

## Comparative Study of the Mating Apparatus of Three Species of Leptophlebiidae (Ephemeroptera)

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Ultrastructural studies carried out on the coupling system of the leptophlebiids, *Habroleptoides confusa*, *Habrophlebia eldae* and *Choroterpes picteti*, identified the fine morphology of the external genital apparatus. In *H. confusa* and *H. eldae* penes consisted of two lobes, each presenting a ventral appendage folded onto itself to form a sperm groove. The structure of the appendages and their merging with the penis lobes differed with the species. By contrast, in *Ch. picteti* appendages were not present and the sperm duct was completely incorporated inside the penes. No peculiar differentiation was observed for the female genitalia of *Ch. picteti*. By contrast, there were found a subgenital plate in *H. confusa* and a tubular ovipositor in *H. eldae*. The morphology of the male and female coupling systems are discussed in view of both paleomorphological traits and aflagellate sperms.

Keywords: sperm groove, aflagellate sperm, ovipositor, coupling system, mayflies.

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### INTRODUCTION

The morphology of the coupling system reflects the mating strategy of insects. In Ephemeroptera several systems of mating facilitation have been evidenced in detail by Brinck (1957). More recently, studies on external genitalia have shown coevolutionary traits of male and female *Tortopus*, which are differentiated to improve clasping for coupling (McCafferty and Bloodgood, 1989).

A detailed study of external genitalia in leptophlebiids is of particular interest because of the aflagellate sperms that constitute a peculiar trait of the representatives of this family (Soldán, 1979; Grimm, 1985).

Ultrastructural investigation on sperm architecture in a few species of leptophlebiids, belonging to *Habrophlebia*, *Habroleptoides* and *Choroterpes*, identified the unusual organization of such immotile gametes consisting in a very simplified structure (Gaino and Mazzini, 1991 a, b).

The mechanisms of egg/sperm encounter in species with immotile sperm are not thoroughly investigated. In leptophlebiids sperm transfer into the female genital apparatus could be enhanced by the differentiation of a peculiar sperm pump, consisting of a thick muscular ring around the terminal part of the vasa deferentia (Grimm, 1985).

We have described under scanning electron microscopy (SEM) the external penial shape and the organization of female gonoduct openings in three species

belonging to *Habroleptooides*, *Habrophlebia* and *Choroterpes*. This work also includes micrographs showing the ultrastructural organization of sperms both in section and in toto.

## MATERIALS AND METHODS

Specimens of *Habroleptooides confusa* Sartori and Jacob (male and female imagines, Piedmont, Capanne di Marcarolo, E. Gaino collection), *Habrophlebia eldae* Jacob and Sartori (male and female imagines, Liguria, along Bisagno river banks, E. Gaino collection) and *Choroterpes picteti* Eaton (male imagines and female subimagines, Liguria, Vara stream, E. Gaino collection) were investigated. Sperms of *Ch. picteti* were observed with a phase contrast light microscope. For transmission electron microscopy (TEM), sperms of *H. eldae* and *Ch. picteti* were fixed in Karnovsky medium (1965) in cacodylate buffer (pH 7.2, 0.1 M), rinsed in the same buffer, postfixed in 1% osmium tetroxide-cacodylate buffer, dehydrated in ethanol series, and embedded in Durcupan resin. Sections obtained with a Reichert ultratome and mounted on formvar-coated copper grids were stained with uranyl acetate and lead citrate and observed in a ZEISS EM9 electron microscope. For scanning electron microscopy (SEM), selected specimens were dehydrated in a graded series of ethanol and critical-point dried in a Pabisch CPD 750 apparatus. Specimens were mounted on stubs and coated with gold-palladium in a Balzers Union evaporator. Observations were carried out with a Philips EM 515 electron microscope. Selected samples were also observed with contrast-phase microscopy.

