

**RESEARCH ARTICLE**

# Morphology and evolution of the wing bullae in South American Leptophlebiidae (Ephemeroptera)

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**Abstract**

Insects were the first animals to take to the skies, and have been flying for over 320 million years. The order Ephemeroptera is, or at least is part of, the most early-diverging lineage of extant winged insects. The extant species present a very short adult life span, mainly dedicated to reproduction and dispersal of eggs. Mating and egg-laying behavior depend on flight. Wings are structures to fly and as such face a number of physical and physiological challenges. The convex curvature along the anterior–posterior axis of the wing generates a camber that must be carefully regulated. One of the most interesting ways of wing bending is provided by the bullae, which have been defined as short sections of flexible chitin, where the flexion lines cross veins. Although the bullae have been frequently used as taxonomic characters, there is no study focused on their morphology, although their prevalence on the wings of mayflies strongly suggests a role in flight. In order to identify evolutionary trends of these structures within Ephemeroptera, we constructed a matrix with comparative anatomy data of the bullae from whole mounts of the wings of 300 specimens belonging to 70 species of several mayfly families, as well as scanning microscopy samples of selected specimens. We also surveyed the number of bullae and their distribution in the wings of the different species within the South American Leptophlebiidae clade. We optimized the characters onto the latest published phylogeny for Leptophlebiidae.

**KEYWORDS**

insect flight, transverse flexion lines, wing morphology

## 1 | INTRODUCTION

Wings are flight structures and as such face a number of physical and physiological constraints. They need to generate the aerodynamic force to stay in flight, which is created by differences in airspeed over and under the wing (Grodnitsky, 1999). In addition, wings move in fluids by generating a vortex as they accelerate the surrounding air (Grodnitsky, 1999). Flight requires thrust and lift of the wings during all phases of the wing beat, and constant changing of the wing curvature to modulate the airflow (Marcus, 2001). The convex curvature along the anterior–posterior axis of the wing generates a camber that must be carefully regulated (Marcus, 2001; Vogel, 1981). Camber regulation can be achieved by varying flexural stiffness, a material property of the wing that makes it independent of the velocity of contraction of its musculature, and is determined by its crossveins. The number, flexibility, thickness, and so forth, of the crossveins

can be altered, modifying thus its flexural stiffness (Ennos, 1989; Marcus, 2001).

The insect order Ephemeroptera contains approximately 3,200 described extant species (Bauernfeind & Soldan, 2012) commonly known as mayflies. Ephemeroptera and Odonata are the only extant orders in the infraclass Paleoptera (Ogden & Whiting, 2003). All other extant insects with wings are in the Neoptera and are characterized by a wing articulation (joint) that allows them to fold their wings back over their abdomens at rest (Carpenter, 1992). The phylogenetic relationships within Ephemeroptera have been frequently addressed, with analyses mostly based on morphology (Dominguez, Ferreira, & Nieto, 2001; Flowers & Dominguez, 1991; McCafferty, 1997, McCafferty, 2004, but see Sun et al., 2006). In spite of these surveys of morphological data in the group, some structures, such as the bullae of the wings, remain poorly studied.

Bullae have been defined as short sections of flexible chitin, where the flexion lines cross veins (Mason, 1986). Although bullae have been frequently used as taxonomic characters (Mason, 1986; McCafferty, 1973; Perrichot, Nel, & Quicke, 2009; Rosen & DeBach, 1979), there is no study focused on their morphology, even though it has been suggested that they might play a main role in the flight of Ephemeroptera (Edmunds Jr. & Traver, 1954). Mason (1986) stated that in Hymenoptera bullae have been similarly named as fenestrae or thyridial area. Nevertheless, there seems to be some confusion about the use of these names. For example, Torre-Bueno (1962):39 states: "bulla (pl. bullae), a blister or blister-like structure; ... in Ephemeridae, a slightly swollen part of the costal area of the wing toward the tip, with more crossveins, practically equivalent to the stigma, q.v.; which are weak spots on some of the wing veins where they are crossed by furrows (Comstock)"; involving two different structures: the bulla and the stigma (or pterostigma). The weak spots, generically called fenestrae, have been noted in several insect orders (Diptera, Trichoptera, Lepidoptera) (Grodznitsky, 1999) but their homology with the mayfly bullae has not been tested.

In this work, we present comparative data on the bullae of whole mounts of the wings of 300 specimens belonging to 70 mayfly species. The specimens were mainly leptophlebiids, but our sample also included members of 10 other families for comparative purposes. We focused on South American Leptophlebiidae because we had a very good taxon sampling, including representatives of all 31 genera. Additionally, a morphological phylogeny for the group is available (Dominguez, 2009). We present data on optical and scanning microscopy samples of the bullae. Finally, we constructed a matrix with characters from the morphology of the bullae and optimized them onto the latest published phylogeny for Leptophlebiidae. Based on that, we identified the evolutionary tendencies of these structures of the wings within the group and inferred some functional aspects of the bullae. We consider this morphological study as a first step in the understanding of the function and biomechanics of the mayfly bullae.

