



## Electroantennogram (EAG) responses of *Microplitis croceipes* and *Cotesia marginiventris* and their lepidopteran hosts to a wide array of odor stimuli: Correlation between EAG response and degree of host specificity?

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### ABSTRACT

In order to test whether the electroantennogram (EAG) response spectrum of an insect correlates to its degree of host specificity, we recorded EAG responses of two parasitoid species with different degrees of host specificity, *Microplitis croceipes* (specialist) and *Cotesia marginiventris* (generalist), to a wide array of odor stimuli including compounds representing green leaf volatiles (GLVs), herbivore-induced plant volatiles (HIPV), ecologically irrelevant (not used by the parasitoid species and their hosts for host location) plant volatiles, and host-specific odor stimuli (host sex pheromones, and extracts of host caterpillar body and frass). We also tested the EAG responses of female moths of the caterpillar hosts of the parasitoids, *Heliothis virescens* and *Spodoptera exigua*, to some of the odor stimuli. We hypothesized that the specialist parasitoid will have a narrower EAG response spectrum than the generalist, and that the two lepidopteran species, which are similar in their host plant use, will show similar EAG response spectra to plant volatiles. As predicted, the specialist parasitoid showed greater EAG responses than the generalist to host-specific odor and one HIPV (*cis*-3-hexenyl butyrate), whereas the generalist showed relatively greater EAG responses to the GLVs and unrelated plant volatiles. We detected no differences in the EAG responses of *H. virescens* and *S. exigua* to any of the tested odor.

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### 1. Introduction

Electroantennogram (EAG), a technique which measures the electrophysiological responses in the insect antennae, provides a general measure of odorant reception at the peripheral level (Roelofs, 1977; Van der Pers and Minks, 1998; Park et al., 2002). EAG responses represent the summed, DC potential response of several different and narrowly tuned olfactory receptor neurons on an insect antenna (Schneider, 1957). The EAG technique has been used in pheromone identification studies, and more recently for identifications of plant volatiles which mediate insect–plant or tritrophic interactions (Cossé et al., 1995; Blight et al., 1997; Honda et al., 1999). However, EAG activity may not necessarily indicate behavioral activity (Park et al., 2001). Thus, the biological role of EAG active compounds must be determined in behavioral bioassays.

There is ample evidence that EAG response of insects to many pheromones can be species-specific (Smith and Menzel, 1989;

Visser and Piron, 1997; Park et al., 2002; Groot et al., 2005) or race-specific (Linn et al., 1999; El-Sayed et al., 2003). Because the specificity of EAG responses of male moth antennae to conspecific pheromone has been instrumental in pheromone identifications, EAG recordings have been used as a diagnostic tool to correlate differences in pheromone detection to genetic differences in several moth species (El-Sayed et al., 2003; Groot et al., 2005). However, EAG response of insects to plant volatiles or other kairomones is not species-specific, and it remains unclear if there is a correlation between EAG response spectra of insects to plant volatiles or host-related volatiles and their diet breadth or host specificity. The existence of such a correlation may imply the potential use of EAG recordings to provide an indication of the diet breadth or host specificity of insects. In that case, one would predict that specialist monophagous or oligophagous insect herbivores should show narrower EAG response spectra than polyphagous generalist herbivores. Similarly, since they utilize fewer hosts and thus are likely to possess a relatively more narrowly tuned (selective) host detection olfactory system, specialist parasitoids should show relatively narrower EAG response spectra to plant volatiles than generalists, which have a broader host range. The need for generalist parasitoids to locate different hosts on a wide variety of plants further suggests that

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they may have evolved the ability to respond to a wider array of plant volatiles than specialists.

Here, we tested the above prediction by recording EAG responses of females of two caterpillar parasitoid species (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson), to a wide array of odor stimuli including green leaf volatiles (GLVs), herbivore-induced plant volatiles (HIPVs), host-specific volatiles (i.e. host sex pheromones, and extracts of host caterpillar body and frass), and ecologically irrelevant plant volatiles (i.e. volatiles not known to be produced by the hosts of the tested insects). *M. croceipes* is a specialist parasitoid of caterpillars of *Heliothis* spp., whereas *C. marginiventris* is a generalist parasitoid of caterpillars in several genera including *Heliothis* spp. and *Spodoptera* spp. The two parasitoid species were selected for this study because they have served as models in previous studies of parasitoid olfaction, and several aspects of their responses to host-related volatiles have been characterized (Loughrin et al., 1994; Cortesero et al., 1997; Röse et al., 1998; Park et al., 2002; Shalit et al., 2003; Gouinguéné et al., 2005; Chen and Fadamiro, 2007; Ngumbi et al., 2009). Additionally we tested EAG responses of adult females of the caterpillar hosts of the parasitoids, *Heliothis virescens* Fab. and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) to most of the above odorants. Both *H. virescens* and *S. exigua* are naturally distributed throughout USA and are important pests of key agricultural crops such as corn and cotton (Pearson, 1982). *Heliothis virescens* is a preferred host of *M. croceipes* (Stadelbacher et al., 1984; King et al., 1985), whereas *S. exigua* is a preferred host of *C. marginiventris* (Jalali et al., 1987) but not a known host for *M. croceipes*. Based on the results of a preliminary study in which we recorded differences in the EAG responses of both parasitoid species to various synthetic host-related volatile compounds (Chen and Fadamiro, 2007) and assuming a correlation between EAG response spectra and host specificity of parasitoids or diet breadth, we hypothesized that the specialist parasitoid, *M. croceipes* will have a narrower EAG response spectrum than the generalist, *C. marginiventris* by showing relatively greater EAG responses to host-specific odor but lower responses to GLVs and ecologically irrelevant plant volatiles. Because the two lepidopteran host species (*H. virescens* and *S. exigua*) are not much different in their diet breadth and host plant use, we hypothesized that both will show similar EAG response spectra to plant volatiles.

## 2. Materials and methods

### 2.1. Insects

The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, GA, USA) and the Department of Entomology, University of Georgia (Tifton campus, contact: John Ruberson), respectively. *Microplitis croceipes* was reared on caterpillars of *H. virescens*, whereas *C. marginiventris* was reared on *S. exigua*. The rearing procedures of both parasitoids were similar to those of Lewis and Burton (1970). Eggs purchased from Benzene Research (Carlisle, PA, USA) were used to start laboratory colonies of the two lepidopteran host species, *H. virescens*, and *S. exigua*. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale, 1965) at  $25 \pm 1$  °C,  $75 \pm 5\%$  r.h. and 14:10 L:D photoperiod. For each parasitoid species, newly emerged adults were collected prior to mating, sexed, and placed in groups of 2 individuals of opposite sex (mated individuals) in a 6-cm diameter plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in

the cap of the tube. About 5 drops (2  $\mu$ l per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. For each lepidopteran species, newly emerged female moths were collected and placed in clear plastic rectangular cages (30 cm  $\times$  30 cm  $\times$  13 cm tall) supplied with water and sugar sources. Water and sugar solution (10%) were provided by filling 25 ml glass cylinder with distilled water or sugar solution (10%) and placing an 8 cm long cotton absorbent wick (Wheat Ridge, CO, USA) at the center. The cylinder was then sealed with parafilm. Mated female moths and parasitoids (aged 3–5 days) were used for EAG recordings.

