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Species Composition and Population Dynamics of Culicidae during their Peak Abundance Period in Three Peri-Urban Aquatic Ecosystems in Northern Spain

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Abstract: For a better understanding of the transmission cycles of mosquito-borne diseases, it is essential to explore the species composition and population dynamics, mainly during their peak abundance period. These investigations do not normally include peri-urban areas, despite their significant epidemiological interest. To address these gaps, an entomological survey was carried out in 2016 and 2017 in three aquatic ecosystems located on the outskirts of the city of Logroño, in northern Spain: the Iregua River and La Grajera (La Rioja) and Las Cañas Reservoirs (Navarra). Mosquitoes were captured using BG-Sentinel traps baited with CO_2 and BG-lure, as well as through the human landing collection method. In total, 6793 mosquito specimens were captured, representing 24 taxa within six genera. A specific PCR based on the ITS2 gene was used to differentiate members of the *Anopheles claviger* complex, and all individuals were identified as *An. claviger* sensu stricto. La Grajera had the most diverse culicid fauna, with 19 taxa, followed by Las Cañas (n = 15) and the Iregua River (n = 13). The composition and abundance of Culicidae varied across the aquatic ecosystem. We observed that the different hydrological management practices of each environment could play a key role in determining the abundance of mosquito genera. The overall risk of mosquito bites in the study area is expected to be relatively low and will depend on the freshwater ecosystem and the time of year.

Keywords: Culicidae; diversity; ITS2; mosquito; La Rioja; *Anopheles claviger* sensu stricto; Upper Ebro Valley

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1. Introduction

The arthropod-borne diseases represent a major risk to humans, livestock, pets and wildlife worldwide [1]. In Spain, there is a wide range of organisms that transmit diseases in our environment, mostly included in the so-called zoonoses [2]. Although reviewing the issue of arthropod-borne diseases in Spain is complex, it is unquestionable that since the beginning of the 21st century, the importance of Diptera has been extraordinary. The burden of some diseases such as bluetongue and leishmaniosis has led to an increase in studies on *Culicoides* biting midges (Ceratopogonidae) and phlebotomine sand flies (Psychodidae), respectively [3,4]. Black flies (Simuliidae) have also become very relevant pests in certain Spanish regions due to the annoyance caused by their painful bites [5].

Despite the above, mosquitoes (Culicidae) continue to be the most studied insects because of their major role as vectors. Invasive mosquito species and the pathogens they transmit represent a serious risk to both animal and public health [6], especially in areas with a Mediterranean climate, where these insects have a major impact throughout much of the year [7]. Therefore, studies of these vectors have not been addressed as much in climate regions such as the north-western, Cantabrian cornice, and inland regions, which are less suitable for their development, although a change in this regard has been observed in the last decade. In fact, since the detection of the Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894), in the Basque Country in 2015 [8], interest in mosquitoes in northern Spain has increased. The recent finding of the invasive *Aedes japonicus* (Theobald, 1901) in Asturias in 2018 and its later detection in Cantabria and the Basque Country in 2019 and 2020, respectively [9,10], again highlighted the importance of conducting mosquito surveys in the northern regions of the country, focusing not only on specific invasive alien mosquitoes but also on native species.

Several studies on mosquitoes have been recently carried out in this part of the Iberian Peninsula, among which faunistic analysis predominated [11–13]. Specifically, since 2016, when a vector surveillance programme (mainly focused on culicids) was implemented in La Rioja region, 25 species have been identified [14–16]. However, these studies have not delved into seasonal population dynamics, which are essential to better understand mosquito-borne disease transmission cycles, identify sources of infection, and successfully control these vectors [17,18].

Most studies in Spain have been carried out in either natural areas or urban environments [19–21]. However, the urban-to-wild gradient is composed of distinct environmental habitats that influence the distribution, diversity, and abundance of mosquito species [20]. The rapid growth of urban areas in Spain has led to an increase in peri-urban habitats, which are areas where urban and rural environments overlap. However, the characteristics of peri-urban habitats differ from those of urban and natural habitats in many ways (e.g., fragmentation, pollution, noise, and light, among others) [22]. Peri-urban areas can provide freshwater ecosystems, such as riverbanks or wetlands, which have high value in terms of ecological diversity. They also enhance the human well-being of urban residents by providing a landscape for recreational activities in addition to their aesthetic value. As a negative counterpart to these aquatic habitats, there is the possibility of disturbance by haematophagous dipterans and their potential for disease transmission [23]. Therefore, it is necessary to monitor the presence of, among others, the members of the family Culicidae in these environments. In this respect, few Spanish studies have examined the mosquitoes that inhabit these particular environments [24–26]. The study of the mosquito fauna associated with peri-urban environments should be of increasing interest, as it is in these often-naturalised environments that the greatest number of encounters between vectors and humans occur.

We aimed to provide data on the species composition and population dynamics of mosquitoes during the peak abundance period in three nearby freshwater ecosystems located in peri-urban areas of Logroño, La Rioja, as well as to detect differences in the frequency of genera and relevant species in each location. Given the ecological aspects studied for the different species detected in the three peri-urban ecosystems, a succinct discussion about the possible public health implications of this mosquito species is provided.

2. Materials and Methods

2.1. Study Area

This study was conducted in the Autonomous Community of La Rioja (northern Spain) and a nearby area of the Chartered Community of Navarra (Figure 1). The entomological surveys were carried out in three sampling sites on the outskirts of the city of Logroño, the capital of the province of La Rioja, lying on the Upper Ebro Valley. This city is 385 m asl;

to the north are the Cantabrian Mountains, to the south the Iberian System. Regarding its climate, it is slightly continental, with quite cold, relatively rainy winters and hot, sunny summers but with some cool and rainy periods. The average temperature of the coldest month (January) was 5 °C; that of the warmest months (July, August) was 20.6 °C. Precipitation amounts to 633 mm per year. It ranges from 29 mm in the driest month (August) to 66 mm in the wettest ones (April, May) [27].

