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The oviposition mechanism in *Habrophlebia eldae* (Ephemeroptera: Leptophlebiidae)

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Females of *Habrophlebia eldae* release eggs into the water by penetrating the surface with the ovipositor. Ultrastructural investigations (SEM, TEM) confirm that at rest the ovipositor of *H. eldae* is composed of a proximal sclerous region and a distal membranous region. During oviposition, the membranous region unfolds a telescopic tube made up of a thin cuticle. At rest, this telescopic structure is kept folded inside the ovipositor. The extension of the structure seems to be passive, caused by the passage of the eggs, pushed out by the peristaltic movements of the net of muscle fibres surrounding the oviducts. The contact of the membranous distal region of the ovipositor with water triggers egg laying through extension of the telescopic tube. Numerous mechanoreceptors, in the form of short bristles, are located ventrally in the membranous region of the ovipositor and seem to be involved in water perception.

Keywords: ultrastructure; mechanoreceptors; ovipositor; aquatic insects; mayfly

Introduction

Adult female insects can predetermine the larval habitat by choosing a specific location for oviposition. In particular, insects with aquatic larvae need to detect suitable sites in water. Egg laying on specific substrates requires adaptations, such as ovipositors with receptors for exploring these substrates (Spänhoff et al. 2003).

The majority of mayflies, including most Leptophlebiidae, oviposit by descending to the water and releasing a few eggs at a time by dipping their abdomen into the water (Brittain 1982).

While the female external genitalia of mayflies have been frequently described (Grandi 1955; Brinck 1957), the involvement of their components in the mechanism of oviposition has been poorly investigated.

Mayflies have no vestiges of the primary ovipositor but in some Leptophlebiidae the pregenital plate forms a tubular process at the opening of the sexual aperture, interpreted as an unpaired secondary ovipositor (Kluge 2004). In particular, in the genus *Habrophlebia* the presence of an evident ovipositor is a distinctive character. The tubular ovipositor of *Habrophlebia eldae* Jacob & Sartori, 1984 (*Habrophlebia fusca* sensu Grandi 1960) has been described by Grandi (1955) and then by Gaino

