

## Specialized olfactory receptor neurons mediating intra- and interspecific chemical communication in leafminer moths *Eriocrania* spp. (Lepidoptera: Eriocraniidae)

Mattias C. Larsson<sup>1,2,\*</sup>, Eric Hallberg<sup>3</sup>, Mikhail V. Kozlov<sup>4</sup>, Wittko Francke<sup>5</sup>, Bill S. Hansson<sup>1,2,†</sup> and Christer Löfstedt<sup>1</sup>

<sup>1</sup>Department of Ecology, Lund University, SE-223 62 Lund, Sweden, <sup>2</sup>Department of Crop Science, Chemical Ecology, Swedish University of Agricultural Sciences, PO Box 44, SE-230 53 Alnarp, Sweden, <sup>3</sup>Department of Zoology, Lund University, SE-223 62 Lund, Sweden, <sup>4</sup>Section of Ecology, Biological Faculty, University of Turku, FIN-20014 Turku, Finland and <sup>5</sup>Institute of Organic Chemistry, University of Hamburg, Martin Luther-King-Platz 6, D-20146, Hamburg, Germany

\*Present address: Department of Crop Science, Chemical Ecology, Swedish University of Agricultural Sciences, PO Box 44, SE-230 53 Alnarp, Sweden (e-mail: mattias.larsson@vv.slu.se)

†Present address: Department of Crop Science, Chemical Ecology, Swedish University of Agricultural Sciences, PO Box 44, SE-230 53 Alnarp, Sweden

Accepted 21 January 2002

### Summary

We performed a physiological and morphological characterization of sensilla auricillica in male *Eriocrania semipurpurella* moths. Each auricillic sensillum contained three olfactory receptor neurons. Responding neurons (87 of 139) could be grouped into five physiological types. Type 1 responded to (*R,Z*)-6-nonen-2-ol and type 2 to its enantiomer (*S,Z*)-6-nonen-2-ol, both of which are pheromone components of *E. semipurpurella*. Type 3 responded to both (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol, which are pheromone components of the sympatric species *E. cicatricella*. Types 4 and 5 responded to the ketones (*Z*)-6-nonen-2-one and/or nonan-2-one, which are found in the pheromone glands of female *E. semipurpurella*.

Field-trapping showed that type 3 receptor neurons mediate strongly antagonistic effects of (*R*)-heptan-2-ol

and (*R,Z*)-4-hepten-2-ol on *E. semipurpurella*, while nonan-2-one should possibly be included as a synergist in the sex pheromone blend of this species. The attraction of *E. cicatricella* and *E. sparrmannella* to compounds mixed with the pheromone blend of *E. semipurpurella* shows that the pheromone components of *E. semipurpurella* have little or no antagonistic effects on these species.

The morphology and physiology of eriocraniid pheromone sensilla are very similar to those found in the order Trichoptera (caddisflies), suggesting a homology between pheromone detection systems in the two sister orders Lepidoptera and Trichoptera.

Key words: pheromone, single sensillum, receptor neurone, antagonist, enantiomer, chirality, behaviour, leafminer moth, *Eriocrania* spp.

### Introduction

The use of long-range pheromones predates the phylogenetic separation of the Lepidoptera (moths and butterflies) from its sister order Trichoptera (caddisflies). The basal lineages of Lepidoptera are similar to those of Trichoptera with regard to pheromone communication (symplesiomorphy), whereas advanced lepidopterans demonstrate significant apomorphic changes (Löfstedt and Kozlov, 1997). In caddisfly species in which pheromone components have been identified, these consist of short-chain (7–9 carbon atoms) secondary alcohols and ketones released from paired glands with their openings on abdominal sternite V (Löfstedt et al., 1994; Bjostad et al., 1996). Similar compounds have been identified as pheromone components in the lepidopteran families Eriocraniidae (Zhu et al., 1995;

Kozlov et al., 1996) and Nepticulidae (Tóth et al., 1995). In the Eriocraniidae, which is the most primitive moth family in which pheromone communication has been confirmed, these pheromones are released from glands apparently homologous to those in the Trichoptera. Unlike most moth pheromones (Arn et al., 1992), eriocraniid pheromone components are usually chiral, and the enantiomeric composition has a profound influence on pheromone activity (Zhu et al., 1995; Kozlov et al., 1996).

In ditrysian moths, comprising the majority of moth species, the male pheromone detection apparatus has been thoroughly studied. These moths use specialized olfactory receptor neurons, housed in long sensilla trichodea, to detect pheromone components released by females. Each component of the

female pheromone blend is typically detected by a corresponding type of receptor neuron on the male antenna (Boeckh, 1984). Except for electroantennographic recordings, no electrophysiological investigations of pheromone receptor neurons have been performed in the Eriocraniidae or other primitive moth families.

Some single-cell data on pheromone reception are available from the sister order Trichoptera. Males and females of the caddisfly *Rhyacophila nubila* have receptor neurons responding to heptan-2-one, heptan-2-ol, nonan-2-one and nonan-2-ol (Larsson and Hansson, 1998), which have been identified from the female sternal glands (Löfstedt et al., 1994). The behavioural significance of these compounds has not been established in *R. nubila* however. In the related species *Rhyacophila fasciata*, in which pheromonal activity has been confirmed (Löfstedt et al., 1994), both sexes also have receptor neurons with response properties similar to those found in *R. nubila* (M. C. Larsson and B. S. Hansson, unpublished results). In both species, the receptor neurons have a high ability for chiral discrimination, although the behavioural effects of the chirality of the compounds are unknown.

We have performed an electrophysiological and morphological investigation of pheromone sensilla in the eriocraniid moth *Eriocrania semipurpurella*. The purpose of our investigation was to study pheromone detection in a primitive moth and to make a comparison with what is known about the detection of structurally similar compounds in caddisflies. As olfactory stimuli, we used a set of ketones and secondary alcohols that function as semiochemicals in *E. semipurpurella* and the sympatric species *E. cicatricella*, *E. sparrmannella* and *E. sangii*. The results from the single-sensillum study prompted us to perform field-trapping experiments to elucidate the behavioural role that the electrophysiologically active compounds play in the chemical communication of *Eriocrania* spp.

## Materials and methods

### *Experimental animal*

*Eriocrania semipurpurella* Stph. is a tiny (wing span 11–14 mm) diurnal moth abundant in the Northern boreal forest zone at the time of the birch leaf flush. Earlier studies have demonstrated that (*R,Z*)- and (*S,Z*)-6-nonen-2-ol are the principal components of the female pheromone in this species; however the most attractive ratio of these two enantiomers exhibited significant geographical variation (Kozlov et al., 1996). Analysis of the Random Amplified Polymorphic DNA (RAPD) variation between individual moths has led to the discovery of large population subdivisions in *E. semipurpurella*: three sympatric 'pheromone races' were discovered in southwest Finland (P. Metcalfe, M. V. Kozlov, W. Francke and C. Löfstedt, unpublished results). Since these races also exhibit morphological differences, they should (most probably) be treated as different species (O. Karsholt, N. P. Kristensen and M. V. Kozlov, unpublished results). Only one race was found in Lund, whereas the material collected from

Turku might include up to three races; this, however, seems unlikely because we used only one enantiomeric composition to collect moths for our study (see below). Since the taxonomic treatment of this species complex is not yet complete, we refer to our study object as *E. semipurpurella* (*sensu lato*).

