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Identification of host-plant chemical stimuli for the European grape berry moth *Eupoecilia ambiguella*

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Abstract. Olfaction is of major importance for survival and reproduction in moths. Males possess highly specific and sensitive olfactory receptor neurones to detect female sex pheromones. However, the capacity of male moths to respond to host-plant volatiles is relatively neglected and the role that such responses could play in the sensory ecology of moths is still not fully understood. The present study aims to identify host-plant stimuli for the European grape berry moth *Eupoecilia ambiguella* Hb. (Tortricidae, Lepidoptera), a major pest of vine in Europe. Headspace volatiles from *Vitis vinifera* L. cv. Pinot Noir, *Vitis vinifera* subsp. *sylvestris* and five other host-plant species comprising five different families are analyzed by gas chromatography linked to electroantennogram (EAG) recording from male *E. ambiguella* antennae and by gas chromatography-mass spectrometry. This procedure identifies 32 EAG-active compounds, among them the aliphatic compounds 1-hexanol, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and 1-octen-3-ol; the terpenes limonene, β-caryophyllene and (*E*)-4,8-dimethyl-1,3,7-nonatriene; and the aromatic compounds benzaldehyde and methyl salicylate. Male and female *E. ambiguella* show similar EAG response amplitudes to individual chemical stimuli and also to mixtures of plant volatiles, as represented by essential oils from ten other plant species. This possibly indicates a common role for plant compounds in the sensory ecology of the two sexes of *E. ambiguella*.

Key words. Electroantennogram, *Eupoecilia ambiguella*, European grape berry moth, host-plant volatiles, pheromone, sensory ecology.

Introduction

To detect female-produced sex pheromones at low concentrations, male moths are equipped with an array of antennal receptor cells located in long sensilla trichodea (Schneider & Steinbrecht, 1968). However, sensilla with receptor cells for plant volatiles (e.g. wall-pored sensilla basiconica) also occur on male moth antennae (Laue *et al*., 1994; Steinbrecht *et al*., 1995; Pophof *et al*., 2005). Until recently, studies on the olfactory system of moths have focused on sex-specific characteristics (Dunkelblum & Gothilf, 1983; Arn *et al*., 1986; Leal, 2005). Studies on plant-volatile detection mainly concentrate on female moths because of their role in finding suitable oviposition sites for the development of the less mobile larvae (Bruce & Cork, 2001; Hern & Dorn, 2002). For both sexes, plant-emitted volatiles may serve as signals to find food (Gregory, 1989) or shelter to protect themselves from desiccation and enemies (Schoonhoven *et al*., 2005). However, they may also play an important role in sexual behaviour as signals for mating sites at which males locate females more efficiently (Landolt & Phillips, 1997). Study of the detection of plant volatiles by male moths is important for understanding the role of such compounds in the sensory ecology of males and also from an applied viewpoint in the integrated management of pest insects. For example, in some moth species, laboratory and field experiments reveal that host-plant volatiles can enhance the response to the sex pheromone (Light *et al*., 1993; Deng *et al*., 2004; Schmidt-Büsser *et al*., 2009), a phenomenon that could be exploited to improve current pheromone-based

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control methods, such as mating disruption. In addition, lures containing pheromone and plant compounds could also serve to attract females, which, in most moth species, are insensitive to their own pheromone.

The European grape berry moth *Eupoecilia ambiguella* Hb. (Tortricidae, Lepidoptera) is an important pest on vines (Vitaceae). However, many other plants belonging to the Araliaceae, Cornaceae, Rhamnaceae, Rosaceae and Oleaceae are reported as host plants for this polyphagous moth (Bovey, 1966; Galet, 1982). The identified ternary mixture of sex pheromone compounds comprises (*Z*)-9-dodecenyl acetate (*Z*9-12:Ac), dodecenyl acetate (12:Ac) and octadecyl acetate (18:Ac) (Rauscher *et al*., 1984; Arn *et al*., 1986) and the sex pheromone is successfully deployed in mating disruption of this pest insect (Charmillot & Pasquier, 2000). Nevertheless, at high pest population densities, mating disruption becomes ineffective. Inclusion of plant volatiles into pheromone dispensers could serve to improve such pheromone-based control methods because male *E. ambiguella* fly upwind faster and in larger numbers to sex pheromone when mixed with plant volatiles in a wind tunnel (Schmidt-Büsser *et al*., 2009).

For a polyphagous species such as *E. ambiguella*, the question arises as to how it can discriminate host plants from nonhost plants using the olfactory system. In general, there are two hypotheses concerning the specificity of host-plant signals for insects: (i) host odour may provide particular information as a result of plant-specific compounds not found in unrelated plant species or (ii) host odour may be rendered specific as a result of particular ratios in which the plant releases compounds that are otherwise generally distributed among plant species (Visser, 1986; Bruce *et al*., 2005). In this context, the present study aims to identify host-plant chemical stimuli for male *E. ambiguella* through a comparative analysis of the odour profiles of different plant parts of *Vitis vinifera* L. cv. Pinot Noir, *Vitis vinifera* subsp. *sylvestris* and of five other host-plant species from different plant families using gas chromatography coupled electroantennogram recording (GC-EAG) from male *E. ambiguella* antennae. In addition, male and female EAG responses are compared using single host-plant compounds and different doses of ten essential oils from plants in different families, representing complex mixtures of volatile products of varying biosynthetic origin, aiming to obtain a better understanding of the role that plant volatiles might play in the sensory ecology of both sexes of *E. ambiguella*.