## 2 | MATERIAL AND METHODS

### 2.1 | Morphological analysis

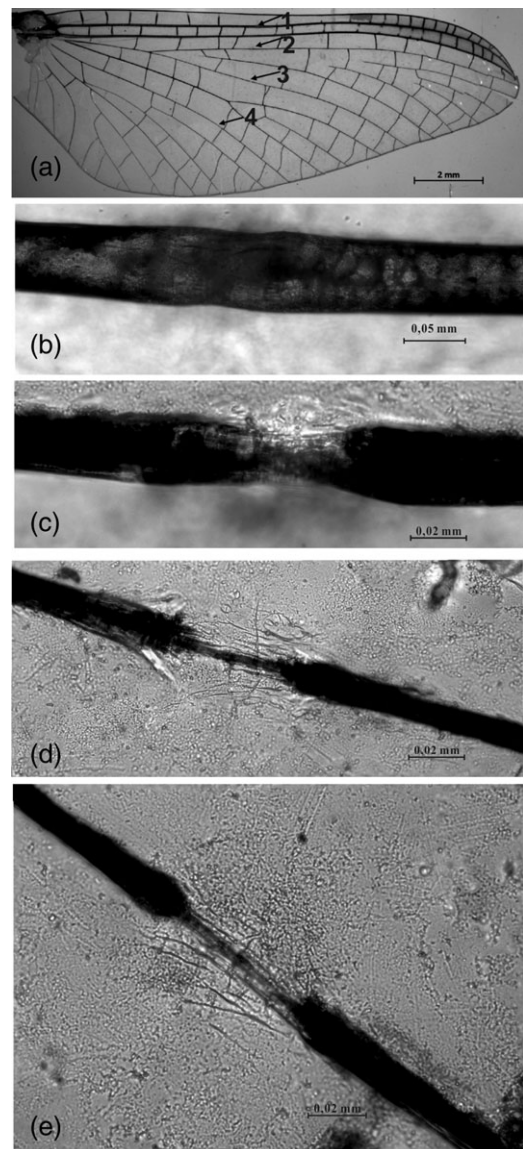
Three hundred specimens belonging to 70 mayfly species, mainly of the South American Atalophlebiinae (sensu Peters, 1980) were studied (Supporting information Appendix S1).

### 2.2 | Optical microscopy

Wings were observed either directly on specimens in alcohol or on wings dissected and mounted dry on slides. Some specimens were stained with different colorants. Photographs were taken with a Zeiss Stemi508 Stereomicroscope (Jena, Germany) and a Zeiss AxioScope. A1 with an Axiocam ICc5 Camera with the Zen<sup>®</sup> dedicated software.

### 2.3 | Scanning electron microscopy

Samples were prepared for scanning view without previous critical-point treatment, and while still wet mounted with double-sided tape

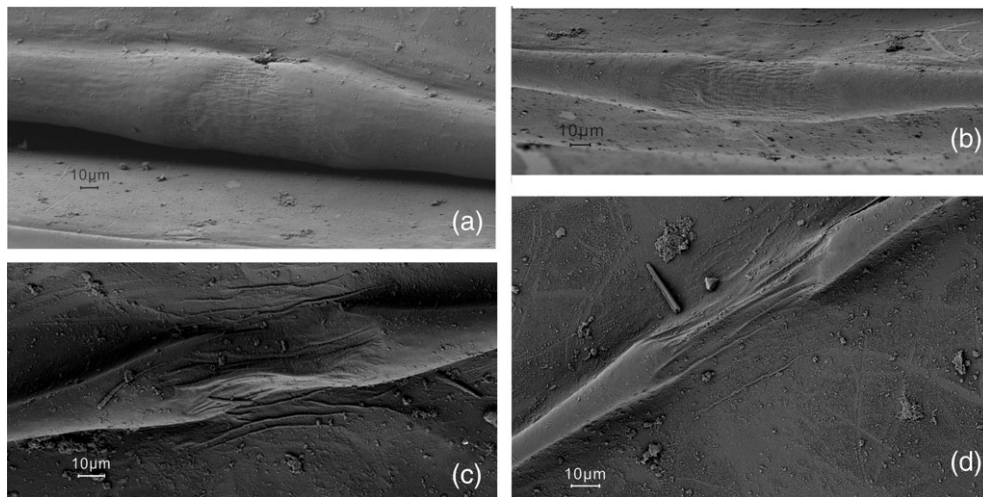


**FIGURE 1** *Penaplebia fulvipes* (Needham & Murphy). (a) General view; (b) Bulla 1 (vein Sc); (c) Bulla 2 (vein R<sub>2</sub>); (d) Bulla 3 (vein R<sub>4</sub> + 5); (e) Bulla 4 (vein MP<sub>1</sub>). Arrows with numbers indicate position of bullae in fore wing. Optical microscope

on SEM stubs. Some samples were sputter-coated with gold, but the results were not satisfactory as the details were masked. The specimens were imaged with a JEOL 35CF Scanning Electron microscope (Tokyo, Japan) at CIME (UNT-CONICET).

