographical location is crucial for disease transfer.

The wide distribution and vector status of *Cx. pipiens* s.l. is essential to understanding the relationships between different habitats or ecological factors that influence its distribution and abundance. Many studies in other countries have studied the effect of climatic variables on WNV cases, mosquito infection rate, and vector abundance (reviewed by Roiz et al. 2014). However, entomological studies of *Cx. pipiens* s.l. in Spain have focused on arbovirus surveillance and host preferences (Busquets et al. 2008, Vázquez et al. 2010, Muñoz et al. 2012), and only studies in coastal areas (Bueno Marí and Jiménez-Peydró 2011, Roiz et al. 2014, Ferraguti et al. 2016) have contributed to the knowledge of the possible factors for the presence of *Cx. pipiens* s.l., disregarding its behavior in inland Spain. These results can be variable, since the coastal areas are regions where human settlements tend to accumulate most, while inland areas have higher mosquito biodiversity and a regional analysis is necessary.

Culex pipiens s.s. has two distinct biological forms, denoted as pipiens form and molestus form (Harbach et al. 1984, 1985). These biological forms are morphologically indistinguishable but

present important ecological and physiological particularities. The pipiens form is characterized as being anautogenous, eurygamous, heterodynamic, and preferably ornithophilic, while the molestus form is autogenous, stenogamous, homodynamic, and preferably mammophilic (Harbach et al. 1984, 1985). In southern Europe, these two forms have a sympatric distribution and hybridization may occur in above-ground habitats (Gomes et al. 2009, Di Luca et al. 2016, Zittra et al. 2016). Traditionally, pipiens and molestus forms showed allopatric distributions (above-ground vs underground) in northern latitudes of Europe and the U.S.A. (Huang et al. 2008). However, recent studies in The Netherlands and Germany found both forms and hybrids in the same breeding sites (Reusken et al. 2010, Rudolf et al. 2013), suggesting a broader sympatric distribution in Europe, although to a lesser extent than in southern latitudes and a more distinct choice of larval habitats, conditioned by climatic conditions (Becker et al. 2012). Hybridization between molestus and pipiens forms may result in a more opportunistic feeding behavior, which may potentiate the role of *Cx. pipiens* s.s. as a bridge vector of WNV (Di Luca et al. 2016). Börstler et al. (2016) detected blood meals from birds, mammals, and human in the pipiens form, indicating that the feeding patterns were not exclusively ornithophilic. On the other hand, a bird-biting population of the molestus form has been described in an area of inter-form hybridization in southern Portugal (Gomes et al. 2013a). Therefore, it is important to identify where pipiens and molestus forms co-exist in sympatry and infer hybridization rates between both forms. However, only two studies from Catalonia addressed the issue of *Cx. pipiens* forms based on isozyme patterns (Chevillon et al. 1995, Eritja and Goula 1999). However, no DNA analyses were used, so knowledge of the distribution of both forms and their hybridization rates are lacking for the majority of the Spanish territory.

As part of a general entomological project of several families of Diptera, the mosquito fauna of Extremadura (the western region of Spain) were morphologically and molecularly characterized in order to (1) update the distribution and seasonal variation of mosquito species in the region; (2) assess ecological determinants of *Cx. pipiens* s.l. abundance to obtain new data which would be useful for future mosquito control programs; and (3) determine form composition of *Cx. pipiens* s.s. populations.

MATERIALS AND METHODS

Study region

The study was conducted in the Extremadura region of western Spain (39°12′N 6°09′W). The region has a Mediterranean warm-temperate climate with dry/hot summers (Csa) according to the Köppen climate classification. Most of the territory has an elevation between 200-600 m a.s.l. The median annual rainfall is 605 mm (400-1,500 mm). Mean annual temperature is 13° C in the north) and 18° C in the south. In winter, average temperatures remain slightly below 7.5° C and in summer average temperatures vary between 22° C and 26° C.

The Extremadura region has a small human population (26 inhabitants/km^2), and about 74% of the territory is classified as an "Important Bird Area of Spain." There are more than 337 bird species, and the region has the largest water reserve of Spain, representing 25% of the dam water in the country that may potentiate larval habitats for some mosquito species as well as attracting bird populations.

Mosquito collection and morphological identification

Mosquito collections were carried out at 21 sampling sites distributed among 13 counties of Extremadura from January, 2012 to December, 2013 using CDC miniature light-traps (Model 512, John W. Hock Company, Gainesville, FL, U.S.A.). Traps were placed approximately 1.5 m from the ground for one 24-h period per month at each sampling site. Morphological identification was based on characters described by Becker et al. (2010). Mosquitoes were transferred to individual, sterilized 1.5 ml vials and stored at -20° C before being processed for DNA extraction.

Ecological and meteorological data

To evaluate the ecological factors associated with the distribution of *Cx. pipiens* s.l., climate variables, temperature (mean, maximum, minimum), and relative humidity (mean, maximum, and minimum) records were made with a portable meteorological station (HOBO Pro v2®) at each sampling site.