### 2.2. Test odor stimuli

Three major categories of odor stimuli were tested for the parasitoids: synthetic host-related plant volatiles, synthetic ecologically irrelevant plant volatiles, and host-specific odor stimuli (host sex pheromones, and extracts of host caterpillar body and frass). Seven host-related plant volatiles were tested in this study: *cis*-3-hexenal, *trans*-2-hexenal, hexanal,  $\beta$ -pinene, *cis*-3-hexenyl acetate, linalool (racemic), and *cis*-3-hexenyl butyrate. The first three compounds (*cis*-3-hexenal, *trans*-2-hexenal and hexanal) are components of green leaf volatiles (GLVs) of most plants, while the remaining four compounds are herbivore-inducible plant volatiles (HIPV) in cotton (*Gossypium hirsutum* L.), and several other plants (Loughrin et al., 1994; McCall et al., 1994; De Moraes et al., 1998; Hoballah et al., 2002). *cis*-3-Hexenyl acetate, a compound from the lipoxygenase pathway, was classified as a herbivore-inducible compound in our study because it has been shown to be induced by caterpillar feeding in cotton plants (Loughrin et al., 1994; McCall et al., 1994; Ngumbi et al., 2009). All selected compounds have previously been reported to elicit antennal and/or behavioral responses in both parasitoids (Li et al., 1992; Park et al., 2001, 2002; Chen and Fadamiro, 2007), and their lepidopteran hosts (Burguiere et al., 2001; Rajapakse et al., 2006). Seven ecologically irrelevant plant volatiles were tested including (arranged in the order of molecular weight) dimethyl disulfide, benzaldehyde, phenyl acetonitrile, phenyl isothiocyanate, geraniol, *trans*-cinnamaldehyde, and pentyl hexanoate. Phenyl acetonitrile and phenyl isothiocyanate are isothiocyanates typically produced by plants in Brassicaceae family, while geraniol (an acyclic monoterpene alcohol found in lemongrass and aromatic herb oils) and *trans*-cinnamaldehyde (a pale yellow viscous liquid occurs naturally in the bark of cinnamon trees and other species of the genus *Cinnamomum*) are essential oils. These compounds were classified as ecologically irrelevant volatiles because they are not known to be produced by the plant hosts of the tested lepidopteran (*H. virescens* and *S. exigua*) species, or used by the tested insects for host location. The ecologically irrelevant plant volatiles were evaluated simply to determine the range of antennal perception in both parasitoids and their moth hosts. All synthetic test compounds were purchased from Sigma<sup>®</sup> Chemical Co. (St. Louis, MO) with purity >97% as indicated on the labels. Each compound was diluted in hexane (HPLC grade) to give 100  $\mu$ g/ $\mu$ l solutions. Further dilutions were made to give 0.1, and 10  $\mu$ g/ $\mu$ l solutions. The solutions were kept in a freezer at  $-20$  °C until used.

Several types of host-specific odor stimuli were also tested for the parasitoids including synthetic host sex pheromones and extracts of host caterpillar body and frass. The sex pheromones of *H. virescens* and *S. exigua* were tested as single components and blends. For *H. virescens*, we tested the major (Z11-16 Ald) and minor (Z9-14 Ald) sex pheromone components, and a blend of the two components in a ratio of 16:1. We also tested *S. exigua* major (Z9E12-14 Ac) and minor (Z9-14 OH) sex pheromone components, and a blend of the two components at a ratio of 10:1. The sex pheromone blend tested for each moth species has been shown to

elicit behavioral responses in conspecific males and could be considered as optimal pheromone blend for each moth species (Mitchell et al., 1978, 1983). Pheromones were purchased from Bedoukian Research (Danbury, CT) and ISCA Technologies, Inc. (Riverside, CA) with 98% purity. Solutions of synthetic test pheromone components were formulated in hexane to obtain 100  $\mu\text{g}/\mu\text{l}$  solutions. All synthetic plant volatiles and pheromones were tested at two doses (1 and 100  $\mu\text{g}$ ), which represented low and high doses, respectively. EAG responses of both parasitoids to extracts of host caterpillar body and frass were also determined. Frass extracts (either with hexane or water) were obtained following the procedures of Mattiacci and Dicke (1995) with some modifications. Briefly, 10 g of fresh frass obtained from caterpillars (*H. virescens* or *S. exigua*) feeding on artificial diet was extracted with 5 ml of hexane or water for 24 h at room temperature. Collected extracts were stored in a freezer at  $-20\text{ }^{\circ}\text{C}$  until use. Body extracts of host caterpillars were obtained following the procedures described by Yasuda and Wakamura (1996) with some modifications. Briefly caterpillars (2nd–3rd instar) of *H. virescens* or *S. exigua* weighing  $\sim 1$  g were extracted with 2 ml of 1:2 mixture of hexane and acetone for 1 h at room temperature. Second and third instar caterpillars were used since they are the stages normally attacked by the parasitoids. The extract was filtered with anhydrous sodium sulphate and silica gel. The filtrate was concentrated to 200  $\mu\text{l}$  under a gentle stream of nitrogen, and was stored in a freezer at  $-20\text{ }^{\circ}\text{C}$  until use. Extracts of host caterpillar frass and body were tested at only one dose (10  $\mu\text{l}$ ).

