

**New contributions to the knowledge of two riparian mosquitoes in northwestern Spain: *Anopheles petragnani* and *Culex mimeticus* (Diptera: Culicidae)**

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## Ecosystem Ecology

# New contributions to the knowledge of two riparian mosquitoes in northwestern Spain: *Anopheles petragrani* and *Culex mimeticus* (Diptera: Culicidae)

Yasmina Martínez-Barciela<sup>\*,</sup>, Alejandro Polina<sup>,</sup>, Josefina Garrido

Departamento de Ecología e Biología Animal, Universidade de Vigo, Vigo, España \*Corresponding author, mail: [ymartinez@uvigo.es](mailto:ymartinez@uvigo.es)

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A study was carried out in 345 water ecosystems (rivers, streams, ditches, lagoons, ponds, puddles, rockpools, and artificial containers) throughout the autonomous community of Galicia (NW Spain) during different seasons between 2020 and 2023. The results revealed the first detections of *Anopheles* (*Anopheles*) *petragrani* Del Vechio, 1939 ( $n = 185$ ) and *Culex* (*Culex*) *mimeticus* Noè, 1899 ( $n = 12$ ) in the territory, allowing to update and expand the overall knowledge about their distribution and ecology. The breeding preferences of the species were analyzed considering habitat characteristics (land use, water body type, and degree of insolation), geographical variables (latitude, longitude, and altitude), and physical–chemical water parameters (temperature, pH, dissolved oxygen, electrical conductivity, salinity, total dissolved solids, and turbidity). In addition, the relationship between the occurrence of these species and other mosquitoes present in the study area was discussed. *Anopheles petragrani* is widely distributed in the region and shows breeding preferences for water bodies of fluvial origin, forest environments, and shaded situations. *Culex mimeticus* was detected sporadically breeding in a pond and in a river in the south of the region, both in industrial and agricultural land uses, and always exposed to the sun. Although the presence of these species may not currently pose a health risk in the region, it is important not neglecting their study since the adequate characterization of their larval biotopes is relevant regarding vector surveillance and control measures.

**Key words:** vector, distribution, ecology, surveillance, Galicia

## Resumen

Se realizó un estudio en 345 ecosistemas acuáticos (ríos, arroyos, canales, lagunas, charcas, charcos, charcas fluviales y recipientes artificiales) a lo largo de la comunidad autónoma de Galicia (NO España) durante diferentes épocas entre 2020 y 2023. Los resultados revelaron las primeras detecciones de *Anopheles* (*Anopheles*) *petragrani* Del Vechio, 1939 ( $n = 185$ ) y *Culex* (*Culex*) *mimeticus* Noè, 1899 ( $n = 12$ ) en el territorio, permitiendo actualizar y ampliar el conocimiento general sobre su distribución y ecología. Se analizaron las preferencias de cría de las especies considerando las características del hábitat (uso del suelo, tipo de cuerpo de agua y grado de insolación), las variables geográficas (longitud, latitud y altitud) y los parámetros físico-químicos del agua (temperatura, pH, conductividad eléctrica, salinidad, total de sólidos en suspensión y turbidez). Además, también se analizó la relación entre la ocurrencia de estas especies y otros mosquitos presentes en el área de estudio. *Anopheles petragrani* está ampliamente distribuido en el territorio y muestra preferencia por aguas de origen fluvial, ambientes forestales y zonas sombreadas. *Culex mimeticus* se detectó puntualmente criando en una charca y un río en el sur de la región, tanto en ambientes industriales como agrícolas, y siempre en zonas soleadas. Aunque actualmente la presencia de estas especies no supone un riesgo sanitario en la región, es importante no descuidar su estudio ya que la adecuada caracterización de sus biotopos larvarios es relevante en términos de vigilancia y control vectorial.



## Introduction

The growing concern and incidence of vector-borne diseases has led to a substantial increase in mosquito surveillance programs in Spain (Ministerio de Sanidad 2023). The first investigations in this regard in Galicia date back to 2005 and 2008 with the entomological surveillance programs for bluetongue and West Nile virus (WNV), respectively (Lucientes and Delacour 2012, Lucientes et al. 2012). In 2017, the Xunta de Galicia, in collaboration with the University of Vigo and the University of Santiago de Compostela, created the “Rede Galega de Vixilancia de Vectores (ReGaViVec)” as a vector surveillance tool at regional level (Martínez-Barciela et al. 2020). The sampling efforts of these initiatives are mainly based on the use of traps for mosquito monitoring, such as ovitraps, BG-Sentinel2, and CDC light traps (European Centre for Disease Prevention and Control [ECDC] 2012). Subsequently, studies in different Galician wetlands expanded the knowledge of mosquitoes in the region (Martínez-Barciela et al. 2021, 2023). As consequence of these researches, more than 20 species and 5 genera of culicids (*Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, and *Culiseta*) have been confirmed in Galicia (Martínez-Barciela et al. 2021). Among them, it is of special relevance the tiger mosquito *Aedes albopictus* (Skuse, 1894) since it was recently detected in the region through a collaborative work between citizens, the citizen science application Mosquito Alert, and the ReGaViVec surveillance network (Martínez-Barciela et al. 2024).

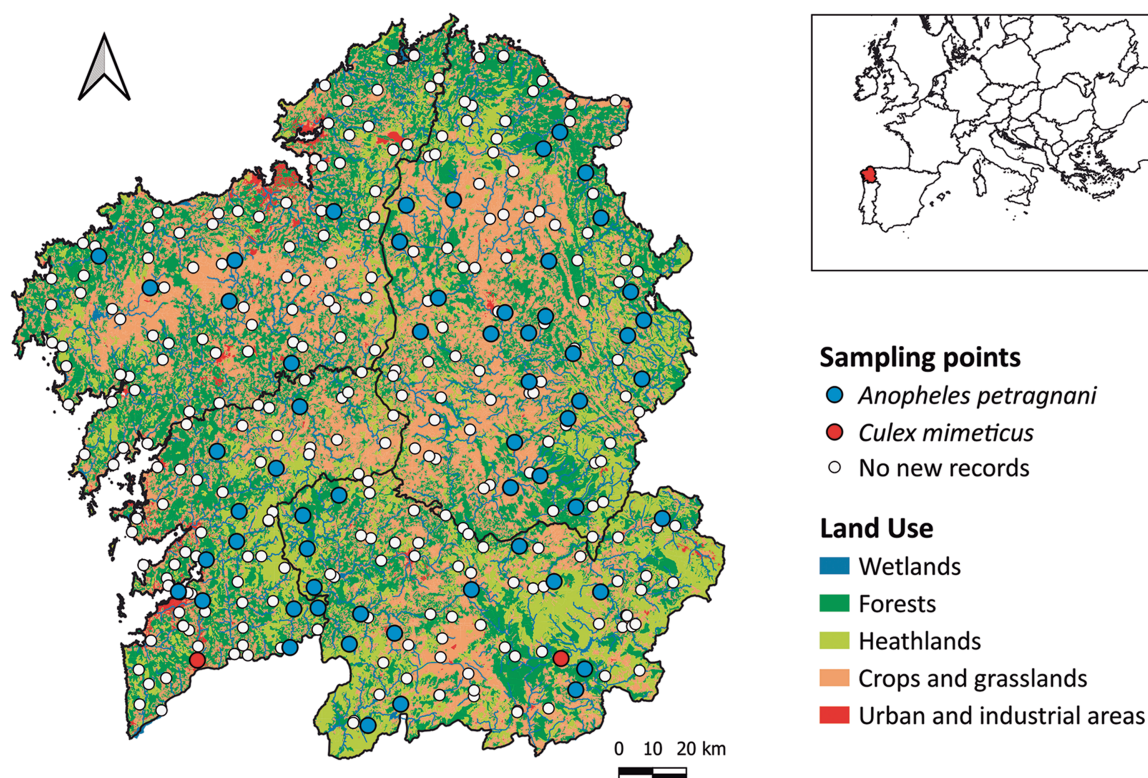
The immature stages of mosquitoes are strictly aquatic, and, depending on the species, the diversity of breeding sites they can inhabit is highly variable (Becker et al. 2020). Galicia harbors a large number and diversity of aquatic ecosystems, being characterized