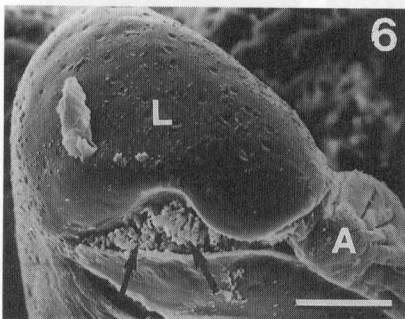
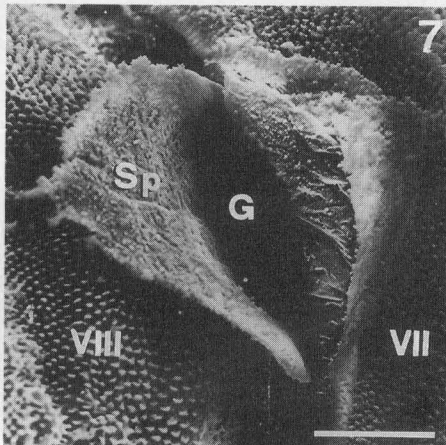
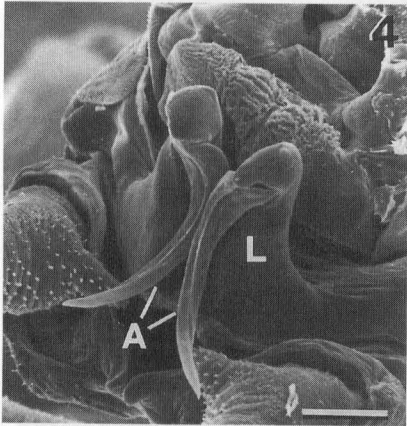
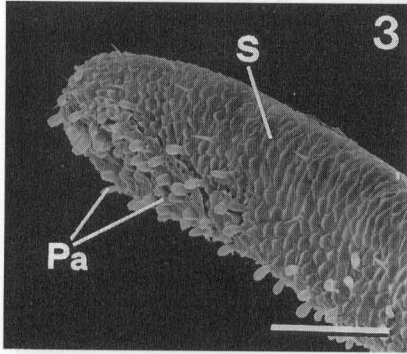
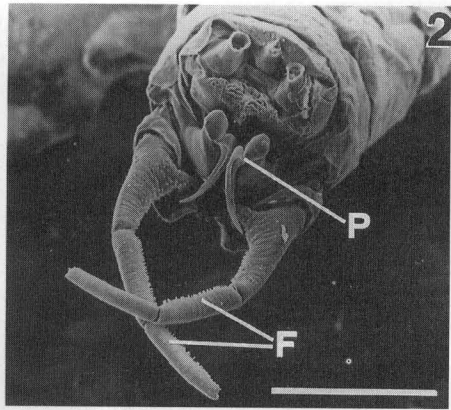
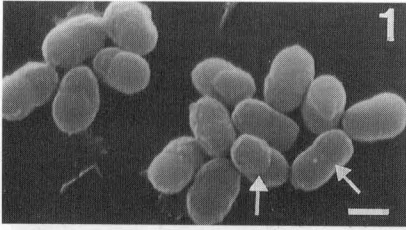
## RESULTS