### 2.3. EAG recordings

The EAG technique and protocols were similar to those previously described by Chen and Fadamiro (2007). The reference electrode consisting of a glass capillary (1.1 mm ID) filled with 0.1 M KCl solution was connected either to the neck of isolated head of an adult female parasitoid or to the base of an excised female moth antenna. The recording electrode consisted of a similar glass capillary connected to the antennal tip (with the last segment of the antenna cut off). Chlorinated silver–silver junctions were used to maintain electrical conduct between the electrode and input of the preamplifier. The analog signal was detected through a probe (INR-II, Syntech<sup>®</sup>, the Netherlands), and was captured and processed with a data acquisition controller (IDAC-4, Syntech<sup>®</sup>, the Netherlands) and analyzed using EAG 2000 software (Syntech<sup>®</sup>, the Netherlands) on a PC. Test compounds diluted in hexane were delivered as 10- $\mu\text{l}$  samples placed on a filter paper (7 mm  $\times$  40 mm, Whatman<sup>®</sup> No. 1). The solvent was allowed to evaporate and the impregnated filter paper was placed into a glass Pasteur pipette ( $\sim 14$  cm in length, Fisher Scientific, Pittsburgh, PA, USA) constituting an odor cartridge. The control stimulus consisted of a similar pipette containing a filter paper impregnated with 10  $\mu\text{l}$  aliquot of hexane. The tip of the pipette was placed about 3 mm into a small hole in the wall of a glass tube (13 cm long, 8 mm diameter) oriented towards the antennal preparation ( $\sim 0.5$  cm away from the preparation). The stimuli were provided as 0.2 s puffs of air (2 ml) into continuous humidified air stream at 1000 ml  $\text{min}^{-1}$  generated by an air stimulus controller (CS-55, Syntech<sup>®</sup>, the Netherlands). At least 2 min interval was allowed between successive stimulations for antenna recovery. Parasitoids and moths aged 3–5 days were tested. Preliminary tests showed that isolated parasitoid head and excised moth antenna preparations lasted up to 40 min with no noticeable decreases in EAG responses observed over this time period at room temperature. Thus, for each category of test compounds, a test series of the same dose (1  $\mu\text{g}/\mu\text{l}$  or 100  $\mu\text{g}/\mu\text{l}$ ) was applied to ten antenna preparations of each parasitoid or moth species in the following order: hexane control, standard stimulus, odorant compounds,

hexane control and standard stimulus. One hundred micrograms of *cis*-3-hexenol was used as the standard stimulus (Chen and Fadamiro, 2007) and presented to an antenna at the beginning and end of a recording series to confirm activity of an antennal preparation. Test compounds were presented in a random sequence. Experiments were carried out in batches replicated in time by testing equal number of individuals of both parasitoids and their lepidopteran hosts daily in a random order.

### 2.4. Statistical analyses

For analysis, EAG response to the solvent control was deducted from the EAG amplitudes elicited by the test odor stimuli. Absolute EAG data met the key assumptions of parametric tests thus were not transformed prior to analysis. Absolute EAG responses to each odorant were compared between the two parasitoid species or the two moth species using the Student's *t*-test ( $P < 0.05$ ; JMP Version 7.01, SAS Institute, 2007). For each parasitoid or moth species, EAG responses to compounds within each odor stimuli category at each dose were analyzed by using analysis of variance (ANOVA) followed by the Tukey–Kramer HSD multiple comparison test ( $P < 0.05$ ; JMP<sup>®</sup> 7.0.1, SAS Institute, 2007) to establish significant differences among the compounds tested.

## 3. Results

### 3.1. EAG responses of parasitoids to host-related plant volatiles

Table 1 shows the results of Student's *t*-test comparison of the two parasitoid species to the different odor stimuli. The three GLVs, *cis*-3-hexenal, *trans*-2-hexenal and hexenal, and two HIPVs,  $\beta$ -pinene and linalool at both doses (1 and 100  $\mu\text{g}$ ) elicited significantly greater EAG responses in the generalist, *C. marginiventris* than in the specialist, *M. croceipes* (Fig. 1a). In contrast, *M. croceipes* showed significantly greater EAG responses to the HIPV, *cis*-3-hexenyl butyrate at both doses, compared to *C. marginiventris* (Fig. 1a). Fig. 1a also shows significant differences in the responses of *M. croceipes* (1  $\mu\text{g}$ :  $F = 11.63$ ,  $df = 6$ ,  $P < 0.0001$ ; 100  $\mu\text{g}$ :  $F = 16.72$ ,  $df = 6$ ,  $P < 0.0001$ ) and *C. marginiventris* (1  $\mu\text{g}$ :  $F = 4.77$ ,  $df = 6$ ,  $P = 0.0005$ ; 100  $\mu\text{g}$ :  $F = 14.27$ ,  $df = 6$ ,  $P < 0.0001$ ) to the seven tested host-related plant volatiles at both doses. *cis*-3-Hexenyl butyrate and *trans*-2-hexenal elicited the highest EAG response in *M. croceipes*, whereas *trans*-2-hexenal elicited the highest EAG response in *C. marginiventris*.  $\beta$ -Pinene and linalool elicited relatively lower EAG responses in both parasitoid species.

### 3.2. EAG responses of parasitoids to ecologically irrelevant plant volatiles

Significant differences were also recorded in the EAG responses of both parasitoid species to the tested ecologically irrelevant plant volatiles (Table 1). All but one of the tested ecologically irrelevant plant volatiles elicited significantly greater EAG responses in the generalist, *C. marginiventris* compared to *M. croceipes*, irrespective of dose (Fig. 1b). The lone exception was phenyl acetonitrile, which elicited only a numerically greater response in *C. marginiventris*. The seven ecologically irrelevant plant volatiles elicited significantly different EAG responses in *M. croceipes* (1  $\mu\text{g}$ :  $F = 4.66$ ,  $df = 6$ ,  $P = 0.0006$ ; 100  $\mu\text{g}$ :  $F = 9.90$ ,  $df = 6$ ,  $P < 0.0001$ ) and *C. marginiventris* (1  $\mu\text{g}$ :  $F = 5.48$ ,  $df = 6$ ,  $P < 0.0001$ ; 100  $\mu\text{g}$ :  $F = 5.88$ ,  $df = 6$ ,  $P < 0.0001$ ; Fig. 1b). At the 100  $\mu\text{g}$  dose, benzaldehyde elicited the highest EAG response in *M. croceipes*, significantly greater than the remaining compounds. Similarly, EAG response of *C. marginiventris* to benzaldehyde was greater than EAG responses to the other compounds. Dimethyl disulfide elicited the lowest EAG response in both parasitoid species (Fig. 1b).

**Table 1**

Results of Student's *t*-test analysis to compare EAG responses of *Microplitis croceipes* and *Cotesia marginiventris* to host-related plant volatiles, ecologically irrelevant plant volatiles, and host sex pheromones at two doses.