### 2.4 | Ancestral state reconstruction

Ten characters of the bullae obtained from whole-mount specimens and ultrastructural samples were included in a matrix (Supporting information Appendix S2) to perform an ancestral state reconstruction. We mapped the characters using parsimony on the Leptophlebiidae cladogram proposed by Dominguez (2009), reduced to the species surveyed. We ran ancestral state reconstructions on a cladogram to create figures illustrating changes in character states; however, we discuss our results in terms of apomorphies, because an ultrametric phylogeny is not available. Optimizations were performed



**FIGURE 2** Wing of *Penaphlebia flavidula* Pescador & Peters. (a) Bulla 1 (vein Sc); (b) Bulla 2 (vein  $R_2$ ); (c) Bulla 3 (vein  $R_4 + 5$ ); (d) Bulla 4 (vein  $MP_1$ ). SEM

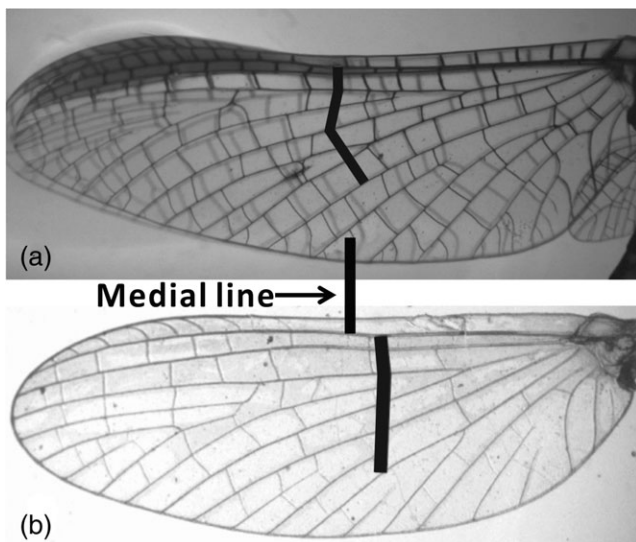
with TNT software (Goloboff, Farris, & Nixon, 2008) and the resulting cladogram was edited with Winclada 1.00.08 software (Nixon, 2002).

### 3 | RESULTS

#### 3.1 | Morphology

We used light microscopy to study the bullae, which are present on the Sc,  $R_2 + 3$ ,  $R_4$ , and  $MP_1$  veins (Figure 1a), named them here 1–4 in the same order, to facilitate the discussion. They decrease in width and increase in the length-to-width ratio from 1 to 4. Bullae 1 and 2 generally look like a cylindrical expansion of a vein, similar to an ampule (Figure 1b,c). Bullae 3 and 4, although exhibiting the same morphology, seem to have thinner walls, sometimes collapsed (Figure 1d,e).

In scanning electron micrographs it appears that all the bullae acquire different shapes owed to their different grade of turgidity

