**Host plant perception and selection in the sibling species Macrolophus melanotoma and Macrolophus pygmaeus (Hemiptera: Miridae)**

# HOST PLANT PERCEPTION AND SELECTION IN THE SIBLING SPECIES *MACROLOPHUS MELANOTOMA* AND *MACROLOPHUS PYGMAEUS* (HEMIPTERA: MIRIDAE)

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**Abstract**

The electroantennogram responses (EAGs) of *Macrolophus melanotoma* and *Macrolophus pygmaeus* (Hemiptera: Miridae) exposed to volatile compounds (VOCs) of host and non-host plants were compared. The VOCs were identified by gas chromatography. Hosts and non-hosts eliciting similar EAGs were tested in olfactory assays against plants without a significant EAGs for the two *Macrolophus* species. No characteristic VOC profile was found for hosts and non-hosts. Terpenes predominated in many hosts and carboxylic acids in non-hosts, but no specific VOCs were characteristic of host plants. Significant EAGs (maximum deflection values in mV) were recorded in plants with very different VOC profiles, both hosts and non-hosts. The EAGs were higher for *M. melanotoma* than for *M. pygmaeus*, and were higher for males than for females. In *M. melanotoma* the EAGs were greater with hosts than with non-hosts, but they were similar in *M. pygmaeus*. The EAGs were correlated with the concentrations of sesquiterpenes and alcohols in both species. In olfactory assays, *M. melanotoma* and *M. pygmaeus* preferred their respective hosts, but they did not discriminate between non-host with and without significant EAGs. According to the results, *Macrolophus* species are expected to rely on ubiquitous VOCs for the identification of their hosts. The variation in the EAGs between *M. melanotoma* and *M. pygmaeus* is attributed to the variation in the proportions of olfactory receptor neurones with different sensitivity to VOCs (e.g. sesquiterpenes). Host plant selection is discussed in the light of the perception of VOCs and the processing of information by the central nervous system.

## Introduction

The process of host plant or prey location in insects is structured in several steps involving peripheral olfactory receptors and the central nervous system (CNS) (Visser 1986; Christensen and Hildebrand 2002; Tohuara and Vosshall 2009). Phytophagous insects, in their evolution with plants, have developed the ability to locate, identify, and select suitable host plants and, for this purpose, they are equipped with olfactory receptors sensitive to plant volatiles (Bernays and Graham 1988; Bernays and Chapman 1994; Dicke 2000). Predators also use the changes in the composition and concentration of volatiles in plants attacked by phytophagous insects to locate their prey (Dicke 1994; Dicke and Loon 2000). Zoophytophagous insects share feeding habits with phytophagous and strictly carnivorous insects and, in consequence, they respond to volatiles emitted by both clean and infested plants (Ingegno et al. 2011; Lins et al. 2014). Mirids (Hemiptera: Miridae) compose one group of insects in which omnivory is widely diffused (Wheeler 2001). Omnivorous mirids,

like many phytophagous insects, have a restricted host plant range (Alomar et al. 1994; Bernays and Chapman 1994; Cassis and Schuh 2012; Ingegno et al. 2008; Martinez-Cascales et al. 2006; Sanchez et al. 2006a; Tavella and Goula 2001; Wheeler 2001). In this group, plant feeding is less nutritiously rewarding than feeding on prey (Sanchez et al. 2004); thus, the intensity of phytophagy increases as the abundance of prey diminishes (Alomar and Albajes 1996; Calvo et al. 2009; Sanchez 2008, 2009; Sanchez et al. 2006b, 2015). Although the performance of omnivorous mirids is better when feeding on prey, plant preference in some species [e.g. *Dicyphus hesperus* Knight (Hemiptera: Miridae)] is nevertheless driven by plant nutritional value (Sanchez et al. 2004).

In the family Miridae, the tribe Dicyphini has been extensively studied in the last few decades for their importance as predators of agricultural pests and for their ecological interest. Many of the species of this tribe may be considered as polyphagous (sensu Bernays and Chapman 1994) because they live and feed on plant species of several families. However, they are stenophagous in relation to the characteristics of the plants they inhabit, as most of their hosts are hairy and/or have sticky glandular hairs (Cassis 1986; Ingegno et al. 2008; Martinez-Cascales et al. 2006; Sanchez et al. 2003, 2006a; Schuh and Slater 1995). *Macrolophus melanotoma* (Costa) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) are two sibling species reported on several plant species, although problems in their identification can make dubious many of the host plant reports before the study of Martinez-Cascales et al. (2006). Castañe et al. (2013) hypothesized that *M. pygmaeus* is the only species that colonizes tomato [*Solanum lycopersicum* L. (Solanaceae)] in the Mediterranean area. *Dittrichia viscosa* (L.) Greuter (Asteraceae) is known as a main host for *M. melanotoma* (Alomar et al. 1994; Perdikis et al. 2003, 2007). *Macrolophus melanotoma* and *M. pygmaeus* share some hosts, such as *Ononis natrix* L. (Fabaceae) and *Paretaria officinalis* L. (Urticaceae) (Martinez-Cascales et al. 2006), but other plant species seem to be specific hosts for *M. melanotoma* (e.g. Salvia officinalis L., Lamiaceae) or *M. pygmaeus* (e.g. *Ballota hirsuta* Bentham, Lamiaceae) (Martinez-Cascales et al. 2006). Although these two *Macrolophus* species have been reported on many plant species, most of the plants sampled in the Mediterranean area are non-hosts and there are few plants where these mirids reach high densities (Sanchez et al. 2003). In environments where most of the plant species present are non-hosts for *Macrolophus* species (Sanchez et al. 2003), these mirids are expected to use plant cues to orientate themselves during host selection. *Macrolophus pygmaeus* has antennae coated with many kinds of sensilla that may be used for the detection of plant volatiles (Ingegno 2011), and it is able to discriminate between air currents carrying volatiles from healthy and infested

plants in several scenarios (Ingegno et al. 2011; Moayeri et al. 2006a, b, 2007a, b). During their evolutionary history, the peripheral olfactory systems of these two species could have evolved differently to accommodate the range of volatile compounds emitted by their host plants. Variations in odor preference result from transformations in the peripheral olfactory receptors in insect populations and species (Visser 1986). The sensory system in insects is known to act as a primary filter of the stimuli present in their surrounding environment (Silbering et al. 2008); therefore, not all plant volatiles are expected to be detected by an insect species, but rather only those providing information relevant for its survival (Visser 1986). The electroantennogram (EAG) technique detects small voltage deflections in olfactory receptors and it has frequently been used to measure the sensitivity of insect antennae exposed to volatiles (Hardie et al. 1995; Park and Hardie 1998; Wohlers and Tjallingii 1983). Odor molecules stimulate odorant receptors in the dendrites of olfactory sensory neurons in the antenna and the electric impulse travels to the CNS (Silbering et al. 2008). The inner processes in the insect brain are difficult to assess, but decisions resulting from interpretation of the information gathered by the olfactory system may be indirectly obtained by behavioral assays (Bruce et al. 2005; Guerrieri et al. 2005). Multiple-choice and olfactometric assays have been performed to determine host plant preferences in several dicyphine species, such as D. errans, D. hesperus, and *M. pygmaeus* (Ingegno et al. 2011, 2013; Moayeri et al. 2006a, b, 2007a, b; Sanchez et al. 2004), but electrophysiological assays have not been performed in this group of insects to date. The aim of the present work was to investigate the mechanism underlying host plant perception and selection in the two sibling species *M. melanotoma* and *M. pygmaeus*. In the first place, we investigated if host and non-host plants of *M. melanotoma* and *M. pygameus* had different volatile profiles and could be differentiated by specific volatile compounds. Secondly, we tested the EAG response of the two Macrolophus species to several host and non-host plants to find out if the two mirids responded differently to hosts and non-hosts or if, in contrast, they responded indistinctly to the volatiles in all the plants. Our hypothesis was that if olfactory receptor neurones were tuned to compounds present at higher concentrations in host than in non-host plants, then the EAG responses to the former would be greater. Thirdly, if both host and nonhost plants elicited a significant EAG response, we wanted to know if the two mirids were able to differentiate hosts from non-hosts in olfactory assays; if they were able to do so, we predicted that the discrimination between host and non-host plants would ultimately take place in the CNS.

## Materials and methods

*Insects and plants*

*Macrolophus melanotoma* was originally collected on *D. viscosa* in the area of Mazarron, Spain (37°3′N, 1°2′W) and *M. pygmaeus* was provided by Bioplanet (Bioplanet s.c.a., Cesena, Italy). The species were identified according to Martinez- Cascales et al. (2006). The two species were reared separately in insect cages on tobacco plants [*Nicotiana tabacum* L. (Solanaceae)], supplemented with eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), ad libitum at 24 ± 1 °C, 16:8 L:D. Ten plant species selected among hosts and non-hosts for *M. melanotoma* and *M. pygmaeus* were used in the electrophysiological assays (see Table 1): *B. hirsuta*, *Calendula officinalis* L. (Asteraceae), *Capsicum annuum* L. (Solanaceae), *Cistus albidus* L. (Cistaceae), *D. viscosa*, *P. officinalis*, *S. officinalis*, *S. lycopersicum*, *Solanum nigrum* L. (Solanaceae), *Vicia faba* L. (Fabaceae).

*Plant volatile analyses*

The volatile blends emitted by clean plants of the 10 species described above were analyzed using a headspace solid-phase microextraction (HS-SPME) device (Supelco, Bellefonte, PA, USA), according to Tholl et al. (2006). The volatile components were adsorbed onto a fused-silica fiber coated with polydimethylsiloxane (Supelco, Bellefonte, PA, USA). For the HS-SPME analysis, each plant in its pot was introduced into a 4-L chamber sealed with a Teflon septum. In order to achieve equilibrium between each sample and its headspace, before HS-SPME, the plants were placed in a glass container heated to 35 °C in a water double boiler for 30 min. Then, the fiber was exposed above the sample headspace for 30 min at 25 °C (the time and temperature needed to achieve equilibrium among the three phases of the system). The same procedure was followed with a pot full of soil, which was used as the control.

