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Bioecology of blackflies (Diptera: Simuliidae) of the water basin of the Huebra River (Western Spain)

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ABSTRACT

Bioecology of blackflies (Diptera: Simuliidae) of the water basin of the Huebra River (Western Spain).

The objectives of this study were to analyse the diversity and geographical distribution of the blackfly species from the Huebra River basin located in Salamanca province, Western Spain. This research significantly contributes to expanding our knowledge of the Simuliidae family. The study not only increases and ameliorates faunal and bioecological data, but also enhances the understanding of blackfly species across the Spanish national territory. The identification of 23 species has led to the addition of six new records to the simuliid fauna of the region: *Simulium (Nevermannia) armoricanum* Doby & David, 1961; *Simulium (Nevermannia) carthusiense* Grenier & Dorier, 1959; *Simulium (Nevermannia) naturale* Davies, 1966; *Simulium (Nevermannia) vernum* Macquart, 1826; *Simulium (Simulium) bezzii* (Corti, 1914) and *Urosimulium faurei* (Bernard, Grenier & Bailly-Choumara, 1972), expanding the previously recorded 19 species to 25. This study also provides valuable insights into the biology and geographical distribution of these species. It sheds light on the ranges of altitude, water velocity, temperature and the depth of watercourses conducive to preimaginal development. Additionally, it offers new information on the abundance of larvae and pupae, revealing the diverse ecological gradients that influence their occurrence and distribution patterns. The results indicate that the diversity of blackfly species varies according to factors such as altitude, water velocity, turbidity, and water temperature.

KEY WORDS: Simuliid species richness, abiotic factors, tolerance ranges, geographical distribution, ematophagy.

RESUMEN

Bioecología de las moscas negras (Diptera: Simuliidae) de la cuenca hidrográfica del río Huebra (Oeste de España).

Los objetivos de este estudio fueron analizar la diversidad y distribución geográfica de las especies de mosca negra de la cuenca del río Huebra, situada en la provincia de Salamanca, en el oeste de España. Esta investigación contribuye significativamente a ampliar el conocimiento de la familia Simuliidae. Este estudio no sólo aumenta y mejora los datos faunísticos y bioecológicos, sino que también repercute en el conocimiento de las especies de mosca negra en todo el territorio nacional español. La identificación de 23 especies ha permitido añadir seis nuevos registros a la fauna de simúlidos de la región: Simulium

(Nevermannia) armoricanum *Doby & David, 1961;* Simulium (Nevermannia) carthusiense *Grenier & Dorier, 1959;* Simulium (Nevermannia) naturale *Davies, 1966;* Simulium (Nevermannia) vernum *Macquart, 1826;* Simulium (Simulium) bezzii *(Corti, 1914) y* Urosimulium faurei *(Bernard, Grenier & Bailly-Choumara, 1972), ampliando a 25 las 19 especies registradas anteriormente. Este estudio también aporta valiosos datos sobre la biología y la distribución geográfica de estas especies, arrojando luz sobre los rangos de altitud, la velocidad del agua, la temperatura del agua y las dimensiones de los cursos de agua propicios para su desarrollo. Además, ofrece nueva información sobre la abundancia de larvas y pupas, revelando los diversos gradientes ecológicos que influyen en su presencia y patrones de distribución. Los resultados indican que la diversidad de especies de mosca negra varía en función de factores como la altitud, la velocidad del agua, la turbidez y la temperatura del agua.*

PALABRAS CLAVE: *riqueza de especies de simúlidos, factores abióticos, rangos de tolerancia, distribución geográfica, hematofagia.*

INTRODUCTION

The study of simuliids (Diptera Simuliidae) in Spain is characterised by fragmentation since the data are quite complete and detailed in some regions, while in others they are scarce or even non existent. Due to this imbalance, every study is of utmost importance, and gathering all the information available on this family of dipterans, from the oldest to the most recent study, is instrumental to gaining a better insight into these insects in the national territory.

The uneven study of simuliids has resulted from blackfly research in Spain traditionally being carried out in areas where the females of this group of insects represented a danger and a nuisance to domestic animals. This is due to their compulsory requirement of blood ingestion to ensure their eggs develop correctly. Besides that, Spain has attracted worldwide research interest owing to its large species richness, which is a consequence of its location, presence of insular territories, and diverse climatology, as highlighted by the Natura 2000 Network (Miteco, 2011), with the target of discovering new species and enhancing our understanding of these nematocerans. Consequently, numerous contributions to the knowledge on blackflies from the general territory of Spain (Strobl, 1900, 1905, Czerny & Strobl, 1909, Grenier & Bertrand, 1954, Grenier & Dorier, 1959, Crosskey & Grácio, 1985, Clergue-Gazeau & Vinçon, 1990, González-Peña, 1990, Belqat & Garrido, 2008), northern Spain (Beaucournu-Saguez, 1975b, Halgoš, 1998), southern Spain (Carlsson, 1969, Beaucournu-Saguez, 1975a, González-Peña et al., 1986, González-Peña et al., 1987, Vinçon & Clergue-Gazeau, 1993, Crosskey & Crosskey, 2000, Gallardo-Mayenco & Toja, 2002), the Balearic Islands (Crosskey, 1991) and the Canary Islands (Bigot, 1892, Becker, 1908, Frey, 1936, Crosskey, 1988, Malmqvist et al., 1993, 1995, Nilsson et al., 1998, Crosskey & Báez, 2004, Reidelbach, 2004, Lüderitz et al., 2010, Seitz, 2021) have been made (note that all of them have not been mentioned here).

In contrast to the situation in regions of the world such as Central Africa, Central America, North America and Central Europe, where blackflies act as vectors of disease to animals and humans (Crosskey, 1990, 1993, Cupp, 1996), in Spain, the occasional incidence of damage derived from the ethology of the females of species with a haematophagous requirement has meant a lower and irregular dedication of efforts to deepen the comprehensive knowledge of the bioecology of these organisms. Nonetheless, recent years have witnessed a reversal of this trend due to various factors that are amplifying the perception of nuisance among farmers and ranchers (Figueras et al., 2011, López-Peña, 2018, López-Peña et al., 2022b), the tourism sector and citizens in general (Gállego et al., 1994, Fuertes Martínez & Salvadó Pascual, 2009, Ruiz-Arrondo et al., 2017, Ruiz Arrondo, 2018, Sánchez-López et al., 2018, Barberá Riera et al., 2018, López-Peña, 2018, 2019, López-Peña et al., 2019, 2021, 2022c), which have motivated their study.