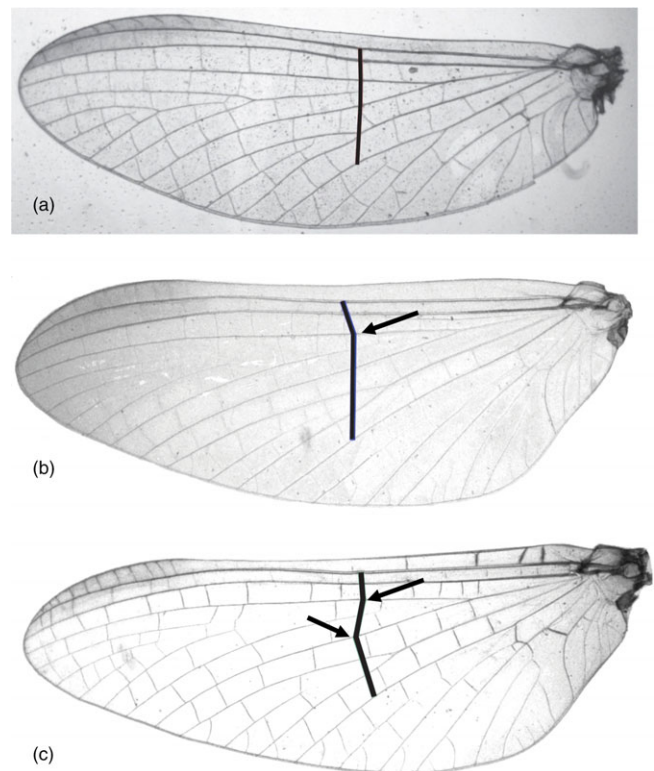


**FIGURE 3** Position of the trajectories (invisible line that connects the bullae). (a) *Massartella brieni* (Lestage), middle of the wing. (b) *Farrodes roundsi* (Traver), base of the wing

(Figure 2). Bullae 1 and 2 (Figure 2a,b), are again wider than the respective vein, and wrinkled. Bullae 3 and 4 show a collapse of their walls (Figure 2c,d). Little folds less complex than the bullae are scattered all over the longitudinal veins (not shown).

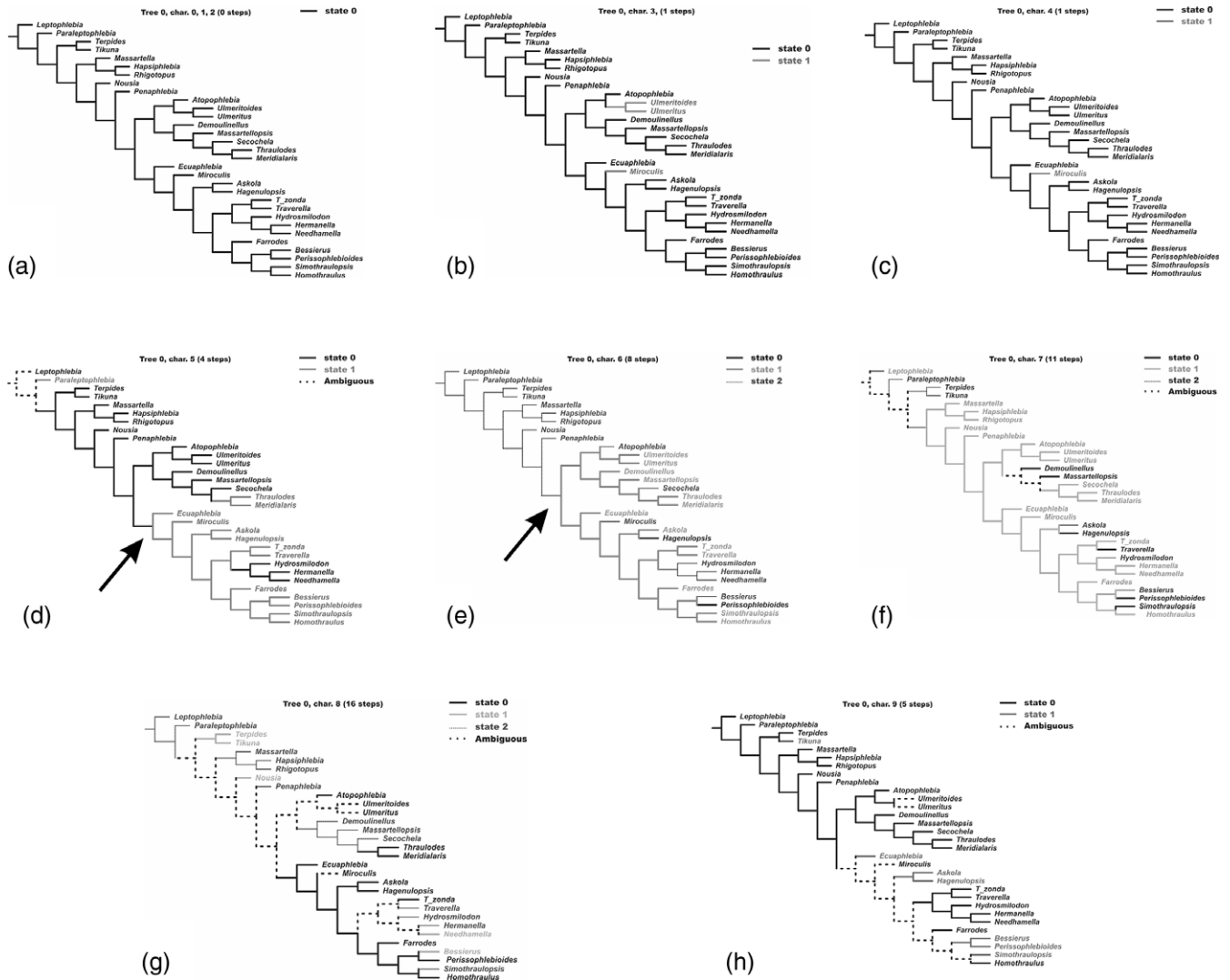
Based on these observations we compiled the following list of characters to be optimized:

- 0) Bullae.
- 0 = Present.
- 1 = Absent.



