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The antennal sensilla of adult mayflies: *Rhithrogena semicolorata* as a case study

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ABSTRACT

The present paper reports on an ultrastructural investigation of the sensilla on the antennal flagellum of the *imago* (SEM and TEM) and *subimago* (SEM) of the mayfly *Rhithrogena semicolorata*. Sensilla coeloconica of two different sizes, named big and small pegs, are distinguishable in both stages. The big pegs are lobe-shaped with a porous cuticle. The small pegs are more or less conical with a smooth cuticle. TEM seriate sections revealed that the big pegs are innervated by three neurons, which enter the peg. The dendrite sheath opens at the entrance to the peg and leaves the dendrites in contact with the outside through pores and pore tubules. This structure is in agreement with that reported for single-walled olfactory receptors. The small pegs may be innervated by two or four neurons. In both cases, two unbranched dendrites enter the peg and are in close association with the peg wall. These show features typical of hygroreceptors. In the small pegs innervated by four neurons, the other two neurons stop short of entering the peg, and one of them branches. This last could constitute the thermoreceptive unit. The function of the fourth neuron is unknown. The presence of olfactory sensilla in adult mayflies, such as Paleoptera, is relevant to the study of evolutionary trends in insect odour perception. Hygro-thermoreceptors are important in such fragile aquatic insects, which need a moist environment for moulting and reproduction.

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1. Introduction

Ephemeroptera are an important component of freshwater ecosystems, in which they constitute a fundamental link in the food chain. While several biological aspects of Ephemeroptera have been thoroughly investigated, little is known about their sensory biology, especially in the two winged stages.

The first ultrastructural study on the fine morphology of the antennal sensilla of *Ephemera* sp. larvae was carried out by Schmidt (1974), who described the pedicel scolopidia which, as it occurs in Johnston's organ of the most advanced insects, detects the movement of the flagellum on the pedicel. In the larvae of *Baetis rhodani*, a very common species belonging to the family Baetidae, a peculiar gustatory sensillum, called "flat-tipped sensillum", was described by Gaino and Rebora (1996, 1997, 1998, 1999).

The antennal sensilla of adult Ephemeroptera have been investigated in some representatives, namely *Stenacron, Cloeon* (Gaino and Rebora, 1997; Gupta, 1998; Gupta and Gupta, 1996; Slifer, 1977), *Caenis* and *Baetis* (Gaino and Rebora, 1997, 1998). However, these observations were limited to the external morphology. In the present study, we carried out an ultrastructural

investigation of the sensilla located on the antennal flagellum of the *imago* (SEM and TEM) and *subimago* (SEM) of *Rhithrogena semicolorata* (Ephemeroptera, Heptageniidae) in order to shed light on the possible function of these sensilla.

As Ephemeroptera are Paleoptera insects, studies on their sensory biology are of particular interest to phylogenetics.

2. Material and methods

Adults of *R. semicolorata* (Curtis, 1834) (Ephemeroptera, Heptageniidae), a common univoltine species in Italy, were obtained in the laboratory from mature larvae collected from the Nera River (Perugia, Central Italy) in May 2007. In the laboratory, the larvae were kept in plastic containers with water, detritus, flora and fauna from the collecting site at 25 ± 2 °C, LD 12:12 h.

Antennal flagella were dissected from anesthetised specimens and processed for SEM and TEM observations, as reported by Rebora et al. (2008).

3. Results

The adult mayfly antenna is made up of a scape, a pedicel and a monoarticulated flagellum. The antennal flagellum of the *imago* of *R. semicolorata* presents sensilla coeloconica located on the



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ventrolateral side. The sensilla are more numerous on the proximal portion of the flagellum, while they tend to disappear on its distal portion (Fig. 1a). On the basis of their size, two kinds of sensilla coeloconica are distinguishable: big pegs (about 2 μ m in width and 3 μ m in length) and small pegs (about 1 μ m in width and 1–2 μ m in length) (Fig. 1a–c). The big pegs are lobe-shaped with a cuticle characterized by pores that are visible on SEM (Fig. 1c). The small pegs are of various shapes, being more or less conical with a smooth cuticle displaying lateral ridges (Fig. 1c).

The *subimago* has similar sensilla, with the same distribution (Fig. 1d): lobe-shaped big pegs (about 2 μ m in width and 3 μ m in length) with a cuticle characterized by pores visible on SEM (Fig. 1e), and cone-shaped small pegs (about 1 μ m in width and 1–2 μ m in length), with a smooth cuticle and lateral ridges (Fig. 1f).

