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Olfaction in dragonflies: Electrophysiological evidence

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ABSTRACT

The problem of olfaction in Paleoptera (Odonata, Ephemeroptera) cannot be considered fully elucidated until now. These insects have been traditionally considered anosmic, because their brain lacks glomerular antennal lobes, typically involved in Neoptera odor perception. In order to understand if the presumed coeloconic olfactory receptors described on the antennal flagellum of adult Odonata are really functioning, we performed an electrophysiological investigation with electroantennogram (EAG) and single cell recordings (SCR), using *Libellula depressa* L. (Odonata, Libellulidae) as a model species. Odors representing different chemical classes such as (Z)-3-hexenyl acetate (acetate ester), (E)-2-hexenal, octanal (aldehydes), (Z)-3-hexen-1-ol (alcohol), propionic acid, butyric acid (carboxylic acids), and 1,4-diaminobutane (amine) were tested. Most of the tested chemicals elicited depolarizing EAG responses in both male and female antennae; SCR show unambiguously for the first time the presence of olfactory neurons in the antennae of *L. depressa* and strongly support the olfactory function of the coeloconic sensilla located on the antennal flagellum of this species.

Electrophysiological activity may not necessarily indicate behavioral activity, and the biological role of olfactory responses in Odonata must be determined in behavioral bioassays. This study represents a starting point for further behavioral, electrophysiological, neuroanatomical and molecular investigation on Odonata olfaction, a research field particularly interesting owing to the basal position of Paleoptera, also for tracing evolutionary trends in insect olfaction.

1. Introduction

In insects, the sense of smell is a complex and highly sensitive modality, governing essential decisions such as choice of mates, food, and oviposition sites. A pair of antennae bearing olfactory sensilla represents the main olfactory organ of hexapods and the olfactory sensory system is organized in a very similar fashion in most insects.

The problem of olfaction in Paleoptera (Odonata, Ephemeroptera) is still an open question. These insects have been traditionally considered anosmic, because their brain lacks glomerular antennal lobes and mushroom body calyces, which in Neoptera are involved in odor perception (Strausfeld et al., 1998; Farris, 2005).

Slifer and Sekhon (1972), in an ultrastructural overview on adult antennal flagellum of some Odonata species, identified coeloconic sensilla located in simple and compound cavities. On the basis of their apparent porous cuticle, the authors hypothesized a chemosensory function for these sensilla.

A recent ultrastructural investigation (SEM, TEM) on the adult of the dragonfly *Libellula depressa* (Odonata: Libellulidae) revealed sensilla located in pits on the lateral–ventral side of the antennal flagellum (Rebora et al., 2008). These sensilla are represented by sensilla coeloconica and by deeply sunken sensilla styloconica. The sensilla coeloconica (Fig. 1) are innervated by three unbranched dendrites entering the peg and they show a dendritic sheath ending at the base of the peg. The peg shows no socket and its cuticle is irregular with wide pore-like structures at the base of which actual pores with pore tubules are evident. The structure of these sensilla is in agreement with that reported for single-walled insect olfactory receptors. The deeply sunken sensilla are represented by two kinds of sensilla styloconica located at the bottom of deep cavities evident on the antennal surface as simple openings (Fig. 1). These sensilla are no-pore sensilla with inflexible socket and unbranched dendrites and, notwithstanding their structural differences, they share common features typical of thermo-hygroreceptors. The presence of putative olfactory and thermo-hygroreceptors has been confirmed in ultrastructural investigations on several Odonata families belonging to the suborders Anisoptera and Zygoptera (Rebora et al., 2009a; Piersanti et al., 2010).

Single cell electrophysiological recordings from adult males and females of *L. depressa*, stimulating the antenna by rapid changes in temperature and humidity, showed the occurrence of a dry, a moist and a cold receptor neurons on the antennal flagellum, probably

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located in the deeply sunken sensilla styloconica (Piersanti et al., 2011).

Odonata are believed to have secondarily invaded freshwater environments. Therefore, the putative olfactory coeloconic sensilla identified on their antennae could be "silent receptors", in which olfactory receptor genes are pseudogenes, as it occurs in secondarily aquatic mammals (Freitag et al., 1998).

On the basis of these data, in order to understand if the coeloconic olfactory receptors located on Odonata antennae represent vestigial organs or are really functioning, we performed an electrophysiological investigation with electroantennogram (EAG) and single cell recordings (SCR), using *L. depressa* as a model species. EAG measures the total amount of electrophysiological responses in the insect antennae, thus providing a general measure of odorant reception at the peripheral level (Schneider, 1957; Roelofs, 1984; Park et al., 2002). When recording the frequency of action potentials in single olfactory receptor neurons using the SCR technique, the specificity in each neuron is revealed (Masson and Mustaparta, 1990). SCR is particularly useful when the number of stimulated neurons is small and gives only a minute EAG (Schiestl and Marion-Poll, 2002).

2. Materials and methods

2.1. Insects

Larvae of *L. depressa*, attributed to the ultimate (F-0) stage were collected in ponds in Central Italy (Perugia, Umbria) in the periods March–April 2010 and 2011. The specimens were kept outdoor in plastic containers (60 × 40 × 40 cm) with water, detritus, flora and fauna from the collecting site, in natural conditions of temperature, humidity and light. The larvae were fed *ad libitum* with plankton (*Daphnia* spp. and *Cyclops* spp.) up to the emergence of the adults. In the experiments male and female of *L. depressa* were used 2 days after their emergence.