In line with this tendency, the main aim of this research has been to enhance the knowledge on simuliids from poorly studied areas of Spain such as the Huebra River hydrographic basin in order to gaing insights into species diversity, geographical distribution and tolerance ranges to abiotic factors, as well as to understand the presence of species of importance for human health and the veterinary sector.

MATERIAL AND METHODS

Study area

The Huebra River is one of the tributaries of the Duero River. It flows through the province of Salamanca (Autonomous Region of Castilla y León) carving granite, slate, quartzite, marl and sandstone materials, and thus shaping ravines and slopes that have resulted in enormous canyons and geographical depressions (MAPA, CHD, 2019a). In addition, its pluvio-nival character has formed a V-shaped valley in some of its sections (Merchán et al., 2022).

The Huebra River is born from the union of several streams that have their source in the surroundings of the Pico Cervero at 1244 m (Antón et al., 2014) which belongs to the Sierra de las

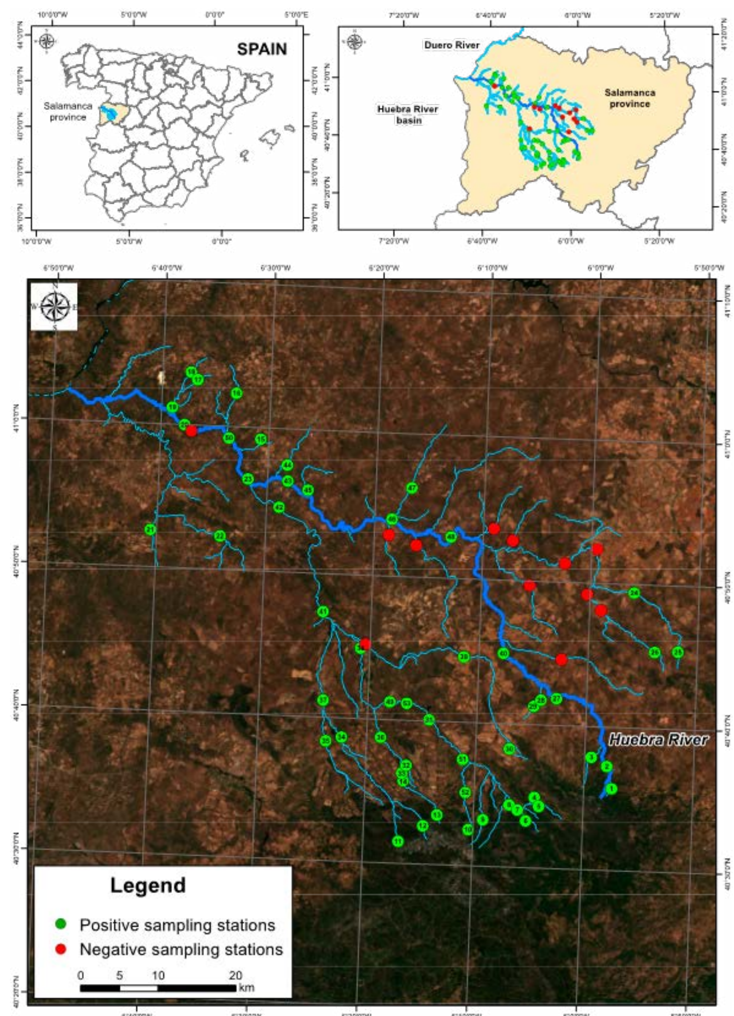

Figure 1. Position of the province of Salamanca in Spain (left), location of the Huebra River basin and the sampling stations (right), detail of the river, its tributaries and each sampling station (below). The numbers refer to the codes of the samples, which are listed in the tables. *Posición de la provincia de Salamanca en España (izquierda), ubicación de la cuenca del río Huebra y las estaciones de muestreo (derecha), detalle del río, sus afluentes y cada estación de muestreo (abajo). Los números se refieren a los códigos de las muestras, que se enumeran en las tablas.*

Quilamas, the northernmost part of the region of Sierra de Francia (CHD, 2019b). This 133.60-km-long river (Antón et al., 2014, CHD, 2019b), with a catchment area of 2808 km² (CHD, 2019b) and an annual average flow of 258.7 hm³/year (CHD, 2019b), flows through the Campo Charro and joins the Duero River, which acts as a natural border with Portugal, at the Salto de Saucelle, a town in the municipality of Saucelle, which is integrated into the Vitigudino region and the sub-region of La Ribera, better known as Las Arribes. The entire hydrographic basin of the Huebra River flows through the province of Salamanca, from its source to its mouth.

This river is characterised by its marked irregularity due to its pluvial-nival regime and its pronounced dry spells, which make it a river of temporary flow (CHD, 2019b). On the southern side, this river receives the waters of the rivers Yeltes and Cámaces, and on the northern side those of the Arganza Stream, and the Oblea River, as well as innumerable streams from both watersheds.

Sample collection

A sample collection campaign was designed to cover the entire hydrographic basin to be studied, which comprised both the main river, Huebra River, its main tributaries, as well as the tributaries that feed them. A total of 65 sampling stations were established, separated from each other at an approximately similar distance, always depending on the accessibility to the bodies of water according to the orography of the terrain (Fig. 1). The sampling, which took place between March and July 1996 (for specific details see Table S1, Supplementary Material, available at https://www.limnetica.net/en/limnetica), were carried out by Manuel Portillo Rubio and María Inmaculada Martín Hernández, collectors from the University of Salamanca, after reviewing rocks, boulders, pebbles, stones, macrophytes, helophytes, branches and leaves of the riverside vegetation in direct contact with the flow of water, and remains of vegetables such as leaves, branches and trunks. In those stations where blackflies were present, a representative sample was collected by obtaining small stones, leaves and fragments of stems on the surface of which the larvae and/or pupae were attached. If this was not viable, the specimens were collected by separating them from the surfaces of the aforementioned adhesion supports using soft entomological tweezers. An attempt was made to dedicate the same effort and sampling time at each station to enable the results obtained to be comparable. The collected specimens were fixed in situ by placing them in plastic containers with 70% ethanol for their transport to the laboratory. After reaching the laboratory, the collectors extracted most of the attachment substrates and stored the entomological material in tubes containing 70% ethanol for preservation.