by inland freshwater bodies and fluvial environments. As mostly of these areas lacked mosquito surveys or have only been sampled by the conventional trapping methods, an intensive larval sampling has been carried out throughout the region. The results of this work have led to the first records of *Anopheles* (*Anopheles*) *petragnani* Del Vecchio, 1939 and *Culex* (*Culex*) *mimeticus* Noè, 1899 in Galicia. These detections represent the most northwestern records of the species in Spain and provide a better understanding of their distribution and ecology. More specifically, their breeding preferences are analyzed as a function of habitat characteristics (land use, water body type, and degree of insolation), as well as geographical variables (latitude, longitude, and altitude) and physical–chemical parameters of the water (temperature, pH, dissolved oxygen, electrical conductivity, salinity, total dissolved solids [TDS], and turbidity). In addition, the relationship between the occurrence of these species and other mosquitoes present in the study area is also discussed.

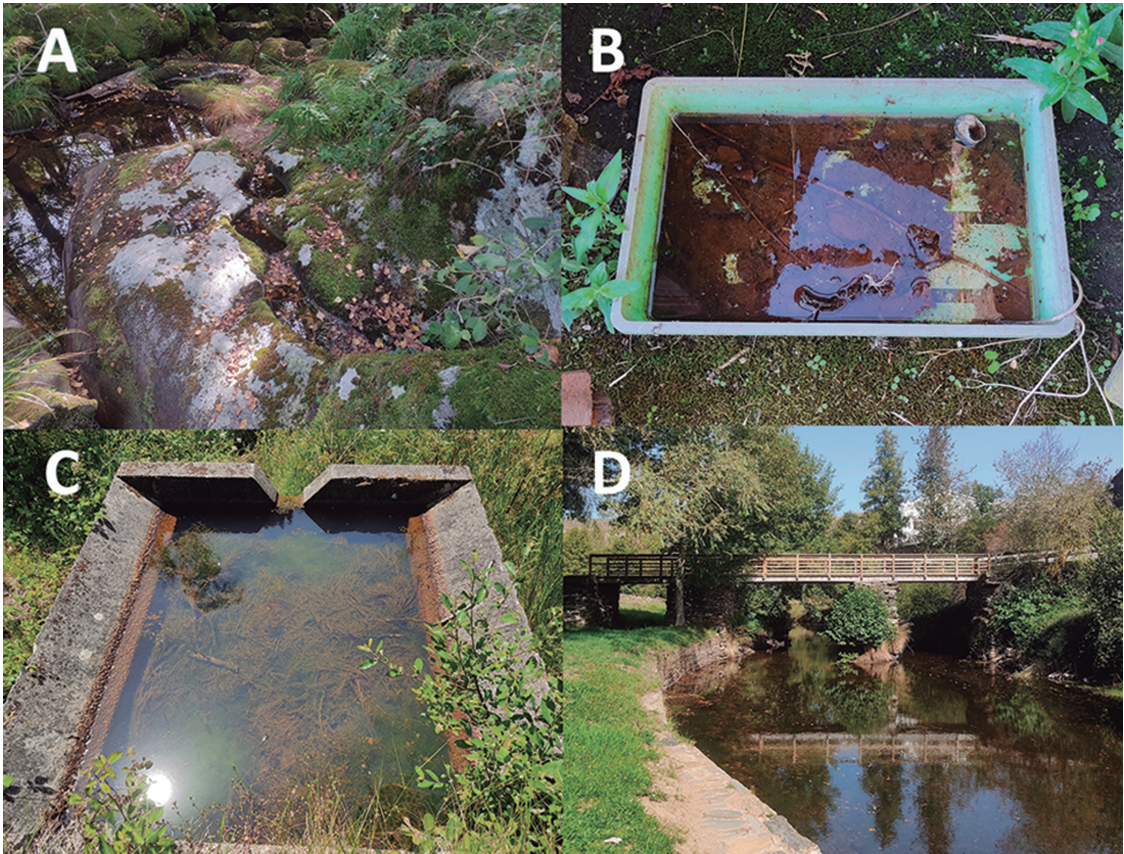
## Materials and Methods

### Study Area

The study was carried out in the Autonomous Community of Galicia, located in the most northwestern region of Spain. The territory covers an area of 29,574 km<sup>2</sup> and encompasses very diverse environments, with altitudes ranging from sea level to 2,000 m. Overall, the climatology of Galicia is characterized by a high relative humidity and rainfall that mellow from the coast inland. According to the Köppen–Geiger climate classification (Köppen 1900), it is divided into 2 predominant temperate climates: the temperate oceanic climate (Cfb), present in the northern and western zone; and the



**Fig. 1.** Sampling points distribution throughout Galicia, northwestern Spain (2020–2023). The locations with new records are indicated. Land use map (SIOSE 2011) extracted and modified from Instituto Geográfico Nacional de España–Xunta de Galicia.



**Fig. 2.** Photographs showing some of the breeding sites of *Anopheles petragrani* (A, B) and *Culex mimeticus* (C, D) in the study area: rockpool of Chandrexa de Queixa (A), artificial container of Arbo (B), pond of Tui (C), and river of Castrolo do Val (D) (Galicia, Spain).

warm-summer Mediterranean climate (Csb), typical of the central and southern region. The first has cold winters, cool summers, and a uniform rainfall between seasons; while the second has cold or mild winters, dry and cool summers, and an irregular rainfall throughout the year.