**FIGURE 4** Breaking angles of the trajectories, and their directions in  $R_2 + 3$  and  $R_4$ . (a) *Hagenulopsis lipeo* Dominguez et al., straight, not crossing MA fork; (b) *Meridialaris diguillina* (Demoulin), one angle in  $R_2 + 3$  basal, crossing MA fork; (c) *Rhigotopus andinensis* Pescador & Peters, two angles,  $R_2 + 3$  basal,  $R_4 + 5$  distal, crossing MA fork





**FIGURE 5** Ancestral state reconstruction onto the reduced phylogeny of Dominguez (2009) with the relationship between the analyzed taxa illustrated. (a) Characters 0, 1 and 2, presence, morphology and width of the bullae; (b) Character 3, number of bullae; (c) Character 4, sexual dimorphism in the number of bullae; (d) Character 5, position of the trajectories (arrow node J); (e) Character 6, breaking angles of the trajectory (arrow node F); (f) Character 7, direction of the breaking angle in  $R_2 + 3$ ; (g) Character 8, direction of the breaking angle in  $R_4$ ; (h) character 9, trajectory line crossing the MA fork

All Leptophlebiidae wings observed present bullae. However, as there are families without bullae (e.g., Oligoneuriidae, Polymtarciidae), we included the absence possibility for future studies with a wider scope.

1) Morphology of the bullae

0 = Cylindrical expansion of the vein similar to an ampule.

Our intensive taxon sampling within Leptophlebiidae allowed us to state that this character state is valid for all the genera examined. A similar morphology was also found in the other families surveyed.

2) Width of the bullae

0 = Decreasing posteriorly.

As expressed above, our taxon sampling showed that this character state is valid for all the Leptophlebiidae and the other families surveyed. With a wider taxonomic scope, it could be possible to quantify different patterns of degrees of diminution.

3) Number of bullae in males

0 = 4.

1 = 3.

All male Leptophlebiidae surveyed present four bullae, with the exception of *Ulmeritus*, *Ulmeritoides*, and *Miroculis* with three.

4) Polymorphism in the number of female bullae

0 = Absent.

1 = Present.

Males and females of most leptophlebiid genera present the same number of bullae (either 4 or 3). However, there are females of some species of *Miroculis* that present different number of bullae (4 or 3), coded here as a polymorphism.

5) Position of the trajectories

0 = Middle (Figure 3a).

1 = Basal (Figure 3b).

We call here “trajectory,” the invisible lines connecting the bullae. We coded this character in this way to reflect the importance of the location of these trajectories as was proposed by Wootton (1992).

6) Breaking angles of the trajectory

0 = 0 (Figure 4a).

- 1 = 1 (Figure 4b).  
 2 = 2 (Figure 4c).  
 7) Direction of the breaking angle in  $R_{2+3}$   
 0 = Absent (Figure 4a).  
 1 = Basal (Figure 4b).  
 2 = Distal (not shown).  
 8) Direction of the breaking angle in  $R_4$   
 0 = Absent (Figure 4a,b).  
 1 = Basal (not shown).  
 2 = Distal (Figure 4c).

These three characters would represent the minimal structural changes that according to Wootton (1992) are responsible for the orientation of the transverse flexion lines.

- 9) Trajectory line crossing the MA fork  
 0 = Yes (Figure 4b,c).  
 1 = No (Figure 4a).

The trajectory line could go through the stem or the bifurcation of a forked vein. A testable hypothesis could be that the location of the trajectory line renders different mechanical responses.