2.2. Chemicals

To understand if the chemoreceptors located on Odonata antennae are really functioning or not, in consideration that at the moment we have no idea of the biological role of these sensilla in dragonfly behavior, we choose a set of generic odors belonging to different chemical classes. In particular: (Z)-3-hexenyl acetate (acetate ester, 98.5% Aldrich, vapor pressure 1.22 mm Hg at 25 °C), (E)-2-hexenal (aldehyde, 98% Aldrich, vapor pressure 4.62 mm Hg at 25 °C), octanal (aldehyde, 99% Aldrich, vapor pressure 2.07 mm Hg at 25 °C), (Z)-3-hexen-1-ol (alcohol, 98% Aldrich, vapor pressure 1.04 mm Hg at 25 °C), propionic acid (carboxylic acid, 99.8% Fluka, vapor pressure 4.23 mm Hg at 25 °C), butyric acid (carboxylic acid, 99.5% Fluka, vapor pressure 1.65 mm Hg at 25 °C), and 1,4-diaminobutane (amine, 99% Aldrich, vapor pressure 2.55 mm Hg at 25 °C).

(Z)-3-hexenyl acetate, (E)-2-hexenal, octanal and (Z)-3-hexen-1-ol were chosen in consideration that they are very common semiochemicals produced by different plants and often used as reference standard in EAG investigations (e.g. Park and Hardie, 1998; Smid et al., 2002; Gouinguene et al., 2005). Propionic acid, butyric acid and 1,4-diaminobutane were chosen in consideration that these chemicals elicited response in single cell electrophysiological investigations in the coeloconic olfactory sensilla of *Drosophila* (Yao et al., 2005).

(Z)-3-hexenyl acetate, (E)-2-hexenal, octanal, and (Z)-3-hexen-1-ol were dissolved in paraffin oil (Fluka), while propionic acid, butyric acid and 1,4-diaminobutane were dissolved in distilled water to obtain 10% (v/v) solutions. When comparing the activity of the different compounds on receptor neurons, no compensation was made for differences in volatility.

For dose–response relationship, serial dilutions of octanal and (E)-2-hexenal in paraffin oil were prepared to obtain 0.01%, 0.1%, 1%, 10% and 50% (v/v) solutions.

The solutions were kept in a freezer at −18 °C until used.

2.3. Electroantennography

2.3.1. Recordings

The antenna of *L. depressa* was carefully excised from the head. Scape and pedicel were removed in order to avoid any noise due to the numerous mechanoreceptors located on these structures. The antennal flagellum bearing the olfactory sensilla was mounted between two glass capillary electrodes (1.5 mm o.d, 1.2 mm i.d) filled with Ringer solution (Beadle and Ephrussi, 1936), containing 5 g/l of polyvinylpyrrolidone (Fluka), in contact with a silver wire. The capillary tubes were drawn to a fine point using a microelectrode puller (Narishige PC-10) to get an inner diameter wide enough to enable insertion of the excised antenna. The base of the flagellum was inserted into the reference glass electrode. The recording electrode was connected to the antennal tip (with the apex of the flagellum cut off). The analog signal was detected through a probe with a high-input impedance preamplifier (10×) (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).

2.3.2. Stimulations

Test compounds diluted in paraffin oil or water were delivered as 20 μl samples placed on a filter paper (15 × 15 mm, Whatman No. 1). The impregnated filter paper was placed into a glass Pasteur pipette (150 mm in length, Volac®) constituting an odor cartridge. The control stimulus consisted of a similar pipette containing a filter paper impregnated with 20 μl aliquot of paraffin oil or distilled water. Fresh stimulus pipettes were prepared every day. The tip of the glass pipette was placed about 3 mm into a small hole in the wall of a 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 air stream at 2500 ml min^{-1} that was flowing over the 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. Preliminary

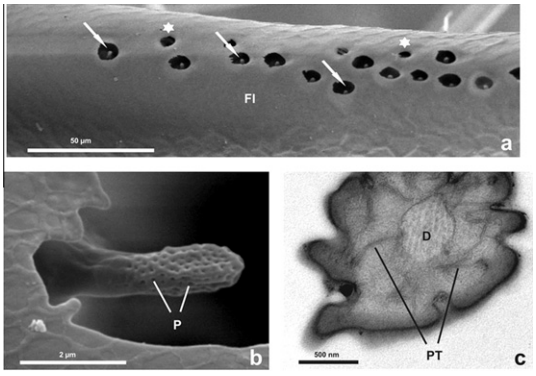

Fig. 1. (a) Antennal flagellum (Fl) of *Libellula depressa* showing the pits hosting coeloconic sensilla (arrows) and the openings of the deep cavities hosting sensilla styloconica (asterisks) under SEM; (b) detail of a coeloconic olfactory sensillum showing pores (P) under SEM; (c) cross section under TEM of a coeloconic sensillum showing pore tubules (PT) typical of olfactory sensilla. D, dendrite.

tests showed dragonfly antenna preparations lasted up to 15 min with no noticeable decreases in EAG responses observed over this time period at room temperature. For the compounds diluted in paraffin oil, a test series of the same dose was applied to 10 antennae from males and to eight antennae from females. For the compounds diluted in distilled water, a test series of the same dose was applied to five antennae from males and to six antennae from females.