Once the analytes had been adsorbed onto the coating, the samples were injected manually into Agilent 6890 N gas chromatographs (GCs) (Palo Alto, CA, USA) equipped with a polar column, DB-Wax 52 CB (polyethyleneglycol-Carbowax) (30 m× 0.32 mm) with a film thickness of 1.0 μm, and an apolar HP-5 column (5 % cross-linked phenyl–methyl siloxane) (30 m× 0.25 mm) with a film thickness of 0.25 μm. Both stationary phases were supplied by Agilent Technologies (Palo Alto, CA, USA). Helium was used as the carrier gas (constant pressure, β-ionone eluted at 27.6 min for the HP-5MS column and at 41.38 min for the DB-Wax column) and the split ratio was set to 10:1.

For both stationary phases, the initial oven temperature was set at 50 °C; it was increased to 60 °C at a rate of 2.0 °C $min^{-1}$, then to 150 °C at a rate of 2.5 °C $min^{-1}$, and finally to 250 °C

with a ramp of 10 °C $min^{-1}$, for a total run time of 51 min. The GCs were coupled to an Agilent 5973 N mass spectrometer (MS) (Palo Alto, CA, USA). The injector was held at 250 °C and the MS transfer line was held at 280 °C. The MS was operated in electron impact ionization mode with an ionizing energy of 70 eV, scanning from m/z 50 to 500 at 3.21 scans/s. The quadrupole temperature was 150 °C and the electron multiplier voltage was maintained at 1300 V. For each species, four plants were analyzed twice in the polar column, while two plants were analyzed in the apolar column without repetition (for the absolute identification of volatile compounds).

The retention indices (RIs) of compounds were determined, relative to the retention times of a series of n-alkanes (C6-C17 n-alkanes), by linear interpolation, using appropriate software (PerkinElmer, Waltham, MA, USA). Single components were identified by the comparison of their RIs, on polar and apolar columns, with those of authentic compounds or literature data (Luning et al. 1994; Couladis et al. 2002; Marongiu et al. 2003; Gazim et al. 2007; Paolini et al. 2008; Farhat et al. 2009; Webster et al. 2010; Ogundajo et al. 2013) and using computer matching with commercial mass spectral libraries (National Institute of Standards and Technology - NIST02, Wiley7n). The compounds imputable to the fiber and pot of soil were subtracted from the data set of volatiles compounds identified in the analyzed plants. Then, the percentage of each compound was calculated by dividing the area of each chromatographic peak by the total chromatogram area. Following every day of recording, cleaning and screening of the residual volatile blend in the fiber were performed.

*EAG trials*

The response of *M. melanotoma* and *M. pygmaeus* to volatiles emitted by the 10 plant species was measured using an EAG device (Syntech Ltd., Hilversum, The Netherlands). The EAG deflection was measured in single antennae of 15 females and 15 males of *M. melanotoma* and 14 females and 10 males of *M. pygmaeus*, individually exposed to air currents carrying odorants of the 10 plant species. A different individual plant was used for each of the insect specimens. The EAG response was replicated four times using the same set of plant individuals (one of each species) for each insect specimen; in each replicate, each of the 10 plant species was offered at random. In each run of the experiment, 1 $cm^2$ (20 mm× 5 mm) of a fresh leaf, cut just before making the measurements, was inserted in a Pasteur pipette (Ø 7 mm× 150 mm); the ends of the pipette were covered with parafilm before and after exposure to the airflow to prevent desiccation of the leaf and evaporation of the plant volatiles. Although EAG studies are generally carried out using pieces of paper impregnated with plant

extracts (Fraser et al. 2003; Sasso et al. 2009), we considered that using pieces of leaves would reflect the spectrum of volatiles more realistically than extracts. Smith and Beck (2013) reported that, although damaged leaves may have a richer emission of volatile organic compounds (VOCs) than undamaged leaves, for both damaged and undamaged plants all plant species could be differentiated from one another by their distinct VOC profiles. Besides, plants damaged by cutting experienced a lesser change in their VOC profile than scratched and punctured plants (Smith and Beck 2013). Mechanical damage is frequent in natural conditions as plants are normally exposed to physical agents (e.g. wind). Following the instructions in the instrument manual (Syntech 2004), a clean strip (20 mm× 0.5 mm) of filter paper inside a Pasteur pipette was used as the negative control. A common green-leaf volatile (20 μL of (Z)-2-hexen-1-ol) provided on a 1 $cm^2$ (20 mm× 0.5 mm) strip of filter paper was used as the positive control, because this substance is known to produce an electrophysiological response in other insect species (Chen et al. 2004; Toshova et al. 2010; Sun et al. 2014). A continuous air flow of 1.2 L $min^{-1}$ and a stimulus flow of 0.6 L $min^{-1}$ were pumped through the pipette during EAG recording. The flow at the end of the tube (1.8 L $min^{-1}$) was ensured by using anti-return valves. The EAGs were recorded using head preparations. Prior to the dissection of the head, the insects were briefly anesthetized by exposing them to $CO_2$ for 10 s. The recording electrode was inserted into the head near the base of the first antennal segment and, subsequently, the other electrode was placed at the distal end of the last antennal segment. The EAG recording began 5 min after insertion of the electrodes, to allow for signal stabilization. The silver electrodes were enclosed in glass capillary tubes, produced with a microelectrode puller (Kopf, Model 720B, David Kopf Instrument, Tujunga, CA, USA), broken at the top and filled with an electrically conductive solution (0.1 M KCl). The antennae of the insects were exposed to the air flow for 1 s and the maximum EAG deflection was recorded; single exposures were separated by a 60-s gap to permit recovery. The negative control (clean filter paper) was tested after each stimulus, while the positive control ((Z)-2-hexen-1-ol) was tested every 10 stimuli to evaluate the antennal vitality. To avoid great differences between measurements and to prevent decreasing antennal sensitivity, a single antenna was tested within an hour of insect dissection.

*Olfactometer bioassays*

Plant choice in the two *Macrolophus* species was investigated by olfactometer bioassays. The plant species used in this experiment were selected based on the results from the EAG assays and on our knowledge of the host plants under natural conditions for the two *Macrolophus*

species. Three plant species which produced a significantly greater EAG response than the negative control were selected for the two-choice experiment (hereafter referred to as the "test plants"). Two of these plant species were known to be specific hosts for either *M. melanotoma* or *M. pygmaeus*; the third species was a non-host for both *Macrolophus* species which produced a significant EAG response. As a control, a fourth plant species (hereafter referred to as the "control"), chosen among the non-hosts for which neither *M. melanotoma* nor *M. pygmaeus* had a significant EAG response, was tested against the other plant species in pairwise choices.

The bioassays were carried out in a vertically-positioned, Y-shaped Pyrex tube (internal Ø 23 mm), consisting of a 250-mm central arm and two 200-mm side arms set at a 70° angle. Each side arm was connected to a glass cylindrical chamber (height 500 mm, Ø 130 mm) containing one whole plant as the odor source, as described by Ingegno et al. (2011). A filtered and humidified airflow of 2.5 L $min^{-1}$ passed through the olfactometer apparatus. Thirty males per species were used for the comparison between the test plants and the control. Males were chosen for these assays because they showed a higher EAG response and are more mobile than females. One-week-old males, reared on tobacco plants and kept without prey in a glass tube (length 120 mm, Ø 23 mm) for 18 h before being used, were individually introduced through a meshed cap at the entrance of the Y-tube central arm. Each male was observed until it had walked at least 6 cm up one of the side arms or until 20 min had elapsed. Males that did not choose a side arm within 20 min were considered as "no choice" and excluded from the statistical analysis. In such cases, new individuals were tested until 30 responses were recorded for each treatment. Each individual was tested only once. The odor sources were hidden behind a black panel and, after testing five individuals, were switched between the left-hand and right-hand side arms to minimize any spatial effect. After testing 10 individuals on the same odor sources the apparatus was cleaned with neutral soap and alcohol (70 % by vol.) and sterilized in an autoclave at 120 °C for 20 min. The bioassays were conducted at 24 ± 1 °C, RH 25–30 %, and 540 ± 30 lx.

*Statistical Analyses*

The VOC composition of each plant species was given as the relative proportions of each compound obtained by peak area normalization after the subtraction of the VOCs due to the silica fiber, the pot, and the collecting chamber. The VOC data used for the statistical analyses, with the areas of each compound for each individual plant, are given in the supplementary table A. The Student's t-test was used to compare the number of VOCs of the

host plants (for either of the two Macrolophus species) and non-hosts. Kruskal's non-metric multidimensional scaling (NMDS), using the Bray-Curtis distance, was applied to reduce the information on VOCs to a lower number of dimensions ($K = 3$). When the concentrations of two compounds were correlated (Pearson's correlation test, $P < 0.05$), the one with the lowest concentration was excluded from the matrix. The compounds included in this analysis are indicated in Table 2. The VOC profiles of host and non-host plants for the two *Macrolophus* species were compared by permutational MANOVA (non-parametric MANOVA). The same kind of analysis was used to compare the VOC profiles of specific host plants for *M. melanotoma* and *M. pygmaeus* (Table 1).

