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Mosquito community composition in two major stopover aquatic ecosystems used by migratory birds in northern Spain

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Abstract

Mosquitoes (Diptera: Culicidae) are common bloodsucking Diptera frequently found in aquatic environments, which are valuable ecosystems for many animal species, particularly migrating birds. Therefore, interactions between these animal species and mosquitoes may play a critical role in pathogen transmission. During 2018–2019, mosquitoes were collected from two aquatic ecosystems in northern Spain using different methodologies and identified using classical morphology and molecular tools. A total of 1529 males and females of 22 native mosquito species (including eight new records for the region) were trapped using CO_2-baited Centers for Disease Control and Prevention (CDC) traps and sweep netting. Among the blood-fed female mosquitoes, 11 vertebrate host species—six mammals and five birds—were identified using DNA barcoding. The developmental sites of eight mosquito species were determined across nine microhabitats, and 11 mosquito species were caught landing on humans. The flight period varied among mosquito species, with some peaking in the spring and others in the summer. Our study highlights the advantages of mosquito sampling using various techniques to comprehensively characterise species composition and abundance. Information on the trophic preferences, biting behaviour and influence of climatic variables on the ecology of mosquitoes is also provided.

KEYWORDS
aquatic ecosystems, blood-meals, diversity, flight activity, host feeding preferences, mosquitoes

INTRODUCTION

Mosquitoes are bloodsucking Diptera of public health relevance because of their capacity to transmit pathogens to humans, wildlife and livestock. The emergence or re-emergence of several pathogens and diseases in Europe, including malaria, dengue, chikungunya, zika, West Nile (WN) and Usutu (Brugueras et al., 2020; Calzolari, 2016; García San Miguel Rodríguez-Alarcón et al., 2021; Vilibic-Cavlek et al., 2019), highlights the need to study mosquito species as potential vectors of the aforementioned pathogens in different scenarios, including natural habitats.

Wetlands are valuable environments that have gained interest because of the growing concern about mitigating climate change and preserving the biodiversity of fauna and flora. These strategies aim to expand these aquatic habitats to reduce the impact of rising sea levels and extreme flooding events, as well as restore drained wetlands worldwide (Dale & Knight, 2008; Medlock & Vaux, 2015a). However, these biotopes provide a wide variety of highly suitable larval sites for the development of immature stages of mosquitoes, which can have substantial health impacts on humans (Hawkes et al., 2020). In Europe, flooded ecosystems, such as freshwater habitats, can favour a massive

Mikel A. González and Fátima Goiri contributed equally to this work.

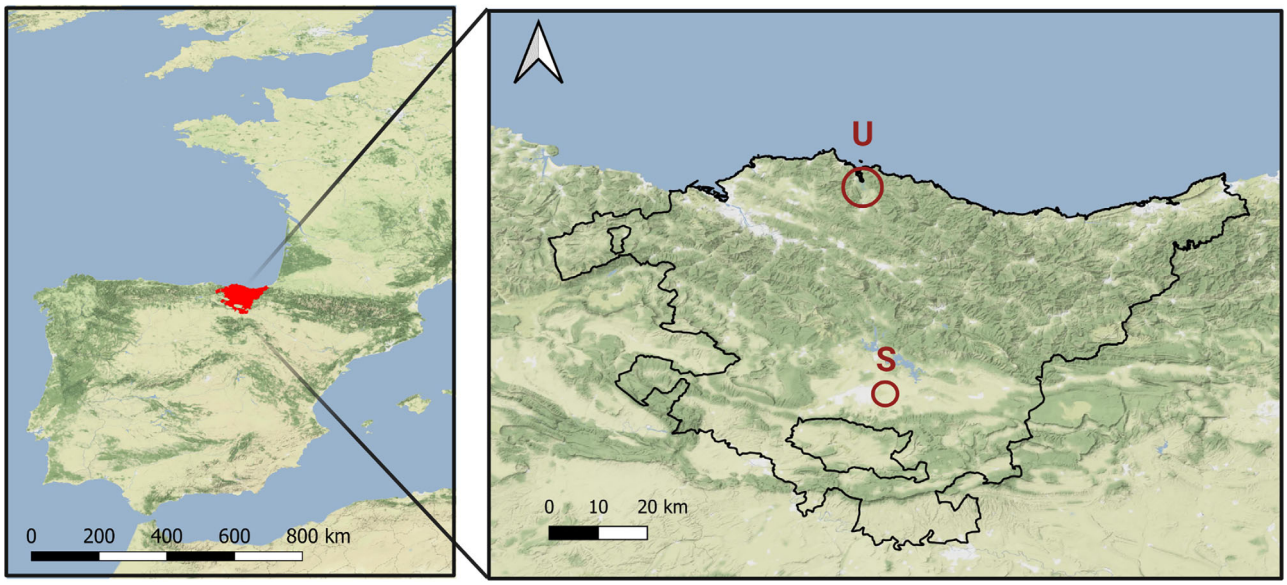

FIGURE 1 Location of the sampling sites. Map of the Basque Country (northern Spain) indicating the two aquatic sites: Urdaibai (U) and Salburua (S).

increase in endemic mosquito species, such as *Aedes caspius*, *Ae. vexans* and *Ae. sticticus*, after natural or unnatural flooding events, which cause severe human nuisance (Dale & Knight, 2008; Medlock & Vaux, 2015a). Moreover, these environments are used by native and migratory birds as resting places in their migratory routes and therefore constitute potential areas of local or allochthonous zoonotic pathogen transmission (Dale & Knight, 2008; Jourdain et al., 2007; Roiz et al., 2015). The study of mosquito diversity and abundance is particularly interesting in freshwater settings (Roiz et al., 2012; Roiz et al., 2015), as many wetland habitats are visited by several bird species susceptible to carrying or transmitting the WN virus (Jourdain et al., 2007), such as waterfowl, storks, raptors or passerines, among others. Studying the host-feeding habits of mosquitoes is therefore a critical step in understanding host-vector-parasite pathways, the role of the fauna as possible reservoirs of pathogens, and their transmission patterns (Rizzoli et al., 2015). Mosquitoes can have either a general preference or feed on a particular group of animals (Brugman et al., 2017); thus, the studies aiming to identify the host-feeding habits of potential vector species have a significant impact in evaluating mosquito vectorial capacity and understanding the transmission cycles.

In Europe, wetland ecosystems have been studied in relation to bloodsucking Diptera, their potential risk of spreading mosquito-borne pathogens, and the impact they can have on these natural landscapes. Considering that several mosquito species can successfully transmit certain pathogens, monitoring of species present in these areas is of great importance for health surveillance and the implementation of appropriate management, if necessary, of these aquatic environments, so that potential disease risk can be balanced against the many environmental and societal benefits of wetlands (Hawkes et al., 2020). Therefore, in this study, we aimed to assess the community composition of mosquitoes in two aquatic ecosystems in northern Spain using different approaches for the collection of both adults and immature stages. Classical morphology, together with mitochondrial cytochrome oxidase subunit 1 (COI) gene-based DNA barcoding tools, was used to identify mosquito species. Factors affecting abundance, as well as their host-blood meal sources, were investigated.

