Diversity and distribution of larval habitats of mosquitoes (Diptera: Culicidae) in northern Spain: from urban to natural areas

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ABSTRACT: Studies of the biodiversity of mosquito larval habitats are important for vector-borne disease control programs and help to improve vector distribution maps. This study was designed to investigate the geographical distribution of mosquito species and their larval habitats in urban, rural, and natural areas in northern Spain. Pre-imaginal stages were collected over two sampling periods (spring and summer) in 2019. In the laboratory, immature specimens were reared to the adult stage for species identification by morphological taxonomy and/or molecular methods. In total, 2,182 specimens belonging to 13 different native mosquito species of five genera were collected from 135 sampling points of which 59.2% harbored larvae. *Culex pipiens* s.l. was the most abundant species (45.1%), followed by *Culex torrentium* (12.3%), *Anopheles maculipennis* s.l. (10.2%), *Culex hortensis* (9.5%), and nine other species with lower frequencies that accounted for less than 25%. By molecular identification, *An. maculipennis* s.s. was identified as the only species within the *An. maculipennis* species complex and *Cx. pipiens pipiens* as the predominant subspecies of the *Cx. pipiens* species complex. Margins in large sunlit water bodies were the most suitable sites for *An. maculipennis* s.l., whereas *Cx. pipiens* s.l. was present in both natural and artificial habitats. The larval site index, species richness, and relative abundance of the mosquitoes were determined based on the characteristics of the sites where they were collected. The public health importance and ecology of some identified mosquitoes is also discussed. ***Journal of Vector Ecology* 46 (2): 173-185. 2021.**

Keyword Index: *Anopheles maculipennis*, biodiversity, larval habitats, *Culex pipiens,* mosquitoes, northern Spain.

INTRODUCTION

Several cases of autochthonous dengue and chikungunya viruses have been reported in Europe since 2007, with an increasing number of cases in Italy, France, and Spain (Jourdain et al. 2020). During the summer seasons of 2018-2020, some unexpected epidemiological events were registered. France reported the first autochthonous cases of Zika virus in Europe, Spain recorded the first autochthonous cases of dengue after several years of epidemiological silence, and between the summer-autumn season of 2020, a major West Nile outbreak was reported in southern Spain (García San Miguel et al. 2021). Although the probability of further local sustained transmission remains low for these arboviruses, the epidemiological alerts show there is vulnerability to these diseases in areas where vector mosquito species are well established.

Among invasive mosquitoes, the Asian tiger mosquito *Aedes albopictus* has received much attention in recent decades in Europe due to its role in the transmission of several arboviruses (Paupy et al. 2009) and the considerable increase in its spread, since the vector is found in almost all the Euro-Mediterranean coastline areas and some of the Atlantic coastal regions of France, Spain, and Portugal. Another relevant exotic vector species is the Asian bush invasive mosquito *Aedes japonicus*. It was first detected in France in the year 2000 (Schaffner et al. 2003) and since then it has been reported frequently in other countries, suggesting a sustained importation and/or spread of the species through the used tire trade or freight transport (Koban et al. 2019). In Europe, there are no confirmed reports of autochthonous pathogen transmission by *Ae. japonicus* in the field, although it is a competent vector of several disease agents in the laboratory (see review of Koban et al. 2019).

Native mosquito species have also gained a great deal of attention due to their role in the transmission of several important viruses. Within the genus *Culex*, the *Culex pipiens* complex is one of the most abundant and well-distributed species in Europe whose role in the transmission of the West Nile virus has been largely examined in competence studies (Vogels et al. 2017). Members of the *Anopheles maculipennis* species complex are also significant vectors of malaria. In fact, since the last case of autochthonous malaria in Spain in 1961, a number of cases of malaria (airport malaria, congenital, and others) have been reported in the past decade, some of them in Aragon, an autonomous community located in northern Spain (Velasco et al. 2017). In addition, some other European native species of mosquitoes, such as *Aedes vittatus* and *Aedes caspius*, among others, should also be considered in epidemiological programs due to their high susceptibility and competence for the transmision of important pathogens (Diez-Fernandez et al. 2018, Gutierrez-Lopez et al. 2019), their abundance, wide distribution, and anthropophilic behavior.

Although the number of bio-ecological studies on potential vector mosquitoes has increased substantially in

most regions of Spain over the last decades, there are still vast areas that have not yet been studied. This is the case in the Basque Country region, a territory in northern Spain with shared boundaries with France. In past years, this territory was not considered to be at epidemiological risk, but this scenario has rapidly changed with the recent arrival of two exotic species to northern regions of Spain. After the detection of *Aedes albopictus* in 2014 in the Basque Country (Goiri et al. 2020) and *Ae. japonicus* in 2018 in Asturias, a region close to the Basque Country (Eritja et al. 2019), this area is now a region at risk of arbovirus outbreaks.

Landscape anthropization causes important ecological changes and could be responsible for the loss in biodiversity and changes in the abundance of some species (Mckinney 2002). In that sense, it has been demonstrated that environmental differences in urban-to-natural gradient influence the diversity and abundance of mosquito species (Ferraguti et al. 2016). Thus, disentangling mosquito community changes between urbanization gradient and types of larval habitats could help to prioritize entomological surveillance strategies. Moreover, information on the diversity and distribution of mosquito species is essential for an effective implementation of mosquito control management. Consequently, the present work aimed to study the species diversity and habitat preferences of immature stages of mosquitoes (with emphasis in those with medical importance) by active collection surveys in urban, rural, and natural areas in the largest province of the Basque Country (northern Spain), an under-studied region in relation to these insects.

MATERIALS AND METHODS

Study area

The study was conducted in Araba (ca. 3,000 km^2), the southernmost of the three provinces of the Basque Autonomous Community in northern Spain (Figure 1). The vast majority of the population (ca. 260,000 citizens) inhabits the capital, Vitoria-Gasteiz (coordinates 42.846409, -2.672463, 525 masl). The province of Araba is an inland territory and features an exceptionally transitional climate between a humid-oceanic climate and a drier and warmer Mediterranean climate, but overall with predominant Atlantic characteristics. The temperature/precipitation fluctuation between winter and summer is high, with cold and wet winters and warm and dry summers. The landscape is dominated by forest (particularly mixed forests) and extensive agricultural fields (e.g. grain: wheat, barley, and oat, and vegetables: potatoes and sugar beet).