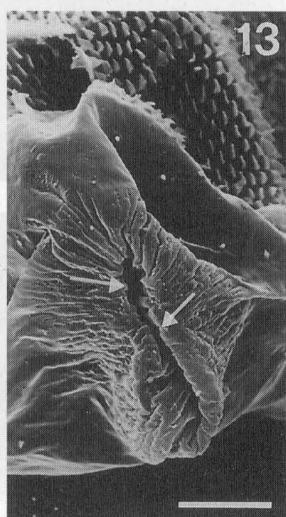
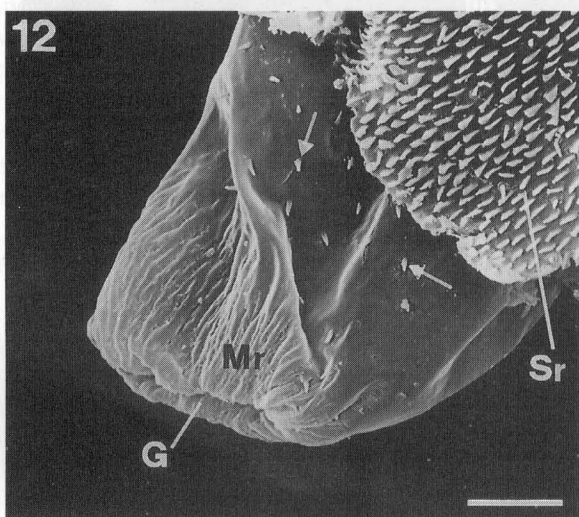
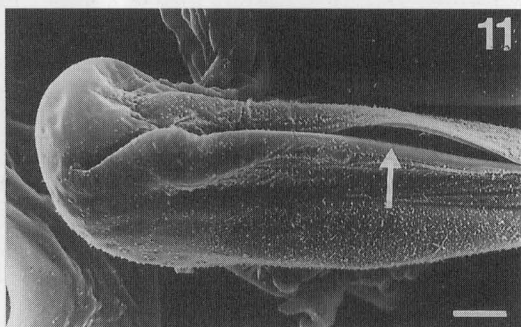
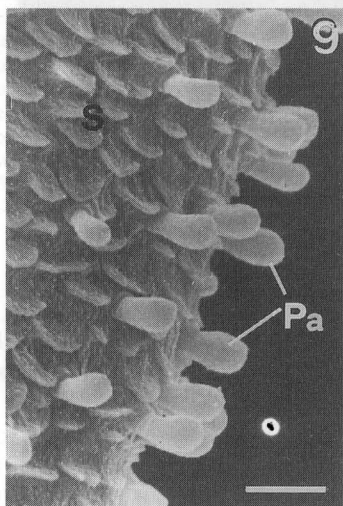
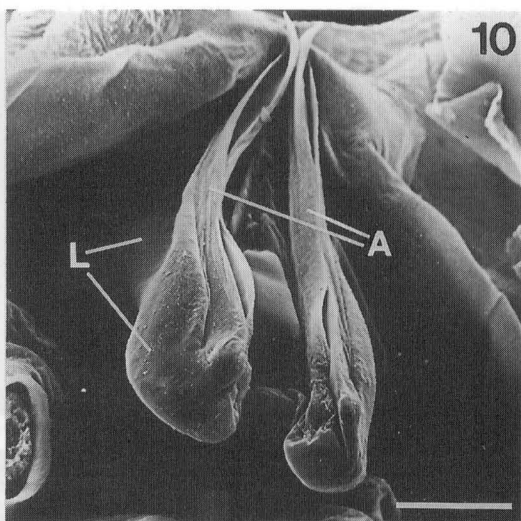
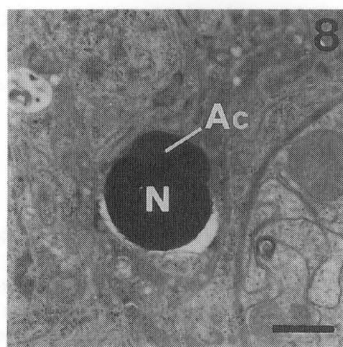
In *Habroleptooides confusa* (Figs. 1-7) the aflagellate sperms have an ellipsoid shape with a central constriction (Fig. 1).