#### 4 | ANCESTRAL STATE RECONSTRUCTION

Three of the included characters, (Presence [1], Morphology [2], and Width [3] of bullae) present just one character state in all taxa (Figure 5a), but we decided to include them to highlight their extreme conservatism. Character 3, state 1 (number of bullae in males: 3) is a synapomorphy of the node *Ulmeritus* + *Ulmeritoides*, independently acquired in *Miroculis* (Figure 5b). Character 4 state 1 (Polymorphism in the number of female bullae: present) is an autapomorphy of *Miroculis* (Figure 5c). Character 5 state 1 (Position of the trajectories: basal) is a synapomorphy of the node J (arrow in Figure 5d, and Dominguez, 2009), independently acquired by *Thraulodes* + *Meridialaris*, with a reversion to the ancestral state (middle) in the clade of *Hydrosmylodon* + [*Needhamella* + *Hermanella*] (Figure 5d). The ancestral state for this character in the whole cladogram is the trajectory in the medial region of the wing. Character 6 state 2 (Breaking angles of the trajectory: 2) is the ancestral one, with a change to state 1 (1) in node F (arrow in Figure 5e, and Dominguez, 2009). Within node F there are five independent reversions to the ancestral state (2) in *Atopophlebia*, *Secochella*, *Miroculis*. The clade *Hydrosmylodon* + [*Needhamella* + *Hermanella*] and *Bessierus*; character state 0 (0) was independently acquired by *Hagenulopsis* and *Perissophlebioides* (Figure 5e). The ancestral state of character 7 (Direction of the breaking angle in  $R_{2+3}$ ) is ambiguous; state 0 (Absent) was independently acquired by *Demoulinellus*, *Masartellopsis*, *Hagenulopsis*, *Traverella*, *Perissophlebioides*, and *Simothraulopsis*; state 2 (Distal) was independently acquired by *Paraleptophlebia*, *Terpides* + *Tikuna*, *Askola*, *Hydrosmylodon*, and *Bessierus* (Figure 5f). The state 2 of character 8 (Direction of the breaking angle in  $R_4$ : Distal) is the ancestral one, with the state 1 (Basal) being a synapomorphy of the node *Terpides* + *Tikuna*, independently acquired by *Nousia*, *Needhamella*, and *Bessierus*; state 0 (Absent) is a synapomorphy of the node *Thraulodes* + *Meridialaris*, independently acquired in *Atopophlebia* and in the base of the node J (Dominguez, 2009) (Figure 5g). The state 0 of character 9 (Trajectory line crossing the MA fork: Yes) is the ancestral one; state 1 (No) is a synapomorphy of the

nodes *Bessierus* + *Perissophlebioides* independently acquired by *Askola* + *Hagenulopsis*, *Tikuna*, *Ecuaphlebia*, and *Simothraulopsis* (Figure 5h).