Materials and methods

Insects

Insects were reared in a climate chamber under an LD 16 : 8 h photocycle at 65% relative humidity (RH) and 25 °C during the photophase, and at 85% RH and 18 °C during the scotophase, as described previously (Schmidt-Büsser *et al*., 2009). For all tests, 2–4-day-old unmated males and females were used.

Odour collection and distillates from host plants and odour collection from the rearing medium

Different techniques were used to collect odours from host plants of *E. ambiguella*: headspace collection with thermal desorption from different plant parts of *V. vinifera* cv. Pinot Noir, a steam distillate of *V. vinifera* cv. Pinot Noir grapes, and headspace collections with solvent extraction from *V. vinifera* subsp. *sylvestris*, from five other host plants and from the rearing medium of the larvae. For the headspace collection of *V. vinifera* cv. Pinot Noir, two leaves or one bunch with either flowers or fruits were cut and placed in a 250-mL airtight glass bottle fitted with inlet and outlet tubes. After 10–20 min at room temperature, volatiles were collected by sucking charcoal-filtered air with a water pump (15 min at 100 mL min^{-1}) over the plant material onto a commercial Tenax™ GR cartridge (17 cm × 0.4 cm inner diameter; Gerstel, Germany). The Tenax™ GR cartridges were previously conditioned in an oven (70 °C, 10 °C min^{-1} until 280 °C, held for 30 min) under a constant flow of N_2 (70 mL min^{-1}). Volatiles trapped on the cartridges were desorbed onto a gas chromatograph column using a thermal desorption system (Gerstel TDS/CIS system; Gerstel; see below). A steam distillate of a cut bunch (750 g) of mature Pinot Noir grapes (twigs included) was obtained using a Clevenger arm distillation apparatus over 6 h. The volatile fraction was recovered in 2 mL of hexane.

Headspace collections of *V. vinifera* subsp. *sylvestris* (cut branches with male and female flowers), *Ligustrum vulgare* L. (Oleaceae; cut branches with flower buds and open flowers), *Olea europaea* L. (Oleaceae; cut branches and shredded leaves) and cut branches of *Viburnum lantana* L. (Caprifoliaceae) and *Rosmarinus officinalis* L. (Lamiaceae) were made during the first generation flight of the grape berry moth in May to June and at the end of the second generation flight in August for *Hedera helix* L. (Araliaceae). One headspace collection was made per plant species and plant part. In addition, headspace odours over 50 g of the larval rearing medium (Rauscher *et al*., 1984) were collected because their behavioural importance has been demonstrated recently in attracting *Lobesia botrana* larvae, a sister species of *E. ambiguella* (Becher & Guerin, 2009). Charcoal-filtered air was pulled at 500 mL min^{-1} for 2 h through a 250 mL gas-wash-bottle containing 20–50 g of plant material or rearing medium, and then through a cartridge containing 50 mg of the porous polymer PorapakQ (80/100 mesh, Alltech, Deerfield, Illinois; conditioned for 90 min at 200 °C under nitrogen before use). Volatiles were desorbed by passing 100 μL of dichloromethane through the cartridge once into glass ampoules that were sealed and held at −20 °C until analysis by GC-EAG recording (see below).

Standard compounds and essential oils

(*Z*)-3-Hexenyl acetate, (*E*)-2-hexen-1-al, *p*-cymene (all from Sigma-Aldrich, Germany), (*Z*)-3-hexen-1-ol, R(+)-limonene, (+)-terpinen-4-ol, linalool, α-terpineol, geraniol,

methyl salicylate (all from Fluka, Switzerland), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT; Givaudan, Switzerland), 1-octen-3-ol (Merck, Germany) and the main *E. ambiguella* pheromone component *Z*9-12:Ac (Plant Research International, The Netherlands) were prepared at three different concentrations (10 ng μL^{-1}, 1 ng μL^{-1} and 100 pg μL^{-1}) in dichloromethane for analysis by GC-EAG detection (see below). These compounds were chosen based on a previous study in this laboratory on chemical stimulants for *E. ambiguella* (S. Connétable & P. M. Guerin, unpublished data) and on published work on host-plant chemical stimuli for other tortricid moths such as the grapevine moth *L. botrana* (Gabel, 1992; Tasin *et al*., 2005, 2006) and the codling moth *Cydia pomonella* (Light *et al*., 1993; Ansebo *et al*., 2004; Yang *et al*., 2004). Essential oils (steam or water distilled) of coriander (Apiaceae), cardamom (Zingiberaceae), *Litsea cubeba* (Lauraceae), *Lantana camara* (Verbenaceae), lemon grass (Poaceae), *Thuja plicata* (Cupressaceae), citronella (Cardiopteridaceae) of three different origins (Ceylon, Java and China) and *Eucalyptus* (Myrtaceae), dissolved in dichloromethane at 1, 10 and 100 $\mu g\ mL^{-1}$, were obtained from Robertet S.A. (Grasse, France) and from Dr S. Mohottalage (Organic Chemistry Laboratory, University of Neuchâtel). This group of essential oils was arbitrarily chosen to provide varied blends of volatile products with which to compare the EAG responses of male and female *E. ambiguella* (see below).