Compound	Dose ( $\mu\text{g}$ )	<i>t</i>	<i>P</i>
Host-related plant volatiles			
cis-3-Hexenal	1	2.33	0.016
	100	2.46	0.002
trans-2-Hexenal	1	2.74	0.008
	100	2.97	0.014
Hexanal	1	3.10	0.004
	100	3.35	0.002
$\beta$ -Pinene	1	3.19	0.003
	100	3.08	0.008
cis-3-Hexenyl acetate	1	0.33	0.371
	100	1.76	0.442
Linalool	1	1.90	0.029
	100	1.98	0.003
cis-3-Hexenyl butyrate	1	3.32	0.004
	100	3.21	0.004
Ecologically irrelevant plant volatiles			
Dimethyl disulfide	1	1.95	0.041
	100	1.88	0.032
Benzaldehyde	1	4.11	0.003
	100	2.09	0.021
Phenyl acetonitrile	1	1.12	0.141
	100	1.13	0.152
trans-Cinnamaldehyde	1	1.99	0.031
	100	2.46	0.032
Phenyl isothiocyanate	1	3.40	0.002
	100	2.49	0.012
Geraniol	1	3.34	0.003
	100	3.25	0.004
Pentyl hexanoate	1	3.80	0.005
	100	3.45	0.005
Sex pheromones			
Z11-16 Ald ( <i>H. virescens</i> )	1	2.90	0.001
	100	2.97	0.010
Z9-14 Ald ( <i>H. virescens</i> )	1	1.79	0.997
	100	1.99	0.062
Blend (Z11-16 Ald/Z9-14 Ald)	1	2.01	0.970
	100	6.30	<.0001
Z9E12-14 Ac ( <i>S. exigua</i> )	1	3.22	0.004
	100	2.23	0.045
Z9-14 OH ( <i>S. exigua</i> )	1	1.23	0.072
	100	1.04	0.322
Blend (Z9E12-14 Ac/Z9-14 OH)	1	1.95	0.963
	100	1.73	0.055

### 3.3. EAG responses of parasitoids to host sex pheromones

Student's *t*-test also revealed significant differences between the two parasitoid species in their EAG responses to host sex pheromones (Table 1). The specialist, *M. croceipes* had significantly greater EAG responses to Z11-16 Ald, the major sex pheromone of its main host, *H. virescens*, at both doses than the generalist, *C. marginiventris* (Fig. 2a). However, EAG responses of both species to Z9-14 Ald (minor sex pheromone component of *H. virescens*) were not significantly different. *M. croceipes* also had greater EAG responses than *C. marginiventris* to the pheromone blend of *H. virescens* (a 16:1 blend of Z11-16 Ald and Z9-14 Ald), but this was significantly different only at the 100  $\mu\text{g}$  dose. In contrast, Z9E12-14 Ac, the major sex pheromone component of *S. exigua*, elicited significantly greater EAG responses in *C. marginiventris* at both doses than in *M. croceipes* (Fig. 2a). *Cotesia marginiventris* also showed numerically (but not significantly) higher EAG responses than *M. croceipes* to Z9-14 OH, a minor component of *S. exigua* pheromone and to the tested *S. exigua* pheromone blend (a 10:1 blend of Z9E12-14 Ac and Z9-14 OH). Significant differences were also recorded in the response of *M. croceipes* (1  $\mu\text{g}$ :  $F = 5.42$ ,  $df = 5$ ,  $P = 0.0004$ ; 100  $\mu\text{g}$ :  $F = 15.91$ ,  $df = 5$ ,  $P < 0.0001$ ) and *C. marginiventris* (1  $\mu\text{g}$ :  $F = 2.28$ ,  $df = 5$ ,  $P = 0.0595$ ; 100  $\mu\text{g}$ :  $F = 1.69$ ,  $df = 5$ ,

$P = 0.0495$ ) to the six different sex pheromone stimuli (i.e. *H. virescens* and *S. exigua* sex pheromone components and blends) (Fig. 2a). For *M. croceipes*, the highest EAG response was elicited by *H. virescens* pheromone blend followed by Z11-16 Ald (*H. virescens* major pheromone component), while the lowest EAG response was elicited by Z9-14 OH (*S. exigua* minor pheromone component). In contrast, *S. exigua* pheromone blend elicited significantly greater EAG response in *C. marginiventris* than either of the major pheromone component of *H. virescens* at both doses and to Z9-14 Ald (minor pheromone component of *H. virescens*) at the 100  $\mu\text{g}$  dose. EAG response of *C. marginiventris* to *S. exigua* pheromone blend was also significantly greater than to Z9-14 OH (*S. exigua* minor pheromone component) at the 1  $\mu\text{g}$  dose, and numerically greater than EAG response to *H. virescens* pheromone blend.