On the basis of preliminary recordings octanal was chosen as reference standard stimulus and presented to each antenna at the beginning, in the middle and at the end of the recording series to confirm activity of the antennal preparation. For each recording series, a pipette with only filter paper was also used to check any contamination of filter paper and to evaluate the air effect in the EAG response. Test compounds were presented in a random sequence.

Two compounds that showed relatively large EAG responses such as (E)-2-hexenal and octanal were tested for their EAG dose–responses in both males and females. For dose–response experiments, 13 antennae from males and 12 antennae from females were tested; the exposure proceeded from lowest to highest concentration with at least 1 min interval between successive stimulations, to minimize the effect of olfactory adaptation by strong stimulation.

2.3.3. Statistical analyses

For evaluating the EAG response, the maximum deflection of the recorded EAG signal after stimulation with an odor was used. In the comparison of the sensitivity of *L. depressa* for different chemical compounds, all recorded EAG responses were expressed as a percentage of the response to the reference standard stimulus (octanal at 10% v/v). This allows compensation for changes of the sensitivity of an antenna during the course of an experiment and the comparison between experiments performed with antennae of different sensitivities. EAG responses for each gender and separately for those compounds dissolved in paraffin oil and in distilled water were compared by 1-way analysis of variance (ANOVA). As post hoc comparisons, Dunnett test was used to compare the response to each compounds to those of the control (paraffin oil or distilled water) (Statistica 6.0, Statsoft Inc., 2001). Data obtained in the dose–response experiments are reported as absolute responses (mv). The responses to the different concentrations were analyzed separately for each gender by 1-way analysis of variance (ANOVA) and by the unequal N Tukey HSD test for multiple comparisons between the means (Statistica 6.0, Statsoft Inc., 2001). Male and female responses to a specific concentration were compared using Student's *t*-test for independent samples (Statistica 6.0, Statsoft Inc., 2001). Before the analysis, Box–Cox transformations were used to reduce data heteroscedasticity (Sokal and Rohlf, 1998).

2.4. Single cell recordings

2.4.1. Recordings

Before testing, each insect was placed inside a channel (diameter 9 mm) drilled through a Plexiglas cube and immobilized by Patafix (UHU, Bostik) and adhesive tape. The antennae, exposed at the top of the holder, were fastened to the Patafix by tungsten hooks. The pits containing the sensilla on the lateral-ventral side of the antenna were well exposed to the stimulation, and easily accessible for microelectrode manipulations from a side. A total of 20 insects, 10 males and 10 females, were used in the experiments.

Nerve impulses from single receptor neurons (RNs) were recorded extracellularly using tungsten microelectrodes sharpened in a highly concentrated solution of KNO_2 to a near tip diameter of 0.2 μm. The recording electrode penetrated the cuticle inside one of the pits located on the lateral-ventral side of the flagellum, using a micromanipulator (Narishige MMO-203) under visual control with a light microscope (WILD M420 connected with a wild zoom 1:5) at 200×. Under stereomicroscope, inserting the recording electrode into the pit, it was not possible to distinguish between the pits hosting sensilla coeloconica (olfactory receptors) and the pits representing the openings of deep cavities hosting the sensilla styloconica (thermo-hygroreceptors). Moreover, in consideration of the incomplete electrical isolation of receptor neurons from neighboring sensilla (Lee and Baker, 2008), each electrode could be picking up spikes from a number of receptor neurons not necessary from the same sensillum. The indifferent electrode was inserted into the eye making contact with the hemolymph. A conventional electrophysiological set-up for extra cellular single cell recording was used. The ring was mounted on anti-vibration table and shielded with a Faraday cage. For data acquisition a 10× gain probe (Universal Single Ended Probe, Type PRS-1, Synthec, Germany) was used. The amplified analog signal was monitored on an audio monitor and captured and processed with a data acquisition controller (IDAC-4, Syntech, Germany).

Spike activity was recorded by the computer software Autospike (Syntech, Germany).

2.4.2. Stimulations

Test compounds and controls were delivered as 15 μl samples using the same procedure described for EAG (see Section 2.3.2).

The tip of the glass pipette constituting the odor cartridge was placed about 3 mm into a small hole in the wall of a plastic pipette (50 mm long, 6 mm inner diameter and 2 mm inner diameter in the tip) oriented towards the antenna (∼5 mm away from the preparation). The stimuli were provided as described for EAG (see Section 2.3.2). Test compounds were presented in a random sequence at intervals of at least 1 min. Each contact was used, replicating stimulations, until it was lost.

The spike trains were analyzed offline by computer software Autospike (Syntech, Germany). To distinguish responses from colocated neurons, the different RNs were sorted into spike populations on the basis of their amplitude. Only spikes clearly separated from noise were evaluated. Responses of individual RNs, identified in each spike trains, were calculated as increase or decrease in the firing rate, relatively to the pre-stimulus rate. The response to a stimulus was obtained by counting action potentials (spikes) during 1 s starting from the time after the stimulation period at which the earliest response for the neuron was found, and deducting the number of action potential during 1 s immediately prior to the response. To achieve net responses we subtracted the value of the response elicited by the control. According to de Bruyne et al. (2001) we considered excitatory or inhibitory responses if the firing frequency exceeded in absolute value approximately twice the mean standard deviation of the firing frequencies of the various neuronal types for the control stimuli, i.e. eight spikes/s.

3. Results

3.1. Electroantennography

Depolarizing EAG responses were recorded in both males and females (Figs. 2–6).