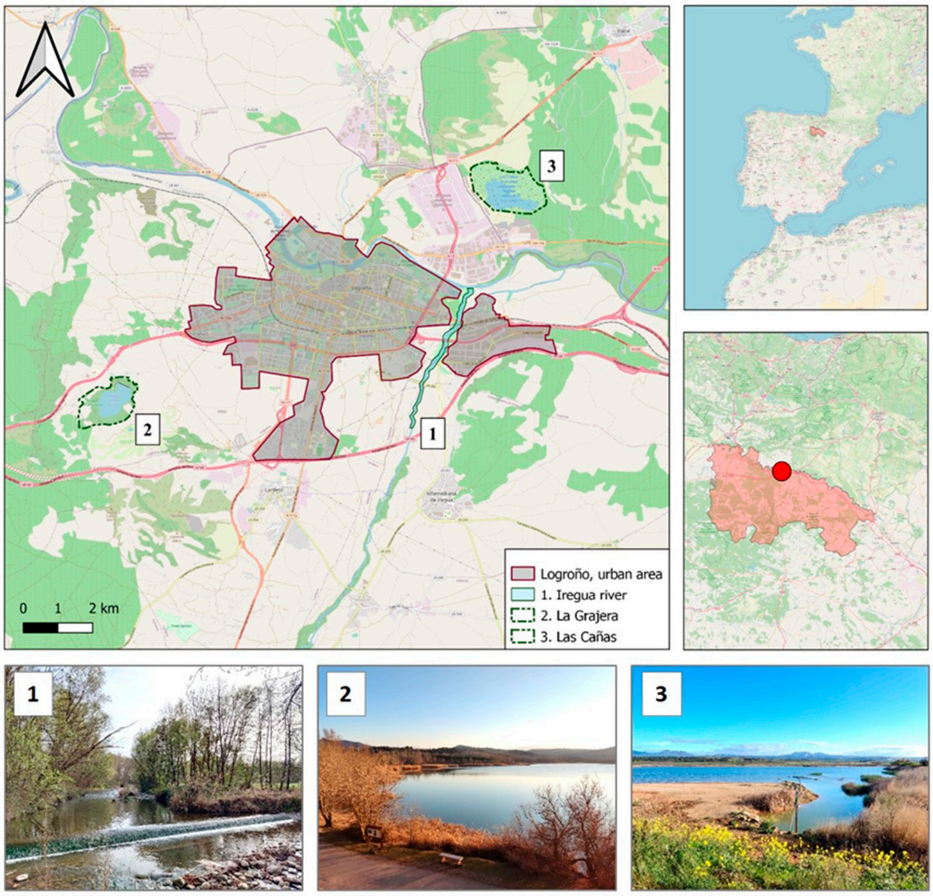

Figure 1. Location of the three peri-urban aquatic ecosystems sampled on the outskirts of the city of Logroño (red dot), northern Spain. (1) Iregua River; (2) La Grajera Reservoir; (3) Las Cañas Reservoir.

The first sampling freshwater habitat was the Iregua River, as it flows through Logroño, near its mouth in the Ebro River. From its source in the Sierra Cebollera to its mouth, this river crosses La Rioja from south to north over a length of 64 km, with a catchment area of 692 km^2 and an average flow rate of 3.32 m^3/s [28]. As it passes through the municipality, the river is mainly characterised by its permanently low flow; the natural vegetation is reduced to some gravel beaches and the slopes that delimit the flood channel. On the former, which were originally submerged, small sea bream and sea bream grow, as well as other shrubs and herbaceous plants of various species. The second peri-urban aquatic ecosystem was La Grajera, one of the few wetlands that exist in La Rioja. This place is currently protected and covers a total area of 87 ha, 32 ha with a permanent sheet of water supplied by a single inlet. It is the last enclave on the Camino de Santiago (Way of St. James) as it passes through Logroño, and it also has an educational classroom, a recreation area, a coffee shop and restaurants, paths around the lagoon, and a golf course with 18 holes [29]. The third and last aquatic environment was La Laguna de las Cañas, a nature reserve covering an area of 178 ha and one of the most important wetlands (80 ha) of Navarra, located in the municipality of Viana, just a few hundred metres from an industrial area of the city of Logroño. This reservoir collects runoff water from a catchment area of 6602 ha through a network of streams and ditches and can reach a volume of dammed water of around 0.7 hm^3 [30]. Las Cañas has several types of environments: crops, Mediterranean scrub in the surroundings, banks, flooded soils, small reed beds in the outer zone and large reed beds in the interior, open waters, islets, and trees, with a changing water level. These areas were chosen according to the presence of mosquitoes, waterflow, and ornithological richness and because they are located in peri-urban areas and frequently visited by people who want to enjoy contact with nature (Figure 1).

2.2. Mosquito Collection and Identification

Mosquitoes were collected from July to September 2016 and from May to September 2017. As can be observed, this study has primarily been carried out during the summer season, as this is the period when mosquitoes concentrate their activity in northern Spain.

The trapping effort was different in the case of the Iregua River, where it started a week later in 2016 and at the end of June in 2017. A total of four or five BG-Sentinel TM traps (BioGents GmbH, Regensburg, Germany) baited with BG-Lure® and 1 kg of CO_2 pellets were set once every two weeks in each location. In total, mosquito traps were run 223 nights during the study period, 81 in 2016 (18 in the Iregua River, 31 in La Grajera, and 31 in Las Cañas) and 143 in 2017 (32 in the Iregua River, 59 in La Grajera, and 52 in Las Cañas). The traps were set at dusk (19:00–21:00) and removed after dawn (8:00–9:00) the following day. The traps located in the two reservoirs were placed in five sampling stations along the entire perimeter of the aquatic ecosystem (separated between 400 and 500 m in La Grajera and 400 and 600 m in Las Cañas). In the case of the Iregua River, four traps were placed along the right bank of the river, covering 1 km of riverbed until it flows into the Ebro River. All sampling stations were selected on the basis of several criteria: covering different locations in the wetland; the presence of some vegetation cover in the vicinity; easy accessibility; and proximity to the aquatic ecosystems' water surface. La Grajera and Las Cañas are 1.7 km and 2 km away from the urban area of Logroño, respectively. The Iregua River separates two urban areas of Logroño, forming a green corridor between 100 and 200 m wide.

During the setting and removal of the traps, host-seeking females were captured by human landing collection (HLC) during the time the collector went from the vehicle to the exact location of the trap, set up the trap, and returned to the car (15 min). The collector exposed their legs and arms and caught landing mosquitoes with a hand-held mouth aspirator. Collection bags containing insects were stored at −80 °C until further processing.

