

Article

Taxonomy, Distribution and Life Cycle of the Maghrebian Endemic *Rhithrogena sartorii* (Ephemeroptera: Heptageniidae) in Algeria

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Abstract: Despite being recorded in Algeria since the nineteenth century, the genus *Rhithrogena* has never been the object of a taxonomical study and no identified species is known from this country. Investigations of the relict mountain streams of El Kala, north-eastern Algeria, have led to the discovery of a *Rhithrogena* population. Morphological and molecular analyses identified the species as the Maghrebian endemic *Rhithrogena sartorii*, so far known only from neighboring Tunisia. We report on the species' distribution, status, and life cycle and discuss its potential role as a bioindicator in environmental monitoring.

Keywords: aquatic insects; conservation; life cycle; limnology; mayfly; North Africa; rivers; streams

1. Introduction

Rhithrogena, a member of the subfamily Rhithrogeniinae (Heptageniidae), is a Holarctic genus with numerous species in the Palearctic region, occupying mainly cold, fast-flowing, and well-oxygenated headwaters [1,2]. Furthermore, isolated populations of *Rhithrogena* in mountainous rivers and streams display high levels of endemism and are often on the IUCN Red List [3].

Despite its ecological importance, the taxonomic status of many *Rhithrogena* species remains a challenge, even in Europe, where taxonomic studies of mayflies are relatively well advanced [4,5]. Based on various nymphal and adult characters, species are lumped into “species groups” [6,7]. However, this grouping remains controversial, marred by cryptic diversity and taxonomic oversplitting [4,5].

The first record of *Rhithrogena* in North Africa occurred at the edge of the Sahara, when, on 19 March 1895, Eaton [8] collected an immature male at Biskra, Algeria. Eaton went on to speculate that the species might have flown south from the deep canyons descending from the Aures Mountains. This specimen and others encountered in various localities across the Maghreb remained unidentified for several decades until the almost synchronous descriptions from Morocco of a series of new species: *Rh. ourika* (High Atlas: 1500 and 2600 m) [9], *Rh. ayadi* (Middle Atlas: 2150 m) [10], *Rh. giudicelliorum* (High Atlas: 2800 m) [11], and *Rh. ryszardi* (Middle Atlas: 1260 m) [12].

Subsequently, Vitte [13] described an additional species, *Rh. mariae*, from the Moroccan Rif. In particular, *Rh. mariae* differed from other North African *Rhithrogena* species by

occurring at a much lower altitude (160 m). Finally, two decades later, Zrelli et al. [14] described a new *Rhithrogena* species, *Rh. sartorii* from Tunisia, which gives a total of six known *Rhithrogena* species in North Africa. Most of the described Maghrebian *Rhithrogena* species are only known as imagos, whereas *Rh. mariae* is known at the nymphal and adult stages, and *Rh. sartorii* at the nymphal and subimaginal stages.

As part of a long-term limnological survey of Algeria, we collected mayfly nymphs from various regions of the country [15], and, in this study, we report the discovery of *Rh. sartorii* in the relict mountain streams of El Kala, the first record for Algeria, and provide information on its distribution. Because knowledge of immature stages, voltinism, and larval growth patterns provide insights into basic life-history traits and is essential to developing and implementing appropriate conservation strategies [16], we also identified the last three nymphal stages and inferred the species' life cycle.

2. Materials and Methods

2.1. Study Area

The Algero-Tunisian border is flanked on its northern part by a mountain range known as Kroumiria, where the Kebir-East River emanates. The watershed of the Kebir-East River is second in size only to the Seybouse River in north-eastern Algeria. Further north, the Oued el Eurg basin drains the hills sandwiched between Kroumiria and the Mediterranean Sea (Figure 1). The climate is typically Mediterranean, with an alternating hot, dry period (May–October) and a rainy season (November–April).

