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Electroantennogram responses of Douglas-fir seed chalcids to plant volatiles

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Abstract

Douglas fir seed chalcids females oviposit specifically into Douglas cones which they locate using olfactory and visual cues. We have tested whether this specialization was correlated with a specialized sensitivity towards cone volatiles. Field collected adults were presented a series of pure volatile chemicals. Electroantennogram responses (EAG) were recorded to generally occurring terpenoids and straight chain alcohols and aldehydes found in flowers and plant leaves. Monoterpenes identified in Douglas cone headspaces (α-pinene, β-pinene, 3-thujene, α- and β-phellandrene, γ-terpinene and myrcene) elicited lower EAG responses than fatty acid derivatives corresponding to green odours. The EAG response profile of females differed significantly from that of males. Females were very sensitive to hexanol-1, heptanal, (Z)-3 hexenol-1, terpineol and terpinen-4-ol. Male responses were significantly higher to humid air, nerolidol, thujan-4-ol, hexanal, carvacrol, piperitone and farnesol. Several compounds (Z- verbenol, carvone, jasminol, geraniol, nerol and eugenol) elicited long lasting electrophysiological responses (over 5 s) in both sexes.

Keywords: Douglas-fir seed chalcid; *Megastigmus spermotrophus*; Hymenoptera; Plant odours; Semiochemicals; Host selection; Olfaction; Electroantennogram; EAG decay; Sexual differences

1. Introduction

Phytophagous insects have evolved different strategies to adapt to their host-plants, and this has placed several constraints on their sensory systems. The peripheral olfactory system should help them to locate suitable plants or vegetation areas, for mating sites and oviposition. It is generally assumed that sensitivity differs according to the host specialization: monophagous species should possess receptors tuned to volatiles that are specific to their host plants, while polyphagous insects would respond to a broader range of chemicals (Dickens, 1984; Visser, 1986; Averill et al., 1988; Raguso et al., 1996).

This "host-specific compounds" hypothesis should be relevant to the Douglas-fir seed chalcids, *Megastigmus spermotrophus* Wachtl. This hymenopteran is a strictly monophagous parasite of Douglas-fir seeds (Hussey, 1955; Lessman, 1971). Adults emerge from cone seeds after a 1 to 4 years diapause. Mated female *M. spermotrophus* use both visual and chemical cues to locate Douglas cones (*Douglas pseudotsuga*, Pinaceae) within the forest environment (Roques, 1986). They oviposit in cones that have reached a proper maturation stage (Roques, 1986). At short range, volatiles released by cones play a major range in orientation and host selection (Roques, 1986). Mature females are attracted in Douglas forests to baits of cone extract (stage: dominant bracts with visible scales), but males never respond under the same conditions (Roques, 1986). These observations suggest that females of *M. spermotrophus* are very sensitive to specific volatiles emitted by Douglas cones during a limited period of development. Conversely, males would be more sensitive to volatiles emitted by shoots and needles where mating is suspected to occur (Hussey, 1955).

In this work, we compared the detection capabilities of males and females of *M. spermotrophus* to terpenoids which represent the dominant class of molecules in Douglas cones headspace trappings (Rappaport et al., 1992), and to generally occurring volatiles including fol-

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iar and floral alcohols aldehydes and ketones using the electroantennogram (EAG) technique. EAG recordings provide an overview of the total responsiveness of the antennae and thus allow for drawing sensitivity profiles to a series of chemicals and differences in sensitivity between sexes. It aims to propose a short list of best detected compounds that may either be attractants or repellents.

2. Material and methods

2.1. Insects

Douglas seeds containing diapausing insects were collected in a seed orchard (La Vercantière, South of France). After emergence (April–May) insects were maintained in a large glass container at 23–25°C, 65–70% r.h. under natural light conditions. Adults had access to water or water + 10% honeydew at their convenience and could mate. The mating status of experimented individuals was not checked.