For identification, the mosquitoes were separated from other insects, enumerated, sexed, and determined at the species level based on external morphological characters and male terminalia using the taxonomic keys of Schaffner et al. [31] and Becker et al. [32]. Males' genitalia were mounted on a slide using Hoyer's medium. A specific study on the molecular identification of different specimens of each species was previously carried out in La Rioja region, in which individuals of *Anopheles maculipennis* s.l. were analysed using the internal transcribed spacer 2 (ITS2) gene and identified as *Anopheles atroparvus* (Van Thiel, 1927) [15]. For this reason, we will henceforth refer to *An. atroparvus* in the present study. However, it had not been possible to determine which species of the Claviger complex were present in the region [15]. Therefore, the specific protocol of Kampen et al. [33] based on the ITS2 gene was used to differentiate between *Anopheles claviger* s.s. (Meigen, 1804) and *Anopheles petragnani* Del Vecchio, 1939. Each species generates PCR products of species-specific lengths: *An. claviger* s.s. at 269 bp and *An. petragnani* at 367 bp. The amplified products were sequenced in both senses using the BigDye R Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forest City, CA, USA) at the Sequencing Unit, Centre for Biomedical Research of La Rioja (CIBIR). The sequences were edited using BioEdit 7.2 software and compared with the sequences published in Kampen et al. [33] and deposited in GenBank.

2.3. Data Analysis

The number of mosquitoes was compared by zone with full-factorial general linear models (GLMs) including zone and period to discard the effect of seasonality. In order to detect differences in the frequency of genera and relevant species depending on the zone, similar GLMs were carried out including the number of mosquitoes as a weighted factor. In case of significant differences between zones, Duncan's *post hoc* test was carried out. A statistical analysis was performed using SPSS 19.0 for Windows (IBM, Armonk, NY, USA), setting the alpha error to 0.05. Figure 1 was created by QGIS V.3.0.1 (QGIS Development Team 2018, available from: http://www.qgis.org, accessed on 15 May 2023). Figure 2 was generated with Microsoft Excel 2013 (Microsoft, Redmond, WA, USA). Figure 3 was created with the software RStudio v. 4.2.0 [34].

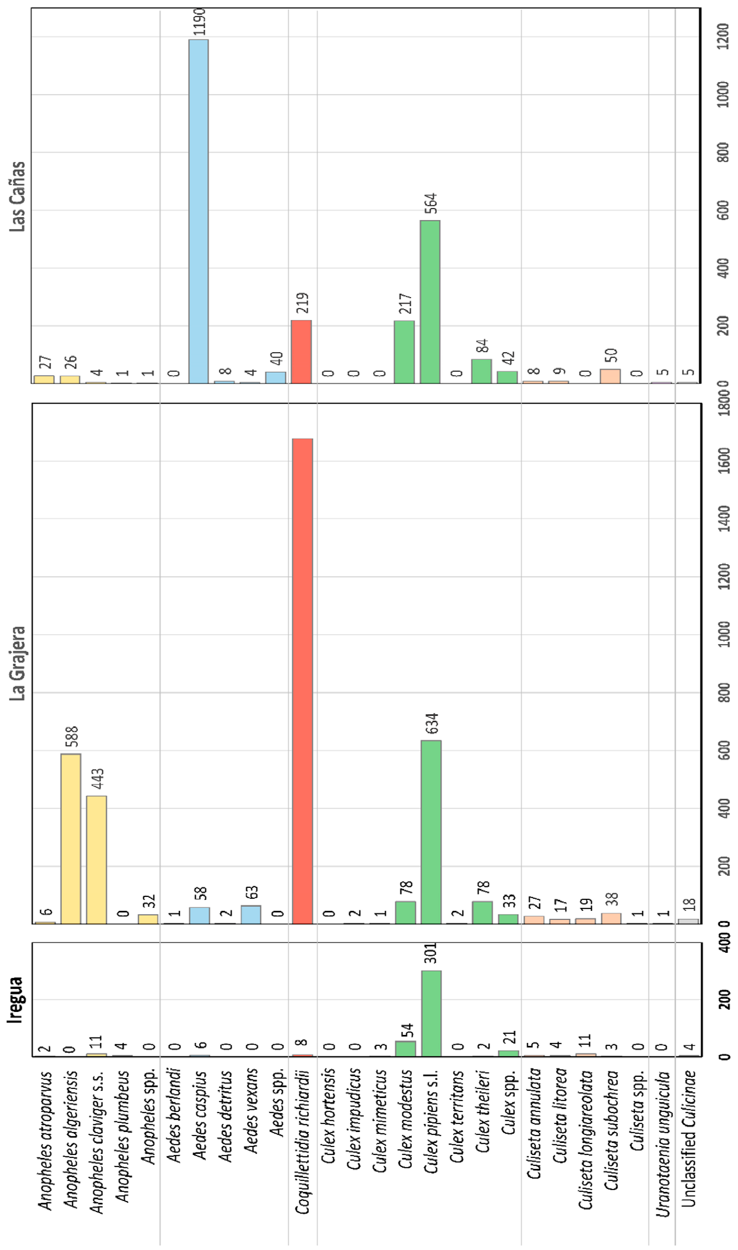

Figure 2. Total number of mosquitoes of each species caught with baited BG-Sentinel in aquatic ecosystems traps in northern Spain, 2016–2017. The specimens categorised as "spp" in each genus could not be assigned to species level because of their poor conservation status. Genera are represented with different colours.