The EAG maximum deflection values (mV) in the four replicates for each insect specimen on the same individual plant were averaged for the statistical analyses. The response of the antennae (i.e. the maximum EAG deflection value, in mV) to each of the 10 plant species and to (Z)-2-hexen-1-ol (positive control) was compared with the response to the negative control using the Student's ttest. The EAGs (i.e. the EAG maximum deflection values, in mV) of females and males were compared by ANOVA for M. melanotoma and M. pygmaeus, for each plant species. The effects of the Macrolophus species and sex on the electrophysiological responses (i.e. the EAG maximum deflection values, in mV) for the 10 plant species were tested by multivariate analysis of variance (MANOVA), according to Crawley (2007). Then, ANOVA was performed individually on each plant species using the EAG maximum deflection values as a dependent variable, to determine the contribution of each plant species to the differences in the EAG response due to species and sex - as determined in the MANOVA. A second MANOVA followed by ANOVA was performed on the data normalized by the positive control. That is, the maximum deflection value for each plant specimen was divided by the maximum deflection value of its respective positive control. This second analysis was performed to determine the differences in perception between *M. melanotoma* and *M. pygmaeus* after normalization in relation to the positive control, to account for the differences in magnitude of the response of each species. The intensity of the EAG response was compared between host and non-host plants, as classified in Table 1, for the two *Macrolophus* species. The relationship between the average EAG response (in mV) and the concentration of the different chemical families of volatiles for each Macrolophus species and sex was determined by linear regression; the EAG values and VOC concentrations were transformed by the natural logarithm to account for heteroscedasticity. The choice of the individuals of the two *Macrolophus* species in the olfactometer bioassays

was analyzed by Chi-square tests; the null hypothesis was that the choice would be distributed 50:50 across the two odor sources.

All statistical analyses were performed using the R software (R Development Core Team 2008).

## Results

*Analyses of Plant Volatiles*

The VOCs for each plant species are reported as the mean percentages (±SE) of theirchromatographic peak area with respect to the total chromatogram area, following the retention index (RI) order on the polar column (Table 2). Generally, more compounds were identified by GC-MS in the host plants than in the non-host plants (Student's *t*-test, *t*-value= 3.923, *df*=38, *P*< 0.001). In fact, the number of compounds found in host plant species (except *P. officinalis*) varied from 11±1 in *S. nigrum* to 34± 1 in *S. officinalis*, and in the non-host plants from 9 ± 1 in *C. albidus* and *V. faba* to 10± 1 in *C. annuum*. Due to the high variability within plant species, the number of VOCs reported in Table 2 is greater than the mean number of compounds found for each plant species. The composition of the volatile phase varied among plant species. Terpenes (mono and sesquiterpenes) predominated in *B. hirsuta*, *C. officinalis*, and *D. viscosa*, while carboxylic acids were the most abundant in *C. annuum*, *C. albidus*, *S. lycopersicum*, *P. officinalis*, and *V. faba*; ketones were highly represented in S. officinalis and *S. nigrum*. In particular, among the host plants, *B. hirsuta*, *C. officinalis*, *D. viscosa*, and *S. officinalis* presented the richest chromatograms, with more than 30 compounds. In *C. officinalis*, monoterpenes and sesquiterpenes accounted for half of the total VOC concentration, comprising 85 % of the identified substances, with α-thujene and α-humulene representing more than 50 %. In S. officinalis, α-thujone together with camphor constituted more than 60 % of the identified volatiles. In B. hirsuta, 82% of the volatiles were sesquiterpenes; in particular, β-cubenene accounted for 35 %. In *D. viscosa*, nerolidol, camphor, and (Z)-α-curcumene constituted more than 50 %. In *S. lycopersicum*, the most abundant compound was hexanoic acid, which constituted 36 % of the total area, while in *S. nigrum* it was fenchone, representing 39 %. *Parietaria officinalis* was the plant species with the lowest number of volatiles (8), of which the most abundant was hexanoic acid. The non-host plant species presented poorer volatile profiles than the host species; the most common substance in non-hosts was hexanoic acid (Table 2). The NMDS showed a highly variable VOC profile within and between plant species (Fig. 1). A high correlation ($R^2$ = 0.99) was observed between the calculated and observed dissimilarities in the NMDS analysis using

three dimensions ($k$ = 3). A number of host plants for the two *Macrolophus* species (i.e. *B. hirsuta*, *C. officinalis*, *S. officinalis*, and *D. viscosa*) were characterized by some specific VOCs, or by compounds present at low frequency in other plant species, which contributed to the segregation of these plants in distinct clusters (Fig. 1). All these species shared many VOCs and were positioned on the negative side of the first dimension in the NMDS, with the exception of two specimens of *D. viscosa* (Fig. 1). This group of host plants had some terpenic hydrocarbons not found in non-host plant species, such as α-muurolene, γ-muurolene, α-copaene, calamenene, α-cubebene, and sabinene (Table 2). In contrast, other host plants (i.e. *S. nigrum* and *P. officinalis*) had low numbers of VOCs and clustered with non-hosts (Fig. 1). Overall, the VOC profile of the host plants for the two *Macrolophus* species was significantly different from that of the non-hosts (Permutational MANOVA: $F$=4.27; $df$=1, 38; $P$< 0.001). Host plants specific for *M. melanotoma* (i.e. S. officinalis and D. viscosa) or *M. pygmaeus* (i.e. *B. hirsuta*, *S. lycopersicum*, and *S. nigrum*) shared in exclusivity very few compounds; the clusters of *M. melanotoma* and *M. pygmaeus* host plants overlapped in the NMDS (Fig. 1). Nevertheless, significant differences were found between the volatile profiles of host plants specific for *M. melanotoma* and *M. pygmaeus* (Permutational MANOVA: $F$=2.60; $df$=1, 18; $P$<0.05). All non-host plants clustered together on the positive side of the first dimension (Fig. 1). The VOCs associated with the cluster of non-host plants (e.g. 2-ethyl-1-hexanol, 2-ethylhexanoic acid, decanal, nonanal) were not specific to the non-host plants and they were also present in the rest of the plant species. The plants in the non-host cluster had in common the absence of most of the terpenic hydrocarbons that characterized the cluster on the negative side of the first dimension in the NMDS.

*EAG Trials*

The typical EAG deflection curves obtained in females and males of *M. melanotoma* and *M. pygmaeus* are shown in Fig. 2. In *M. melanotoma*, the average EAG values ranged from -0.153± 0.026 mV (negative control, mean± SE) to -0.647± 0.046 mV [(Z)-2-hexen-1-ol)] in females, and from -0.191± 0.029 mV (negative control) to - 0.789 ± 0.090 mV [(Z)-2-hexen-1-ol] in males. The greatest deflections were recorded with *B. hirsuta* ((-0.617 ± 0.083 mV) for females, and with *D. viscosa* (-0.660 ± 0.085 mV) for males (Fig. 3). *Vicia faba* elicited the smallest response, in both females (-0.154 ± 0.021 mV) and males (-0.191 ± 0.029 mV) (Fig. 3). For all the plants, with the exception of C. *albidus* and *V. faba*, the EAG responses in *M. melanotoma* (females and males) differed significantly ($P$<0.1) from the negative control (Table 3). Significant differences in the EAG responses between females and males of *M.*

*melanotoma* were found for *C. annuum* ($F$=6.83; $df$=1, 28; $P$<0.05), *P. officinalis* ($F$=6.00; $df$=1, 28; $P$<0.05), and S. *nigrum* ($F$=5.14; $df$=1, 28; $P$<0.05). In *M. pygmaeus*, the average EAG values ranged from -0.084± 0.024 mV (negative control) to -0.573 ± 0.071 mV [(Z)-2-hexen-1-ol)] in females, and from -0.081 ± 0.021 mV (negative control) to -0.483 ± 0.050 mV [(Z)-2-hexen-1-ol)] in males (Fig. 3). *Dittrichia viscosa* produced the biggest deflections, in both females (-0.380 ± 0.032 mV) and males (-0.376 ± 0.058 mV), while *V. faba* caused the smallest deflections (females = -0.097± 0.018 mV; males= -0.108 ± 0.022 mV) (Fig. 3). The EAG responses in *M. pygmaeus* were significantly different from the negative control, with the exception of *C. albidus*, *P. officinalis*, *S. nigrum*, and *V. faba* in females, and *C. albidus* and *V. faba* in males (Table 3). Significant differences in the EAG responses between the females and males of *M. pygmaeus* were found for *C. annuum* ($F$=13.33; $df$=1, 22; $P$ = 0.01), *P. officinalis* (F=5.94; df = 1, 22; P<0.05), and *S. nigrum* ($F$=4.98; $df$=1, 22; $P$<0.05).

The MANOVA denoted a significant difference in the perception of volatiles between the two *Macrolophus* species ($F_{approx}$=4.76; $df$=1, 50; $P$< 0.001) and the two sexes ($F_{approx}$=2.46; $df$=1, 50; $P$< 0.05). All the stimuli [plants, (Z)-2-hexen-1-ol, and negative control], with the exception of *S. lycopersicum* ($F$=0.424; $df$=1, 50; $P$= 0.518), contributed significantly to the differences in perception between the two *Macrolophus* species. *Capsicum annuum* ($F$=12.99, $df$=1, 50, $P$<0.001), *P. officinalis* ($F$=9.25, $df$=1, 50, $P$<0.01), and *S. nigrum* ($F$=8.58, $df$=1, 50, $P$< 0.01) contributed significantly to the difference between the sexes. The EAG values were normalized, in relation to the positive control, to test for the differences in perception between the two *Macrolophus* species independently of the magnitude of the response to the volatiles. When the MANOVA was run on the normalized data set, there were still significant differences in the perception of stimuli between the two *Macrolophus* species ($F_{approx}$ = 7.50; $df$ = 1, 50; $P$ < 0.001) and the two sexes ($F_{approx}$ = 2.28; $df$ = 1, 50; $P$< 0.05 ); the species-sex interaction was not significant ($F_{approx}$=1.02; $df$=1, 50; $P$=0.446). *Ballota hirsuta*, *P. officinalis*, *S. lycopersicum*, and *S. nigrum* were the plant species that contributed significantly to the differences in EAG response between *M. melanotoma* and *M. pygmaeus* (Table 4), while *B. hirsuta*, *C. annuum*, *P. officinalis*, and *S. nigrum* contributed to the EAG differences between sexes (Table 4). In *M. melanotoma* the EAG signal was stronger with host than with non-host plants ($F$=25.83, $df$=1, 296, $P$< 0.001). In contrast, in *M. pygmaeus* no significant differences in the intensity of the response were found between host and non-host plants ($F$=1.10, $df$=1, 296, $P$= 0.296). The EAG response was correlated with the concentration of sesquiterpenes, in both *M. melanotoma* ($F$=10.52, $df$=1, 18, $P$<0.01) and *M. pygmaeus* ($F$=7.60, $df$=1, 18, $P$<0.05). The EAG response was also significantly correlated with the