The geographic coordinates and altitude (< 600 m a.s.l, > 600 m a.s.l) were determined by GPS. The information about the presence of sylvatic fauna (deer, foxes, hares, and wild boars) and domestic animals (dog and cat) within a radius of 50 m around the traps were recorded by direct visualization and epidemiological findings. Farms (poultry, porcine, bovine, ovine, caprine) were considered as present whenever their location fell within a 500 m radius from a trapping point.

Habitats were classified as urban (≥40 inhabitants/km^2), rural (<40 inhabitants/km^2), and sylvatic, according to human density and activities (collected from municipal data; IEEX, 2016, http://estadistica.gobex.es/). Urban habitats were divided into (a) urban center, within the agglomeration, or (b) peri-urban when outside the city center (discontinuous urban fabric). The rural habitats were considered for collections performed in areas with <40 habitants/km^2 and where farming activities occurred. Sylvatic areas were considered as those where collections were performed in forested areas of natural parks, with water resources in the vicinity and where human activities were very limited or nonexistent.

DNA extraction and molecular identification

Genomic DNA was extracted by the method of Collins et al. (1988). DNA was resuspended in 100 µl of TE buffer (pH 7.0) and stored at -20° C. Deionized water was used as the negative control for each extraction procedure. Species identification of four members of the *Anopheles maculipennis* complex (*An. atroparvus*, *An. labranchiae*, *An. maculipennis* s.s., and *An. melanoon*) was performed by a PCR-RFLP assay targeting polymorphisms in the Internal Transcribed Spacer 2 (*ITS-2*) of the ribosomal DNA (Vicente et al. 2011).

Molecular identification of the *Cx. pipiens* complex was carried out by a multiplex PCR assay targeting polymorphisms in the intron-2 of the acetylcholinesterase-2 (Ace-2) gene (Smith and Fonseca 2004). Specific primers were used to identify *Cx. pipiens* s.s., *Cx. quinquefasciatus,* and *Cx. torrentium*. To differentiate between molestus and pipiens forms, PCR amplification of the flanking region of microsatellite CQ11FL was performed (Bahnck and Fonseca 2006).

Amplicons from both PCR methods were separated by 2% agarose gel electrophoresis, using ethidium bromide as staining solution, and with a 100 bp DNA ladder as a molecular weight marker (Gene Ruler 100 bp DNA Ladder; Thermo Fisher Scientific).

Statistical data analyses

Statistical analyses were performed using SPSS version 20 and R statistical software version 3.2.4. Mosquito density was calculated as the arithmetic mean (AM) of the mosquitoes captured per trap-night. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to analyze data for normality, while Levene's test was used for homogeneity of variance. Due to the lack of normality of the data, large standard deviations, and lack of homogeneity of variance, non-parametric tests were used. Mann-Whitney U (M-W U) was used for comparing mosquito densities among provinces (Cáceres and Badajoz) and Kruskal-Wallis (KW) tests for mosquito densities between the habitats. When significant differences were found, multiple comparisons were performed using the Dunn-Bonferroni (DB) pairwise comparisons. Mosquito densities for the two years of study (only the data of collections in the same locations, $N = 186$ for each year) were compared by Willcoxon Signed Ranks (Z) paired samples test, and correlation analyses between mosquito densities and climatic variables were performed with Spearman rho (r_s). Pearson's Chi-square or Fisher test, were used to compare proportions of *Cx. pipiens* forms between populations from different habitats.

A negative binomial generalized linear model (NBGLM) was implemented using the MASS package (Modern Applied Statistics with S", 4th edition, 2002) in order to examine associations between *Cx. pipiens* s.l. abundance (number captured per trap-night) and environmental variables. The model uses log link in order to estimate the incidence rate ratio (IRR) of the supposedly dependent variable (number of captured *Cx. pipiens* s.l.) for each independent variable category with respect to the reference category. For continuous variables, such as temperature and relative humidity, IRR represents the expected change of the dependent variable (mosquito abundance) for a one unit change in these predictors. All factors were subjected to bivariate analysis, followed by a backward stepwise model selection procedure, starting with variables showing *P*-values lower than 0.2, to construct the multivariate model. Hence, variables with $P \leq 0.05$ were retained. The possibility of interaction and/or confusion between different variables was examined by constructing different logistic regression models compared by log likehood values, Akaike's information criterion (AIC), and the Vuong test.

RESULTS

Species identification and relative abundance

A total of 1,495 mosquitoes of 16 species was captured, totaling 881 females (58.9%) and 614 males (41.1%) with a sex ratio of 1.43 females per male. *Culex pipiens* s.l. was the most prevalent, representing 69.5% of the total sample, followed by *Cx. theileri* (9.8%) and *Culiseta longiareolata* (9.6%) (Table 1). Morphological identification of males yielded four new mosquito records for Extremadura: *Orthopodomyia pulcripalpis* (N = 34), *Aedes (Ochlerotatus) punctor* (N = 1), *Culex europaeus* (N = 3), and *Culex modestus* (N = 2). Within the *Anopheles maculipennis* complex, through *ITS-2* assay, *An. atroparvus* and *An. maculipennis* s.s. were also found, while in *Cx. pipiens* s.s. by CQ11FL assays, pipiens form and molestus form were detected. The presence of *Culex univittatus* was confirmed in another study (Mixão et al. 2016).