Sampling design

The study was conducted in and around a maximum 25 km radius (1,963.49 km) from the most populated territory including urban, rural, and natural areas (Figure 1). The study was performed in 2019 over the course of two different periods. The "1st period" included samplings from 15-May to 1-July (late spring and early summer), in which mild temperatures (mean 14.6 ^{o}C) and abundant rainfall (91 liters/m^2) were recorded. The same sampling sites were examined during the "2nd period" from 15-August to 1-October (late summer and early fall), characterized by lower precipitation (48 liters/m^2) and warmer temperatures (mean 20.3 ^{o}C) (https://www.euskalmet.euskadi.eus). The same researcher carried out the samplings in both study periods.

Selection of sampling sites

Sampling areas were selected according to the following criteria: potential presence of larval habitats, such as wet zones (backwaters, irrigation pools, ponds, and lagoons),

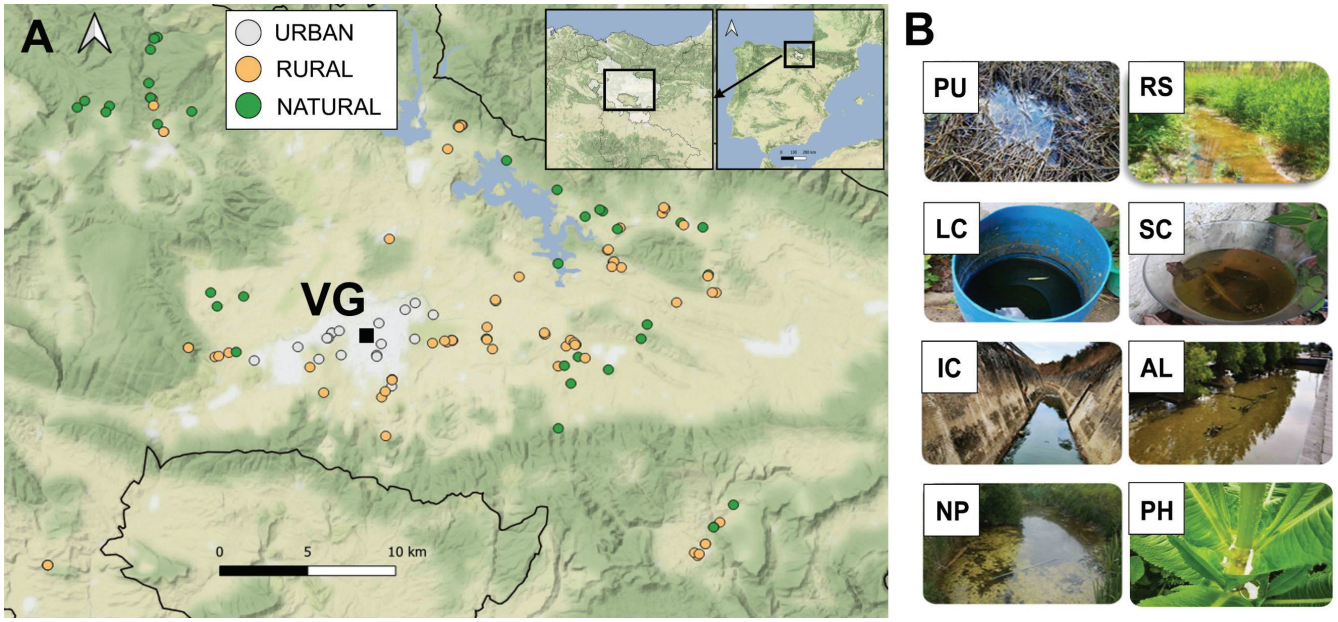

Figure 1. Map showing the 135 sampling sites in and around (25 Km buffer) the city of Vitoria-Gasteiz (VG) (Araba, Basque Country) in northern Spain. (A) Sampling sites are categorized as urban, rural, and natural; (B) Larval habitats categories are illustrated [PU, puddle; RS, river shore; LC, large container; SC, small container; IC, irrigation canal; AL, artificial lake; NP, natural pond; and PH, phytotelmata].

small villages (vegetable gardens, irrigation canals, fountains, and ditches), green natural protected areas and parks, and farm-associated habitats, meadows, and other places with livestock or animals. This task was achieved by 3D representation of satellite imagery displayed on Google Earth. The sampling itinerary was planned in order to visit as many sampling places as achievable in a day, and as far as possible under favorable weather conditions. However, due to the distinct terrain conditions and distance between places, it was impracticable to cover all the territory uniformly. When a type of larval habitat was detected several times in the same municipality, such as an accumulation of discarded tires, it was sampled a maximum of three per municipality.

Field sampling

Almost all types of lentic aquatic habitats are suitable places for a wide range of mosquito species. We defined a potential larval habitat as a place with "standing water or stagnant freshwater" using as an example other rearing sites already described in Spain (Bueno-Marí 2010, Ph.D. thesis, Chordá-Olmos 2014, Ph.D. thesis). We established the criterion of time to obtain unbiased standardization and reasonable qualitative data (approx. 10 min) for the search and collection of mosquito immature stages (larvae and/or pupae) in water-filled sites. Homogeneous sampling was attempted at each larval habitat. Muddy, large water bodies or inaccessible places were sampled by using a plastic homemade dipper (600 mL volume capacity) along with careful collection of the specimens with a plastic pipette. In small water bodies and/or easily accessible places, a large plastic pipette (20 ml) was used. Dipping and pipetting were performed where larvae usually develop, around margins of water bodies, in shallow areas, and around aquatic vegetation. Given that the field sampling was done by the same operator, we considered that the sampling effort was evenly proportional in all the studied sites. Immature stages were collected with their own water and then transferred to hermetic plastic containers (400 ml volume), labeled, and stored inside polystyrene boxes for their preservation until laboratory processing.