EAG

For EAG analysis (Schneider, 1957), an antenna (male or female) of *E. ambiguella* was mounted between two glass capillary electrodes filled with 0.1 M KCl and positioned 1 cm from the outlet of a glass water-jacketed tube (inner diameter 7 mm) that served to direct test compounds into a charcoal-filtered humidified air stream (95% RH) at 1 m s^{-1} on to the antenna. The recording electrode containing a silver wire, and on which the antennal tip was mounted, was connected to a high impedance preamplifier (gain × 10). The recorded potential was fed to an amplifier (gain × 100; Syntech, The Netherlands). The responsiveness of the antenna was tested at the start and end of each stimulation sequence with an air puff from a 5-mL stimulus syringe containing 10 µg of (+)-terpinen-4-ol on a filter paper strip introduced into the glass water-jacketed tube through a rubber septum-covered hole in the glass wall at 22 cm from the antennal preparation (Taneja & Guerin, 1997). If the antennal response to this 1-s air puff (1 mL s^{-1}) was less than 0.4 mV, it was discarded and another antenna was mounted. This stimulus syringe stimulation system was used to obtain EAG responses to essential oils by placing an aliquot (10 µL) of each concentration on a filter paper strip that was placed in the stimulus syringes after evaporation of the solvent. All EAG responses were expressed as a percentage of the average response to the reference product [10 µg of (+)-terpinen-4-ol]. Essential oils were tested in a random order starting with the lowest dose. For each essential oil and dose, three to six female and male antennae were used.

Headspace volatile collections and solutions containing test chemical stimuli were analyzed by means of GC-EAG analysis (Arn *et al*., 1975). The gas chromatograph (Carlo Erba Instruments 5160, Mega Series; Carlo Erba Instruments, Italy) was equipped with a precolumn (fused silica capillary tubing, length 1 m, inner diameter 320 µm, deactivated with OV-1701-OH; BGB Analytik, Switzerland) and an apolar column (SE-30, length 30 m, inner diameter 0.25 mm, film thickness 0.15 µm; BGB Analytik) to analyze the volatiles emitted by *V. vinifera* cv. Pinot Noir and with a polar column (FFAP, length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm; BGB Analytik) to analyze extracts of the other host-plants. H_2 was used as carrier gas. The column effluent was split in two (50 : 50; GRAPHPACK metal splitter, Gerstel) with one outlet directed to the flame ionization detector (FID) and the second, via an outlet heated to 230 °C in the wall of the chromatograph, into the air stream of the glass water-jacketed tube to the biological detector (see above). The FID and EAG responses were recorded on a personal computer using GC-EAG software (Syntech). Compounds eliciting an EAG response of at least 0.05 mV in the headspace volatile analyses from at least two antennae were considered as valid and their Kovats retention indices (KRI, for temperature programmed chromatography) were calculated. For each odour sample, three to five antennae were used.

Volatiles of *V. vinifera* cv. Pinot Noir trapped on the Tenax™ GR cartridges were desorbed onto the GC column using the Gerstel TM TDS/CIS System (Gerstel). The thermal desorption system (TDS) was heated from 30 to 200 °C at 30 °C min^{-1} and held for 1 min to transfer volatiles to the cooled injection system (CIS). During thermal desorption, the CIS (cooled with liquid nitrogen) was held at −80 °C and then heated at 12 °C s^{-1} to 220 °C in splitless mode (1 min) to pass the volatiles onto the GC column. For all other samples, 2 µL of the headspace collection extracts and the test product solutions were injected on-column. The GC oven was held at 40 °C for 5 min, then heated to 230 °C at 10 °C min^{-1} and held at this temperature for 5 min.

Identification of EAG-active compounds by gas chromatograph linked mass spectrometry

Headspace collections analyzed by GC-EAG were subsequently analyzed by GC-coupled mass spectrometry (GC-MS; HP5890 series II chromatograph) linked to a HP 5971A mass selective detector (Hewlett Packard, Palo Alto, California) with the same column and conditions as for the GC-EAG analysis (above). Helium was used as carrier gas. Biologically active components of extracts located by the GC-EAG analysis described above were relocated by GC-MS using KRIs and by comparison of chromatogram profiles. Identification of an EAG-active peak in an extract was first based on the match of its mass spectrum with a reference mass spectrum in a library (Nist98) and by interpretation of the mass spectrum. The KRI of the chemical stimulant was then compared with that of a standard of the compound proposed by the library (when available) injected under the same conditions.

Biological activity of commercially available analogues was also established by GC-EAG using male *E. ambiguella* antennae. Enantiomeric forms were not determined for compounds with a chiral center.

Statistical analysis

The EAG responses of female and male *E. ambiguella* to 10 and 100 ng of standard products and to three different concentrations of the essential oils were compared using three-way analysis of variance (ANOVA) with sex, stimulus and stimulus quantity as parameters. Statistical tests were applied with the statistical package R, version 2.4.1 (http://www.r-project.org).

