ORIGINAL PAPER

Olfaction in phytophagous ladybird beetles: antennal sensilla and sensitivity to volatiles from host plants in *Chnootriba elaterii*

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Abstract
Olfaction and chemical ecology in phytophagous ladybird beetles have been largely ignored so far. The present paper describes for the first time basiconic, grooved, and trichoid porous olfactory sensilla on the antennae of the phytophagous ladybird *Chnootriba elaterii* (Epilacnini) and demonstrates the sensitivity of the antennae to Volatile Organic Compounds (VOCs) from host plants, belonging to common and less common Cucurbitaceae crops and wild species (*Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita pepo*, *Cucurbita moschata*, *Lagenaria siceraria*, *Luffa cylindrica*, and *Ecballium elaterium*). Epilachnini ladybirds are phytophagous, with adult and larvae feeding on various Cucurbitaceae, Fabaceae, and Solanaceae. Notwithstanding some species are serious pests in crops, their chemical ecology has been largely disregarded so far. The present paper discusses the ultrastructure of antennal sensilla (scanning electron microscopy-SEM) and the electroantennographic recordings (EAG) from the olfactory sensilla responding to the proposed chemicals, also considering the literature on chemical ecology and olfaction of aphidophagous ladybugs. In particular, antennal sensilla are similar to those described in predatory species but in Epilachnini they are much more numerous on the apical flagellum, probably because involved in host plant location and selection. This research can be considered a first step in developing semiochemical-based and sustainable control strategies for melon ladybird in crops.

Keywords African melon ladybug beetle · VOCs · Cucurbitaceae · Chemical ecology · Ultrastructure · Electroantennography

Introduction

Insect-plant interaction includes a wide range of relationships, such as pollination, and plant provision of food and shelter to insects (Beck and Reese 1976). Coevolution among insects and plants strongly influenced plant production of chemicals, not only to attract pollinators but also to

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defend against herbivores (rev by Chen et al. 2008; Mauch-Mani et al. 2017). Simultaneously, phytophagous insects learned to use volatile compounds (VOCs) produced by specific plants as secondary metabolites, to locate and recognize them as suitable food (Finch and Collier 2000). Many ecologists believe that the great diversity of current plants and insects is in large part due to their coevolution (Bernays and Chapman 2007). Chemical ecology plays a key role in the interaction between insects and plants. In particular, the complete system of host plant selection involves three linked steps, the first step is governed by volatile plant chemicals, a central step is related to visual stimuli, and a final step which involves non-volatile plant chemicals (Finch and Collier 2000). Here, plant odors and their perception by phytophagous insects can be considered a crucial aspect of the management of agricultural pests, as the plant odor is the first sensory cue driving plant selection for feeding and oviposition (Finch and Collier 2000).

The species in the Coccinellidae family exhibits a broad trophic diversity that ranges from specialized predation to

strict herbivory (Tomaszewska and Szawaryn 2016). The coccinellid tribe Epilachnini encompasses 27 genera (Szawaryn et al. 2015), including important pests of Solanaceae and Cucurbitaceae crops, the maize ladybug *Chnootriba similis* (Thunberg), and the Mexican bean beetle *Epilachna varivestis* (Mulsant) (Tomaszewska and Szawaryn 2016). In particular, the melon ladybird beetle *Chnootriba elaterii* (Rossi) is an oligophagous multivoltine species, widespread in Eurasia and representing a serious pest of cucurbit crops in Southern Europe, as well as in Near East, Middle East and North Africa (Akandeh and Shishehbor 2011; Liotta 1964). The larva and adult of the melon ladybird beetle feed on leaves of pumpkin, sweet gourd, bitter gourd, cucumber, etc.; sometimes flowers or even fruits are also destroyed and seedlings of late sowings can be entirely consumed (Al-Digail et al. 2012). Damaged leaves die after a short time (Talhouk 1969) and damaged fruits keep badly, decaying in 30–40 days (Al-Digail et al. 2012). In Saudi Arabia, *C. elaterii* is one of the most injurious pests (Al-Digail et al. 2012).

Numerous studies have been performed on the chemical ecology of Coccinellid beetles, for example, the harlequin ladybug *Harmonia axyridis* is well known for its aggregation behaviour driven by long-chain cuticular hydrocarbons (Durieux et al. 2012), and the long-distance attraction of males toward females is the result of highly volatile sex pheromones (Fassotte et al. 2014). Considering the economic importance of Epilachnini pests, our knowledge of these pests appears to be quite limited (Tomaszewska and Szawaryn 2016). In a study on the chemical factors involved in the selective feeding of *E. varivestis* on *Phaseolus vulgaris*, researchers identified sucrose as the main phagostimulant fraction in the seeds (Augustine et al. 1964). In different studies (Abe and Matsuda 2000; Augustine et al. 1964; Endo et al. 2004; Hori et al. 2010), pests belonging to the genus *Epilachna* were strongly stimulated to feed by plant chemicals, such as methyl linoleate and methyl linolenate acting synergistically with fructose, glucose, and sucrose (Hori et al. 2010). Moreover, cucurbitacins, produced by cucurbitaceous plants as deterrents for herbivorous insects, are commonly used by specialized feeding insects as an attractant (Abe and Matsuda 2000). Otherwise, in Epilachnini, the role of plant volatile organic compounds (VOCs) in insect-plant interaction has been poorly investigated. Bouquets of volatile organic compounds, and in particular long-chain fatty acids, seem to attract *E. varivestis* (Ballhorn et al. 2013) and *E. dodecastigma* (Sarkar et al. 2013) in olfactometer choice trials, but no data are available on the antennal sensilla, potentially involved in the long-distance plant recognition, or on the peripheral sensory responses.

In this regard, the present study investigates, from a morphological and electrophysiological perspective, the potential role of volatile cues in host plant selection by *C. elaterii*. In particular, the paper describes the antennal olfactory sensilla in adult males and females and tests their ability to respond to VOCs blend emitted by Cucurbitaceae belonging to common and less common crops (watermelon, melon, cucumber, zucchini, pumpkin, and loofah and calabash) and the wild main host plant (squirting cucumber). The electrophysiological screening on the antennae also encompasses standard volatiles reported in the literature as emitted by Cucurbitaceae. Investigations on the chemical ecology of *C. elaterii*, with particular attention to host plant selection, can offer numerous basal and applied research perspectives, also in developing integrated pest management programs.

Materials and methods

Insects

Adults of *C. elaterii* were collected in the field in Perugia surrounds in September 2020. Insects were kept in a culture room under controlled conditions (25 ± 2 °C, $45 \pm 15\%$ RH, photoperiod 14L:10D) inside net cages $30 \times 30 \times 30$ cm. Insects in different developmental stages (eggs, larvae, and adults) were maintained in separated cages, and they were nourished with species of cucurbits not used in the experiments. The eggs, mainly laid on the abaxial surface of leaves, and the plants with the eggs were gently moved in a new cage. As the larvae emerged, they were carefully moved in a larval cage with plants, until adult emergence.

Plant materials

Eight species of Cucurbitaceae belonging to six genera as reported in Table 1 were grown for VOCs collection. These eight species were selected because they are particularly common in Italy, and are representatives of common cucurbit crops (watermelon, melon, cucumber, zucchini, pumpkin), less common cucurbit crops (calabash, loofah), and not edible wild species (squirting cucumber) (Chomicki et al.