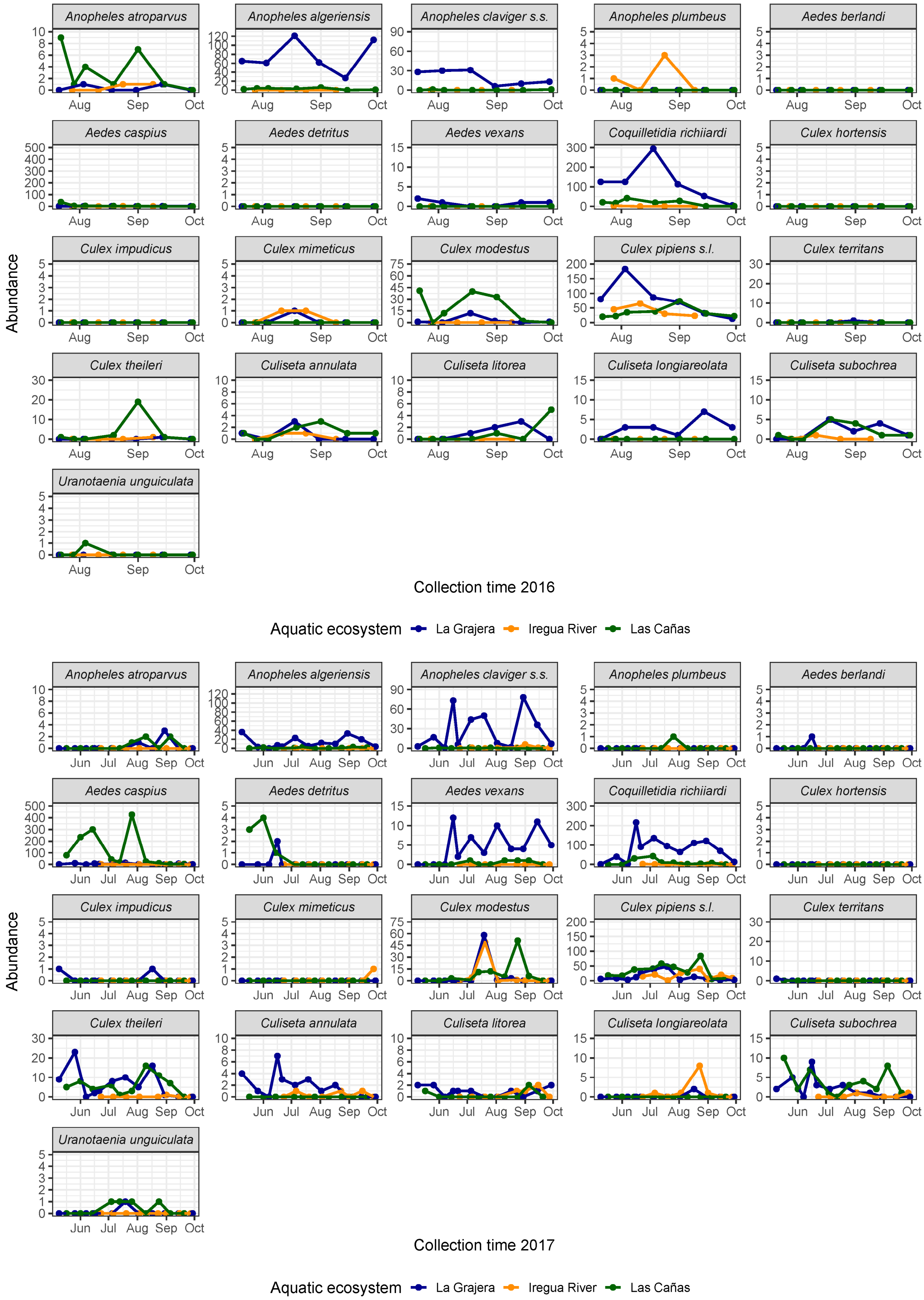

Figure 3. Population dynamic of each species caught by aquatic ecosystem with baited BG-Sentinel traps in aquatic ecosystems in northern Spain, 2016–2017 (Jun: June; Jul: July; Aug: August; Sep: September; Oct: October).

2.4. Ethics Approval and Consent to Participate

Ethical clearance for HLC was obtained from the regional ethics committee (Comité de Ética de Investigación con Medicamentos de La Rioja, Ref. CEImLAR PI-688). All procedures were in accordance with the ethical standards of the research committee and with the 1964 Helsinki Declaration and its later amendments.

3. Results

The morphological characteristics and genetic approach to the Claviger complex identified at least 24 taxa of mosquitoes arranged in six genera: *Aedes* (7 species), *Anopheles* (4 species), *Coquillettidia* (1 species), *Culex* (7 species), *Culiseta* (4 species), and *Uranotaenia* (1 species). The species and the number of specimens found in each aquatic ecosystem are displayed in Figure 2.

3.1. Molecular Identification of the Claviger Complex

An analysis of 17 *An. claviger* s.l. yielded 10 positive identifications for the species *An. claviger* s.s. as all bands were below 300 bp. The subsequent sequencing and alignment with the sequences published by Kampen et al. [33] reconfirmed that identification. Our sequences of *An. claviger* s.s. (amplicon length sequences between 235 and 238 bp) showed an identity of 96.93–98.92% with respect to the *Anopheles claviger* s.l. from Italy (MK625346). In turn, our sequences had a 95.61–96.89% similarity to the sequence of *An. claviger* s.s. (AY129232) deposited by Kampen et al. [33] and 86.67–87.56% similarity to the unique sequence of *An. petragnani* (AY129233.1) deposited in GenBank [33]. The detailed specimen records and sequence information of *An. claviger* s.s. were submitted to the GenBank public database under the following accession numbers: OQ955587–OQ955589.

3.2. Species Composition and Abundance

A total of 6735 mosquitoes were captured in the BG-Sentinel traps, of which 6479 were females and 256 males. The species composition and abundance varied depending on the aquatic ecosystem. La Grajera was the environment with the highest total catches and also with the highest relative abundances in relation to the sampling effort (n = 3801; 42.23 specimens/trap), followed by Las Cañas (n = 2499; 30.11 specimens/trap) and the Iregua River (n = 435; 8.70 specimens/trap) (Table 1). The most abundant genus was *Culex*, followed by *Coquillettidia richiardii* (Ficalbi, 1889), *Aedes* sp., and *Anopheles* sp.

Table 1. Total number of mosquitoes of each genus caught with baited BG-Sentinel traps in aquatic ecosystems in northern Spain, 2016–2017.

Genus	Iregua River (n = 50)	La Grajera (n = 90)	Las Cañas (n = 83)	Total (n = 223)
Anopheles	17	1069	59	**1145**
Aedes	6	124	1242	**1372**
Coquillettidia	8	1677	219	**1904**
Culex	381	828	907	**2116**
Culiseta	23	102	67	**192**
Uranotaenia	0	1	5	**6**
Unidentified	4	18	5	**27**
Total	**435**	**3801**	**2499**	**6735**

n indicates the sampling effort represented by the total number of traps used per aquatic ecosystem.