At each sampling station, the values of the following variables were recorded: sample number, date, elevation (m), water temperature (º C), depth (m), adhesion substrate (vegetation, stones, concrete), water velocity (m/s), and water coloration (according to the following qualitative scale: very clear, clear, yellowish, darkened) (Table S2, Supplementary Material, available at https://www.limnetica.net/en/limnetica). Water velocity was measured by estimating the time, with the help of a stopwatch, that it took a piece of cork to travel a 5-m-long transect delimited by a rope tied to two wooden stakes. Geographical coordinates of the sampling stations were not recorded numerically; however their exact location was represented on a map, the recreation of which is shown in figure 1.

Species identification

The biological material from 53 sampling stations underwent processing, which involved the removal of plant structures from many samples to detach adhered blackfly specimens. Blackfly larvae were distinguished from pupae and housed in Petri dishes containing 80% alcohol. Following this, immature larvae were differentiated from mature ones, with the acknowledgment that identifying young larval instars can be challenging as they lack the level of morphological development necessary for taxonomic classification. Pupae and mature larvae were then identified using morpho-anatomic-based taxonomic keys from various sources (Knoz, 1965, González-Peña, 1997, Bass, 1998, Jedlička et al., 2004, Rivosecchi et al., 2007, Kúdela et al., 2022). Finally, both immature larvae and identified specimens in mature larval and pupal stages were tallied. These procedures were conducted under Wild M8 Heerbrugg and Leica M80 stereomicroscopes.

Data analysis

As a first step, we explore the correlations between the environmental variables of the sampling stations. Only those pairs of variables for which Pearson's r was lower than 0.8 were retained for a Principal Component Analysis (PCA), which was used to explore the differences between sampling stations on standardised values of the environmental variables measured.

We performed a Canonical Correspondence Analysis (CCA; Borcard et al., 2011) using the log abundance data for mature larvae (immature larvae could not be identified morphologically with sufficient taxonomic resolution) and pupae of the identified species. CCA was chosen after assuming a unimodal model response of blackfly species to the gradients defined by the environmental variables. A previous Detrended Canonical Correspondence Analysis (DCCA) provided support for this (i.e., the maximal length of the first two axes of the DCCA ordination > 3 SD; Leps & Smilauer, 2003). For CCA, we followed a forward selection procedure that allowed the identification of the environmental variables that best explained the variability in the blackfly assemblages of the sampling stations. The significance of the environmental variables introduced at each step was inferred from Monte Carlo permutation tests (999 permutations, p-value < 0.05).

Finally, we used Weighted Average (WA) regression (Birks, 1995) to estimate the distribution of the mature larval and pupal stages of the blackfly species identified in the study along each of the environmental gradients of the variables selected in the CCA. WA regression estimated the value with the highest probability of occurrence with respect to a quantitative environmental variable for a given species based on the weighted average of the values of the variable of interest in those sampling stations where the species was present. Weights were the relative abundances of each species at each sampling station.

All statistical analyses were carried out using the free software R version 3.3.3 from The R Foundation for Statistical Computing (R Development Core Team, 2017; https://cran.r-project.org). PCA was performed using the prcomp function from the 'stats' package. DCCA and CCA analyses were performed using the decorana, cca and ordistep functions from the 'vegan' package (Oksanen et al., 2022). The functions optima, tolerance and caterpillarPlot within the 'analogue' package (https://cran.r-project.org/web/packages/analogue/index.html) were used in WA regression to depict the values with highest probability of occurrence and ecological amplitude for environmental variables of the mature larvae and pupae of the identified simuliid species.

Creation of maps

The sampling stations's location figure as well as the figures for the distribution of the blackfly species recorded for the first time from the province of Salamanca were generated employing the geographic information system (GIS) programme ArcMapTM 10.5, ESRI's ArcGIS ® software (Redlands, CA, USA).

RESULTS

Species occurrence and geographical distribution

In total, 53135 immature larvae, 1980 mature larvae and 3099 pupae were examined from the collected and processed material. These specimens belong to three genera, *Metacnephia* Crosskey, 1969, *Prosimulium* Roubaud, 1906, and *Urosimulium* Contini, 1963, plus five subgenera within the genus *Simulium* Latreille, 1802: *Boophthora* Enderlein, 1921, *Eusimulium* Roubaud, 1906, *Nevermannia* Enderlein, 1921, *Simulium* Latreille, 1802, and *Wilhelmia* Enderlein, 1921. Note that according to Day et al. (2010) the characters of larvae and pupae of the species of subgenus *Eusimulium* can show slight differences or be variable. Therefore, it is recommended to identify the species *S. angustipes*, *S. aureum*, *S. petricolum*, and *S. rubzovianum* only to subgenus level when using identification keys based on morphological characters.

Consequently, 22 species were identified from the studied area: *Metacnephia nuragica* Rivosecchi, Raastad & Contini, 1975; *Prosimulium latimucro* (Enderlein, 1925); *Prosimulium tomosvaryi* (Enderlein, 1921); *Simulium (Boophthora) erythrocephalum* (De Geer, 1776); *Simulium (Eusimulium) angustipes* Edwards, 191; *Simulium (Eusimulium) aureum* Fries, 1824; *Simulium (Eusimulium) petricolum* (Rivosecchi, 1963); *Simulium (Eusimulium) rubzovianum* (Sherban, 1961); *Simulium (Nevermannia) armoricanum* Doby & David, 1961; *Simulium (Nevermannia) carthusiense* Grenier & Dorier, 1959; *Simulium (Nevermannia) cryophilum* (Rubtsov, 1959); *Simulium (Nevermannia) naturale* Davies, 1966; *Simulium (Nevermannia) vernum* Macquart, 1826; *Simulium (Simulium) bezzii* (Corti, 1914); *Simulium (Simulium) intermedium* Roubaud, 1906; *Simulium (Simulium) ornatum* Meigen, 1818; *Simulium (Simulium) trifasciatum* Curtis, 1839; *Simulium (Wilhelmia) equinum* (Linnaeus, 1758); *Simulium (Wilhelmia) lineatum* (Meigen, 1804); *Simulium (Wilhelmia) pseudequinum* Séguy, 1921; *Simulium (Wilhelmia) sergenti* Edwards, 1923 and *Urosimulium faurei* (Bernard, Grenier & Bailly-Choumara, 1972), which means that the species identified represent 42% of the simuliid species reported from Spain (Table 1).