2.2. Electroantennogram recording

EAG responses were recorded from 2–3 day old adults (15 females and 11 males) using a classical EAG set up. Severed heads with their 2 antennae were mounted by connecting the right antenna between 2 glass pipettes filled with saline solution and containing chlorided silver wires (Gabel et al., 1992). Records were obtained by inserting one these electrodes into the head, the other pipette contacting the tip of the antenna (Fig. 1).

2.3. Odour stimuli

Chemicals were obtained from commercial suppliers or kindly offered as GC-MS standard by Dr Jean Luc Le Quéré (INRA des Arômes, Dijon, France). All of them were > 95% pure except for the 3 mixtures of isomers (linalool (+,−), α-, β- phellandrene and α-, β-thujone). Thirty nine test compounds were selected as belonging to different chemical groups (Table 1). Test chemicals were choosen for one of the following reasons: (i) linear alcohols, aldehydes and ketones including so called "green leaf" volatiles (LA), (ii) hydrocarbon terpenoids produced by different coniferous trees (HCT), including (iii) those produced by Douglas cones (DT), (iv) oxygenated terpenoids (OT) frequently occuring as floral scent constituents, and (v) compounds including distilled water as test chemicals. Odours were diluted in paraffin oil (Prolabo), 10 μl in 1 ml (1% dilution). Paraffin oil solutions (20 μl) were pipetted immediately before experiments onto 4 cm × 0.5 cm filter papers inserted into a glass Pasteur pipette. Olfactory stimulations were delivered by a valve for 2 sec (1 ml.sec^{-1}) in a continuous airflow (10 ml.sec^{-1}; humidified compressed air), and 1 min elapsed between 2 consecutive stimulations. Odour cartridges were replaced every 5 stimulations. In order to compensate for the decrease in the antennal responsiveness, hexanol-1 was delivered at the beginning of the experiment and after each group of 5 odours as a standard stimulus.

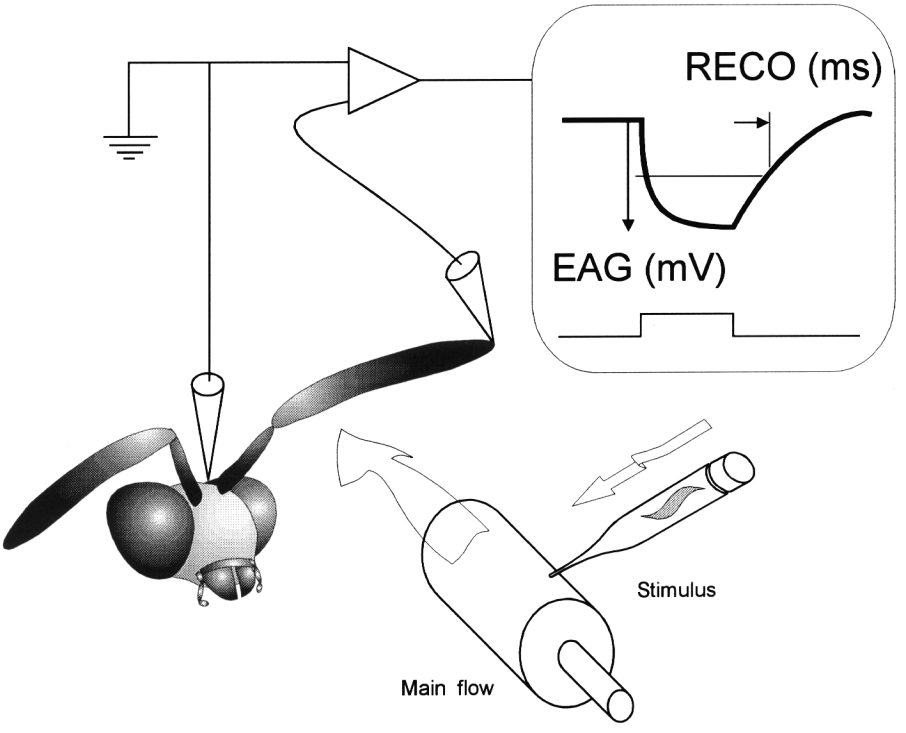

Fig. 1. Classical electroantennogram set up with the procedure for measurement of amplitude and half recovery time (RECO).