### *Collection of experimental animals*

For electrophysiological studies, we caught live *E. semipurpurella* males in hollow containers baited with a blend of 50 µg of (*R,Z*)-6-nonen-2-ol and 50 µg of (*S,Z*)-6-nonen-2-ol. The animals were caught in 1995, 1996 and 1997 at two field sites with birch stands outside Lund, Sweden, and outside Turku, Finland. For morphological studies, we caught *E. semipurpurella* males in Lund by netting.

### *Chemicals*

The nine compounds used in the electrophysiological and behavioural experiments are listed in Tables 1 and 2. (*R*)-heptan-2-ol and (*S*)-heptan-2-ol were purchased from Aldrich Chemical Co. The synthesis of all other compounds followed the procedures described previously, and chemical and enantiomeric purity were checked by chiral gas chromatography (Kozlov et al., 1996). The chemical purity of all compounds was at least 99%. The enantiomeric excess for all the chiral compounds was 98% or greater, i.e. no more than 1% of the antipode was present. The compounds were dissolved in hexane and then diluted in decadic steps.

### *Electron microscopy*

For scanning electron microscopy, antennae from male *E. semipurpurella* were fixed in 70% ethanol, dehydrated and air-dried. The antennae were mounted on holders with white glue and sputter-coated with gold/palladium. The specimens were studied in a JEOL T330 scanning electron microscope operated at 10 kV. For transmission electron microscopy, the antennae were excised, cut into smaller pieces and immersed in 4% glutaraldehyde in 0.15 mol l<sup>-1</sup> cacodylate buffer for 8 h. Postfixation was carried out in 1% osmium tetroxide for 2 h. After dehydration, the specimens were embedded in Epon resin and polymerized. Sections cut on a diamond knife were stained with uranyl acetate and lead citrate in an LKB ultrastainer. The sections were examined in a JEOL 1200 EX transmission electron microscope. Olfactory sensilla were classified according to morphological criteria, i.e. the structure of pores (Altner, 1977).

### *Electrophysiology*

Odour stimuli for electrophysiological experiments were prepared by pipetting 10 µl samples of hexane with dissolved compound onto small pieces of filter paper (approximately 10 cm × 15 mm) placed in Pasteur pipettes and letting the solvent evaporate. The amount of compound deposited in each pipette ranged from 10 pg to 100 ng in decadic steps for the dose/response trials. A blank cartridge containing only filter paper plus solvent was also prepared. For screening, we used 1 µg in each pipette. The test stimuli were kept at

Table 1. Eriocrania species for which semiochemicals have been identified in previous field-trapping studies

Species	Compound	Ratio	Role
<i>E. semipurpurella</i> <sup>1</sup>	( <i>R,Z</i> )-6-nonen-2-ol	50	Pheromone component
	( <i>S,Z</i> )-6-nonen-2-ol	50	Pheromone component
	Nonan-2-one	250	Antagonist?
	( <i>Z</i> )-6-nonen-2-one	3700	Antagonist?
<i>E. sangii</i> <sup>1</sup>	( <i>R,Z</i> )-6-nonen-2-ol	Trace <sup>3</sup>	Antagonist
	( <i>S,Z</i> )-6-nonen-2-ol	100	Pheromone component
	Nonan-2-one	50	Antagonist?
	( <i>Z</i> )-6-nonen-2-one	1500	Antagonist
<i>E. cicatricella</i> <sup>2</sup>	( <i>R</i> )-heptan-2-ol	15	Pheromone component
	( <i>R,Z</i> )-4-hepten-2-ol	100	Pheromone component
	( <i>Z</i> )-4-hepten-2-one	0.5	Pheromone component
<i>E. sparrmannella</i> <sup>2</sup>	( <i>S</i> )-heptan-2-ol	?	Attractant <sup>4</sup>
	( <i>S,Z</i> )-4-hepten-2-ol	?	Attractant <sup>4</sup>

All nine compounds shown have been used as electrophysiological and/or behavioural stimuli in the present study. Note that some compounds are duplicated for more than one species.

The table also shows the ratios at which the compounds have been found in females of the respective species (ratios only valid within species). Relative amounts are based on the 50 µg+50 µg loads of the major pheromone components of *E. semipurpurella* used for field-trapping in the present study.

<sup>1</sup>Kozlov et al. (1996); <sup>2</sup>Zhu et al. (1995); <sup>3</sup>found in trace amounts, according to electroantennogram responses from male antennae; <sup>4</sup>attracts males to traps, but female glands have not been examined.

–20 °C when they were not in use to minimize evaporation of the test compounds. Each test cartridge was replaced within 2 days.

For the single-sensillum recordings, moths were restrained in holders cut from small, plastic micropipette tips (Tamro MedLab, Mölndal, Sweden), and the head and antennae were fixed with dental wax. A thin silver wire, serving as a ground electrode, was inserted into the abdomen. Contact was established with receptor neurons by means of tungsten microelectrodes, sharpened electrolytically in KNO<sub>2</sub>-solution and inserted near the base of olfactory sensilla (Boeckh, 1962). A binocular microscope with up to 300× magnification and two Leitz micromanipulators were used to position the animals and the recording electrode.

A charcoal-filtered and humidified air stream was blown over the antenna at a constant velocity of 0.5 m s<sup>-1</sup> through a glass tube with the outlet approximately 15 mm from the antenna. Stimulation was performed by inserting the tip of the test pipette into a hole, 15 cm from the outlet of the glass tube,

and blowing air (2.5 ml over 0.5 s) through the pipette. The stimulus air puffs were generated by a Syntech SFC-1/b stimulus controller (Hilversum, the Netherlands).

Upon contact with a sensillum (or sensilla), distinguished by spontaneous or penetration-induced action potentials ('spikes') from receptor neurons, we stimulated the antenna with the blank plus all the test compounds at the screening dose. If a neuron responded to any of the test compounds with a change in spike frequency different from that to the blank, a dose/response trial was performed in which all active compounds were presented to the antenna at all doses. All stimuli were delivered by dose level, starting with the lowest dose. At each dose level, the stimuli were delivered at random with an interval of at least 20 s, except at the higher levels, where the stimuli showing the lowest response were delivered first. At these high levels, it was also necessary to allow longer intervals between stimuli to let the neurons recover their normal spontaneous activity. Some neurons were classified only according to their responses to the screening stimuli.

The signal was amplified using a low-pass/high-pass high-impedance amplifier constructed in our laboratory. During the experiments, the neural responses were visualised on an oscilloscope and stored on videotape for later processing. The filtering of the signal through the amplifier did not suffice to remove enough low-frequency noise from the recordings, resulting in low signal-to-noise ratios for action potentials with low amplitudes. The signal from the videotape was therefore filtered a second time through a Syntech UN-06 amplifier with better filtering capacity before being transferred to a Compaq ProLinea 4/66 computer for analysis with the program Syntech Auto Spike v.3.0. The identification of individual neurons was based on differences in the amplitudes of their action potentials. The action potentials were counted, and the net response to a stimulus was calculated as the number of spikes over 1 s after stimulation minus the number of spikes over 1 s before stimulation. The net response to the blank was subtracted to avoid any non-specific responses, for example to the air puff.