As a result of this study, six species were re-

Table 1. Taxonomic classification of the identified blackfly species of this study and of the previous studies carried out in Salamanca province, Western Spain. *Clasificación taxonómica de las especies de simúlidos identificadas en este estudio y en los anteriores llevados a cabo en la provincia de Salamanca, España occidental.*

Genus	Subgenus	Species-group	Species
Metacnephia Crosskey, 1969			*M. blanci* (Grenier & Theodorides, 1953)
			M. nuragica Rivosecchi, Raastad & Contini, 1975
Prosimulium Roubaud, 1906		*hirtipes* species-group	*P. latimucro* (Enderlein, 1925)
			P. tomosvaryi (Enderlein, 1921)
Simulium Latreille, 1802	*Boophthora* Enderlein, 1921		*S.* (*Boophthora*) *erythrocephalum* (De Geer, 1776)
	Eusimulium Roubaud, 1906		*S.* (*Eusimulium*) *angustipes* Edwards, 1915
			S. (*Eusimulium*) *aureum* Fries, 1824
			S. (*Eusimulium*) *petricolum* (Rivosecchi, 1963)
			S. (*Eusimulium*) *rubzovianum* (Sherban, 1961)
	Nevermannia Enderlein, 1921	*vernum* species-group	*S.* (*Nevermannia*) *armoricanum* Doby & David, 1961
			S. (*Nevermannia*) *carthusiense* Grenier & Dorier, 1959
			S. (*Nevermannia*) *cryophilum* (Rubtsov, 1959)
			S. (*Nevermannia*) *naturale* Davies, 1966
			S. (*Nevermannia*) *vernum* Macquart, 1826
	Simulium Latreille, 1802	*bezzii* species-group	*S.* (*Simulium*) *bezzii* (Corti, 1914)
		ornatum species-group	*S.* (*Simulium*) *intermedium* Roubaud, 1906
			S. (*Simulium*) *ornatum* Meigen, 1818
			S. (*Simulium*) *trifasciatum* Curtis, 1839
		reptans species-group	*S.* (*Simulium*) *reptans* (Linnaeus, 1758)
		variegatum species-group	*S. (Simulium) monticola* Friederichs, 1920
	Wilhelmia Enderlein, 1921	*equinum* species-group	*S.* (*Wilhelmia*) *equinum* (Linnaeus, 1758)
			S. (*Wilhelmia*) *lineatum* (Meigen, 1804)
			S. (*Wilhelmia*) *pseudequinum* Séguy, 1921
			S. (*Wilhelmia*) *sergenti* Edwards, 1923
Urosimulium Contini, 1963			*U. faurei* (Bernard, Grenier & Bailly-Choumara, 1972)

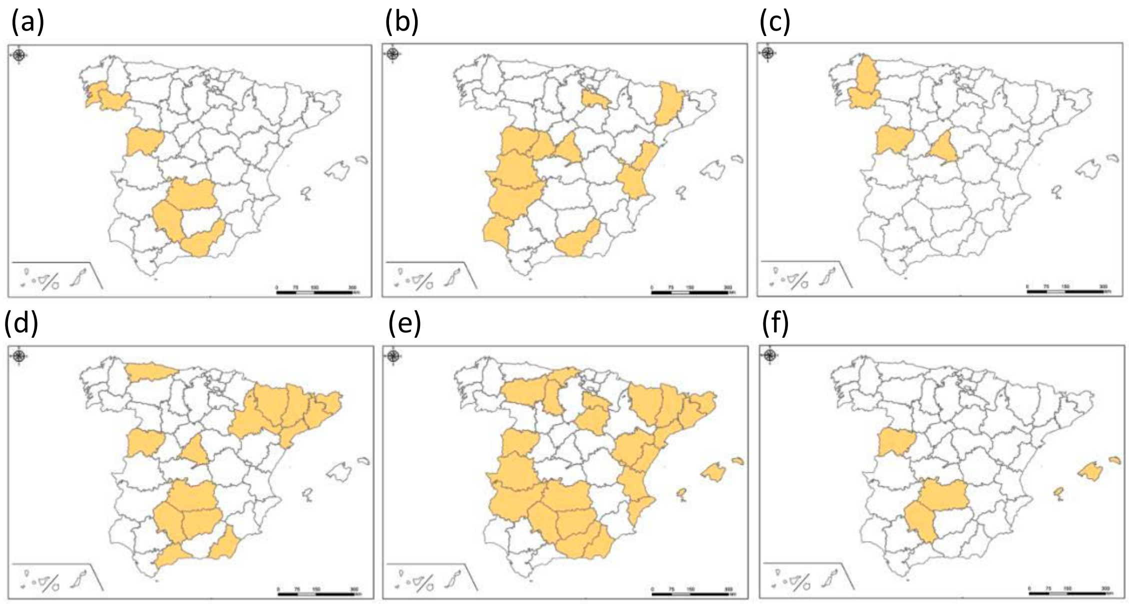

Figure 2. Provincial distribution maps for each of the six species recorded for the first time from the province of Salamanca based on this study: *S. armoricanum* (a), *S. carthusiense* (b), *S. naturale* (c), *S. vernum* (d), *S. bezzii* (e) and *U. faurei* (f). The rest of the shaded provinces indicate previous reports of these species prior to this study. *Mapas de distribución provincial para cada una de las seis especies registradas por primera vez en la provincia de Salamanca basado en este estudio:* S. armoricanum *(a),* S. carthusiense *(b),* S. naturale *(c),* S. vernum *(d),* S. bezzii *(e), and* U. faurei *(f). El resto de las provincias sombreadas indican registros previos de estas especies antes de este estudio*.

corded for the first time for the area of the province of Salamanca: *S. (N.) armoricanum*, *S. (N.) carthusiense*, *S. (N.) naturale*, *S. (N.) vernum*, *S. (S.) bezzii*, and *U. faurei*. Consequently, their known geographical distributions (López-Peña & Jiménez-Peydró, 2017) have been enlarged and updated (Fig. 2).

Pupae of *S. ornatum* and *S. intermedium* were present in more than 50% of the sampling sites (75.5% and 66% of sampling stations, respectively), followed by *S. rubzovianum* (in 43.4% of the sampling stations), *S. angustipes* and *S. petricolum* (both present in 34% of sampling stations). The remaining identified species had less than ten sightings, primarily being detected in 1, 2, or 4 stations. Among the species with the narrowest distribution, *P. latimucro*, *S. armoricanum*, *S. aureum*, *S. bezzii*, *S. carthusiense* and *S. naturale* were present only in one sampling station (that is in 1.88% of sampling stations).