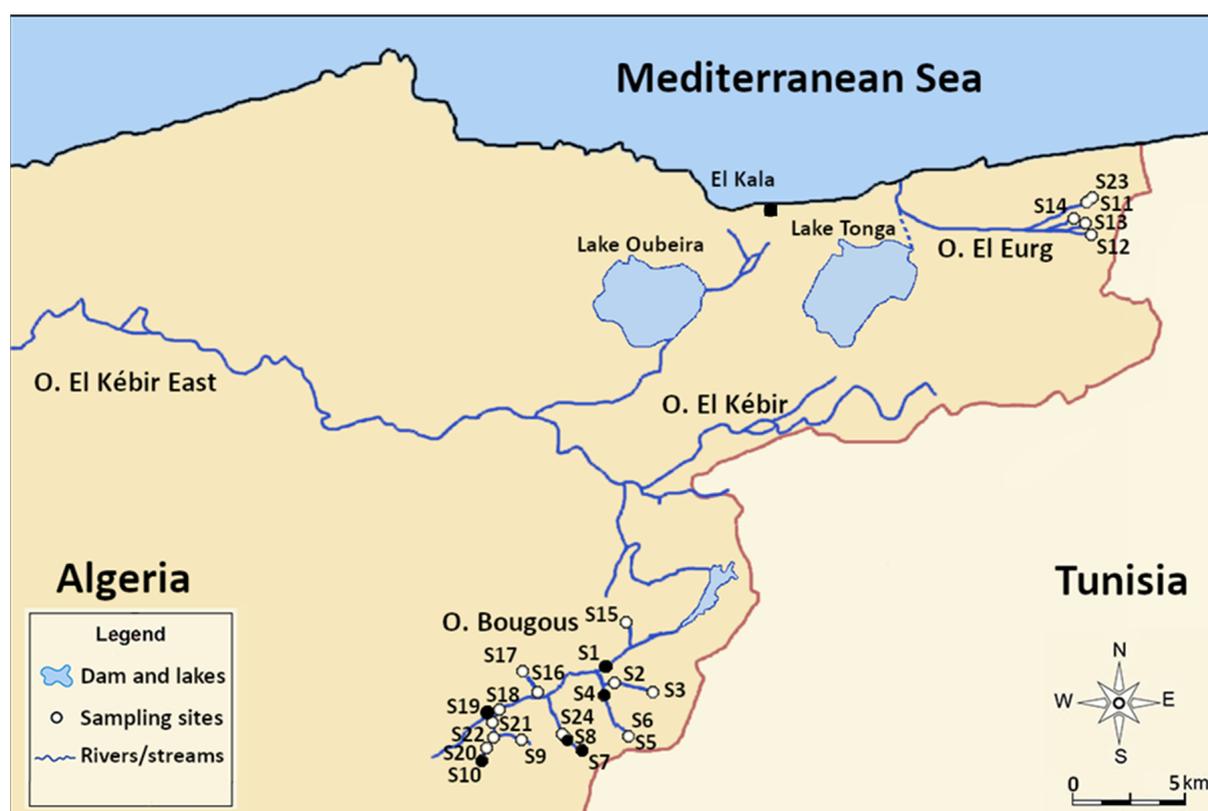


Figure 1. Study area with sampling sites. Dark circles indicate localities where *Rhithrogena sartorii* has been recorded.

2.2. Sampling

A set of 24 localities (S1–S24), distributed across both O. Bougous, the main tributary of O. El Kebir, and the O. El Eurg watershed, were sampled monthly from November 2018 to June 2021 [15,17]. Mayfly nymphs were collected using a dipnet (500 µm mesh size, 35 cm diameter) and by walking slowly and repeatedly across all micro-habitats (aquatic

vegetation, rocks, leaf litters, riffles, runs, pools, flats, etc.) for ten minutes at each locality, as described in [18–20].

2.3. Molecular Analyses

To complement morphological examinations, we compared mitochondrial DNA sequences of specimens from this study to Tunisian topotype specimens. Specifically, we generated a 658-bp fragment of the cytochrome c oxidase subunit I (COI) gene from five newly-sequenced specimens (two from Algeria and three from the type locality in Tunisia) using LCO1490 and HCO2198 primers [21]. For all specimens, we followed the non-destructive DNA extraction procedure described in [4]. The DNA was extracted using the BioSprint 96 extraction robot (Qiagen Inc., Hilden, Germany). Polymerase Chain Reaction (PCR) was conducted in a volume of 30 μ L, consisting of 9 μ L (unknown concentration) of template DNA, 1.5 μ L (10 μ M) of each primer, 0.24 μ L (25 mM) of dNTP solution (Promega, Madison, WI, USA), 6 μ L of 10X buffer (Promega, Madison, WI, USA) containing 7.5 mM of $MgCl_2$, 3 μ L (25 mM) of $MgCl_2$, 1.5 U of Taq polymerase (Promega, Madison, WI, USA), and 8.46 μ L of sterile ddH₂O. Optimized PCR conditions included initial denaturation at 95 °C for 5 min, 38 cycles of denaturation at 95 °C for 40 s, annealing at 50 °C for 40 s, and extension at 72 °C for 40 s, with a final extension at 72 °C for 7 min. The purification and automated sequencing were carried out in Microsynth (Balgach, Switzerland). We further included one published COI sequence from [5], also corresponding to a topotype specimen (Table 1). The sequences were aligned using MAFFT [22] as implemented in Jalview 2.11.1.4 [23]. MEGAX [24,25] was used to visualize the alignment, calculate the number of variable sites, define two groups (one group with the two sequences from Algeria, one group with the four topotype sequences from Tunisia), and calculate K2P [26] mean distances within and between groups.

Table 1. Codes and origin of specimens examined in the COI analysis. For each specimen, the GBIF code, the sampling information (country, locality, coordinates, and date of sampling), the GenBank accession number of the COI sequence, and the corresponding publication source are provided. All specimens from Tunisia are from the type locality (topotypes).