Classification of sampling sites

Biological characteristics of the larval habitats were annotated in order to conduct two classification systems, at micro-scale based on the type of aquatic larval habitat (eight categories) and at mesoscale according to the different degree of human intervention (three habitats). In the first classification, larval habitats were linked to one of the eight categories within "aquatic habitat types" described by Chordá-Olmos (2014, Ph.D. thesis) but with some modifications: RS (river shore, stream, or run-off), NP (natural pond, semi-natural pond, swamp, or lagoon), AL (artificial lake for irrigation or artificial pond), LC (large container > 10 liter volume: trough, spring, swimming pool, tank, etc.), SC (small container < 10 liter volume: tire, bucket, can, packaging, etc.), PU (puddle, standing pool water, or backwater), IC (irrigation canal, sewer, ditch both open or underground), and PH (phytotelmata environments offering plant-held water bodies). Secondly, habitats were classified as urban (medium to high density of inhabitants/km^2 including peri-urban areas), rural (low density of inhabitants/km^2 usually with farming activities), and natural (no human habitation and/or human activities very limited or non-existent).

Laboratory processing

Pre-imaginal stages of mosquitoes were transferred alive into "Mosquito Breeders" (Bioquip Products, U.S.A.) with the water from their larval habitat to be reared until adult stage (ca. three weeks) in an insectary room (24º C ± 3; 60-80% RH) under controlled photoperiodic conditions. Possible predators accidentally introduced in the breeders were removed in order to avoid loss of mosquito larvae. Every week, a few ground flakes of commercial fish food were added to each breeder. Emerged adult mosquitoes were drawn every two to three days by unscrewing the upper section of the breeders and placing them in the freezer for 10 min at -30º C. Afterward, adult mosquitoes were separated by sex, and males were stored in 2 ml vials filled with 70% alcohol while females were kept desiccated with a bottom of silica gel and cotton in a drilled plastic labeled container (volume of 50 ml). All larvae and pupae were counted prior to being transferred into breeders (field-caught collection) and adults were counted after their emergence (laboratory collection). This difference was recorded as the mortality rate.

Morphological identification

Male genitalia were mounted on glass slides (Hoyer´s medium), whereas females were glued (right thorax side) on a small cardboard in entomological pins. Both sexes were morphologically identified with the electronic interactive keys (Schaffner et al. 2001, https://www.medilabsecure.com/moskeytool.html) and a dichotomous identification key (Becker et al. 2010).

Molecular identification

A PCR based on the cytochrome *c* oxidase I subunit (*COI*) DNA barcoding was carried out, following procedures already described (Simon et al. 1994), to identify damaged or morphologically indistinguishable mosquito specimens. DNA amplicons were sent for sequencing to external services (Eurofins Genetics) and the sequences were compared using BLAST against those available in the NCBI (www.ncbi.nlm.nih.gov) and BOLD (http://boldsystems.org) databases. BLAST results with sequence similarity ≥98% were considered as valid species. For those belonging to the *An. maculipennis* and *Cx. pipiens* species complexes, only a random subsample of each larval habitat (from one to five specimens depending on availability) was analyzed. *Cx. pipiens* s.l. and *An. maculipennis* s.l. sibling species were identified by amplification of the flanking region of the CQ11 microsatellite loci and Internal Transcribed Spacer 2 (ITS-2) of the ribosomal DNA, respectively, following procedures described elsewhere (Bahnck and Fonseca 2006, Vicente et al. 2011). Since not all the collected specimens were analyzed, both *Cx. pipiens* and *An. maculipennis* are named throughout the text as sensu lato (s.l.). The *Anopheles claviger* complex composed of *An. claviger* s.s. and *Anopheles petragnani* were

not differentiated, and therefore, *An. claviger* specimens were also referred to as s.l.

Collection of data, index calculation, and statistical analysis

Geographical coordinates were recorded with the software QGIS 3.4.12-Madeira (QGIS Geographic Information System, Open Source Geospatial Foundation Project) to represent sampling sites (Figure 1). The level of infestation by larvae and/or pupae was estimated using the larval site index (R), which was calculated as the number of water-holding biotopes positive for culicids/total number of sampled sites. The R index was categorized as low (0-0.25), medium (0.25-0.50), high (0.50-0.75), or very high (0.75-1.00). Relative abundance (RA) was calculated as the number of each species of Culicidae x 100/total number of specimens in the study. The larval density was calculated as the mean number (± SE) of larvae per 10 min of sampling. Those habitats without water were considered as negative. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to analyze mosquito (emerged adults) data for normality. After determining that the data were not normally distributed, a Mann-Whitney test was performed to compare the mosquito abundance between both study periods. Moreover, Kruskall-Wallis tests were used to measure the differences in the abundance of mosquitoes between the type of urbanization degree (urban, rural, and natural) and type of aquatic larval habitats (RS, NP, AL, LC, SC, PU, IC, and PH). When significant differences were found, a Wilcoxon pairwise rank sum test with a Bonferroni correction was used to compare the differences between groups. The proportion of mosquito species between the 1st study and 2nd study were compared using the Chi Squared test or Fisher's exact test. Statistical analyses were performed using SPSS™ version 22.0 software (alpha level at 0.05).

In addition to the non-parametric analysis, multivariate Generalized Linear Models (GLM) were run to evaluate the associations between mosquito abundance and species richness related to habitat and study period (based on emerged adults). Larval habitat was not evaluated by GLM due to the low number (or lack) of observation for some larval habitat types. Due to the over-dispersion and excess of zeros, zero-inflated negative binomial (ZINB) (for abundance) and zero-inflated Poisson (ZIP) (for species richness) generalized linear models were carried out using "pscl" R package (Zeileis et al. 2008). For zero-inflated models, log-link and logit-link were used for the count component and zero component, respectively (Zuur et al. 2009). Interaction between habitat and study period was included in the full models. In addition to overall mosquito abundance, *Cx. pipiens* s.l., *Culex torrentium,* and *An. maculipennis* s.l abundance were also evaluated separately. The best model was selected using the "dredge" function from the "MuMIn" package of the R software (Barton 2009, http://r-forge.r-project.org/projects/mumin/) based on Akaike Information Criterion corrected to sample size (AICc). The overall fit of the model was evaluated with the likelihood ratio test, comparing the best model with the null model.