Results

EAG-active compounds in host-plant volatile collections and distillate

Each host-plant headspace collection and the distillate of *V. vinifera* cv. Pinot Noir was analyzed by GC-EAG with three to five male *E. ambiguella* antennae. Only compounds eliciting an EAG response in at least two antennae were considered as valid chemical stimuli for *E. ambiguella* males. In total, over 50 constituents of host-plant headspace collection extracts evoked EAG responses from male *E. ambiguella* and, in general, the response pattern was consistent among individuals (Fig. 1). Twenty-seven compounds evoked EAG responses in the headspace collection that was thermally desorbed onto the GC-column and in the steam distillate of different parts of *V. vinifera* cv. Pinot Noir (Table 1) and 22 of these were identified by GC-MS on the basis of matching mass spectra, matching KRI and matching EAG activity of known standards. Most of the identified EAG-active compounds were present in headspace collections from the different parts of *V. vinifera* cv. Pinot Noir analyzed (i.e. grapes, flowers and leaves). The predominating volatiles identified from these plant parts were green leaf volatiles and terpenes. No EAG responses were recorded to aromatic compounds in the headspace collection from the grapevine plant parts but, in the steam distillate of grapes, phenylacetaldehyde induced an EAG response. In addition, (*E*)-2-hexen-1-ol, linalool oxide, linalool, furfural, terpinen-4-ol and α-terpineol identified in the steam distillate of grapes elicited EAG responses from male *E. ambiguella*, although these compounds were not found in the headspace collections of grapes, flowers and leaves. Three chemical stimuli were identified as common to headspace collections of all plant parts of *V. vinifera* and the grape steam distillate, namely (*Z*)-3-hexen-1-ol, nonanal and β-caryophyllene.

Only compounds eliciting an EAG response in three of the six extracts were the subject of identification in the headspace collections with solvent extraction of *V. vinifera* subsp. *sylvestris* and five other host-plants. In this manner,

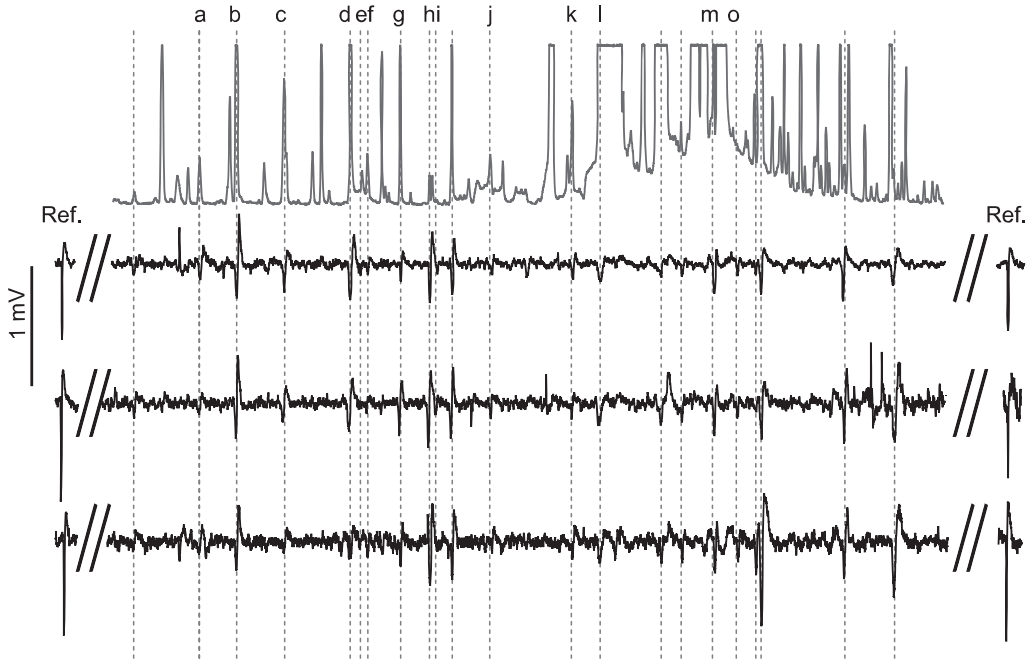

Fig. 1. Gas chromatography coupled electroantennogram (EAG) traces of three male *Eupoecilia ambiguella* (lower three traces) to products in the headspace collection extract of *Vitis vinifera* subsp. *sylvestris* (upper trace). Labelled volatiles found to elicit EAG responses in at least three of the plant extracts tested were identified as: a, limonene; b, *(E)*-2-hexenal; c, ocimene; d, *(E)*-4,8-dimethyl-1,3,7-nonatriene; e, *(Z)*-3-hexenyl acetate; f, 6-methyl-5-hepten-2-one; g, 1-hexanol; h, *(Z)*-3-hexenol; i, nonanal; j, 1-octen-3-ol; k, benzaldehyde; l, β-caryophyllene; m, α-farnesene; o, methyl salicylate (Table 2). Ref. represents the response to an air puff applied to the antennal preparation at the start and end of each analysis from a 5-mL syringe containing 10 µg (+)-terpinen-4-ol (see Materials and methods); mV scale common to EAG recordings.

Table 1. Compounds eliciting electroantennogram (EAG) responses from *Eupoecilia ambiguella* males identified in volatile collections of grapevine leaves, fruits, flowers (headspace collection with thermal desorption) and a steam distillate of a bunch of grapes (Dist.) of the grapevine *Vitis vinifera* cv. Pinot Noir (for details of methods, see text).