GBIF Code	Country	Locality	Latitude	Longitude	Date	GenBank ID	Source
GBIFCH00671210	Tunisia	Ennour	36.8018	8.6568	28.IV.2010	LN868554	Vuataz et al. (2016)
GBIFCH00671211	Tunisia	Ennour	36.8018	8.6568	28.IV.2010	MZ433256	This study
GBIFCH00671212	Tunisia	Ennour	36.8018	8.6568	28.IV.2010	MZ433257	This study
GBIFCH00671213	Tunisia	Ennour	36.8018	8.6568	28.IV.2010	MZ433258	This study
GBIFCH00673108	Algeria	Guitna inf	36.6379	8.3652	06.VI.2019	MZ433260	This study
GBIFCH00673114	Algeria	Guitna inf	36.6181	8.3462	06.VI.2019	MZ433259	This study

2.4. Morphometry

Rhithrogena nymphs from two localities, Guitna sup (S7) (Figure 2) and Guitna inf (S8), were selected for measurements. With one exception (see results), measurements were lumped together after inspection of density plots, and Mann–Whitney U tests did not reveal any differences between the two localities. Body length (BL), head width (HW), and length of the mesonotum + wing pad (mn + wsl) were measured using a Precision Steel Rule to the nearest 0.1 mm. The criteria for instar assignment were BL, HW, mn + wsl, and the ratio (mn + wsl)/HW, hereafter referred to as the “Ratio” [27]. Only the last three instars (F-0, F-1, and F-2) were identified; all other stadia were designated as “smaller nymphs”. Instars were determined through graphical plots and statistical analyses. The sex of each nymph in the last two instars was determined according to the presence (male) or absence of genital forceps (gonostyli) on the ventral surface of the ninth abdominal segment. Presence in F-0 nymphs of dark wing pads was assumed as evidence of imminent emergence.

2.5. Statistical Analysis

A fast, density-based clustering analysis of BL, HW, mn + wsl, and Ratio was performed using DBSCAN (density-based spatial clustering of applications with noise) [28] to identify the last three instars, F-0, F-1, and F-2. The algorithm attempts to identify the structure in the spatial data set by aggregating objects into similar subgroups [29]. All statistical tests were conducted using R software [30].



Figure 2. View of Guitna sup., a typical habitat of *Rhithrogena sartorii* during winter (a) and summer (b).

3. Results

3.1. Distribution and Phenology

During the study period, *Rhithrogena sartorii* nymphs were recorded between January and June at six localities: Pont Bougous (S1), Zitoun Meftah (S4), Guitna sup (S7), Guitna inf (S8), Nouazi (S10), and Kherrata (S19). Nymphs were found in streams that had a substrate made up of cobbles, stones, and boulders and in microhabitats with a relatively cold, fast-flowing current. The nymphal growth and development occurred in winter and spring with marked differences between years: Both in 2019 and 2021, nymphs were first recorded in March, whereas, in 2020, nymphs were first collected in January. In all years, no nymphs were recorded beyond June (Figure 3).