The abundance of the different genera varied according to the freshwater ecosystem. The genera *Anopheles* and *Coquillettidia* were dominant in La Grajera ($p < 0.001$), while the genera *Aedes*, *Culex*, and *Uranotaenia* dominated in Las Cañas ($p < 0.001$, $p = 0.007$, and $p = 0.047$, respectively) (Table 2). Catches of the genus *Culiseta* did not differ significantly by aquatic ecosystem. The average number of captures of the genera *Anopheles*, *Aedes*, and *Coquillettidia* showed large differences between the area with the highest number of captures and the rest of the areas. This was not the case for the genus *Culex*, where the averages were more homogeneous between the different zones.

Table 2. Mean ± standard deviation of catches of each mosquito genus with BG-Sentinel traps in aquatic ecosystems in northern Spain, 2016–2017.

Genus	Iregua River (n = 50)	La Grajera (n = 90)	Las Cañas (n = 83)	p-Value
Anopheles	0.34 ± 0.72 [a]	11.88 ± 16.26 [b]	0.71 ± 1.31 [a]	<0.001
Aedes	0.12 ± 0.33 [a]	1.38 ± 2.14 [a]	14.96 ± 36.70 [b]	<0.001
Coquillettidia	0.16 ± 0.47 [a]	18.63 ± 25.43 [b]	2.64 ± 3.90 [a]	<0.001
Culex	7.62 ± 8.73 [a]	9.20 ± 12.93 [ab]	10.93 ± 10.73 [b]	0.007
Culiseta	0.46 ± 1.16	1.13 ± 1.86	0.81 ± 1.57	0.080
Uranotaenia	0.00 ± 0.00 [a]	0.01 ± 0.11 [ab]	0.06 ± 0.24 [b]	0.047
All	8.70 ± 8.99 a	42.23 ± 42.41 c	30.11 ± 38.26 [b]	<0.001

n indicates the sampling effort represented by the total number of traps with mosquito catches used per aquatic ecosystem. Different superscripts for each gender indicate significant differences according to Duncan's *post hoc* test ($p < 0.05$).

Table 3 shows the percentage distribution of mosquitoes in each zone to see which was dominant relative to the total number of mosquitoes captured in each zone. For instance, in the Iregua River, the dominant genus was *Culex* sp. (87.6%) compared to the rest of the genera ($p < 0.001$). Although there were more *Culex* specimens in the other two aquatic ecosystems in absolute terms (Table 2), in relative terms they were less frequent (21.8% in La Grajera and 36.3% in Las Cañas) (Table 3). According to this analysis, the genera *Anopheles* and *Coquillettidia* remained dominant in relative terms in La Grajera ($p < 0.001$) and the genus *Aedes* in Las Cañas ($p < 0.001$). *Coquillettidia* was the genus with the highest relative captures in La Grajera (44.1%) and *Aedes* sp. in Las Cañas (49.7%).

Table 3. Mean ± standard deviation of the percentage of catches of each genus over the total catches per area (only if catches were present and weighted by the number of catches per trap) with baited BG-Sentinel traps in aquatic ecosystems in northern Spain, 2016–2017.

Genus	Iregua River (n = 47)	La Grajera (n = 88)	Las Cañas (n = 82)	p-Value
Anopheles	3.9 ± 8.8 [a]	28.1 ± 21.3 [b]	2.4 ± 5.2 [a]	<0.001
Aedes	1.4 ± 5.3 [a]	3.3 ± 5.0 [a]	49.7 ± 39.4 [b]	<0.001
Coquillettidia	1.8 ± 5.8 [a]	44.1 ± 24.1 [b]	8.8 ± 13.3 [a]	<0.001
Culex	87.6 ± 16.2 [c]	21.8 ± 20.2 [a]	36.3 ± 34.3 [b]	<0.001
Culiseta	5.3 ± 11.3	2.7 ± 4.0	2.7 ± 6.6	0.187
Uranotaenia	0.0 ± 0.0	0.0 ± 0.1	0.2 ± 1.0	0.226

n indicates the sampling effort represented by the total number of traps used per aquatic ecosystem. Different superscripts for each gender indicate significant differences according to Duncan's *post hoc* test ($p < 0.05$).

Table 4 shows the most captured species with respect to the captures of other species of the same genus in each sampling point. For instance, *Aedes caspius* (Pallas, 1771) represents all the captures of this genus in the Iregua River and 95.8% in Las Cañas ($p < 0.001$), while the Claviger complex represents 64.7% of the captures of the genus *Anopheles* in the Iregua River and 41.4% in La Grajera ($p < 0.001$). *Culex modestus* Ficalbi, 1890 accounted for 23.9% of the captures of *Culex* sp. in Las Cañas ($p = 0.007$). *Culex pipiens* s.l. Linnaeus, 1758 was the most abundant species of this genus in the three ecosystems, but it did not show significant differences according to the freshwater environment (p = 0.129). The same occurred with *Anopheles algeriensis* Theobald, 1903, being the most abundant *Anopheles* species in La Grajera and Las Cañas with 55% and 44.1%, respectively.

Table 4. Mean ± standard deviation of the percentage of catches of the most representative mosquito species with respect to catches of its genus (only if there were catches of that genus and weighted by the no. of catches of the corresponding genus per trap) with baited BG-Sentinel traps in aquatic ecosystems in northern Spain, 2016–2017.

	Iregua River		La Grajera		Las Cañas		
Species	***n***	**Avg ± SD**	***n***	**Avg ± SD**	***n***	**Avg ± SD**	***p*-Value**
Anopheles algeriensis	12	0.0 ± 0.0	77	55.0 ± 34.0	30	44.1 ± 42.0	0.123 [1]
Anopheles claviger s.s.	12	64.7 ± 49.3 [b]	77	41.4 ± 34.9 [b]	30	6.8 ± 21.0 [a]	0.032
Aedes caspius	6	100.0 ± 0.0 [b]	46	46.8 ± 39.5 [a]	51	95.8 ± 5.8 [b]	<0.001
Culex pipiens s.l.	47	79.0 ± 33.3	73	76.6 ± 31.4	79	62.2 ± 26.5	0.129
Culex modestus	47	14.2 ± 33.2 [a]	73	9.4 ± 20.1 [a]	79	23.9 ± 25.8 [b]	0.007

[1] The Iregua River was excluded from statistical analysis. *n* indicates the sampling effort represented by the total number of traps with catches of each genus per aquatic ecosystem. Different superscripts for each species indicate significant differences according to Duncan's *post hoc* test ($p < 0.05$).