The magnitude of the response to the standard stimulus (octanal at 10% v/v) was similar in the two sexes (males, −0.014 mV ± 0.002 SE; females, −0.011 mV ± 0.001 SE (t = 0.90; d.f. = 29; P = 0.374).

3.1.1. Chemicals dissolved in paraffin oil

In males (E)-2-hexenal and octanal elicited EAG activity significantly higher in comparison with the EAG activity recorded in

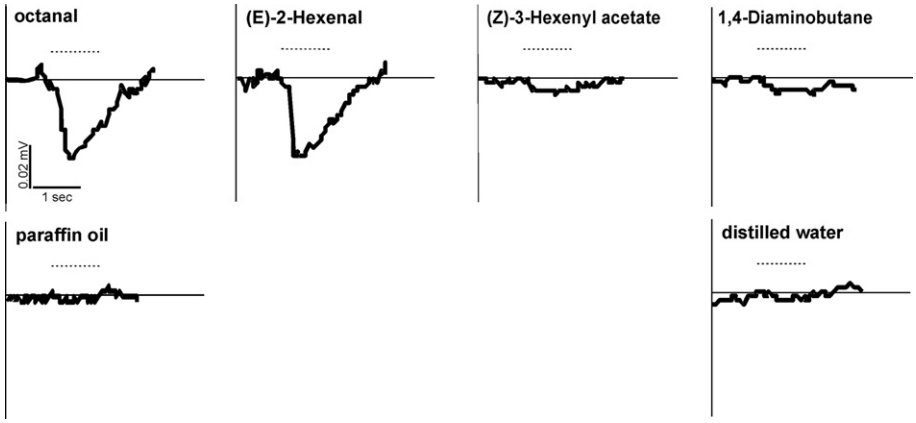

Fig. 2. Examples of EAG response waveforms of a female of *L. depressa* to 20 μl of different chemicals compared with the response to the controls (paraffin and distilled water). Dotted line corresponds to 1 s stimulus.

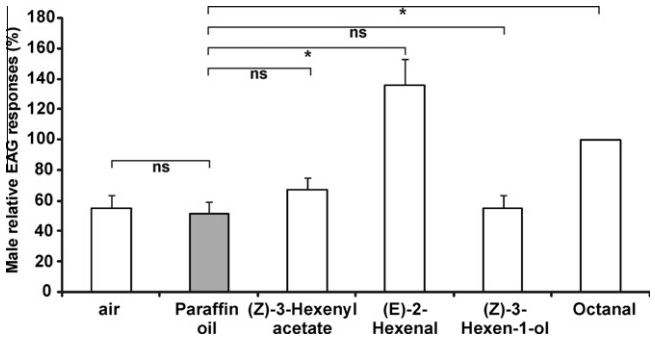

Fig. 3. EAG responses (mean ± SE) of male antennae of *L. depressa* to synthetic compounds dissolved in paraffin oil at 10% (v/v) concentration. White bars are compared with gray bar (control) (*$P < 0.05$; ns, not significant; ANOVA, Dunnett test). EAG responses have been normalized to the octanal responses.

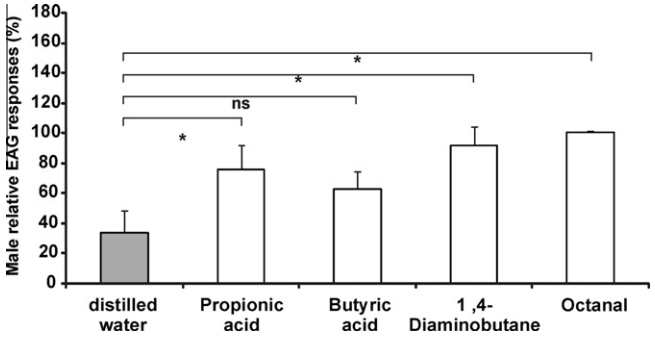

Fig. 5. EAG responses (mean ± SE) of male antennae of *L. depressa* to synthetic compounds dissolved in distilled water at 10% (v/v) concentration. White bars are compared with gray bar (control) (*$P < 0.05$; ns, not significant; ANOVA, Dunnett test). EAG responses have been normalized to the octanal responses.

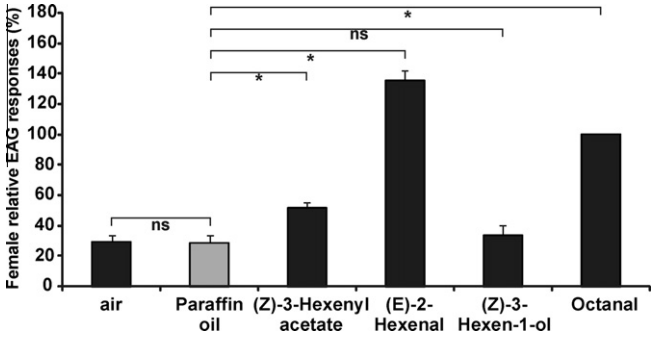

Fig. 4. EAG responses (mean ± SE) of female antennae of *L. depressa* to synthetic compounds dissolved in paraffin oil at 10% (v/v) concentration. Black bars are compared with gray bar (control) (*$P < 0.05$; ns, not significant; ANOVA, Dunnett test). EAG responses have been normalized to the octanal responses.