Collections exhibited a wide variation during the two-year period, ranging from nil to very high numbers (Figure 1). The overall relative abundance of mosquito species varied according to year, being higher in 2013 than in 2012 ($Z = -3.245$, $P < 0.001$), with peak densities of 3.81±12.88 mosquitoes/trap-night (AM±SD) for April, 2012, and 22.11±48.95 mosquitoes/trap-night for August, 2013.

Total mosquito density was different across the sampled habitats (K-W = 19.3, $P < 0.001$), mainly due to lower densities in the sylvatic (1.32±7.58 mosquitoes/trap-night), in comparison to other habitats, urban center (2.02±2.61), peri-urban (3.40±12.78), and rural (4.39±18.65) (DB pairwise comparisons, $P < 0.05$), but was not significantly different among the other habitats.

Culex pipiens s.l. densities were also different across categories of habitats (K-W = 16.7, $P < 0.01$), also due to lower densities in the sylvatic (0.26±0.94 mosquitoes/trap-night) in comparison to the urban center (1.44±1.99) and peri-urban (2.41±9.55) (DB pairwise comparisons, $P < 0.01$), but were not significantly different to the rural (3.16±14.34) or among the other habitats. *Culex theileri* densities were not significantly different among habitats (0.34±2.49) (K-W = 4.6, $P > 0.05$).

The difference with respect to year extended to the two most abundant taxa, *Cx. pipiens* s.l. ($Z = -3.398$, $P < 0.001$) and *Cx. theileri* ($Z = -0.310$, $P < 0.001$). However, the densities were not significantly different between the provinces of Cáceres and Badajoz, either in respect to total mosquitoes, *Culex pipiens* s.l. or *Cx. theileri* (M-W U ≥ 20.138, $P > 0.05$, for all).

Total mosquitoes, *Cx. pipiens* s.l. and *Cx. theileri* densities, showed a positive association with the mean temperature ($r_s \geq 0.23$, $P < 0.0001$, for all) but were negatively correlated with mean humidity ($r_s \geq -0.16$, $P < 0.0001$, for all) and with large variations in elevation ($r_s \geq -0.11$, $P < 0.0001$), with the exception of *Cx. theileri*.

***Culex pipiens* s.l. and ecological factors**

Bivariate analysis. Registered temperature and humidity showed significant negative linear correlations with one another ($r_s = -0.68$, $P < 0.001$). This was important to identify collinearity effects potentially leading to erroneous identification of relevant predictors in multivariate analysis. Accordingly, the IRR of *Cx. pipiens* s.l. captures by °C in relation to maximum, minimum, and mean temperatures were similar, with 95% confidence limits between 1.11-1.28° C, whereas IRR ranges with respect to humidity variables were 0.94-0.98% RH for an increase of 1% humidity. The relationship between observed mean temperature and mean humidity with the amount of captured of *Cx. pipiens* s.l. can be observed in box and whiskers plots (Figure 2).

Using dichotomous factors as unique predictors of *Cx. pipiens* s.l. counts, nine of 12 variables had significant values ($P<0.2$) in multivariate analysis. Of these, six had significant associations, yielding IRR >1 for capturing *Cx. pipiens* s.l. in the vicinity (<500 m) of ovine livestock ($P<0.001$), the proximity of human settlements ($P<0.01$), as well as swine farm and manure ($P<0.05$).

Table 1. Total adult mosquitoes and relative abundances of each species, as well as their distribution by province and biotypes within Extremadura, Spain, during 2012-2013.

	(n)			Confirmed presence (Province)		Habitats (n)			
Species	Female	Male	%	Cáceres	Badajoz	Urban	Peri-urban	Rural	Sylvatic
Anopheles atroparvus [a]	2	1	0.20	✓	✓		1	2	
Anopheles claviger s.l.	1		0.07	✓				1	
Anopheles maculipennis s.s.[a]	1		0.07	✓ *				1	
Aedes (Ochlerotatus) caspius	20	3	1.54	✓	✓	1	3	18	1
Aedes (Ochlerotatus) punctor		1	0.07	✓*			1		
Culex europaeus‡		3	0.20	✓*	✓*		1	2	
Culex hortensis hortensis	4		0.27	✓				4	
Culex laticinctus	1		0.07	✓					1
Culex modestus		2	0.13		✓*		1	1	
Culex pipiens s.l.	604	435	69.50	✓	✓	65 (x,z)	352 (y,z)	610 (y,z)	12 (z)
Culex pipiens forms ¥	459	8		✓	✓				
pipiens form	302	6		✓	✓	53.8%	67.4%	65.2%	80.0%
molestus form	39			✓	✓	26.9%	7.6%	7.3%	0.0%
hybrid	118	2		✓	✓	19.2%	25.0%	27.5%	20.0%
Culex theileri	100	47	9.83	✓	✓	4	52	91	
Culex univittatus	15		1.00	✓	✓	1	13		1
Culex spp.	63	6	4.62	✓	✓	2	26	41	
Culiseta annulata	7		0.47	✓	✓		3	4	
Culiseta longiareolata	61	82	9.57	✓	✓	10	34	53	46
Culiseta subochrea	2		0.13	✓	✓			1	1
Orthopodomyia pulcripalpis		34	2.27	✓*	✓*	6	9	19	
	881	614	100.0	✓		89	472	832	62

✓= Presence.