Table 1
List of test compounds used as odours. (1) Justification for inclusion in study: Dictionary of organic compounds, 6th edition, Merck index, 8th edition. Chemicals in italics are produced by Douglas fir trees (Rappaport et al., 1992; Dickens et al., 1983, 1984)

Compounds (class)	*Formula*	*Justification*(1)
Fatty acid derivatives (FADA)		
(incl. "green leaf" volatiles)		
hexanol-1	$C_6H_{14}O$	"green tissues" volatiles, leaves stems and flowers
hexanol-2	$C_6H_{14}O$	idem
(Z) 3- hexen-1-ol	$C_6H_{12}O$	idem
(E) 3- hexen-1-ol	$C_6H_{12}O$	idem
hexanal	$C_6H_{12}O$	idem
heptanal	$C_7H_{14}O$	idem
heptanone-2	$C_7H_{14}O$	idem
heptanone-4	$C_7H_{14}O$	idem
Hydrocarbon monoterpenes (HCT and DT)		
d-limonene	$C_{10}H_{16}$	needles and twigs of Pinaceae (*Abies*, *D. pseudotsuga*)
α- pinene	$C_{10}H_{16}$	needles and cones of many Pinaceae and Cupressaceae
β-pinene	$C_{10}H_{16}$	like α pinene, occurs in smaller amounts
γ-terpinene	$C_{10}H_{16}$	produced by cones of *D. pseudotsuga*
α-, β-phellandrene	$C_{10}H_{16}$	Pinaceae (*Abies,Douglass Pinus* ssp), a in eucalyptus
camphene	$C_{10}H_{16}$	Pinaceae (*Abies and Cupressus* ssp) Myristicacea,
3-thujene	$C_{10}H_{16}$	Fresh tops of *Juniperus* (Cupressaceae)
b myrcene	$C_{10}H_{16}$	Douglass, Myrtacea and many essential oils
p-cymene	$C_{10}H_{16}$	Labiatae, Umbelliferae and various plants
α-copaene	$C_{10}H_{16}$	
δ-3-carene	$C_{10}H_{16}$	several Pinacea (*Pinus, Picea* and *Abies* ssp)
α-terpinene	$C_{10}H_{16}$	Origanum, cardamone
terpinolene	$C_{10}H_{16}$	various species of Pineceae and other coniferous
Oxygenated monoterpenes (OT)		
linalool (+,−) mix	$C_{10}H_{18}O$	Labiate (*Lavandula*), Umbelliferae, Rutaceae
geraniol	$C_{10}H_{18}O$	many plants and flowers
(Z) verbenol	$C_{10}H_{18}O$	
carvone	$C_{10}H_{14}O$	Umbelliferae (Anethum ssp), Labiate (*Mentha spicata*)
α-terpineol	$C_{10}H_{18}O$	Myrtaceae and where α terpinene occurs
nerol	$C_{10}H_{18}O$	Often occurs with geraniol, several Asteraceae
terpinen-4-ol	$C_{10}H_{14}O$	Asteracea (tansy flowers)
α-, β- thujone	$C_{10}H_{16}O$	Cupressaceae (*Thuja* ssp), Asteraceae (tansy, *artemisia*)
menthol	$C_{10}H_{20}O$	Labiate (several *Mentha* species)
carvacrol	$C_{10}H_{14}O$	Several Labiates (*Origanum vulg*., Thyme)
thujan-4-ol	$C_{10}H_{18}O$	Asteracea (tansy flowers)
piperitone	$C_{10}H_{16}O$	Gramineae, Labiate (Mentha ssp), Eucalyptus
Sesquiterpenoids		
β-caryophylene	$C_{15}H_{24}$	
(E,Z) farnesol (mix)	$C_{15}H_{26}O$	
nerolidol	$C_{15}H_{26}0$	flower scent (myrtaceae, Leguminosaea)
Other		
distillied water	H_2O	
eugenol	$C_{10}H_{12}0_2$	
jasmone	$C_{11}H_{18}O$	flower scent (jasmine)