Big pegs were seen to be more numerous in male specimens than in females, while no difference in the number of small pegs was observed between the sexes. However, more specimens will have to be observed in order to clarify this aspect. No difference in



Fig. 1. SEM (a-f) and TEM (g and h) of the antennal flagellum of the *imago* (a-c, g and h) and *subimago* (d-f) of *Rhithrogena semicolorata*; (a) ventrolateral side of the flagellum; note the sensilla coeloconica (arrows). Scale bar = 20 μ m; (b) detail showing the two kinds of sensilla coeloconica: big and small pegs. Scale bar = 10 μ m; (c) close-up view; note the cuticular pores (arrows) on the big pegs. Scale bar = 2 μ m; (d) ventrolateral side of the flagellum showing the sensilla coeloconica (arrows). Scale bar = 25 μ m; (e) big peg with cuticular pores (arrows). Scale bar = 1 μ m; (f) small peg. Scale bar = 1 μ m; (g) cross-section of the flagellum at the level of the emergence of two big pegs (arrows). Scale bar = 5 μ m; (h) cross-section showing a small and a big peg. Scale bar = 100 nm. BP, big peg; C, cuticle; SP, small peg.

the number or distribution of the two kinds of sensilla was observed between the two winged stages.

In cross-section, the antennal flagellum of the *imago* of *R*. *semicolorata* shows sensilla coeloconica on its ventrolateral side (Fig. 1g). Big and small pegs are clearly distinguishable on TEM (Fig. 1h).

Seriate sections enabled the organization of the sensilla to be reconstructed. The big pegs are innervated by three neurons, whose outer dendritic segments enter the peg (Fig. 2a–h). These three neurons are enveloped by three accessory cells, the thechogen, trichogen and tormogen cells, which are connected to one another by septate junctions and desmosomes (Fig. 2c–h).



Fig. 2. TEM of the antennal flagellum of the *imago* of *R. semicolorata*: sections of big pegs; (a) longitudinal section of a peg showing three dendrites immersed in the sensillum liquor; the dendrite sheath opens at the entrance of the peg, leaving the dendrites in contact with the outside by means of pores on the peg cuticle. Scale bar = 500 nm; (b) detail of the peg showing pore tubules in the sensillum liquor between pores (arrows) and dendrites. Scale bar = 250 nm; (c) cross-section of the outer dendritic segments enveloped by a thick dendrite sheath. Scale bar = 500 nm; (d, e and f) seriate cross-sections of the outer dendritic segments at a more proximal level, showing the thecogen cell gradually secreting the dendrite sheath. The accessory cells are connected to one another by septate junctions (arrow heads) and desmosomes (arrows). Scale bar = 500 nm; (d) cross-section at the level of the ciliary constrictions separating the inner and outer dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments. Scale bar = 500 nm; (h) cross-section at the level of the inner dendritic segments; ILC, inner sensillum liquor; MI, mitrochondria; MI, microtubules; N, nucleus; OD, outer dendritic segments; P, pores; PT, pore tubules; SL, sensillum liquor; Th, thecogen cell; To, tormogen cell.

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Fig. 3. TEM of the antennal flagellum of the *imago* of *R. semicolorata*: sections of small pegs; (a) cross-section of a peg showing two dendrites (1 and 2) in close contact with the peg cuticle; note the irregular clefts of the cuticle (arrow heads); (b) cross-section at the insertion of the peg; note the cuticle, which penetrates deep into the sensillum and fuses with the dendrite sheath. At this level, a third dendrite (3) is visible; (c) longitudinal section of the peg showing the peg cuticle, which penetrates deep into the sensillum and fuses with the dendrite sheath; (d–f) seriate cross-sections at the level of the four outer dendritic segments (1, 2, 3 and 4) showing the secretion of the dendrite sheath by the thecogen cell; note the presence of small dendrite branches (arrow). The accessory cells are connected to one another by septate junctions (arrow heads); (g and h) seriate cross-sections at a more proximal level, showing the transition between the ciliary constrictions and the outer dendritic segments in the inner sensillum lymph cavity; note that one ciliary constriction (arrow) is separated from the others and at a different level; (i) more proximal cross-section showing the four inner dendritic segments; (j–m) seriate cross-sections of a small peg innervated by two neurons. The peg (not shown) has the same morphology shown in (a). (j) Insertion of the peg. At this level the cuticle fuses with the dendrite sheath; (k) more proximal section showing the dendrite sheath surrounding the two outer dendritic segments; (l and m) transition between the ciliary constrictions and the ucer dendritic segments; D, dendrite second by the thecogen cell, which has a scolopale-like structure. The accessory cells are connected to one another by septate junctions (arrow heads). Scale bars = 500 nm. C, cuticle; CC, ciliary constrictions; D, dendrite; DS, dendrite sheath; ID, inner dendritic segments; ILC, inner sensillum lymph cavity; Mt, mitochondria; Mt, microtubules; OD, outer dendritic segments; OLC, outer se