### 3.4. EAG responses of parasitoids to host frass and caterpillar body extracts

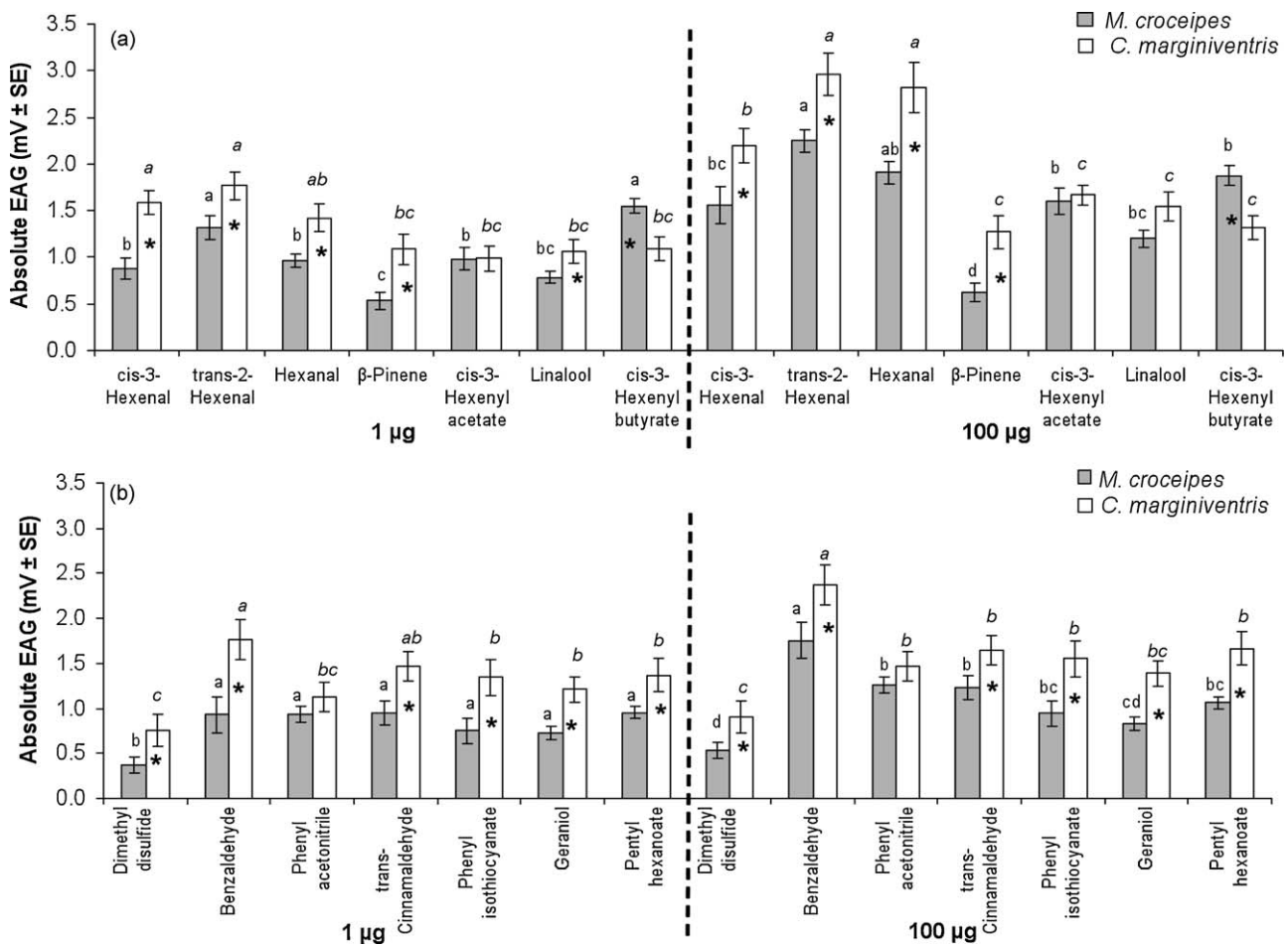
Student's *t*-test also revealed significant differences in the responses of both parasitoid species to the different host-specific stimuli (Table 1), with each species showing relatively greater EAG responses to odor stimuli of its preferred host. The specialist, *M. croceipes* showed significantly greater EAG responses than *C. marginiventris* to *H. virescens* caterpillar frass hexane extract ( $t = 2.50$ ,  $df = 1$ ,  $P = 0.012$ ), frass water extract ( $t = 4.31$ ,  $df = 1$ ,  $P = 0.0003$ ), and body extract ( $t = 2.84$ ,  $df = 1$ ,  $P = 0.0056$ ; Fig. 2b). In contrast, *C. marginiventris* showed significantly greater EAG responses than *M. croceipes* to *S. exigua* caterpillar frass hexane extract ( $t = 2.57$ ,  $df = 1$ ,  $P = 0.0248$ ), and body extract ( $t = 2.70$ ,  $df = 1$ ,  $P = 0.0148$ ; Fig. 2b). However, no significant difference was recorded in the response of both species to *S. exigua* caterpillar frass water extract ( $t = 0.15$ ,  $df = 1$ ,  $P = 0.8804$ ). Comparing the response of each species to the six host-specific stimuli, *H. virescens* frass hexane extract elicited the highest EAG response in *M. croceipes* followed by *H. virescens* caterpillar body extract ( $F = 21.09$ ,  $df = 5$ ,  $P < 0.0001$ ). For *C. marginiventris*, *S. exigua* frass hexane extract elicited significantly greater EAG response compared to the other five stimuli ( $F = 14.68$ ,  $df = 5$ ,  $P < 0.0001$ ). In general, frass water extracts of *H. virescens* and *S. exigua* elicited the lowest EAG responses in both parasitoid species (Fig. 2b).

### 3.5. EAG responses of *H. virescens* and *S. exigua* to host-related plant volatiles

Table 2 shows the results of Student's *t*-test comparison of the two moth species to the different odor stimuli. All of the tested plant volatiles elicited EAG responses in females of *H. virescens* and *S. exigua* at the two tested doses (1 and 100  $\mu\text{g}$ ) (Fig. 3a). In general, higher EAG responses were recorded at the 100  $\mu\text{g}$  dose than at the 1  $\mu\text{g}$  dose. However, no significant differences were recorded in the EAG responses of *H. virescens* and *S. exigua* to any of the tested volatiles, irrespective of dose (Fig. 3a). At the 1  $\mu\text{g}$  dose, all seven host-related plant volatiles elicited similar EAG responses in *H. virescens* ( $F = 1.42$ ,  $df = 6$ ,  $P = 0.2202$ ), and *S. exigua* ( $F = 1.49$ ,  $df = 6$ ,  $P = 0.1944$ ). At the higher 100  $\mu\text{g}$  dose, however, trans-2-hexenal and hexanal elicited significantly greater EAG responses than the remaining compounds in *H. virescens* ( $F = 11.89$ ,  $df = 6$ ,  $P = 0.0001$ ), and *S. exigua* ( $F = 10.92$ ,  $df = 6$ ,  $P = 0.0001$ ; Fig. 3a).

### 3.6. EAG responses of *H. virescens* and *S. exigua* to ecologically irrelevant plant volatiles

Both *H. virescens* and *S. exigua* showed significant EAG responses to the ecologically irrelevant plant volatiles but the responses were not clearly dose dependent (Fig. 3b). Furthermore, no significant differences were recorded in EAG responses of both



**Fig. 1.** Absolute EAG responses (mV ± SE,  $n = 10$ ) of *Microplitis croceipes* and *Cotesia marginiventris* to (a) host-related plant volatiles, and (b) ecologically irrelevant plant volatiles at two doses (1 µg and 100 µg). \*Significant difference between the two species ( $t$  test,  $P < 0.05$ ). Means for the same species and dose having no letter in common are significantly different among compounds (ANOVA, Tukey HSD test,  $P < 0.05$ ). Letters in italics are for *C. marginiventris*.