3.3. Population Dynamics in the Peak Abundance Period

Different patterns in the population dynamics of culicids were observed depending on the species and aquatic ecosystem (Figure 3).

Two of the most abundant species in La Grajera, *An. algeriensis* and *An. claviger* s.s., had a constant presence during the study period, even presenting similar dynamics with three peaks of abundance in July, August, and October 2016 and another three peaks of abundance in May–June, July, and September–October 2017. Within the Maculipennis complex, *An. atroparvus* was the only species previously identified in the study area [15]. This species was the only anopheline species with a very limited temporal distribution, between mid-July and September in La Grajera and Las Cañas. The abundance of *Anopheles plumbeus* Stephens, 1828 was so low that no clear patterns in its population dynamic could be observed, except that it was present during July and August.

Aedines did not have a constant presence in the aquatic ecosystems studied, but rather had peaks of abundance depending on the time of year. For example, there were hardly any captures of *Ae. caspius* in Las Cañas in 2016, but there were two notable peaks of abundance between June and August 2017. *Aedes detritus* (Haliday, 1833) was a species that concentrated its presence in the late spring and early summer in 2017 in the two swamps where it was identified. The presence of *Aedes vexans* (Meigen, 1830) seemed to be constant from June onwards in La Grajera.

The presence of the most abundant species at La Grajera, *Cq. richiardii*, was constant during the two years of this study. However, its abundance peaks differed between the two years. The main peak in 2016 was in August, while in 2017 we found four peaks from May to September. Despite its lower presence in Las Cañas, two peaks were observed during August and September 2016 and a single peak in June–July 2017.

Culex pipiens s.l. was the only species present throughout the study period in all three habitats. A similar pattern was observed in all three studied ecosystems, with a peak in July and a peak in August. In Las Cañas, a third peak was also observed during September. Adults of *Cx. modestus* appeared in June, with two peaks of abundance in July and August–September. *Culex theileri* Theobald, 1903, also showed several peaks of abundance depending on the aquatic ecosystem, with a clear peak in August–September in all habitats.

Adults of *Culiseta* species were also active throughout the study period, except for the species *Culiseta longiareolata* (Macquart, 1838). The other species, either because they were secondary species or because of their own population patterns, had a temporal distribution that was not constant over time.

Uranotaenia unguiculata Edwards, 1913 appeared mainly from July to September.

3.4. Human Landing Collection

Six species were collected in the HLC: *An. plumbeus* (*n* = 1, Iregua River); *Ae. caspius* (*n* = 26, Las Cañas; *n* = 2, La Grajera); *Ae. detritus* (*n* = 1, Las Cañas; *n* = 19, La Grajera); *Ae. vexans* (*n* = 1, La Grajera); *Cx. modestus* (*n* = 2, Iregua River); and *Cq. richiardii* (*n* = 2, Las Cañas; *n* = 4, La Grajera). Specimens of *Ae. detritus* were identified biting humans in La Grajera on 24 May 2016 during the first aquatic ecosystem awareness visit.

4. Discussion

Understanding the risk of transmission of mosquito-borne diseases requires knowledge of the culicid fauna associated with environments frequented by humans, such as urban and peri-urban areas. This knowledge serves as a fundamental tool and cornerstone of the "One Health" approach. Therefore, this study highlights the large mosquito fauna that inhabit peri-urban environments in northern Spain.

In Spain, a total of 65 mosquito species from seven different genera have been described, including *Aedes*, *Anopheles*, and *Culex* [9,35], the main vectors of pathogens in Europe. In La Rioja, the culicid fauna identified include 25 species in six genera: *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, and *Uranotaenia* [15,16,36–38]. In Navarra, 21 species belonging to the same six genera have been recorded [14,35].

4.1. Molecular Identification of the Claviger Complex

PCR has been employed to specifically identify members of the Claviger complex, marking a novel application in Spain. We also provide sequences of *An. claviger* s.s. for the country. Moreover, the identification of this anopheline implies its incorporation into the list of human malaria vectors in La Rioja, together with *An. atroparvus* and *An. plumbeus*. Although *An. claviger* s.s. is regarded as a secondary malaria vector in Europe [39], with a preference for biting humans and large mammals outdoors after dusk [40], its vector competence should not be overlooked. In the case of *An. petragnani*, although we did not capture any adults in our study, it has previously been described in the larval stage in La Rioja [37].

4.2. Species Composition and Abundance

The composition and abundance of mosquito fauna in these three closely spaced habitats varied according to the type of aquatic ecosystem and the hydrological management practices employed. La Grajera, a naturalised reservoir with minimal human intervention, had the highest number of mosquito captures. In Las Cañas, naturalised conservation of the swamp is carried out by managing the reeds and promoting the conservation of native fish species and amphibians, which exert some degree of control over the pre-imaginal populations of mosquitoes. The Iregua River, on the other hand, lacks standing water bodies, so its mosquitoes depend mainly on the floods that allow parts of its groves to be flooded. The genera *Anopheles* and *Coquillettidia* dominated in La Grajera due to its stable water level during the summer season. *Coquillettidia richiardii* is also a dominant species in other wetlands of northern Spain [18]. In contrast, *Aedes* was the predominant species in Las Cañas due to its high-water fluctuation, which results in a considerable drop in water levels during summer. *Aedes* species like *Aedes rusticus* (Rossi, 1790) are also dominant in other wetlands in northern Spain [18]. The most abundant genus, *Culex*, was found to be well represented in the two reservoirs, despite being dominant only in the Iregua River. This may be attributed to the fact that *Cx. pipiens* s.l. is a species adapted to breeding in different types of natural breeding sites, including riverbanks, extremely eutrophic and polluted waters, habitats with abundant invasive hydrophytes [41], and even artificial reservoirs in urban areas [42].