#### Field-trapping

We conducted the field-trapping experiments in 1998, at a field site outside Lund, using Delta traps. Within each replicate, the traps were randomly placed at least 5 m apart, suspended 1–2 m above the ground from the branches of young birch trees. The traps were emptied each day, except at the end of the second trial (see below), when they were left for a whole week to catch the last stray moths at the end of the field season. After each sampling time, the position of each trap was changed to minimize the effects of habitat heterogeneities. Odour dispensers for field-trapping experiments were prepared from red rubber septa (Arthur H. Thomas Co.; Catalogue no. 1780-J07) by pipetting 100 µl samples of hexane containing blends of dissolved compounds onto each rubber septum.

During the first trial, conducted on 23–28 April, the effects of adding electrophysiologically active compounds to the previously reported pheromone blend of *E. semipurpurella*



were tested. In each replicate, a reference bait containing 50 µg each of (*R,Z*)-6-nonen-2-ol and (*S,Z*)-6-nonen-2-ol was compared with six other baits containing the same blend plus 5 µg of another compound. The other compounds tested were (*R*)-heptan-2-ol, (*S*)-heptan-2-ol, (*R,Z*)-4-hepten-2-ol, (*S,Z*)-4-hepten-2-ol, (*Z*)-6-nonen-2-one and nonan-2-one. Treatments were compared with the reference bait using a Mann–Whitney *U*-test for pairwise comparisons.

In the second trial, conducted from 29 April to 7 May, the effects of adding different amounts of compounds to the pheromone blend of *E. semipurpurella* were tested. The same type of reference bait as in the first trial was used, while seven other baits containing the pheromone blend plus (*R*)-heptan-2-ol (0.5 and 5 µg), (*S,Z*)-4-hepten-2-ol (0.5, 5 and 50 µg), (*Z*)-6-nonen-2-one (0.5 µg) and nonan-2-one (0.5 µg) were tested. All treatments were compared using a Kruskal–Wallis analysis of variance followed by a Mann–Whitney *U*-test for pair-wise comparisons.

## Results

### Electron microscopy

According to morphological criteria, there were two types of olfactory sensilla on the antennae of the males of *E. semipurpurella*: trichoid and auricillic sensilla. The antenna was covered with scales, forming a coat above the surface, but these scales were often dislodged from their sockets (Fig. 1A,B).

Auricillic sensilla were the most numerous, with their leaf-shaped cuticular hairs arranged along the whole antennal surface. Each auricillic sensillum was 7–10 µm long and 4–5 µm wide and had a short shaft connecting it to the antennal surface. Its surface was provided with longitudinal furrows in which the pores were found (Fig. 1A). The numerous pores were found on both sides of the flattened cuticular hair. The cuticular walls of the hair had a maximal thickness of approximately 100 nm, and the pores were marked by invaginated less-electron-dense areas of the hair cuticle. Each auricillic sensillum was innervated by three sensory cells ( $N=27$ ), with branched dendritic outer segments within the hair structure. In cross sections of the hairs, there were approximately 100 branches with a diameter of approximately 50 nm (Fig. 1C).

The trichoid sensilla were found only on the ventral part of the antennae in groups of approximately 10 on the proximal part of

each flagellomere. The slightly curved cuticular hairs were approximately 25 µm long, and their distal parts were bent towards the antennal surface (Fig. 1B). The cuticular walls (thickness approximately 120 nm) were provided with pores, but the density of pores was considerably lower than in the auricillic sensilla. Each trichoid sensillum was innervated by two sensory cells ( $N=10$ ), which had branched dendritic outer segments. The branching of the dendritic outer segments was moderate compared with that of the auricillic sensilla: in cross sections, there were 4–5 branches in the middle part of the hair (Fig. 1C).

### Electrophysiology

We obtained 83 recordings from 26 male moths that lasted long enough for a characterization with all screening stimuli.

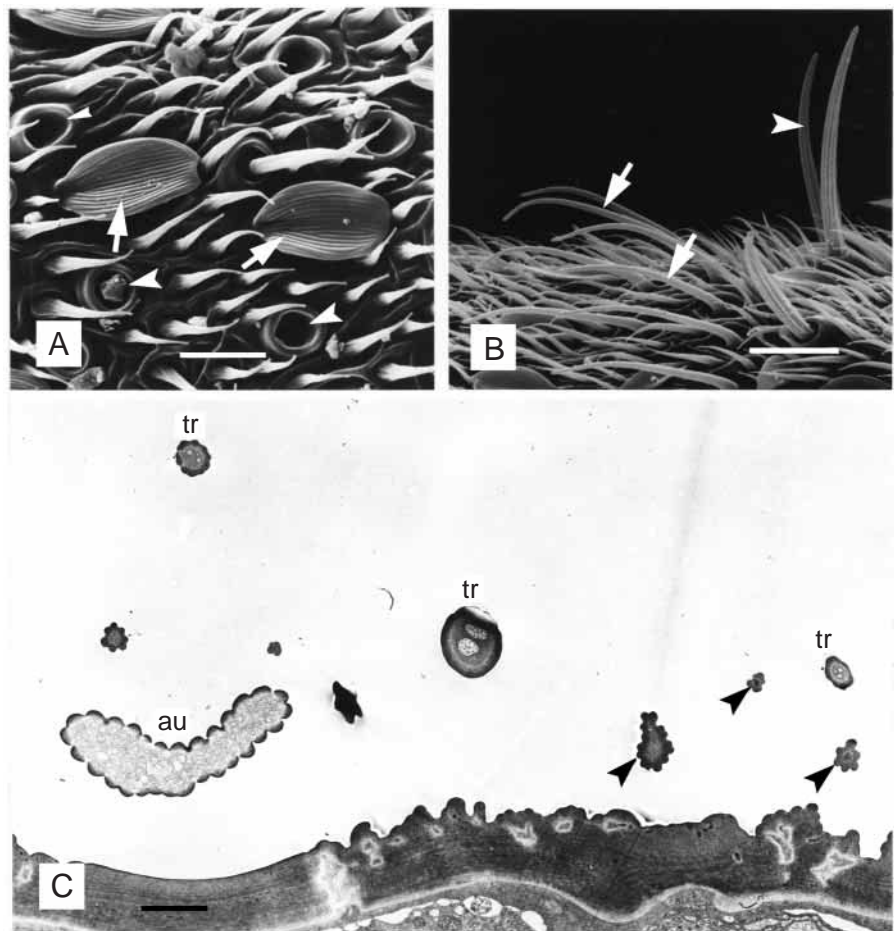
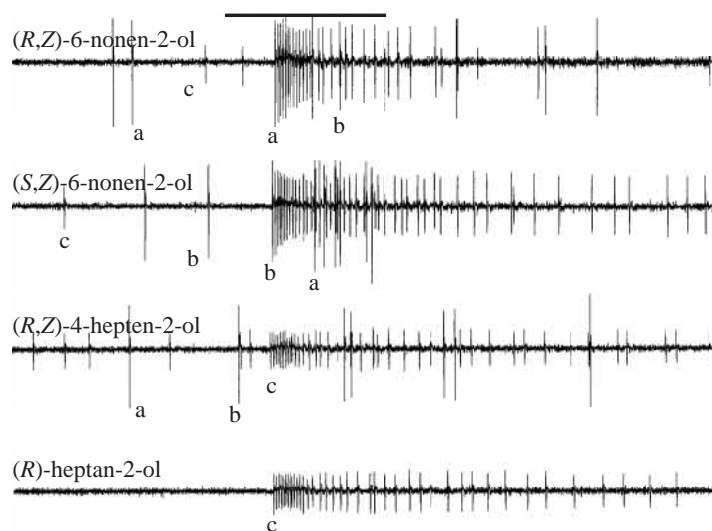