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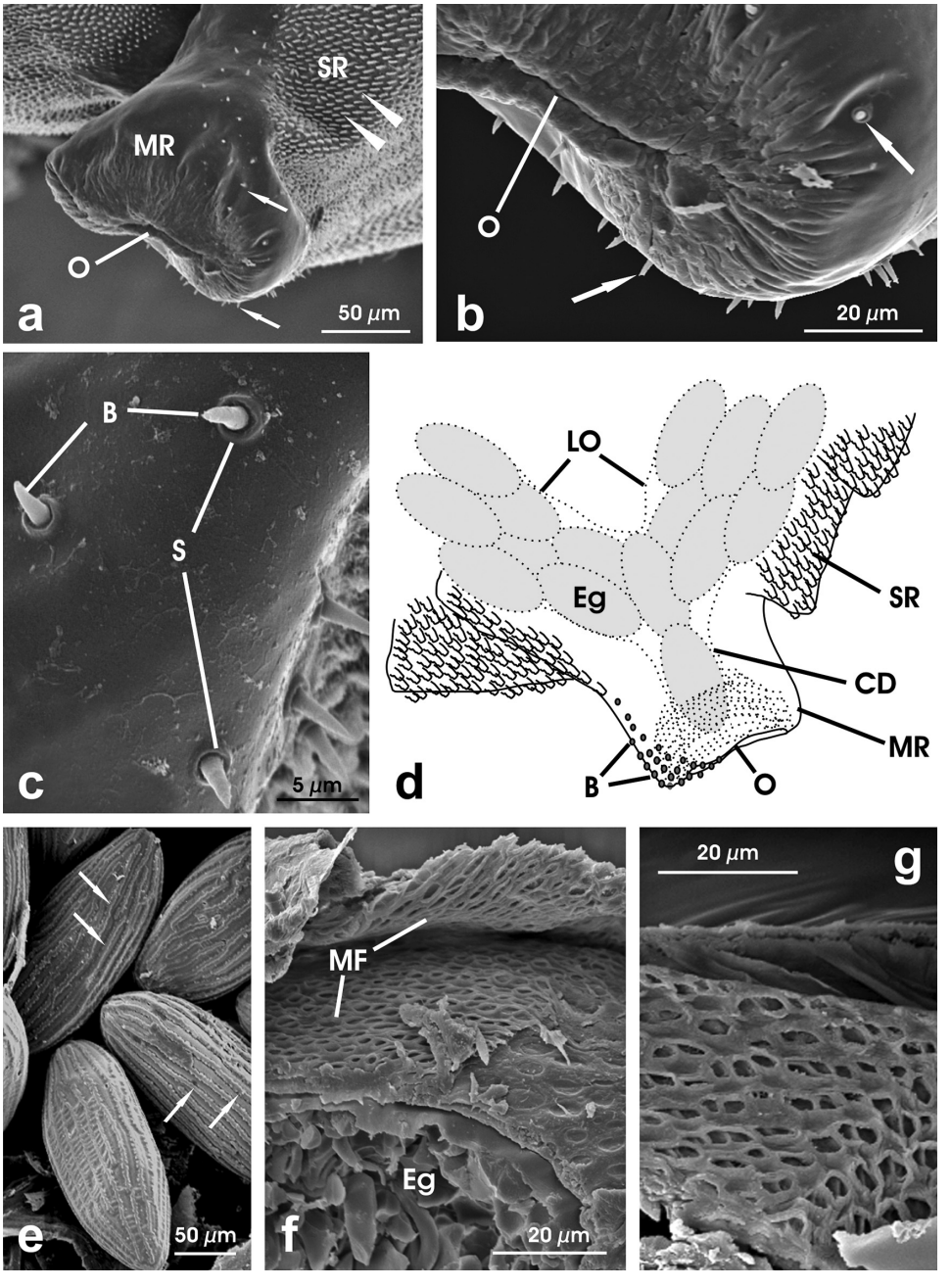
and Rebora (1995), in a comparative analysis of the mating apparatus in some Leptophlebidae. These papers described the ovipositor of *H. eldae* in its rest position, as a tubular structure containing a common oviduct in which the paired oviducts join. No data have been reported about the morphology and functioning of the ovipositor during egg laying.



In the light of these papers, the aim of the present study is to describe the fine morphology (SEM, TEM) of the ovipositor of *H. eldae* during egg laying. Particular attention has been paid to the mechanism of egg release and to the occurrence of sensory structures involved in water perception.

Materials and methods

Adult females of *H. eldae* were obtained in the laboratory from mature larvae collected in Oscano stream (Perugia, Umbria Region, Central Italy) in Spring 2007. The specimens where kept in water provided with supplementary oxygen, stones, and detritus from the collecting site, at 25 $\pm$ 2°C, LD12:12 h light conditions.

Functional observations

In order to observe the mechanism of oviposition, 10 adult females were observed under a stereomicroscope. Egg laying was triggered by repeatedly dipping their abdominal apex into Petri dishes filled with water.