Anopheles atroparvus was the least commonly trapped *Anopheles* species; nevertheless, multiple resting captures at various locations in La Grajera suggest that it may be more prevalent (Ruiz-Arrondo, unpublished data). Regarding *An. algeriensis*, this species historically inhabits the Mediterranean coast, where it commonly breeds in coastal marshy areas and saline or oligosaline channels with abundant helophytic vegetation [43]. Although this anopheline species has also been found in the province of Teruel [35], its detection in northern Spain is of particular interest, as it is far from its known distribution area [15].

Aedes caspius appeared as the most abundant species of *Aedes* in both the Iregua River and Las Cañas, although it did not represent more than 50% of the total of aedines in La Grajera due to the presence of *Ae. vexans* and *Ae. detritus*. *Aedes caspius* is one of the most abundant species on the Spanish Mediterranean coast, and *Ae. detritus* is also frequent [44]. Regarding the invasive *Aedes* mosquitoes, these species have adapted to breed in a variety of man-made and disused containers in urban/rural areas [45]. Although natural ecosystems are not favourable for the development of certain species such as *Ae. albopictus*, proactive surveillance remains crucial in preventing potential introductions and effectively managing mosquito-related risks. In fact, in La Rioja and Navarra regions, specific entomological surveillance programmes employ ovitraps and citizen science applications for invasive *Aedes* mosquito detection [14,16].

A potential limitation of this study was the use of a single trap model, namely BG-Sentinel traps, to investigate the diversity and abundance of mosquito populations in these aquatic ecosystems. In addition to the HLC method, other approaches such as the capture of adult resting mosquitoes and sampling of immature stages should be employed to address this limitation and obtain a more comprehensive understanding of the local mosquito fauna.

4.3. Population Dynamics in the Peak Abundance Period

The two most abundant anophelines, *An. algeriensis* and *An. claviger* s.s., showed a constant abundance throughout the summer season. *Anopheles claviger* s.l. has also been observed as a multivoltine species in northern Spain [18]. Schaffner et al. [31] described *An. claviger* s.s. as bivoltine, once in the early spring and once in the late summer. According to our findings, this species would have one more generation between those two periods. The same was observed for *An. algeriensis*, where the peak population periods occur during the spring and autumn [31], and yet our observations point to another peak of abundance during the summer.

The population dynamic of certain aedine species might be influenced by the availability of suitable breeding sites, especially those that are temporarily flooded, as is the case with *Ae. caspius*, *Ae. detritus*, and *Ae. vexans*, which are commonly referred to as "open floodwater species". The alternation of flooding and drought creates favourable conditions for the high-density hatching of these mosquitoes, so environmental factors such as rainfall significantly influence their population dynamics [46]. In fact, the amount and distribution of rain have a powerful effect on mosquitoes' population dynamics in species that overwinter as drought-resistant eggs. *Aedes caspius* preferentially oviposits in moister sites with abundant vegetation, while *Ae. detritus* oviposits in more saline soils [47]. However, their larvae can also be found on the margins of permanent bodies of water such as rivers, along with in trees, reeds, and canes [48]. Although rarely, we also captured adult *Ae. caspius* in the Iregua River in our study. In the Levante region, both species are closely linked to temporary bodies of water, especially in the coastal area. For example, it can be observed that the peaks of *Ae. caspius* capture, a multivoltine species (with an annual average of 14 generations in Huelva, Spain [44]), coincide with the periods close to the equinoxes, when the highest rainfall occurs in this area of eastern Spain. In the case of *Ae. detritus,* the difference between the spring and autumn capture peaks is more pronounced [49], a phenomenon also observed in other southern European countries, suggesting a possible univoltinism of the species [31]. However, on the south-western coast of Spain, they have a multivoltine character, with an annual average of five to seven generations between the spring and autumn [44]. Despite the low capture rates of this species, our results support this hypothesis, as only one peak of activity was observed between June and July in the two lotic water bodies sampled and bites occurring on human bait by *Ae. detritus* in spring 2016 in La Grajera. The same flight range for *Ae. detritus* has been noted in northern Spain [18]. In the marshy areas typical of the Valencian Community, after prolonged periods of rainfall, there are waterlogged soils with salinity conditions that greatly favour the hatching of larvae of both species, although with a predominance of *Ae. caspius*, with an increase in the abundance of this species observed in May and September [50], similar to what was observed in 2017 in La Grajera and Las Cañas. Regarding *Ae. vexans*, the seasonal population dynamic of this floodwater-associated aedine species shows a typical multivoltine pattern of abundance in European countries [32,51] and specifically in Spain [48], which is in line with our results.

Coquillettidia richiardii has several generations per year in La Grajera, a behaviour described in southern European populations [32]. This species shows at least four peaks of abundance during the summer, although a reduction in the population is observed at the end of the summer [31]. Nevertheless, Gonzalez et al. [18] noted its univoltine character in an aquatic ecosystem in northern Spain.

Culex pipiens s.l., a multivoltine species, shows the highest population peak in August, with the number of individuals decreasing progressively on each capture date. Although *Cx. modestus* is not widely represented in the wetland, two distinct peaks of abundance are observed during the summer, in line with the observations of other authors [18,31,32]. *Culex theileri* is a polycyclic species whose larvae occur in the spring [32]. In our environment, its abundance differs from one year to another; however, in 2017, its multivoltine character was observed with three distinct maxima in the two reservoirs. Meanwhile, in other Spanish regions such as Doñana and the area of influence of the Lower Guadalquivir rice fields (south-western Spanish coast), it is by far the most frequent and abundant species in the spring months [44].

The few individuals of *Ur. unguiculata* captured were concentrated during the central part of the summer, although Becker et al. [32] explained that this species is more abundant in the late summer.

We did not evaluate the population dynamics of Culicidae during May and June in 2016; therefore, the population dynamics of the species is not completed for the warm season of this year.