RESULTS

Captures, species richness, relative abundance, and distribution

During both study periods, 80 out of the 135 sites inspected (R = 0.59) tested positive for the presence of immature stages of mosquitoes. A total of 1,401 (Mean ±

Table 1. Relative abundance of mosquitoes rearing in aquatic environments in both study periods during the summer of 2019 in Araba (northern Spain).

Species*	1 st study				2 nd study				Both studies			
	♀	♂	Total	%	♀	♂	Total	%	♀	♂	Total	%
Cx. pipiens s.l.	104	97	201	**32.90**	186	223	409	**51.77**	290	320	610	**43.54**
Cx. torrentium	92	68	160	**26.19**	14	19	33	**4.18**	106	87	193	**13.78**
An. maculipennis s.l.	19	29	48	**7.86**	53	42	95	**12.03**	72	71	143	**10.21**
Cx. hortensis	54	45	99	**16.20**	14	20	34	**4.30**	68	65	133	**9.49**
Cs. longiareolata	6	6	12	**1.96**	41	51	92	**11.65**	47	57	104	**7.42**
Cx. territans	7	11	18	**2.95**	42	42	84	**10.63**	49	53	102	**7.28**
Cs. annulata	23	25	48	**7.86**	2	2	4	**0.51**	25	27	52	**3.71**
An. claviger s.l.	9	9	18	**2.95**	6	13	19	**2.41**	15	22	37	**2.64**
Cx. mimeticus	0	0	0	**0.00**	6	4	10	**1.27**	6	4	10	**0.71**
Ae. vexans	0	0	0	**0.00**	3	5	8	**1.01**	3	5	8	**0.57**
Ae. cantans	2	2	4	**0.65**	0	1	1	**0.13**	2	3	5	**0.36**
Cx. theileri	1	2	3	**0.49**	0	0	0	**0.00**	1	2	3	**0.21**
An. plumbeus	0	0	0	**0.00**	1	0	1	**0.13**	1	0	1	**0.07**
Total specimens	317	294	611		368	422	790		685	716	1,401	

*Specimens included are those that emerged in the laboratory breeders.

SE = 10.3 ± 1.68) immature stages of the 2,182 (Mean ± SE = 16.0 ± 2.16) specimens collected in the field developed to the adult stage in the laboratory breeders. This represented 35.8% of mortality in the immature stages. No exotic invasive mosquitoes (*Ae. albopictus* or *Ae. japonicus*) were found in any of the sampling sites studied.

Thirteen autochthonous mosquito species belonging to four genera (*Culex, Anopheles, Culiseta,* and *Aedes*) were found in the aquatic habitats analyzed. *Culex pipiens* s.l. was the most abundant species (43.5%), followed by *Cx. torrentium* (13.8%), *An. maculipennis* s.l. (10.2%), and *Cx. hortensis* (9.5%). Nine other species accounted for less than 25% of the total catches (Table 1).

Molecular analysis determined *An. maculipennis* s.s. (n = 32, 100%) was the unique member species within the *An. maculipennis* species complex. Within the *Cx. pipiens* complex, only two ecological forms were identified, *Cx. pipiens pipiens* (n = 66, 88.0%) and *Cx. pipiens/molestus* (hybrid) (n = 6, 8.0%), and the ecological form could not be determined in three specimens (4.0%).

Twenty-six *COI* sequences (around 460 bp) were obtained to allow the confirmation of some doubtful specimens (one *Aedes vexans,* one *Culex mimeticus*, two *Cx. pipiens* s.l., three *An. claviger* s.l., 19 *Cx. torrentium*). From those sequences, a selection of 12 sequence types has been deposited in the GenBank database with the accesion numbers: MZ027401-MZ027411 and MZ053467.

As expected, the sex ratio was roughly equal (females/males = 685/716 = 1/1.04). In terms of the genus distribution, out of the 80 positive sites, *Culex* was present in 58 sites (72.5%), *Anopheles* in 29 (36.3%), and *Culiseta* in 19 (23.8%), whereas *Aedes* were only present in one (1.3%). Regarding the co-existence of the most relevant species, *Cx. pipiens* s. l. and *Cx. torrentium* co-existed in ten out of the 18 larval habitats where *Cx. torrentium* was present, while *An. maculipennis* s.l. and *An. claviger* s.l. species developed together only in three of the 29 *Anopheles* habitats.

Distribution of mosquitoes between both study periods

The 1st study period yielded lower abundance of emerged specimens (mean ± SE = 4.51 ± 0.99), larval site index (R = 0.37), species (S = 9), and mortality (M = 28.2%) in comparison with the 2nd studied period (5.61 ± 1.27; R = 0.42, S = 13, and M = 41.7%). However, there were no significant differences between these periods (W = 8757.5; p = 0.5197). Despite that, there were significant differences in the proportions of the relative abundance of the most commonly trapped species between both study periods, e.g., the proportions of *Culiseta annulata* (χ^2 = 4.50; p = 0.033), *Cx. hortensis* (χ^2 = 7.20; p = 0.007), and *Cx. torrentium* (χ^2 = 16.13; p ≤ 0.001) were significantly higher during the 1st period compared to the 2nd one. On the contrary, the proportions of *Cx. pipiens* s.l. (χ^2 = 4.76; p = 0.029), *Culex territans* (χ^2 = 4.57; p = 0.032), and *Culiseta longiareolata* (χ^2 = 7.10; p = 0.007) were significantly lower during the 1st period in comparison to the 2nd one. The proportions of *An. maculipennis* s.s. (χ^2 = 0.80; p = 0.371) and *An. claviger* s.l. (χ^2 = 0.4; p = 0.527) did not vary significantly between both study periods (Table 1).

Distribution of mosquitoes by type of urbanization degree

Of the 135 larval habitats, 19 were classified as urban (R = 0.31), 39 as rural (R = 0.64), and 77 as natural (R = 0.63) (Table 2). The largest species richness and relative abundance were recorded in rural environments (Table 2). The urban habitat had a significantly lower abundance of mosquitoes compared to rural and natural habitats (H = 6.7971, df = 2; p = 0.0334), significant differences were found only between rural and urban habitats (p = 0.046). The R index was also the lowest in the urban environment. In the urban habitats, most of the catches belonged to three species: *Cx. hortensis, Cx. pipiens* s.l., and *Cs. longiareolata,* whereas in both rural and natural habitats the most predominant species was *Cx. pipiens* s.l., followed by *Cx. torrentium* (rural environment) or by *An. maculipennis* s.s. (natural environment) (Figure 2). The species identified in each habitat and shared species between habitats are shown in Figure 3.