391 concentration of alcohol, in both *M. melanotoma* ($F$=7.15, $df$=1, 18, $P$<0.05) and *M.*
392 *pygmaeus* ($F$=5.21, $df$=1, 18, $P$<0.05).
393
394 *Olfactometer Bioassays*
395 *Solanum lycopersicum*, *D. viscosa*, and *C. annuum* were selected out of the plant species
396 eliciting a significantly higher EAG response than the control (Table 3) in both *Macrolophus*
397 species, according to a dichotomic criterion. These three plant species were tested in
398 olfactometer bioassays against *V. faba*, which provided EAG values similar to those of the
399 negative control - in both the females and males of the two *Macrolophus* species. In the Y-
400 tube olfactometer assays (Fig. 4) for the comparison of *S. lycopersicum* with *V. faba*, *M.*
401 *melanotoma* chose the two odor sources with equal frequency ($\chi^2$=0.00; $df$=1; $P$=1.00), while
402 *M. pygmaeus* showed a higher preference for tomato plants ($\chi^2$=3.33; $df$=1; $P$= 0.07). In the
403 comparison of *D. viscosa* with *V. faba*, *M. melanotoma* showed a strong preference for *D.*
404 *viscosa* ($\chi^2$=4.80; $df$=1; $P$= 0.028), whereas no significant difference was found in the choice
405 of *M. pygmaeus* ($\chi^2$=0.13; $df$=1; $P$= 0.72). In the comparison of *C. annuum* with *V. faba*, the
406 similar distribution of individuals in the two arms of the olfactometer, for both *Macrolophus*
407 species, indicated no preference for either plant species (*M. melanotoma*: $\chi^2$=2.13; $df$=1;
408 $P$=0.14. *M. pygmaeus*: $\chi^2$=0.00; $df$=1; $P$ = 1.00). Only two males of *M. melanotoma* and one
409 of *M. pygmaeus* did not respond in the olfactometer bioassays.
410
411 **Discussion**
412 A large number of volatile compounds were identified, with great variation in the chemical
413 profiles among the plant species. Other authors have also reported great variability in plant
414 volatiles, even within genotypes grown under the same environmental conditions (Webster et
415 al. 2010). Host plants for the *Macrolophus* species had, in general, a greater number of VOCs
416 than non-host plants, but there were no characteristic VOC profiles for hosts and non-hosts.
417 Some of the host plants for the two *Macrolophus* species (*B. hirsuta*, *C. officinalis*, *D.*
418 *viscosa*, and S. *officinalis*) were very rich in VOCs and showed a distinct profile, but others
419 (S. *nigrum* and *P. officinalis*) had a low number of VOCs and a profile similar to that of non-
420 host species. At the species level, no differences between the VOC profiles of *M. melanotoma*
421 and *M. pygmaeus* specific host plants were evident.
422 In the absence of specific compounds that differentiate host from non-host plants, these two
423 *Macrolophus* species are expected to rely on ubiquitous compounds for the identification of
424 their hosts. The use of ubiquitous compounds seems to be more widely diffused than the use

425 of specific compounds for host recognition in insects (Bruce and Pickett 2011; Webster et al.
426 2010). It is considered that reliance on ubiquitous rather than specific plant volatiles makes
427 the odor-coding system flexible and allows species to adapt to environmental changes (Bruce
428 and Pickett 2011). In agreement with this hypothesis, significant EAGs in *M. melanotoma* and
429 *M. pygmaeus* were registered for plant species with very different VOC spectra. In contrast,
430 olfactory receptor neurones in insects are generally specific to a small number of the volatile
431 compounds emitted by their hosts (Webster et al. 2010). In the present work, the maximum
432 deflection values in the EAGs were correlated with the concentrations of sesquiterpenes and
433 alcohols in the volatile phase. Olfactory receptor neurones might have specificity to some of
434 the sesquiterpenes (i.e. α-muurolene, γ-muurolene, α-copaene, calamenene, α-cubebene, and
435 sabinene) common to some of the host plants (i.e. *B. hirsuta*, *C. officinalis*, *S. officinalis*, and
436 *D. viscosa*). Besides, olfactory receptor neurones may be tuned to some of the alcohols
437 present in certain host and non-host plants. The lack of EAG response to some of the plant
438 species (e.g. *V. faba*) could be due to the absence of volatile compounds that stimulate
439 olfactory receptor neurones.
440 The EAG assay measures the sum of all the action potentials and, thus, it varies in function of
441 the abundance of olfactory receptor neurones with differing specificity to volatile compounds
442 in the insects' antenna (Bernays and Chapman 1994). Therefore, the differences in the *M.*
443 *melanotoma* and *M. pygmaeus* EAGs between some of the plant species could be due to
444 variation in the proportions of olfactory receptor neurones with differing sensitivity to the
445 volatile compounds present in each plant species. The abundance and specificity of olfactory
446 receptors may be related to the range of host plants of these two mirid species. This is in
447 agreement with the higher EAGs registered for hosts than for non-hosts in *M. melanotoma*,
448 something which was not observed in *M. pygmaeus*. Differences in the composition of the
449 olfactory receptors in sensory organs may have ecological implications. For instance, tomato
450 is not a host for *M. melanotoma* and a reduction in the level of perception of the volatiles
451 emitted by tomato would be expected to have little impact on the performance of this mirid in
452 its environment. By contrast, tomato is one of the main hosts for *M. pygmaeus* and the ability
453 of this mirid to detect its volatiles may confer evident advantages in the process of host
454 location. In the same way, the plants which did not elicit a significant EAG, such as *V. faba*,
455 are not used as hosts by either of the two *Macrolophus* species and, therefore, detection of the
456 volatiles emitted by these plant species could be irrelevant to orientation during host-plant
457 location. In contrast, *Aphis fabae* Scopoli (Hemiptera: Aphididae) responds significantly to
458 the volatiles emitted by *V. faba*, its host plant species (Webster et al. 2008).

In cases where olfactory receptors are stimulated by compounds that are not unique to their host plants, the host recognition depends on the particular blend of volatiles (Bruce and Pickett 2011). Because similar EAG responses may be generated by different blends of volatiles, the determination of plant identity depends on the processing of the information conveyed by the different types of neuroreceptor ("labeled lines") in the CNS (Bernays and Chapman 1994; Ryan 2002; Webster et al. 2010). In fact, in spite of the high variation in plant VOCs and the similarity of the EAG response between some host and non-host plants, the olfactometer assays showed that *M. melanotoma* and *M. pygmaeus* are able to discriminate between host and non-host species. For instance, *M. melanotoma* had a significant EAG response to the volatiles emitted by both hosts (e.g. *D. viscosa*) and non-hosts (e.g. *C. annum* and *S. lycopersicum*), but in the olfactometer assays its highest preference was for *D. viscosa*, its main host in nature. Similarly, *M. pygmaeus* had a significant EAG response to both hosts (e.g. *S. lycopersicum*) and non-hosts (e.g. *D. viscosa* and *C. annum*), but its highest preference was for *S. lycopersicum*, one of its main hosts in nature. In all these cases, the peripheral olfactory system in the two *Macrolophus* species was stimulated by the blend of volatiles present in both host and non-host plants; therefore, discrimination among plant species is expected to take place ultimately in the CNS. The similar olfactory perception in two forms of *A. fabae* associated with different plant species also suggests that host discrimination does not take place in the periphery but rather in the CNS (Hardie et al. 1995). Other studies have recorded a higher preference of *M. pygmaeus* for tomato volatiles than for clean air currents (Ingegno et al. 2011).

The EAG response was generally higher in males than in females, with significant differences between the sexes, curiously, for the two *Macrolophus* species in the same plants (i.e. *C. annuum*, *P. officinalis*, and *S. nigrum*). These similarities suggest that males may use some of the compounds released by plants as cues to find females, or that there is a resemblance between those compounds and sex pheromones. Moayeri et al. (2007a) argued that some of the volatiles induced in tomato plants were used by males of *M. pygmaeus* as cues for mate location. Groot et al. (1999) found that some of the esters that elicited EAG responses that were greater in males than in females of *Lygocoris pabulinus* (L.) (Hemiptera: Miridae) were components of sex pheromones. The greater sensitivity of males than of females might be related to the different role of each gender in reproduction; males have to find females and their greater olfactory sensitivity may confer on them some advantages in the process of mate location. Dicyphine females are less likey to fly than males, especially with high egg-loads (personal observations). Due to their greater tendency to move, it is quite likely that males

play the main role in gene flow among populations, so their higher sensitivity to VOCs may increase the probability that they locate the plants where females are established. Males from another mirid species [i.e. *Lygus rugulipennis* Poppius (Heteroptera: Miridae)] are known to respond to plant volatiles, which could be used as cues to locate feeding sites and to increase the probability of finding females (Frati et al. 2008). Another possibility is that males act as pioneers, enhancing the probability of mate encounters; Frati et al. (2008) found that *L. rugulipennis* females were attracted to plants with mated conspecific males in wind tunnels. Although sex pheromones are expected to be implicated in mate location, plant volatiles are quite likely to occur in the environment at higher concentrations and may be more reliable for orientation at long distances than sex pheromones. Studies on other insects (i.e. cerambycid beetles) indicate that host plant volatiles are used to locate mates at long distances, while pheromones may operate at short and medium distances (Lacey et al. 2004; Ginzel and Hanks 2005).