At the level of the outer dendritic segments, the thechogen cell secretes the dendrite sheath (Fig. 2c–f) that opens at the entrance to the peg, leaving the dendrites immersed in the sensillum liquor (Fig. 2a and b). In the peg, the neurons are in contact with the outside through pores in the cuticle (Fig. 2a and b). Pore tubules are located between the pores and dendrites in the sensillum liquor (Fig. 2b). No socket is visible at the insertion of the peg (Fig. 2a). In the inner sensillum lymph cavity, three ciliary constrictions separating the inner and the outer dendritic segments are clearly visible (Fig. 2g). At this level, the thecogen cell shows microtubules (Fig. 2g). The inner dendritic segments contain mitochondria, which usually have a swollen appearance (Fig. 2h).

The small pegs are innervated by four neurons (Fig. 3a-i). Two of these are unbranched and enter the peg (Fig. 3a and c); the other two stop short of the peg and one of them is branched (Fig. 3b and d-f). The outer dendritic segments are enveloped by a thick dendrite sheath secreted by the thecogen cell (Fig. 3d–f). The distal portion of the dendrite sheath is surrounded by an outer sensillum lymph cavity (Fig. 3d). At the base of the peg, the dendrite sheath fuses with the peg cuticle, which penetrates deep inside the sensillum (Fig. 3b and c). In the peg, the dendrites are in close contact with the thick cuticle, which shows clefts (Fig. 3a). There is no connection between the dendrites and the outside of the peg (Fig. 3a). No socket is visible at the insertion of the peg (Fig. 3c). In the inner sensillum lymph cavity, four ciliary constrictions separating the inner and outer dendritic segments are clearly visible (Fig. 3h). These ciliary constrictions are not all at the same level and one is separated from the others by the thecogen cell (Fig. 3h). In more distal sections the separate dendrite is joined to the others inside the inner sensillum lymph cavity (Fig. 3g). At the level of the ciliary constrictions, the thecogen cell contains a scolopale-like structure in the form of intracellular bundles of longitudinally oriented microtubules (Fig. 3g and h). The four inner dendritic segments are separated by the thecogen cell and contain mitochondria and numerous microtubules (Fig. 3i). In some cases, the small pegs are innervated by only two unbranched neurons (Fig. 3j-m). The outer dendritic segments of these neurons enter the peg, whose internal structure (not shown in the figure) is like that previously described in the small pegs innervated by four neurons (Fig. 3a). As in the above-described sensilla, the dendrite sheath enveloping the outer dendritic segments stops at the base of the peg, where it is fused with the peg cuticle, which penetrates deep inside the sensillum (Fig. 3j and k). In these small pegs, only two ciliary constrictions in the inner receptor lymph cavity are visible (Fig. 2m).

In some sections, a campaniform sensillum (not shown in the figures) was identified.

4. Discussion

The present ultrastructural investigation on the antennal flagellum of the two winged stages of the mayfly *R. semicolorata* identified two kinds of sensilla coeloconica in the form of big and small pegs. TEM micrographs of the *imago* stage revealed that the big pegs are unsocketed, with a porous cuticle showing pore tubules similar to those described by Steinbrecht (1997). These sensilla have a dendrite sheath, which stops at the entrance to the peg, leaving the dendrites immersed in a medium electron-dense sensillum liquor. These features are consistent with those described in chemoreceptors and, in particular, in single-walled olfactory sensilla (Altner, 1977; Altner and Prillinger, 1980).