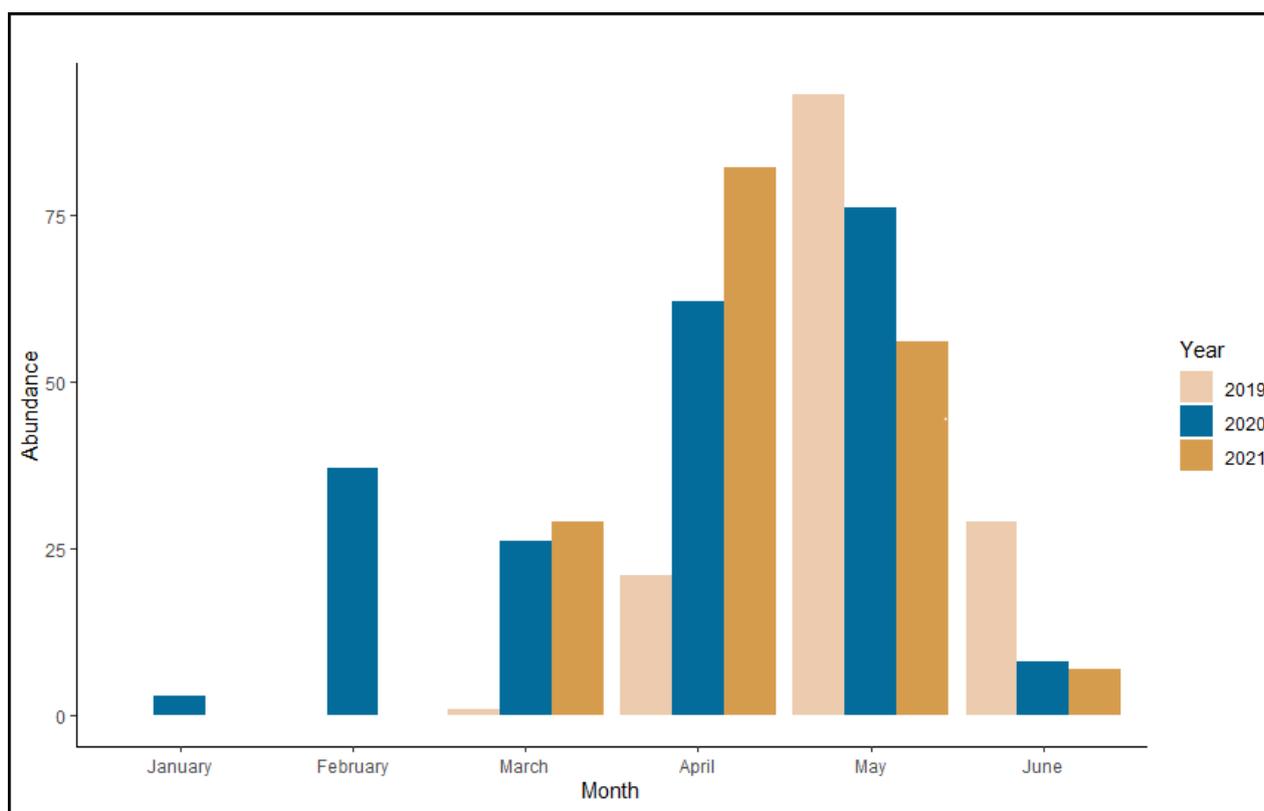


Figure 3. Histogram of the number of *Rhithrogena sartorii* specimens sampled each year in the study area (January 2019–June 2021).

3.2. Taxonomy

Rhithrogena sartorii nymphs are characterized by the following combination of characters: (1) All gills are crenulated (Figure 4A–C); (2) compared to *Rh. insularis* (Figure 5A), the plica of the dorsal face of the first gill is well expressed, clearly triangular, the leading edge somewhat concave (Figure 5B); (3) the lateral sclerites of the first sternite are slightly turned backward, sometimes perpendicular to the body axis (Figure 4C); (4) the upper face of femora of all legs has a well-expressed rounded blackish hypodermal macula (Figure 4B); (5) the crown of the galea-lacinia has 9–11 comb-shaped bristles, each with 6–7 teeth (Figure 5C).

3.3. Molecular Analyses

There were no missing data, gaps, or ambiguous sites in the COI alignment, and a total of four variable sites were recorded. The K2P mean distances within groups were 0.25% and 0.15% for Tunisian (topotype) and Algerian sequences, respectively. The K2P mean distance between groups was 0.23% (maximum distance: 0.61%). Two Tunisians and one Algerian specimen shared the same COI haplotype.



Figure 4. *Rhithrogena sartorii*, habitus in dorsal view (A), lateral view (B), and ventral view (C). The arrow points to the lateral sclerites of the first sternite. Scale bar: 1 mm.