	Extraction method						
	Headspace			Dist.			
Compound	Fruit	Flower	Leaf	Grapes	Identification criteria	KRI (SE-30)[a]	KRI (FFAP)[a]
Aliphatic compounds							
Hexanal	+	+	+	–	MRE	Undefined	–
(E)-2-Hexenal	+	+	+	–	MRE	828	–
(Z)-3-Hexenol	+	+	+	+	MRE	840	1359
(E)-2-Hexenol	–	–	–	+	MRE	–	1380
Heptanal	+	–	–	–	MRE	882	–
Octanal	+	+	+	–	MRE	983	–
(Z)-3-Hexenyl acetate	+	–	+	–	M	993	–
(E)-2-Hexenyl acetate	+	+	–	–	M	1021	–
Hexyl acetate	+	+	+	–	M	1044	–
Nonanal	+	+	+	+	MRE	1094	1375
(Z)-3-Hexenyl butyrate	+	–	+	+	MRE	1172	1581
Terpenes							
DMNT	+	+	+	–	M	1116	–
Decanal	+	+	+	–	MRE	1193	–
ß-Caryophyllene	+	+	+	+	MRE	1400	1558
α-Humulene	+	+	+	–	MRE	1437	–
β-Farnesene	+	+	+	–	M	1449	–
Linalool oxyde	–	–	–	+	M	–	1441
Furfural	–	–	–	+	M	–	1444
Linalool	–	–	–	+	MRE	–	1524
α-Terpineol	–	–	–	+	MRE	–	1667
Terpinen-4-ol	–	–	–	+	MRE	–	1563
Aromatic compounds							
Phenylacetaldehyde	–	–	–	+	MRE	–	1597
Unknown compounds							
Unidentified #1	–	+	–	–	–	1054	–
Unidentified #2	–	+	–	–	–	1083	–
Unidentified #3	+	–	–	–	–	1167	–
Unidentified #4	–	–	–	+	–	–	1514
Unidentified #5	–	–	–	+	–	–	1745

[a] Se-30 and FFAP represent apolar and polar gas chromatography phases, respectively (see text).
Identification criteria were: M, matching mass spectra; R, matching retention time with standard product; E, matching EAG activity with standard product; KRI, Kovats retention index; DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene.

14 chemical stimuli were identified that can be divided in three chemical classes: the aliphatics 1-hexanol, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, 1-octen-3-ol, 6-methyl-5-hepten-2-one and nonanal; the terpenes limonene, ocimene, β-caryophyllene, DMNT and α-farnesene; and the aromatics benzaldehyde and methyl salicylate (Table 2). All 14 identified compounds were present in *V. vinifera* subsp. *sylvestris*, whereas only seven were found in *H. helix*. Some were also identified in the headspace collection extract of the larval rearing medium, namely 1-hexanol, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, nonanal, 6-methyl-5-hepten-2-one, limonene, β-caryophyllene and methyl salicylate. Both methyl salicylate and (*Z*)-3-hexen-1-ol were found in all of the extracts and, remarkably, strong EAG responses were recorded to these two compounds, even at just 100 pg present in the 1-µL aliquot of extract injected (Table 2). When comparing the compounds identified as chemical stimuli in headspace collections from different parts of *V. vinifera* cv. Pinot Noir (Table 1) and from *V. vinifera* var. *sylvestris* and the five other host-plants (Table 2), six compounds emerge as common, namely (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, nonanal, β-caryophyllene and DMNT.

EAG responses of male and female E. ambiguella to host-plant volatiles standards

EAG responses of male *E. ambiguella* were recorded to defined amounts (100 pg, 1 ng and 10 ng) of 12 known plant volatiles and the pheromone compound *Z*9-12:Ac injected onto the same polar phase used to analyze the host-plant volatile collections. Male *E. ambiguella* showed EAG responses to all compounds at the highest dose (10 ng; Fig. 2). Responses were still recorded to (*Z*)-3-hexen-1-ol, 1-octen-3-ol, *p*-cymene, DMNT, (+)-terpinen-4-ol, R(+)-limonene, linalool, methyl

Table 2. Compounds eliciting electroantennogram (EAG) responses in male *Eupoecilia ambiguella*, present in at least three headspace collections (with solvent extraction) from six host-plants and the rearing medium.

KRI_{STD}	KRI_{HSP}	Compound	*Vitis vinifera* subsp. *sylvestris*	*Viburnum lantana*	*Ligustrum vulgare*	*Olea europaea*	*Rosmarinus officinalis*	*Hedara helix*	Rearing medium	Identification criteria
Aliphatic compounds										
1350	1354	1-Hexanol	+	+	+	+	–	–	+	MR
1203	1213	*(E)*-2-Hexenal	+	–	+	+	–	–	–	MRE
1370	1380	*(Z)*-3-Hexenol	+	+	+	+	+	+	+	MRE
1314	1323	*(Z)*-3-Hexenyl acetate	+	+	+	+	+	–	–	MRE
1430	1446	1-Octen-3-ol	+	+	–	+	+	–	+	MRE
1335	1342	6-Methyl-5-hepten-2-one	+	–	+	+	–	+	+	M
1393	1388	Nonanal	+	+	+	+	+	–	+	MR
Terpenes										
1188	1190	Limonene	+	+	–	–	+	+	+	MRE
1256	1254	Ocimene	+	+	+	+	+	+	–	M
1590	1589	β-Caryophyllene	+	+	–	+	+	+	+	MRE
1303	1313	DMNT	+	+	+	+	–	–	–	MRE
1746	1747	α-Farnesene	+	+	+	+	–	–	–	MR
Aromatic compounds										
1525	1528	Benzaldehyde	+	+	+	–	+	+	–	MRE
1783	1778	Methyl salicylate	+	+	+	+	+	+	+	MRE

KRI, Kovats retention index established for standards (KRI_{STD}) and for EAG-active products in headspace samples (KRI_{HSP}) identified by gas chromatography-mass spectrometry. Identification criteria were: M, matching mass spectra; R, matching retention time with reference standard; E, matching EAG activity with reference standard; DMNT, *(E)*-4,8-dimethyl-1,3,7-nonatriene.

salicylate and *Z*9-12:Ac at 100 pg eluting from the column, with the strongest response to the pheromone product (Fig. 2a). The responses to the highest dose of the most EAG-active host-plant compound injected [10 ng (+)-terpinen-4-ol] was only as strong as the response of male *E. ambiguella* to 100 pg *Z*9-12:Ac (Fig. 2a). One antennal preparation remained stable over 3 h and EAG responses to all the three test doses could be recorded (once for each dose) and compared (Fig. 2b). The EAG response amplitude to *Z*9-12:Ac did not increase between 1 and 10 ng, indicating saturation of the antennal receptor cells beyond 1 ng. The responses of this antenna to the plant volatiles decreased with decreasing doses injected.