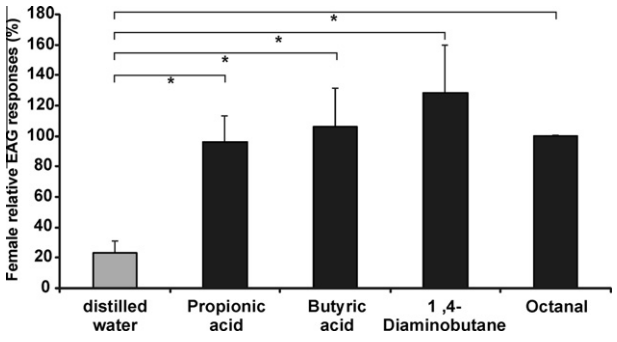

Fig. 6. EAG responses (mean ± SE) of female antennae of *L. depressa* to synthetic compounds dissolved in distilled water at 10% (v/v) concentration. Black bars are compared with gray bar (control) (*$P < 0.05$; ns, not significant; ANOVA, Dunnett test). EAG responses have been normalized to the octanal responses.

response to paraffin oil, while no significant difference was present between the EAG activity recorded in response to (Z)-3-hexenyl acetate and to (Z)-3-hexen-1-ol and the EAG activity recorded in response to paraffin oil ($F = 16.99$; d.f. = 5, 131; $P < 0.001$) (Fig. 3).

In females, (E)-2-hexenal, octanal and (Z)-3-hexenyl acetate elicited EAG activity significantly higher in comparison with the EAG activity recorded in response to paraffin oil, while no significant difference was detected between the EAG activity recorded in response to (Z)-3-hexen-1-ol in comparison with the EAG activity recorded in response to paraffin oil ($F = 65.06$; d.f. = 5, 107; $P < 0.001$) (Fig. 4).

3.1.2. Chemicals dissolved in distilled water

In males, propionic acid and 1,4-diaminobutane elicited EAG activity significantly higher in comparison with the EAG activity recorded in response to distilled water while no significant difference was present between the EAG activity recorded in response to butyric acid and the EAG activity recorded in response to distilled water ($F = 5.63$; d.f. = 4, 25; $P = 0.002$) (Fig. 5).

In females, propionic acid, butyric acid and 1,4-diaminobutane elicited EAG activity significantly higher in comparison with the EAG activity recorded in response to distilled water ($F = 9.71$; d.f. = 4, 34; $P < 0.001$) (Fig. 6).

The response to distilled water was significantly lower than the response to the standard stimulus (octanal) in both sexes (Figs. 5 and 6).

3.1.3. Dose–response experiments

Two compounds that showed relatively large EAG responses such as (E)-2-hexenal and octanal were tested for their EAG dose–responses in both males and females (Figs. 7 and 8).

EAG responses in *L. depressa* generally increased in amplitude with increasing doses of the two chemicals. EAG dose–response curve to octanal (Fig. 7) showed that male antennae reached saturation at 10% concentration, while female reached saturation at 1%. In males, 10% concentration was the sole concentration that elicited an EAG response statistically different from that elicited by paraffin oil, while concentrations of octanal of 0.01% and 0.1% were not statistically different from that elicited by paraffin oil, and 50% was intermediate ($F = 6.73$; d.f. = 5, 54; $P = 0.001$). In females, response to concentrations of octanal of 1%, 10% and 50% were statistically different from that elicited by paraffin oil, while responses to 0.01% and 0.1% concentrations were not statistically different from that elicited by paraffin oil ($F = 10.89$; d.f. = 5, 46; $P < 0.001$). The concentration of 1% was the only concentration where a significant difference was present between male and female EAG response (paraffin oil: $t = 0.547$; d.f. = 17; $P = 0.591$; 0.01%: $t = 1.82$; d.f. = 9; $P = 0.102$; 0.1%: $t = 1.47$; d.f. = 9; $P = 0.176$; 1%: $t = 2.79$; d.f. = 18; $P = 0.012$; 10%: $t = 0.90$; d.f. = 29; $P = 0.374$; 50%: $t = 1.17$; d.f. = 18; $P = 0.259$).

EAG dose–response curve to (E)-2-hexenal (Fig. 8) showed that male and female antennae reached saturation at 10% concentration. In males ($F = 5.02$; d.f. = 5, 45; $P = 0.001$) and females ($F = 6.96$; d.f. = 5, 40; $P < 0.001$), 10% and 50% concentrations elicited an EAG response statistically different from that elicited by paraffin oil, while concentrations of 0.01%, 0.1% and 1% elicited a response not statistically different from that of paraffin oil and not statistically different from the responses to concentration of 10% and 50%.

We observed a significant difference between male and female EAG response to (E)-2-hexenal at concentrations of 0.01% ($t = 2.37$; d.f. = 9; $P = 0.042$) and 0.1% ($t = 2.26$; d.f. = 8; $P = 0.049$), while no statistical difference was present between the male and female EAG response to paraffin oil ($t = 0.55$; d.f. = 17; $P = 0.591$) and at concentrations of 1% ($t = 0.35$; d.f. = 15; $P = 0.724$), 10% ($t = 0.85$; d.f. = 18; $P = 0.408$) and 50% ($t = 0.19$; d.f. = 18; $P = 0.853$).