Table 1 Plant materials

Species	Common name
Citrullus lanatus (Thunb) Matsum. & Nakai	Watermelon
Cucumis melo L.	Melon
Cucumis sativus L.	Cucumber
Cucurbita pepo L.	Zucchini
Cucurbita moschata Duchesne	Pumpkin
Lagenaria siceraria (Molina) Standl	Calabash
Luffa cylindrica (L.) M. Roem	Loofah
Ecballium elaterium (L.)	Squirting cucumber

Latin and common names of the eight Cucurbitaceae plants tested in the experiments

2020). In addition, the selected species represent both annual (watermelon, melon, cucumber, zucchini, pumpkin, loofah) and perennial (squirting cucumber and calabash) Cucurbitaceae with different phylogenetic relationships within the family (Chomicki et al. 2020).

Commercial seeds (Rosi Sementi, Italy) were used to obtain plants, except for squirting cucumber whose seeds were collected in October 2020 from spontaneous plants in Perugia surrounds. The seeds were sown into individual plastic pots ($8 \times 8 \times 9$ cm for watermelon, melon, cucumber, calabash, loofah, and squirting cucumber, and $9 \times 9 \times 13$ cm for zucchini and pumpkin, which have a larger size and more rapid growth), in sterile topsoil (Patzer Einheitserde, Manna Italia, Bolzano, Italy), except squirting cucumber that was grown in a draining mixture soil (1/3 of topsoil, 1/3 of sand and 1/3 of Agriperlite) because this species suffers from water stagnation.

Plants were grown for approximately 30 days in a controlled climate chamber (25 ± 2 °C, $45 \pm 15\%$ RH, photoperiod 14L:10D) equipped with lights with a photosynthetic photon fluence rate of 200 µmol m^{-2} s^{-1} placed above the foliage. Water was supplied by sub-irrigation.

VOCs collection

VOCs collection was carried out from all the eight Cucurbitaceae species (watermelon, melon, cucumber, zucchini, pumpkin, calabash, loofah, and squirting cucumber). Plants about 30 days old were used for VOCs collection. Plants with 4/5 leaves, with the pot wrapped by food aluminium foil, were placed in a cylindrical glass chamber (5 l volume). For squirting cucumber, we used 4 plants together for each extract, to obtain a leaf surface similar to the other species, which typically have faster growth. An air stream purified by passing through a charcoal filter was pumped through the chamber at 500 ml/min. A glass cartridge (10×0.5 cm) containing a plug of 100 mg of Porapak Q (80–100 mesh; Sigma-Aldrich) was used to collect the VOCs. After collecting for 24 h, at 25 °C, the traps were eluted with 800 µl of hexane and concentrated to ~200 µl under a gentle nitrogen stream, the extracts from 4 traps (4 plants) for each species were mixed. Extracts were stored in a freezer at −18 °C, in glass vials with Teflon cap liners, until use. All replicates were carried out under controlled conditions (25 ± 2 °C, $50 \pm 10\%$ RH, and photoperiod 14L:10D). After each collection, the chambers were washed with water and fragrance-free detergent, rinsed with hexane and acetone, and baked overnight at 150 °C.

Scanning electron microscopy

To investigate the antennal olfactory sensilla in *C. elaterii*, male and female antennae were dissected from anesthetized adults and fixed for 12 h in 2.5% glutaraldehyde in cacodylate buffer (Electron Microscopy Sciences), pH 7.2, to be observed under scanning electron microscopy (SEM).

Twenty fixed antennae for each sex were repeatedly rinsed in the same buffer and then dehydrated by using ascending ethanol gradients (20%, 50%, 70%, 80%, 95%, 100%), followed by drying in an oven at 40 °C for 3 days. The morphology of the samples was analyzed by field emission scanning electron microscopy FE SEM LEO 1525 (ZEISS). The sample was deposited on aluminum support using adhesive tape. Before the analysis, the samples were metalized with a thin layer of chromium (8 nm) using a Q150 T ES (Quorum) sputter coater for 25 s. Measurements were carried out using an In-lens detector at 5 kV. The number and distribution of the olfactory sensilla on the last 3 flagellar segments of 6 males and 4 females were evaluated using the software Adobe Photoshop 2020. Sensilla are named as in *H axyridis* (Sevarika et al. 2020), to enhance clarity and comparability between phytophagous and aphidophagous ladybirds.

Electroantennography (EAG)

Electroantennography recordings were performed to test the responses of male and female antennae to the VOCs blend collected from the eight Cucurbitaceae species selected (watermelon, melon, cucumber, zucchini, pumpkin, calabash, loofah, and squirting cucumber), as above reported in Table 1. The sensitivity of the antennae to standard volatiles reported as emitted by Cucurbitaceae plants was also investigated.

Chemicals

Because no data are available in the literature about olfactory responses from *C. elaterii* antennae, to increase the probability of testing standard volatiles able to elicit responses, and potentially involved in host plant selection, the literature on VOCs emitted by Cucurbitaceae was revised (Andersen 1987; Chatterjee et al. 2018; Fernando and Grn 2001; Ferrari et al. 2006; Karmakar et al. 2016, 2018, 2020; Karmakar and Barik 2016; Metcalf and Lampman 1989; Mitchell et al. 2015; Mukherjee and Barik 2016; Mukherjee et al. 2014; Njuguna et al. 2018; Sarkar and Barik 2015; Sarkar et al. 2013, 2016; Shapiro et al. 2012). Thirteen widespread compounds, reported in more than one paper as emitted by leaves of *Momordica*, *Cucumis*, and *Cucurbita* species were selected among 234 compounds reported as emitted by leaves and/or flowers from *Momordica*, *Cucumis*, *Cucurbita*, *Luffa*, *Solena*, and

Linnaeosicoys species. In detail, the selected compounds were: E2-exenal, heptanal, nonanal, decanal, methyl salicylate, toluene, (−)—linalool, (−)—alfa pinene, Z3-hexen-1-ol, pentanol, hexanol, nonanol, and octanol. In addition, we tested Octanal as green leaf volatile commonly eliciting responses in phytophagous insects and pre-tested as an active compound in *C. elaterii*.

Odorants were from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest grade available (P 98%). To prevent rapid evaporation of test compounds, they were dissolved in paraffin oil to obtain 10% (v/v) solutions. Antennal responses were pre-tested by octanal solutions at different concentrations, constructing a dose–response curve and showed that a 10 µl dose produced a significant response and a 40 µl dose elicited the greatest response.

To obtain dose–response curves, paraffin oil solutions (0.01, 0.1, 1, 10, 20, and 50% v/v) of Z3-hexen-1-ol and hexanol, used as standard compounds, were prepared.

To prevent compound degradation, all the test solutions were kept in a freezer at − 18 °C, in glass vials with Teflon cap liners, until used.