EAG responses to eight of the 12 host-plant compounds tested above and to the pheromone injected at 1 and 10 ng were also recorded from female *E. ambiguella* and compared with male responses. The EAG response of male and female *E. ambiguella* to different doses of these host-plant compounds did not differ significantly (Fig. 2; three-way ANOVA: $F_{(1,71)} = 0.902$, $P = 0.346$). This contrasts with the response to the pheromone component *Z*9-12:Ac, where no EAG response was recorded from female antennae even at the highest dose of 10 ng (Fig. 2).

EAG responses of male and female E. ambiguella to essential oils

Vapours from essential oils were blown over the antennae of male and female *E. ambiguella* as a 1-s air puff from a 5-mL syringe containing 1, 10 and 100 µg on a filter paper strip. Confirming that recorded for single host-plant volatiles (above), the EAG responses of males and females to these complex mixtures of volatiles were not different (Fig. 3; three-way ANOVA: $F_{(1,168)} = 0.032$, $P = 0.858$).

Discussion

Analysis of the headspace collection extracts of *V. vinifera* cv. Pinot noir, *V. vinifera* subsp. *sylvestris* and five other host plants and examination of EAG responses of *E. ambiguella* to compounds that evoke EAG responses in other tortricids shows that the olfactory system of male *E. ambiguella* is sensitive to several host-plant volatiles, namely short chain alcohols, aldehydes and esters, terpenes and aromatic products. In total, 32 host-plant compounds serve as chemical stimuli for male *E. ambiguella*. The effects of 11 of these products on the behavioural responses of male *E. ambiguella* to the blend of *Z*9-12:Ac, 12:Ac and 18:Ac that constitutes its pheromone are reported elsewhere (Schmidt-Büsser *et al*., 2009). Four of these host-plant products, namely (*Z*)-3-hexen-1-ol, (+)-terpinen-4-ol, (*E*)-β-caryophyllene and methyl salicylate, increase the flight response of male grape berry moths presented with suboptimal levels of their sex pheromone in a wind tunnel (Schmidt-Büsser *et al*., 2009).

In general, EAG responses provide an image of the combined depolarization of several olfactory receptor cells activated by a particular stimulus (Boeckh *et al*., 1965). A low response threshold for a product in the EAG assay, such as for the sex pheromone of moths, may indicate a key