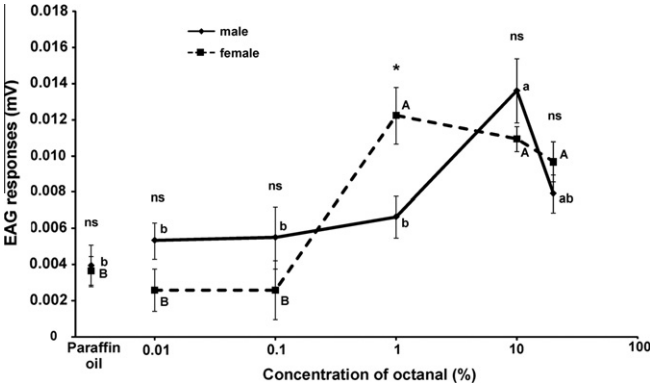

Fig. 7. Dose–response relationships for stimulation of *L. depressa* with octanal. Data represent means ± SE. Points with different letters are significantly different at $P < 0.05$ (ANOVA, Tukey's test). For comparisons for each concentration between male and female: $^{*}P < 0.05$; ns, not significant (Student *t*-test for independent samples). The concentrations are reported in a log 10 scale but values are in %.

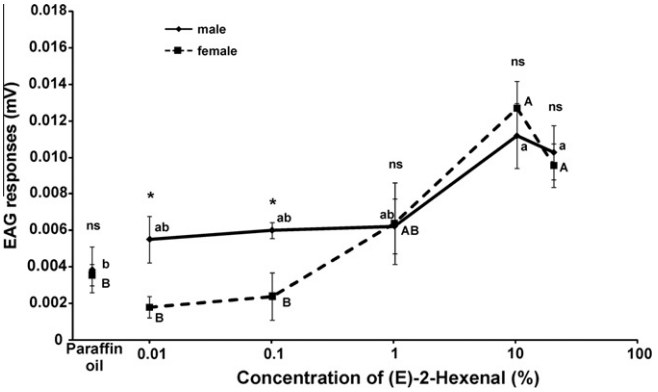

Fig. 8. Dose–response relationships for stimulation of *L. depressa* with (E)-2-hexenal. Data represent means ± SE. Points with different letters are significantly different at $P < 0.05$ (ANOVA, Tukey's test). For comparisons for each concentration between male and female: $^{*}P < 0.05$; ns, not significant (Student *t*-test for independent samples). The concentrations are reported in a log 10 scale but values are in %.

3.2. Single cell recordings

Successful contacts were obtained from RNs in 16 insects (eight males and eight females). Only neurons clearly responding to odors were analyzed. Responses to odors were obtained from 14 dragonflies, but due to a low quality of the signal (low signal to noise ratio) only the responses from seven insects are presented here. Co-located RNs were effectively sorted on the basis of their spike amplitude (Fig. 9).

The recordings showed the presence of seven ORNs responding with seven different pattern to the tested odors (Table 1). Each of the seven ORNs has been recorded once, except ORN4 recorded twice. All the tested chemicals elicited response from at least one ORN except 1,4 diaminobutane that elicited no response. (Z)-3-hexen-1-ol caused only an inhibitory response. Octanal elicited response in 4 ORNs, (Z)-3-hexenyl acetate, (E)-2-hexenal and propionic acid elicited response in three ORNs, while (Z)-3-hexen-1-ol and butyric acid elicited response only in one ORN (Table 1).

The intensity of the background firing varied among the different ORNs (Table 1, Fig. 10).

The ORNs responded in a relatively phasic-tonic manner, maintaining tonic firing for several seconds following stimulations to all odors except (Z)-3-hexenyl acetate that elicited short phasic responses (Fig. 10). In any case, a certain variability has been noted in the responses to the same odorant in different neurons and to the different odorants in the same neuron. Further electrophysiological investigations are necessary to describe the physiology of these ORNs.

4. Discussion

Our results show that some chemicals, belonging to different classes, elicit EAG activity in the antennae of *L. depressa*. In

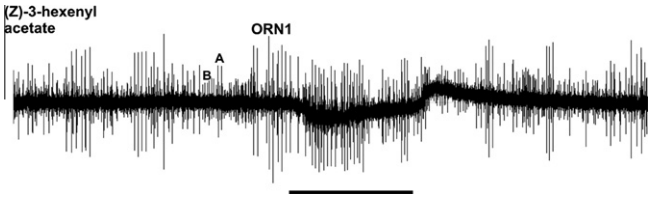

Fig. 9. Example of recording from one olfactory neuron (ORN1) co-located with two neurons (A and B) not responding to the tested odors. The co-located neurons are distinguished on the base of their spike-amplitude. Bar represents 1 s stimulations.

Table 1
Identified ORNs (ORN1–ORN7) in *Libellula depressa*, responding with seven different patterns to the tested odors. For the tested odors, we report only the average net response (mean ± SE) with the firing frequency exceeding in absolute value approximately twice the mean standard deviation of the firing frequencies of the various neuronal types for the control stimuli (de Bruyne et al., 2001), i.e. eight spikes/s. In italics it is reported the response (spikes/sec) (mean ± SE) for the controls. The background firing is expressed as mean firing rate of 40 s (spikes/s) (mean ± SE).