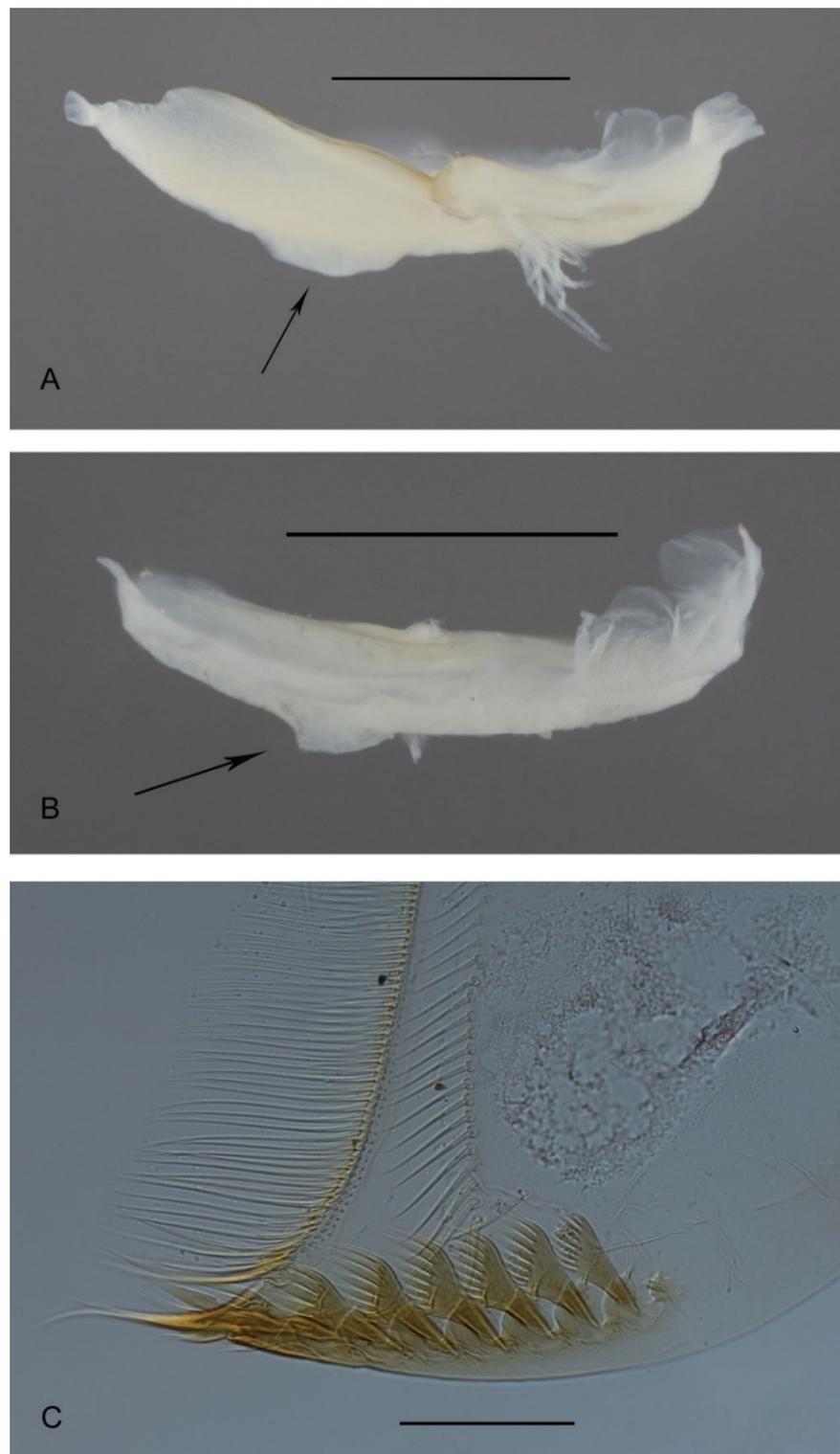


Figure 5. *Rhithrogena* spp. Latero-dorsal view of the first gill to emphasize the shape of the plica (arrow) in *Rh. insularis* (A) and *Rh. sartorii* (B), Scale bar: 1 mm. Crown of the galea-lacinia of *Rh. sartorii* (C). Scale bar 0.1 mm.

3.4. Morphometry

A total of 524 nymphs were measured. Overall, nymphal body length (BL) ranged from 2.0 to 10.8 mm, while the ranges of head width (HW) and mesonotum length + wing pad length (mn + wsl) were 0.8–3.4 and 0.2–5.2 mm, respectively. Females' BL were

marginally longer than males' (one-way ANOVA: $F_{1,164} = 3.6, p = 0.06$). All other measured morphometric characters did not differ between the sexes. Assignment of the last three final instars suggested the presence of three clusters corresponding to F-0: Ratio ≥ 1.1 , F-1: $1.1 > \text{Ratio} \geq 0.75$, F-2: $0.75 > \text{Ratio} \geq 0.5$. The rest may be grouped into the category "smaller instars" (Figure 6a). The allometric growth of wing pads (mn + wsl) at the F-0 instar contrasting sharply with the isometric growth of BL and HW (Figure 6a,b) is noteworthy.