Fig. 1. Electron micrographs of antennae of male *Eriocrania semipurpurella*. (A) Scanning electron micrograph showing two sensilla auricillica (arrows) and sockets from dislodged scales (arrowheads). The short hairs present on the antennal surface are non-innervated cuticular structures (compare C). Scale bar, 5 µm. (B) In addition to the auricillic sensilla, trichoid sensilla (arrows) occur basally on the flagellomeres. Stout chaetic sensilla (arrowhead) are found on the distal parts of the flagellomeres. Scale bar, 10 µm. (C) Transmission electron micrograph showing a transverse section of sensilla above the antennal surface (bottom) of a male *E. semipurpurella*. The auricillic sensillum (au) contains numerous branches of the dendritic outer segments, whereas the trichoid sensilla (tr) have moderately branched dendritic outer segments. Non-innervated hairs (arrowheads) are present between the sensilla. Scale bar, 1 µm.



Contacts were obtained mainly from the ventral side of each antennae, in the medial parts of the flagellomeres. The sensilla from which we recorded could not be identified with certainty in the light microscope, but the location of the recording electrode showed that the recordings stemmed from auricillic sensilla. In each recording, we simultaneously registered spikes from 1–3 neurons with different spike amplitudes. The smaller spikes could not be clearly distinguished during the experiments, but after filtering through a second amplifier they were clearly distinguishable from the baseline noise (Fig. 2). All responses to test stimuli were characterized by an increase in spike frequency relative to the spontaneous level of activity. We recorded from 139 receptor neurons, of which 87 responded to one or several of our test stimuli with a higher spike frequency than to the blank. Blank stimuli generally elicited no spikes or very few spikes above the spontaneous level of activity. Responding neurons could all be grouped into five separate types depending on their response characteristics (Fig. 3; Table 2). We found all five types of receptor neuron in moths from both Sweden and Finland.

The two most common types of receptor neuron, designated type 1 and type 2, responded to the two pheromone components of *E. semipurpurella*. Type 1 neurons responded selectively to (*R,Z*)-6-nonen-2-ol, while type 2 neurons responded selectively to (*S,Z*)-6-nonen-2-ol. Both types of receptor neuron had a high ability for chiral discrimination, with the sensitivity to the key compound being at least 100 times higher than to the respective enantiomer (Fig. 3; Table 2).

Another set of receptor neurons, designated type 3, responded identically to two different compounds, (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol, which have not been identified in extracts of *E. semipurpurella*. The type 3 neurons had an ability for chiral discrimination similar to that of the type 1 and type 2 neurons, i.e. a 100-fold difference in sensitivity between the two key compounds and their respective enantiomers (Fig. 3; Table 2).

Type 4 neurons responded to both nine-carbon ketones, with

Fig. 2. Simultaneous recordings from three neurons with different spike amplitudes (marked with a, b and c; see Table 3) stimulated with four different compounds (source dose 1 ng). The a neuron (type 1) responded to (*R,Z*)-6-nonen-2-ol, the b neuron (type 2) to (*S,Z*)-6-nonen-2-ol and the c neuron (type 3) to both (*R,Z*)-4-hepten-2-ol and (*R*)-heptan-2-ol. The horizontal bar shows the duration of the stimulus period (0.5 s).

a preference for (*Z*)-6-nonen-2-one over nonan-2-one, while type 5 neurons responded slightly to nonan-2-one alone, but only at the highest doses (100 ng to 1 µg).

Specific combinations of 2–3 neurons were frequently encountered (Table 3). Presumably these neurons resided in the same sensillum, although some cases of recordings from more than one sensillum cannot be excluded. Neurons may also occasionally have been damaged by the penetrating electrode and therefore never recorded or not responded. When two or more neurons were found together, the relationship between their spike amplitudes was usually, but not always, similar between different recordings. Receptor neurons for the two pheromone components usually had the highest spike amplitudes, with type 1 > type 2, while other physiological types had lower amplitudes. Non-responding neurons typically had the lowest spike amplitudes (Table 3).

#### Field-trapping

In the first series of field-trapping experiments, (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol dramatically suppressed the catch of *E. semipurpurella* males when added to the *E.*

Table 2. Frequency and response spectra of different types of olfactory receptor neuron encountered on the antennae of male *Eriocrania semipurpurella*

Stimulus	Receptor neuron type					No response
	1	2	3	4	5	
( <i>Z</i> )-4-hepten-2-one	0	0	0	0	0	0
( <i>R</i> )-heptan-2-ol	0	0	++++	0	0	0
( <i>S</i> )-heptan-2-ol	0	0	++++	0	0	0
( <i>R,Z</i> )-4-hepten-2-ol	0	0	++++	0	0	0
( <i>S,Z</i> )-4-hepten-2-ol	0	0	++++	0	0	0
Nonan-2-one	0	0	0	+++	+	0
( <i>Z</i> )-6-nonen-2-one	0	0	0	++++	0	0
( <i>R,Z</i> )-6-nonen-2-ol	++++	+	(+)	0	0	0
( <i>S,Z</i> )-6-nonen-2-ol	++	++++	(+)	0	0	0
Frequency	37	25	8	12	5	52

Receptor neuron sensitivity is defined by their approximate response thresholds to the stimuli (amounts in the stimulus pipettes): +++++, 10 pg; +++++, 100 pg; +++, 1 ng; ++, 10 ng; +, 100 ng; (+), 100–1000 ng in some cases; 0, no response.

Responses were characterized by an increase in action potentials. No response means a response not differing from the blank response, which was typically very low.