Distribution of mosquitoes by larval habitat categories

The majority of the larval habitat categories exhibited a high to very high R index (PU, RS, SC, IC, LC, NP) with the exception of two (AL and PH) that had a lower R index (Table 2). The largest species richness was recorded in the PU category, followed by IC, NP, and LC, whereas low species richness was observed in the categories RS, SC, and AL. No mosquito larvae were found in PH (Table 2). The analysis showed statistical differences in the immature mosquito abundance among the eight larval habitat categories (H=23.842; df = 7; p = 0.001), where the categories PU (p = 0.024), IC (p = 0.011), and NP (p = 0.001) showed a significantly higher abundance when compared to AL (Table 2). Most of the immature stage catches were collected in three of the categories (PU, LC, IC), whereas the categories NP, RS, SC, AL, and PH only accounted for 30.5% of immature stages (Table 2). Concerning the relative abundance of the mosquito species, *Cx. pipiens* s.l. was the most abundant species in four (PU, LC, IC, RS) of the eight habitat categories (Figure 2). *An. maculipennis* s.l. was the predominant species in two categories (NP, AL), whereas *Cx. torrentium* was the most common species trapped in the SC category (Figure 2).

Multivariate models for urbanization degree and study period

The total mosquito abundance (ZIBN count component) slightly decreased from urban to natural habitats (Estimate ± Standard Error [E ± SE] = -0.94 ± 0.55; p = 0.08), and the occurrence probability (ZIBN zero component) increased (excess of zeros decreased) from urban to rural and natural habitats (-1.13 ± 0.48; p = 0.01 and -1.36 ± 0.53; p = 0.01, respectively). The occurrence probability for *Cx. pipiens* s.l. presence slightly increased (excess of zeros decreased) (-0.81 ± 0.45; p = 0.07) in the second period. Due to the absence of *An. maculipennis* s.l. and *Cx. torrentium* in urban areas, only "rural" and "natural" categories were included in those models. The occurrence probability for *An. maculipennis* s.l. increased (excess of zeros decreased) from rural to natural environment (1.48 ± 0.66; p = 0.02), whereas the abundance of *Cx. torrentium* decreased (-1.33 ± 0.69; p = 0.05) from the

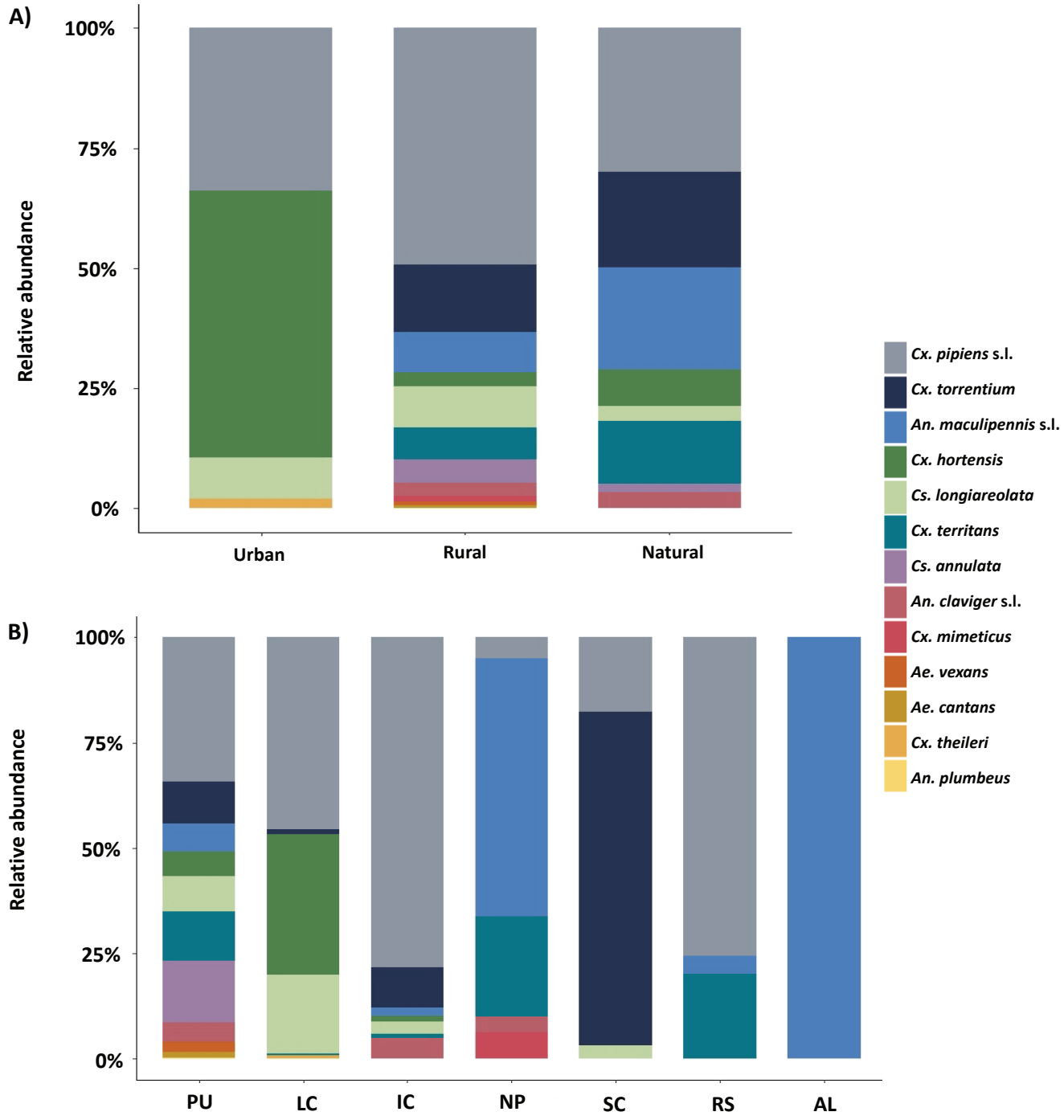

Figure 2. Relative abundance of mosquito species in each of the three types of urbanization degree (urban, rural and natural) (A); and in the larval habitats categories (B) [PU, puddle; RS, river shore; LC, large container; SC, small container; IC, irrigation canal; AL, artificial lake; NP, natural pond; and PH, phytotelmata].