Recordings

EAG recordings were carried out using excised antennae from males and females of *C. elaterii*. The antennal flagellum was carefully excised from the head of insects and anesthetized at a cold temperature (3 min at − 18 °C), using microsurgical scissors under a stereomicroscope. Scape and pedicel were removed to avoid any noise due to the numerous mechanoreceptors located on these segments. The antennal flagellum was mounted between two glass capillary electrodes with an inner diameter of 1.2 mm filled with Ringer solution (Ephrussi and Beadle 1936) containing 5 g/l of polyvinylpyrrolidone (Fluka), in contact with a silver wire. The capillary tubes were modelled up to have a fine tip using the microelectrode puller PC-10 (Narischige, Tokyo, Japan). The basal antennomere of the flagellum was inserted into the reference glass electrode. The recording electrode was connected by inserting a big sensillum chaeticum or trichodeum on the tip of the antenna into the recording electrode himself. Dissection and mounting of the flagellum never required more than 2 min. The insect was kept under a constant stream of humidified and filtered air at 200 ml/min which guaranteed the antenna functionality for almost 40 min. The analogue signal was detected through a probe with a high-input impedance preamplifier (10x) (EAG Kombiprobe, Syntech, Germany), and was captured and processed with a data acquisition controller (IDAC-4, Syntech, Germany), and analyzed using EAG 2000 software (Syntech, Germany).

Stimulations

40 µl of each VOCs extract were absorbed on a filter paper strip (15 mm × 15 mm, Whatman No. 1) and placed into a glass Pasteur pipette (150 mm in length, Volac®) to constitute an odor cartridge. Standard compounds were delivered as 10 µl samples placed on the filter paper. The control stimuli consisted of similar pipettes containing a strip of filter paper impregnated with the same aliquot of solvent (40 µl of hexane for VOCs extracts and 10 µl of paraffin oil for standard compounds). Fresh stimulus pipettes were prepared every day. The tip of the pipette was placed about 3 mm into a small hole in the wall of an L-shaped glass tube (130 mm long, 12 mm diameter) oriented towards the antennal preparation (~ 5 mm away from the preparation). The stimuli were provided as 1-s puffs of purified, charcoal-filtered air into a continuous humidified main airstream at 200 ml/min that was over the flowing antennal preparation at a velocity of 50 cm/s generated by an air stimulus controller (CS-55, Syntech, Germany). At least 1 min interval was allowed between successive stimulations for antenna recovery. Based on preliminary recordings, octanal was chosen as a reference standard stimulus and presented to the antenna every 6/7 stimulations in the recording series, to confirm and monitor the activity of the antennal preparation. Test compounds and VOCs extracts were presented in a random sequence. Fifteen female antennae and 15 male antennae were used to test the EAG responses to standard chemicals, and 18 females and 18 males were used to testing the EAG responses to VOCs extracts. Only one set of stimulations was performed for each antenna.

For dose–response experiments, 10 antennae from females and 8 antennae from males were tested with compounds that showed a relatively large response, Z3-hexen-1-ol and hexanol. The exposure proceeded from lowest to highest concentration for each chemical, with at least 2 min intervals between successive stimulations, to minimize the effect of olfactory adaptation by strong stimulation.

Statistical analyses

Two-way ANOVA, considering the side and the sex as factors were used to compare the number of sensilla in males and females and on the dorsal and ventral side of the antennae.

For evaluation of EAG responses, the maximum deflection of the recorded EAG signal after stimulation with a test compound was used. Antennal sensitivity to the different chemicals was recorded as a percentage of all recorded EAG responses concerning the reference standard stimulus (octanal 10% v/v), in detail, responses to volatiles have been normalized concerning the estimated responses to octanal during the experiment (estimated responses to octanal have been

obtained by interpolation between the 3 recorded responses). This procedure allows compensation for antennal sensitivity changes during the experiment and the comparison between experiments performed with antennae of different sensitivity. The responses to standard chemicals, to plant extracts, and to the different concentrations of Z3-hexen-1-ol and hexanol were analyzed using a two-way ANOVA, considering the sex and the cues or the different concentrations as factors. As a post hoc comparison, the Dunnett test, was used to compare the response to each test compound to those of the control (paraffin oil for standard VOCs and Hexane for extracts) (Statistica 6.0, Statsoft Inc. 2001). Before all the analysis, Box-Cox transformations were used to normalize the data (Sokal and Rohlf 1998).

Results

Sensilla on antennal flagellum

Antennae of male and female adults of *C. elaterii* are frontally located in the head and slightly divergent (Fig. 1a). They are approximately 1350 μm long, constituted of a large scape with an evident bulge on the latero-ventral side, a cylindric pedicel with a small bulge in the same position, and a flagellum articulated in 9 flagellomeres (Fig. 1a, b). No difference is reported between males and females in the flagellum length (950–1050 μm). Flagellomeres 1 to 6 are cylindric, the first one is twice as long as the others that appears very similar in size (about 80–90 μm long and 60–70 μm diameter) and shape. Differently, the last 3 flagellomeres are expanded (Fig. 1a, b). Flagellomeres 7 and 8 are very similar (about 100 μm long and 150 μm diameter) and show a swelled region rich in sensilla on the latero-ventral side (Figs. 2, 3), while the apical flagellomere is longer (about 140–150 μm) and ends with two flat surfaces, one on the dorsal and the other on the ventral side, which hosts the highest number and variety of sensilla in the antenna (Figs. 2, 3 and Table 2). The antennal cuticle is scaly and some small holes are widespread along the flagellum (Figs. 1c, 2, 3).

Sensilla trichodea (ST)

The flagellum is rich of sensilla trichodea (Figs. 1a, b, 2 3). These hairs (Fig. 1c) are long (between 40 and 90 μm) and thin (diameter at the base about 2,5 μm, decreasing from the base to the tip), with a pointed apex and longitudinal grooves alongside its entire length, no pore has been observed (Fig. 1c, f). These sensilla insert into the cuticle with an angle of less than 30° and sit in an articulated socket, which appears as a hole among the cuticular scales (Fig. 1c). Sensilla trichodea are particularly abundant in the last three

flagellomeres (Figs. 1c, 2, 3), but are widespread alongside the antenna, on both sides and both sexes without differences (Table 2).

Sensilla chaetica (SC)

Together with sensilla trichodea, the 3 apical flagellomeres also bring some very long (between 90 and 140 μm) and thick (diameter at the base about 5 μm, decreasing from the base to the tip) sensilla chaetica (Fig. 1c). These sensilla are much less abundant than trichodea (Table 2); they also have a socket, longitudinal grooves, and no pore on the cuticular surface, but they are distinguishable because of a blunt tip bearing an evident pore in the middle (Fig. 1e). In addition, the insertion angle of these sensilla is around 70° (Figs. 1b, 2, 3). Sensilla chaetica are confined to the last three segments, except flagellomeres 1 and 3, which occasionally bring one cheta on the latero-ventral side, and scape and pedicel, which bring some chetae (less than 10 in total) on their bulges (Fig. 1b). No difference is present in the distribution between the two sides of the antenna both in males and females (Table 2).

Sensilla conica (SCo)

Male and female antennae bear 2–3 sensilla conica (Fig. 1d). located only on the dorsal surface of the apical flagellomere (Table 2). They are smooth and small cones (about 5 μm long and with a basal diameter of 2.5 μm, decreasing from the base to the tip), inserted into the antennal surface through a bulged socket whose diameter is more than twice the diameter of the cone base. The cone tip is blunt and no pore is observed on the cuticular surface. No difference is reported in the number and distribution of these sensilla between the two sexes (Table 2).