4.4. Human Landing Collection: Epidemiological Implications of the Identified Species

Human landing collection is considered the current "gold standard" for estimating human-biting rates, but it is labour-intensive and implies the risk of exposure to infectious mosquito bites [52]. This could explain why this technique has been so scarcely used in Spain to date [18], this being one of the first works to incorporate it. However, this technique has been used for the study of other Diptera such as simulids in the country [53]. The chance of being bitten by a mosquito in the three aquatic ecosystems studied is not very high, except during the spring hatching of *Ae. detritus* at La Grajera and the peaks of *Ae. caspius* abundance in the summer at Las Cañas.

The species most abundantly caught using HLC were *Ae. caspius* (n = 28) and *Ae. detritus* (n = 20), two crepuscular and anthropophilic mosquito species. These aedines are known for their aggressive biting behaviour towards humans and animals, both during the day and at night [54–56]. In Spain, there are serious biting nuisances for humans in certain regions such as the Levante, the eastern region of the Iberian Peninsula, where these anthropophilic species breed in flooded lagoons and coastal marshes near touristic areas [57]. Although *Ae. caspius* exhibits a preference for mammalian hosts, it has been demonstrated that they can also feed on birds, albeit when alternative host classes are not readily available [58]. In relation to disease transmission, invasive *Aedes* are of the greatest importance, as they are responsible for autochthonous cases of arbovirus, such as dengue virus (DENV) [59]. However, the potential role of indigenous species in the transmission of other pathogens of medical and veterinary importance in Spain should not be underestimated. Previous studies revealed that *Ae. caspius* populations from the Camargue (region of southern France) possess the potential to act as vectors of chikungunya virus (CHIKV) [60] and Rift Valley fever virus (RVFV) [61]. However, *Ae. caspius* populations from the Camargue and Andalusia regions (Spain) were incapable of transmitting West Nile virus (WNV) [62] and Zika virus (ZIKV) [58,63].

Coquillettidia richiardii was the third most abundant species caught with HLC (n = 6). This member of the Mansoniini feeds opportunistically on vertebrates and can naturally transmit Batai virus (BATV), Tahyna Orthobunyavirus (TAHV), Sindbis virus (SINV), and WNV [31]. With respect to its anthropophily, *Cq. richiardii* is confirmed as a species that eventually bites humans in Spain. Its anthropophily in our study is not as marked as in the region of the Danube Delta [64] or in southern England, where this species was responsible for the highest recorded biting rate in an HLC assay in the UK, with up to 161 bites per hour [65].

The low capture rate of *Culex*, with only two specimens of *Cx. modestus* caught, may be attributed to the species' preference for nocturnal activity over crepuscular activity (which was when the captures were made). Despite its abundance in the BG traps, no *Cx. pipiens* s.l. was captured in the HLC. However, it is essential not to underestimate the anthropophilic preferences and vectorial capacity of *Culex* mosquitoes. For example, the recent WNV outbreak in south-western Spain in 2020 highlighted the central role played by this genus in the transmission of this zoonotic arboviral disease [66]. Specifically, *Culex perexiguus* Theobald, 1903 and *Cx. pipiens* s.l. played a central role in the transmission of this arbovirus [67]. The apparent ornithophilic character of this latter species in these aquatic ecosystems means that the risk of human cases of WNV by this bridge vector might be lower.

As far as the genus *Anopheles* is concerned, only one specimen of *An. plumbeus* was captured in the HLC. This species is an aggressive and persistent biter, feeding principally on mammalian hosts [52] but also on birds and reptiles. Some populations even show a strong anthropophilic preference [68]. In fact, this species is usually found in urban environments, where immatures develop in tree holes in gardens and parks, causing a serious nuisance in some countries of Central Europe [31]. In our peri-urban environment, *An. plumbeus* does not pose a great risk due to its limited abundance. However, because it was the only species of anopheline captured in the HLC, it could be the species attributed as a vector in the second case of autochthonous malaria in the country, which occurred in the study area in 2014 [14]. It should be noted that despite the relative abundance of *An. claviger* s.s. in our study, it has not been a species implicated in human disturbance as González et al. [18] noted in their study in northern Spain. This behaviour might be attributed to the possibility that they are, in fact, *An. petragnani* rather than *An. claviger* s.s.

Another *Anopheles* species that in principle does not pose an obvious risk to the human population in the study area is *An. maculipennis* s.l., more especially, *An. atroparvus* as the only member of this complex identified molecularly in this peri-urban area [15]. This species played an important role in human malaria transmission in Europe. In fact, it was the species responsible for the first autochthonous case of malaria that occurred in northern Spain in early 2010 [69].

Public reports of mosquito bites in the urban area are rare and, in all cases, have been due to localised *Aedes* breeding sites. This means that a priori the mosquito populations in the ecosystems studied do not cause nuisance in the urban area. The reasons could be several: (1) the large population of mammals and birds in the two reservoirs could provide a food source for the mosquitoes without them having to look for hosts outside the area of influence of the aquatic ecosystems; (2) the low productivity of its breeding sites in the Iregua River prevents the massive emergence of species that could cause problems in the urban area; (3) in the case of Las Cañas, there is an industrial area between the reservoir and the urban environment which may be acting as a barrier to the natural dispersal of the mosquito population; and (4) not all species detected in the HLC have enough flight range to reach the urban area, with the exception of some aedine species.

These results on mosquito species composition and population dynamics during the peak abundance period contribute to a better understanding of the transmission risk of pathogenic agents of medical and veterinary importance. This study demonstrates the importance of monitoring the mosquito fauna in naturalised environments in areas very close to the city, such as peri-urban environments. The large influx of people enjoying leisure time in these environments and the constant presence of several species with vectorial potential in the summer season favours encounters between vectors and their hosts, highlighting the importance of including peri-urban areas in arthropod vector monitoring programmes.

5. Conclusions

This study highlights the large culicid fauna that inhabit peri-urban environments in northern Spain. Among the 24 taxa identified in the three aquatic environments, at least 5 are of medicalveterinary importance dueto their aggressiveness, distribution, and abundance. The different hydrological management practices could play a key role in determining the abundance of mosquito genera. Our study can provide valuable information to guide public health policies and strategies aimed at controlling mosquito populations and preventing disease outbreaks in the future. Moreover, peri-urban areas are believed to pose a higher risk of zoonotic disease transmission to humans due to their location on the fringes of urban areas and their proximity to farmland, freshwater ecosystems, and other wildlife habitats, as observed in our study. This underscores the significance of such investigations for public health.

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