With regard to species abundance, considering both mature larvae and pupae, *S. ornatum* emerged as the most populous species, with 2117 individuals (837 larvae and 1280 pupae), followed by *S. intermedium* with 984 (258 larvae and 726 pupae), *S. rubzovianum* with 503 (331 larvae and 172 pupae) and, finally, *P. tomosvaryi* with 484 individuals (56 larvae and 428 pupae). The abundances of the pupae and larvae of the other identified blackfly species were comparatively lower (Table S3, Supplementary Material, available at https://www.limnetica.net/en/limnetica).

Characterisation of larval and pupal habitats and ecological distinctiveness of blackfly species

The habitats for mature larvae and pupae of the identified blackfly species were characterised using a PCA on the main physico-chemical environmental variables. The first two axes of the ordination explained 48.57% and 28.16% of the total variance, respectively, among the sampling stations (Fig. 3). PCA1 was positively related to water temperature (loading = 0.631) and negatively to elevation (loading = -0.648). PCA2 was negatively related to water velocity (loading = -0.835) and depth (loading = -0.467). These four

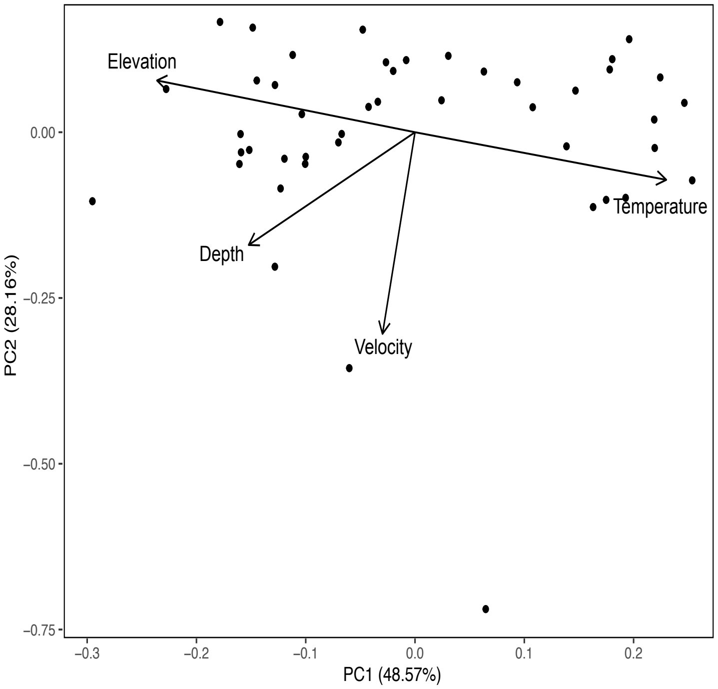

Figure 3. Principal Components Analysis (PCA) biplot of environmental variables in the sampling stations of the Huebra River. *Análisis de Componentes Principales (ACP) sobre las variables ambientales de las estaciones de muestreo del río Huebra.*

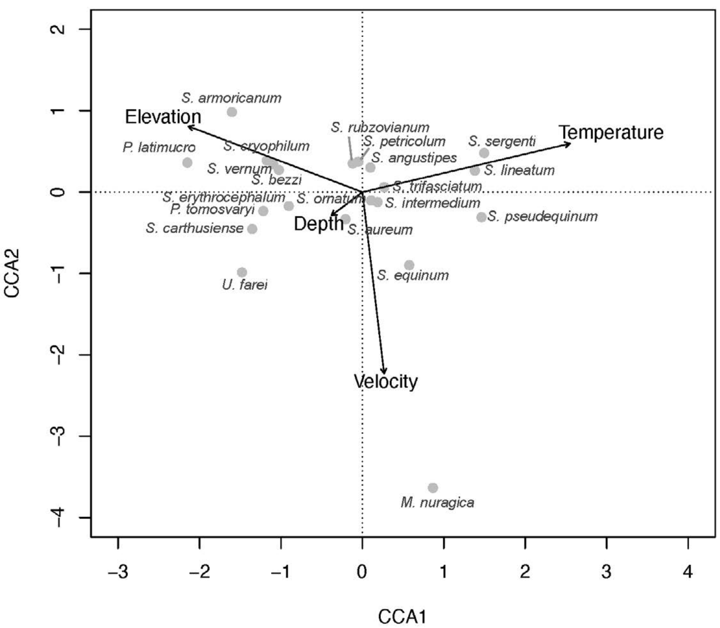

Figure 4. Canonical Correspondence Analysis (CCA) ordination biplot of blackfly mature larvae and pupae assemblages. Scores for blackfly species are represented by black dots placed at the centroid (i.e., weighted average) of the sampling points where each species occurs. Environmental variables showed as vectors were incorporated using forward selection and Monte Carlo permutation tests. *Análisis Canónico de Correspondencias (ACC) de los ensamblados de larvas maduras y pupas de simúlidos en los puntos de muestreo, aquí representados como círculos grises. Las coordenadas en el plano de ordenación para las especies de simúlidos se representan por cruces de color negro situadas en el centroide (i.e., la media ponderada) de los puntos de muestreo donde se ha en-*contrado *cada especie. Las variables ambientales se muestran como vectores y se incorporaron a la ordenación mediante un proceso de selección por pasos y pruebas de permutación basadas en el método de Monte Carlo.*

variables were retained for further analyses.

The CCA explained a significant percentage of total inertia (20%) in the variability of blackfly assemblages (F = 2.319; df = 4, 37; *p*-value = 0.004). Further, a small subset of environmental variables was enough to retain most of the explained variability after applying forward selection. Hence, blackfly pre-imaginal assemblages were mainly explained by a first CCA axis positively related to temperature and negatively to elevation, introduced sequentially, and a second CCA axis negatively related to water velocity (Fig. 4). Depth appeared to have a minor effect on blackfly assemblages. Blackfly species in figure 4 are placed at the centroids (weighted average) of the sampling stations where each species occurred. Accordingly, *S. armoricanum*, *P. latimucro* and *S. cryophilum* appear to occur at high elevation and low temperatures. On the other hand, *S. sergenti* or *S. lineatum* are revealed as species with higher temperature optima and as preferentially inhabiting low-land areas. The species clearly associated with the highest water velocity was *M. nuragica*.

Optima and tolerance values for the four environmental variables found to be relevant for blackfly pupae and larvae assemblage composition (elevation, temperature, depth and velocity) provided more comprehensive information regarding niche specificity of the pre-imaginal stages of these species (Fig. 5).