Olfactory sensilla (SB, PST, SGP)

Three types of putative olfactory sensilla (Table 2) have been identified in *C. elterii*, sensilla basiconica, porous sensilla trichodea and grooved pegs, located in the apical region of the antenna (flagellomeres 7 to 9) and significantly more abundant in the dorsal (154.8 ± 6.7) than in the ventral side (106.0 ± 6.4) and in males (139.2 ± 9.8) than in females (115.1 ± 9.3) (Side: $F = 47.69$: df = 1, 15; $P < 0.001$. Sex: $F = 14.24$: df = 1, 15; $P = 0.002$. Side × sex: $F = 1.11$; df = 1, 15; $P = 0.308$). Putative olfactory sensilla are described in detail below.

Sensilla basiconica (SB) shows a porous cuticle, particularly rich in pores in the distal portion (Fig. 4a, b and insets). These sensilla are located on both sides of the antenna, mainly in the flat apical regions of the last flagellomere and in the swelled regions of the subapical ones
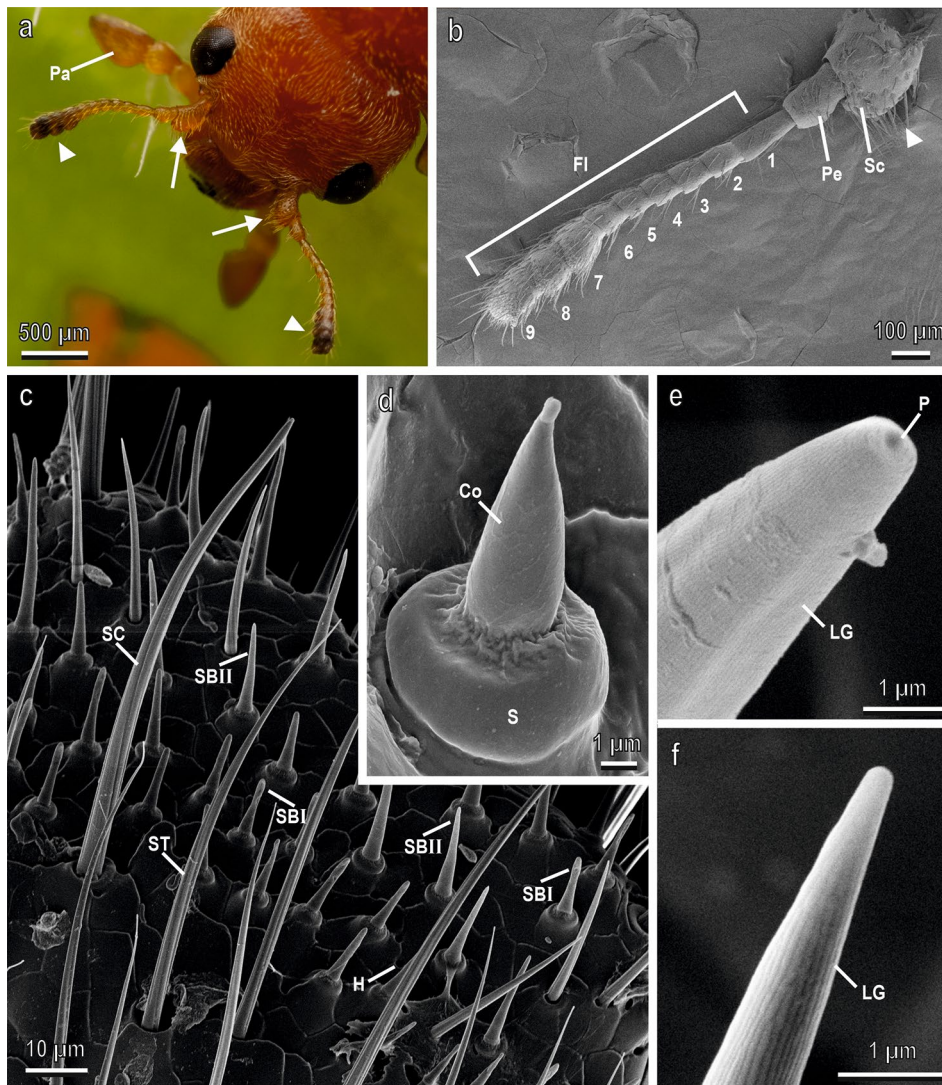
Fig. 1 Antennae of *Chnootriba elaterii* under an optical microscope (**a**) and SEM (**b–f**). In an adult head under an optical stereo microscope, note the evident bulge of the scapus in a medio-ventral position (arrows) and the enlarged profile of the three apical flagellar segments (arrowheads). Pa, palp. **b** right antenna (medio-dorsal view) under SEM composed of a scape (Sc), a pedicel (Pe), and a flagellum (Fl) of 9 segments (1–9). Sensilla are visible along the antennal flagellum, more concentrated in the last flagellar segments; note the sensilla chaetica (arrowhead) on the bulge of the scapus. **c** the apex of the last flagellar segment, note the long and thick sensillum chaeticum (SC), with a blunt apex, and the thin and pointed sensillum trichodeum (ST), they are widespread between sensilla basiconica type I (SBI) and type II (SBII), small holes (H) are visible among the cuticular scales. **d** sensillum conicum, constituted by a bulged socket (S) and a smooth cone (Co) with some ridges. **e** the blunt apex of a sensillum chaeticum, note the apical pore (P) and the longitudinal grooves (LG). **f** pointed apex of a sensillum trichodeum, no pores are visible on the cuticle while longitudinal grooves (LG) are evident

(Figs. 2, 3). They are the most abundant olfactory sensilla on the antennae, without differences between sexes (Table 2). Sensilla basiconica can be distinguished into two types: type I (SBI) is long between 8.5 and 15 μm, with a diameter at the base of about 2 μm, decreasing from the base to the tip. It shows a blunt tip and the cone is normally flattened in the distal two-thirds, the socket is evident, but not bulged (Fig. 4a). In this sensillum pores are numerous and evident (around 45 nm) (Fig. 4a and

inset). Type II basiconic sensillum (SBII) is long between 18 and 25 μm, with a diameter at the base of about 2.5 μm, decreasing from the base to the tip. The tip is pointed and pores are so small (around 20 nm) that the cuticle seems smooth at low magnification (Fig. 4b and inset). The socket is evident and bulged (Fig. 4b). Type II sensilla are very few, compared with type I, and they are mainly located in the apical flagellomere, without difference between males and females (Table 2 and Figs. 2, 3). Some
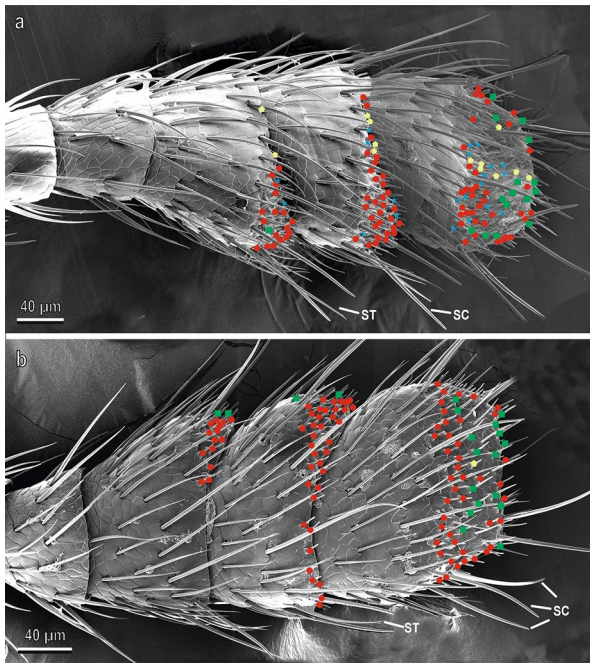
Fig. 2 Detail of the last three flagellomeres of one left antenna of *Chnootriba elaterii* female under SEM, in a medio-dorsal (**a**) and latero-ventral (**b**) view. The four types of olfactory sensilla have been pointed with different colors, according to their morphology, to show their number and distribution. Sensilla basiconica are red dots (type 1) and green squares (type II), porous sensilla trichodea are yellow pentagons, and sensilla grooved pegs are blue stars. *ST* sensilla trichodea, *SC* sensilla chaetica