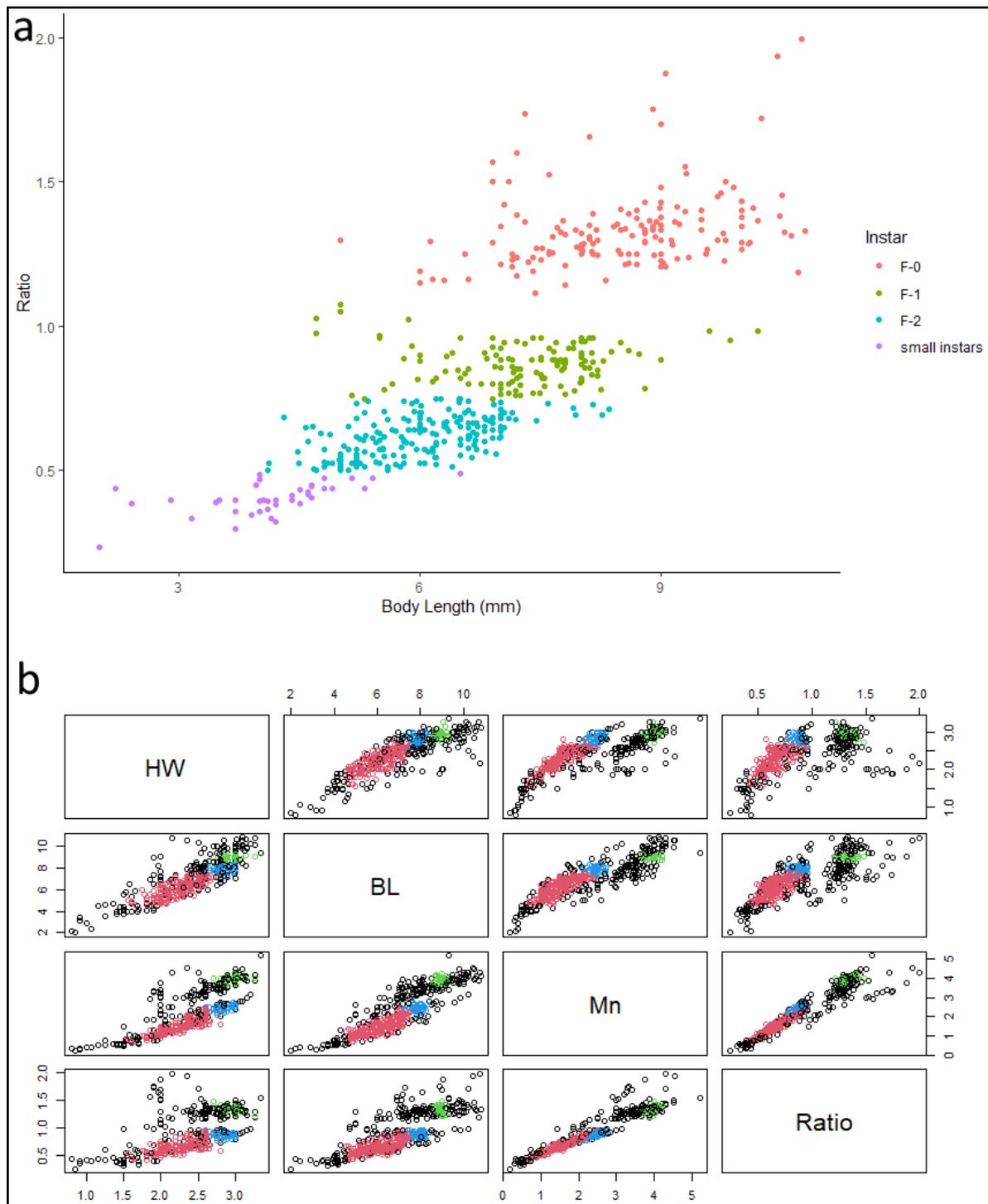


Figure 6. (a) Plot of Ratio (mesonotum length + wing pad length (mn + wsl)/head width (HW)) versus body length (BL) showing the assignment of F-0 (green), F-1 (blue), and F-2 (red); (b) Multiplot of the DBSCAN clustering indicating three classes (colored dots) corresponding to the last three nymphal instars. BL, HW, and Mn (mn + wsl) units are in mm.

The density-based clustering algorithm, DBSCAN, using the following parameters: eps (maximum radius between two neighbors belonging to the same cluster) = 0.35, and MinPts (minimum number of neighbors required to form a cluster) = 15, and the variables: BL, HW, mn + wsl, and Ratio, confirmed the preliminary visual inspection by assigning 318 nymphs into three clusters corresponding to F-0, F-1, and F-2 (Figure 6b). The rest (206) corresponded to “smaller nymphs” and noise.

3.5. Life Cycle

Both in 2019 and 2021, the nymphs first appeared in March, and development proceeded quickly with F-0 nymphs with pigmented wing pads occurring from April to June (Figure 7a–d). In 2020, nymphal development was more protracted, with nymphs collected from January to June, but in all three years, the emergence spanned April to June, coinciding with the drying up of streams in early summer. The size difference between the two sampling sites in May 2020 (BL: $\log_e(W_{\text{mann-Whitney}}) = 5.44$, $N = 76$, $p = 2.09 \times 10^{-6}$; HW: $\log_e(W_{\text{mann-Whitney}}) = 5.20$, $N = 76$, $p = 1.19 \times 10^{-7}$) and the persistence of “small instars” nymphs in June 2020 at Guitna inf (S8) while Guitna sup (S7) dried up is noteworthy.