The external genitalia of *H. confusa* consist of a pair of penis lobes and a pair of gonopodes or forceps, a typical feature of mayflies (Fig. 2). The surface of the forceps is covered by scales and papilla-like structures, these latter located along the ventral side (Fig. 3). The penes consist of a bilobated structure, with a common basal portion and two diverging extremities of round shape (Fig. 4). A ventral appendage departs from the apex of each penis lobe (Fig. 4). Each appendage consists of a thin sheet folded on itself to delimit a cavity (Fig. 5). The tip of each sheet is open, showing the seminal groove formed by the proximal portion (Fig. 5). The attachment of the appendage to its penis lobe is different on each side. On the inner portion the appendage completely merges into the penis, whereas on the outer portion the attachment is uneven. Such arrangement delimit-

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Figs. 1-7. Scanning electron micrographs, *Habroleptooides confusa*: 1, sperms have an ellipsoid shape with a central constriction (arrows) (bar= 1 $\mu$ ); 2, dorsal view of the terminal part of male abdomen. Note external genitalia consisting of a pair of penes (P) and forceps (F) (bar= 500 $\mu$ ); 3, terminal pad of the forceps with scales (S) and papilla-like structures (Pa) (bar= 50 $\mu$ ); 4, detail of penes. Note the penis lobes (L) and the ventral appendages (A) for sperm transfer (bar= 100 $\mu$ ); 5, detail of the appendages consisting of thin sheets folded to realize seminal grooves (arrows) (bar= 50 $\mu$ ); 6, detail of the connection between penis lobe (L) and appendage (A). Sperm accumulation (arrows) is evident in the area where the appendage merges into the penis lobe (bar= 25 $\mu$ ); 7, female seventh (VII) and eighth (VIII) sternum. Note the triangular-shaped subgenital plate (Sp) leading to the gonopore (G) (bar= 50 $\mu$ ).





its a space between the apical part of the penis lobe and its appendage, which allows sperm descent (Fig. 6). The distal portions of the appendages diverge laterally (Figs. 2-4-5).

The female of *H. confusa* presents a triangle-shaped subgenital plate emerging along the intersegmental area between the seventh and the eighth sterna, protruding over the eighth segment. The plate leads to the gonopore (Fig. 7).

In *Habrophlebia eldae* (Figs. 8-13), sections reveal that sperms have a very simple organization and consist of a nucleus with an overlying acrosome (Fig. 8). Apices of forceps, similar to those observed in the previous species, show with SEM scales and papilla-like structures on their surfaces (Fig. 9). The penes also present two different portions, lobes and appendages (Fig. 10). In *H. eldae* appendages do not diverge distally and each appendage follows its lobe to the apical region (Fig. 10). The appendages are folded and form a seminal groove along their whole length (Fig. 11).

Females present a characteristic tubular structure protruding from the seventh sternum and overlying part of the eighth segment (Fig. 12). The female genital apparatus, as in *H. confusa*, consists of paired ovaries and oviducts, which join to form a common duct lying inside the tubular structure (Grandi, 1955; Gaino and Mazzini, 1990). This complex is called ovipositor or egg guide. The external shape of the ovipositor includes a sclerotized region in its proximal portion and a membranous tract distally (Fig. 12). A cuticle with uniformly distributed thorn-shaped projections characterizes the sclerous region. Similar projections are more sparse on the membranous tract (Fig. 12), which presents a folded border delimiting the gonopore (Fig. 13).