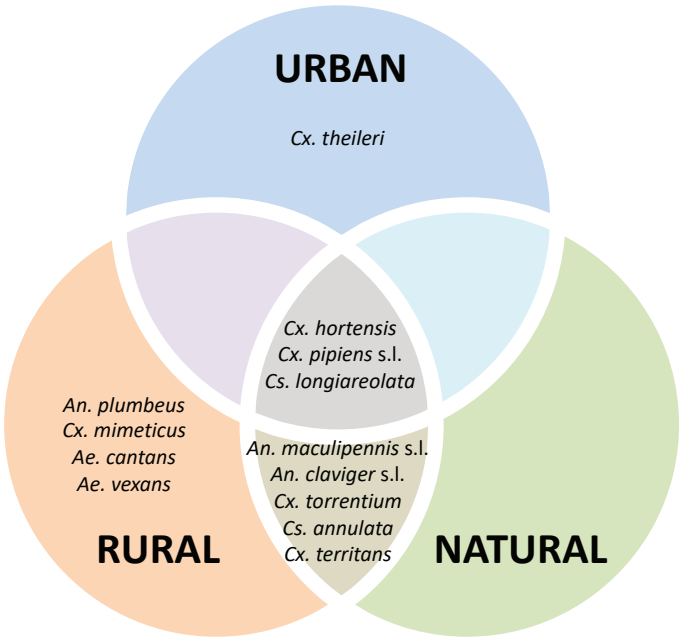

Figure 3. Venn diagram showing the mosquito species found in urban, rural, and natural habitats.

first study period to the second one. Species richness (ZIP count component) increased in the second study period (0.84 ± 0.26; p = 0.002). A summary of the best models is presented in Table 3.

DISCUSSION

To the best of our knowledge, this study represents the most comprehensive characterization of mosquito larval habitats in northern Spain. With the exception of the research on larval habitat preferences of mosquitoes extensively carried out in Valencia (eastern Spain) (Bueno-Marí 2010 Ph.D. thesis, Chordá-Olmos 2014 Ph.D. thesis), little data are available for other Spanish regions. This study also contributed to the number of mosquito species described in the territory of Araba (Gonzalez et al. 2020, Gonzalez et al. 2021), with the increase from 14 to 19 by the addition of five new records (*Cx. territans*, *Cx. mimeticus, Culex theileri, Ae. vexans,* and *Ae. cantans*). Mosquitoes were present in a wide variety of micro-habitats during the entire study. In particular, the genus *Culex* appeared in 72.5% of the mosquito-infested sites.

The absence of exotic mosquito species in the studied sampling sites is noteworthy, given the presence of *Ae. japonicus* and *Ae. albopictus* in surrounding territories (Eritja et al. 2019, Goiri et al. 2020). Unfortunately, during the sampling prospection there were some difficulties in accessing potential larval habitats of *Aedes* invasive mosquito species, e.g., drinking points (troughs) of livestock in private farms and fenced meadows and other *Ae. albopictus*-preferred larval habitats, such as water-filled containers, such as pools, and abandoned objects, that were abundant on private properties.

The results showed the presence of several suitable environments for the proliferation of potential vectors of arboviruses. The well-known and ubiquitous *Cx. pipiens* s.l. was the predominant species in both rural and natural environments in all larval habitat types except natural ponds (NP), small containers (SC), and artificial lakes (AL). In urban habitats, *Cx. hortensis*, particularly abundant in rainwater drums in periurban settlements, overcame *Cx. pipiens* s.l. collections. The analysis of males by morphology of genitalia and/or females by molecular analysis allowed the separation of *Cx. pipiens* s.l. from the morphologically similar species *Cx. torrentium,* allowing us to infer the ecological requirements of these sympatric species. Both species were well adapted to various larval habitat types, however, *Cx. pipiens* s.l. was more broadly distributed in comparison to *Cx. torrentium*, the latter being largely predominant in small artificial water-filled containers (<10 liter volume) and, to a lesser extent, in diverse types of natural puddles as proposed elsewhere (Weitzel et al. 2014) and irrigation chanels. The sympatric coexistence of *Cx. pipiens* s.l. and *Cx. torrentium* has been largely reported in Europe (Boukraa et al. 2016, Hesson et al. 2014, Luhken et al. 2015). Other members of the genus *Culex* were also found; *Cx. hortensis* was collected both in rainwater large artificial containers and urban sites, whereas *Cx. territans* was almost exclusively found on natural water bodies, such as puddles, standing water, river shore, natural ponds, etc. in accordance with other studies (Becker et al. 2010, Boukraa et al. 2016).

It was interesting to note that within the genus *Anopheles,* there was a widespread presence of both *An. maculipennis* s.l. and *An. claviger* s.l. in aquatic environments. Unfortunately, there is not much information available on the characteristics and ecology of the larval habitats of *An. maculipennis* s.s. in comparison to its congenerical species *An. atroparvus,* due to its minor relevance as a malaria vector, as well as its restricted distribution in Europe (Hertig 2019). *Anopheles maculipennis* s.s. was observed on the edges of open large permanent sunlit fresh pools with vegetation (mostly *Potamogeton, Berula, Lemna,* and algae) and/or on other detritus such as natural ponds, lakes, and lagoons. Despite not having been analyzed

Table 2. Summary of mosquitoes collected based on the type of habitat and the type of larval habitat during the summer of 2019 in Araba, northern Spain.