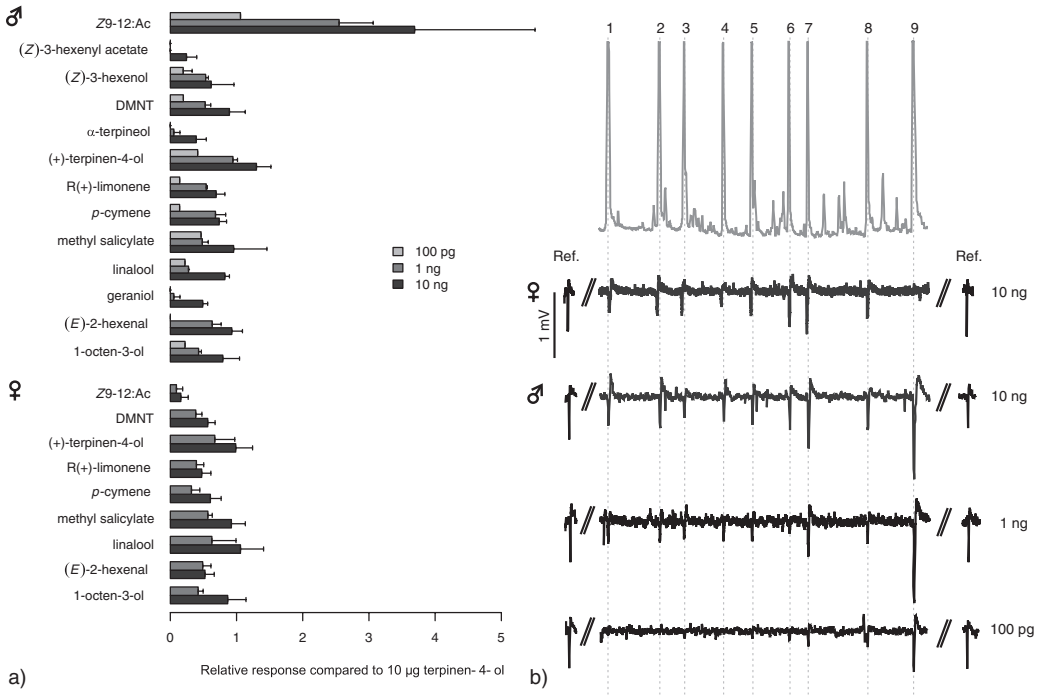

Fig. 2. (a) Mean relative electroantennogram (EAG) responses (% of the response to standard with standard deviations, see text) of *Eupoecilia ambiguella* males (top) and females (bottom) to host-plant compounds and the pheromone product *Z*9-12:Ac each injected at 10 ng, 1 ng and 100 pg μL^{-1} (only males) onto a gass chomatography (GC) column (see Materials and methods). (b) GC-flame ionization detector trace (upper trace; 10 ng μL^{-1} injected) of eight plant compounds (1–8) and the pheromone product *Z*9-12:Ac (9) and the corresponding EAG responses (lower traces) of a female antenna to 10 ng and of a male antenna to 100 pg, 1 ng and 10 ng injected. No difference between male and female EAG responses to plant compounds was found (analysis of variance: $P = 0.346$). Note the strong EAG response of male antennae to *Z*9-12:Ac even at 100 pg compared with the plant volatiles. By contrast to males, the female EAG response to the pheromone is minimal. 1, R(+)-limonene; 2, *p*-cymene; 3, *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT); 4, *(E)*-2-hexen-1-al; 5, 1-octen-3-ol; 6, linalool; 7, (+)-terpinen-4-ol; 8, methyl salicylate; 9, *Z*9-12:Ac. Ref. represents the response to an air puff from a 5-mL syringe containing 10 µg (+)-terpinen-4-ol on a filter-paper strip applied to the antennal preparation at the start and end of each analysis; mV scale common to EAG recordings.

stimulus for the species in question (Visser, 1979; Mayer *et al*., 1987). In the present study, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, linalool, R(+)-limonene, DMNT, (+)-terpinen-4-ol, *p*-cymene and methyl salicylate prove to be strong chemical stimuli for *E. ambiguella* males eliciting EAG responses at a dose of 100 pg. In behavioural tests, three of these products, namely (*Z*)-3-hexen-1-ol, (+)-terpinen-4-ol and methyl salicylate, along with β-caryophyllene, are reported to be behaviourally active in combination with the pheromone in male *E. ambiguella* (Schmidt-Büsser *et al*., 2009) and are in agreement with the low EAG response threshold–key stimulus hypothesis. Further behavioural experiments with different doses of compounds showing low EAG-response thresholds would be necessary to test this hypothesis more rigorously.

The plant volatiles evoking EAG responses from *E. ambiguella* are commonly occurring secondary plant products emitted by a range of plants from different families (Knudsen *et al*., 1993). The chemical stimuli (*Z*)-3-hexen-1-ol, derived from linoleic acid by lipoxygenase activity (Hatanaka, 1993), and methyl salicylate, derived from salicylic acid (Lee *et al*., 2005), are found in all the headspace collection extracts tested. These two products are released in larger amounts by many plants upon damage. Headspace odour collection from cut plant material here may partly explain the presence of these compounds in all the extracts. Nevertheless, behavioural studies demonstrate an important role for these generally-occurring plant compounds for arthropods: methyl salicylate is reported to be implicated in the tritrophic interactions between the lima bean *Phaseolus lunatus* (Fabaceae), the phytophagous mite *Tetranychus urticae* and the predatory mite *Phytoseiulus persimilis* (De Boer & Dicke, 2004). Methyl salicylate inhibits oviposition in *Mamestra brassicae* (Ulland *et al*., 2008) and is synthesized as a pheromone by the tick *Amblyomma variegatum* (Diehl *et al*., 1991), providing evidence for the widespread use of this product as a chemostimulant in both insects and arachnids. (*Z*)-3-Hexen-1-ol enhances male *C. pomonella* and *Spodoptera exigua* flight responses to the respective sex pheromones in wind tunnel tests (Deng *et al*., 2004; Yang *et al*., 2004). Both (+)-terpinen-4-ol and *p*-cymene are emitted by the tansy flowers *Tanacetum vulgare*, a species of temperate regions often found in vineyards (Gabel, 1992). These flowers are reported to be regularly visited by the female grapevine moths *L. botrana*, and may serve as food sources (Gabel, 1992). *p*-Cymene is found in rosemary extracts by Katerinopoulos *et al*. (2005) but not in the rosemary headspace collections in the present study.