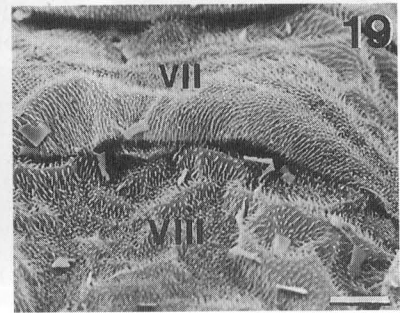
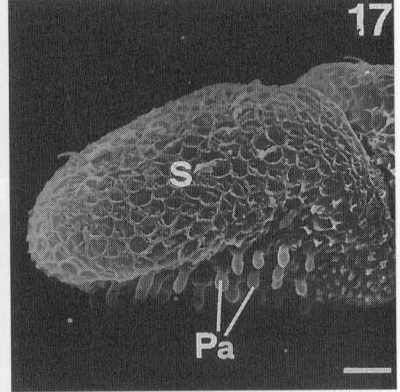
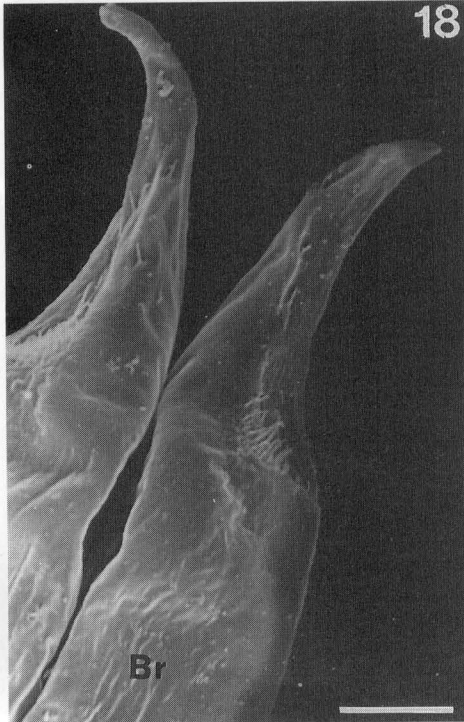
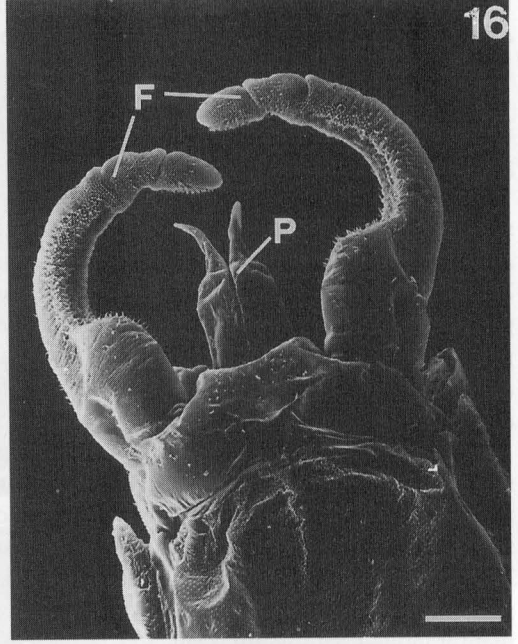
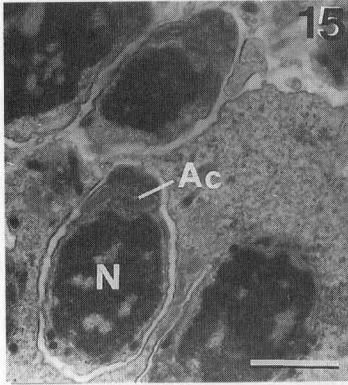
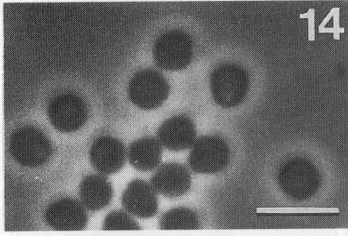
In *Choroterpes picteti* (Figs. 14-19), sperms show a "coccolidal shape" with phase-contrast light microscope (Fig. 14). With TEM they present an acrosome in their anterior region (Fig. 15).

Forceps and penes of the coupling apparatus are shown in Figure 16. The surface of the forceps is provided with scales and numerous papilla-like structures gathered along the ventral surface of the terminal pad of the forceps (Fig. 17). The paired penes are composed of two lobes, each consisting of a basal region that tapers towards its apical part (Fig. 18). The paired basal regions run tightly together and adhere to each other. The distal portions slightly diverge toward a laterally bent apex (Fig. 18). The lumen for the transfer of aflagellate sperms is completely incorporated inside the penes.