MATERIALS AND METHODS

Study area

The study was conducted in two aquatic environments in the Basque Country (northern Spain) (Figure 1). The wetlands of Salburua (Alava Province; 42°51′ N, 02°39′ W; 512 m above sea level) and the marsh of Urdaibai (Biscay Province; 43°22′ N, 02°40′ E; 2 m above sea level) are two protected areas that provide crucial wintering and migratory resting places for a wide range of bird species. Both environments are frequently visited by students in schools, walkers and ornithologists.

Salburua is a natural wetland formed by the emergence of subterranean water from a Quaternary aquifer. It extends over 2.2 km^2 and includes two main shallow water reserves of freshwater surrounded by meadows and a small oak grove. The wetland is partially bordered by a residential district of approximately 7000 residents. Wetlands are predominant habitats for numerous wildlife species. The most frequent potential host communities are birds (Anseriformes, Gruiformes, Passeriformes and Ciconiiformes), mammals (Carnivora and Artiodactyla) and walkers accompanied by dogs. The climate oscillates during the year, with a dry and hot summer and a wet and cold winter; consequently, the lagoons are full during the winter and dry gradually in the summer (Frías-Sáez, 2022). During the course of the year, the temperature generally varies from 1°C to 26°C and rarely drops below −4°C or rises above 34°C.

The marsh in Urdaibai is located in the Bay of Biscay. It covers a vast area of approximately 220 km^2 and consists of marshlands, meadows, green oak forests and leafy wood. Twelve municipalities are integrated into this territory, with approximately 45,000 inhabitants. This highly preserved environment hosts hundreds of vertebrate species, including birds (Anseriformes, Gruiformes, Passeriformes and Podicipediformes), mammals (Carnivora, Artiodactyla and Perissodactyla) and humans (ornithologists and walkers). It has a mild temperate and humid climate with frequent rainfall, even during the summer. During the year, the temperature generally varies from 6°C to 24°C and rarely falls below 1°C or rises above 28°C. The marsh remains flooded throughout the year, with periodic oscillations during the summer. Depending on the location, the marsh water is either fresh, salty or mixed (Hernández & Caño, 1999).

Sampling approach

Two sampling sites were selected in each natural area in 2018 and three in 2019 as previously described (González et al., 2022). Briefly, the collection sites consisted of woodlands, pastures and vegetated ecosystems with permanent and temporary water bodies (lagoons, lakes, pools, ponded streams, slow flowing streams and puddles). In 2018 (July–October, 8 samplings at each site) and 2019 (May–October, 13 samplings at each site), mosquitoes were captured using CO_2-baited Centers for Disease Control and Prevention (CDC) traps and a sweep net. In addition, host-seeking female mosquitoes approaching the operator were collected to identify human landing species. In 2019 (May–October), mosquito larval sites were examined to collect immature stages in the vicinity of the CDC traps.

Carbon dioxide (CO_2)-baited CDC-traps and sweep net

CDC miniature traps (model 1212, John Hock, Gainesville, FL) equipped with incandescent light and baited with approximately 1.5 kg of dry ice (CO_2) were used. The traps were deployed at each site fortnightly for 24 h (one CDC trap per sampling site, set early in the morning and recovered the next day), suspended on tree branches at 1–1.5 m, and protected against sunlight and wind exposure. After placing CDC traps, Culicidae resting on the vegetation were collected with a polyester long-handled net (38 cm diameter, 0.8 mm mesh size, Bioquip, Compton, CA) between 09.30 and 11.00 a.m. The net was swept for approximately 4 min over the surface of herbaceous and shrubby vegetation around a 25 m radius area from each CDC-trap position. The collected mosquitoes were transported to the laboratory and immediately stored at −20°C to kill them before morphological identification.

Human-landing catches (HLC)

Host-seeking female mosquitoes landing on the operator were collected by aspiration (pooter-style aspirator, Bioquip, Compton, NC). An operator in the upright position collected mosquitoes from both the exposed and non-exposed human parts. HLC was conducted for 15 min during the morning period (10.00 a.m.–12.00 p.m.) near the CDC traps at the same dates as the examination of larval breeding sites. The catches were stored dry at −30°C until further morphological identification.

Collection of immature stages

Potential larval development sites, including stagnant water ditches, ponded streams, puddles, artificial containers, the edges of water bodies and aquatic shallow areas, were sampled around a 200 m radius for each CDC trap position. The selected larval habitats were systematically sampled monthly by dipping (600 ml cup dipper) multiple times for 15–20 min in the morning, which is considered an acceptable effort for the active search, collection of immature stages and suitable estimation of the mosquito community (Bueno-Marí, 2010; González, Cevidanes, et al., 2021). In addition, in those environments with aquatic vegetation (i.e., *Typha* sp.), roots were manually extracted and shaken vigorously with water on a white tray to dislodge attached larvae of the genus *Coquillettidia* (Batzer, 1993). A maximum of five root samples were extracted per date/habitat. In the field, the collected material was transferred into 500 ml plastic flasks with its own water (with aquatic vegetation, if needed) and transported to the laboratory as soon as possible. Subsequently, the immature stages were transferred to mosquito breeders (Bioquip, Compton, CA) and reared for a maximum of 3 weeks (fish food was provided in low amounts) at room temperature (20–22°C) and low light incidence to obtain adult mosquitoes. The upper portions of the mosquito breeders with emerged adults were stored at −20°C for further morphological identification.

Morphological identification of mosquitoes

In the laboratory, mosquitoes were separated by sex and females were classified according to their physiological status (blood-fed, gravid and unfed). Species identification was based on the morphological features of the female and male genitalia using taxonomic keys (Becker et al., 2020; Schaffner et al., 2001). For further identification of blood-meal host species, abdomens of blood-engorged and gravid females were removed and individually stored in 2 ml screw-top vials at −20°C after being classified according to the scale of Sella (II–VI) by observation of the degree of digestion of the blood and gravid stage (containing eggs) (Detinova, 1962).

Molecular analysis

Molecular identification of mosquitoes

Damaged or morphologically indistinguishable mosquito specimens ($n = 104$; 56.7% in Urdaibai and 43.3% in Salburua) were identified

via molecular methods using the primers described by Delgado-Serra et al. (2021) and a PCR targeting the COI gene (Hernández-Triana et al., 2017). After DNA extraction from mosquito thorax using a commercial kit (QIAamp DNA Mini kit, Qiagen, Hilden, Germany), PCR amplicons were purified using ExoSAP-IT (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania) and subjected to Sanger sequencing (Eurofins Genomics, Germany). The sequences obtained were analysed using BioEdit software (v.7.2.5) (Hall, 1999) and compared with the GenBank database by nucleotide sequence homology searches at the network server of the National Center for Biotechnology Information using the BLAST tool. Nucleotide sequence type (ntST) evaluation was carried out using DnaSP 6, and a phylogenetic tree was constructed by Neighbour-Joining (based on Kimura 2-parameter genetic distance model) with MEGA 6.0, resampled 1000 times to generate bootstrap values (Tamura et al., 2013).