* = New report in this province in Extremadura.

[a] = Molecular differentiation between *Anopheles atroparvus* and *Anopheles maculipennis* s.s. was specifically conducted for this article.

‡ (Ramos et al., 2003).

¥ in a subsample of 467 specimens of *Cx. pipiens* s.l. Group proportions differing significantly according to Pearson's Chisquare test are marked with a different letter (x, y, or z).

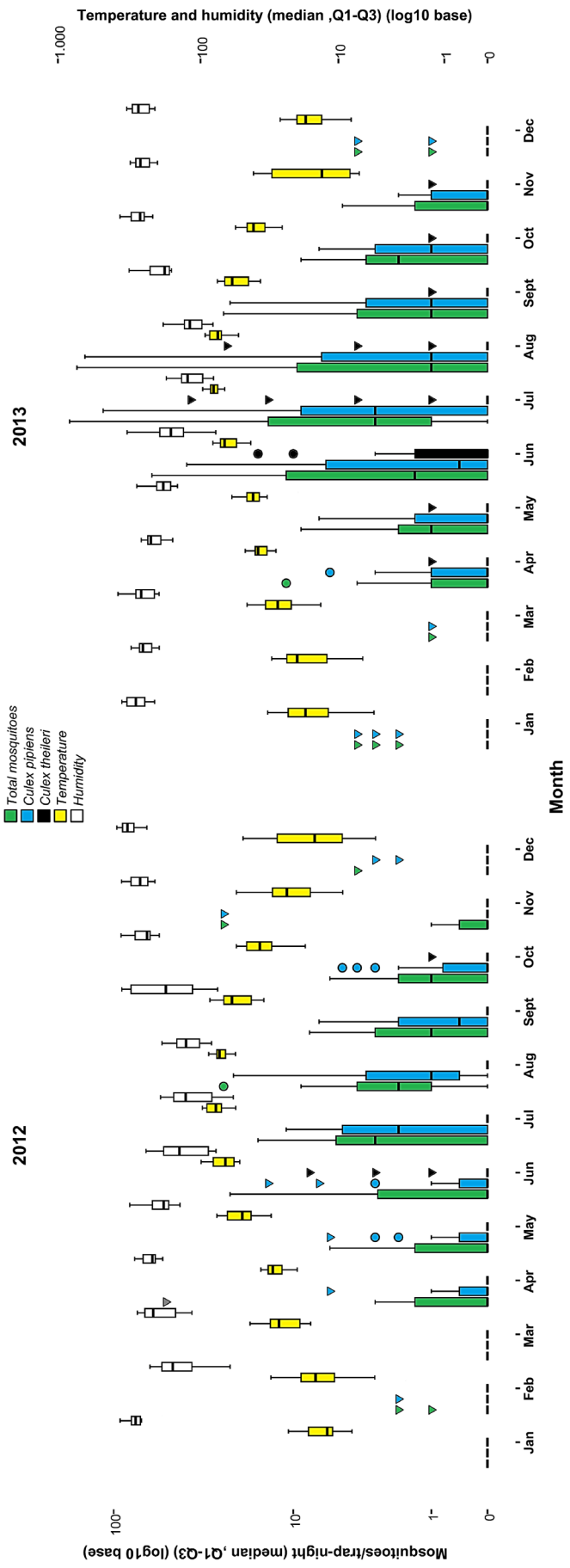

Figure 1. Seasonal distribution and median density of mosquitoes (mosquitoes/trap-night) collected by month, in 2012 and 2013, in 21 samples of stations of Extremadura, Spain. The circles represent outliers that are high and low values that fall out of the range of typical values (1.5xIQR). The triangles represent the extreme outliers that are defined with a higher upper limit and a smaller lower limit, so they are fewer in number than outliers (3xIQR).