Fig. 3 Detail of the last three flagellomeres of one left (**a**) and one right (**b**) antenna of *Chnootriba elaterii* male under SEM, in a medio-dorsal (**a**) and latero-ventral (**b**) view, respectively. The four types of olfactory sensilla have been pointed with different colours to show their number and distribution. Sensilla basiconica are red spots (type 1) and green squares (type II), porous sensilla trichodea are yellow pentagons and sensilla grooved pegs are blue stars. *ST* sensilla trichodea, *SC* sensilla chaetica

sensilla basiconica present an intermediate morphology that makes them difficult to be categorized as type I or type II.

Porous sensilla trichodea (PST) are very short (less than 10 μm), with an evident bulged socket and a conical shape, with a base diameter of 3–4 μm and a pointed tip (Fig. 4c). The hair cuticle shows many pores, interspersed in a wrinkled surface (Fig. 4c and inset). These sensilla are the less numerous among olfactory sensilla (around 10–15 on the antenna) and are mainly located on the dorsal side, without any difference between sexes (Table 2 and Figs. 2, 3).

Sensilla grooved peg (SGP) are present in low numbers and only on the dorsal side of the last three flagellar segments, both in males and females (Table 2 and Figs. 2, 3). They are characterized by a long peg inserted into a bulged socket, whose diameter is almost as long as the length of the peg (6–8 μm) (Fig. 4d). The base of the peg is smooth, about 2 μm wide, and conically shaped, while the distal half is pointed and shows digitiform processes giving to this sensillum a typical grooved morphology (Fig. 4d). Pores are not visible on the surface, but typically this kind of sensilla are double-walled olfactory sensilla with pores located between the digitiform processes (Sevarika et al. 2020).
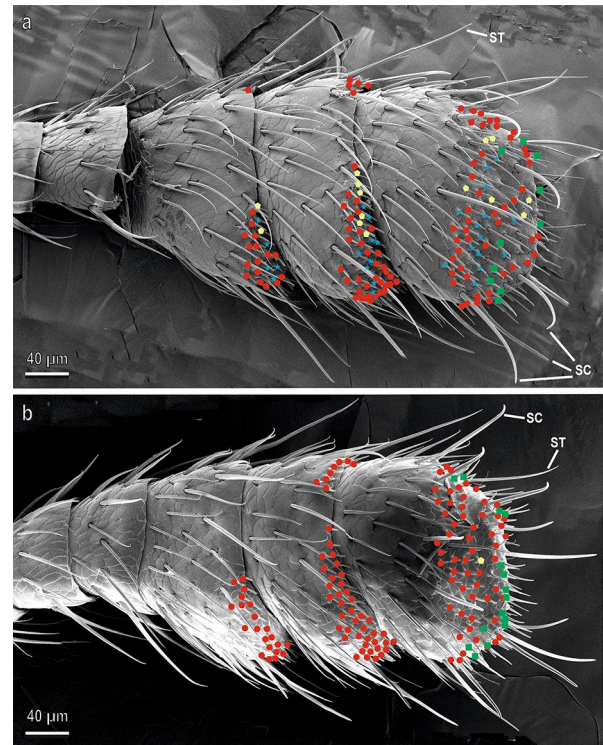
EAG responses to standard VOCs

Response of male and female antennae of *C. elaterii* to the standard VOCs tested, except Toluene (reported in *Cucurbita* leaves) and (−)- alfa pinene (reported in *Momordica* leaves), is significantly different from the control paraffin oil) (Fig. 5). So E2-exenal, heptanal, nonanal, decanal, methyl salicylate, -(−) linalool, Z3-hexen-1-ol, pentanol, hexanol, nonanol, and octanol can be defined as "active compounds" (Fig. 5). No significant difference has been highlighted between the sexes.

In the EAG dose-responses tests, males and females showed a no different responses to increasing concentrations of both Z3-hexen-1-ol and hexanol. Both males and females showed a significant response to Z3-hexen-1-ol in comparison with the solvent (paraffin oil), at 0.01%, 0.1%, 1%, 10%, 20%, 50% concentrations (Fig. 6a). The response to hexanol was significantly higher than the response to the solvent at 0.1%, 1%, 10%, 20%, and 50% concentration, while it was not significant at the lowest concentration

Table 2 Antennal sensilla

	Sensilla type	Female	n	Male	n
Medio-dorsal	<u>SBI</u>	87.8 ± 5.3	4	115.0 ± 5.3	5
	<u>SBII</u>	15.3 ± 2.6	4	14.2 ± 3.0	5
	<u>PST</u>	13.3 ± 1.7	4	13.4 ± 1.7	5
	<u>SGP</u>	19.5 ± 4.3	4	27.2 ± 2.4	5
	SC	15.0 ± 0.9	4	18.0 ± 1.6	5
	SCo	2.8 ± 0.3	4	3.2 ± 0.6	5
	ST	135.3 ± 12.5	4	148.4 ± 4.7	5
Latero-ventral	<u>SBI</u>	76.0 ± 8.5	4	96.7 ± 7.4	6
	<u>SBII</u>	17.3 ± 1.9	4	15.3 ± 1.2	6
	<u>PST</u>	1.3 ± 0.5	4	1.5 ± 0.7	6
	<u>SGP</u>	–	4	–	6
	SC	13.3 ± 1.9	4	15.2 ± 1.1	6
	SCo	0.3 ± 0.3	4	0.5 ± 0.3	6
	ST	131.8 ± 11.5	4	133.0 ± 6.6	6

Number (mean ± SEM) and distribution (medio-dorsal or latero-ventral side) of the different sensilla (olfactory sensilla are underlined) in the last three flagellar segments of *Chnootriba elaterii* female and male antennae

SBI sensilla basiconica type I, *SBII* sensilla basiconica type II, *PST* porous sensilla trichodea, *SGP* sensilla grooved pegs, *SC* sensilla chaetica, *SCo* sensilla conica, *ST* sensilla trichodea

(0.01%). The highest responses for both Z3-hexen-1-ol and hexanol were recorded at 10% concentration (Fig. 6b).

EAG responses to extracts of Cucurbitaceae

Male and female antennae of *C. elaterii* show a significant response to the extracts of VOCs collected from, melon, cucumber, calabash, zucchini, and pumpkin, so all of them can be defined as "active extracts". On the contrary, antennae do not respond to extracts from squirting cucumber and loofah and only female antennae show a significant response to watermelon (Fig. 7). The responses of males (36.1 ± 1.4) are significantly higher than female ones (28.9 ± 1.4).