2.4. Data acquistion and analysis

Electrical signals were continuously recorded and analyzed using a computer and a custom programme designed to this purpose (Marion-Poll, 1986; Marion-Poll and Tobin, 1991; Marion-Poll and Thiéry, 1996). From these recordings, peak amplitude as well as half recovery time (RECO) were measured, and stored in ASCII files. The EAG amplitude was computed as the difference between the baseline level just before stimulation and the maximum reached during the stimulation. RECO was measured as the time necessary to return to half of the EAG amplitude (Fig. 1). In order to compensate for the decay in the EAG amplitude along the experiment, responses were expressed as the % of the amplitude to the standard. Assuming a linear decay of the response between 2 consecutive standard stimulations, responses were computed as follows:

$$C = R \times 100/(S1 + ((S2 - S1) \times n/6))$$

Where C = relative response to hexanol in %, R =

raw response to the stimulus, *S1* = raw response to the preceding standard, *S2* = raw response to the following standard, *n* = rank between 2 consecutive standards (1 to 5). This formula was applied for calculating relative amplitudes and half recovery times.

2.5. Statistics

Values of amplitudes and RECO were analyzed usind identical procedures: relative values were Log10 transformed and then each was subjected to a 2 way ANOVA (sex X chemicals) (SAS 5, Glm procedure). When an effect of chemicals or sexes, and/or interaction of these 2 factors was found, a more detailed analysis was applied. First, within each sex, a one way ANOVA was computed on relative values (Statistica 4.0) and contrasts between chemicals were examined by the Schéffé's contrast method. Second, for each chemical, we compared between sexes the relative amplitudes and RECO by a Student *t*-test. Third, we calculated for each individual a Spearman's rank correlation coefficient between absolute values of amplitude and RECO (Siegel, 1956). Like in Raguso et al. (1996), we compared grand mean relative amplitudes obtained by pooling and averaging responses for chemicals grouped in 4 select structural chemical classes: FADA (fatty acid derivatives, alcohols and aldehydes), OT (oxygenated terpenoids), DT (terpenoids produced by Douglas cones) and HCT (hydrocarbon terpenoids). These grand mean values were compared by a Student *t*-test adjusted for multiple comparisons with the sequential Sidak method (Scherrer, 1984). Amplitudes are expressed in the text as means of *n* responses ± SD.

3. Results

3.1. EAG peak amplitude

Responses obtained by classical EAG recording reached 4–6 mV of amplitude in both sexes with a background noise of 250–500 μV rms. Low pass filtering with a Bessel filter eliminated most high frequency noise and allowed us to sample between 25 and 200 Hz. An acceptable baseline stability, which is an essential prerequisite for long duration recording, was improved by reducing the dissection time and the connection to less than 1 min, and bathing the preparation with moist air.

The mean responses of the 15 females to the first stimulation by hexanol (standard) was 2.99 ± 1.03 mV, while it was lower in males 1.89 ± 0.72 mV. This response almost linearly decreased during the experiment in both sexes, reaching 1.48 ± 0.47 mV in females and 1.42 ± 1.18 mV in males after 45 min.

The normalized peak response to the other chemicals varied in females from 30 ± 7% (α-copaene) to 107 ± 15% (heptanal) and in males from 39 ± 14% (α-copaene) to 108 ± 21% (terpineol) (Figs 2 and 3). Peak amplitude obtained with parafin oil was almost similar in females 30% ± 15% response to the standard and in males 32 ± 13%.

The 2-way ANOVA indicated strong differences between chemical (F = 21.46, 38 df, $P < 0.0001$), between sexes (F = 28.1, 1 df, $P < 0.0001$) and a significant interaction sex/chemicals (F = 3.24, 36 df, $P < 0.0001$). A one-way ANOVA on each sex indicated important differences among the different chemicals tested, F = 23.1 (38 df, $P < 10^{-10}$) in females and F = 7.45 (36 df, $P < 10^{-10}$) in males. The analysis of contrasts by the Schéffé procedure indicates differences within each sex (Fig. 3).