		No. sites	Immatures collected (%)	R* (%)	Mean (±SE)§	Species (S)†
Type of habitat	Urban	19	10.7	0.32	7.9 (5.5)	4
	Rural	77	68.4	0.63	12.4 (3.3)	12
	Natural	39	20.7	0.64	7.4 (2.2)	8
Type of larval habitat	PU - Puddle, standing pool water, or backwater	22	25.1	0.82	15.9 (4.5)	9
	LC - Large container >10 liters: through, spring, swimming pool, tank	32	23.0	0.59	10.1 (3.5)	5
	IC - Open or underground irrigation canal, sewer, ditch, canal	19	21.6	0.68	17.1 (6.8)	7
	NP - Natural-pond, semi-natural pond, swamp or lagoon	24	11.4	0.58	6.6 (2.5)	6
	RS - River shore, stream, or run-off	8	11.3	0.75	11.7 (8.1)	3
	SC - Small container <10 liters: Tire, bucket, can	13	6.7	0.69	12.1 (4.9)	3
	AL - Artificial lake for irrigation or artificial pond	14	0.9	0.70	0.8 (0.8)	1
	PH - Phytotelmata	3	0.0	0.0	0	0

*R, Larval site index; § Mean (± SE), average of the mumber of immature mosquitos yielded per 10 min sampling (± standard error); †S, species richness.

here, the presence of these macrophytes seemed to be an important factor linked with the presence of this species, as it has been observed for other *Anopheles* species (Azari-Hamidian 2005, Azari-Hamidian 2011, Bergey et al. 1992, Orr and Resh 1989). *Anopheles maculipennis* s.s. is frequently associated with rice fields (Azari-Hamidian 2011, Calzolari et al. 2021) but this particular habitat was not present in the studied areas. Albeit less frequently, *Anopheles maculipennis* s.s. has also been collected in ephemeral and large man-made habitats, including ditches, irrigation channels, and river bed pools as mentioned in other parts of Europe (Calzolari et al. 2021, Dakic et al. 2008, Kavran et al. 2018). Moreover, *An. claviger* s.l. was less widespread and abundant, being recorded mainly in margins of ditches, canals, scrapes, and on small rocky puddles as reported in other studies (Becker et al. 2010, Boukraa et al. 2016, Hawkes et al. 2020). *Anopheles plumbeus* was rarely collected in the present study. No immature instars of *Anopheles* spp. were found in artificial containers with low water volume as observed elsewhere (Roiz et al. 2007).

Regarding *Culiseta* mosquitoes, two clear ecological patterns were recorded. While the widespread *Cs. longiareolata* preferred man-made water-filled containers as suitable habitats for larval development, *Cs. annulata* was found in temporary forest-associated habitats. This pattern had not been observed in other studies where *Cs. annulata* were present in both artificial and natural sites (Azari-Hamidian 2005, Boukraa et al. 2016). However, *Cs. longiareolata* is reported to be primarily found in artificial containers (Becker et al. 2010, Becker and Hoffmann 2011,; Seidel et al. 2013). Drinking troughs in meadows and depth pools of water with high content of organic matter were particularly productive for *Cs. longiareolata* and *Cs. annulata*, respectively.

Aedes species were scarce despite the availability of favorable developmental sites, and only two species (*Ae. vexans* and *Ae. cantans*) were recorded in organic-rich puddles in forested micro-habitats as previously reported (Bueno-Marí 2010 Ph.D. thesis). None of the three other remaining genera currently present in the Spanish territory (*Uranotaenia*, *Orthopodomiya,* and *Coquillettidia*) were collected in this study. The first two are generally infrequent genera with a single-generic species, while the latter requires the use of specific sampling methods to be effectively

Table 3. Summary of the best zero-inflated negative binomial (ZINB) models for total mosquitoes, *Culex pipiens*, *Anopheles maculipennis* s.l., and *Culex torrentium* abundance, and zero-inflated poisson (ZIP) for Species Richness.

	Total abundance			*Cx. pipiens* abundance			*An. maculipennis* s.l. abundance			*Cx. torrentium* abundance			Species Richness		
	Est ± SE	z	p	Est. ± SE	z	p	Est ± SE	z	p	Est ± SE	z	p	Est ± SE.	z	p
Count component															
Habitat [Urban]	Ref.			Ref.			Not in.			Not in.			Ref.		
Habitat [Rural]	-0.31±0.52	-0.59	0.55	Not sel.			Ref.			Ref.			Not sel.		
Habitat [Natural]	-0.94±0.55	-1.70	0.08	Not sel.			-0.65±0.54	-1.19	0.23	Not sel.			Not sel.		
Study period [First]	Ref.			Ref.			Ref.			Ref.			Ref.		
Study period [Second]	Not sel.			0.11±0.58	0.19	0.85	Not sel.			-1.33±0.69	-1.92	0.05	0.84±0.26	3.14	0.002
Zero component															
Habitat [Urban]	Ref.			Ref.			Not in.			Not in.			Ref.		
Habitat [Rural]	-1.13±0.48	-2.36	0.01	Not sel.			Ref.			Ref.			Not sel.		
Habitat [Natural]	-1.36±0.53	-2.52	0.01	Not sel.			-1.48±0.66	-2.22	0.02				Not sel.		
Study period [First]	Ref.			Ref.			Ref.			Ref.			Ref.		
Study period [Second]	Not sel.			-0.81±0.45	-1.80	0.07	Not sel.			0.30±0.55	0.54	0.59	1.08±0.77	1.39	0.16
Observations	270			270			232			232			270		

Est ± SE, Estimate ± Standard Error; z, statistic z-value; p, p-value; Ref., reference category; Not in., Not included in the full model; Not sel., Not selected for the best model.

captured, as this genus is associated with fixed aquatic plants (Rueda et al. 2015).