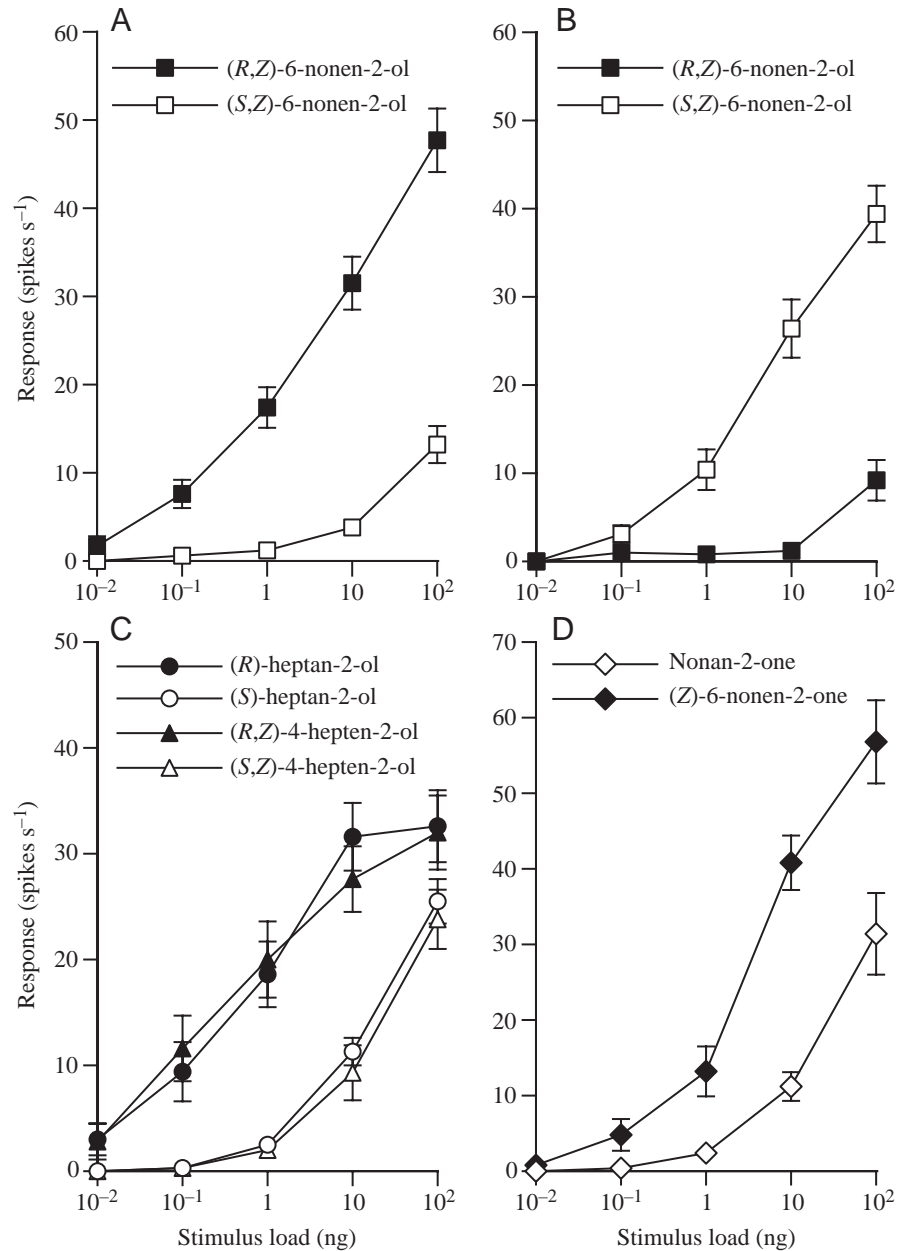


Fig. 3. Dose/response relationships of four types of olfactory receptor neuron. Values are the number of spikes over 1 s (means  $\pm$  S.E.M.). (A) Type 1 neurons responded to the pheromone component (*R,Z*)-6-nonen-2-ol, with the (*S*)-enantiomer having a 100-fold higher response threshold ( $N=13$ ). (B) The response pattern of the type 2 neurons is a mirror image of that of the type 1 neurons; they responded to the pheromone component (*S,Z*)-6-nonen-2-ol ( $N=11$ ). (C) Type 3 neurons responded to two compounds, (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol, with the response threshold for the respective (*S*)-enantiomers being approximately 100 times higher ( $N=5$ ). (D) Type 4 neurons responded to both nine-carbon ketones with a preference for (*Z*)-6-nonen-2-one over nonan-2-one ( $N=5$ ). Dose/response curves are not shown for type 5 neurons because these neurons responded only to nonan-2-one and only at high concentrations.

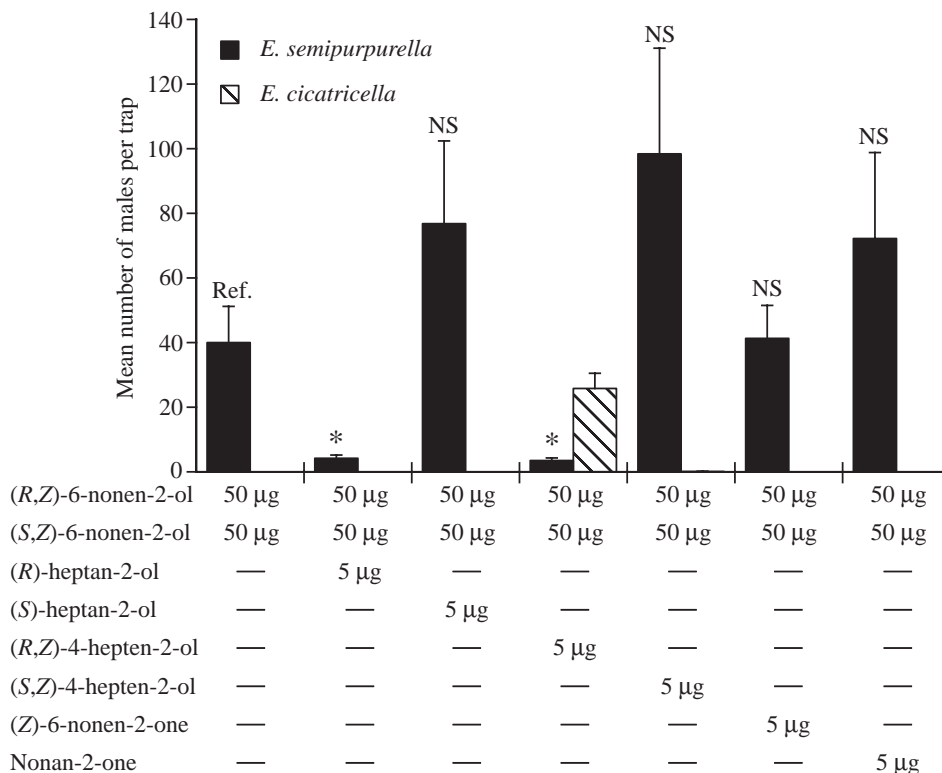
Table 3. Combinations of different physiological types of receptor neuron found together

Amplitude	Sensillum category							
	1	2	3	4	5	6	7	8
a	Type 1	Type 1	Type 1	Type 1	Type 1	Type 2	Type 4	Type 4
b	Type 2	Type 2	Type 3	Type 4	Type 4	Type 1	Type 1	Type 5
c	NR/–	Type 3	NR/–	NR/–	Type 5	NR/–	NR/–	NR/–
Frequency	14	6	2	5	2	1	3	1

Presumably, these neurons reside in the same sensillum, although some cases of recordings from more than one sensillum cannot be excluded. Neurons may also occasionally be damaged by the penetrating electrode and therefore never be recorded or counted as not responding.

a, b and c represent neurons of different spike amplitude, from the highest to the lowest amplitude (see Fig. 2). NR/– indicates a non-responding neuron or that no neuron was found in this position (i.e. no spikes with this relative amplitude). The table does not include all neurons, since some contacts yielded just a single neuron.

Fig. 4. Results from the first field-trapping experiment, 23–28 April. In each treatment, 5 µg of an electrophysiologically active compound was added to the pheromone blend of *Eriocrania semipurpurella* [reference blend (Ref.) 50 µg of (*R,Z*)-6-nonen-2-ol and 50 µg of (*S,Z*)-6-nonen-2-ol]. The graph shows the number of males of the two species *E. semipurpurella* and *E. cicatricella* caught per trap. Values are means + s.e.m. ( $N=15$ ). \*Significantly different from the reference blend (Ref.) ( $P<0.001$ ) (Mann–Whitney *U*-test); NS, no significant difference from the reference blend.