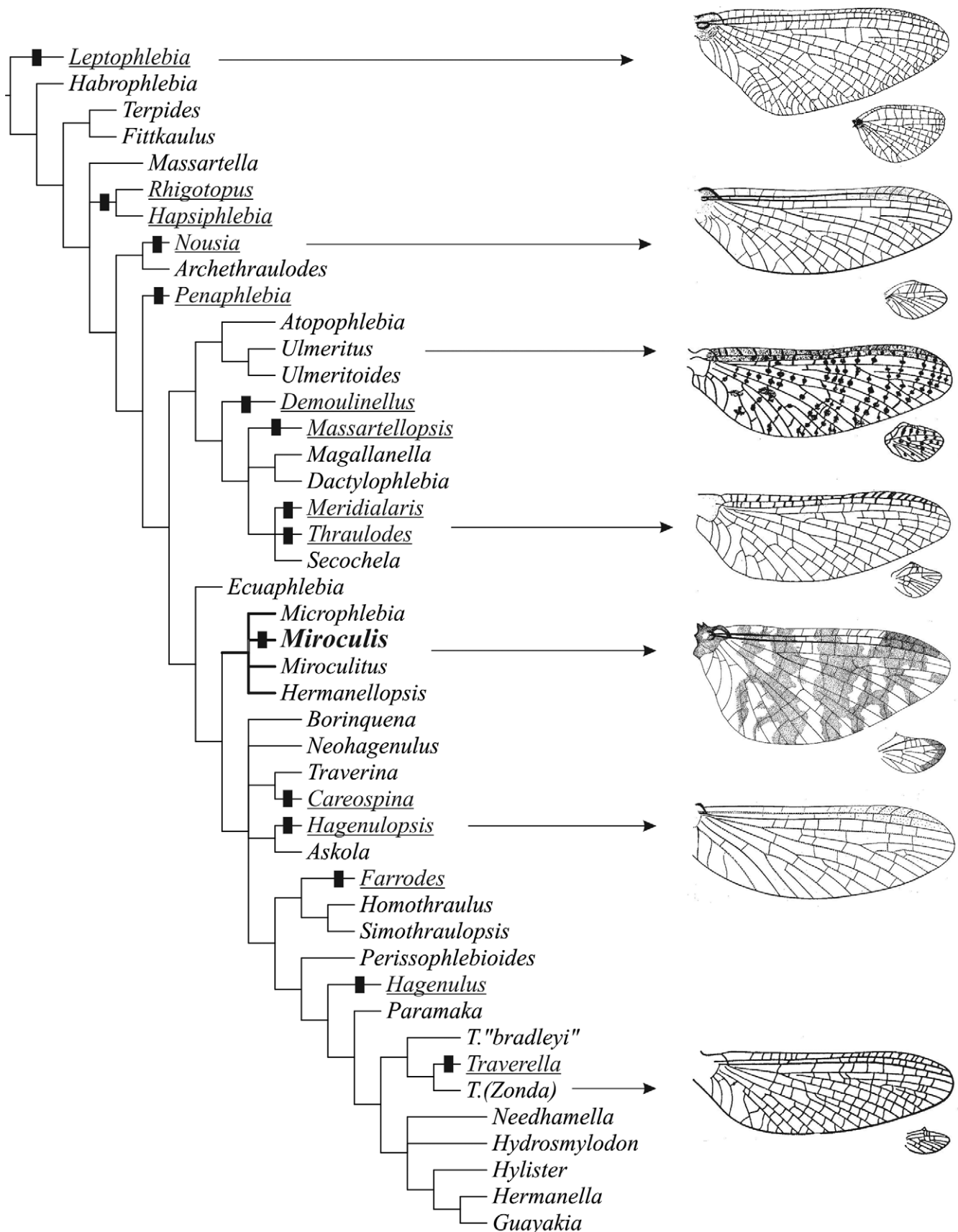
#### 5 | DISCUSSION

Edmunds Jr. and Traver (1954) wrote a pioneering article on the wing, the wing venation, the distribution of the bullae and their probable function in Ephemeroptera. They focused in *Siphonurus* (Siphonuridae), and surveyed several other mayfly families, but provided no details on the bullae structure characteristics. In relation with the fluttering of the membrane, they stated that the bullae are always on concave or negative veins. Also, they hypothesized that the line of bullae would allow the tip of membrane to bend in the upstroke, diminishing the pressure, and offering less resistance. On the contrary, in the downstroke the wing would remain rigid and get a maximum of lift and propulsion. Nevertheless, they offered no experimental information to support their inferences. Our data on the bullae show that they do not look like weakened spots, but rather as ampule or blister-like structures, as have been defined by Grasse (1949). These new observations on the size and morphology of the bullae do not support Edmunds Jr. and Traver's (1954) interpretation of wing bending in mayflies. If the C and Sc veins are stronger and more turgid than the veins in the posterior region of the wing, we hypothesize that the curvature of the wing is opposite of that predicted by Edmunds & Traver: less pronounced toward the anterior margin and more pronounced toward the posterior margin. This would certainly be an important issue to test experimentally.

Grodnitsky (1999) described structures similar to the bulla, such as the thyridium and arculus, in caddisflies. These are desclerotized spots or fenestrae located on particular veins of the wing. He traced a trajectory that would pass through these fenestrae, along which the wing would bend. We also called "trajectory" the invisible lines connecting the bullae. According to Grodnitsky (1999), these structures generate a mechanism able to alter the wing profile, enhancing their flexibility, although this point has not been proven experimentally. Grodnitsky (1999) reported fenestrae, including the thyridium, and arculus, in several insect groups such as Myrmeleontida and Panorpidia; and their absence in some Diptera. A clear relationship between these structures and some functional/adaptive factor has not been discovered yet. Wootton (1979, 1981, and 1992) has repeatedly mentioned and described the flexion lines of the insect wings, distinguishing between the longitudinal and transverse flexion lines and explaining that transverse flexion lines are mainly related to ventral bending of the wings. We posit that the position of the bullae determines the location of the transverse flexion lines. This is in accordance with Wootton (1992) who stressed that the orientation of the flexion lines can be altered with minimal structural changes. These structures show a conservative morphology within Leptophlebiidae, with variations recorded in relation to size, number and location on the wing.

##### 5.1 | Ancestral state reconstruction

The optimization of the first three characters (Presence [1], Morphology [2], and Width [3] of bullae) results in the same state in all taxa. From a cladistic point of view, these characters would be irrelevant.



**FIGURE 6** Cladogram of the South American Leptophlebiidae. Black marks show the genera in which the mating flight was described in at least one species. An outline of the wings is included to show the general shape and venation and whether one or two wings are present

However, they convey information about the bauplan of Leptophlebiidae wing in relation to bullae: all Leptophlebiidae have bullae, with similar shape, and decreasing in size posteriorly. These apomorphies suggest a number of questions such as why they are so extremely conserved in the group. Although the possibility that they were simply inherited from their ancestors can not be dismissed with our data, they could achieve importance if could be linked to some functional property of the family wings, related to the flight itself or other entirely different function.

Four is the plesiomorphic number of bullae in males (character 3). In two unrelated clades, the bullae of vein  $MP_1$  are lost; therefore only three bullae are present. Bulla four is lost in both sexes of all the species surveyed in the clade *Ulmeritus-Ulmeritoides* and this character state is also a putative synapomorphy of these two sister genera.