The differences in the intensity of the EAG responses between females and males might be due to the variation in the number of sensillae in the antennae of the two sexes. Ingegno (2011) reported that the number of sensillae in the second antennal segment of *M. pygmaeus* was significantly higher in males than in females. An EAG registers the sum of the fall in voltage of all the stimulated olfactory sensory neurones in the antenna (Park and Hardie 1998); therefore, greater voltage deflections are correlated with a higher number of neurones responding to the signal (Bernays and Chapman 1994; Ryan 2002). No information is available on the number of sensillae in the antennae of *M. melanotoma*, but we predict that they will be more numerous in this species than in *M. pygmaeus*, and in males relative to females. In other mirid species, such as *L. pabulinus*, the number of sensillae in males was found to be almost double that in females (Groot et al. 1999).

Other results, like the non-significant EAG response of *M. pygmaeus* females to some of the hosts reported for this species (e.g. *S. nigrum*), may reflect differences in the perception of the volatiles among populations of *Macrolophus* species depending on the host plants present in their distribution area. *Macrolophus pygmaeus* is common on *S. nigrum* in Greece, Italy, and northern Spain (Alomar et al. 1994; Lykouressis et al. 2000; Tavella and Goula 2001), but not in southern Spain (Sanchez et al. 2003). Differences in perception could reflect variations in the sensitivity of the peripheral olfactory systems among populations. Sanchez et al. (2012) found genetic differences among populations of *M. pygmaeus* on the Iberian, Italian, and Balkan peninsulas, which may be associated with divergences in the biological traits of the populations. Variations in the response to plant odors have been found in different

populations of other insect species such as *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) and *Leptinotarsa decemlineata* L. (Coleoptera: Chrysomelidae) (Fuyama 1978; Visser 1986). In the same way, the differences in perception between the two Macrolophus species could be associated with modifications of the peripheral olfactory system following the divergence of the two species through their particular evolutionary pathways. *Macrolophus melanotoma* and *M. pygmaeus* are sympatric species that may have segregated spatially on different hosts to avoid competition; thus, their olfactory systems may, over time, have become tuned to the compounds most relevant to each species.

In this study, electrophysiological assays, performed for the first time in the dicyphine tribe, showed significant differences in the perception of plant volatiles between the two sibling species *M. melanotoma* and *M. pygmaeus*. This is also the first time that the composition of plant volatiles has been linked to the EAGs and behavior of these insects. The results of the electrophysiological and behavioral assays indicate that both the peripheral olfactory and central nervous systems are involved in the process of host plant selection in *M. melanotoma* and *M. pygmaeus*. The volatiles of some of the plant species not used as hosts in nature did not elicit an EAG response in either of the two *Macrolophus* species; therefore, the peripheral olfactory system seems to act as a primary filter of information, and host choice may be limited by the particular perception of the environment by each species. Besides, the two *Macrolophus* species had similar EAG responses to plants with very different VOC profiles; thus, the discrimination between host and non-host plants is expected to take place in the CNS. Further research should be performed to deepen our knowledge of the peripheral olfactory system in both *Macrolophus* species, by means of scanning and transmission electron microscope analyses and the study of EAG responses in single sensillae, and of the perception of single volatile compounds, using GC coupled with EAG.

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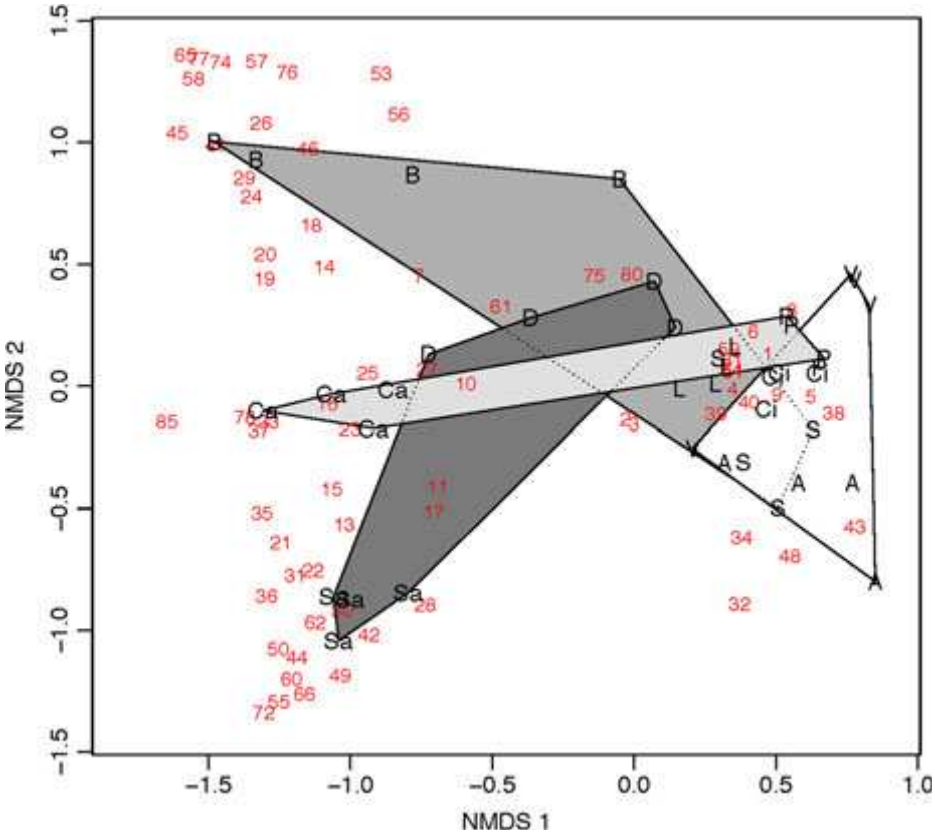
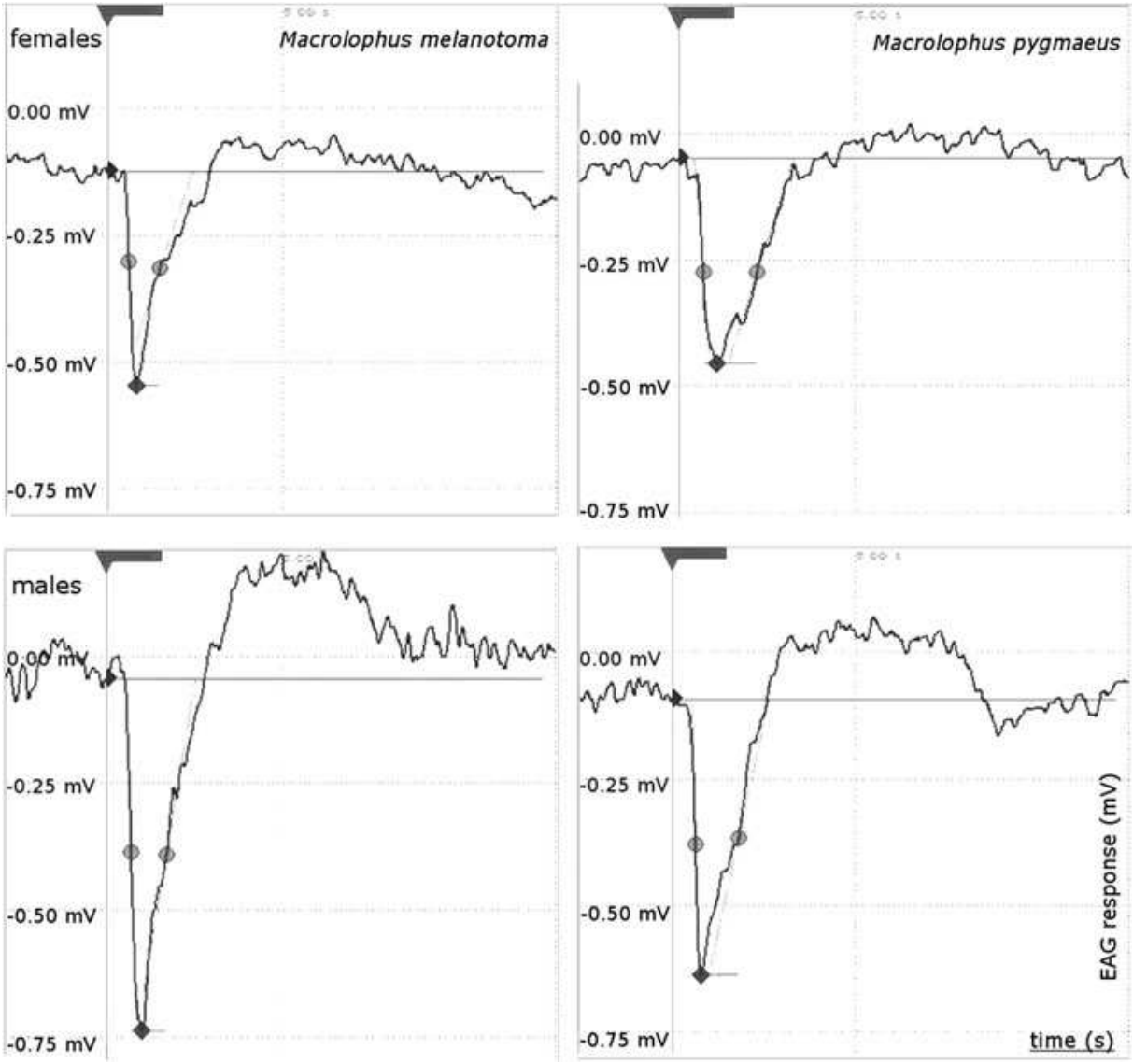



710

711 **Fig. 1** Plot of the two first components in the NMDS analysis. Plant codes: B (*Ballota hirsuta*), Ca (*Calendula*
712 *officinalis*), A (*Capsicum annuum*), Ci (*Cistus albidus*), D (*Dittrichia viscosa*), L (*Solanum lycopersicum*), P
713 (*Parietaria officinalis*), Sa (*Salvia officinalis*), S (*Solanum nigrum*), V (*Vicia faba*). The volatile codes are given
714 by numbers (see Table 2). The polygons connect the outer individuals in specific M. melanotoma and M.
715 pygmaeus hosts, shared host plants, and non-host plants.