3.2. Rank orders of EAG responses

In females, the largest peak amplitudes were obtained respectively with heptanal, terpineol, 1-hexanol, terpinen-4 ol and 2 heptanone. The amplitudes obtained with the first 17 chemicals ranked by decreasing relative amplitude (Fig. 3), were not statistically different from the best stimulant (heptanal), while the 17 remaining chemicals elicited smaller peak amplitudes ($P < 0.05$).

In males, terpineol, heptanal, 1-hexanol, 2-heptanone and hexanal were respectively the most effective chemicals. Despite the global difference found by ANOVA, we could only find a significant contrast between the 2 extremes, terpineol and α-copaene ($P < 0.05$).—The least effective chemicals were α-copaene and thujan-ol in females and α copaene and carvone in males; their amplitude were similar to that obtained with paraffin oil alone.

3.3. Chemical class comparisons

Pooling and averaging female responses to selected chemicals allows for comparison between groups of structural chemical classes (Fig. 4). Grand mean EAG amplitude to fatty acid derivatives was significantly higher to that of other classes (Student *t* test, $P < 10^{-7}$). Oxygenated terpenoids (OT) produced higher amplitudes than hydrocarbon ones (HCT) (Student *t*-test, $P < 2.10^{-8}$). Responses to hydrocarbon monoterpenes (DT) produced by Douglas cones were higher than those to HCT (Student *t* test $P < 10^{-8}$) (Fig. 4). Also in males, FADA was the most stimulating group, but there were no differences between the 3 other classes (Fig. 4).

3.4. Half recovery time, RECO

Values of RECO were automatically measured with a stable baseline level, and complete sets of data could be obtained in 13 females and 11 males. We found that absolutes amplitude and RECO were correlated in 10