DNA of those specimens identified as *Anopheles maculipennis* s.l. upon sequencing ($n = 6$) was submitted to a PCR-RFLP assay targeting polymorphisms of the internal transcribed spacer 2 (ITS-2) of the ribosomal DNA (Vicente et al., 2011) to identify individual members of the *An. maculipennis* complex. Specimens identified as *Culex pipiens* s.l. upon sequencing ($n = 22$) were analysed using a PCR targeting the flanking region of the CQ11 microsatellite (Bahnck & Fonseca, 2006) to identify the *Cx. pipiens* female form (*Cx. pipiens pipiens*, *Cx. pipiens molestus* and hybrids).

Host blood-meal analysis

Vertebrate host species of blood-fed ($n = 39$) and gravid ($n = 14$) females were investigated at the Centre for Biodiversity Genomics, University of Guelph (Guelph, ON, Canada). The abdomen was removed for DNA purification (Ivanova et al., 2006), and mosquito species identification was performed by amplification of the COI gene (Hernández-Triana et al., 2014). The amplicons were submitted for Sanger sequencing as described in Estrada-Franco et al. (2020) and González et al. (2020). Host feeding patterns were identified using a metabarcoding-like approach with next-generation sequencing (NGS) technology as previously described (Estrada-Franco et al., 2020; González et al., 2020). The raw sequence reads were filtered and cleaned. Reads passing these filters were clustered into operational taxonomic units (OTUs) with 97% identity and a minimum of 10 reads per OTU. Each OTU sequence was compared using the BLAST tool and BOLD systems, which are composed of global vertebrate COI sequences. Identification was considered valid only when the query sequence matched the reference sequence with at least 95% nucleotide identity. Detailed specimen records and sequence information were uploaded to the Barcode of Life Database (BOLD-http://www.boldsystems.org) and can be found within the Working Group 1.4 Initiative 'Human Pathogens and Zoonoses' container. The digital object identifier (DOI) for publicly available projects in BOLD is doi:dx.doi.org/10.5883/DS-MQBMBC.

Data analysis

Statistical analyses were performed using the R statistical software version 3.6.1 (R Core Team, 2019). Generalised linear models (GLM) were used to compare mosquito catches by sampling strategies (CDC traps vs. sweep net). Multivariate GLM were used to evaluate the associations between culicid abundance (catch/trap/night) related to year (2018 and 2019), months of sampling (July–October, shared period for both years) and aquatic setting (Salburua and Urdaibai). Owing to data overdispersion, negative binomial generalised linear models (NBGLM) were applied (O'Hara & Kotze, 2010) using the MASS package (Venables & Ripley, 2002). The best model was selected with the 'MuMIn' package using the 'dredge' function (Barton, 2020), which is based on the Akaike Information Criterion and corrected to the sample size (AICc). The overall fit of the model was evaluated using the likelihood ratio test to compare the best model with the null model. Species richness (S) and Shannon-Wiener's diversity index (H′) were calculated to compare biodiversity among wetlands, using the 'diversity' function of the R package 'vegan'. Due to the fact that not all the specimens were analysed to identify the species within the *An. maculipennis*, *Anopheles claviger* and *Cx. pipiens* complexes, each complex was considered a species group for the analysis of diversity. Graphical representation of seasonality in mosquito population flight periods was carried out using catches obtained in 2019 using CDC traps, comprising a six-month sampling period (May–October).

The mean climatic variables (mean, maximum and minimum temperatures [°C], relative humidity [%], and precipitation [mm]) were retrieved for 7, 14, 7–14, and 7–21 days before each sampling night from the meteorological stations of Arkaute (42°51′15.4″N, 2°37′18.6″W) and Gautegiz-Arteaga (43°20′49.2″N, 2°39′25.7″W), located less than 2 km from sampling points in Salburua and Urdaibai, respectively. These variables were evaluated for collinearity using correlograms and the Spearman's correlation test. Associations between mosquito abundance and climatic variables were analysed using generalised additive models (GAM). These models can fit data with non-linear relationships using spline smoothing operators and have been broadly used to study species distribution and environmental conditions, for example, in vector ecology studies (Roiz et al., 2015). A negative binomial distribution was assumed in the GAM analysis to deal with overdispersion. The percentage of the explained deviance was estimated for the best model. The explained deviance is equivalent to the unadjusted R^2 for non-Gaussian families and is an indicator of the explanatory power of the model (Zuur, 2009).

RESULTS

Species composition and abundance

A total of 1529 adult mosquitoes (254 males and 1275 females) were collected from the two aquatic environments of Basque Country (northern Spain) using CO_2-baited CDC traps and sweep netting. A

TABLE 1 Adult mosquitoes (Diptera: Culicidae) trapped with CDC traps baited with CO_2 (CDC) and sweep netting (SN) in 2018 and 2019 in the two aquatic settings from northern Spain.

	Salburua				Urdaibai				Total	
Culicidae species	**CDC**	**SN**	**Total**	**%**	**CDC**	**SN**	**Total**	**%**	**Total**	**%**
Ae. cantans	75	14	89	17.5	0	1	1	0.1	90	5.9
Ae. caspius	0	0	0	0	34	29	63	6.2	63	4.1
Ae. detritus	0	0	0	0	43	68	111	10.9	111	7.3
Ae. rusticus	174	46	220	43.3	0	0	0	0	220	14.4
Ae. sticticus	3	0	3	0.6	0	0	0	0	3	0.2
Ae. vexans	1	0	1	0.2	0	0	0	0	1	<0.1
An. claviger s.l.	46	5	51	10.0	53	5	58	5.7	109	7.1
An. maculipennis s.l.	4	1	5	1.0	6	1	7	0.7	12	0.8
An. plumbeus	0	0	0	0	1	0	1	0.1	1	<0.1
Cq. buxtoni	0	0	0	0	98	18	116	11.4	116	7.6
Cq. richiardii	19	0	19	3.7	253	107	360	35.3	379	24.8
Cs. annulata	15	3	18	3.5	16	5	21	2.1	39	2.6
Cs. fumipennis	2	0	2	0.4	3	0	3	0.3	5	0.3
Cs. litorea	2	1	3	0.6	1	0	1	0.1	4	0.3
Cs. morsitans	0	0	0	0	10	6	16	1.6	16	1.0
Cs. subochrea	1	0	1	0.2	0	0	0	0.0	1	<0.1
Cx. hortensis	0	1	1	0.2	0	0	0	0	1	<0.1
Cx. modestus	1	0	1	0.2	140	2	142	13.9	143	9.4
Cx. pipiens s.l.	71	14	85	16.7	93	10	103	10.1	188	12.3
Cx. territans	0	2	2	0.4	2	7	9	0.9	11	0.7
Cx. theileri	1	2	3	0.6	0	0	0	0	3	0.2
Cx. torrentium	0	0	0	0	1	2	3	0.3	3	0.2
Not identified	1	2	3	0.6	3	3	6	0.6	9	0.6
Total	416	92	508		757	264	1021		1529	

total of 32 and 78 CDC trap/night contents were examined in 2018 and 2019, respectively. Twenty-two native mosquito species: six *Culex* spp., five *Culiseta* spp., three *Anopheles* spp., six *Aedes* spp. and two *Coquillettidia* spp. were identified (Table 1). Overall, the most abundant species was *Coquillettidia richiardii* ($n = 379$, 24.8%), followed by *Aedes rusticus* ($n = 220$, 14.4%), *Cx. pipiens* s.l. ($n = 188$, 12.3%) and *Culex modestus* ($n = 143$, 9.4%) (Table 1). The species composition varied depending on the aquatic setting, and both habitats shared 38.1% of the mosquito species captured by CDC traps. *Aedes rusticus* was the most abundant species in Salburua (43.3% of the total catches), whereas *Cq. richiardii* was the predominant species in Urdaibai (35.3%). Molecular analysis of 104 female mosquitoes generated 94 barcoding COI sequences of 405–514 bp in length with 98.7% to 100.0% homology when compared with GenBank sequences. A selection of these sequences ($n = 87$ and 17 species) was deposited in GenBank under accession numbers OQ361883 to OQ361929, and a phylogenetic tree was constructed (Figure S1).