Discussion

The data collected in the present study demonstrate, for the first time, the olfactory abilities of phytophagous ladybugs, in particular, describe the putative olfactory sensilla along the antennal flagellum and the antennal sensitivity to volatiles emitted by Cucurbitaceae, as potential cues to locate host plants in *C. elaterii*. Epilachnini is a cosmopolitan group including only species whose adults and larvae feed upon leaves, flowers, small fruits, and seedlings (Tomaszewska and Szawaryn 2016), with species-specific relationships between insects and host plants (e.g. *Epilachna varivestris* feeding on Fabaceae or *Henosepilacna vigintioctopuntata*

feeding on Solanaceae) (Szawaryn et al. 2015). Despite their significant economic importance as pests of crops, Epilachnini has been completely neglected regarding their sensory equipment and chemical ecology, compared to other large and economically important families of phytophagous beetles, such as Chrysomelids (Bartlet et al. 1999).

Antennal morphology

The morphology and segmentation of *C. elaterii* antennae are quite similar to other ladybugs described in former studies (Hao et al. 2020; Sevarika et al. 2020). In particular, antennae have 11 segments, as in the most Coccinellids (Hao et al. 2020), clavate shape with the last segments enlarged and rich in sensilla, and there is no significant difference in shape and length between males and females, similarly to *Hippodamia convergens* (Hamilton et al. 1999), *Pseudoscymnus tsugae* (Broeckling and Salom 2003) and *Harmonia axyridis* (Sevarika et al. 2020), but different from *Propylea dissecta*, whose females antennae are longer than males (Pervez et al. 2020).

As in aphidophagous *H. axyridis*, whose fine structure of antennal sensory organs has been described by Sevarika et al. (2020), the antennae of *C. elaterii* are scaly and present small holes scattered between the scales. Similar holes have been reported, erroneously interpreted as sensilla coeloconica, in other Coccinellid species (cf. review in Hao et al. 2020) and they have been demonstrated to correspond to class II and class I exocrine glands, based on Noirot and Quennedey (1974) classification (Sevarika et al. 2020). Different functions have been proposed for exocrine antennal glands in insects, such as pheromone secretion, sensilla protection, and social signalling (Di Giulio et al. 2009; Rebora et al. 2015; Romani et al. 2008; Sevarika et al. 2020). In Coccinellids, the presence of these glands in a phytophagous species supports the hypothesis that they are involved in biological aspects different from predation, such as contact recognition of species or production of a lubricant/protective substance for the sensilla (Sevarika et al. 2020).

Antennae of *C. elaterii* present 6 different types of sensilla (3 of which with a supposed olfactory function). Long sensilla trichodea with no pores, longitudinal grooves, and a pointed tip are the most numerous sensilla along the flagellum and have a supposed mechanosensory function because of their morphology (Keil 1997). They are distinguishable from the less numerous sensilla chaetica, which are thicker, insert into the antenna with an angle close to 70°, and are characterized by a pore in the middle of their blunt tip, which suggests a gustatory function (Altner and Prillinger 1980). Sensilla chaetica and trichodea of *C. elaterii* correspond to those described in *H. axyridis* (Sevarika et al. 2020) but are much longer (often more than twice), and much more numerous (more than 250 trichodea and 25 chaetica in *C.*

Fig. 4 Olfactory sensilla on the last flagellar segment of *Chnootriba elaterii* under SEM. **a**, **b** and insets, sensilla basiconica type I (**a** and inset) and type II (**b** and inset); note the pores (P), in type II (**b** and inset) they are visible only at high magnification in the inset; the not articulated socket (arrow) is bulged in type II (**b**) and the tip is flattened in type I (**a**). **c** and inset, porous sensillum trichodeum, note the small pores (P) under a wrinkled cuticular surface, detailed in the inset. BS bulged socket. **d** sensillum grooved peg, the base of the peg is smooth and conically shaped, while the distal portion is pointed and characterized by digitiform processes (DP). BS bulged socket
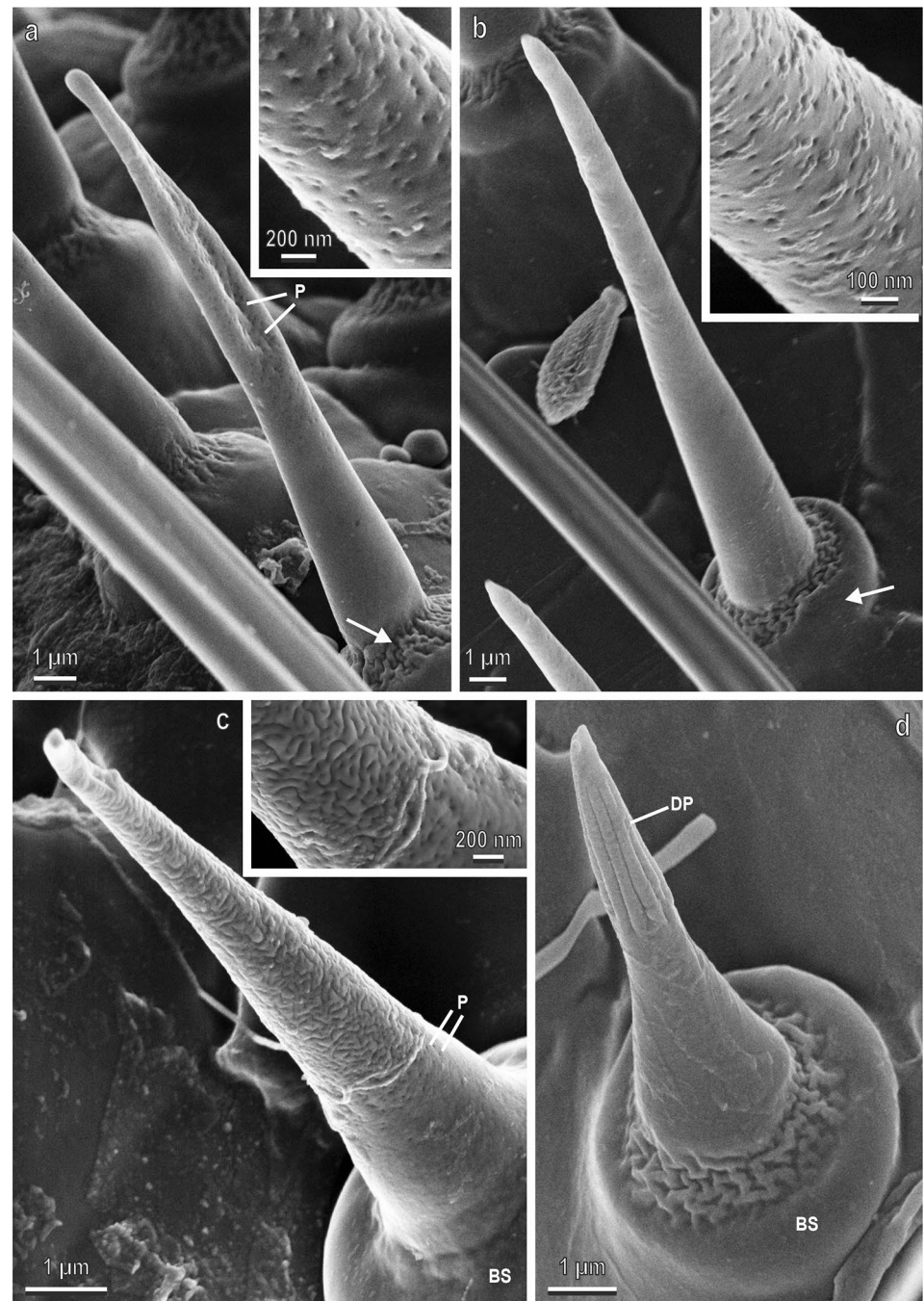
elaterii respect to less than 20 trichodea and less than 10 chaetica in *H. axyridis*), giving a hairy aspect to the flagellum of the melon ladybirds. Maybe not all putative sensilla trichodea of *C. elaterii* are innervated (Mustaparta 1973), as supposed for the hairy antennae of the Chrysomelidae *Psylliodes chrysocephala* (Bartlet et al. 1999), but it is interesting to note that numerous long mechanosensory and gustatory sensilla could be very useful for the melon ladybirds, to perceive their host plants extremely rich of trichomes of different shape, length, and hardness (Ali and Fahad 2011).