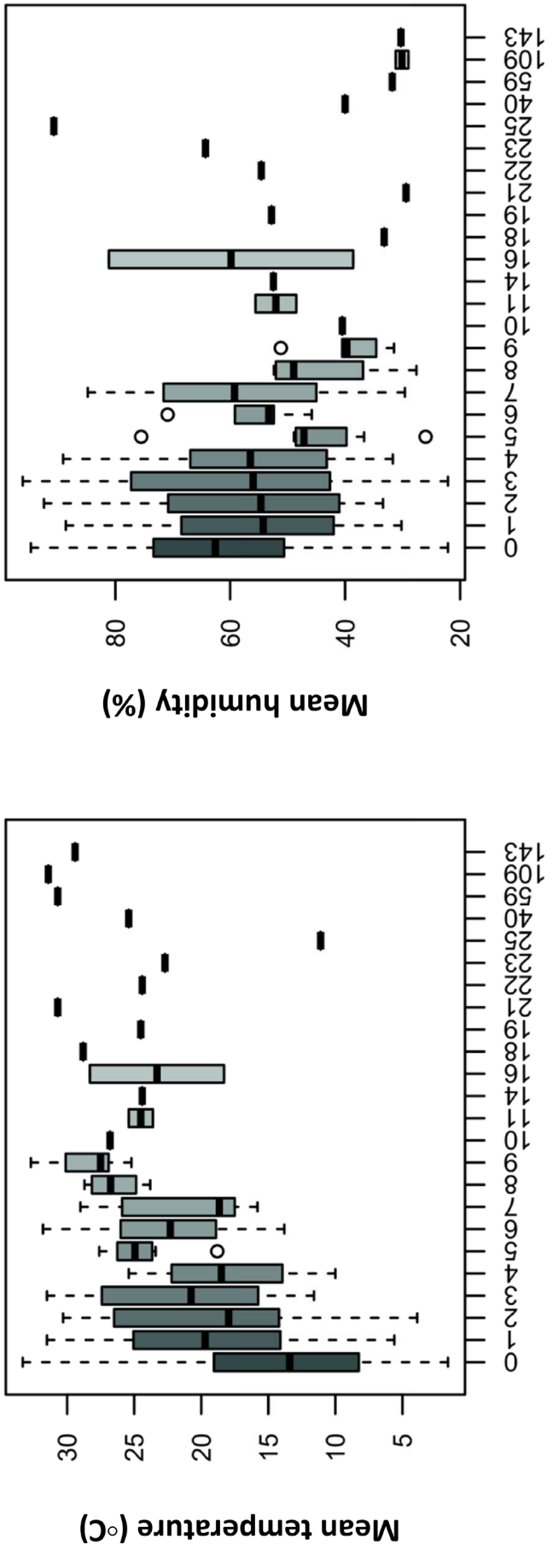

Figure 2. Boxplot and whisker plot showing the relationship between observed mean temperature and mean humidity with the numbers of captured of *Culex pipiens* s.l. Temperature and relative humidity were calculated through data collected during the day and night during the monthly captures.

The estimated IRR was low at elevations above 600 m a.s.l or in the proximity of bovine farms ($P < 0.001$).

In order to analyze the influence of human settlements in the abundance of *Cx. pipiens* s.l., sylvatic habitat was taken as a reference. In fact, in all the habitat categories, *Cx. pipiens* s.l. densities obtained the highest IRR ($P < 0.001$) when compared with sylvatic areas.

Multivariate analysis. Due to collinearity of climatic variables, only maximum temperature was considered in the final model, which was reduced to seven predictors (Table 2). The IRR of maximum temperature remained almost unchanged and therefore a meaningful predictor, mainly representing the expected increase of mosquito counts for each increase of one ºC.

The proximity of bovine and caprine farms had no influence on a higher density of *Cx. pipiens* s.l., unlike in the proximity of human settlements, and more strongly in ovine farms. With respect to the captures of *Cx. pipiens* s.l. in different habitats, the highest IRR were found in urban center and peri-urban and less so in rural ones, albeit with a broad range in all the categories.

Culex pipiens forms identification

In order to determine the relative composition of *Cx. pipiens* forms, a subsample of 467 females identified as *Cx. pipiens* s.s. of 20 (I-XX) stations, representing the four habitats (urban center N = 26, peri-urban N = 129, rural N = 302, and sylvatic N = 10, as the remaining ten were males which were not used for biotype analysis) were screened by CQ11FL assay. A total of 308 (66%) were identified as the pipiens form, 39 (8.4%) as the molestus form and 120 (25.7%) displayed a hybrid pattern.

Sympatric presence of both forms was detected in 12 out of 20 stations (Figure 3) which, alongside hybrids, co-existed in all habitats. Pipiens form was the most represented for all habitats, always > 50% (Table 1, Figure 3), although in the sylvatic biotope, only two females and eight males, eight pipiens form and two hybrids, were identified. When the distribution of forms in the four habitats was analyzed, there were no significant differences. On the other hand, molestus form tended to concentrate in urban center areas, as opposed to peri-urban and rural, while hybrid rates had lesser variation among urban center, peri-urban and rural habitats, hence, the distribution of forms was significantly different between the urban center and either the peri-urban (χ^2= 8.1, P= 0.03) or rural habitats (χ^2= 11.5, P= 0.01).

Analysis of the distribution of *Cx. pipiens* forms by month indicated that the pipiens form was predominant, followed by hybrids and to a lesser extent by the molestus form.

DISCUSSION

Species identification

In our study of the mosquito fauna of the Extremadura region, 16 species were identified, accounting for 25% of the 64 taxa recorded to date for Spain (Bueno-Mari et al. 2012). In this region, rural and peri-urban habitats showed the highest abundances and number of species. The finding of five previously unreported species in Extremadura, namely, *Or. pulchripalpis*, *Ae. (Och.) punctor, Cx. europaeus, Cx. modestus*, and *An. maculipennis* s.s., improves the catalog record for the mosquito distribution of Spain. The most important record of this group is the finding of

Table 2. Multiple regression NBGL models, using backward stepwise for the cross-sectional study. The results of the risk factors IRR (Incidence-Rate Ratio), and their 95% confidence intervals, associated with densities of *Culex pipiens* s.l. for various environmental factors in Extremadura, Spain, are depicted.