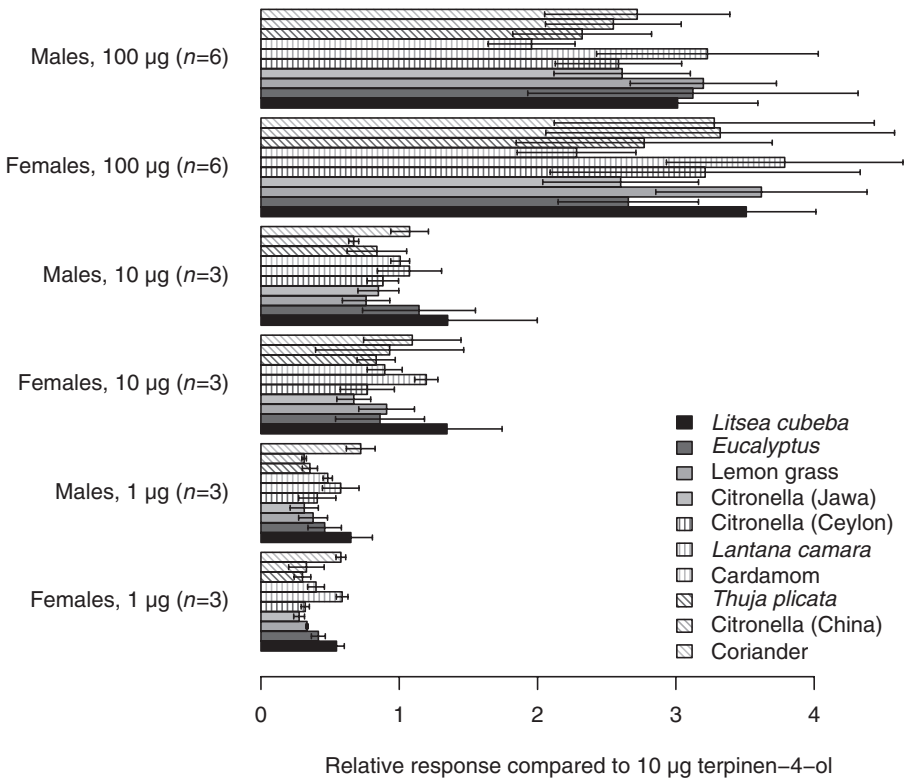

Fig. 3. Mean relative electroantennogram responses by *Eupoecilia ambiguella* (% of the response with standard deviations to an air puff from a 5-mL syringe containing 10 µg (+)-terpinen-4-ol on a filter paper strip) to ten essential oils presented at three different doses (1-s air puff from a 5-mL syringe containing 1, 10 and 100 µg on a filter paper strip). There are no differences between the responses of males and females at any of the doses of the essential oils tested (analysis of variance: $P > 0.05$).

The presence of specific receptor neurones for such ubiquitous volatiles in many insect species further emphasizes an important role of such compounds. Olfactory receptor cells are reported for (*E*)-2-hexen-1-al, 1-octen-3-ol, limonene and methyl salicylate in the fruit fly *Drosophila melanogaster* (de Bruyne *et al*., 2001), for (*Z*)-3-hexen-1-ol and ocimene in the parasitoid wasp *Microplitis croceipes* (Ochieng *et al*., 2000) and for green-leaf volatiles in the Colorado potato beetle *Leptinotarsa decemlineata* (Ma & Visser, 1978), as well as in the fruit chafer *Pachnoda marginata* (Stensmyr *et al*., 2001). That no chemical stimulant specific to a plant species or family is found for *E. ambiguella* in the present study is not surprising for a polyphagous species compared with the sensitivity to host-plant specific products in monophagous insects (Guerin *et al*., 1983). By definition (Bernays & Chapman, 1994), polyphagous species exploit host plants belonging to different families that are likely to have quite different odour profiles. However, some specificity can be conferred by the particular ratios between compounds in the bouquet from a given plant species (Visser, 1986; Bruce *et al*., 2005). In male *E. ambiguella*, a 10 : 1 ratio of the host-plant chemical stimuli β-caryophyllene and (*Z*)-3-hexen-1-ol increases the response to the sex pheromone more than at other ratios of these chemical stimuli (Schmidt-Büsser *et al*., 2009). Female *L. botrana* are attracted to a mixture of β-caryophyllene, DMNT and β-farnesene at a ratio identified from grape but not at a ratio found in the nonhost, apple *Malus domestica* (Tasin *et al*., 2006). In addition, the absence of inappropriate or non-volatile products on the plant surface may play a role in host-plant selection (Bernays & Chapman, 1994). In general, naturally occurring ratios of plant volatiles are quite difficult to determine precisely because headspace vapour profiles are dependent on the selectivity of the collection method (Tholl *et al*., 2006).

Strong EAG responses in male *E. ambiguella* to the pheromone compound *Z*9-12:Ac are recorded even at 100 pg, whereas, in females, a response is absent even at levels that are 100-fold higher. This difference between the response of the sexes to their own sex pheromone is described for many moth species, with just a few exceptions (Hansson *et al*., 1989; Ljungberg *et al*., 1993). However, the capacity to sense plant compounds between the sexes is reported for only a few moth species (Fraser *et al*., 2003; Pophof *et al*., 2005; Casado *et al*., 2006). Male *E. ambiguella* show EAG responses to host-plant compounds that are similar to those of females. This is established by comparing EAG responses to single plant compounds and to blends of plant volatiles as represented in the present study by essential oils. Ansebo *et al*. (2004) only report marginal differences in the EAG responses of male and female *C. pomonella* to host-plant volatiles but Casado *et al*.

(2006) report significant differences between male and female *C. pomonella* EAG responses to six plant compounds, with an overall tendency of larger responses from male compared with female antennae. Quantitative but not qualitative differences in EAG responses to plant compounds are reported for male and female *Manduca sexta* (Fraser *et al*., 2003), where females tend to show higher EAG response amplitudes than males. Røstelien *et al*. (2005) identify the same functional types of olfactory receptor cells for plant compounds in male and female *Helicoverpa armigera*. In *Bombyx mori*, Wanner *et al*. (2007) identify olfactory receptor genes common to females and males, although some genes are expressed at higher levels in females than in males and others are absent in males. The authors suggest that female *B. mori* are equipped with olfactory receptor cells tuned to food and mating site stimuli with other cells sensitive to products indicating oviposition sites. The similarity in the EAG responses of male and female *E. ambiguella* suggests that plant volatiles play a role in the sensory ecology of both sexes, serving as cues to find shelter or for mating on plants. When *E. ambiguella* adults emerge in April, vegetation on the grapevine is still sparse (Bovey *et al*., 1972), such that surrounding plants may serve as shelter. Indeed, *E. ambiguella* is often found on typical hedge plants such as *L. vulgare*, *V. lantana* and *H. helix*, and all are considered as host plants of this insect (Galet, 1982), and represent the plants from which chemical stimuli are identified in the present study.

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