Elevation and temperature settled the main en-

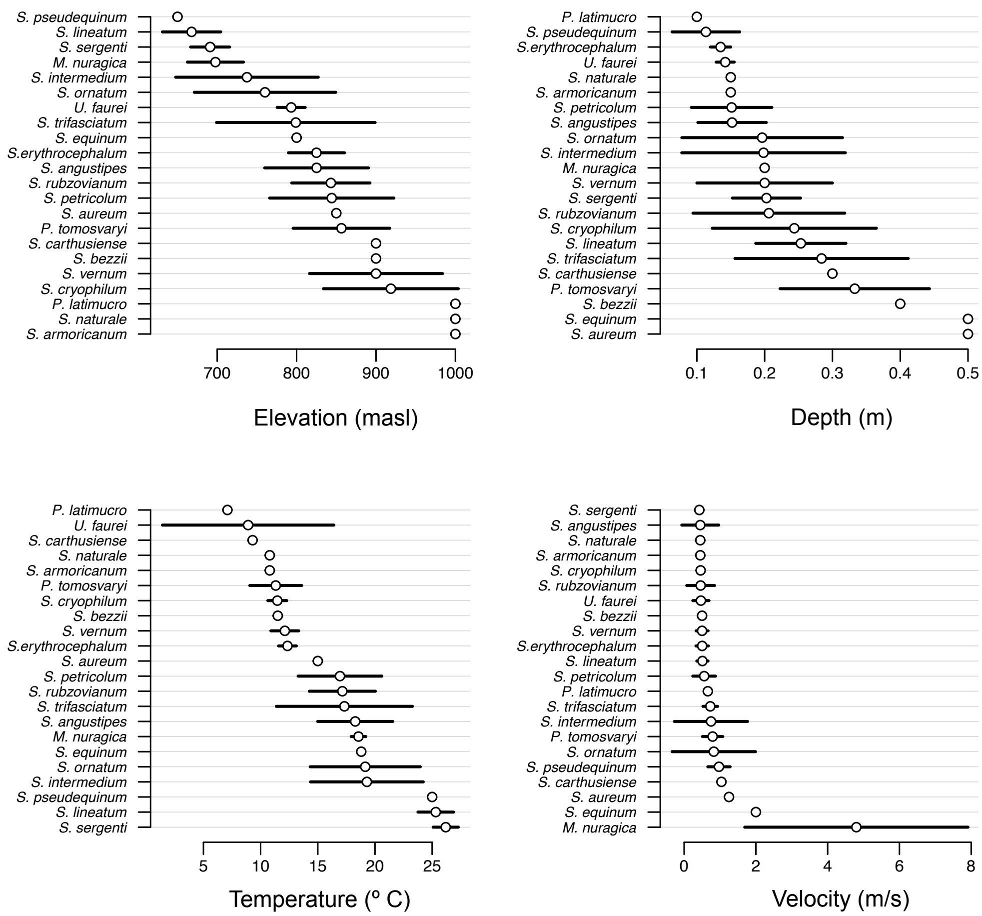

Figure 5. Optima (modes) and tolerances (error bars) for (a) elevation, (b) depth (c) temperature and (d) velocity for the mature larvae and pupae of the blackfly species found in this study. Species are arranged on the y axis by increasing optima for each variable. *Óptimos y tolerancias para (a) altitud), (b) profundidad, (c) temperatura y (d) velocidad de la corriente de las larvas maduras y pupas de las especies de simúlidos identificadas en este estudio. Las especies se han ordenado en el eje y de manera ascendente según sus óptimos para cada variable.*

vironmental gradient in our study. Several species had their optimum above 900 m a.s.l. For example, *P. latimucro*, *S. armoricanum*, and *S. naturale* were gathered at 1000 m a.s.l., although the latter two species were recorded from single sampling stations. Other species found at high elevation and collected at several stations were *S. vernum*, and *S. cryophilum*, a fact that has allowed for better defining the ranges at which they occur. Contrarily, *S. pseudequinum* and *S. lineatum* were found at the lowest elevations. The species with the widest altitudinal range was *S. trifasciatum*. The distribution of simuliid species along an elevational gradient correlated negatively with their thermal optima. Half of the identifies species had thermal optima for pupae and larvae below 15º C, whereas three species appeared to be particularly well prepared to survive under warmer waters: *S. pseudequinum*, *S. lineatum* and *S. sergenti*, all with tolerances exceeding 25º C. *U. faurei* stood out from the rest of the species with the widest thermal ranges (1.2-11.8 º C).

Overall, we observed wide tolerances for the depth range in which pupae and larvae were found in the set of species studied. Of course, our data are limited because some species were found at only one sampling station. Finally, the analysis of optima and tolerances confirms the rheophilic character of *M. nuragica*. No other species seemed to cope with water velocities above 2 m/s.

Elevation, depth, temperature and velocity ranges of the watercourse of the species recorded for the first time in the province of Salamanca

The aquatic habitats of the six newly recorded species (*S. armoricanum*, *S. carthusiense*, *S. naturale*, *S. vernum*, *S. bezzii*, and *U. faurei*) show interesting differences and particularities regarding the elevation, depth, temperature and flow velocity of their freshwater breeding sites. These nuances bring to light the ranges of the aforementioned abiotic factors. Interestingly *S. armoricanum*, *S. bezzii*, *S. carthusiense* and *S. naturale* were recorded from single sampling stations, although typically at high elevation. *S. armoricanum* and *S. naturale* were collected at an elevation of 1000 m a.s.l. at sampling station 4, located in the surroundings of the municipality of La Bastida, *S. bezzii* at 900 m at sampling station 5, from the waters of del Zarzoso stream and *S. carthusiense* also at 900 m at sampling station 10, from Maíllo River. Contrarily, the species *S. vernum* and *U. faurei* were collected in several places, a fact that has allowed for stimating the altitudinal ranges they inhabit. In this way, the species with the widest altitudinal range, of 150 m, was revealed to be *S. vernum* as a consequence of its collection between 850 m – at sampling station 6, from a stream in Parque La Piñuela, and 9 from del Zarzosillo stream –, and 1000 m, at sampling station 4. On the other hand, *U. faurei* showed the narrowest altitudinal range. This species was found between 775 m and 800 m at sampling station 2, from Huebra River, and 3, near Tejeda y Segoyuela municipality.