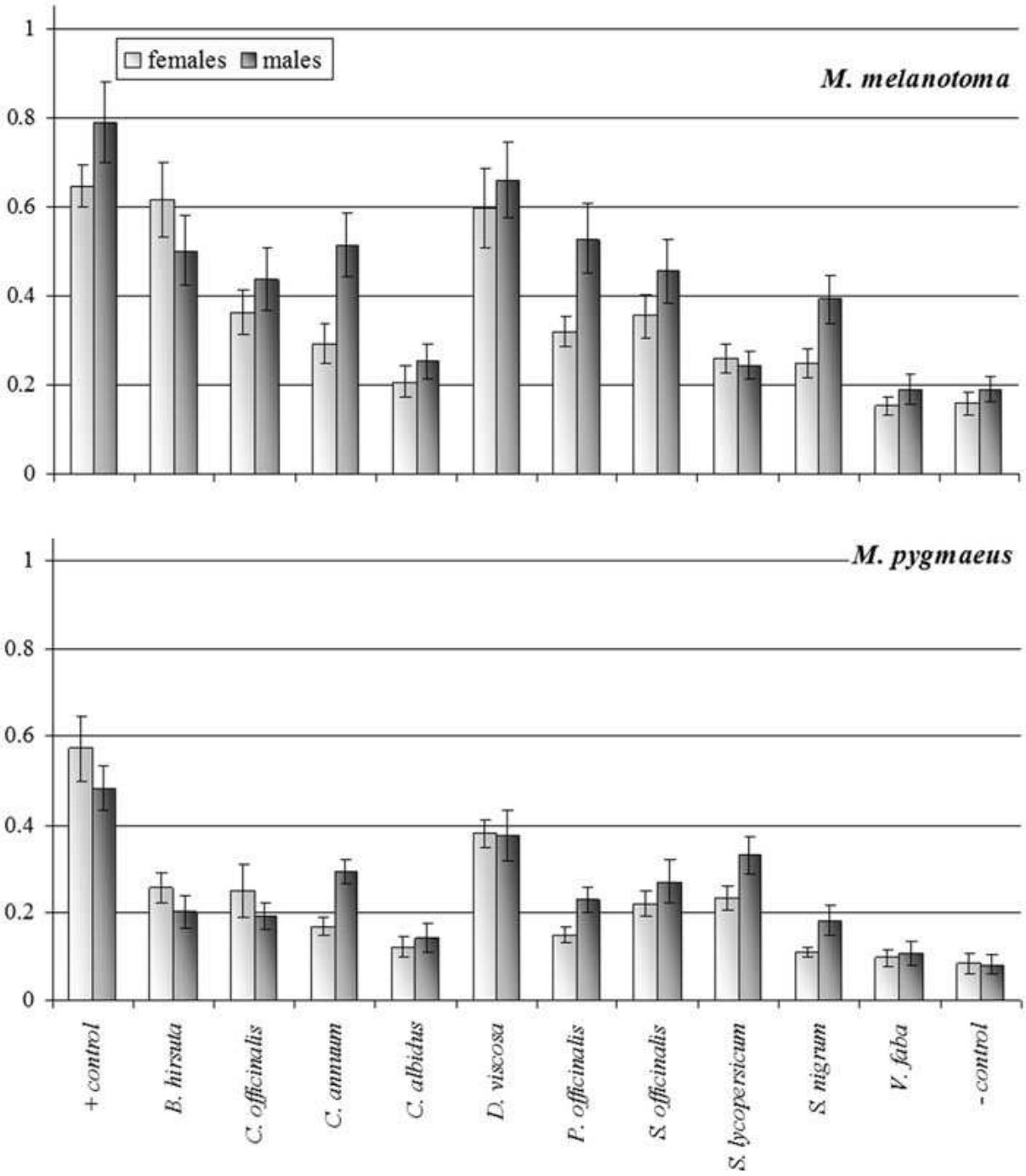
716




717

718 **Fig. 2** EAG response waveforms of a female (top) and a male (bottom) of *Macrolophus melanotoma* (left) and
719 *M. pygmaeus* (right), for an active odor stimulus (*Dittrichia viscosa*)

720

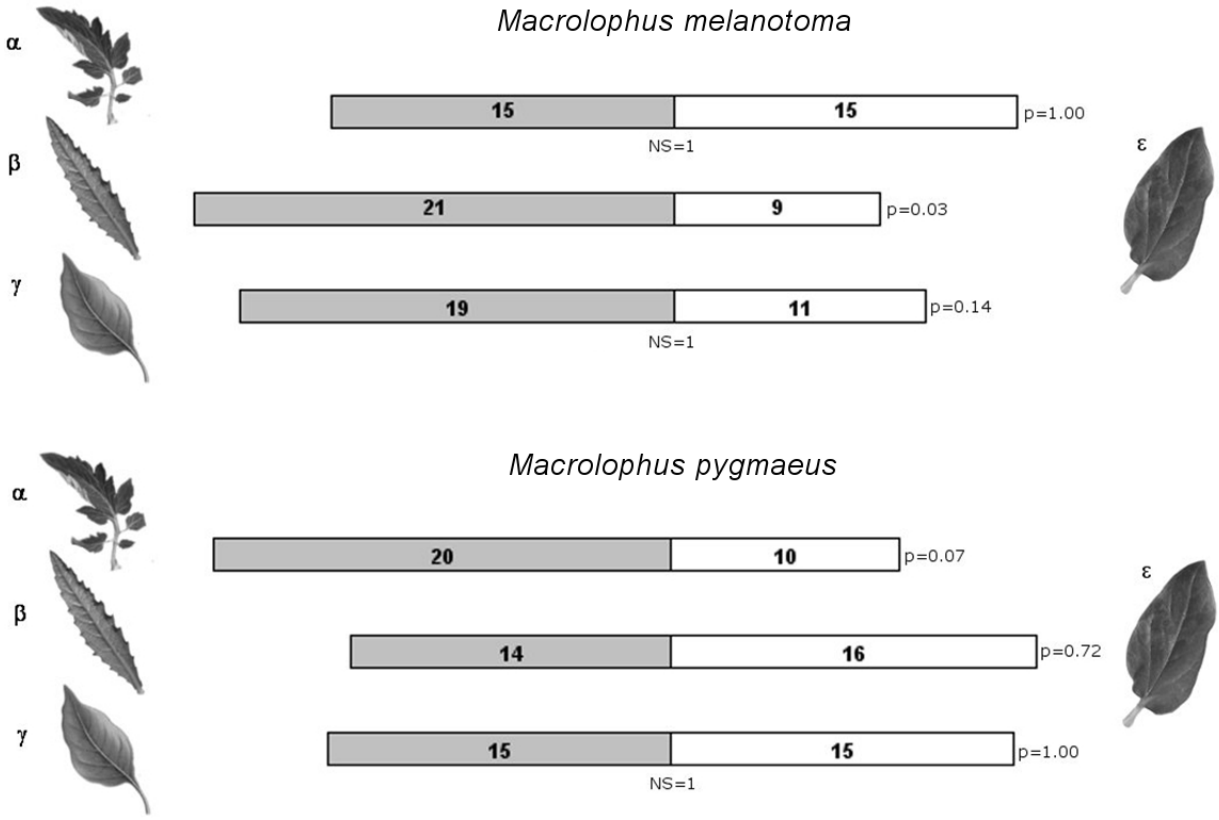



721

722 **Fig. 3** Absolute average EAG response (± standard error) to each odor stimulus in females (light gray bars)

723 and males (dark gray bars) of *Macrolophus melanotoma* (upper graph) and *M. pygmaeus* (lower graph)

724




725

726 **Fig. 4** Responses of *Macrolophus melanotoma* and *M. pygmaeus* [no. of responding males, in bars, and of non-
727 responding males (NS)] in a Y-tube olfactometer to the odors of α = *Solanum lycopersicum*; β = *Dittrichia*
728 *viscosa*; γ = *Capsicum annuum*, compared with ε = *Vicia faba*; the $\chi 2$ statistics tested the hypothesis that the
729 distribution of side-arm choices deviated from a null model in which odor sources were chosen with equal
730 frequency

731

732 **Table 1** Plant species used in the EAG assays, reported as hosts and non-hosts of *M. pygmaeus* and/or *M.*
733 *melanotoma*

| Species | Host type | Plant species | References |
|---|---|---|---|
| *M. melanotoma* | Host | *Calendula officinalis* | Alomar et al. 2006 |
| | | *Dittrichia viscosa* | Alomar et al. 1994; Castañe et al. 2013; Goula 1989; Lykouressis et al. 2000; Martinez-Cascales et al. 2006; Perdikis et al. 2003; 2007 |
| | | *Parietaria officinalis* | Lykouressis et al. 2000 |
| | | *Salvia officinalis* | Ingegno B.L. unpublished data |
| | Non-host | *Ballota hirsuta* | Martinez-Cascales et al. 2006 |
| | | *Capsicum annuum* | Sanchez et al. 2003 |
| | | *Cistus albidus* | Sanchez et al. 2003 |
| | | *Solanum lycopersicum* | Perdikis et al. 2008; Castañe et al. 2013; Sanchez J.A. unpublished data |
| | | *Solanum nigrum* | Sanchez J.A., unpublished data |
| | | *Vicia faba* | Sanchez et al. 2003 |
| *M. pygmaeus* | Host | *Ballota hirsuta* | Martinez-Cascales et al. 2006 |
| | | *Calendula officinalis* | Tavella and Goula 2001 |
| | | *Parietaria officinalis* | Tavella and Goula 2001 |
| | | *Solanum lycopersicum* | Castañe et al. 2013; Goula and Alomar 1994; Lykouressis et al. 2000; Tavella and Goula 2001; Martinez-Cascales et al. 2006 |
| | | *Solanum nigrum* | Goula and Alomar 1994; Lykouressis et al. 2000; Tavella and Goula 2001 |
| | Non-host | *Capsicum annuum* | Sanchez et al. 2003 |
| | | *Cistus albidus* | Sanchez et al. 2003 |
| | | *Dittrichia viscosa* | Lykouressis et al. 2000; Castañe et al. 2013; Sanchez J.A. unpublished data |
| | | *Vicia faba* | Sanchez et al. 2003 |