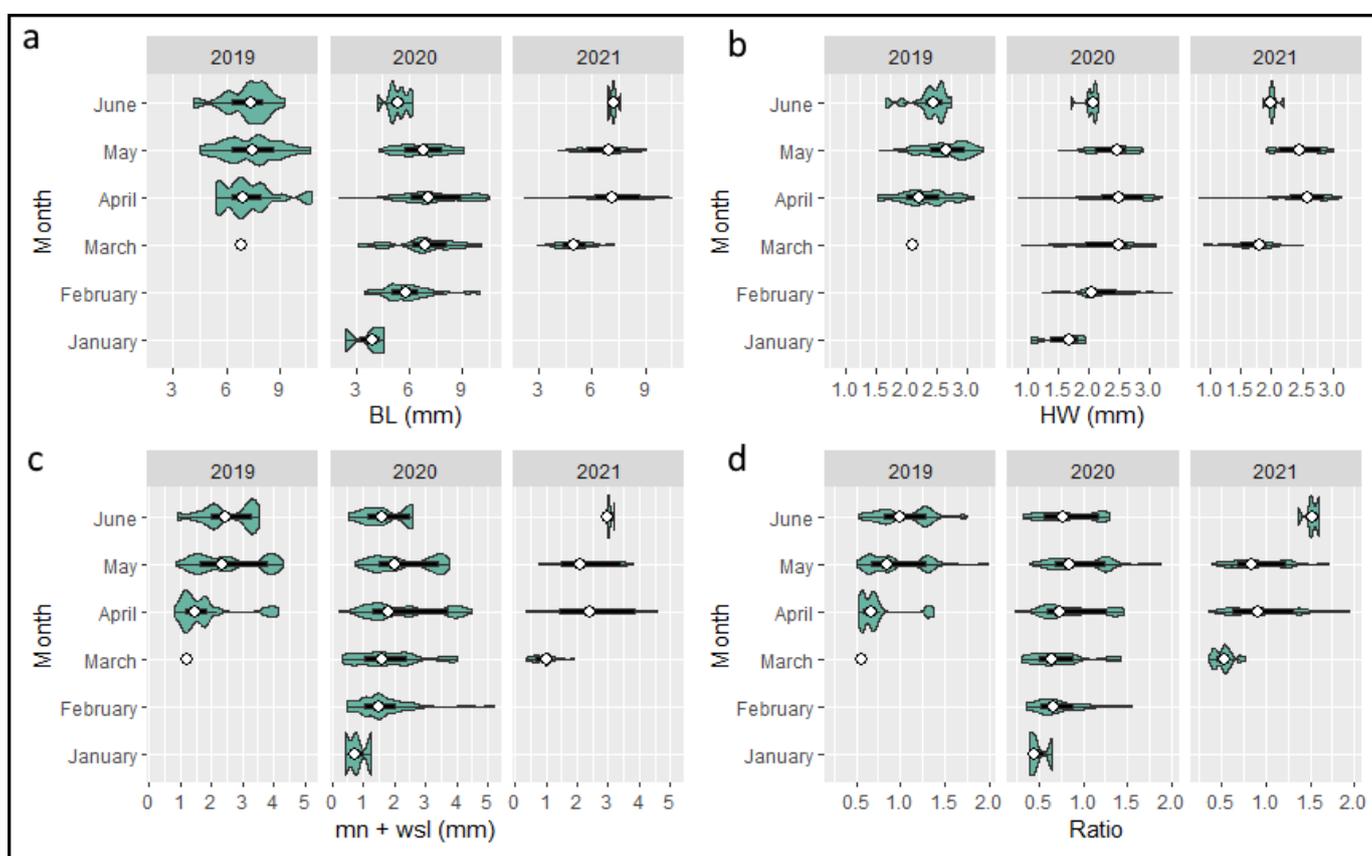


Figure 7. Seasonal changes in size–frequency distribution of *Rhithrogena sartorii* (2019–2021) for BL (a), HW (b), mn + wsl (c), and Ratio (d).

4. Discussion

4.1. Distribution

Both in Tunisia [14] and in Algeria, the distribution of *Rhithrogena sartorii* was restricted to the Kroumiria mountain range. Overall, the species seems confined to the metarhithral and parapotal river reaches. The lower end of the altitudinal range of the species’ habitats (200–650 m), almost matching *Rh. mariae*, which is able to colonize lower stretches (160 m) in Morocco, is noteworthy [13].

4.2. Taxonomy

Recently described from Tunisia, *Rh. sartorii* was thought to belong to the *insularis*-species group [14]. However, after preliminary investigations reported in [5], a careful re-examination of these nymphs conducted here confirmed that this species is more related to species of the so-called *sowai*-group [6]. Specifically, the shape of the plica, which is clearly concave (always less prominent and convex in species of the *hybrida*-group—Figure 5A), and the lateral sclerites of the first sternite, which can be slightly turned backward (always perpendicular to body axis in *hybrida*-group). Species of the *sowai*-group are poorly known; seven species have been described with only one in the nymphal stage [31], although an unnamed species has been described at the nymphal stage from Portugal [32]. All of these species are restricted to the Mediterranean basin. Finally, the lack of true affinities with species of the *hybrida*-group is demonstrated by the quite isolated position *Rh. sartorii* occupies in the phylogeny of European species of the genus based on mitochondrial and nuclear markers [5].

With a maximum of 0.61%, the COI K2P distances between sequences from Algeria and topotypes from Tunisia are very low, typically corresponding to intraspecific divergence in previous mayfly barcoding studies (e.g., [33–36]). Moreover, given that one of our specimens has the same COI haplotype as the two topotype specimens and that the combination of morphological characters fully fits the *Rh. sartorii* description, we can be confident in our identification. This is not surprising, as both populations are located in the same mountain range (Kroumiria), only c. 30 km distant from each other.