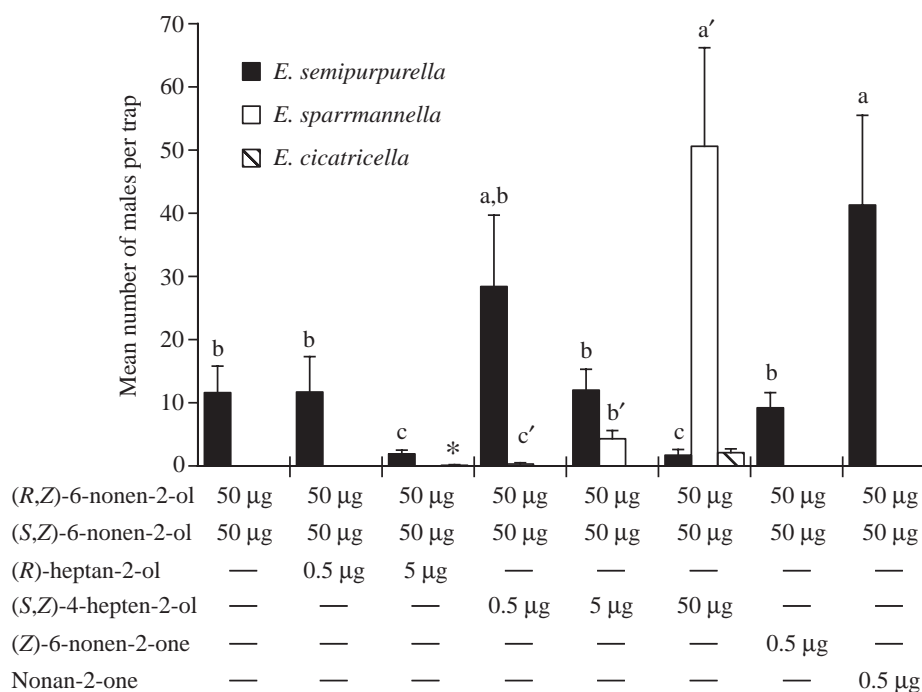


*semipurpurella* pheromone blend. Other compounds had no significant effects, although traps containing one of the two corresponding (*S*)-enantiomers or nonan-2-one caught high numbers of *E. semipurpurella* males compared with the reference traps (Fig. 4).

In the later series of field-trapping experiments, testing different doses of the potentially active compounds, (*R*)-

heptan-2-ol turned out to be antagonistic to *E. semipurpurella* at the 5 µg dose (ratios 1:1:0.1) but not at the 0.5 µg dose (ratios 1:1:0.01). (*S,Z*)-4-hepten-2-ol had no significant effect at 0.5 µg or 5 µg, but significantly reduced the catch at the 50 µg dose. The ketone (*Z*)-6-nonen-2-one had no effect, while 0.5 µg of nonan-2-one significantly increased the trap catch when added to the blend (Fig. 5).

Fig. 5. Results from the second field-trapping experiment, 29 April to 7 May. Different amounts of potential semiochemicals were added to the pheromone blend of *Eriocrania semipurpurella* [50 µg of (*R,Z*)-6-nonen-2-ol and 50 µg of (*S,Z*)-6-nonen-2-ol]. The graph shows the number of males of the three species *E. semipurpurella*, *E. sparrmannella* and *E. cicatricella* caught per trap. Values are means + s.e.m. ( $N=15$ ). One *E. cicatricella* male was caught in a trap containing 5 µg of (*R*)-heptan-2-ol (indicated with an asterisk). Columns labelled with the same letters are not significantly different (a,b,c refer to *E. semipurpurella*; a',b',c' refer to *E. sparrmannella*) (Kruskal–Wallis analysis of variance followed by pairwise comparisons with a Mann–Whitney *U*-test;  $P<0.05$ ).





In the first series of field-trapping tests, a large number of *E. cicatricella* Zett. males were caught in the traps with 5 µg of (*R,Z*)-4-hepten-2-ol (Fig. 4). In the second series, several males of this species were also caught in the traps with 50 µg of (*S,Z*)-4-hepten-2-ol (Fig. 5). During the second series of field trials, *E. cicatricella* were caught primarily at the beginning of this series. No *E. cicatricella* males were caught during May (data not shown).

The addition of (*S,Z*)-4-hepten-2-ol to the blend had a dramatic effect on the species *E. sparrmannella* Bosc., which had not been caught in traps containing this compound during the first series of trials (Figs 4, 5). Increasing the dose of (*S,Z*)-4-hepten-2-ol from 0.5 to 50 µg led to a steep increase in the catch of *E. sparrmannella* males. *E. sparrmannella* males were predominantly caught during the later part of the second series of field trials (data not shown).

### Discussion

Our study shows that antennal sensilla auricillica are the pheromone-detecting sensilla in *E. semipurpurella*. Sensilla auricillica are the only olfactory sensilla on the central part of the antennal flagellomeres, where most responding neurons were recorded. The presence of 1–3 receptor neurons in our recordings agrees with morphological data showing the presence of three neurons in each sensillum auricillicum. The function of the second type of olfactory sensillum, the trichoid sensillum, remains unclear. Neither coeloconic nor basiconic sensilla, two other sensillum types ubiquitous in moths (Keil, 1999), were found in *E. semipurpurella*. Sensilla auricillica are found exclusively in moths of the suborder Glossata, which comprises all moths from the family Eriocraniidae, as its most primitive member, to the higher moths (Kristensen, 1984) (see also Davis, 1978). In more advanced moths such as noctuids, sensilla auricillica apparently function as plant odour detectors (Anderson et al., 2000). They bear no resemblance to the trichoid sensilla typically containing pheromone-detecting receptor neurons in moths, but instead show striking similarities to sensilla placodea and other related sensilla found in several families of Trichoptera, including *Rhyacophila* species (Denis, 1985; Hallberg and Hansson, 1999). No attempts to link physiological types of receptor neuron to specific sensilla have been successful in the Trichoptera, however.

The olfactory receptor neurons found here explain the detection mechanisms behind electroantennographic responses to female extracts previously measured from male antennae (Kozlov et al., 1996). In *E. semipurpurella*, each of the two most common types of receptor neuron (type 1 and type 2) responded to one of the two identified pheromone components, (*R,Z*)-6-nonen-2-ol and (*S,Z*)-6-nonen-2-ol, respectively. These two types of neuron perform the chiral discrimination necessary to differentiate the pheromone blends of several 'pheromone races' or sibling species within *E. semipurpurella* (*sensu lato*). Both types of pheromone receptor neuron had a high ability for chiral discrimination, with the key stimulus

being at least 100 times more potent than its respective enantiomer. The dose/response characteristics of the pheromone receptor neurons in *E. semipurpurella* closely match those of receptor neurons in the two caddisfly species *R. nubila* and *R. fasciata*, in which the same or structurally related compounds were tested (Larsson and Hansson, 1998; M. C. Larsson and B. S. Hansson, unpublished results). The ability for chiral discrimination is high and appears to be of approximately the same magnitude in the *Rhyacophila* and *Eriocrania* species. However, responses to more than one enantiomer could be caused by enantiomeric impurities in the stimuli, thus reflecting the purity of the test compounds rather than the true discriminatory abilities of the receptor neurons, which in reality could be considerably higher. Studies of pheromone detection in scarab beetles, using enantiomers of exceptionally high purity, have indeed demonstrated chiral receptor neurons in which the corresponding enantiomers are almost completely inactive (Wojtasek et al., 1998; Larsson et al., 1999).