734 **Table 2** Volatile organic compounds of 10 plant species, presented as the mean percentage (±SE) of their chromatographic peak area and reported according to their RI order
735 in a polar column. Volatile code (Code). * indicates that the compound was included in the NMDS analyses

| Code | * | Volatile compounds | RI polar | RI apolar | *Ballota hirsuta* | *Calendula officinalis* | *Capsicum annuum* | *Cistus albidus* | *Dittrichia viscosa* | *Parietaria officinalis* | *Salvia officinalis* | *Solanum lycopersicum* | *Solanum nigrum* | *Vicia faba* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Terpenic Hydrocarbons** | | | | | | | | | | | | | | |
| | | Monoterpenes | | | | | | | | | | | | |
| 72 | * | tricyclene | 1086 | | | | | | | | 0.1±0.0 | | | |
| 17 | * | α-pinene | 1098 | 1004 | 0.2±0.2 | | | | 3.6±0.7 | | 4.3±1.2 | | | 13.3±0.0 |
| 37 | * | α-thujene | 1098 | 1000 | 0.3±0.0 | 35.3±3.9 | | | | | 3.9±0.0 | | | |
| 28 | * | camphene | 1129 | 1013 | | | | | 0.3±0.2 | | 2.9±0.6 | | | 18.5±0.0 |
| 12 | | β-pinene | 1168 | 1034 | | 0.2±0.1 | | | 1.6±0.2 | | 1.6±0.3 | | | 7.1±0.0 |
| 16 | * | sabinene | 1179 | 1034 | | 2.2±0.3 | | | 0.5±0.2 | | | | | |
| 51 | * | 4-carene | 1191 | | | | | | | | | 5.7±2.9 | | |
| 33 | * | 3-carene | 1207 | 895 | | 0.4±0.1 | | | | | | | | |
| 42 | * | myrcene | 1224 | 1048 | | | | | 0.3±0.0 | | 0.9±0.2 | | | 1.4±0.0 |
| 25 | * | α-phellandrene | 1224 | 1058 | 0.1±0.0 | 2.4±0.2 | | | | | | 0.7±0.2 | | |
| 80 | * | cumene | 1238 | | | | | | 0.7±0.2 | | | | | |
| 21 | * | α-terpinene | 1241 | 1069 | | 0.2±0.1 | | | 0.3±0.0 | | 0.1±0.0 | | | |
| 3 | * | limonene | 1262 | 1081 | 1.5±1.3 | 1.4±0.2 | 5.7±1.9 | 6.0±3.0 | 1.2±0.4 | 1.6±0.3 | 2.6±0.6 | 5.1±1.4 | 2.2±0.6 | 2.7±2.3 |
| 59 | * | β-phellandrene | 1273 | 1081 | | | | | | | | 5.7±1.4 | | |
| 35 | * | (*E*)-β-ocimene | 1304 | 1104 | | 0.2±0.0 | | | | | | | | |
| 15 | * | γ-terpinene | 1316 | 1004 | 0.1±0.0 | 1.8±0.5 | 1.4±0.0 | | | | 0.3±0.0 | 0.4±0.0 | | |
| 47 | | (*Z*)-β-ocimene | 1324 | 1000 | | 0.1±0.0 | | | | | | | | |
| 11 | * | p-cymene | 1346 | 1013 | 0.1±0.0 | 1.2±0.3 | | 0.2±0 | | | 0.5±0.1 | | 1.7±1.4 | |
| 44 | * | terpinolene | 1359 | 1034 | | | | | 0.3±0.0 | | 0.7±0.1 | | | |
| | | Sesquiterpenes | | | | | | | | | | | | |
| 24 | * | α-cubenene | 1545 | 1427 | 1.6±0.5 | 0.3±0.1 | | | 0.7±0.4 | | | | | |
| 64 | * | δ-elemene | 1560 | | | | | | | | | 4.9±1.2 | | |
| 45 | * | ylangene | 1573 | 1446 | 1.0±0.3 | 0.1±0.0 | | | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | * | α-copaene | 1581 | 1450 | 2.5±0.5 | 1.6±0.3 | | | 2.2±0.7 | | | | | |
| 27 | * | α-gurjunene | 1620 | 1479 | | 3.6±0.7 | | 0.7±0.0 | 6.8±1.2 | | | | 2.0±0.0 | |
| 41 | * | germacreneD | 1630 | 1463 | 1.9±0.1 | 0.3±0.1 | | | | | | | | |
| 83 | | zingiberene | 1650 | | 1.0±0.6 | | | | | | | | | |
| 8 | * | longifolene | 1658 | 1517 | 2.4±2.4 | | 1.2±0.5 | | 0.5±0.2 | 6.1±1.2 | | 0.7±0.1 | 1.9±0.7 | 6.2±1.9 |
| 76 | * | β-elemene | 1671 | 1466 | 0.7±0.7 | | | | | | | | | |
| 46 | * | α-bergamotene | 1679 | 1480 | 0.4±0.2 | | | | 0.4±0.2 | | | | | |
| 58 | * | β-gurjunene | 1683 | | 4.7±0.1 | 0.2±0.0 | | | | | | | | |
| 10 | * | *(E)*-β-caryophyllene | 1689 | 1487 | 1.6±0.9 | 4.7±1.2 | | 2.1±1.3 | 1.9±0.5 | | 0.9±0.2 | 4.3±1.4 | | 0.7±0.0 |
| 56 | * | aromadendrene | 1699 | 1524 | 0.5±0.4 | 0.1±0.0 | | | | | | | | |
| 29 | * | isoledene | 1731 | 1542 | 1.9±0.3 | 0.2±0.1 | | | 0.5±0.2 | | | | | |
| 23 | * | alloaromadendrene | 1735 | 1554 | | 0.2±0.0 | | | 1.7±0.2 | | | | | |
| 77 | * | β-sesquiphellandrene | 1737 | | 0.7±0.0 | | | | | | | | | |
| 13 | * | α-humulene | 1759 | 1536 | | 13.4±1.9 | | | | | 4.0±0.1 | 0.9±0.2 | 1.9±0.0 | 1.1±0.0 |
| 65 | * | epibicyclosesquiphellandrene | 1759 | 1496 | 1.4±0.1 | | | | | | | | | |
| 61 | * | (*Z*)-β-farnesene | 1761 | 1528 | | | | | 6.4±2.8 | | | | | |
| 52 | | α-amorphene | 1765 | 1541 | 1.5±0.3 | 0.4±0.0 | | | | | | | | |
| 18 | * | γ-muurolene | 1779 | | 4.9±0.4 | 1.4±0.3 | | | 3.0±0.9 | | 0.1±0.1 | | | |
| 36 | * | viridiflorene | 1785 | | | 0.1±0.0 | | | | | | | | |
| 26 | * | β-cubebene | 1797 | | 33.9±8.5 | 2.1±0.7 | | | 0.8±0.0 | | | | | |
| 78 | * | cedren.9.ene | 1810 | | | 0.2±0.1 | | | | | | | | |
| 57 | * | β-bisabolene | 1818 | 1573 | 1.1±0.3 | | | | | | | | | |
| 19 | * | α-muurolene | 1826 | 1598 | 1.0±0.4 | 0.9±0.3 | | | 1.5±0.7 | | | | | |
| 74 | * | α-cedrene | 1830 | 1592 | 2.1±0.8 | | | | | | | | | |
| 38 | * | azulene | 1832 | 1247 | | | 1.3±0.4 | 2.7±1.6 | | | | | | |
| 7 | * | δ-cadinene | 1847 | 1585 | 14.2±2.5 | 8.5±2.6 | 1.1 | 0.5±0.3 | 6.3±3.0 | | 0.1±0.1 | 1.0±0.4 | 1.7±0.1 | 0.4±0.2 |
| 75 | * | α-curcumene | 1861 | | | | | | 6.9±4.8 | | | | | |
| 79 | | (*Z*)-α-bisabolene | 1863 | 1608 | 1.4±0.5 | | | | | | | | | |
| 43 | * | squalene | 1895 | | | | 15.4±5.4 | | 1.9±1.1 | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | * | calamenene | 1918 | | 0.4±0.1 | 0.1±0.1 | | | 0.3 | | | | 0.9 | |
| 85 | * | γ-gurjunene | 1988 | 1654 | | 0.2±0.0 | | | | | | | | |
| | | Alcohols | | | | | | | | | | | | |
| 1 | * | 2-ethyl-1-hexanol | 1587 | 1085 | 3.7±3.1 | 1.9±0.7 | 10.3±1.7 | 27.61±7.4 | 6.3±2.6 | 29.6±10.7 | 0.1±0.0 | 13.9±3.1 | 17.7±7.2 | 21.6±7.3 |
| 71 | | p-cymen-8-ol | 1940 | | | | | | | | 0.1±0.0 | | | |
| 31 | * | (*Z*)-sabinenehydrate | 1558 | 1121 | | 0.2±0.1 | | | 0.3±0 | | 0.2±0.0 | | | |
| 62 | * | (*Z*)-β-terpineol | 1644 | 1159 | | | | | 0.4 | | 0.2±0.0 | | | |
| 32 | * | linalool | 1648 | | | | 10.2±3.3 | | | | 0.3±0.1 | | | |
| 50 | * | terpinen-4-ol | 1699 | | | 0.4±0.0 | | | | | 0.3±0.0 | | | |
| 48 | * | isoborneol | 1763 | | | | 2.2±0.4 | | | | | | | 1.0±0.0 |
| 54 | | α-terpineol | 1791 | 1266 | | | | | | | 0.2±0.0 | | | |
| 30 | * | borneol | 1795 | 1232 | | | | | 0.7±0.1 | | 2.8±0.5 | | | |
| 69 | | myrtenol | 1882 | | | | | | | | 0.2±0.0 | | | |
| 63 | | (*Z*)-carveol | 1924 | 1296 | | | | | | | 0.1±0.0 | | | |
| 81 | * | farnesol | 1895 | 1649 | | | | | | | | 0.8±0.0 | | |
| 70 | | nerolidol | 2042 | 1632 | | | | | 24.3±7.1 | | | | | |
| 53 | * | α-bisabolol | 2104 | | 6.4±2.5 | | | | | | | | | |
| | | Aldheydes | | | | | | | | | | | | |
| 6 | * | nonanal | 1485 | 1142 | 0.5±0.4 | 1.3±0.4 | 5.9±0.7 | 18.0±6.5 | 6.4±2.6 | 12.1±3.2 | | 7.8±2.4 | 15.8±0.0 | 10.2±2.1 |
| 4 | * | decanal | 1595 | 1181 | 0.3±0.3 | 0.8±0.2 | 3.2±0.7 | 4.2±0.8 | 3.3±1.3 | 5.3±0.7 | 0.1±0.1 | 2.8±0.7 | 6.6±1.2 | 1.6±0.0 |
| | | Ketones | | | | | | | | | | | | |
| 34 | * | fenchone | 1485 | 1142 | | | | | | | 0.6±0.4 | | 38.9±8.5 | |
| 55 | * | α -thujone | 1511 | 1175 | | | | | | | 40.8±4.3 | | | |
| 60 | * | β -thujone | 1533 | 1164 | | | | | | | 7.6±2.0 | | | |
| 2 | * | camphor | 1610 | 1202 | 11.1±8.9 | 10.9±7.0 | 12.3±1.0 | 12.7±3.9 | 16.3±6.5 | 5.9±0.4 | 20.9±3.6 | 9.5±0.4 | 15.3±4.4 | 14.1±3.8 |
| 67 | | isocamphopinone | 1640 | 1223 | | | | | | | 0.1±0.0 | | | |
| 9 | * | geranylacetone | 1942 | | | | 1.4±0.1 | 1.2±0.1 | 1.0±0.3 | 0.7±0.2 | | 1.5±0.3 | 2.6±0.9 | 1.0±0.6 |
| 40 | * | cuminone | 1944 | | | | | | | | | 0.6±0.1 | 1.8±1.2 | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Carboxylic acids | | | | | | | | | | | |
| 5 | * | hexanoic acid | 2006 | 1161 | | 38.8±7.1 | 31.3±10.9 | 0.4±0.2 | 41.6±15.8 | 0.7±0.1 | 35.7±5.8 | 14.8±4.7 | 33.4±6.1 |
| | | Ether | | | | | | | | | | | |
| 22 | * | eucalyptol | 1270 | 1082 | 1.6±0.5 | | | 0.4±0.2 | | 2.4±0.5 | | | |
| | | Epoxide | | | | | | | | | | | |
| 68 | | linaloloxide | 1564 | 1128 | | | | | | 0.1±0.0 | | | |
| | | Ester | | | | | | | | | | | |
| 49 | * | L-bornylacetate | 1677 | 1127 | | | | | | 1.8±0.5 | | | 1.2±0.0 |
| 82 | | myrtenylacetate | 1781 | 1411 | | | | | | 0.2±0.1 | | | |