These six species revealed certain preferences with regard to the depth of the sites chosen for their growth and development. *S. armoricanum* and *S. naturale* were both collected at the sampling station 4 at 0.5 m depth, *S. bezzii* sampling station 5 at 0.4 m depth and *S. carthusiense* at sampling station 10 at 0.3 m depth. In constrat, *P. latimucro* was found at the shallowest depth. Regarding depth tolerances, *S. vernum* showed the broadest depth range, from 0.15 m to 0.4 m, at sampling stations 4 and 6 and 5 and 9 respectively. The species with the narrowest depth range was *U. faurei*, a specialist of shallow waters (0.12-0.15 m).

In relation to the ranges of water temperature in the lotic habitats of these six species, *U. faurei* showed a range 1.2-11.8 ºC having been found at sampling station 2 in Escurial de la Sierra municipality and station 3 in the surroundings of Tejeda y Segoyuela. Among the studied species, it was by far the most tolerant to temperature variation. As noted above, many of the other species were found at single sampling station, so tolerance ranges could not be estimated, and the temperature optima shown here are certainly tentative.

Regarding water velocity, most of these six species showed similar optima, although we again stress that these values are tentative. *S. armoricanum* and *S. naturale* were collected from waters of 0.45 m/s, similarly to *S. bezzii* and *U. faurei*, which were found at ~0.5 m/s. Interestingly, *U.*

faurei exhibited the narrowest range of water velocity (0.38-0.7 m/s). Regarding maximum tolerated water velocity, *S. carthusiense* and *S. vernum* were found in waters running at 1.04 m/s. This last species also showed the widest range of water velocity (0.45-1.04 m/s), as revealed by the measurements acquired at sampling stations 4 and 6 in La Bastida and Cereceda de la Sierra, and station 10 from Maillo River in El Maillo.

DISCUSSION

Species occurrence and geographical distribution

The simuliid fauna of the province of Salamanca has previously been studied by both national and international experts (González-Peña, 1990, Vinçon & Clergue-Gazeau, 1993, González, 1997, Crosskey & Crosskey, 2000, Crosskey & Báez, 2002, López-Peña et al., 2022a), who reported the presence of 19 species (Table 2). Except for three previously recorded, *Metacnephia blanci* (Grenier & Theodorides, 1953), and *Simulium reptans* (Linnaeus, 1758), both listed by González-Peña (1990), and *Simulium monticola* Friederichs, 1920 listed by Vinçon & Clergue-Gazeau (1993), the other 16 were also collected in the present study in addition to six other first records for this region. Our study shows that the distribution and abundance of the immature stages of blackfly species depend on physico-chemical factors of water such as elevation, velocity, depth of the aquatic habitat, and temperature.

Update of ecological factor ranges for the identified blackfly species

Our study has allowed us to update the ecological ranges of the identified blackfly species for the four key ecological factors explaining the distribution of their breeding sites, at least in the province of Salamanca. Our results, if not confirmatory, have allowed us to estimate more accurately the ecological range of many of these species (see Table 2), which can assist in the management of biosecurity.

For many species, our results slightly increased the altitudinal ranges previously described and although they are essentially confirmatory, it is also true that they provide greater confidence in these values. Note that the altitudinal ranges of *M. nuragica* (650-700 m), *P. latimucro* (1000-1800 m), *S. aureum* (775-850 m), and *S. sergenti* (660-850 m) are enlarged essentially because their presence was previously reported from single sampling stations. It is worth mentioning that our study involved a considerable sampling effort of the territory under investigation. As shown in Table 2, the widest increases in altitudinal range as a result of our study are those of *P. tomosvaryi* (775-1800 m) and *S. cryophilum* (800-1800 m).

Temperature is also one of the parameters that better discriminate the distribution of blackflies, since it directly influences their metabolism, affecting their eggs' embryonic development (Cupp, 1981), egg hatching, larval growth and development, pupation of the cocoon, and adult emergence (Thorup, 1974, Morky, 1976, Ross & Meritt, 1978, Colbo, 1979, Neveu & Lapichin, 1979, Merritt et al., 1982, Post, 1983). Hence, it is very important to identify the temperature ranges at which blackfly species grow and develop. As a result of the present study, the temperature ranges for several of the species reported in the province of Salamanca have also been updated (Table 2). For some species, the increase in temperature ranges, especially by higher maximum values, has been notable. For instance, the maximum recorded temperature for *S. lineatum*, *S. ornatum* and *S. petricolum* has been increased by 2.1 ºC, and for *S. intermedium* and *S. pseudequinum*, by 2 °C. However, the most striking increase was observed in *S. ornatum*, which was found in extraordinarily cold waters.

The update of the depth at which breeding sites of the different blackfly species occur is highly important because this variable changes throughout the year according to the rainy and dry seasons. Here we followed the so-called space-for-time substitution approach as an alternative to a long-term study accounting for the variation in depth conditions of particular breeding sites. The approach is justified by the number of sampling stations included in this study. In this regard, the species for which the depth range at which their larvae and pupae are found has increased the most are *S. equinum* and *S. pseudequinum* (0.05-1.7 m)

(Table 2). The minimum value of the range is evidence that these two species can live in very shallow waters. This, together with the preference of *S. equinum* and *S. pseudequinum* for high temperatures, supports the idea that they could complete their life cycle in temporary waters, a scenario that is likely to become more frequent in the near future.

Regarding water velocity, we highlight the information regarding the update for several species and other relevant insights (Table 2). A few species have experienced an increase in their range of water velocity, namely *M. nuragica*, *S. equinum* and *S. lineatum*, after being gathered from fast-water sampling stations (between 2-5 m/s). Notably our results suggested that most of the studied species tolerate slow water flows. The presence of blackflies has been described in practically lentic waters, with little discharges (Van Someren, 1944, Crosskey, 1969), characterised by being temporary, shallow water courses, and with very weak currents (Beaucournu-Saguez, 1972, Grácio, 1985, González et al., 1987). A clear example of this involves the species of the subgenus *Eusimulium*, which can remain in reduced water flows with little current and which therefore have a wide distribution in the rivers of the Mediterranean basin (Jarry, 1973, Grácio, 1985). In our study, the species *S. angustipes*, *S. petricolum*, and *S. rubzovianum* belonging to this subgenus were documented in the sampling stations with the records of lowest water velocity measurements, in the first case in flows with a minimum of 0.25 m/s like that of sampling station 39 and in the second and third cases with readings of 0.125 m/s water course speed, like those of sampling station 21. However, they were also found in habitats with higher speeds at up to 5