The type 3 receptor neurons identified in our study detect secondary alcohols not present in the pheromone blend of *E. semipurpurella*, but which are part of the pheromone blend of the sympatric species *E. cicatricella*. These neurons respond identically to the two compounds (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol. Nevertheless, they have the same ability for chiral discrimination as the type 1 and type 2 neurons, i.e. both key stimuli are 100 times more potent than their respective (*S*)-enantiomers (but see above). The field-trapping tests showed that both (*R*)-heptan-2-ol and (*R,Z*)-4-hepten-2-ol work as pheromone antagonists, strongly suppressing the attraction of *E. semipurpurella* to its pheromone blend when added at a dose of 5 µg (ratios 1:1:0.1) (Figs 4, 5).

Inhibition of pheromone attraction by compounds not present in the conspecific pheromone blend is a common phenomenon among insects, e.g. in the noctuid moths *Trichoplusia ni* and *Pseudoplusia includens* (Linn et al., 1988; Grant et al., 1988). These antagonist compounds have often been found in pheromone blends of sympatric species sharing some pheromone components. Pheromone antagonists are detected by specialized receptor neurons that apparently function as fail-safes to avoid attraction between heterospecifics in cases where mistakes could easily be made. Examples of specialized antagonist detectors can be found among noctuid moths (Grant et al., 1988; Almaas and Mustaparta, 1991; Cossé et al., 1998) and among beetles from several families (Mustaparta et al., 1977, 1980; Okada et al., 1992; Wojtasek et al., 1998), in which chirality often determines the difference between pheromone components and antagonists. It is worth noting that, in some cases, heterospecific pheromone components can be synergistic, as when bark beetles or longhorn beetles are attracted to heterospecific bark beetle semiochemicals that may indicate weakened tree hosts (Tømmerås et al., 1984; Allison et al., 2001).

The pheromone blends of *E. semipurpurella* and *E. cicatricella* are based on different compounds, and there seems to be no need for additional heterospecific antagonists to avoid



cross-attraction. The specialized receptor neurons and the antagonistic effect on attraction may, however, have evolved primarily for the avoidance of other *Eriocrania* species whose pheromone blends are not yet known. Antagonistic effects of heterospecific pheromone components are not always reciprocal, as is apparent in the study of Grant et al. (1988) and the present study. Both *E. cicatricella* and *E. sparrmannella* presumably lack receptor neurons for *E. semipurpurella* pheromone components because they are highly attracted to synthetic blends in which their own specific attractants make up only a fraction of the total blend. Kozlov et al. (1996) showed that non-reciprocal inhibitory effects may exist between *E. cicatricella* and *E. sparrmannella*, however.

The antagonist neurons found in *E. semipurpurella* share some features with antagonist neurons in other insect species. These neurons often have broader response spectra than pheromone neurons, which could represent an economic way of detecting several compounds mediating the same stop signal (Wojtasek et al., 1998; Baker et al., 1998; Cossé et al., 1998). Antagonist neurons are often co-localized with pheromone neurons in the same sensilla. This may have an adaptive significance for the precise timing necessary to separate odour filaments of different origin in the air (Baker et al., 1998; Fadamiro et al., 1999). Conceivably, the type 3 neurons in our investigation may actually be two neurons with identical spike amplitudes but different key stimuli. At the time of the dose/response trials, we did not perform reciprocal adaptation experiments since the nature of the type 3 neurons became firmly established only through additional filtering during data analysis. However, the absence of double spikes and other interference in the spike pattern of the type 3 neurons (Fig. 2) and the fact that both physiological recordings and morphological studies of sensilla auricillica revealed a maximum of three neurons strongly suggest that these recordings stem from single neurons.

*Eriocrania* moths sometimes appear to make mistakes in chiral discrimination, in spite of their selective receptor neurons, although the concentrations applied here may have exceeded those that the moths would normally encounter in nature. *E. cicatricella* was somewhat attracted to 50 µg doses of (*S,Z*)-4-hepten-2-ol, the antipode of its pheromone component (Fig. 5). The same compound acted as an antagonist to *E. semipurpurella* when added to the pheromone blend at a dose of 50 µg (Fig. 5). This matches the physiological response properties of the type 3 neurons, in that high doses of (*S,Z*)-4-hepten-2-ol have electrophysiological as well as behavioural effects. An alternative explanation for the electrophysiological and/or behavioural effects of (*S,Z*)-4-hepten-2-ol in these two moth species could be the presence of the enantiomer (*R,Z*)-4-hepten-2-ol as an impurity (see above).

Two types of receptor neuron (types 4 and 5) responded to ketones found in the pheromone glands of female *E. semipurpurella*. Type 4 receptor neurons responded to both (*Z*)-6-nonen-2-one and nonen-2-one (with lower sensitivity). Type 5 neurons responded to nonen-2-one, but only at high doses, which adds some doubt as to whether this compound is

really the key stimulus for these neurons. The role of the two ketones in the pheromone communication of *E. semipurpurella* is unclear. The ketones are the most abundant compounds in the pheromone gland extracts of *E. semipurpurella* and *E. sangii*. When added to the blend according to the ratios found in the females, the ketones act as inhibitors to *E. sangii* and apparently to *E. semipurpurella* as well (Kozlov et al., 1996). Our field-trapping data indicate that nonan-2-one, but not (*Z*)-6-nonen-2-one, could actually be a synergist to *E. semipurpurella* at low doses, which is difficult to explain in the context of our electrophysiological results unless we postulate the existence of other, as yet unidentified, types of receptor neuron. A definite answer to whether any of the ketones is a true pheromone component requires a re-examination of the pheromone blend, including measurements of the amounts actually released from females.

Our study has revealed a complex interplay between various semiochemicals taking place within and among different *Eriocrania* species, and it has shown how this interplay may be mediated at the level of the antennal receptor neurons. The results of our field-trapping studies further suggest a temporal separation between different *Eriocrania* species, with *E. sparrmannella* appearing later in the season than *E. semipurpurella* and *E. cicatricella*. Different 'pheromone races' of *E. semipurpurella* have also demonstrated slight temporal shifts during other experiments in Turku (P. Metcalfe, M. V. Kozlov, W. Francke and C. Löfstedt, unpublished data). The evidence obtained here suggests a close homology between Trichoptera and primitive Lepidoptera not only in their systems of pheromone release but also in the antennal systems responsible for pheromone detection. As new gland structures and pheromone components arose later in more advanced Lepidoptera, so the task of pheromone detection may have shifted to new types of sensilla housing different physiological types of receptor neuron. The possibility that these two shifts occurred in parallel is intriguing but not yet substantiated.

We would like to thank Rita Wallén for help with electron microscopy and Junwei Zhu, Fredrik Östrand, Glenn Svensson and Erling Jirle for help with field work. This work was supported by the Swedish Natural Science Research Council and the Knut and Alice Wallenberg Foundation.