Habitat Characterization

The field research was conducted in 345 hydric ecosystems evenly distributed throughout the region (Fig. 1). The habitats were characterized according to 3 variables: land use, categorized as urban and industrial, crops and grasslands, heathlands, forests, and wetlands; water body type, such as rivers, streams, ditches, lagoons, ponds, puddles, rockpools, and artificial containers; and degree of insolation, classified as open sun (completely exposed to sunshine), half shade (partially shaded throughout the day), and shade (completely shaded). Generally, the sampling points had abundant vegetation in the surroundings, whether aquatic plants (algae and macrophytes) or terrestrial plants (moss, shrubs, or trees) (Fig. 2). Physical–chemical data (temperature, pH, dissolved oxygen, electrical conductivity, salinity, TDS, and turbidity) were measured in situ with a multiparameter water quality meter (HI 9829-HANNA Instruments) (Leighton Buzzard, Bedfordshire, England) at 313 sampling points.

Mosquito Sampling and Processing

Sampling was performed once per location throughout different seasons between 2020 and 2023, most of them performed in the summer of 2021 and 2022. Immature forms of mosquitoes (larvae

**Table 1.** Absolute (N) and relative (N%) abundance of mosquito species captured in the study area

| Species                                           | N     | N%   |
|---------------------------------------------------|-------|------|
| <i>Anopheles claviger</i> s.s. (Meigen, 1804)     | 37    | 0.7  |
| <i>Anopheles maculipennis</i> s.l. (Meigen, 1804) | 175   | 3.1  |
| <i>Anopheles petragrani</i> Del Vecchio, 1939     | 185   | 3.3  |
| <i>Anopheles plumbeus</i> Stephens, 1828          | 7     | 0.1  |
| <i>Culex hortensis</i> Ficalbi, 1889              | 632   | 11.1 |
| <i>Culex impudicus</i> Ficalbi, 1890              | 183   | 3.2  |
| <i>Culex mimeticus</i> Noè, 1899                  | 12    | 0.2  |
| <i>Culex pipiens</i> s.l. Linnaeus, 1758          | 2,749 | 48.5 |
| <i>Culex territans</i> Walker, 1856               | 688   | 12.1 |
| <i>Culex theileri</i> Theobald, 1903              | 34    | 0.6  |
| <i>Culex torrentium</i> Martini, 1925             | 650   | 11.5 |
| <i>Culiseta annulata</i> (Schränk, 1776)          | 12    | 0.2  |
| <i>Culiseta longiareolata</i> (Macquart, 1893)    | 303   | 5.3  |
| <i>Culiseta subochrea</i> (Edwards, 1921)         | 3     | 0.1  |

and pupae) were collected through the standardized dipping technique (Service 1993) introducing a 500 ml dipper in those areas of the water body where mosquitoes are more likely to be present (edges, shallow spots, and surroundings of aquatic vegetation). Following the criteria of Becker et al. (2016), 10 dips were taken per sampling point as long as the volume of the breeding site allowed it (otherwise, sampling was conducted by pipetting). Mosquito larvae and pupae were transported to the laboratory in plastic jars with water from their respective breeding grounds (containing organic material and remains of aquatic vegetation) and were reared at room



**Table 2.** Capture data of *Anopheles petragrani* and *Culex mimeticus*, including province (Pr.) (C: A Coruña, L: Lugo, O: Ourense, P: Pontevedra), place names, type of water body, UTM coordinates (Datum WGS 84), altitude in meters (Alt. [m]), collection date, and number of specimens (N)

| Species               | Pr. | Place               | Water body | UTM (29T)       | Alt. (m) | Date         | N  |
|-----------------------|-----|---------------------|------------|-----------------|----------|--------------|----|
| <i>An. petragrani</i> | P   | Vigo                | Ditch      | 524738, 4678500 | 34       | 14-VII-2020  | 1  |
|                       | P   | A Cañiza            | Puddle     | 558991, 4673354 | 510      | 30-VII-2021  | 1  |
|                       | O   | Ramirás             | Pond       | 578782, 4671831 | 363      | 10-VIII-2021 | 13 |
|                       | O   | A Bola              | River      | 588831, 4666153 | 467      | 10-VIII-2021 | 1  |
|                       | P   | Soutomaior          | Pond       | 533003, 4687925 | 86       | 19-VIII-2021 | 1  |
|                       | P   | Ponte Caldelas      | Rockpool   | 542029, 4693348 | 397      | 19-VIII-2021 | 1  |
|                       | P   | Cerdedo-Cotobade    | Rockpool   | 542743, 4702268 | 466      | 19-VIII-2021 | 1  |
|                       | O   | Beariz              | Stream     | 561654, 4701050 | 696      | 23-VIII-2021 | 2  |
|                       | O   | Avión               | River      | 562948, 4691195 | 579      | 23-VIII-2021 | 4  |
|                       | O   | Melón               | Rockpool   | 564992, 4679746 | 720      | 24-VIII-2021 | 9  |
|                       | L   | Friol               | River      | 601823, 4765463 | 505      | 30-VIII-2021 | 1  |
|                       | L   | Vilalba             | River      | 606305, 4794553 | 464      | 30-VIII-2021 | 1  |
|                       | L   | Mondoñedo           | Stream     | 633004, 4809703 | 188      | 30-VIII-2021 | 2  |
|                       | L   | Lourenza            | River      | 637849, 4814639 | 112      | 30-VIII-2021 | 1  |
|                       | L   | A Pontenova         | Stream     | 645529, 4802595 | 208      | 31-VIII-2021 | 6  |
|                       | L   | A Fonsagrada        | Stream     | 650048, 4789201 | 694      | 31-VIII-2021 | 1  |
|                       | L   | Pol                 | River      | 634582, 4776408 | 495      | 31-VIII-2021 | 18 |
|                       | L   | Castroverde         | Stream     | 633610, 4760019 | 517      | 31-VIII-2021 | 3  |
|                       | L   | O Corgo             | River      | 628594, 4755147 | 432      | 31-VIII-2021 | 1  |
|                       | L   | Sarria              | Pond       | 628745, 4740677 | 517      | 31-VIII-2021 | 3  |
|                       | L   | Bóveda              | Stream     | 624439, 4722720 | 404      | 01-IX-2021   | 4  |
|                       | L   | A Pobra do Brollón  | River      | 631955, 4712796 | 420      | 01-IX-2021   | 1  |
|                       | L   | Monforte de Lemos   | Puddle     | 623242, 4709295 | 321      | 01-IX-2021   | 4  |
|                       | P   | Cuntis              | River      | 536207, 4720058 | 196      | 08-IX-2021   | 2  |
|                       | O   | Quintela de Leirado | Lagoon     | 575420, 4662926 | 862      | 16-IX-2021   | 2  |
|                       | O   | Lobios              | Rockpool   | 580982, 4638818 | 822      | 16-IX-2021   | 3  |
|                       | P   | Forcarei            | River      | 553745, 4715029 | 612      | 21-IX-2021   | 4  |
|                       | O   | O Irixo             | River      | 572362, 4707042 | 576      | 21-IX-2021   | 4  |
|                       | O   | Maceda              | Pond       | 611623, 4679066 | 550      | 29-IX-2021   | 1  |
|                       | C   | Paderne             | Pond       | 570847, 4791126 | 215      | 06-X-2021    | 1  |
|                       | L   | Lugo                | Stream     | 617432, 4754862 | 507      | 18-X-2021    | 1  |
|                       | L   | Lugo                | River      | 621577, 4761165 | 431      | 18-X-2021    | 4  |
|                       | P   | Arbo                | Container  | 557829, 4661933 | 60       | 10-V-2022    | 17 |
|                       | C   | Vimianzo            | Pond       | 501170, 4777885 | 166      | 14-VI-2022   | 1  |
|                       | O   | Riós                | Pond       | 642570, 4649383 | 827      | 18-VII-2022  | 3  |
|                       | O   | Rubiá               | River      | 668225, 4700148 | 401      | 19-VII-2022  | 1  |
|                       | L   | Quiroga             | River      | 642641, 4703487 | 245      | 20-VII-2022  | 2  |
|                       | O   | Calvos de Randín    | Pond       | 590663, 4645130 | 916      | 28-VII-2022  | 2  |
|                       | L   | Palas de Rei        | River      | 596510, 4755509 | 522      | 03-VIII-2022 | 1  |
|                       | L   | Guitiriz            | River      | 590334, 4782151 | 452      | 03-VIII-2022 | 1  |
|                       | L   | Guitiriz            | River      | 592326, 4792988 | 495      | 03-VIII-2022 | 5  |
|                       | P   | Silleda             | Pond       | 560736, 4733274 | 285      | 22-VIII-2022 | 2  |
|                       | C   | Touro               | River      | 558262, 4746143 | 246      | 22-VIII-2022 | 1  |
|                       | C   | Santa Comba         | River      | 516348, 4768509 | 328      | 23-VIII-2022 | 1  |
|                       | C   | Cerceda             | River      | 541450, 4776639 | 280      | 23-VIII-2022 | 1  |
|                       | C   | Trazo               | Pond       | 539819, 4764535 | 319      | 23-VIII-2022 | 2  |
|                       | L   | Baralla             | River      | 641784, 4749023 | 471      | 15-IX-2022   | 1  |
|                       | L   | A Fonsagrada        | Pond       | 658865, 4767302 | 725      | 16-IX-2022   | 3  |
|                       | L   | Navia de Suarna     | River      | 662713, 4758880 | 301      | 16-IX-2022   | 1  |
|                       | L   | Becerreá            | River      | 657963, 4754371 | 345      | 16-IX-2022   | 2  |
|                       | L   | Cervantes           | Stream     | 662190, 4741487 | 558      | 16-IX-2022   | 6  |
|                       | L   | Triacastela         | Stream     | 643715, 4734963 | 656      | 16-IX-2022   | 1  |
|                       | L   | O Incio             | Puddle     | 640262, 4729786 | 928      | 16-IX-2022   | 12 |
|                       | O   | A Teixeira          | Rockpool   | 625846, 4691934 | 619      | 03-X-2022    | 1  |
|                       | O   | Chandrexa de Queixa | Rockpool   | 636139, 4681475 | 1,070    | 03-X-2022    | 6  |
|                       | O   | Manzaneda           | River      | 649948, 4678391 | 790      | 04-X-2022    | 3  |
|                       | O   | A Gudiña            | River      | 645210, 4655558 | 727      | 04-X-2022    | 1  |
|                       | P   | Crecente            | Pond       | 566165, 4673631 | 162      | 16-VIII-2023 | 4  |
|                       | P   | Redondela           | Container  | 531909, 4675778 | 210      | 12-IX-2023   | 2  |
| <i>Cx. mimeticus</i>  | P   | Tui                 | Pond       | 530372, 4658160 | 36       | 29-VI-2021   | 6  |
|                       | O   | Castrelo do Val     | River      | 638195, 4658665 | 883      | 04-X-2022    | 6  |