Two species within the *An. maculipennis* complex (*An. atroparvus* in Urdaibai, $n = 4$, and *An. maculipennis* s.s. in Salburua, $n = 2$) and three ecoforms of *Cx. pipiens* s.l. (*Cx. pipiens pipiens*, $n = 19$; *Cx. pipiens molestus*, $n = 2$; and *Cx. pipiens* hybrids, $n = 1$) were determined using molecular methods.

Assessment of adult mosquito trapping

CO_2-baited CDC traps collected 1173 specimens (4.6% males and 95.4% females), whereas netting on vegetation retrieved only 356 specimens (56.2% males and 43.8% females). Although CDC traps collected approximately three times more specimens than the sweep net, these differences were not significant in the overall mosquito catches ($p = 0.83$) or in the case of species such as *Cq. richiardii*, *Ae. rusticus or Cx. pipiens* s.l. However, netting was more effective for collecting *Culex territans* and *Ae. detritus* ($p < 0.001$ and $p < 0.05$, respectively), while CDC-traps were more effective for collecting *Cx. modestus* and *An. claviger* s.l. ($p < 0.01$ and $p < 0.05$, respectively) (Table 1). Furthermore, male mosquito catches were significantly higher for sweep nets than CDC traps ($U = 1213$, $p < 0.001$). Blood-fed mosquitoes ($n = 53$) were trapped by CDC traps (31.4%) as well as sweeping (68.6%).

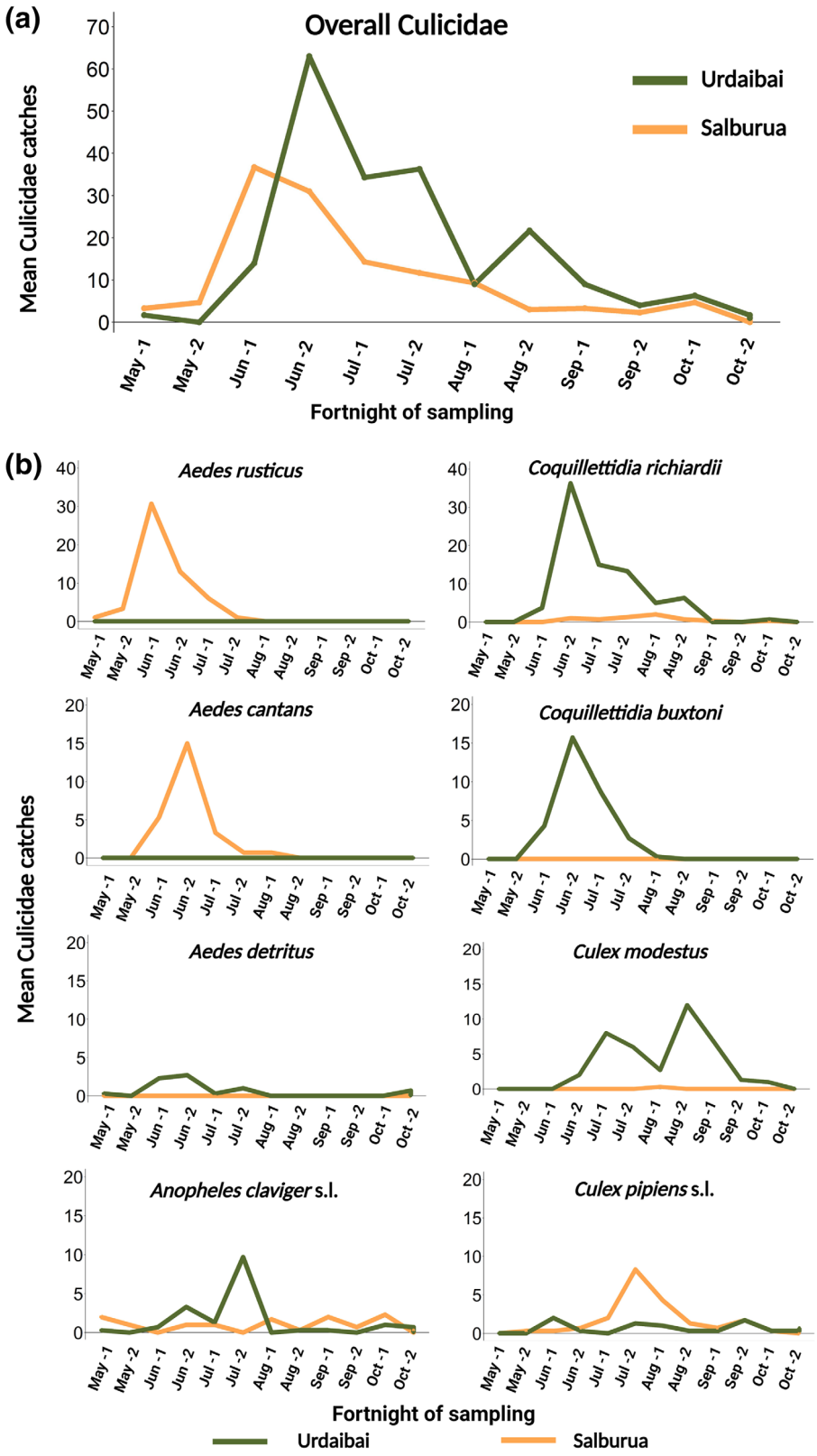

FIGURE 2 Seasonal flight activity of Culicidae species at both aquatic environments in 2019. (a) overall Culicidae and (b) most abundant species.