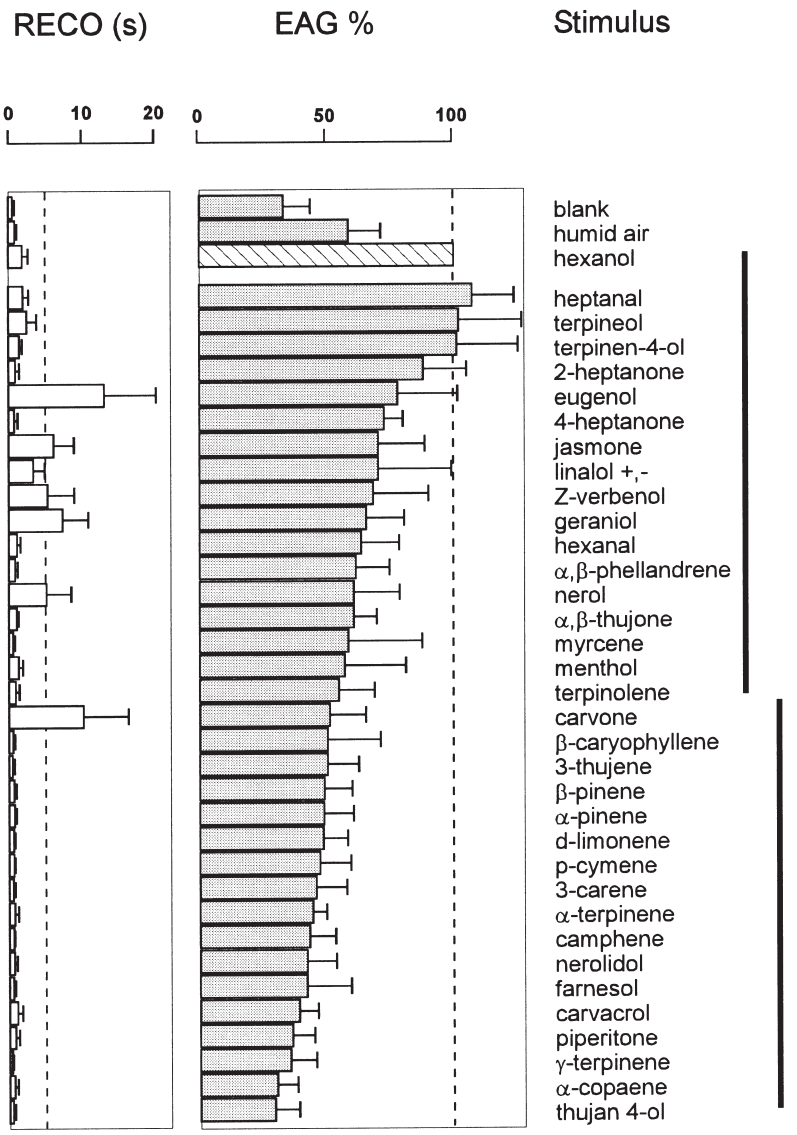

Fig. 2. Electroantennogram (EAG) responses obtained in *Megastigmus spermotrophus* females, ranked according to decreasing amplitude. Grey bars correspond to normalized peak amplitudes (mean ± SD of 15 EAG expressed as % of the response to 1-hexanol) and white bars to the duration of half-recovery time (RECO) expressed in s. Statistics: chemicals joined by a vertical line to the height did not elicit significantly different EAG responses (Anova and Shéffé's methods of contrasts). Differences with males are indicated in Fig. 3. Differences between RECO are reported in the results section.

from 13 females (rs, $P < 2.10^{-4}$) and 9 from 11 males (rs, $P < 5.10^{-4}$). The 2-way ANOVA (sex X chemicals) indicates that RECO differed between chemicals and sex (sex: $F = 12.78$, 1 df, $P < 4.10^{-4}$, chemicals: $F = 16.49$, 38 df, $P < 10^{-5}$) but no interaction was found ($F = 0.7$, 36 df). One-way ANOVA indicated that several chemicals strongly differed from each other ($F = 25.07$, df 38, $P < 10^{-14}$) and males ($F = 4.50$, df 36, $P < 10^{-14}$). Seven odours induced half recovery time lasting over 5 s in females, (decreasing order: eugenol, Z-verbenol, geraniol, nerol, carvone and jasmone) (Fig. 2). The same chemicals, except Z-verbenol, also induced long lasting EAGs in males (Fig. 3). In females, eugenol contrasted from 28 chemicals ($P < 10^{-2}$) while jasmone differed only from 10 chemicals ($P < 3.10^{-2}$); no statistical difference could be found in males by this method.

4. Discussion

EAG experiments represent an attractive and convenient method to assess the overall sensitivity of insects to a range of compounds at physiological relevant concentrations, under the rationale that the peripheral olfactory system has evolved sensitivity to behaviourally important odours. This approach is however limited. Considering that plant scents are composed of tens to