The female of *Ch. picteti* does not show any particular differentiation between

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Figs. 8-13. Transmission (8) and scanning (9-13) electron micrographs, *Habrophlebia eldae*: 8, sperm organization consisting of nucleus (N) and acrosome (Ac) (bar= 0,5 $\mu$ ); 9, surface of forceps provided with scales (S) and papilla-like structures (Pa) (bar= 10 $\mu$ ); 10, penes each consisting of lobe (L) and appendage (A) (bar= 50 $\mu$ ); 11, detail of one appendage. Note the seminal groove (arrow) (bar= 10 $\mu$ ); 12, Female ovipositor exhibiting the sclerous basal portion (Sr) and its membranous region (Mr). Note the different distribution of thorn-shaped projections (arrows) at the top of the ovipositor. The gonopore (G) is visible (bar= 25 $\mu$ ); 13, Detail of the female gonopore with its folded rim (arrows) (bar= 25 $\mu$ ).



its seventh and eighth sterna that may identify the opening outside of the genital apparatus (Fig. 19).

## DISCUSSION

Our work has demonstrated the occurrence of penis grooves in *Habroleptoides confusa* and *Habrophlebia eldae*. This finding is quite surprising, since according to Grandi (1960) ejaculatory ducts in Ephemeroptera are included inside the penis lobes. In our experience only *Ch. picteti* exhibits such a location. On the basis of paleomorphological studies, Kukalová-Peck (1991) has shown that distinct grooves occur in Paleozoic Ephemeroptera, even though they are borne by gonapophyses rather than by penes. Gonapophyses constitute a small genital armature which is probably lost by apomorphy. As a consequence, the occurrence of grooves seems to be an ancestral condition, probably inherited by some living species, including those mentioned above. If additional investigations of several mayfly groups evidence the presence of grooves in the penis, as suggested by several drawings of genitalia of *Siphonurus*, *Paraleptophlebia*, *Leptophlebia*, it seems acceptable to consider the seminal groove a plesiomorphic state.

On the basis of the number and morphology of the Malpighian tubes, Landa (1970) has distinguished different groups within leptophlebiids. A furcation within the recent Leptophlebiidae into the lineage of Leptophlebiinae and Atalophlebiinae has been proposed by Peters (1980). *Choroerpes* belongs to Atalophlebiinae, a subfamily that includes genera sharing a higher number of derived characters than the Leptophlebiinae, which include *Habrophlebia* and *Habroleptoides*. The change of external seminal grooves into internal lumina is in agreement with a more specialized condition and supports the attribution of *Choroerpes* to a distinct lineage. By contrast, females of *Ch. picteti* show lateral oviducts opening separately with two gonopores, a primitive condition occurring only in a few mayfly families (Brinck, 1957). This organization is peculiar to leptophlebiids, and is due to the acquisition of a unique gonopore by the representatives of this family (Grandi, 1955; Brinck, 1957).

The occurrence of a well-developed ovipositor is considered one of the characteristics that distinguish *Habrophlebia* from *Habroleptoides* (Peters, 1979). The projection of the genital forceps is pointed in *Habroleptoides* and blunt in *Habrophlebia* (Peters, 1979; Jacob and Sartori, 1984; Sartori, 1986). A common

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Figs. 14-19. Optical view (14), transmission (15) and scanning (16-19) electron micrographs, *Choroerpes picteti*: 14, live sperms under phase-contrast microscope (bar= 3 $\mu$ ); 15, sperm organization consisting of nucleus (N) and acrosome (Ac) (bar= 0.6 $\mu$ ); 16, dorsal view of the terminal part of male abdomen. Note external genitalia consisting of a pair of penes (P) and forceps (F) (bar= 100 $\mu$ ); 17, terminal pad of the forceps with scales (S) and papilla-like structures (Pa) (bar= 25 $\mu$ ); 18, Enlargement of the penes. Penes consist of a basal region (Br) that tapers towards the apical part (bar= 10 $\mu$ ); 19, seventh (VII) and eighth (VIII) sternum of the female. No subgenital devices leading to gonopores are present (bar= 50 $\mu$ ).