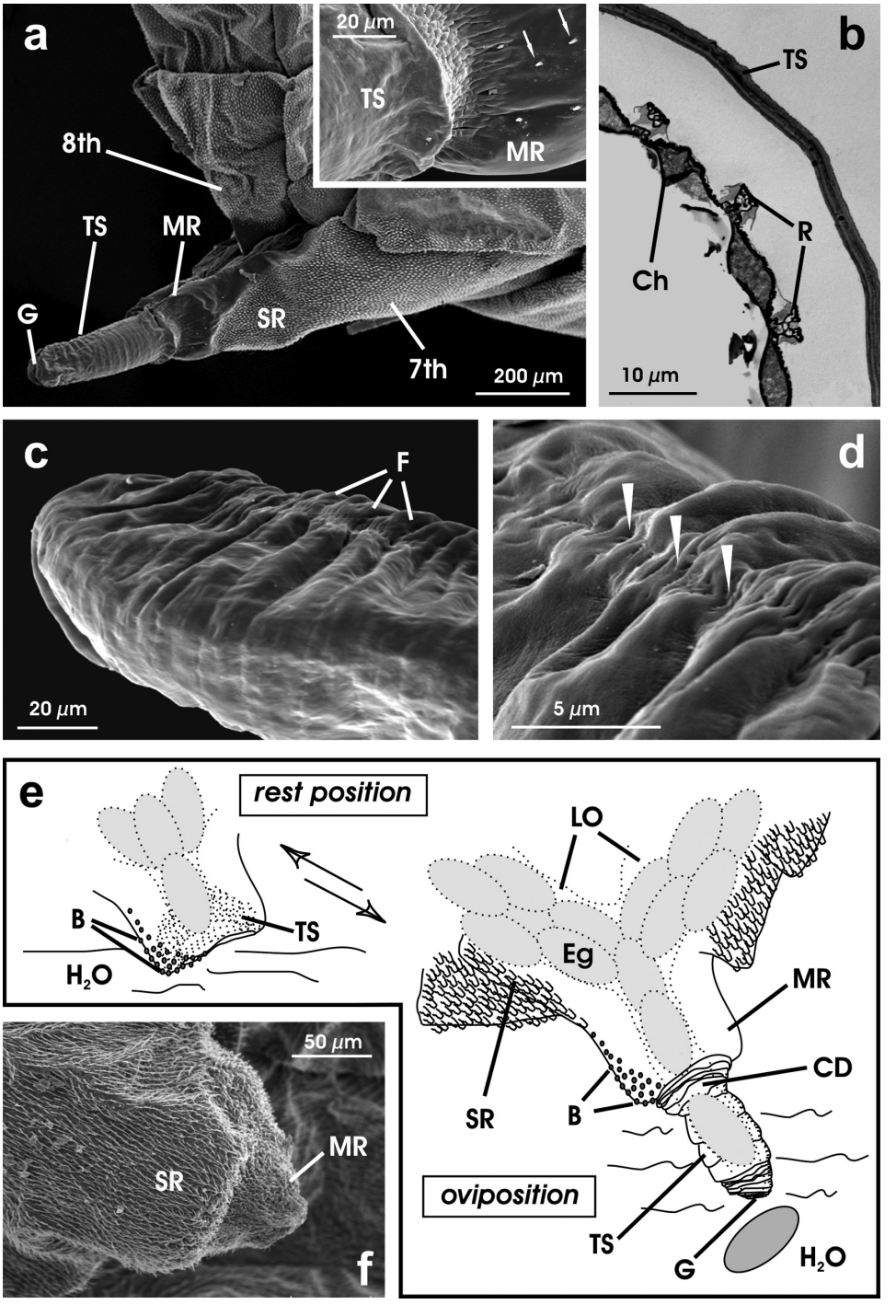
Ultrastructural investigations (SEM, TEM)

The female genitalia were dissected from anaesthetised imagines and subimagines and fixed for 12 hours in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2. Genitalia of alive females were fixed during oviposition by repeatedly dipping the abdominal apex in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2.

For scanning electron microscopy (SEM) analysis, the fixed material was repeatedly rinsed in the same buffer, then dehydrated by using ethanol gradients, followed by critical-point drying in a critical-point dryer CPD 030 Bal-Tec (Bal-Tec Union Ltd., Balzers, Liechtenstein). Specimens were mounted on stubs with silver conducting paint, sputter-coated with gold-palladium in a sputterer Emitech K550X (Emitech, Ashford, UK), and observed with a Philips XL30 (Philips, Eindhoven, Netherlands), at an accelerating voltage of 18 kV. For transmission electron microscopy (TEM), the fixed genitalia were repeatedly rinsed in cacodylate buffer and post-fixed for 1 hour at 4°C in 1% osmium tetroxide in cacodylate buffer. Afterwards the material was repeatedly washed in the same buffer, dehydrated by using ethanol gradients, and finally embedded in an Epon-Araldite mixture resin. Ultrathin sections, cut on a Leica EM UC6 ultracut (Leica Microsystem GmbH,