Seasonal activity

The flight-period activity of the mosquito species extended over the entire sampling season (May–October 2019) (Figure 2). Overall, the population dynamics of most mosquito species showed a single large peak of activity, followed by a smaller peak in some species. In Salburua, mosquitoes peaked in the first half of June (1st–15th June), driven mainly by the emergence of *Ae. rusticus* and the start

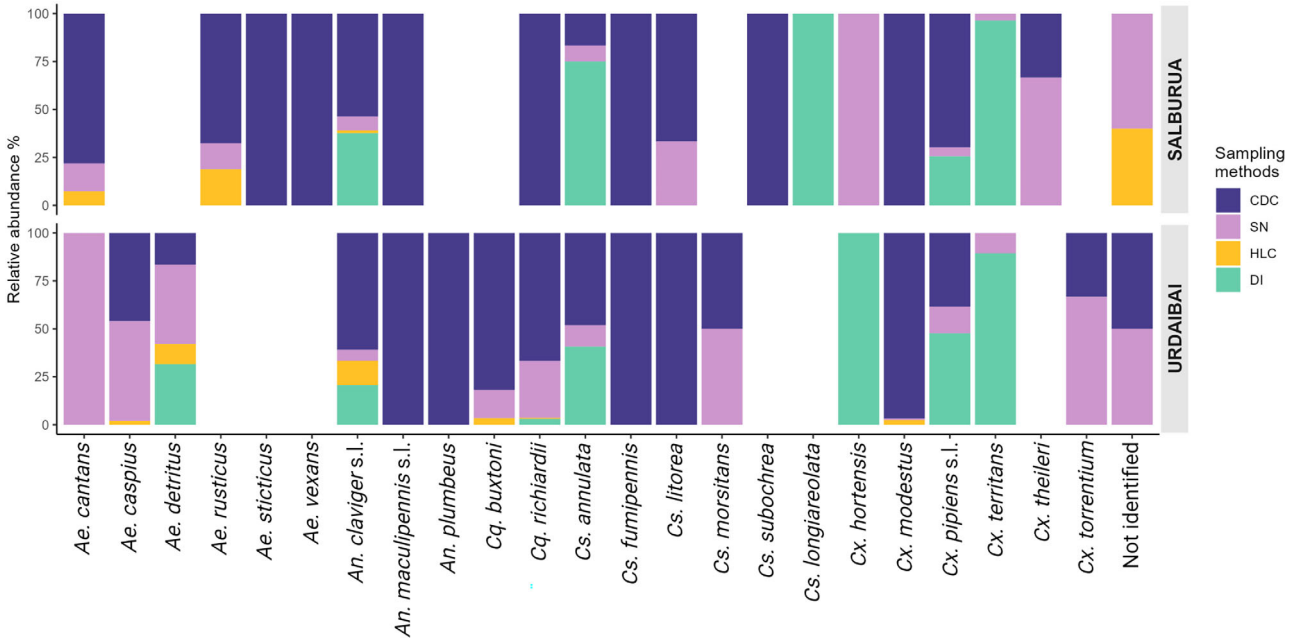

FIGURE 3 Relative abundance of different mosquito species trapped with the four sampling methods during 2019 in both aquatic ecosystems. CDC, CDC light traps baited with CO_2; SN, sweep netting; HLC, human landing catches; DI, dipping.

of the season of *Ae. cantans* (Figure 2). Following this period, the mosquitoes were active but at lower emergence levels. In Urdaibai, two peaks (bimodal pattern) were observed, with the highest abundance of mosquitoes occurring in the second half of June (16th–30th June), driven by *Coquillettidia* spp., followed by a minor peak in the second half of August (16th–31st August), driven by *Cx. modestus* (Figure 2).

Human landing catches (HLC)

A total of 85 host-seeking females of eight species were caught landing at the human operator (Figure 3; Table S1). *Aedes rusticus* was the species more commonly collected on humans ($n = 46$; 54.1% of the total catches), followed by *An. claviger* s.l. ($n = 11$, 12.9%), *Aedes detritus* ($n = 8$, 9.4%) and *Aedes cantans* ($n = 7$, 8.2%). Host-seeking females in Salburua showed a maximum peak in activity in May ($n = 25$), followed by a progressive decrease during the study period (June, $n = 19$; July, $n = 9$; August, 0 catches; September, $n = 2$; and October, 0 catches). In contrast, host-seeking females in Urdaibai showed consistent activity from May to September (May, $n = 4$; June, $n = 4$; July, $n = 7$; August, $n = 4$; September, $n = 9$; and October, $n = 1$). A higher number of mosquitoes landing on the operator was observed in Salburua (56 catches) than in Urdaibai (29 catches). Some of these anthropophilic mosquito species were not found at the breeding sites (*Ae. rusticus*). Conversely, some mosquito species abundant at the larval sites (*Cx. pipiens* s.l., *Cx. territans*, *Cx. hortensis* and *Cs. annulata*) were not caught landing on the operator (Figure 3; Table S1).

Larval rearing sites

The developmental sites of eight mosquito species ($n = 130$ in Salburua and $n = 204$ in Urdaibai) were identified across the nine microhabitats inspected (Table 2). The most abundant species was *Cx. territans* ($n = 112$), followed by *Cx. pipiens* s.l. ($n = 53$) and *An. claviger* s.l. ($n = 44$). Within natural habitats, vast permanent pools were particularly productive to *Cs. annulata*, *An. claviger* and *Cx. territans*, whereas *Culex*, *Anopheles*, and *Aedes* species preferred temporary pools, ephemeral puddles or pools and ponded ditches or streams, respectively (Table 2). *Coquillettidia richiardii* was successfully collected in *Typha* roots in the marsh, but they were unable to reach the adult stage (Table 2). Artificial ponds and troughs are prone to host artificial container breeding species, such as *Cx. pipiens* s.l., *Culiseta longiareolata* and *Culex hortensis*. Notably, *Cs. longiareolata* was not captured with CDC traps or by net sweeping in Salburua; however, a few specimens were observed at the larval sites (Figure 3; Table S1). Mosquito immature stages were active during the six-month trapping period; however, the inconsistency of the water levels (flushing and drainage) prevented further analysis.

Analysis of diversity and variables affecting mosquito abundance

The Shannon Index (H′) value for Salburua was 1.83 and for Urdaibai was 2.09, while the species richness (S) for both sites was 17. Multivariate models revealed that total mosquito abundance was positively

TABLE 2 Summary of the mosquito species collected by dipping in both natural and artificial aquatic environments during the year 2019 (May–October) in northern Spain.

		Type of breeding site[a]									
Aquatic ecosystem	**Species**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**Total**
Salburua	*An. claviger* s.l.	3	0	0	9	16	-	-	-	-	**28**
	Cs. annulata	0	0	14	5	8	-	-	-	-	**27**
	Cs. longiareolata	0	0	2	0	0	-	-	-	-	**2**
	Cx. pipiens s.l.	0	8	11	1	2	-	-	-	-	**22**
	Cx. territans	0	0	26	10	15	-	-	-	-	**51**
Urdaibai	*Ae. detritus*	-	-	-	-	-	22	24	0	0	**46**
	An. claviger s.l.	-	-	-	-	-	15	1	0	0	**16**
	Cq. richiardii[b]	-	-	-	-	-	0	11	0	0	**11**
	Cs. annulata	-	-	-	-	-	11	0	0	0	**11**
	Cx. hortensis	-	-	-	-	-	0	0	0	28	**28**
	Cx. pipiens s.l.	-	-	-	-	-	3	28	0	0	**31**
	Cx. territans	-	-	-	-	-	41	17	3	0	**61**
	Total	**3**	**8**	**53**	**25**	**41**	**92**	**81**	**3**	**28**	**334**

[a]Sites A–E = (A) ditch, (B) artificial pond, (C) puddle, (D) forest temporary pool, (E) river edge. Sites F–I = (F) diverse pools (shallow pools in the woodland and pools with vegetated margins in open pastures) (G) natural shallow channels of water (maximum width and depth 2 and 0.4 m, respectively), (H) open-marsh with *Typha* sp., (I) artificial container.
[b]Death larvae were also counted.