Stimuli	ORN1	ORN2	ORN3	ORN4	ORN5	ORN6	ORN7
(Z)-3-hexenyl acetate	15	43 ± 1.5	26 ± 4.5				
(E)-2-hexenal		11 ± 5.5	34 ± 7.0	18 ± 3.6			
(Z)-3-hexen-1-ol					−10		
Octanal			51 ± 3.5	24 ± 4.6	31		26
Paraffin oil	*1*	*0 ± 1.0*	*−8 ± 2.0*	*5 ± 1.7*	*2*		*−2*
Propionic acid	−11					25 ± 10.2	22
Butyric acid						27 ± 3.0	
1,4-Diaminobutane							
Distilled water	*2*					*2 ± 1.5*	*0*
Background firing	8	16 ± 0.6	19 ± 2.2	4 ± 1.0	7	9 ± 0.5	6
Number of stimulations	1	2	2	10	1	4	1
Number of cells	1	1	1	2	1	1	1
Insect gender	♂	♂	♀	♀	♀	♂	♀

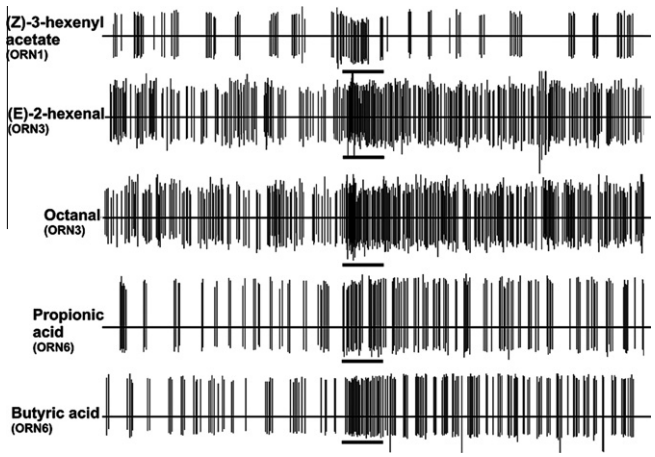

Fig. 10. Examples of analyzed recordings (SCR) showing excitatory responses of different ORNs to odors. Bars represent 1 s stimulations.

particular, in females all the tested chemicals elicited response except (Z)-3-hexen-1-ol, while in males the tested chemicals elicited response except (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol and butyric acid. Differences between male and female antennae have been highlighted also in the EAG dose–response curves to octanal. In both sexes EAG responses generally increased in amplitude with increasing doses of the chemicals, but saturation was reached at a concentration of 10% in (E)-2-hexenal in both sexes, while in octanal only males reached saturation at a concentration of 10% whereas females reached saturation at a lower concentration (1%). After saturation, the response tends slightly to decrease (even if there is no significant statistical difference between the response at saturation and the higher concentrations for both chemicals), maybe owing to the decreased volatility of the compounds at the higher dose or to an olfactory adaptation at high concentrations or to unexplained physiological phenomena, as reported in other EAG investigations (e.g. Sant'Ana and Dickens, 1998; Zhou et al., 1999).

The magnitude of the EAG response in *L. depressa* is similar in males and females and fairly weak if compared with that shown by other insects. This observation is consistent with the low number of olfactory sensilla located on the antennae of this species, about 40 on each antenna (each sensillum is innervated by three neurons leading to a total of only 120 olfactory receptor neurons (ORNs) in each antenna) (Rebora et al., 2008). Weak absolute EAG responses have been recorded in other insects with a low number of olfactory sensilla such as *Scaphoideus titanus* (Cicadellidae) showing an absolute EAG response to odors from the host plant of about 0.09 mV (Mazzoni et al., 2009) while absolute EAG responses to some chemicals with an amplitude of voltage of about 1–4 millivolt are typical of Hymenoptera, Diptera, Lepidoptera (e.g. Park et al., 2002; Ngumbi et al., 2010).

Since the activity in a restricted number of neurons may result in weak EAG responses (Wibe, 2004), we decided to carry out SCR from the antennal flagellum of *L. depressa*. Olfactory coeloconic sensilla have been studied in *Drosophyla melanogaster* (Yao et al., 2005). In this regard, it is worth to remember the technical difficulty of obtaining high-quality electrophysiological recordings from coeloconic sensilla. In addition to their small size, they are more susceptible to damage by the electrode and yield recordings with lower signal to noise ratios than the other types of sensilla (Yao et al., 2005). In our insects, unfortunately, it was not possible to ascertain if the recordings were made from sensilla coeloconica, as the pits containing these sensilla are indistinguishable from those hosting the deeply sunken sensilla styloconica (thermo-hygroreceptors) (see Section 2.4.1). In addition, owing to the incomplete electrical isolation of receptor neurons from neighboring sensilla, our spikes trains cannot be certainly referred to only one sensillum. On this account, it is interesting to note that frequently we recorded from sensory neurons not responding to the tested odors. These neurons could be olfactory neurons belonging to coeloconic sensilla responding to other odors or could belong to the deeply sunken thermo-hygroreceptors described in *L. depressa* (Piersanti et al., 2011; Rebora et al., 2008). However, we must consider that different association patterns of sensory specificity could exist; as reported for *Drosophila*, in which an olfactory coeloconic sensillum may respond to humidity changes (Yao et al., 2005), or *Periplaneta*, whose antennae are equipped with two different cold receptors, one present in a thermo-hygroreceptive sensillum and the other associated with an olfactory sensillum (Nishikawa et al., 1992).

That being stated, our SCR data identify in *L. depressa* seven ORNs responding with seven different patterns to the tested odors. As in EAG investigations, where octanal showed relatively larger EAG responses, in SCR octanal elicited response in four ORNs, while (E)-2-hexenal, (Z)-3-hexenyl acetate and propionic acid elicited response in three ORNs and butyric acid elicited response only in one ORN. (Z)-3-hexen-1-ol, which in EAG did not elicit any response, in SCR elicited only one inhibitory response. A striking difference between the results obtained with the two techniques concerns 1,4 diaminobutane that in EAG elicited response both in males and females, while in SCR did not elicit any response. This difference could be due to the low number of identified ORNs and must be further investigated; in any case, it has been reported that the

two recording techniques can give different results, both quantitatively and qualitatively (Wibe, 2004).