Figure 1. (a–c) Ovipositor of *H. eldae* in its resting position; (e–g) internal view of the oviducts under SEM; drawing (d) shows the internal organisation of the female genitalia. (a) Ovipositor composed of a sclerous basal region (SR), rich with cuticular projections (arrowheads), and a membranous distal region (MR) with a folded border delimiting a central opening (O). Note the short bristles (arrows) on the membranous region. (b) Detail of the ventral region of the folded border. Note the short bristles (arrows) close to the opening (O). (c) Enlarged view showing the short bristles (B) set in an evident socket (S). (d) Drawing of the genitalia showing two lateral oviducts (LO) which join in a common short duct (CD) inside the membranous region (MR) of the ovipositor; B, short bristles; Eg, egg; O, opening; SR, sclerous region. (e) Eggs inside a lateral oviduct. Note the ribs (arrows) on the chorionic surface. (f) Thick net of muscle fibres (MF) surrounding the common duct. Note the eggs (Eg) inside the duct. (g) Enlarged view showing the arrangement of the muscle fibres.

Vienna, Austria), were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 (Philips, Eindhoven, Netherlands).



Results

As previously reported (Gaino and Rebora 1995), at rest the ovipositor of *H. eldae* is a characteristic tubular structure protruding from the seventh sternum and overlying the middle of segment eight (Figure 1a). The ovipositor is constituted of a proximal sclerous region, rich of cuticular projections, and a membranous distal region, fairly smooth, which presents a folded border delimiting a central opening (Figure 1a). Numerous short bristles are located ventrally in the membranous distal region of the ovipositor (Figures 1a, b). These short bristles are about 3 μm long and are set in an evident socket (Figure 1c).

The internal genitalia of *H. eldae* have two lateral oviducts, which join in a common short duct inside the ovipositor (Figure 1d). Mature eggs gradually fill the oviducts (Figure 1e). The terminal portion of the paired oviducts and the common duct are surrounded by a thick net of muscle fibers (Figures 1f, g).

The observations of ovipositing females under stereomicroscope allowed the oviposition mechanism to be outlined and to give evidence of the remarkable modification of the ovipositor during egg laying (Figures 2a, e). In fact, the eggs of *H. eldae* are released in water through a telescopic structure, extending from the folded opening of the ovipositor membranous region (Figure 2a and inset). At the apex of the extruded telescopic structure the presence of coalescent folds makes the gonopore hardly visible (Figure 2a). As seen in histological sections, the telescopic structure is made up of a very thin cuticle, leaving a limited space between it and the egg chorionic surface (Figure 2b).

The telescopic structure shows a pleated smooth surface (Figure 2c), in which the folds are closely arranged along the dorsal and the ventral region (Figure 2d). Sensory structures are not present.

At rest, the telescopic structure is kept folded at the base of the ovipositor; the contact of the ovipositor with water triggers the extrusion of this structure and, consequently, the egg laying (Figure 2e). The eggs are released one by one and after each laying the telescopic structure returns to its resting position inside the ovipositor (Figure 2e).

In the subimago the ovipositor is rough, covered by microtrichia, and the different components are hardly distinguishable (Figure 2f).

Figure 2. External genitalia of *H. eldae* during egg laying, under SEM (a, c, d) and under TEM (b), together with reconstruction of the oviposition mechanism (e), and ovipositor of the subimago (f). (a) Ovipositor in its extended configuration. Note the telescopic structure (TS) extruding from the opening of the membranous region (MR). The gonopore (G) is hardly visible at the apex of the telescopic structure. SR, sclerous region; 8th, 8th abdominal segment; 7th, 7th abdominal segment. In the inset, the connection between the telescopic structure (TS) and the membranous region (MR) with short bristles (arrows). (b) Thin section of the telescopic structure (TS) during the egg passage trough. Note the ribs (R) on the egg chorionic surface (Ch). (c) A portion of the telescopic structure showing its smooth pleated surface with regularly spaced folds (F). (d) Enlarged view showing the folds closely arranged along the dorsal region (arrowheads). (e) Drawing of the oviposition mechanism showing the transition from rest position to egg laying position. Egg laying is triggered by the contact of the short bristles (B) with water (H_2O). CD, common duct; Eg, egg; G, gonopore; LO, lateral oviducts; MR, membranous region; SR, sclerous region; TS, telescopic structure. (f) In the subimago, the sclerous region (SR) and the membranous region (MR) are hardly distinguishable.