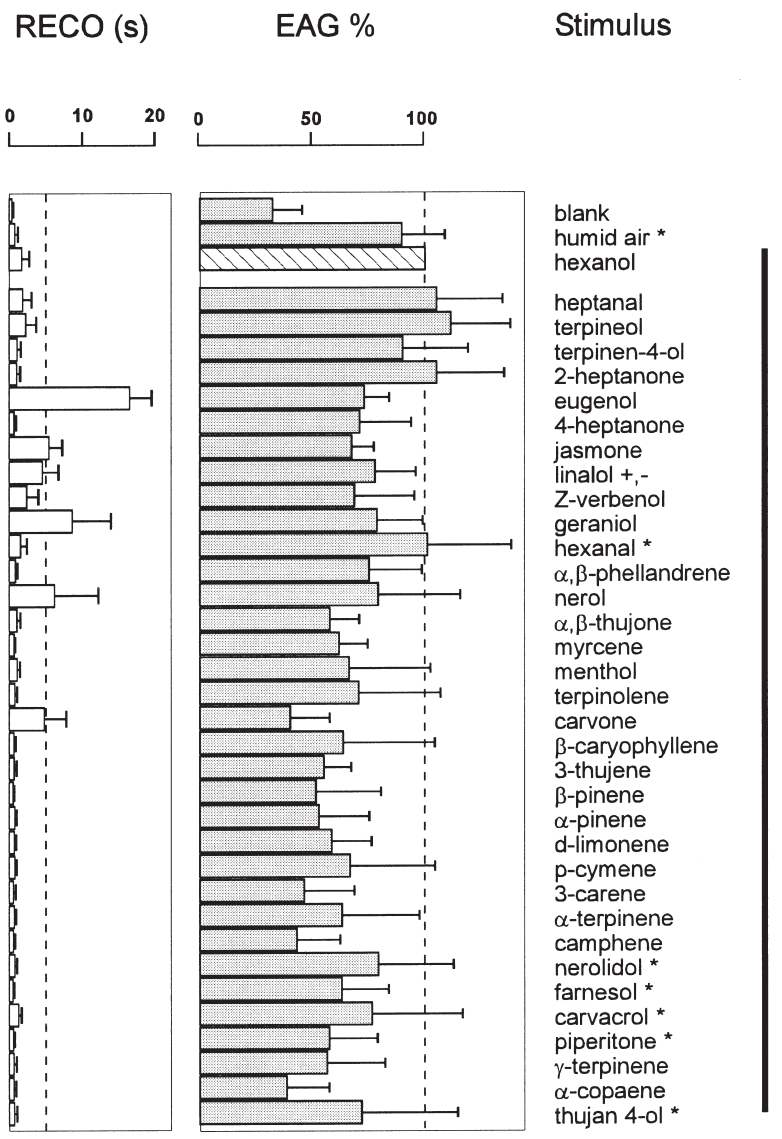

Fig. 3. Electroantennogram (EAG) responses obtained in *Megastigmus spermotrophus* males (chemicals ranked as in females). Grey bars correspond to normalized peak amplitudes (mean ± SD of 11 EAG expressed as % of the response to 1-hexanol) and white bars to the duration of half-recovery time (RECO)expressed in s. Statistics: Chemicals joined by a vertical line to the hight did not elicit significantly different EAG responses (Anova and Shéffé's methods of contrasts). An asterisk indicates chemicals which elicited different relative amplitudes between males and females. Differences between RECO are reported in the results section.

hundreds of chemicals, any series of compounds represents only a very small subset of the odours that insects might experience in the field. Additional limits include the EAG technique and the experimental protocol. EAG is a rough estimate of the activities elicited simultaneously within a mosaic of receptors. Mass effects may therefore mask activities from small groups of receptors tuned to particular volatiles. Also the number of molecules reaching the antennae vary, according to the volatility of each compound and its affinity to paraffin oil, filter paper or its adherence to the stimulus cartridge (Dickens et al., 1991). Nevertheless, strong stimulus effectiveness discrepancies should show up within such an experimental protocol.

Within these limits, our results indicate that *M. spermotrophus* can detect most of the odours we have used and that sexual differences can be obtained with several chemicals. Nerolidol, thujan-4-ol, hexanal, carvacrol, piperitone, farnesol and moist air evoked higher peak amplitude in males than in females. Considering these observations, behavioural differences between sexes are not likely to be explained by gross differences in the antennal receptor physiology, but rather by central nervous system connectivity.

Surprisingly, the largest peak amplitudes were obtained in both sexes with aliphatic alcohols and aldehydes, while the lowest amplitudes were obtained with hydrocarbon monoterpenes. Douglas fir volatiles such as