Variables	***Culex pipiens* s.l.**		
	p-value	IRR[a]	95% C.I.
Maximum temperature (°C) (Continuous)	<0.001	1.13***	1.10-1.16
Human settlements (ref. > 1km)	0.02	8.66*	1.24-83.18
Manure[b]	0.06	1.74	0.98-3.16
Presence of farm animals			
None	Ref.		
Caprine	<0.001	0.19***	0.09-0.38
Ovine	<0.001	4.81***	2.41-9.79
Bovine	<0.001	0.05***	0.01- 0.18
Habitat			
Sylvatic	Ref.		
Urban center	<0.001	30.45***	5.46-248.57
Periurban	<0.001	18.56***	3.36-133.57
Rural	0.02	7.42*	1.66-75.07

[a]IRR= Incidence-Rate Ratio.
[b]Reference category = No presence.
Ref.= Reference category.
CI=95% confidence interval. *≤0.05, **≤0.01, ***≤0.001

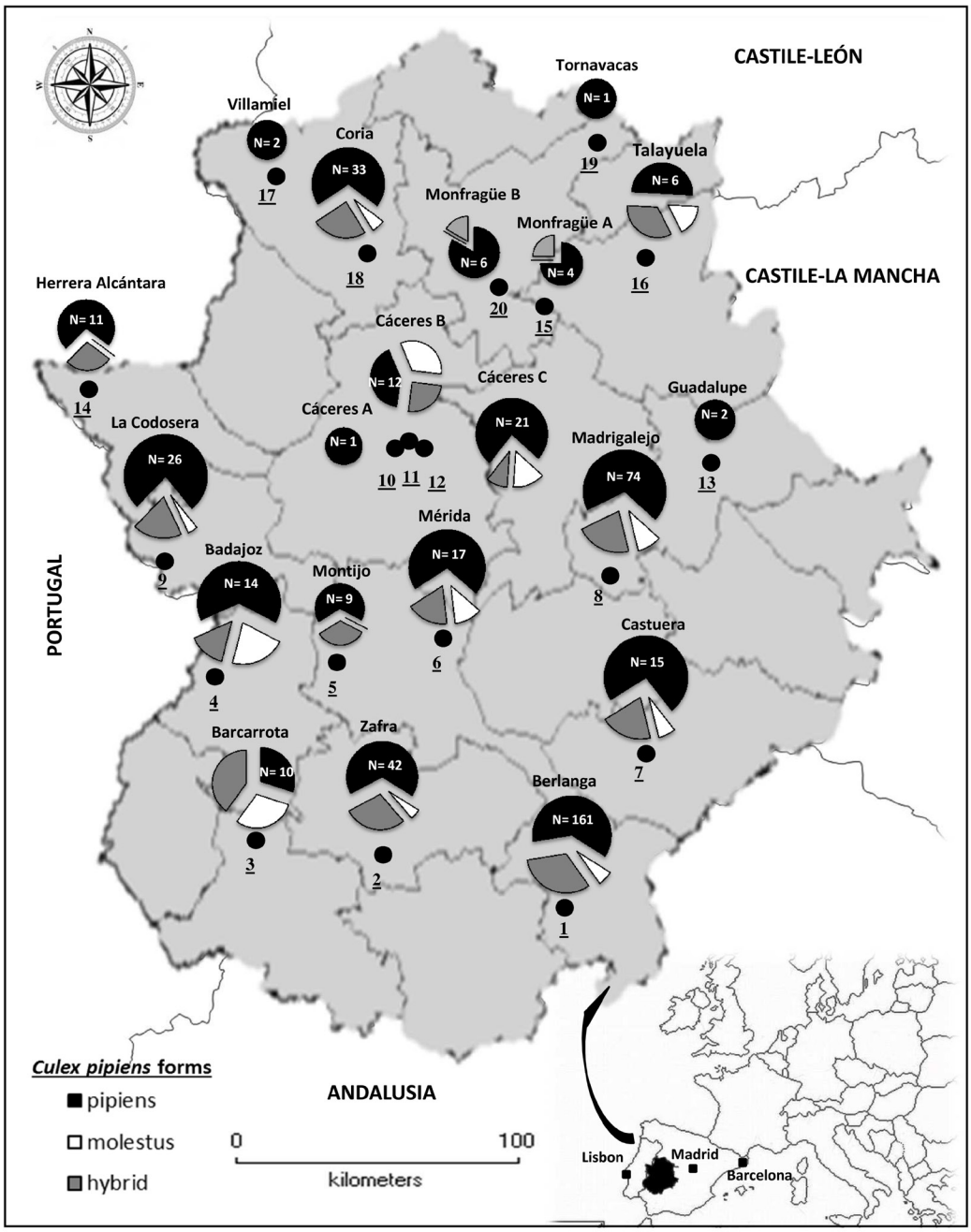

Figure 3. Graphical representation of distribution of *Culex pipiens* forms analyzed by CQ11FL assay of 20 sampling stations in Extremadura (Spain). Black (pipiens form), white (molestus form), and grey (hybrid). The number of the sampling station is underlined.