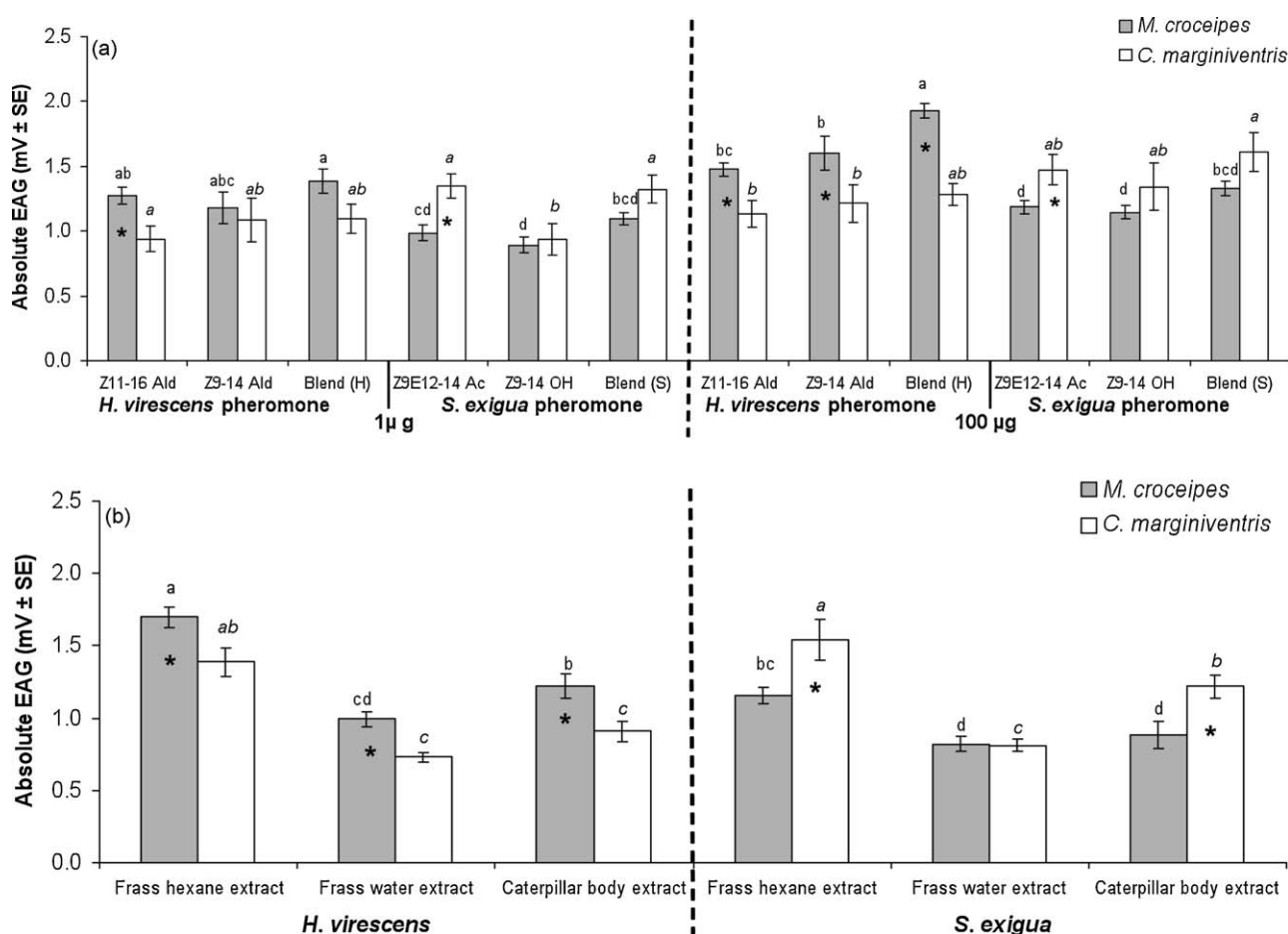
moth species to any of the ecologically irrelevant plant volatiles, irrespective of dose (Fig. 3b). Significant differences were recorded in the response of *H. virescens* to the seven ecologically irrelevant plant volatiles (1 µg:  $F = 2.53$ ,  $df = 6$ ,  $P = 0.0296$ ; 100 µg:  $F = 4.99$ ,  $df = 6$ ,  $P = 0.0003$ ). Benzaldehyde elicited the highest EAG response in *H. virescens* at both doses, while dimethyl disulfide elicited the lowest EAG responses. *Spodoptera exigua* also showed significantly different responses to the ecologically irrelevant plant volatiles (1 µg:  $F = 2.99$ ,  $df = 6$ ,  $P = 0.0125$ ; 100 µg:  $F = 4.49$ ,  $df = 6$ ,  $P = 0.0008$ ). Pentyl hexanoate elicited the highest EAG response at the 1 µg dose, while benzaldehyde elicited the highest EAG response at the 100 µg dose. Dimethyl disulfide also elicited the lowest EAG response in *S. exigua* at both doses (Fig. 3b).

#### 4. Discussion

Our results revealed intriguing differences in the EAG responses of both parasitoid species to the tested odor stimuli. As predicted, the generalist parasitoid, *C. marginiventris* showed a wider EAG response spectrum to odor than the specialist, *M. croceipes*. While the generalist showed greater EAG response than the specialist to most green leaf volatiles (GLVs) and ecologically irrelevant plant volatiles, the specialist showed relatively greater responses to host-specific odor stimuli such as host sex pheromones and host caterpillar frass and body extracts, and to *cis*-3-hexenyl butyrate, a herbivore-induced plant volatile (HIPV). These results are fairly consistent with those reported in a preliminary study in which we

compared the EAG responses of *M. croceipes* and *C. marginiventris* to two GLVs (*cis*-3-hexenol and hexanal) and three HIPVs (*cis*-3-hexenyl acetate, linalool, and (*E,E*)- $\alpha$ -farnesene). In that study, *C. marginiventris* also showed relatively greater EAG responses than *M. croceipes* to the two GLVs (Chen and Fadamiro, 2007).

We are not aware of any published studies which compared the behavioral response of both parasitoid species to a wide range of volatiles, as evaluated in the present EAG study. However, our data are consistent with those which demonstrated higher behavioral response of *C. marginiventris* to GLVs and volatiles from freshly damaged plants than to volatiles from plants with old damage (Cortesero et al., 1997; Hoballah et al., 2002; D'alessandro and Turlings, 2005; Hoballah and Turlings, 2005). It would seem adaptive for generalist parasitoids to show greater response than specialists to GLVs and a wider array of plant volatiles since they attack numerous hosts on different host plants. In contrast, specialist parasitoids are likely to have evolved the ability to respond to a narrower range of volatiles, while showing greater olfactory response to the volatiles which are specifically linked to their hosts, such as host frass, body odor, and host sex pheromones. *cis*-3-Hexenyl butyrate was the only tested HIPV which elicited significantly greater EAG response in *M. croceipes* compared to *C. marginiventris*. This compound is a major HIPV component emitted by cotton plants damaged by both *H. virescens* and *S. exigua* caterpillars (Loughrin et al., 1994; McCall et al., 1994; Ngumbi et al., 2009), and has been shown to elicit behavioral response in *M. croceipes* (Whitman and Eller, 1992). A recent study showed that