736 **Table 3** T-test comparison of the EAG values of the tested plant species with the negative control (clean filter

737 paper), for females and males of *Macrolophus melanotoma* and *M. pygmaeus*

| Plant species | Sex | *M. melanotoma* | | | *M. pygmaeus* | | |
|---|---|---|---|---|---|---|---|
| | | T-statistic | *DF* | *P* | T-statistic | *DF* | *P* |
| cis-2-hexen-1-ol | female | -12.77 | 14 | 0.000** | -6.72 | 13 | 0.000** |
| *B. hirsuta* | female | -5.47 | 14 | 0.000** | -3.47 | 13 | 0.004** |
| *C. officinalis* | female | -3.85 | 14 | 0.002** | -1.88 | 13 | 0.082° |
| *C. annuum* | female | -3.64 | 14 | 0.003** | -1.95 | 13 | 0.074° |
| *C. albidus* | female | -1.10 | 14 | 0.289 | -0.15 | 13 | 0.886 |
| *D. viscosa* | female | -4.89 | 14 | 0.000** | -5.94 | 13 | 0.000** |
| *P. officinalis* | female | -4.59 | 14 | 0.000** | -0.89 | 13 | 0.388 |
| *S. officinalis* | female | -3.61 | 14 | 0.003** | -2.50 | 13 | 0.027* |
| *S. lycopersicum* | female | -2.42 | 14 | 0.030* | -2.72 | 13 | 0.018* |
| *S. nigrum* | female | -2.13 | 14 | 0.052° | 0.25 | 13 | 0.806 |
| *V. faba* | female | 0.15 | 14 | 0.879 | 0.64 | 13 | 0.532 |
| cis-2-hexen-1-ol | male | -8.09 | 14 | 0.000** | -6.78 | 9 | 0.000** |
| *B. hirsuta* | male | -4.23 | 14 | 0.001** | -2.26 | 9 | 0.050° |
| *C. officinalis* | male | -4.05 | 14 | 0.001** | -3.16 | 9 | 0.012* |
| *C. annuum* | male | -4.49 | 14 | 0.001** | -3.98 | 9 | 0.003** |
| *C. albidus* | male | -1.54 | 14 | 0.147 | -1.53 | 9 | 0.161 |
| *D. viscosa* | male | -6.05 | 14 | 0.000** | -4.27 | 9 | 0.002** |
| *P. officinalis* | male | -4.05 | 14 | 0.001** | -2.63 | 9 | 0.027* |
| *S. officinalis* | male | -3.44 | 14 | 0.004** | -3.17 | 9 | 0.011* |
| *S. lycopersicum* | male | -1.95 | 14 | 0.071° | -4.09 | 9 | 0.003** |
| *S. nigrum* | male | -3.55 | 14 | 0.003** | -1.93 | 9 | 0.085° |
| *V. faba* | male | 0.00 | 14 | 0.997 | -0.60 | 9 | 0.562 |

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740 **Table 4** ANOVA table for the EAG responses of females and males of *Macrolophus melanotoma* and

741 *M. pygmaeus* to plant stimuli, using values normalized to the positive control ((Z)-2-hexen-1-ol)

| Plant species | Factor | df | MS | F | P |
|---|---|---|---|---|---|
| *B. hirsuta* | Species | 1 | 1.175 | 10.427 | 0.002** |
| | Sex | 1 | 0.598 | 5.302 | 0.025* |
| | Species × Sex | 1 | 0.059 | 0.522 | 0.473 |
| | Residuals | 50 | 0.113 | | |
| *C. officinalis* | Species | 1 | 0.124 | 1.896 | 0.175 |
| | Sex | 1 | 0.009 | 0.137 | 0.713 |
| | Species × Sex | 1 | 0.007 | 0.102 | 0.751 |
| | Residuals | 50 | 0.065 | | |
| *C. annuum* | Species | 1 | 0.109 | 0.907 | 0.345 |
| | Sex | 1 | 1.193 | 9.923 | 0.003** |
| | Species × Sex | 1 | 0.004 | 0.035 | 0.853 |
| | Residuals | 50 | 0.120 | | |
| *C. albidus* | Species | 1 | 0.044 | 1.515 | 0.224 |
| | Sex | 1 | 0.043 | 1.466 | 0.232 |
| | Species × Sex | 1 | 0.045 | 1.531 | 0.222 |
| | Residuals | 50 | 0.029 | | |
| *D. viscosa* | Species | 1 | 0.075 | 0.476 | 0.493 |
| | Sex | 1 | 0.072 | 0.457 | 0.502 |
| | Species × Sex | 1 | 0.036 | 0.227 | 0.636 |
| | Residuals | 50 | 0.157 | | |
| *P. officinalis* | Species | 1 | 0.510 | 3.404 | 0.071° |
| | Sex | 1 | 0.919 | 6.137 | 0.017* |
| | Species × Sex | 1 | 0.014 | 0.093 | 0.762 |
| | Residuals | 50 | 0.150 | | |
| *S. officinalis* | Species | 1 | 0.004 | 0.036 | 0.850 |
| | Sex | 1 | 0.060 | 0.535 | 0.468 |
| | Species × Sex | 1 | 0.001 | 0.009 | 0.925 |
| | Residuals | 50 | 0.112 | | |
| *S. lycopersicum* | Species | 1 | 0.847 | 11.509 | 0.001** |
| | Sex | 1 | 0.076 | 1.027 | 0.316 |
| | Species × Sex | 1 | 0.307 | 4.175 | 0.046* |
| | Residuals | 50 | 0.074 | | |
| *S. nigrum* | Species | 1 | 0.396 | 6.179 | 0.016* |
| | Sex | 1 | 0.460 | 7.180 | 0.010** |
| | Species × Sex | 1 | 0.001 | 0.012 | 0.912 |
| | Residuals | 50 | 0.064 | | |
| *V. faba* | Species | 1 | 0.011 | 0.226 | 0.636 |
| | Sex | 1 | 0.054 | 1.097 | 0.300 |
| | Species × Sex | 1 | 0.012 | 0.234 | 0.631 |
| | Residuals | 50 | 0.050 | | |

742 Probability, $**P < 0.01$; $*P < 0.05$; $°P < 0.10$