Cx. modestus, even if at low density, due to its medical relevance as a vector of arboviruses (e.g., WNV, Tahyna virus), heartworm (*D. immitis*), or bacteria (*Francisella* spp.) (Schaffner et al. 2010, Bueno-Marí 2011). Because it is spreading throughout Europe, its ability to act as a secondary vector makes it more relevant (Bødker et al. 2015, Vaux et al. 2015). *Culex pipiens* s.s. was undoubtedly the most abundant species in this survey and particularly in urban habitats. This is in agreement with its widespread distribution on the southern Iberian Peninsula (Almeida et al. 2008, Bueno-Marí and Jiménez-Peydró 2011, Pérez-Bote 2012). It was detected year-round, with higher densities from May to October that follow a classic thermophilic trend: adaptability to environmental conditions and continuous reproductive development throughout the year, according to previous studies in Spain (Roiz et al. 2012, 2014). On the other hand, *Cx. theileri* was the second most abundant species collected in the study area, especially in rural areas, similar to other parts of the Iberian Peninsula (Almeida et al. 2008, Ferraguti et al. 2016). This mosquito is a vector of *Dirofilaria* sp., WNV, Rift Valley fever, and Sindbis viruses (Ferreira et al. 2015) and should be taken into account for future control and surveillance programs in rural areas due to its preference for feeding on farm animals but also biting readily on humans (Almeida et al. 2008, Muñoz et al. 2012, Osório et al. 2014).

The use of mini CDC light traps is common for monitoring

programs in Europe and they have been shown to be effective for different WNV vectors, such as *Cx. pipiens*, *Cx. perexiguus/univittatus*, *Cx. modestus,* and *Cx. theileri* (Roiz et al. 2012). However, some studies have indicated that abundance data from CDC traps underestimate local mosquito species such as *An. maculipennis* s.l. and invasive mosquito species such as *Ae. aegypti* and *Ae. albopictus* (Almeida et al. 2008, Roiz et al. 2012). Due to budgetary constraints, alternative methods such as CO_2 baited traps, BG sentinel traps, indoor resting collections, and larvae sampling could not be included in the present survey. These would be convenient to use in future studies to complement our results.

Ecological factors of *Culex pipiens* s.l.

Results from our study show that *Cx. pipiens* s.l. density varies across weather and habitats patterns. *Culex pipiens* appears to be more sensitive to temperature changes. Several studies have shown that high temperatures favor greater abundances of *Cx. pipiens* (reviewed by Roiz et al. 2014) and can reduce development time (Gardner et al. 2012), as well as differentially affect vector competence of *Cx. pipiens* forms and their hybrids to WNV (Deichmeister and Telang 2011, Vogels et al. 2016). Atmospheric humidity is considered a secondary predictor due to its strict relation to other meteorological parameters (rainfall, soil moisture, wind, temperature, etc.) (Carrieri et al. 2014), and in this area of study we observed an inversely proportional relationship with temperature. The importance of precipitation is indicated by many authors (Becker et al. 2010, Lebl et al. 2013), noting that more days of rainfall at the beginning and end of the year will lead to increased formation and persistence of larval mosquito habitats and mosquito populations remaining steady during the year (Rosà et al. 2014). This factor may adversely affect the development of mosquitoes according to the intensity of precipitation and type of larval habitat (Carrieri et al. 2014).

On the other hand, mosquito densities were markedly different in the two survey years, being lower in 2012 than in 2013, although no differences could be registered in the mean temperature and mean humidity in our sampling sites from one year to the other (M-W U test, $p>0.05$ for both variables). However, the year 2012 was drier than normal in most of Spain, registering the most pronounced rainfall deficit in this region. These dry conditions, along with high temperatures, are likely to have caused a decline of larval habitats that adversely affect mosquito populations, as shown in other studies in Croatia and Italy (Bogojević et al. 2009, Rosà et al. 2014). Rainfall is an important variable that is positively related to annual abundances of *Cx. pipiens*, which are very sensitive to drought (Roiz et al. 2014). Therefore, our data suggest that if higher temperatures are not accompanied by sufficient water sources, it cannot be assumed that this will necessarily result in a homogeneous increase of *Cx. pipiens* populations. However, it may favor an increase of WNV transmission by promoting a greater proximity between mosquito populations and their vertebrate hosts along the same permanent water sources (Bowden et al. 2011, Roiz et al. 2014). In order to establish that higher temperatures caused by climate change may bring higher mosquito densities, other environmental factors, which also affect the abundance of mosquitoes, such as precipitation, evaporation, and soil cover, must be considered (Wang et al. 2011).

In our study, elevation above 600 m was a negative factor in the bivariate analysis, with the highest densities of *Cx. pipiens* s.l. observed between 200-500 m as reported by other workers in Spain (reviewed by Alarcón-Elbal et al. 2012). Higher *Cx. pipiens* s.l. densities were found in urban (30×), peri-urban (18×), and rural (7×) areas, in comparison to sylvatic areas. These results are in agreement with previous studies in the Iberian Peninsula, where *Cx. pipiens* s.l. is the species that was more associated with urbanized areas of higher population density and, consequently, with more human activity pressure (Bueno Marí and Jiménez-Peydró 2011, Osório et al. 2014, Ferraguti et al. 2016). Modification of the environment by human activities, such as irrigation areas, canals, and water storage, promotes mosquito proliferation, and *Cx. pipiens* s.s., particularly of the molestus form, may find sites suitable for larval development over the winter (Vinogradova 2000). Its great abundance in urban areas and its vector competence for numerous pathogens can lead to an increase in disease transmission to humans.