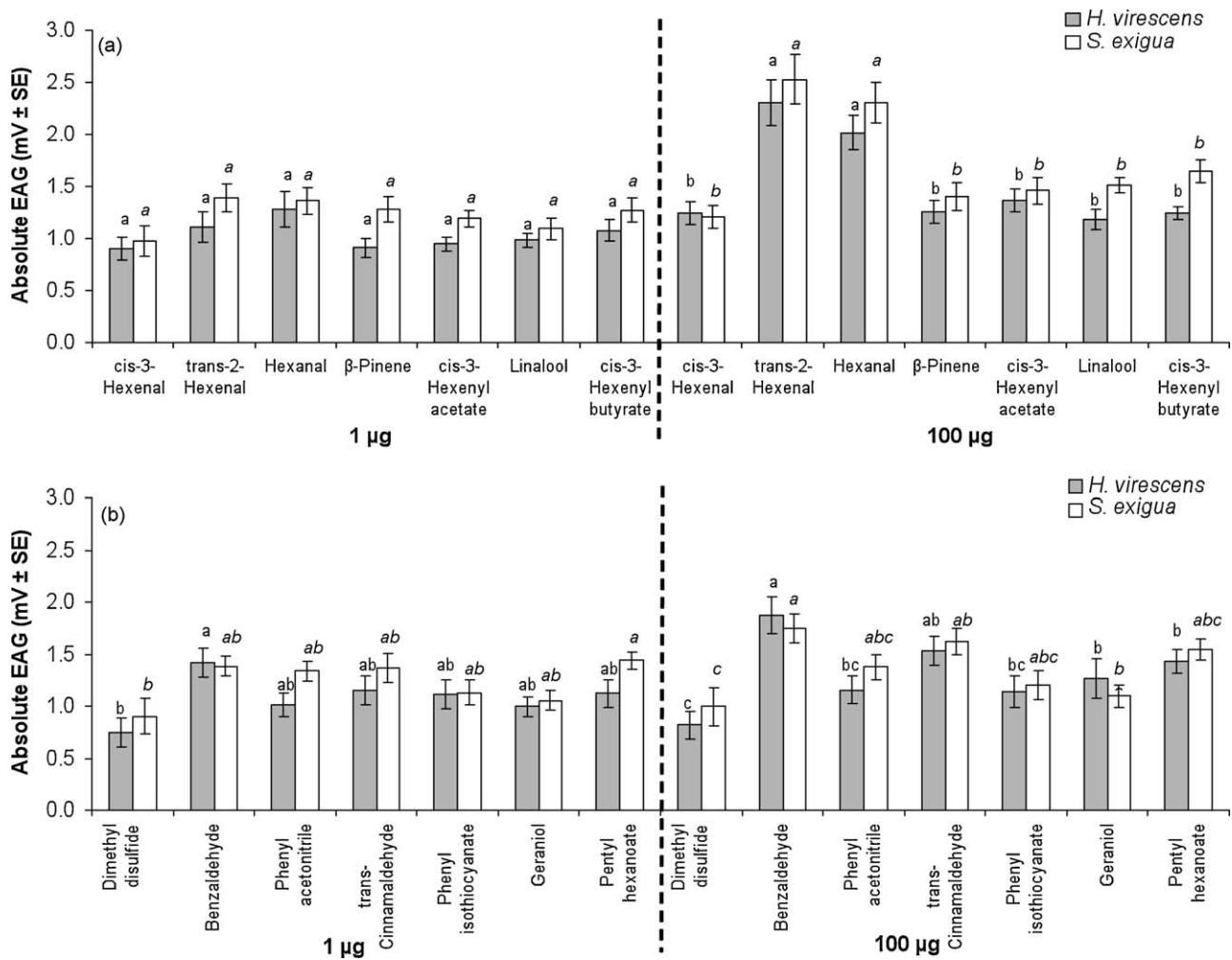
**Fig. 2.** Absolute EAG responses (mV ± SE,  $n = 10$ ) of *Microplitis croceipes* and *Cotesia marginiventris* to (a) host sex pheromones, at two doses (1 µg and 100 µg) and (b) host-specific stimuli (caterpillar body and frass extracts) at one dose (10 µl). Blend (H) = *H. virescens* pheromone blend (Z11-16 Ald + Z9-14 Ald in the ratio of 16:1). Blend (S) = *S. exigua* pheromone blend (Z9E12-14 Ac + Z9-14 OH in the ratio of 10:1). \*Significant difference between the two species ( $t$  test,  $P < 0.05$ ). Means for the same species and dose having no letter in common are significantly different among odor stimuli (ANOVA, Tukey HSD test,  $P < 0.05$ ). Letters in italics are for *C. marginiventris*.

*cis*-3-hexenyl butyrate is emitted in greater amounts by plants damaged by *H. virescens* compared to plants damaged by *S. exigua* (Ngumbi et al., 2009), suggesting that this compound could play an important role in host location behavior of *M. croceipes*. In contradiction to our hypothesis and previous GC-EAD results (Ngumbi et al., 2009), the HIPV, linalool elicited significantly greater EAG response in *C. marginiventris* compared to *M. croceipes*, as was reported earlier by Chen and Fadamiro (2007). These contradictory results with racemic linalool, which comprised of the (+) and (−) enantiomers, may be related to differences in the concentration of linalool reaching the antenna in the EAG versus GC-EAD tests. A recent study showed that the two enantiomers of linalool were perceived in different parts of the brain of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Reisenman et al., 2004). Thus, it is possible that the observed differential electrophysiological responses of our test parasitoids to racemic linalool may be related to concentration. Future studies will test this hypothesis and attempt to resolve our contradictory EAG and GC-EAD results with linalool.

Compared to *C. marginiventris*, *M. croceipes* showed relatively greater EAG response to frass hexane and water extracts and body extract of caterpillars of *H. virescens*, its preferred host, whereas *C. marginiventris* showed comparatively greater EAG response to frass hexane extract and body extracts of caterpillars of *S. exigua*, one of its key hosts. These results imply the ability of parasitoids to use host-specific odor stimuli such as frass and host body odor to discriminate between preferred and non-preferred host species,

and are consistent with previous findings by other authors. For instance, *M. croceipes* has been reported to use host frass as a host location cue (Jones et al., 1971; Eller et al., 1988; Lewis and Tumlinson, 1988). Frass volatiles represent a source of host-specific information which allows specialist parasitoids such as *M. croceipes* to discriminate host and non-host species from a distance (Alborn et al., 1995; Cortesero et al., 1997). Previous studies also demonstrated attraction of *C. marginiventris* to frass hexane extract of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) and the actual (unextracted) frass of *S. exigua* (Loke and Ashley, 1984). More recently, *C. marginiventris* was found to be attracted to chemical footprints of its host, *S. frugiperda* on infested plants (Rostas and Wölfling, 2009).