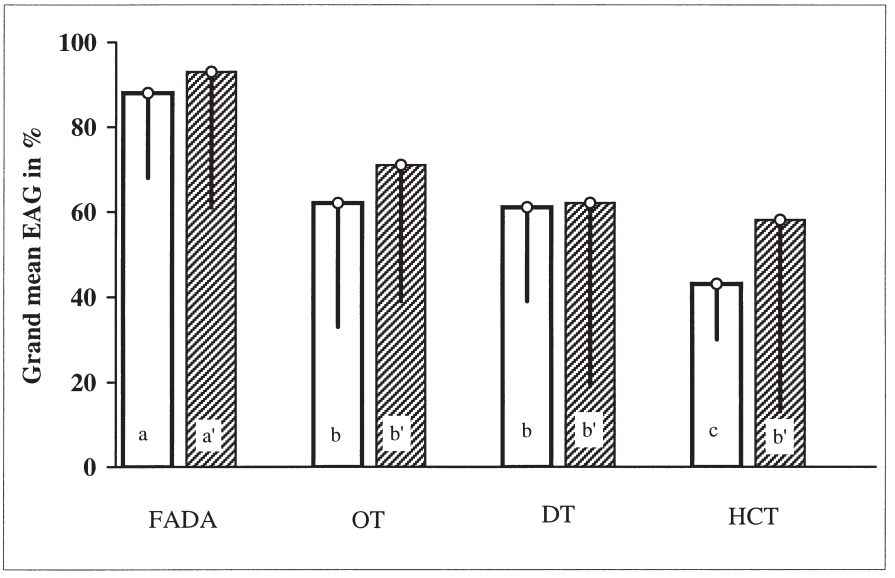

Fig. 4. Averaged EAG responses of *Megastigmus spermotrophus* to 4 different chemical classes, fatty acid derived alcohols and aldehydes (FADA), oxygenated monoterpenes (OT), Douglas produced hydrocarbon monoterpenes (DT) and other hydrocarbon monoterpenes (HCT). White bars represent females, and grey bars males. Relative responses are expressed as percentage of response to 1-hexanol. Different letters indicate differences within each sex at $P < 10^{-5}$ (Student *t*-test).

α-pinene, camphene and limonene (Dickens et al., 1983, 1984) and additional cones produced volatile (β-pinene, 3-thujene, α-, β-phellandrene and γ-terpinene and myrcene) (Rappaport et al., 1992); these were amongst the less effective stimulants. Again these compounds elicited responses that were close to 50% of the standard. Females were more responsive to the group of HC monoterpenes produced by its oviposition site than to the other HC monoterpenes, but not the males. By comparison, oxygenated terpenoids produced higher EAG amplitudes in both sexes than non-oxygenated ones. Similar differences were also noted between oxygenated and hydrocarbon terpenoids in other unrelated insects including *Leptinotarsa decemlineata* (Visser, 1979), *Psila rosae* (Guerin and Visser, 1980), *Rhynchaenus quercus* (Kozlowski and Visser, 1981), *Yponomeuta* sp. (Van der Pers, 1981) and *Anthonomus grandis* (Dickens, 1984).

Six compounds elicited EAG responses lasting over 5 s after stimulation in both sexes: eugenol, Z-verbenol, geraniol, nerol, carvone and jasmone. Among those, carvone elicited the smallest peak EAG while producing a long lasting response. The meaning of the EAG duration was recently proposed to be linked to the duration of deactivation processes of the receptor sites (Dickens et al., 1993; Hardie et al., 1995). Due to the long lasting effect of these compounds on the olfactory receptors, these compounds may have an impact on behaviour, either as stimulants or deterrents. Their biological value to *M. spermotrophus* however, remains uninvestigated.

The large responses obtained with "green odours" and oxygenated terpenoids which are frequently found in flowers or buds may suggest feeding from nectar sources. *M. spermotrophus* has often been observed on broom flowers (*Sarothamnus scoparius* Leguminosae) blooming at the time of adult emergence.

In conclusion, these experiments represent a partial answer to the initial question concerning the "specific host odour" hypothesis. Scents produced by Douglas cones elicit responses in the range of other terpenoids. The responses to the "green odour-compounds" suggest that the olfactory system of *M. spermotrophus* is not only tuned to detect its specific host plant odours as mentioned for other phytophagous insects, irrespective to their host range (Raguso et al., 1996; Hardie et al., 1995; Dickens et al., 1983; Visser, 1979; Jactel et al., 1996). It may indicate that other plants could be important to females and males or that a contrast of host chemicals from a background of non-host chemicals could be useful to them. Our preliminar observations using a GC as stimulation opens up the possibility of testing odour blends trapped in the near vicinity of the cones.

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Insects conference held at Beijing during the XIX International Congress of Entomology.

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