Wootton (1992) highlighted the consequences that the position of the flexion lines has for the aerodynamic behavior of the wing. He posited that the more basal the flexion line, the higher the possibility of twisting of the leading edge. Our data show that Leptophlebiidae present an evolutionary trend toward a basal position of the trajectory in the wing (character 5).

In the genus *Miroculis*, all the males of the species studied have three bullae, meanwhile in the females, one species also has three bullae, and the others have four. We studied several *Miroculis* mating flights; and none of them correspond to any type described in the comprehensive article by Brodsky (1973). Their flight, although presenting several differences among species, exhibit a rather static hovering over a determined place of the river, with no up and down primitive phase. In one case (*M. fittkai*), the males were able to shoot up to a higher place, but in no case similar to the active phase of the up and down type A flight (Brodsky, 1973). As noted above, *Miroculis* males have three bullae and a basal trajectory. *Ulmeritus-Ulmeritoides* also have three bullae, but a medial trajectory, although no details of their mating flight have been described in so far. It would be very interesting to observe the details of their flight to test the importance of the medial trajectory in wings with three bullae. We observed that two species of the dipterous *Hagenulopsis* (*H. lipeo* and *H. sp.* from Colombia), with four bullae and straight trajectory, presented a mating flight quite similar to that of their four-winged congeners. If the number of bullae is relevant to the maneuverability of the wing and flight performance, then it could be predicted that *Ulmeritus-Ulmeritoides* (and their sister group) will present also some kind of differences in their flight with the four-bearing bullae species. Likewise, it would be relevant to compare the flight of the three versus four bullae bearing females of *Miroculis*. Unfortunately, no information on these cases are available so far.

Following Wootton (1992) ideas we could infer that twisting possibility was a main factor driving wing morphology in this family. When the breaking angles of the trajectories are considered (character 6), our data show that the ancestral leptophlebiid wing presented two angles, with a tendency to reduce them to 1, with multiple reversions. The extreme, without angles and a straight trajectory is achieved independently in two dipterous genera: *Hagenulopsis* and *Perissophlebioides*.

The remaining characters convey information about the direction of the breaking angles in  $R_{2+3}$  (character 7) and in  $R_4$  (character 8), and whether or not the trajectory lines cross the MA fork (character

9). Our optimizations suggest several instances of homoplasy. The relevance of these complex patterns cannot be assessed with the current knowledge of the details of mating flight. Recent advances on the morphology of the wing veins (Zhou, 2007, but see also Edmunds Jr. & Traver, 1954. p. 391) postulates that the presence of a costal brace results in a common movement of C, Sc, and R. This characteristic, coupled with the apical flexibility of the wing provided by the bullae could result in a highly versatile wing.

## 5.2 | Bullae and mating flight

We superimposed on the cladogram of South American Leptophlebiidae a black mark showing the genera in which the mating flight was described in at least one species (Figure 6). We also included an outline of the wings, to show the general shape and venation and whether one or two wings were present. All the species we checked have bullae, and all the mating flights described were of the up and down type (Type A flight of Brodsky, 1973). Savage (1983) in a well-documented study on the shape evolution of *Miroculis* wings stated that the fore wings of *Miroculis* retained the ancestral marginal intercalaries, and hypothesized that their wings should be of the rigid vertical flight mode. Type A of flight (Brodsky, 1973), would be correlated with the ancestral wing as described by Edmunds Jr. and Traver (1954). They stated that this wing was corrugated, with alternating convex and concave longitudinal veins. This would make the wing rigid enough to provide support, but bullae would allow the concave veins (and the tip of the wing) to bend during the upstroke while the whole wing remains rigid during the downstroke. Type D, active in both directions has been recorded for *Leptohyphes*, *Tricorythodes* (Leptohyphidae), and *Caenis* (Caenidae). Although all these genera present an apparently similar up and down flight, Leptohyphidae has three bullae, and Caenidae none. So, the relevance of bullae to this type of flight remains as an open question.

We examined *Lachlania* (Oligoneuriidae) and *Campsurus* (Polymitarciidae), that present a patrolling flight, and despite the difference in venation (few in oligoneurids, many in polymitarciids), neither one has bullae.

We consider that in order to be able to correlate wing characters with nuptial flight characters, more precision in flight descriptions is needed. Bullae alone seem not to be entirely responsible for drastic changes in flight. Another interesting possibility cannot be discarded with the data at hand. As mayflies are the only winged insects that undergo a moulting of the wings from subimago to imago, the bullae could also participate in facilitating the extraction of the imaginal wing out of the subimaginal one.

Probably, the flight pattern is a result of a complex combination of macro structures such as hind wings presence or absence, wing shape, venation, and microstructures such as bullae.

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## SUPPORTING INFORMATION

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

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