Both parasitoids showed EAG response to the sex pheromones of the adult form (moth) of their hosts even though caterpillars are their actual hosts. Furthermore, *M. croceipes* showed significantly greater EAG response than *C. marginiventris* to host sex pheromones, suggesting that specialist parasitoids may have evolved greater ability than generalists to associate host (adult moth) sex pheromones with host (caterpillar) availability. These results are not surprising given that host sex pheromones have been shown to attract many parasitoid species, mainly egg parasitoids (Nordlund et al., 1983; Noldus and Van Lenteren, 1985; Colazza et al., 1997; Powell, 1999). The larval parasitoid, *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae) was also shown to be attracted to the sex pheromone of its host *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Reddy et al., 2002).



**Fig. 3.** Absolute EAG responses (mV ± SE,  $n = 10$ ) of *Heliothis virescens* and *Spodoptera exigua* to (a) host-related plant volatiles, and (b) ecologically irrelevant plant volatiles at two doses (1 µg and 100 µg). Means for the same species and dose having no letter in common are significantly different among compounds (ANOVA, Tukey HSD test,  $P < 0.05$ ). Letters in italics are for *S. exigua*. No significant differences were recorded between the two moth species in their responses to any of the tested compounds ( $t$  test,  $P < 0.05$ ).

In contrast to the results obtained for the parasitoids, we found no major differences in the EAG response spectra of the two moth species, *H. virescens* and *S. exigua*, to all tested plant volatiles. This finding is not surprising given that caterpillars of both species are generalist herbivores with similar diet breadth. The GLVs (*trans*-2-hexenal, and hexanal) elicited the highest EAG responses in both moth species, as has previously been reported for *S. exigua* (Dickens et al., 1993) and *S. frugiperda* (Malo et al., 2004). Similarities in the response of parasitoids and their hosts (moths) to plant volatiles have also been reported (Salkeld, 1959; Guerin and Visser, 1980). Similar results were also obtained in the present study, in which *trans*-2-hexenal and hexanal elicited the largest EAG responses in the parasitoids as well as their host moths. Our data which showed EAG responses of both moth species to the tested ecologically irrelevant plant volatiles are also not astounding. Using GC-EAD (coupled gas chromatography electroantennographic-detection) and single cell recordings, Jönson and Anderson (1999) showed that *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) responded to cotton plant volatiles with the ability to discriminate between damaged and undamaged host plants. Rajapakse et al. (2006) reported that *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) showed EAG responses to its common host plants, pigeon pea (*Cajanus cajan* L.), tobacco (*Nicotiana tabacum* L.), cotton, and bean (*Phaseolus vulgaris* L.), as well as to non-host plants such as lantana (*Lantana camara* L.) and oleander (*Nerium oleander* L.). Results from a related study that investigated receptor

neurons in three heliothine moth species (Lepidoptera: Noctuidae) with different degrees of host specificity, *H. virescens* (oligophagous), *Heliothis armigera* (Hübner) (polyphagous), and *Helicoverpa assulta* (Guenée) (oligophagous), revealed the presence of similar types of plant odor receptor neurons in all three species, suggesting that functionally similar olfactory receptors are conserved in related species despite the evolution of polyphagy and oligophagy (Stranden et al., 2003). Taken together, the above findings and our data suggest that polyphagous/oligophagous herbivorous insects such as the moth species tested in the present study likely use a broad suite of volatiles common to many plants for host location. Therefore, the EAG technique which measures gross olfactory response to odor may not be robust enough to provide an indication of the diet breadth of moths. Nevertheless, further studies with moth models of different diet breaths (monophagous versus polyphagous) are necessary to confirm this prediction.

The generalist parasitoid, *C. marginiventris* showed greater EAG responses than *M. croceipes* to all tested ecologically irrelevant plant volatiles, whereas both moth species showed similar EAG responses to the ecologically irrelevant plant volatiles. Among the ecologically irrelevant plant volatiles, benzaldehyde elicited the highest EAG response while dimethyl disulfide elicited the least EAG response in both parasitoid species. Benzaldehyde also elicited the highest and dimethyl disulfide the lowest EAG responses in both moth species, although the differences between the compounds were not as clear as those recorded for the

**Table 2**

Results of Student's *t*-test analysis to compare EAG responses of *Heliothis virescens* and *Spodoptera exigua* to host-related plant volatiles and ecologically irrelevant plant volatiles at two doses.

Compound	Dose (μg)	<i>t</i>	<i>P</i>
Plant volatiles			
cis-3-Hexenal	1	0.39	0.649
	100	0.38	0.723
trans-2-Hexenal	1	0.70	0.754
	100	1.54	0.796
Hexanal	1	1.15	0.868
	100	2.20	0.973
β-Pinene	1	0.87	0.802
	100	1.12	0.745
cis-3-Hexenyl acetate	1	0.53	0.700
	100	0.87	0.882
Linalool	1	2.64	0.991
	100	0.80	0.757
cis-3-Hexenyl butyrate	1	3.14	0.996
	100	3.24	0.765
Ecologically irrelevant plant volatiles			
Dimethyl disulfide	1	0.54	0.700
	100	0.79	0.781
Benzaldehyde	1	0.63	0.745
	100	0.43	0.300
Phenyl acetonitrile	1	0.37	0.682
	100	1.24	0.884
trans-Cinnamaldehyde	1	0.48	0.798
	100	0.53	0.681
Phenyl isothiocyanate	1	0.28	0.700
	100	0.36	0.608
Geraniol	1	1.35	0.659
	100	0.78	0.225
Pentyl hexanoate	1	0.88	0.765
	100	0.80	0.781

parasitoids. Benzaldehyde has also been reported to elicit EAG response in *M. croceipes* (Li et al., 1992; Park et al., 2002), and in the moths *H. armigera* (Burguiere et al., 2001), and *Choristoneura rosaceana* (Harris) (Stelinski et al., 2003). The ability of parasitoids and moths to show notable EAG responses to ecologically irrelevant plant volatiles could be as a result of the fact that they encounter these volatiles in their habitats (Vinson, 1976; Powell and Poppy, 2001). Dimethyl disulfide is a component of larval frass of the diamond back moth, *P. xylostella*, and is among the three disulfides reported to play a role in the host searching behavior of the parasitoid, *Diadromus pulchellus* (Auger et al., 1989). Thus, our data in which both *M. croceipes* and *C. marginiventris* showed very low EAG responses to dimethyl disulfide are not surprising, given that both parasitoids are not known to attack *P. xylostella*.

In summary, our results demonstrated a correlation between EAG response spectra of parasitoids and their degree of host specificity, supporting our hypothesis that specialist parasitoids will have a narrower EAG response spectrum than generalists. This EAG study represents an initial attempt to test if the EAG response spectrum of an insect can give an indication of its degree of host specificity or diet breadth. Future studies with other appropriate parasitoid and moth models are needed to confirm the present results.

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