The possible presence of olfactory sensilla in mayfly adults is intriguing. Neuroanatomical studies on insect brain have prompted researchers to hypothesize that paleopteran insects, such as Ephemeroptera and Odonata, are probably all anosmic with respect to airborne odours; indeed, they lack glomerular antennal lobes, which typically receive olfactory receptor neurons in Neoptera, and mushroom body calyces, which are probably important for olfactory processing (Farris, 2005; Strausfeld et al., 1998). A recent neuroanatomical study on the ground plan of the insect mushroom body suggested that the simplicity of these structures in mayflies may not be entirely attributable to a primitive character state, but rather related to the highly derived lifestyle of Ephemeroptera, with aquatic larvae and short nonfeeding adults, in which olfaction is redundant (Strausfeld et al., 2009). At present, we have no data on the actual role of the abovedescribed olfactory sensilla in mayfly biology. On account of the short lifespan of the adult stage, in which the main task is reproduction, it seems plausible that these sensory structures might be involved in mating behaviour (we observed a difference in the number of putative olfactory sensilla between males and females, even though more specimens will have to be observed in order to clarify this aspect). On the other hand, considering that mayflies are believed to have secondarily invaded freshwater environments, the porous sensilla identified on the antennae of R. semicolorata could be "silent receptors" in which odorant receptor genes are not expressed, or are pseudogenes, as occurs in secondarily aquatic mammals (Freitag et al., 1998). In any case, it would be astonishing if such a complex organization as that of the coeloconic sensilla described in the present paper had been preserved during evolution without being associated to an actual olfactory function. Given that recent ultrastructural investigations have also identified olfactory sensilla in Odonata adults (Rebora et al., 2008), further molecular, electrophysiological, and behavioural studies will be necessary in order to shed light on this controversial field of research into the supposed ability of Paleoptera to perceive odours.

The small pegs are aporous sensilla with an inflexible socket. They may be innervated by two or four neurons. In both cases, only two unbranched dendrites enter the peg, and are in close association with the thick peg wall. This characteristic, together with the peg cuticle, which penetrates deep into the sensillum and proximally fuses with the dendrite sheath, and the thecogen cell containing a scolopale-like structure, suggests that these two neurons may perform a hygroreceptive function (reviews in Altner and Loftus, 1985; Steinbrecht, 1998; Tichy and Loftus, 1996; Yokohari, 1999).

In the small pegs innervated by four neurons, in addition to the previously described hygroreceptive units, two neurons stop short, without entering the peg. One of these latter has a branched outer dendritic segment. This branched neuron could constitute the thermoreceptive unit associated to the two hygroreceptive ones, as in the typical physiological triad of thermo-hygrosensitive sensilla (Yokohari, 1999). Indeed, digitiform branches commonly represent the tendency toward surface enlargement of thermoreceptive dendrites in hemimetabolic insects (Steinbrecht, 1998), such as Locusta (Altner et al., 1981) and Periplaneta (Yokohari, 1981). In Periplaneta in particular, hygro-thermoreceptive sensilla very similar to those of R. semicolorata have been described. Probably, these are innervated by four receptor cells: two unbranched neurons entering the peg (hygroreceptors), one branched neuron that terminates below the base of the cuticular apparatus (thermoreceptor) and a fourth unbranched neuron, additional to the typical triad. This last terminates en route to the cuticular apparatus, but physiological data do not enable a specific function to be attributed it (Yokohari, 1999). In the hygro-thermoreceptors of R. semicolorata, one of the ciliary constrictions was seen to be located at a different level from the others, from which it was separated by the thecogen cell. However, it was not possible to determine whether this constriction belongs to the fourth unit. The function of this fourth unit in the hygro-thermoreceptors of R. semicolorata is uncertain, as in Periplaneta.

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This is the first ultrastructural evidence of hygro-thermoreceptors in Ephemeroptera. Although the need to perceive temperature and humidity is obvious in such fragile insects, which can dry out quickly during moulting and swarming (Kriska et al., 1998), almost nothing has so far been established with regard to hygro/thermo-sensitivity in Ephemeroptera. As far as temperature perception is concerned, a behavioural study on the main stimuli involved in Ephemeroptera swarming and egg-laying reported that these insects must have thermal sensitivity in order to perceive the optimal temperature for swarming (above 14– 15 °C); moreover, slight temperature changes affect the duration of the swarming period (Kriska et al., 1998).

The presence of similar sensilla coeloconica in the two winged stages of *R. semicolorata* suggests that olfactory and hygro-thermosensitive sensilla are the main receptors on the antennal flagellum in the *subimago*, too. The presence of hygro-thermo-receptors could be related to the need of this intermediate stage to remain close to a humid environment for the final moult. The role of olfactory sensilla in the *subimago*, if indeed they really do perceive odours, is difficult to hypothesize, given the extremely short lifespan of this intermediate stage, which represents an archaic condition (Ogden and Whiting, 2003) whose real function is still a matter of debate.

5. Conclusions

The present study is the first ultrastructural investigation on the internal organization of antennal sensilla in adult mayflies, and provides the first ultrastructural evidence of olfactory and hygro–thermoreceptors in these insects. These data contribute to our knowledge of the non-visual sensory ability of Paleoptera insects and raise new questions regarding the evolution of insect olfaction.

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