Two–three sensilla conica without pores and with a bulged socket are reported on the antennae of *C. elaterii*; these sensilla are located on the medio-dorsal side in both sexes and probably have a thermo-hygroreceptive function (reviews in Helmut Altner and Loftus 1985; Yokohari 1999), as supposed in *H. axyridis* (Sevarika et al. 2020).

Fig. 5 EAG responses, as a percentage to the reference standard stimulus (octanal 10% v/v), of males and females of *Chnootriba elaterii* to synthetic VOCs, reported in the literature as emitted by leaves of Cucurbitaceae. The table inset shows the statistical parameters of two-way ANOVA. Bars indicate the means ± s.e.m. *$P < 0.05$, ns not significant; Dunnet test comparing the responses with those to control (paraffin oil used as control, see grey bars)
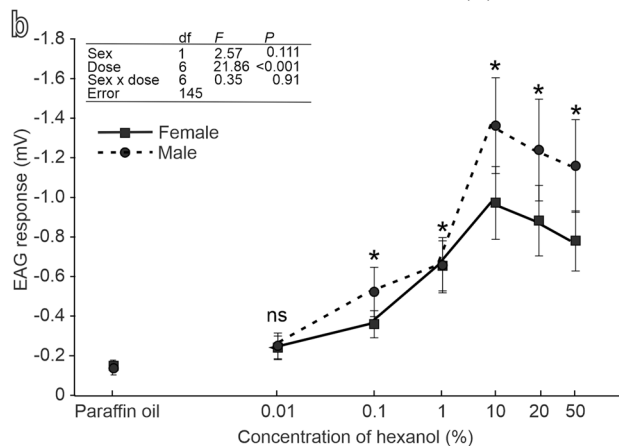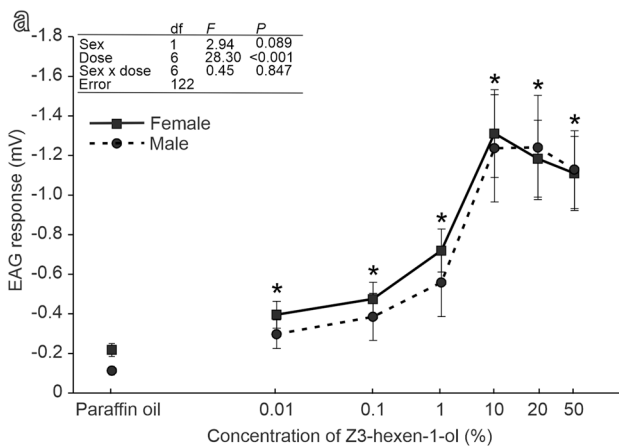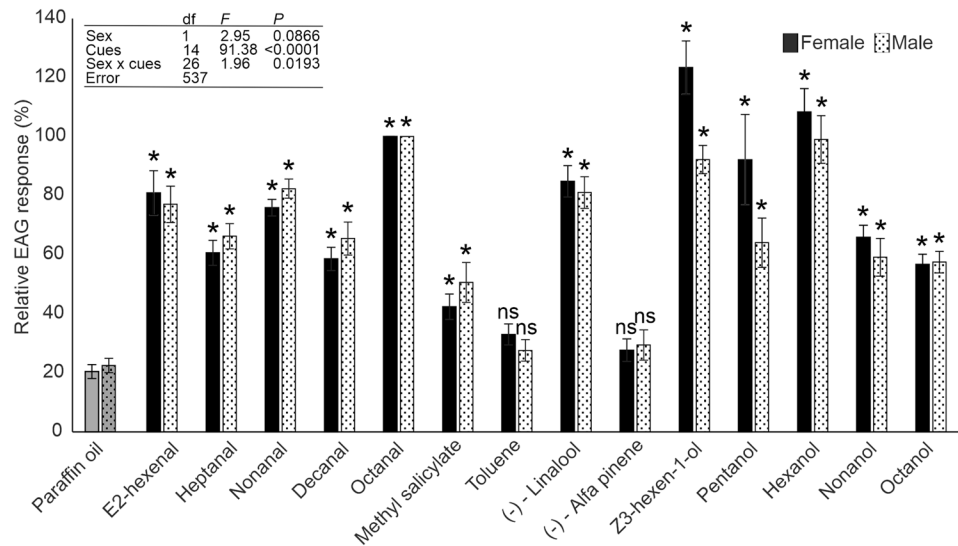

Fig. 6 Dose–response relationships for stimulation of male and female antennae of *Chnootriba elaterii* with Z3-hexen-1-ol (**a**) and hexanol (**b**) solutions in paraffin oil. The table inset shows the statistical parameters of two-way ANOVA. Bars indicate the means ± SEM of absolute responses (mV). *$P < 0.05$, ns not significant; Dunnet test comparing the responses with those to control (paraffin oil used as control)
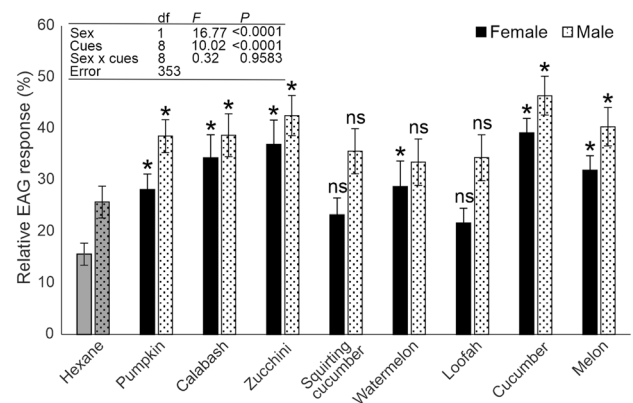
Fig. 7 EAG responses of males and females of *Chnootriba elaterii* to VOCs extracts (eluted in hexane) from eight Cucurbitaceae species. The table inset shows the statistical parameters of two-way ANOVA. Bars indicate the means ± SEM. *$P < 0.05$, *ns* not significant; Dunnet test comparing the responses with those to control (hexane used as control)

Olfactory sensilla

Chnootriba elaterii antennae present different types of porous sensilla whose morphology suggests an olfactory function (Keil and Steinbrecht 1984), namely basiconic sensilla (SBI and SBII), porous sensilla trichodea (PST), and grooved pegs (SGP). As in other Coccinellids (Hao et al. 2020), they are located in the distal part of the antenna, mainly on the flat surfaces on the medio-dorsal and latero-ventral side of the last flagellomere. These sensilla are more numerous in males than in females and on the medio-dorsal side than on the latero-ventral one. Sexual dimorphism has been often reported in Coleoptera antennae (Colgoni and Vamosi 2006; Jourdan et al. 1995; Tanaka et al. 2006) and sexual pheromones have been described in *H. axyridis*

(Fassotte et al. 2014), but further studies are needed in *C. elaterii*, whose males spend days on females during the reproductive period (Liotta 1964). The higher number of olfactory sensilla on the medio-dorsal side of *C. elaterii* antennae is probably related to their better exposition to long-distance volatiles in the environment, emitted by plants, predators, or conspecifics. These chemoreceptors are much more numerous than sensilla chaetica, probably because in Coccinellids gustatory abilities are moved to the maxillary palps while antennae are mainly olfactory organs (Sevarika et al. 2020). On this account, only a few reports have been found for mouthpart sensilla in aphidophagous ladybugs (Hao et al. 2019) and more investigations are needed in phytophagous ones (Barbier et al. 1996).