TABLE 3 Summary of best negative binomial regression models for total number of Culicidae and species richness per trap and night. The variable 'year of sampling' was not selected for the best models.

	Abundance per trap/night		
Variables	**Est ± SE**	***z***	***p*-value**
Water setting			
Salburua	Ref.		
Urdaibai	1.10 ± 0.26	4.24	<0.001
Month of sampling			
July	Ref.		
August	−0.81 ± 0.33	−2.40	0.016
September	−1.43 ± 0.35	−4.09	<0.001
October	−1.98 ± 0.36	−5.52	<0.001

Note: Est ± SE = estimate ± standard error; z = statistic *z*-value; *p* = *p*-value; Ref. = reference category.

associated with the water ecosystem, with the total abundance of mosquitoes in the marsh of Urdaibai being significantly higher than that in the wetland of Salburua (Table 3). Moreover, the number of mosquito catches was positively associated with the sampling period, with high catches at the beginning of summer and decreasing progressively during the following months (Table 3).

Regarding the climatic variables analysed, the full model was obtained using collinearity analysis fitted with the mean temperature, relative humidity and precipitation. Generally, these climatic variables explained a high percentage of the deviance of overall mosquito abundance in Salburua (Table 4), whereas in Urdaibai, the explanatory power of these climatic variables was lower. The best model for *Ae. rusticus* was based on climatic data 14 days before sampling, which explained 53.5% of the deviance and depended mostly on mean temperatures ($p = 0.0052$) and precipitation ($p = 0.0049$). For *Ae. cantans*, the model that considered the interval 7–14 days before sampling explained 72% of the deviance, with relative humidity ($p = 0.0014$) and precipitation ($p = 0.0042$) being the more significant variables compared with mean temperatures ($p = 0.0236$). For *Coquillettidia* spp., the best models were built with observations in the interval 7–21 days before sampling, and the percentage of deviance explained in the models was 35.0% and 41.8% for *Cq. richiardii* and *Cq. buxtoni*, respectively.

Relative humidity was the primary factor for both *Cq. richiardii* ($p = 0.0121$) and *Cq. buxtoni* ($p = 0.0119$); however, for *Cq. buxtoni*, precipitation was also a significant variable ($p = 0.0418$). These results suggest that for *Aedes* spp., the mean temperature was a consistently significant variable for all time periods, whereas for *Coquillettidia* spp. it was not significant.

Host DNA blood-meals

Among the 53 female mosquitoes analysed (39 blood-fed and 14 gravid females), 47 yielded a COI DNA barcode sequence and host DNA sequences were retrieved from 32 mosquito specimens (60.4% yield). Thus, high success in host DNA identification was recorded in blood-fed female mosquitoes, regardless of their blood digestion status (Sella scale 2: 15/15 [100%]; scale 3: 2/3 [67%]; scale 4: 3/4 [75%]; scale 5: 3/4 [75%]; and scale 6: 6/13 [46%]). The host DNA was successfully identified in only two of the 14 (14%) early gravid

TABLE 4 Results of the models (generalised additive models) for mosquito abundance in the wetlands with regard to climatic variables.

		Independent climatic variables					
		Mean temperature		Relative humidity		Precipitation	
Dependet variables	Deviance explained (%)	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
7 days before sampling							
Overall (Urdaibai + Salburua)	9.03%	1.681	0.1976	1.045	0.2986	2.86	0.0936
Overall (Urdaibai)	19.00%	0.49	0.676	1.536	0.327	0.829	0.346
Overall (Salburua)	52.40%	3.632	0.0132*	2.934	0.0126*	1.064	0.308
Ae. cantans (Salburua)	60.40%	7.178	2.13e-05***	2.368	0.0716	8.821	0.0048**
Ae. rusticus (Salburua)	50.70%	2.886	0.0340*	2.688	0.0207*	0.945	0.3363
Cq. buxtoni (Urdaibai)	4.03%	0.018	0.971	0.192	0.674	0.72	0.4
Cq. richiardii (Urdaibai)	11.50%	0.296	0.718	0.551	0.545	0.622	0.424
14 days before sampling							
Overall (Urdaibai + Salburua)	8.47%	0.009	0.9243	1.445	0.2556	5.684	0.0189*
Overall (Urdaibai)	27.80%	0.46	0.6888	1.352	0.2505	3.891	0.0163*
Overall (Salburua)	45.10%	3.493	0.0137*	0.177	0.6759	3.469	0.0120*
Ae. cantans (Salburua)	45.10%	4.665	0.00291**	0.063	0.80314	3.27	0.01751*
Ae. rusticus (Salburua)	53.50%	4.471	0.00515**	2.454	0.09103	3.926	0.00485**
Cq. buxtoni (Urdaibai)	23.00%	0.991	0.368	0.816	0.3709	3.015	0.0411 *
Cq. richiardii (Urdaibai)	18.70%	0.319	0.6637	0.532	0.4694	2.756	0.0696
7 to 14 days before sampling							
Overall (Urdaibai + Salburua)	5.68%	0.145	0.704	1.395	0.234	2.272	0.135
Overall (Urdaibai)	24.00%	0.185	0.6695	0.307	0.7244	3.874	0.0398*
Overall (Salburua)	46.60%	1.088	0.31748	2.917	0.03024*	4.344	0.00464**
Ae. cantans (Salburua)	72.00%	3.619	0.02361*	11.592	0.00136**	4.297	0.00422**
Ae. rusticus (Salburua)	44.70%	3.619	0.02361*	11.592	0.00136**	4.297	0.00422**
Cq. buxtoni (Urdaibai)	24.80%	0.305	0.6229	0.175	0.678	3.059	0.0252*
Cq. richiardii (Urdaibai)	17.80%	0.036	0.8498	0.213	0.6468	2.441	0.0519
7 to 21 days before sampling							
Overall (Urdaibai + Salburua)	2.19%	0.744	0.39	0.906	0.343	2.271	0.135
Overall (Urdaibai)	37.70%	0.135	0.7149	3.231	0.0137*	1.622	0.2089
Overall (Salburua)	7.94%	2.87	0.0963	0.115	0.7362	0.476	0.4935
Ae. cantans (Salburua)	55.50%	3.542	0.01275*	0.295	0.69957	3.397	0.00441**
Ae. rusticus (Salburua)	20.60%	5.219	0.00949**	0.764	0.38626	0.369	0.54646
Cq. buxtoni (Urdaibai)	41.80%	3.051	0.0871	3.524	0.0119*	4.373	0.0418*
Cq. richiardii (Urdaibai)	35%	0.552	0.4612	3.539	0.0121*	1.277	0.2641

***<0.0001.
**<0.001.
*<0.05.

specimens. Blood-fed mosquitoes were trapped in May ($n = 1$), June ($n = 12$), July ($n = 8$), August ($n = 17$), September ($n = 5$) and October ($n = 10$).