temperature until reaching the IV instar larvae and adult stage, respectively. The jars were checked daily and those specimens that did not survive were removed and identified immediately, being included in the final count. Both the larvae and the adults were fixed in 70% ethanol and identified to species level under a binocular magnifier and an optical microscope according to the morphological criteria of Becker et al. (2020). Subsequently, they were stored in the scientific collection of the Aquatic Entomology Laboratory at the University of Vigo (Vigo, Galicia, Spain).

### Data Analysis

The frequency and abundance (absolute and relative) of the species were calculated to determine their distribution and dominance in the study area, paying special attention to habitat characteristics (land use, water body type, and degree of insolation). The normality of the data (both biological and physical–chemical) was analyzed using the Shapiro–Wilk test. Since these metrics did not follow a normal distribution, the comparison of medians through the Kruskal–Wallis test was applied to determine differences between groups at a significance level of 0.05 according to the test parameters ( $H$ : test statistics,  $df$ : degrees of freedom,  $P$ :  $P$ -value) (Kruskal and Wallis 1952) and, subsequently, a Tukey's range test (post-hoc test) was applied to identify which groups were significantly different from others.

Pairs of boxplots were deployed to represent the environmental data of habitats with absence and presence of *An. petragani* (with statistical differences indicated according to the Kruskal–Wallis test results). The relationship between the larval abundance of the species (response variable) and environmental variables (explanatory variables) was analyzed by applying negative binomial regression as it is an appropriate method for databases with numerous zeros. Data were standardized and a manual forward-stepwise approach was employed for model selection, resulting in the inclusion solely of those explanatory variables with statistically significant information for the response variable ( $P < 0.05$ ). These analyses were not performed on *Cx. mimeticus* due to lack of data.

In order to determine the relationship between pairs of species occurring in the same larval habitat, the index of affinity (Fager and

McGowan 1963) and the index of association (Silver 2008) were calculated. The affinity index is defined by  $[J/(nA + nB)^{1/2}] - 1/2(nB^{1/2})$ ; where  $J$  = the number of joint occurrences of both species,  $nA$  = total number of occurrences of species  $A$ , and  $nB$  = total number of occurrences of species  $B$ , so that  $nA \leq nB$ . Pair of species with index results equal or higher than 0.5 are considered to show affinity (Fager and McGowan 1963). The association index is defined by  $2(J/A + B - 0.5)$ ; where  $J$  = the number of individuals of both species in samples occurring together,  $A$  = the number of individuals of species  $A$  in all samples, and  $B$  = the total individuals of species  $B$  in all samples. This index has a range of +1 to –1 (Silver 2008).

The data analysis was run using the BiodiversityR, pgirmess, and MASS packages of version 4.2.0 of the R software (Kindt and Coe 2005). The characterization of the study area and the development of the map were applied using version 3.8 of Quantum GIS (QGIS) (QGIS Development Team 2009).