Discussion

The ovipositor of *H. eldae* consists of two parts: an external portion, previously described by Gaino and Rebora (1995), and an internal telescopic portion, which becomes evident as a long extension only during egg deposition.

The telescopic portion seems to recall the membranous egg guide mentioned for the leptophlebiid *Simulacala massula* (Peters et al. 1990).

Thin sections, observed under TEM, revealed that the telescopic structure of the ovipositor of *H. eldae* is a thin cuticular layer lacking muscle fibres. Therefore, the distention of this structure seems to be passive, caused by the passage of the eggs. In particular, we suppose that mature eggs are pushed through the telescopic structure by peristaltic movements of the net of muscle fibres, which surround the distal portion of the lateral oviducts and the common duct.

In this regard, it is remarkable that Gaino and Mazzini (1990) described a thick outer layer of muscle cells in the posterior region of the oviducts, as well as in the common duct, of larvae of *H. eldae*. These authors hypothesised that, in the adults, peristaltic movements of the muscles may facilitate sperm–egg interaction after the copula, because leptophlebiids typically have immotile sperm (Soldàn 1979; Gaino and Mazzini 1991), which are pushed into the female genitalia by a sperm pump (Grimm 1985).

The present study suggests that the thick net of muscle fibres around the terminal oviducts of *H. eldae* can play a central role in oviposition, other than facilitating fertilisation. In *H. eldae* the eggs are released one by one and after each laying the telescopic structure returns to its rest position, folded inside the membranous region of the ovipositor. Even this mechanism of retraction seems to be passive, due to the pleated arrangement of the cuticle that tends to return in its rest position.

Our functional observations demonstrated that in *H. eldae* oviposition is triggered by the contact of the ovipositor with water. This mechanism seems to be linked to the mechanical stimulation of the short bristles, located in the ventral area of the ovipositor, when in contact with the liquid surface. In fact, the external shape of these short bristles suggests their role as mechanoreceptors (Keil 1997). It is worth stressing that some fixation procedures, by repeatedly dipping the abdominal apex of alive females in the fixative medium, gave inception to egg laying.

A similar mechanism, where the stimulation of the ovipositor mechanoreceptors induces muscle contraction in the oviduct and consequent egg release, has been hypothesised for a South American decapitating fly, which parasitises workers of *Solenopsis* sp. (Zacaro and Porter 2003).

Ovipositor sensilla involved in the activation and regulation of the egg laying process have been described in several insects: parasitoid species frequently possess chemoreceptors to perceive stimuli in the host haemolymph (Brown and Anderson 1998); some pest insects use touch and taste sensilla to select the host plant (Baker and Ramaswamy 1990; Hummel et al. 2006); in endophytic species of Odonata, sensilla located in the female external genitalia are responsible for the control of precise egg positioning in plant stems (Matushkina and Gorb 2002); in Trichoptera, mechanoreceptors on the ovipositor have been described in a species that oviposits into cracks on submerged wood (Spänhoff et al. 2003).

The present research is the first description of sensilla on the ovipositor of Ephemeroptera and could represent a premise for further physiological and behavioural studies.

Mayflies express different oviposition behaviour, releasing all their eggs splashing on the water surface, dropping the eggs from the air, dipping the abdomen multiple times releasing a few eggs at time, landing on rocks and ovipositing on the undersides or floating downstream while releasing their eggs (Encalada and Peckarsky 2007). In this regard, studies combining functional and morphological approaches could contribute to widen the knowledge on the different oviposition mechanisms in these insects.

Acknowledgements

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