duct is occasionally present in mayflies (Landa et al., 1980). In *H. eldae* it is included in a tubular extension protruding on the eighth sternum and forming an ovipositor or egg-guide. The presence of an ovipositor has been described in a number of lineages in leptophlebiids (Peters, 1969, 1971; Peters and Edmunds, 1970, 1984), although gross morphology works based on old observations show variability of this structure in many cases. Morrison (1919) outlined a trend of complexity from a simple subgenital plate (egg-valve) to a tubular formation. This structure is seen in *Hagenulus* and determines elongation of the seventh abdominal sternum (*Paraleptophlebia praepidita* Eaton) or specialization of both seventh and eighth sterna (*Habrophlebiodes americana* Banks). A trend of increasing complexity can be also envisaged in several other Atalophlebiinae of the West Indies (Peters, 1971).

In Ephemeroptera the terms ovipositor/egg guide seem to comprise different structures. The ovipositor is a new structure since in Paleoptera the primitive ovipositor was a carrying device, its different parts being moved by muscles (Kukalová-Peck, 1991). It is likely that in mayflies the egg guide a new acquisition even in view of the fact that females oviposit in water (review in Brittain, 1982). In *H. eldae* the ovipositor also constitutes the only passageway for sperms at copulation. The presence of a well developed ovipositor may affect both the morphology of male genitalia and the mating strategy. Indeed, Peters (1971) stressed the correlation between long ovipositors together with elongated male genital forceps and styliiger plates. In *Tortopus* a pair of discrete grooved excavations was found in the eighth abdominal sternite of females receiving male parastyli during copulation (McCafferty and Bloodgood, 1989). Specialization in the coupling system increases success of copulation. In this context, the presence of scales and papilla-like structures on the forceps of the leptophlebiid species described in this paper might perform both a mechanical function, by improving the cohesion with the female cuticle, and a sensory activity.

Although several mayflies possess an egg guide, the only ultrastructural data on this formation concern *H. eldae*. In this species the presence of a thick muscle layer around both terminal parts of the internal paired oviducts and common duct has been described (Gaino and Mazzini, 1990). At present, it is impossible to assess whether this organization constitutes a characteristic of species with a tubular ovipositor.

Leptophlebiids are characterized by aflagellate sperms (Soldán, 1979; Gaino and Mazzini, 1991 a, b). Grimm (1977, 1985) assumed that the occurrence of a muscular ring around vasa deferentia constitutes a sperm pump enabling the transfer of immotile sperms into female apparatus. This is probable but the presence of two types of sperm pump in *Hexagenia*, a species which shows flagellate sperm, remains unexplained (Phillips, 1969).

In *H. eldae* peristaltic movement of the wide muscle sheath enveloping the ovipositor may facilitate sperm/egg interaction (Gaino and Mazzini, 1990). A similar mechanism has been previously proposed in Psychodidae, a dipteran family including species with motionless sperms (Burrini and Dallai, 1975). We



can speculate that even when an ovipositor is lacking, as in *H. confusa* and *Ch. picteti*, sperm progression may be achieved by the peristaltic activity of the muscle that surrounds the oviducts. These data suggest that the architecture of the female genitalia may have a pivotal role in egg/aflagellate sperm encounter.

In conclusion, in the reproductive apparatus of leptophlebiids the penis grooves and the pair of female gonopores are ancestral characteristics, whereas the ovipositor or egg guide is a new acquisition. Aflagellate sperm constitute a specialized structure since lack of a flagellum is viewed as a final stage of evolution (Baccetti, 1972).

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