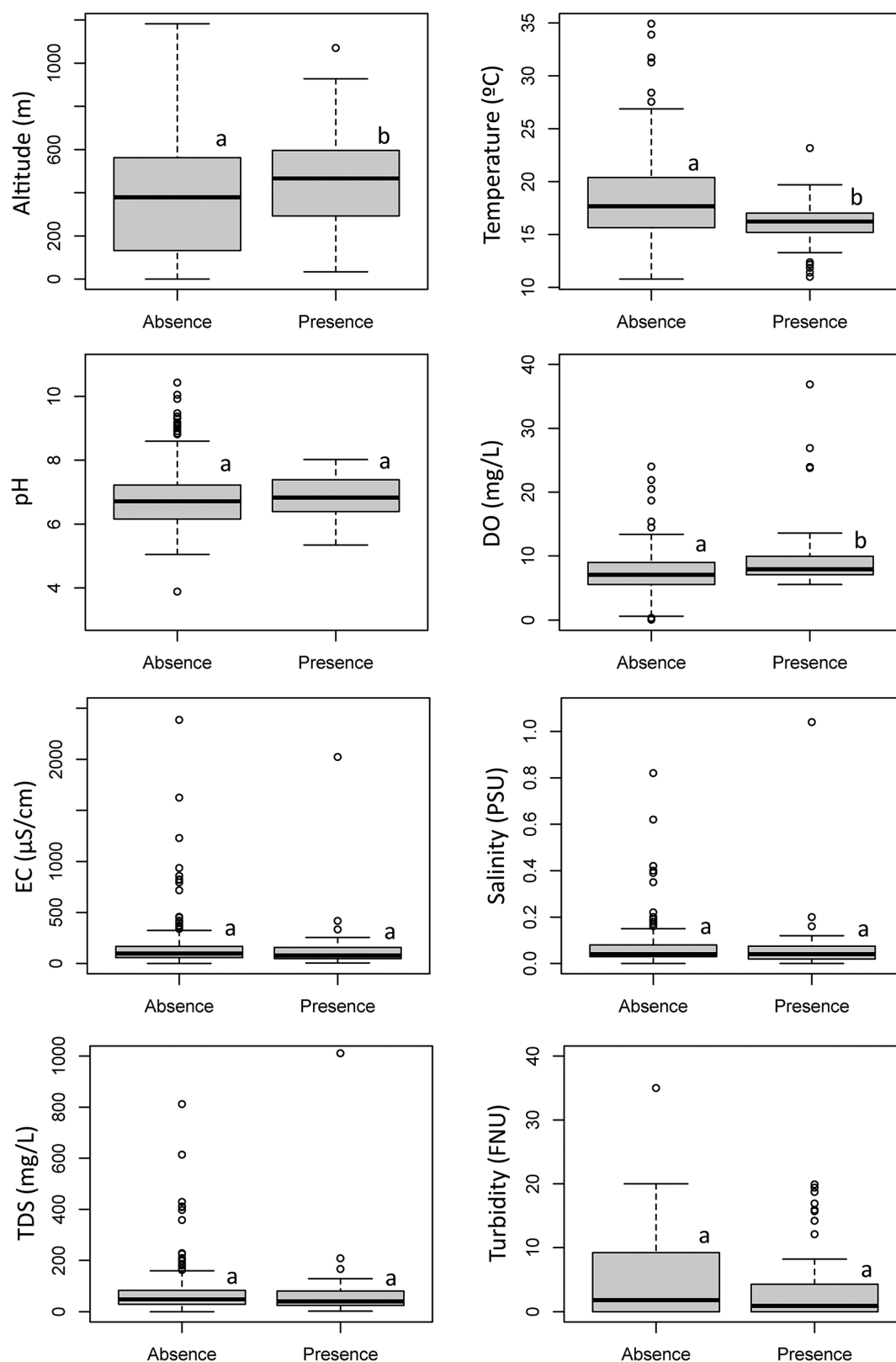
### Results

During the study, 5,670 mosquitoes belonging to 14 species of the genus *Culex* (87.3%), *Anopheles* (7.1%), and *Culiseta* (5.6%) were identified (Table 1). Mosquito larvae were collected in most of the sampling points (64%), with 27% of them showing the presence of *An. petragani* (59 locations) and 0.9% the presence of *Cx. mimeticus* (2 locations) (Table 2). While the former is widely distributed throughout Galicia, the latter has only been detected in the south of the region (Fig. 1). The frequency and abundance of *An. petragani* varied according to habitat characteristics, suggesting a preference for breeding in forests environments, fluvial water bodies (rivers, rockpools, and streams), and shaded situations (Table 3, Fig. 2). However, statistically significant differences were only detected between water bodies, with rivers and streams having the highest larval abundance on average (Table 3). On the other hand, *Cx. mimeticus* was found breeding in a pond and in a river, both in industrial and agricultural land uses, and always exposed to the sun (Fig. 2).

*Anopheles petragani* larvae have shown tolerance over a wide altitudinal and physical–chemical ranges, yet their breeding sites

**Table 3.** Data related to *Anopheles petragani* breeding sites considering habitat characteristics. Number of total sampling points ( $n$ ), number of samples with presence of the species ( $n+$ ), frequency in the study area [ $F = (n+/n) \cdot 100$ ], total ( $N$ ) and relative ( $N\%$ ) abundance. The medians (Me) correspond to the middle abundance value of the species considering the total number of mosquito breeding sites for each habitat characteristic. The Kruskal–Wallis test results (K–W) regarding medians comparison for each group are given ( $H$ : test statistics,  $df$ : degree of freedom,  $P$ :  $P$ -value), with different superscript letters indicating groups with statistically differences according to Tukey's range test ( $<0.05$ )

| Habitat characteristics |                      | $n$ | $n+$ | $F$ | $N$ | $N\%$ | Me               | K–W                                           |
|-------------------------|----------------------|-----|------|-----|-----|-------|------------------|-----------------------------------------------|
| Land use                | Urban and industrial | 27  | 2    | 7%  | 2   | 1%    | 0                | $H = 8.21$<br>$df = 4$<br>$P = 0.08$          |
|                         | Crops and grasslands | 205 | 34   | 17% | 124 | 67%   | 0                |                                               |
|                         | Heathlands           | 22  | 3    | 14% | 17  | 9%    | 0                |                                               |
|                         | Forests              | 67  | 19   | 28% | 40  | 22%   | 0                |                                               |
|                         | Wetlands             | 24  | 1    | 4%  | 2   | 1%    | 0                |                                               |
| Water body              | Artificial container | 40  | 2    | 5%  | 19  | 10%   | 0 <sup>a</sup>   | $H = 51$<br>$df = 7$<br>$P = 9 \cdot 10^{-9}$ |
|                         | Puddle               | 47  | 3    | 6%  | 17  | 9%    | 0 <sup>a</sup>   |                                               |
|                         | Pond                 | 90  | 12   | 13% | 36  | 19%   | 0 <sup>a</sup>   |                                               |
|                         | Lagoon               | 30  | 1    | 3%  | 2   | 1%    | 0 <sup>a</sup>   |                                               |
|                         | Rockpool             | 19  | 6    | 32% | 21  | 11%   | 0 <sup>a</sup>   |                                               |
|                         | Ditch                | 8   | 1    | 13% | 1   | 1%    | 0.5 <sup>a</sup> |                                               |
|                         | Stream               | 34  | 9    | 26% | 26  | 14%   | 2 <sup>b</sup>   |                                               |
|                         | River                | 77  | 25   | 32% | 63  | 34%   | 1 <sup>b</sup>   |                                               |
|                         |                      |     |      |     |     |       |                  |                                               |
| Degree of insolation    | Open sun             | 117 | 8    | 7%  | 38  | 21%   | 0                | $H = 17.6$<br>$df = 2$<br>$P = 0.06$          |
|                         | Half shade           | 109 | 18   | 17% | 30  | 16%   | 0                |                                               |
|                         | Shade                | 119 | 33   | 28% | 117 | 63%   | 0                |                                               |