Olfactory sensilla of *C. elaterii* are very similar in number and shape to those described in aphidophagous ladybugs (Hao et al. 2020; Sevarika et al. 2020), except for the porous sensilla trichodea that have never been distinguished from basiconica in other species. Porous sensilla trichodea and sensilla basiconica are probably single-walled olfactory sensilla, while grooved pegs show the typical external morphology of double-walled olfactory sensilla (Steinbrecht 2007). Grooved pegs are not numerous in *C. elaterii*, less than 30 per antenna, and located only on the medio-dorsal side of the apical flagellomere, more numerous in males than in females. The same sensilla are widespread in insects, frequently called coeloconic and interpreted as olfactory, but also as thermo-hygroreceptors (Rebora et al. 2008; Ruchty et al. 2009; Yao et al. 2005). Similar sensilla are reported in aphidophagous coccinellids (Sevarika et al. 2020), where sometimes are called sensilla basiconica (Hamilton et al. 1999; Hao et al. 2020), and this suggests the need for universal naming of sensilla useful for comparative analysis.

Relationships of antennal sensilla with feeding habits have been studied in several insects and in many cases, the number and distribution of certain sensilla can be more related to host preference than phylogeny (Isberg et al. 2013). Phylogeny of Coccinellidae strongly suggests the origin of predatory behaviour in the ancestor as critical innovations leading to the evolutionary success of the taxon, while phytophagous habits of Epilachnini can be considered the result of a later divergence, and an ancestral character of the tribe (Seago et al. 2011; Szawaryn et al. 2015). On this account, comparative studies on phytophagous ladybugs could be useful to confirm the correlation between type and number of antennal sensilla, feeding preferences, and evolutionary processes.

Electroantennographic responses

The present results demonstrate that male and female antennae of *C. elaterii* are sensitive to several compounds emitted by Cucurbitaceae, in detail E2-exenal, heptanal, nonanal, decanal, methyl salicylate, -(−) linalool, Z3-hexen-1-ol, pentanol, hexanol, nonanol, and octanol; only toluene and (−)- alfa pinene did not elicit any significant response. These last compounds were selected because emitted by leaves of zucchini (Shapiro et al. 2012) and cucumber (Njuguna et al. 2018), respectively, but it is interesting to note that *C. elaterii* antennae respond to several other compounds from the same host plants, namely methyl salicylate, decanal, nonanal, (−)- linalool from Cucumber, and Z3-hexen-1-ol, heptanal, nonanol, E2-hexenal and pentanol from zucchini. In agreement with these results, VOCs extract from zucchini and cucumber elicits significant responses both in males and females. Antennae of *C. elaterii* clearly respond also to extracts from melon, calabash, pumpkin, and watermelon, while they do not respond to extracts from loofah and *E. elaterii*.

As far as loofah is concerned, species belonging to the genus have never been reported as host plants for the melon ladybird (Liotta 1964) and in feeding laboratory experiments (El-Abdin and Siragelnour 1991) using *Citrullus*, *Cucumis*, *Cucurbita*, *Lagenaria*, and *Luffa*, the insects never feed on *Luffa*. More difficult is to explain the absence of responses of *C. elaterii* antennae to the VOCs extracted from *E. elaterii*, which is a typical wild host plant for the melon ladybird, responsible for its specific epithet "*elaterii*" (Liotta 1964). Considering the coevolution history that typically characterizes phytophagous insects and their host plants (Hougen-Eitzman and Rausher 1994; Thompson 1988), it is possible that squirting cucumber, being the wild and favourite host for melon ladybirds (Liotta 1964), has evolved strong defenses from this phytophagous, such as unrecognizable VOCs. These defenses could be particularly effective in "young" plants (1–2 months old, as we used to obtain extracts), which are particularly injured from the feeding activity of the phytophagous (Liotta 1964), while VOCs from perennial "old" plants could be able to attract the insects. Crops Cucurbitacea could have a different characterization of VOCs pattern during their development, also considering that they are annual plants (Chomicki et al. 2020). This hypothesis needs to be tested with additional behavioural and electrophysiological experiments but is supported by preliminary personal data. In laboratory experiments, indeed, larvae of melon ladybirds did not develop on "young" *E. elaterii* plants but perfectly accomplish their cycle on leaves of "old" plants. At the same time, in feeding laboratory experiments *C. elaterii* larvae seem to prefer "old" leaves of *E. elaterii* (more than 8 months) concerning "young" ones. Differences in EAG responses to VOCs extracted from different developmental stages of the same plant have been reported in other pests, such as *Eurydema oleracea* tested for VOCs emitted by *Eruca sativa* and *Brassica oleracea* (Piersanti et al. 2020). Otherwise, we cannot exclude that the absence of EAG responses of *C. elaterii* to squirting cucumber could

be due to the extraction procedure, which could not allow for extracting all the active VOCs.

Considering that electroantennography measures the total amount of electrophysiological responses by olfactory neurons in the insect antennae (Park et al. 2002; Schneider 1957), higher EAG depolarizing responses to plant extracts and standard VOCs in *C. elaterii* male can be simply related with the higher number of olfactory sensilla present on this sex, as above reported. These olfactory sensilla, or at least part of them, could be tuned both to host plant volatiles and sexual pheromones, as reported in other insects such as *Sesamia inferens* (Zhang et al. 2014) and *Rhynchophorus palmarum* (Saïd et al. 2003). On this account, it is also interesting to consider that in Chrysomelidae, such as *Crioceris duodecimpunctata*, pioneer males initiate the colonization and for this reason, they are often more sensitive than females to the host plant VOCs (Pistillo et al. 2022). More accurate behavioural and physiological studies are needed to investigate these hypotheses.

Conclusions

The present study confirms the presence of olfactory sensilla in the antennae of melon ladybird *C. elaterii*, similar to those described in aphidophagous coccinellids, but much more numerous for each sensilla type (Hao et al. 2020; Sevarika et al. 2020). Considering that EAG-active compounds are common of ecological significance (Germinara et al. 2017), the present recordings provide strong evidence of the functionality of the antennal olfactory sensilla and confirm a crucial role of antennae in host plant location and selection by the melon ladybird. The present results provide then a rich basis for future research, such as chemical investigations aimed at identifying biologically active compounds, useful for semiochemical-based control strategies of this important crop pest, particularly in the Mediterranean Basin and Arabian Peninsula. Understanding insect chemical ecology from an ecological perspective is then crucial to balance control and conservation, supporting insect ecosystem services such as pollination, fertilization, or pest control (Haverkamp et al. 2018).

Declarations

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