At least 11 different vertebrate host species, including six mammals and five birds, were identified from 11 mosquito species (Table 5). *Aedes* spp. ($n = 18$) and *Coquillettidia* spp. ($n = 3$) fed on ungulates, whereas *Culiseta* spp., *Culex* spp. and *An. claviger* s.l. fed primarily on birds (Table 5). A distinct host-feeding pattern was observed depending on the aquatic setting; mosquitoes fed predominantly on red deer and birds in Salburua, whereas they fed on a great variety of domestic (horses, cattle and sheep) and wild ungulates (wild boar) as well as birds and humans in Urdaibai.

DISCUSSION

As a critical component of disease surveillance programmes and ecosystem health assessments, we studied mosquito community

TABLE 5 Host DNA blood-meals determined in mosquito species from two water ecosystems investigated in 2018 and 2019 in northern Spain.

	Salburua		Urdaibai	
Mosquito species	**No.**[a]	**Host DNA**	**No.**	**Host DNA**
Aedes cantans	1	*Cervus elaphus* (1)[b]		
Aedes caspius			3	*Sus scrofa* (1)
Aedes detritus			9	*Bos taurus* (6), *Ovis aries* (2)
Aedes rusticus	12	*Cervus elaphus* (8)		
Anopheles claviger s.l.	1	*Meleagris gallopavo* (1)	2	—
Coquillettidia buxtoni			5	*Sus scrofa* (1)
Coquillettidia richiardii			3	*Equus caballus* (1), *Sus scrofa* (1)
Culex modestus			3	*Anas platyrhynchos* (1), *Turdus* sp. (1), *Homo sapiens* (1)
Culex pipiens s.l.	2	*Parus major* (1)	1	*Turdus philomelos* (1)
Culiseta annulata	2	*Meleagris gallopavo* (2)	6	*Bos taurus* (1)
Culiseta litorea			1	—
Culiseta morsitans			2	*Turdus merula* (1)

[a]Number of mosquitoes analysed.
[b]In parenthesis the number of mosquitoes in which host DNA was identified.

composition and feeding preferences in two crucial stopovers for migratory birds in northern Spain. Different collection methods are required to obtain a better representation of mosquito species composition in a given area (ECDC, 2018). Therefore, our study employed a multi-trapping approach (CO_2-baited CDC traps, sweep nets, HLC and larvae collection) to obtain comprehensive information on mosquito fauna. A wide set of mosquito species was found to be in line with what is expected in natural areas compared with urbanised areas (Wilke et al., 2021). Thus, the number of mosquito species in the Autonomous Community of the Basque Country (Eritja et al., 2021; Goiri et al., 2020; González et al., 2020; González, Cevidanes, et al., 2021; González, Goiri, et al., 2021) increased to 28 species after the incorporation of the eight new species (*Cq. buxtoni*, *Cx. modestus*, *Culiseta subochrea*, *An. atroparvus*, *Ae. caspius*, *Ae. detritus*, *Ae. rusticus* and *Ae. sticticus*) collected in this study.

The collection of flying mosquitoes using baited traps is less time-consuming than other trapping methods; however, different traps vary in their ability to catch certain species. Since some species move long distances from their larval sites and others are difficult to collect using adult suction traps, the direct collection of immature stages in aquatic habitats should be pursued (Wilke et al., 2022). In this study, *Cx. hortensis*, *Cx. territans*, *Cs. annulata* and *Cs. longiareolata* were collected at immature stages, but were scarce or absent in the CDC catches. In contrast, larvae of several mosquito species were not found in the breeding sites examined, even though adult mosquitoes were trapped using CDC traps and HLC (*Ae. rusticus*, *Cx. modestus* and *Cq. buxtoni*). The reasons for these contradictory findings could be found in the biases of CDC traps, the dispersal patterns of emerging adults, or their resting behaviours, among others (ECDC, 2014). In addition, the complementary technique of sweep netting is a valuable tool for collecting blood-fed and gravid mosquitoes, males, or species not attracted by CDC traps (ECDC, 2018), as observed in this study with *Cx. territans* and *Ae. detritus*. These observations highlight the importance of using several techniques simultaneously to accurately characterise and define mosquito biodiversity in specific habitats, which is critical to achieving reliable and actionable results (Reisen et al., 1999).

The affinity of mosquito species to bite humans was evaluated in both aquatic areas using opportunistic HLC, which is considered the gold standard approach for the collection of anthropophilic mosquitoes. In this study, eight species were recorded biting the human collector, although these numbers might be increased by trapping over the crepuscular period (Becker et al., 2020; Brugman et al., 2017); for example, *Culex* species bite predominantly at dusk (Hawkes et al., 2020). In Salburua, *Ae. rusticus* was the most common species collected on both humans and suction traps, particularly at the end of the spring season (May–June). This species was responsible for the mosquito bite complaints that caused the hospitalisation of two people working in Salburua Park (Oier Quesada, personal communication). In Italy, *Ae. rusticus* is a problematic species that causes severe biting during local tourism in the Toscana region (Mosquitoweb Difenditi Dalle Zanzare, 2022). In Urdaibai, several mosquito species (*An. claviger* s.l., *Ae. detritus*, *Cq. richiardii*, *Cq. buxtoni* and *Cx. modestus*) showed some degree of propensity to bite humans, which is in accordance with other studies conducted in Britain (Brugman et al., 2017; Hawkes et al., 2020). Other mosquito species (*Cx. pipiens* s.l., *Cx. territans*, *Cx. hortensis* and *Cs. annulata*) were not collected on humans, possibly as they are typically crepuscular, ornithophilic or do not disperse far from larval sites.

Our study determined the larval habitats of eight mosquito species. The most common species found breeding was *Cx. territans* (33.5%), followed by *Cx. pipens* s.l. (16.2%) and *An. claviger* s.l. (13.2%). Most species were retrieved from ditches, puddles and small pools; however, large water pools were considerably unproductive for harbouring immature stages of mosquito species, except those of *Cx.*

territans that are commonly trapped in open pools of water holdings (Bueno-Marí, 2010). Notably, larval sites of monocyclic *Ae. rusticus* were not spotted even though their typical larval sites, including woodland pools and ditches of various origins with vegetation, were inspected (Grego & Zamburlini, 2021). Mosquito species such as *Cx. modestus*, *An. atroparvus*, *Ae. detritus* and *Ae. caspius*, which usually breed in brackish and saline water (Hawkes et al., 2020; Medlock & Vaux, 2015a; Roiz et al., 2015), were exclusively present in the marsh, whereas other species linked to temporary freshwater pools in wet woodlands, such as *Ae. rusticus* and *Ae. cantans* (Hawkes et al., 2020; Medlock & Vaux, 2015a) were present in the wetland. *Coquillettidia richiardii*, the most abundant species identified in this study, is a relatively common species that breeds in permanent water with abundant vegetation (Hawkes et al., 2020); however, its congeneric *Cq. buxtoni* seems to have a limited range distribution in Europe (Becker et al., 2020), and in this study they were spotted only in the marsh. Pre-imaginal stages of *Coquillettidia* spp. are not easily detected (Medlock & Vaux, 2015b) unless aquatic plant stems and root systems are removed and examined (Johnson & Russell, 2019), which explains the low records of these species in other studies. Notably, species frequently observed in Mediterranean wetlands in southwestern Spain (Roiz et al., 2012; Roiz et al., 2015), such as *Culex theileri* and *Culex perexiguus*, are rare or absent in the two aquatic systems studied in northern Spain and other Atlantic climatic regions (Hawkes et al., 2020). In contrast, other species with high ecological plasticity, such as *Cx. pipiens* s.l. were found in both habitats, as in other studies (Joyce et al., 2018).