Table 2. Bibliographic and updated data on the ranges of elevation, water temperature, depth, and water velocity of 16 of the simuliid species identified in the province of Salamanca. ND (No data), WC (No changes). 1 (Crosskey & Crosskey, 2000), 2 (González Peña, 1990), 3 (González et al., 2002), 4 (López-Peña et al., 2022a), 5 (Vinçon & Clergue-Gazeau, 1993). *Datos bibliográficos y actualizados de los rangos de altitud, temperatura del agua, profundidad y velocidad del flujo de agua de 16 de las especies de simúlidos identificadas en la provincia de Salamanca. ND (Sin datos), WC (Sin cambios). 1 (Crosskey & Crosskey, 2000), 2 (González Peña, 1990), 3 (González et al., 2002), 4 (López-Peña et al., 2022a), 5 (Vinçon & Clergue-Gazeau, 1993).*

Species	Elevation (m)		Temperature (º C)		Depth (m)		Water velocity (m/s)		References for previous data
	Bibliographic range	Updated range	Bibliographic range	Updated range	Bibliographic range	Updated range	Bibliographic range	Updated range	
M. blanci	570	WC	ND	ND	ND	ND	ND	ND	2, 1
M. nuragica	675	650-700	16.9	16.9-18.6	0.15	0.15-0.2	0.714	0.6-5	4
P. latimuro	1800	1000-1800	ND	7.1	ND	0.1	ND	0.66	5
P. tomosvaryi	1300-1800	775-1800	ND	1.2-13	ND	0.1-0.5	ND	0.38-1.08	5
S. angustipes	190-950	WC	9.2-25	9.2-25.5	0.3-5	0.05-5	0.1-0.833	0.1-5	2, 4
S. aureum	775	775-850	20.5	15-20.5	0.15	0.15-0.5	0.333	0.333-1.25	4
S. cryophilum	1300-1800	800-1800	ND	10.8-13	ND	0.15-0.4	ND	0.38-0.5	5, 1
S. equinum	750-850	WC	19.2-25	18.8-25	0.15-1.7	0.05-1.7	0.416-0.833	0.416-2	2, 1, 4
S. erythrocepahlum	750-1080	WC	19.2-23	11.8-23	0.15-0.7	0.12-0.7	0.416-0.833	0.38-0.833	2, 4
S. intermedium	525-950	525-1000	11.5-25	1.2-27	0.02-1.7	WC	0.166-1.25	0.125-5	4
S. lineatum	190-822	190-850	12-24.9	11.8-27	0.15-0.7	0.05-0.7	0.166-0.833	0.166-2	2, 1, 4
S. monticola	1300-1800	WC	ND	ND	ND	ND	ND	ND	5, 1, 3
S. ornatum	650-950	WC	11.5-24.9	1.2-27	0.03-1.5	WC	0.166-1.25	0.125-5	4
S. petricolum	700-950	650-950	9.2-24.9	9.2-27	0.03-5	WC	0.1-0.833	0.1-1.66	4
S. pseudequinum	800-814	650-900	21-23	21-25	0.4-1.7	0.05-1.7	0.454-0.702	0.454-1	4
S. reptans	850	WC	ND	ND	ND	ND	ND	ND	2, 3
S. rubzovianum	700-950	650-950	9.2-24.9	9.2-25.5	0.03-5	WC	0.1-0.833	0.1-5	4
S. sergenti	700	660-850	24.9	24-27	0.2	0.05-0.3	0.416	0.35-0.56	4
S. trifasciatum	700-950	650-950	21-24.9	12.3-25	0.05-0.4	WC	0.333-0.714	0.333-0.66	4

m/s, at sampling station 23 on the Huebra River.

It is known that water flow velocity also determines the dominant substrate type of the riverbed (Vogel, 1981). Moreover, both the type of substrate and water velocity are known to affect the geographical distribution of the preimaginal stages of blackflies (Ross & Merritt, 1987). It is not surprising that in the headwaters of rivers, simuliids adhere to stone surfaces, while in the intermediate and lower areas they can also be found on surfaces of plant origin (González-Peña, 1990). Furthermore, it has been shown that the mountainous sections of rivers, due to their greater heterogeneity of microhabitats as a consequence of the different velocities of water flow and different types of substrates available, constitute areas of great diversity of simuliid species (Colbo & Moorhouse, 1979). This was observed in the present study, where the stations located at altitudes of 700-800 m (stations 3, 42 and 46) presented the highest diversities of simuliids (with larvae and pupae of 7-8 different co-occurring species). The relationship between substrate heterogeneity and blackfly diversity, and the association between the larvae and pupae of particular simuliid species to specific substrates are matters of further research, towards which we are making new efforts.

CONCLUSIONS

This research contributes substantially to expanding the knowledge of Simuliidae, not only increasing and enhancing the faunal and bioecological data of the blackfly species present in the study area of the Huebra River basin of Salamanca province (Western Spain), but also in the Spanish national territory as a whole. As a result of the occurrence of the 23 species identified, the simuliid fauna reported from this region have increased by six new records (*S. armoricanum*, *S. bezzii*, *S. carthusiense*, *S. naturale*, *S. vernum*, and *U. faurei*), which means that the record of 19 species in the literature has been expanded to 25. Likewise, this study brings to light valuable information that contributes to better understanding the biology of these species and their geographical distribution, providing new insights for some of them and updating the ranges of abiotic factors for others. These factors include the altitudinal ranges where they tend to be found, the water velocity and water temperature typical of their specific breeding habitats, as well as the depth of the watercourses where they habitually develop and grow. In addition, our study provides new knowledge regarding their larvae and pupae abundances, laying bare the variety of ecological gradients that define their occurrence and distribution patterns. In particular, our results demonstrate that the diversity of blackfly species swings according to the elevation, water velocity, depth and temperature.

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AUTHOR CONTRIBUTIONS

D.L.P: Fund acquisition, Processing and identification of samples, Data processing, Preparation of the original draft, Review, Editing and Validity. **M.S.P.R.**: Conceptualization, Sampling methodology, Guidance, Review, Editing and Validity. **E.M.G.R**: Data processing, Figures creation, Participation of the original draft preparation, Review, Editing and Validity. **A.L.C**: Figures creation, and Validity. **J.V.F.G**: Project supervision, Review, Editing and Validity.

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