**Fig. 3.** Pairs of boxplots representing the data distribution of the different environmental variables recorded in habitats with absence and presence of *Anopheles petragani*. Statistically significant differences between both groups are indicated by different letters ( $P < 0.05$ ) according to the Kruskal-Wallis test results for altitude ( $H = 5.67$ ,  $df = 1$ ,  $P = 0.017$ ), water temperature ( $H = 19.26$ ,  $df = 1$ ,  $P = 0.00001$ ), and DO ( $H = 8.22$ ,  $df = 1$ ,  $P = 0.004$ ). DO, dissolved oxygen; EC, electrical conductivity; PSU, Practical Salinity Units; TDS, total dissolved solids; FNU, Formazin Nephelometric Units. Note: for easier visualization of the data, some outliers have been removed from the “Absence” group for the variables EC (5 values  $> 6,000$   $\mu\text{S}/\text{cm}$ ), salinity (6 values  $> 3$  PSU), and TDS (3 values  $> 3,000$   $\text{mg}/\text{L}$ ).

**Table 4.** Negative binomial regression model results for *Anopheles petragrani* larval abundance, including the parameter estimates ( $\beta$ )  $\pm$  standard error (SE), Z-value (Z), and P-value (P) for each explanatory variable, as well as the percentage of deviance explained (DE) and the Akaike information criterion (AIC) of the global model. Model: Larval abundance = Temperature + Dissolved oxygen + Latitude + Insolation

| Explanatory variables           | $\beta \pm SE$   | Z     | P     | DE    | AIC   |
|---------------------------------|------------------|-------|-------|-------|-------|
| Intercept                       | 0.52 $\pm$ 1.55  | 0.34  | 0.73  |       |       |
| Temperature                     | -0.22 $\pm$ 0.08 | -2.81 | 0.004 |       |       |
| Dissolved oxygen                | 0.14 $\pm$ 0.05  | 2.80  | 0.005 |       |       |
| Latitude                        | -0.58 $\pm$ 0.27 | -2.15 | 0.03  |       |       |
| Degree of insolation (open sun) | 0.93 $\pm$ 0.69  | 1.34  | 0.19  |       |       |
| Degree of insolation (shade)    | 1.77 $\pm$ 0.62  | 2.85  | 0.004 |       |       |
| Global model                    |                  |       | 0.02  | 25.7% | 245.8 |

**Table 5.** Affinity (AFI) and association (ASI) indexes of *Anopheles petragrani* with the different species of mosquitoes with which it shared breeding sites

| Species                            | AFI   | ASI  |
|------------------------------------|-------|------|
| <i>Anopheles claviger</i> s.s.     | 0.65  | 0.44 |
| <i>Anopheles maculipennis</i> s.l. | 0.19  | 0.04 |
| <i>Culex hortensis</i>             | 0.27  | 0.24 |
| <i>Culex impudicus</i>             | 0.74  | 0.53 |
| <i>Culex pipiens</i> s.l.          | 1.08  | 0.18 |
| <i>Culex territans</i>             | 2.32  | 0.81 |
| <i>Culex torrentium</i>            | 0.46  | 0.23 |
| <i>Culiseta annulata</i>           | -0.01 | 0.02 |
| <i>Culiseta longiareolata</i>      | 0.19  | 0.17 |

differ significantly from other aquatic habitats (Fig. 3). Larvae were frequently found at relatively high altitudes (>400 m) ( $H = 5.67$ ,  $df = 1$ ,  $P = 0.017$ ), cold water temperatures (<17°C) ( $H = 19.26$ ,  $df = 1$ ,  $P = 0.00001$ ), and well-oxygenated waters (>7 mg/L) ( $H = 8.22$ ,  $df = 1$ ,  $P = 0.004$ ). The results of the negative binomial regression showed a statistically significant relationship between the species abundance and 4 environmental variables: latitude, temperature, dissolved oxygen, and degree of insolation (only in the case of “shaded” habitats) (Table 4). No statistically significant differences were detected among the other variables ( $P > 0.05$ ). *Culex mimeticus* larvae showed a wide altitudinal range (36–883 m) (Table 2), but its tolerance range for the physical–chemical variables could not be determined due to lack of data.

*Anopheles petragrani* larvae were found breeding with 9 different species of mosquitoes, showing affinity with *Cx. territans*, *Cx. pipiens* s.l., *Cx. impudicus*, and *An. claviger* s.s., and association with *Cx. territans* and *Cx. impudicus* (Table 5). *Culex mimeticus* was only found with larvae of *Cx. impudicus* and *Cx. territans*, with affinity and association indexes close to zero.