From an epidemiological point of view, *Anopheles* spp. and *Cx. pipiens* are possibly the most important mosquitoes collected. *Anopheles atroparvus* is currently considered the major malaria vector in Europe. Although malaria was officially eradicated in Spain in 1964, imported cases have been registered in the country in the last decades, and uncommonly, autochthonous malaria cases might also occur (Bueno-Marí and Jiménez-Peydró 2008, Sousa et al. 2020). Among the collected *An. maculipennis* individuals, ca. 22% were molecularly analyzed and confirmed as *An. maculipennis* s.s. This *Anopheles* species, together with *An. claviger* s.l., seems to be less competent (both species are considered refractory to *Plasmodium* spp. transmission) in transmitting malaria in Europe (Tagliapietra et al. 2019), and therefore their relevance as vectors is minor. Interestingly, its congeneric *An. atroparvus* was spotted in a region near the Basque Country territory (Gonzalez et al. 2020, Ruiz-Arrondo et al. 2019). On the other hand, *Cx. pipiens* s.l. is a well-distributed species in the Iberian Peninsula, where it causes a notable overnight biting nuisance in humans. The recent outbreak of West Nile virus (WNV) that caused 77 cases and seven deaths in southern Spain was associated with *Cx. pipiens* and/or other *Culex*-related species (García San Miguel et al. 2021). Based on the climatic conditions and the human-activity-related environmental predictors, *Cx. pipiens* seems to be less abundant in northern Spain (Gangoso et al. 2020). Our study showed the widespread presence of *Cx. pipiens* s.l. in most of the sampled habitats. Traditionally, the *Cx. pipiens pipiens* form is commonly found in above-ground and rural habitats, whereas the *Cx. pipiens molestus* form is usually found in underground urban environments (Becker et al. 2012, Byrne and Nichols 1999, Farajollahi et al. 2011). However, in our study there was a clear dominance of *Cx. pipiens pipiens* in the urban, rural, and natural habitats, with a lower representation of hybrids and the absence of *Cx. pipiens molestus*, as previously reported in the same territory (Gonzalez et al. 2020, 2021). The few analyzed specimens from the underground sewer systems were also identified as *Cx. pipiens pipiens*. Further studies on the habitat preferences of the different subspecies of this species complex would help to clarify the proportion of each ecological form. The broad habitat diversification of *Cx. pipiens* s.l. poses an epidemiological threat for the inhabitants of this region.

The R index is widely used as a measure in several ecological studies, especially for the preference of biotopes and mosquito infestation patterns. The highest R index, species richness, and abundance were recorded in the category of PU. This type of temporary habitat was very suitable for nine mosquito species and represented the 25% of the total collections. Within this category, margins of watercourses, riverbeds, muddy puddles, and water obstacles were widely used by *Culex* species. Despite not being reflected in field-caught collections, the most productive habitats (>100 larvae/10 min sampling) were an irrigation channel with slow running water and the margin of a stream, both highly infested by *Cx. pipiens* s.l.. The lowest R index, species richness, and abundance were unvegetated artificial ponds and irrigation rafts, which seemed to be largely inappropriate for immature instars. This is relevant, as public artificial ponds are part of the landscape of urban and peri-urban settings. The presence of predators, as well as the lack of vegetation that would serve as refuge and protection from predation, might explain the absence of mosquito immature stages in those settings (Bergey et al. 1992, Bond et al. 2005, Orr and Resh 1989). Even though tree-cavities and other phytotelmic environments might be favorable as rearing sites to both native and non-native species (Bueno-Marí et al. 2016), phytotelmata (mostly leaves of *Dipsacus fullonum* forming a cup-like depression) did not harbor mosquitoes in our study. However, this type of larval habitat was scarcely represented (n = 3), thus, further studies are needed to confirm the current observations.

Our analysis revealed seasonal variation in mosquito collections, with the warmer season (2nd study period) having higher species diversity, abundance of mosquitoes, and R index compared to the 1st study period. The distribution and dynamics of mosquitoes is a very complex interaction system that relies on several biotic components (i.e., wind speed, temperature, rainfall, relative humidity, solar radiation, photoperiod, evapotranspiration, land cover, vegetation type, etc.) and abiotic (physical and chemical conditions) factors. One possible explanation for the higher mosquito collection is the extra 6° during the second study period, since higher temperatures favor mosquito abundance (Bravo-Barriga et al. 2017, Gardner et al. 2012, Roiz et al. 2014). However, there are also other plausible explanations, such as the modification of the environment during the summer season, e.g. the water flow in streams and/or channels becomes slower offering more suitable lentic habitats. Moreover, the higher solar exposure boosts the growth of floating algae, then the site becomes more suitable for some mosquito species (*Anopheles* spp.) (Bergey et al. 1992). These observations suggested that any action towards mosquito control may have to be intensified during the summer period. The environmental conditions between both study periods could be more pronounced in rural and natural habitats than in urban habitats. However, although occurrence probability was higher in rural and natural habitats compared with urban ones, none of the best multivariate models included the interaction between habitat and study period.

There are a few limitations to be taken into account for future studies. A high level of mortality was observed in immature stages inside the breeders (ca. 37% did not reach adult stage) despite having kept the larvae in the water of the habitat itself to provide nutrients and microorganisms, such as bacteria and algae (Goselle et al. 2018). We also noticed that some highly polluted water substrates decomposed quickly, causing the sudden death of the immature instars. Secondly, it is well-known that some genera such as *Culiseta* are voracious feeders of other younger instars of different mosquito species (Shaalan 2012). Particularly younger preys were usually more susceptible to predation, so this phenomenon might explain also the high mortality observed inside breeders. Overcrowding can decrease fitness (Kauffman et al. 2017)

and depredation is expected to be higher in reduced spaces (Ramos de Andrade 2015 Ph.D. thesis). Alternatively, species identification based on larvae (L3-L4 stage) could be an option despite its complexity and the requirement of entomological experts (Nebbak and Almeras 2020). Finally, even though dipping can be used as an easy tool for the determination of mosquito abundance and species richness, the extensive number of dipping techniques available together with other variables such as the target species, water depth, presence of vegetation or debris, time of sampling, number of dips, and the volume of water extracted makes this method a non-standardized tool that hampers the comparison of quantitative data among studies.

We updated the biodiversity of mosquitoes in Araba (northern Spain), a territory where until recently, Culicidae species had not been considered a threat. It is necessary to promote biological studies, not only to broaden the faunistic knowledge of this important Diptera group, but also to detect the establishment of new invasive species and to elaborate predictive maps of epidemiological interest. The surveillance of these disease vectors of major public health significance is essential for the prevention and control of emerging and re-emerging zoonoses in Europe such as malaria, dengue or West Nile fever.

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