On the whole, our EAG and SCR data show unambiguously for the first time the presence of olfactory neurons in Odonata antennae and strongly support the olfactory function of the coeloconic sensilla located on the antennal flagellum of this species.

In consideration of the widespread presence of olfactory coeloconic sensilla in Anisoptera and Zygoptera antennae (Rebora et al., 2009a; Piersanti et al., 2010), we can attribute the ability to perceive odors to all the whole order. Odonata are generalist predators, mainly visually oriented in catching prey as well as in mate recognition and oviposition. As reported by Corbet (1999), these insects detect more general cues visually and the final, more specific cues, by means of other sensory modalities. In this regard, studies on the chemical ecology of dragonflies and damselflies could shed light on aspects of their sensory biology largely disregarded so far, thus offering a new insight in insects chemical ecology.

Notwithstanding the lack of differences in the number and morphology of the olfactory sensilla in males and females of *L. depressa* (Rebora et al., 2008), our EAG data identified some differences between the two sexes. Thus, in general, female antennae look like more sensitive to the tested chemicals but, in consideration that these EAG investigations are simply directed to test the presence of functional olfactory sensilla on Odonata antennae, at present no hypothesis can be put forward on the biological significance of this difference. Electrophysiological activity may not necessarily indicate behavioral activity (Park et al., 2001), and the biological role of olfactory responses in Odonata must be determined in behavioral bioassays coupled with further electrophysiological investigations.

The identification of olfactory neurons in Odonata antennae is relevant not only for the biology of this insect group but, in consideration of its basal position, also for tracing evolutionary trends in insect olfaction. In this regard, it is interesting to note that similar olfactory coeloconic sensilla have been recently described in Ephemeroptera (Rebora et al., 2009b, 2010), which, together with Odonata, are Paleoptera, the oldest winged insects. The presence of coeloconic sensilla on the antennae of Paleoptera is coherent with the old origin of this kind of receptors (Steinbrecht, 1997). Coeloconic olfactory sensilla have been described in *Drosophila* where single cell electrophysiological investigations (Yao et al., 2005) suggested that the specificities of these olfactory receptor neurons may reflect the basic need of an ancestral insect. In particular, among other chemicals, the ORNs of *Drosophila* coeloconic sensilla respond to some aldehydes, acids and amines (Yao et al., 2005), likewise the antennal receptors of both sexes of *L. depressa*.

The presence of functioning olfactory receptors in Odonata raises questions about the processing of the olfactory information at brain level. Indeed neuroanatomical studies on insect brain (Strausfeld et al., 1998, 2009; Farris, 2005) – relating the ability to perceive odors to the complexity of the antennal lobe and the dimension of the calices of the mushroom bodies – hypothesized that paleopteran insects, such as Ephemeroptera and Odonata, are probably all anosmic with respect to airborne odors because these insects lack glomerular antennal lobes and well developed calices. In this regard, some recent neuroanatomical studies revealed that some Hemiptera, showing on their antennae a very sparse sensillar setup (with a low number of ORNs) (Kristoffersen et al., 2006), but able to perceive odors according to electrophysiological investigations (Kristoffersen et al., 2008b), show aglomerular antennal lobes (Kristoffersen et al., 2008a). This condition of aglomerular antennal lobes in insects able to perceive odors has been presented as unique but could be common to other insects such as Odonata. Neuroanatomical investigations on Paleoptera olfactory pathway could help to have insight into the organization of primary olfactory centers in insects. Among basal insects, detailed neuroanatomical data on the antennal lobe are available for Archaeognatha (Mißbach et al., 2011) and some preliminary observation has been published for Zygentoma (Schachtner et al., 2005), but no detailed studies on Paleoptera antennal lobes have been published so far.

Another intriguing aspect of Odonata olfaction is related to the presence of odorant receptor (OR) genes. Starting from their discovery in *D. melanogaster* (Vosshall et al., 1999), OR genes have been identified in several species (see reviews in Jacquin-Joly and Merlin, 2004; de Bruyne and Baker, 2008; Kaupp, 2010). Although an increasing number of insect OR sequences have been identified, investigations have been mainly focused on insect species having an economical impact, such as crop pests or vector diseases (all Neoptera) (Walton et al., 2005), whereas key species relevant for an evolutionary perspective, such as Odonata, have been disregarded. A subfamily of ionotropic glutamate receptors, the ionotropic receptors (IRs) has been recently identified in coeloconic olfactory sensilla of *D. melanogaster* as a new class of olfactory receptors (Benton et al., 2009). IRs are expressed in olfactory organs across Protostomia (encompassing arthropods, nematodes and molluscs) indicating that they represent an ancestral protostome chemosensory receptor family (Croset et al., 2010). Paleoptera antenna, bearing coeloconic sensilla and not basiconic or trichoid sensilla (housing OR-expressing neurons in all other insects examined), could potentially house IR-expressing neurons (Croset et al., 2010). Using *L. depressa* as insect model, the sequenced antennal transcriptome could shed light on the molecular basis of olfaction in Paleoptera and could contribute to trace evolutionary trends in insect odor perception and OR origin.

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