## Discussion

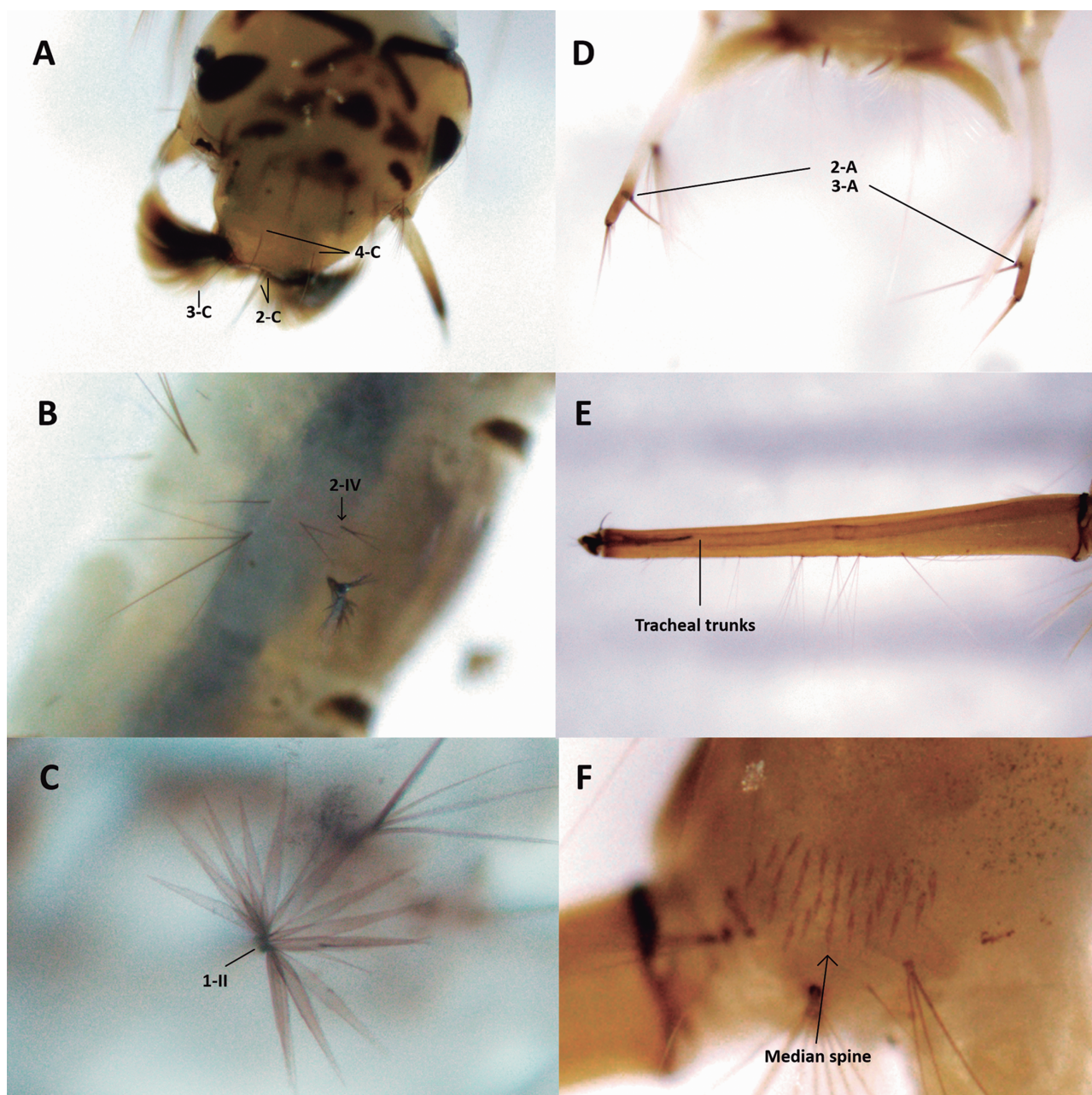
As a member of the *Anopheles Claviger* Complex, *An. petragrani* can only be morphologically differentiated from its sibling species *An. claviger* s.s. by partial overlapping characteristics of immature stages (Becker et al. 2016). *Anopheles petragrani* was initially described as a variety of *An. claviger* s.s. (Del Vecchio 1939) and eventually classified as a separate species due to morphological differences detected in larvae and pupae by Coluzzi (1960), Kampen et al. (2003), and Becker et al. (2016). Thanks to these studies, fourth-instar larvae of both species can now be distinguished by chaetotaxy of the head and abdomen. More specifically, larvae of *An. petragrani* were identified according to the following characteristics (Becker et

al. 2020): absence of siphon in abdominal segment VIII, frontal setae (5-C to 7-C) long and pinnate, inner clypeal setae (2-C) situated close together, outer clypeal setae (3-C) and postclypeal setae (4-C) simple (rarely bifid) (Fig. 4A), antepalmate setae on abdominal segments IV and V (2-IV and 2-V) with 2-3 branches (if 3-branched, the median is shorter than the others) (Fig. 4B), and palmate setae on abdominal segment II (1-II) with more than 15 leaflets (Fig. 4C). The main morphological differences with *An. claviger* s.s. are the chaetotaxy of the 4-C setae (which instead of being simple has 2-5 branches), the 2-IV and 2-V setae (which have 3-5 branches instead of 2-3 and, if 3-branched, all are the same length), and the 1-II setae (with fewer than 15 leaflets) (Becker et al. 2016, 2020).

While *An. claviger* s.s. occurs across most of Europe, up to Middle East and North Africa, the known distribution of *An. petragrani* seems to be restricted to the western Mediterranean sub-region, including Italy, France, Spain, Portugal, and North Africa (Becker et al. 2016). Similarly, the known distribution of the former species in Spain is wider than the latter, whose detection until now was limited to 8 Autonomous Communities (Aragón, Castilla y León, Castilla-La Mancha, Cataluña, Comunidad Valenciana, Extremadura, Islas Baleares, and La Rioja) (Encinas Grandes 1982, Bueno-Marí et al. 2012), to which Galicia has now been added. Since *An. petragrani* has shown a widely distribution throughout the territory (Fig. 1), it is surprising that its presence was not detected previously (Martínez-Barciela et al. 2020, 2023). Precisely because this species is native in the country and relatively frequent in the study area (Table 3), a recent introduction is discarded. Rather, this suggests that sampling methods based on the use of traps for mosquito monitoring (Martínez-Barciela et al. 2020) are not the most suitable for detecting this species, either because it is not attracted to them or because, even if the adults did so, their identification by morphological means would not be possible (Becker et al. 2016). On the other hand, the fact that no larvae have been captured in previous samplings in Galician wetlands through the use of an entomological net (Martínez-Barciela et al. 2021, 2023) suggest that this method is not the best for their capture either, unlike what has been observed with the dipping technique (Table 3).

*Anopheles petragrani* can be found from sea level to above 1,700 m (Ribeiro et al. 1988), being considered a common species in mountainous environments (Bueno-Marí et al. 2009). Similar to the observations made by Pires et al. (1982) and Ribeiro et al. (1988) in Portugal, most of the detections in Galicia have occurred between 400 and 600 m altitude, being exceptionally found above 1,000 m (Table 2, Fig. 3). Although this species can be found in coastal areas, most of the records are from inland ones (Fig. 1), in such a way that larval abundance slightly decreases with latitude (towards the north of the region) (Table 4). Larvae were most frequently found in cold (16°C–17°C), neutral (pH 6.5–7), well-oxygenated (7–8 mg/L),





**Fig. 4.** Photographs of the taxonomic characters used for the identification of *Anopheles petragrani* (A–C) and *Culex mimeticus* (D–F). A) Arrangement and shape (single branches) of the head setae (2–4C). B) Antepalpal setae on abdominal segments IV with 2 branches. C) Palmate setae on abdominal segment II with more than 15 leaflets. D) Insertion of the subapical setae (2–3A) about 2/3 of the distance between antennal setae (1–A) and tip of the antenna. E) Narrow main tracheal trunks. F) Comb with 24 scales with a distinct median spine longer than the others.

and clear (0–1 Formazin Nephelometric Units [FNU]) waters with low values of electrical conductivity ( $<100 \mu\text{S}/\text{cm}$ ), salinity ( $<0.1$  Practical Salinity Units [PSU]), and TDS ( $<50 \text{ mg}/\text{L}$ ) (Fig. 3). However, only the physical–chemical variables of temperature and dissolved oxygen have shown a statistically significant relationship with the presence and abundance of this species, so that the number of larvae decreases with temperature and increases with dissolved oxygen concentration (Table 4). Aside from one exception ( $24^\circ\text{C}$ ), all temperature values fall within the optimal range described for the species ( $11\text{--}20^\circ\text{C}$ ) (Schaffner et al. 2001). Consistent with the literature (Pires et al. 1982, Bueno-Mari et al. 2009, Becker et al. 2016), *An. petragrani* has been found breeding preferably in river margins, rockpools, and streams, as well as in natural and rural environments

(Table 3), showing a positive relationship between larval abundance and shaded situations (Table 4). Although it has been already documented (Romi et al. 1997), the recent detection of this species breeding in artificial containers in highly anthropized areas (urban and industrial) is noteworthy (Table 3, Fig. 2).