4.3. Eaton's *Rhithrogena*

In contrast to the relatively straightforward identification of *Rh. sartorii*, Eaton's *Rhithrogena* still remains shrouded in mystery. Unless the specimen is retrieved, we will probably never know which species of *Rhithrogena* Eaton [8] collected in Algeria, but we can safely rule out many of the known North African species on the basis of their limited distribution and ecology. Indeed, four species (*Rh. ourika*, *Rh. ayadi*, *Rh. giudicelliorum*, and *Rh. ryszardi*) occupy an altitudinal range between 1260 and 2800 m. Likewise, based on the location, Biskra, where Eaton has recorded the specimen, we can safely assume that the species was able to stand high temperatures in one phase of its life cycle. North African rheophilic *Rhithrogena* species, such as *Rh. mariae*, are present at low altitudes, but due to their localized distribution in the Rif, this latter is unlikely to represent a good candidate (but see [13]) for Eaton's *Rhithrogena*, which once inhabited the edge of the desert.

In addition, based on the flight period (late winter) of Eaton's specimen, we can also exclude that it was *Rh. sartorii* that emerges in late spring. The delayed nymphal growth and development of *Rh. sartorii* are suggestive of a univoltine winter/spring life cycle, whereas nymphal development in Eaton's species probably occurred in late autumn and winter, similar to the life cycle of *Rh. germanica* [37,38]. Based on all these elements and the extensive surveys of mayflies of the Aures Mountains (unpublished), we hypothesize that Eaton's *Rhithrogena* has probably gone extinct.

4.4. Life Cycle

Nymphal development of *Rh. sartorii* occurred during winter and spring, but there was considerable annual variation (January–March) in the first records of nymphs, probably linked to the close relationship between egg development and water temperature [39,40]. For instance, eggs of the cold stenothermal *Rh. loyolaea* and *Rh. nivata* rarely hatch at temperatures above 10 °C, thus restricting the species to cold streams [41,42]. If this was the case for *Rh. sartorii*, this threshold would limit hatching to winter months. In addition, once hatching is underway, the differential growth rates in small nymphs may be responsible for the extended period of their presence [41].

Rhithrogena sartorii managed a single generation per year, with the nymphal stage spread over winter and spring. According to Clifford's classification [43], the species exhibited a seasonal univoltine cycle (Us-Uw) where the egg stage and part of the nymphal

stage overwinters. Nevertheless, the univoltine life cycle of *Rh. sartorii* is quite unusual, with a winter and vernal growth and a long embryonic diapause during the warm months. It is somewhat distinct from the life cycle of *Rh. germanica*, a univoltine winter species, which emerges in Central Europe between February and April, undergoes a summer embryonic diapause with eggs hatching once the temperature drops in October [37].

Furthermore, the presence of small nymphs of *Rh. sartorii* in June may either suggest a protracted egg hatching period or a proclivity for the species to undertake a second generation if environmental conditions are adequate. In all three years, the habitats dried up, and thus, this question deserves further investigation. Although *Rhithrogena* species are known to be mainly univoltine [43], plasticity in voltinism has been demonstrated, ranging from semivoltinism for *Rh. loyale* [44] to partial bivoltinism [45], and even bivoltinism [46] for *Rh. semicolorata* and *Rh. diaphana*, respectively.

4.5. Conservation

Although species of the genus *Rhithrogena* may be perceived as less threatened, as their rhithral habitats may be contending with lesser anthropogenic pressures than downstream habitats, they are highly sensitive to various environmental factors [47,48]. In addition, due to historical factors (transboundary region and previous war zone), the El Kala district has been relatively maintained as a hotspot of freshwater biodiversity. However, despite its status as a Man and the Biosphere Reserve, the area is now under severe anthropogenic pressures fueled by a burgeoning population [49,50]. With its restricted distributional range encompassing the Tunisian and Algerian Kroumiria, *Rh. sartorii* is clearly an endemic species of conservational concern. Moreover, in most sites and during the three-year study, the species was never abundant. Thus, the limited range, low abundance, and narrow ecological niche (rheobiont associated with riffles) make this threatened species and its habitat vulnerable to various natural and anthropogenic stressors (climate change, pollution, land conversion, etc.). *Rhithrogena sartorii* may act as a useful bioindicator of such scarce habitats and an umbrella species for the conservation of the unique freshwater biodiversity hosted by the Kroumiria mountain range that spans north-eastern Algeria and north-western Tunisia [15,51,52]. Unless urgent steps are taken to lessen human encroachment on its habitats, this imperiled Maghrebian microendemic may rapidly go extinct.

5. Conclusions

A survey of the highlands of the El Kala region, north-eastern Algeria, has led to the discovery of a species of *Rhithrogena* that occupied the hyporhithral and parapotamal river reaches. Molecular and morphological analyses identified the species as *Rh. sartorii*, a Maghrebian microendemic confined to the Kroumiria mountain range and environs on the Algero-Tunisian border. The species exhibited a univoltine life cycle (Us-Uw) with emergence spread between April and June. *Rhithrogena sartorii* is threatened due to the species' limited range and the mounting anthropogenic pressures (water abstraction, fire, pollution, etc.) in the region.

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Data Availability Statement: Data is available from the corresponding author upon request.

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