We also found that areas near ovine farms have four times higher *Cx. pipiens* s.l. densities than other animal farms, and this is similar to that reported in Italy (Tran et al. 2014). Ovine farms are an important economic resource in Spain and animals are mainly kept outside during the night, providing feeding opportunities when mosquitoes are active. This, coupled with the availability of water resources for larval development (e.g., animal drinking water containers), make small ruminant farms favorable sites for mosquito proliferation and this is where entomological surveillance should be focused. Similar results were found by Sánchez-Murillo et al. (2014) in a study of insect fauna on farms in Extremadura, where most of the catches were *Culex* spp., but unfortunately, species composition was not specified. Despite the importance of WNV for horses, we have not found a correlation between the presence of this animal and *Cx. pipiens* s.l. density, possibly because we did not sample specific farms of this host, recording only the presence of horses in the vicinity of the traps. Boukraa et al. (2016) indicated that *Cx. pipiens* s.l. and *Cx. torrentium* were the most collected species in equestrian farms in a more northern European country (Belgium). More studies are necessary to understand the relationship between *Cx. pipiens* s.l. and equines in this region and evaluate the risk of WNV for this host.

Identification of *Culex pipiens* forms

In our study, adults of pipiens and molestus forms presented a sympatric distribution in urban, peri-urban, and rural aboveground habitats. This study reports for the first time the coexistence of these two forms in southwestern Spain, confirming a common framework across Europe (Chevillon et al. 1995, Eritja and Goula 1999, Gomes et al. 2009, Osório et al. 2014, Di Luca et al. 2016, Zittra et al. 2016) and northern Africa (Amraoui et al. 2012, Krida et al. 2015). Moreover, a continuous presence of both forms and hybrids is likely to occur throughout the year. However, our low sampling size did not allow us to confirm distribution differences between pipiens and molestus forms or to confirm the presence of adults from the molestus form and hybrids during the winter.

The hybridization rate found in this study by CQ11FL (25.7%) is higher than those reported in Portugal, Greece, Italy,

and Austria (Gomes et al. 2013a,b, Osório et al. 2014, Di Luca et al. 2016, Zittra et al. 2016), but are similar to those found in northern Africa (Amraoui et al. 2012). Although we have detected large hybridization rates in all habitats studied, these were higher in rural areas, consistent with other studies, namely in Austria where these are reported in agricultural and non-irrigated arable land (Zittra et al. 2016). In the literature, hybridization between molestus biotype (preferentially mammophylic) and pipiens biotype (preferentially ornithophylic) is associated with an opportunistic feeding behavior that can potentiate a bridge vector role of *Cx. pipiens* to transmit WNV to mammals (Huang et al. 2009, Farajollahi et al. 2011, Muñoz et al. 2012). Hybridization has also been linked to asymmetric introgression between biotypes *pipiens* and *molestus* in Portugal and in Greece (Gomes et al. 2009, 2013b). This phenomenon may impact the ecology and behavior of the *Cx. pipiens* forms since gene exchange may promote shifts in host preferences or other traits (Kilpatrick et al. 2007).

The assay based on locus CQ11FL has been designed to identify both forms of *Cx. pipiens* (pipiens form and molestus form) and their hybrids. Most of the studies characterizing *Cx. pipiens* forms in Europe have been based on this single genetic assay (Reusken et al. 2010, Osório et al. 2014, Di Luca et al. 2015, Zittra et al. 2016, Boukraa et al. 2016). The CQ11FL assay may produce misleading results where *Cx. quinquefasciatus* are sympatric with *Cx. pipiens* s.s. (Shaikevich et al. 2016), given that a PCR-product of the same size is obtained for both *Cx. quinquefasciatus* and molestus form. Therefore, we took into account the results of both methods, Ace-2 and CQ11FL, as is recommended (Smith and Fonseca 2004). Moreover, this molecular assay is only partially effective as a diagnostic marker in regions with hybridization, and results should be interpreted only at the population level (Bahnck and Fonseca 2006). While the CQ11FL is a good indicator of the sympatric presence of molestus and pipiens biotypes in southern Europe (Gomes et al. 2009), additional studies with alternative methodologies, such as clustering analysis based on microsatellites loci (Gomes et al. 2009, 2013a) and screen of wPip strains (Shaikevich et al. 2016), would be desired to clarify hybridization and its possible behavioral impacts in this region.

This study contributes to the knowledge of the distribution of mosquitoes in Spain with the first report of *An. maculipennis* s.s., *Or. pulchripalpis*, *Ae. (Och). punctor*, *Cx. europaeus*, and *Cx. modestus* in the western region of Spain. *Culex pipiens* s.l. population density reflects a complex interplay among climates, urban habitats or areas near human settlements, and presence of ovine farms in rural areas. These data, along with the sympatric presence of pipiens and molestus forms, as well as hybrids in all sampling habitats, may facilitate accidental transmission of virus to mammalian hosts, including humans, due to its wide distribution. Further studies are needed to explore whether other predictor variables can improve the prediction. Knowledge about the environmental determinants and *Cx. pipiens* forms distribution are important to support WNV surveillance activities, especially in areas related to migration routes of birds and of livestock farming. These results are a first approximation to help contribute to a better approach and knowledge of this species in Spain in order to carry out more effective preventive programs.

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