*Anopheles petragrani* larvae were found between May and October (more frequently in August and September) (Table 2), along with other mosquito species in the 75% of the cases (Table 5). Among them, it showed high affinity indexes ( $>0.5$ ) with *Cx. territans* (2.32), *Cx. pipiens* s.l. (1.08), *Cx. impudicus* (0.74), and *An. claviger* s.s. (0.65), which means that it is very likely that they share similar environmental needs and, therefore, occur together (Fager and McGowan 1963). On the other hand, *An. petragrani*

only showed high association indexes ( $>0.5$ ) with *Cx. territans* (0.81) and *Cx. impudicus* (0.53), so that the larval abundance of one seems to favor the other (Silver 2008). Regarding the feeding patterns, females are strongly zoophilic and shows host preference for livestock such as pigs and cows (Becker et al. 2020), biting humans accidentally in the vicinity of their larval habitats (Encinas Grandes 1982). So far, this species is not related to disease transmission, being an anopheline with no apparent role in malaria transmission (Becker et al. 2020).

*Culex mimeticus* was originally described by Noë (1899) and subsequently included in the Mimeticus Complex by Sirivanakarn (1976). As the only species of the complex present in Europe, the larvae were identified as having the following morphological characteristics (Becker et al. 2020): long siphon (siphonal index at least 4.0) with several pairs of siphonal tufts, subapical setae (2-A and 3-A) inserted about 2/3 the distance between antennal setae (1-A) and tip of the antenna (Fig. 4D), main tracheal trunks narrow (less than half as wide as the siphon at its apical 1/3) (Fig. 4E), and comb with 20–35 scales with a distinct median spine longer than the others (Fig. 4F).

The distribution of *Cx. mimeticus* covers the southern Palearctic and Oriental regions, from the Mediterranean subregion to eastern and southeastern Asia (Schaffner et al. 2001). In Spain, it has been recorded in 11 Autonomous Communities (Andalucía, Aragón, Castilla y León, Castilla-La Mancha, Cataluña, Comunidad Valenciana, Extremadura, La Rioja, Madrid, País Vasco, and Región de Murcia) (Encinas Grandes 1982, Bueno-Marí et al. 2012), among which Galicia is now added. The species shows a limited presence in the territory, being detected only in 2 locations more than 100 km apart in the south of the region (Fig. 1). Precisely because of the long distance between the found populations and the native nature of the species in the country (Encinas Grandes 1982), a recent introduction is unlikely. Rather, these facts suggest that the sampling methods previously applied (Martínez-Barciela et al. 2020, 2023) have not been the most adequate to detect this species in the low densities that it seems to occur in the study area (Table 2).

Although *Cx. mimeticus* is considered an orophilic species (Schaffner et al. 2001, Bueno-Marí et al. 2009), in Galicia it has been found at low (36 m) and moderate altitudes (883 m) (Table 2). These results are similar to those recorded in Portugal (150–1,100 m) (Ribeiro et al. 1988) and Comunidad Valenciana (127–1,155 m) (Bueno-Marí 2010). Physical–chemical data could only be measured at one of the 2 sampling points where the species was detected, which was characterized by cold (11.9°C) and slightly acidic (pH 6.3) water with low levels of electrical conductivity (43  $\mu$ S/cm), salinity (0.02 PSU), TDS (22 mg/L), and turbidity (0.1 FNU). These data are partially consistent with the observations of Ribeiro et al. (1977), who note that larvae are common in clear waters, although the pH was slightly higher than the optimum range documented for the species (5.2–6.0). *Culex mimeticus* is usually found breeding in shallow pools at the margins of streams, either strongly or weakly flowing (Encinas Grandes 1982, Becker et al. 2020). In the study area, larvae were collected in the backwater of a river with a rocky substrate and little flow, as well as in a rocky pond of anthropogenic origin (Fig. 2), matching the records of Bueno-Marí (2010) in Comunidad Valenciana. Although it has been documented that this species is predominantly rural (Schaffner et al. 2001), it has been observed both in agricultural and industrial areas, being close to an industrial estate. Due to lack of data, no general conclusions can be drawn about their breeding preferences.

*Culex mimeticus* larvae were found in late June and early September (Table 2) sharing breeding site with *Cx. territans* and *Cx. impudicus*, with which affinity and association indexes could

not be determined due to the scarcity of available data. Regarding the feeding patterns, females are zoophilic with preference for birds (Schaffner et al. 2001), although may eventually bite humans (Rioux 1958). Its sanitary interest is not negligible since it can be infected by the WNV (Schaffner et al. 2001), being considered a potential vector for the maintenance of the disease among the avian population (Jiménez-Peydró et al. 2023).

Both *An. petragani* and *Cx. mimeticus* are species that have shown a preference for breed in rural and fluvial environments (Schaffner et al. 2001, Becker et al. 2016, 2020). Although the former has a wide distribution throughout Galicia, it does not pose a health risk since it is not associated with disease transmission or biting pressure (Becker et al. 2020). However, it is important not neglecting its study since its epidemiological interest may undergo changes over time and future research. *Anopheles petragani* larvae has shown high affinity with *Cx. pipiens* s.l. and *An. claviger* s.s., the main vector of WNV and a secondary vector of malaria in Spain, respectively (Jiménez-Peydró et al. 2023). This relationship could be of special interest when providing information on the characterization of larval biotopes regarding vector surveillance measures. On the other hand, *Cx. mimeticus* is a secondary vector itself in the transmission of WNV that could have a relevant role in the maintenance of the disease in bird populations. The scarce detections and the apparently reduced distribution of this species do not imply a current problem in the region. Nevertheless, considering that Galicia has numerous wetlands that host a rich avian diversity, it is necessary to monitor the population evolution of this species in order to foresee potential health risks.

Precisely because of the importance of keeping a good public and environmental health conditions, it is essential to implement stable and continuous vector surveillance programs at regional level. In this regard, the most appropriate sampling methods and preferred larval habitats by the different species should be considered when applying surveillance and vector control measures.

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## Author Contributions

Yasmina Martínez-Barciela (Conceptualization [Lead], Data curation [Equal], Formal analysis [Lead], Funding acquisition [Supporting], Investigation [Lead], Methodology [Equal], Writing—original draft [Lead], Writing—review & editing [Equal]), Alejandro Polina (Conceptualization [Supporting], Data curation [Equal], Investigation [Supporting], Methodology [Equal], Writing—original draft [Supporting], Writing—review & editing [Equal]), and Josefina Garrido (Conceptualization [Supporting], Funding acquisition [Lead], Project administration [Lead], Resources [Lead], Supervision [Lead], Writing—review & editing [Supporting])

## Data Availability

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



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