The study of mosquito population dynamics is important in understanding the periods of activity of each species with the aim of predicting biting risks for humans and animals. In the present study, the earliest species that emerged in spring, after the temperatures in the autumn decreased and the cold winter period was set in, was *Ae. rusticus* (Becker et al., 2020). This snowmelt species showed a well-defined unimodal peak in activity at the beginning of June, as previously reported (Becker et al., 2020). Other univoltine species, including *Ae. cantans*, *Cq. richiardii* and *Cq. buxtoni*, peaked at the end of June, whereas other culicids such as *Cx. pipiens* s.l., *Cx. modestus*, *An. claviger* s.l. and *Ae. detritus* show a bivoltine or multivoltine pattern (Medlock & Vaux, 2015b).

The differences in diversity and mosquito abundance between the two aquatic systems could be attributed to abiotic and biotic factors. The Salburua wetland, which is located in the transition zone between Atlantic and Continental Mediterranean climates, has more extreme climatic conditions than Urdaibai, which is located on the coast and is influenced by the mild Atlantic climate. During the summer, unlike Urdaibai, the Salburua wetland dries up completely; therefore, precipitation (together with relative humidity and average temperatures) considerably explains the variations in mosquito abundance. Precipitation is a critical variable for *Ae. cantans*. This species lays eggs in dried-up hollows under periodic flooding and is typically found in pools in shaded forested areas (Hawkes et al., 2020). In contrast, in Urdaibai, other factors may have more weight in explaining the differences in the abundance of mosquitoes. For example, the level of the sea tide is associated with the emergence and abundance of species such as *Ae. detritus* and *Ae. caspius* that benefit from the high tides that flood the salt marshes and create larval breeding sites (Roiz et al., 2014). In relation to other species abundant in the marsh of Urdaibai, such as *Cq. buxtoni*, our analysis showed that they are dependent on rainfall rather than temperature for the development of their life cycle. Regarding biotic factors (not included in this study), the aquatic community of predators that feed on mosquito larvae is reported to modulate mosquito abundance (Shaalan & Canyon, 2009). In addition, the capacity of wetlands to retain water during the year (permanent or temporary wetlands) determines the wildlife species inhabiting them (Wellborn et al., 1996).

The feeding habits of mosquitoes are critical factors in the transmission of vector-borne pathogens (Montgomery et al., 2011; Yan et al., 2021). Some species feed opportunistically on a wide range of hosts, whereas others feed on a limited range of hosts (Gibson & Torr, 1999). Owing to the low number of blood-fed specimens recorded in our study, it is difficult to establish more robust conclusions. Nevertheless, the host DNA from mosquito blood meals reflects the vertebrate species present in each aquatic ecosystem. *Aedes* spp. and *Coquillettidia* spp. primarily fed on ungulates, whereas *Culex* and *Culiseta* spp. fed on birds and mammals. The feeding habits of both genera showed strong ornithophilic preferences along an urban-natural gradient near the region of this study (González et al., 2020). Humans were uncommon hosts, and only a single specimen of *Cx. modestus* contained human blood, despite the relatively high frequency of walkers in both natural settings. Considering that *Cx. modestus* is a major human-biting mosquito species recorded as a WNV bridge vector in wetlands in France (Ponçon et al., 2007), it would be interesting to assess whether the population of *Cx. modestus* present in these aquatic environments harbours this virus.

Several other mosquito species found in the study area have been described as potential vectors of the West Nile virus (*Culex* spp.), Sindbis virus (*Culex* spp.), Usutu virus (*Cx. torrentium*, *Cx. pipiens* s.l. and *An. maculipennis* s.l.), Tahyna virus (*Ae. vexans*, *Cs. annulata* and *Ae. cantans*), protozoa causing malaria (*Anopheles* spp.) and filarioid nematodes (*Culex* spp. and *Ae. vexans*) (Becker et al., 2020; Calzolari et al., 2013; Hawkes et al., 2020; Medlock & Vaux, 2015a; Schaffner et al., 2001). However, other species such as *An. claviger* s.l., *Ae. rusticus*, *Coquillettidia* spp. and *Culiseta* spp. do not pose a concern to human health. However, the feeding behaviour of some of these species on humans or birds indicates that they are putative bridge vectors of arboviruses (Hawkes et al., 2020; Wang et al., 2021).

The interaction of mosquitoes with birds and wild animals is potentially frequent in these aquatic ecosystems, a condition that favours WNV emergence and amplification. Thus, considering that both aquatic systems are located along one of the major migratory routes between Europe and Africa and wild birds are able to carry and introduce WNV over large distances into new areas, there is a need to reinforce surveillance and early detection of WNV in animals (Jourdain et al., 2007). Therefore, the information provided here might be critical, as some mosquito species can act as bridge vectors between birds and humans.

CONCLUSION

This study highlights the importance of studying mosquito diversity. Mosquito sampling strategies that use techniques complementary to ordinary adult mosquito surveillance systems can potentially supplement the sampling power of the surveillance programmes. The data obtained here provided local information on the ecology of several mosquito species, such as their biting behaviour, choice of resting sites, aquatic habitats and the highest relative abundance levels of immature mosquito stages. Based on the recorded data, and compared with the wetland of Salburua, the Urdaibai Marsh showed a greater abundance of mosquitoes and more species regarded as potential vectors of pathogens. The results presented here can be used by local health authorities to design the best management and sanitary strategies for mosquito control operations because the socio-economic relevance of mosquito control requires considering nuisance, vector-borne diseases and environmental effects together.

AUTHOR CONTRIBUTIONS

Mikel A. González: Methodology; investigation; data curation; writing – original draft; writing – review and editing. **Fátima Goiri:** Methodology; formal analysis; investigation; data curation; writing – original draft. **Aitor Cevidanes:** Formal analysis; investigation; writing – original draft; writing – review and editing. **Luis M. Hernández-Triana:** Investigation; resources; writing – review and editing; funding acquisition. **Jesús F. Barandika:** Conceptualization; methodology; investigation; data curation; writing – review and editing. **Ana L. García-Pérez:** Conceptualization; resources; writing – original draft; writing – review and editing; supervision; project administration; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Phylogenetic tree based on the COI gene (378 bp).

Table S1. Comparison of mosquito catches obtained in 2019 by CO_2 baited CDC traps, sweep netting (SN), human landing catches (HLC) and captures of larvae by dipping (DI).

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