## References

- Allison, J. D., Borden, J. H., McIntosh, R. L., de Groot, P. and Gries, R. (2001). Kairomonal response by four *Monochamus* species (Coleoptera: Cerambycidae) to bark beetle pheromones. *J. Chem. Ecol.* **27**, 633–646.
- Almaas, T. J. and Mustaparta, H. (1991). *Heliothis virescens*: Response characteristics of receptor neurons in sensilla trichodea type 1 and type 2. *J. Chem. Ecol.* **17**, 953–972.
- Altner, H. (1977). Insect sensillum specificity and structure: an introduction to a new typology. In *Olfaction and Taste IV* (ed. J. Lemagnen and P. MacLeod), pp. 295–303. London: Information Retrieval.
- Anderson, P., Hallberg, E. and Subchev, M. (2000). Morphology of antennal sensilla auricillica and their detection of plant volatiles in the Herald moth, *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae). *Arthropod Struct. Dev.* **29**, 33–41.

- Arn, H., Tóth, M. and Priesner, E. (1992). *List of Sex Pheromones of Lepidoptera and Related Attractants*. Paris: International Organisation for Biological Control – Western Palearctic Regional Section (IOBC – WPRS).
- Baker, T. C., Fadamiro, H. Y. and Cosse, A. A. (1998). Moth uses fine tuning for odour resolution. *Nature* **393**, 530.
- Bjostad, L. B., Jewett, D. K. and Brigham, D. L. (1996). Sex pheromone of caddisfly *Hesperophylax occidentalis* (Banks) (Trichoptera: Limnephilidae). *J. Chem. Ecol.* **22**, 103–121.
- Boeckh, J. (1962). Electrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen des Totengräbers (*Necrophorus*, Coleoptera). *Z. Vergl. Physiol.* **46**, 212–248.
- Boeckh, J. (1984). Neurophysiological aspects of insect olfaction. In *Insect Communication* (ed. T. Lewis), pp. 83–104. New York: Academic Press.
- Cossé, A. A., Todd, J. L. and Baker, T. C. (1998). Neurons discovered in male *Helicoverpa zea* antennae that correlate with pheromone-mediated attraction and interspecific antagonism. *J. Comp. Physiol. A* **182**, 585–594.
- Davis, D. R. (1978). A revision of the North American moths of the superfamily Eriocraniioidea with the proposal of a new family Acanthopteroctetidae (Lepidoptera). *Smithson. Contr. Zool.* **251**, 1–231.
- Denis, C. (1985). Étude comparée des microstructures antennaires des trichoptères adultes. *Bull. Soc. Sci. Bretagne* **57**, 103–114.
- Fadamiro, H. Y., Cossé, A. A. and Baker, T. C. (1999). Fine-scale resolution of closely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea*. *J. Comp. Physiol. A* **185**, 131–141.
- Grant, A. J., O'Connell, R. J. and Hammond, A. M. (1988). A comparative study of pheromone perception in two species of noctuid moths. *J. Insect Behav.* **1**, 75–95.
- Hallberg, E. and Hansson, B. S. (1999). Arthropod sensilla: morphology and phylogenetic considerations. *Microsc. Res. Tech.* **47**, 428–439.
- Keil, T. A. (1999). Morphology and development of the peripheral olfactory organs. In *Insect Olfaction* (ed B. S. Hansson), pp. 5–47. Berlin, Heidelberg: Springer Verlag.
- Kozlov, M. V., Zhu, J., Philipp, P., Francke, W., Zvereva, E. L., Hansson, B. S. and Löfstedt, C. (1996). Pheromone specificity in *Eriocrania semipurpurella* (Stephens) and *E. sangii* (Wood) (Lepidoptera: Eriocraniidae) based on chirality of semiochemicals. *J. Chem. Ecol.* **22**, 431–454.
- Kristensen, N. P. (1984). Studies on the morphology and systematics of primitive Lepidoptera (Insecta). *Steenstrupia* **10**, 141–191.
- Larsson, M. C. and Hansson, B. S. (1998). Receptor neuron responses to potential sex pheromone components in the caddisfly *Rhyacophila nubila* (Trichoptera: Rhyacophilidae). *J. Insect Physiol.* **44**, 189–196.
- Larsson, M. C., Leal, W. S. and Hansson, B. S. (1999). Olfactory receptor neurons specific to chiral sex pheromone components in male and female *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J. Comp. Physiol. A* **184**, 353–359.
- Linn, C. E., Jr, Hammond, A., Du, J. and Roelofs, W. L. (1988). Specificity of male response to multicomponent pheromones in noctuid moths *Trichoplusia ni* and *Pseudoplusia includens*. *J. Chem. Ecol.* **14**, 47–58.
- Löfstedt, C., Hansson, B. S., Petersson, E., Valeur, P. and Richards, A. (1994). Pheromonal secretions from glands on the 5th abdominal sternite of hydropsychnid and rhyacophilid caddisflies (Trichoptera). *J. Chem. Ecol.* **20**, 153–169.
- Löfstedt, C. and Kozlov, M. (1997). A phylogenetic analysis of pheromone communication in primitive moths. In *Insect Pheromone Research – New Directions* (ed. R. T. Cardé and A. K. Minks), pp. 473–489. New York: Chapman & Hall.
- Mustaparta, H., Angst, M. E. and Lanier, G. N. (1977). Responses of single receptor cells in the pine engraver beetle, *Ips pini* (Say) (Coleoptera: Scolytidae) to its aggregation pheromone, ipsdienol and the aggregation inhibitor, ipsenol. *J. Comp. Physiol.* **121**, 343–347.
- Mustaparta, H., Angst, M. E. and Lanier, G. N. (1980). Receptor discrimination of enantiomers of the aggregation pheromone ipsdienol, in two species of *Ips*. *J. Chem. Ecol.* **6**, 689–701.
- Okada, K., Watanabe, A., Mori, M., Shimazaki, K., Chuman, T., Mochizuki, F. and Shibuya, T. (1992). Olfactory responses to the sex pheromone component and its behavioural inhibitor in the male cigarette beetle, *Lasioderma serricorne*. *J. Insect Physiol.* **38**, 705–709.
- Tømmerås, B. Å., Mustaparta, H. and Gregoire, J.-C. (1984). Receptor cells in *Ips typographus* and *Dendroctonus micans* specific to pheromones of the reciprocal genus. *J. Chem. Ecol.* **10**, 759–769.
- Tóth, M., Szöcs, G., Van Nieuwerkerken, E. J., Philipp, P., Schmidt, F. and Francke, W. (1995). Novel type of sex pheromone structure identified from *Stigmella malella* (Stainton) (Lepidoptera: Nepticulidae). *J. Chem. Ecol.* **21**, 13–27.
- Wojtasek, H., Hansson, B. S. and Leal, W. S. (1998). Attracted or repelled? – A matter of two neurons, one pheromone binding protein and a chiral center. *Biochem. Biophys. Res. Commun.* **250**, 217–222.
- Zhu, J. W., Kozlov, M. V., Philipp, P., Francke, W. and Löfstedt, C. (1995). Identification of a novel moth sex pheromone in *Eriocrania cicatricella* (Zett.) (Lepidoptera: Eriocraniidae) and its phylogenetic implications. *J. Chem. Ecol.* **21**, 29–43.