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Comparative GC-EAD Responses of A Specialist (*Microplitis croceipes*) and A Generalist (*Cotesia marginiventris*) Parasitoid to Cotton Volatiles Induced by Two Caterpillar Species

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Abstract Plants emit volatile blends that may be quantitatively and/or qualitatively different in response to attack by different herbivores. These differences may convey herbivore-specific information to parasitoids, and are predicted to play a role in mediating host specificity in specialist parasitoids. Here, we tested the above prediction by using as models two parasitoids (Hymenoptera: Braconidae) of cotton caterpillars with different degree of host specificity: *Microplitis croceipes*, a specialist parasitoid of *Heliothis* spp., and *Cotesia marginiventris*, a generalist parasitoid of caterpillars of several genera including *Heliothis* spp. and *Spodoptera* spp. We compared GC-EAD (coupled gas chromatography electroantennogram detection) responses of both parasitoid species to headspace volatiles of cotton plants damaged by *H. virescens* (a host species for both parasitoids) vs. *S. exigua* (a host species for *C. marginiventris*). Based on a recent study in which we reported differences in the EAG responses of both parasitoids to different types of host related volatiles, we hypothesized that *M. croceipes* (specialist) would show relatively greater GC-EAD responses to the herbivore-induced plant volatile (HIPV) components of cotton headspace, whereas *C. marginiventris* (generalist) would show greater response to the green leaf volatile (GLV) components. Thirty volatile components were emitted by cotton plants in response to feeding by either of the two caterpillars, however, 18 components were significantly elevated in the headspace of *H. virescens* damaged plants. Sixteen consistently elicited GC-EAD responses in both parasitoids. As predicted, *C. marginiventris* showed significantly greater GC-EAD responses than *M. croceipes* to most GLV components, whereas several HIPV components elicited comparatively greater responses in *M. croceipes*. These results suggest that differences in the ratios of identical volatile compounds between similar volatile blends may be used by specialist parasitoids to discriminate between host-plant and non-host-plant complexes.

Keywords *Microplitis croceipes* · *Cotesia marginiventris* · *Heliothis virescens* · *Spodoptera exigua* · GC-EAD · Green leaf volatiles · Herbivore-induced plant volatiles

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Introduction

Plants emit blends of volatile compounds in response to insect herbivory (Turlings et al. 1990; Loughrin et al. 1994; McCall et al. 1994; De Moraes et al. 1998). This production is triggered by substances present in the oral secretion of herbivores (Dicke et al. 1993; Turlings et al. 1993). The volatile compounds released from herbivore-damaged plants can be sub-divided into two major groups: constitutive compounds, and inducible or herbivore-induced plant volatiles (HIPVs). Constitutive compounds are present constantly in plants and are released immediately in response to mechanical damage or at the beginning of herbivore feeding. They include green leaf volatiles (GLVs) such as *cis*-3-hexenal, hexanal, and *cis*-3-hexen-1-ol (Turlings et al. 1990; Dicke et al. 1993; Loughrin et al. 1994; McCall et al. 1994; Cortesero et al. 1997; Smid et al. 2002; Gouinguené

et al. 2005). In contrast, HIPVs are emitted as a delayed response to herbivore feeding damage. HIPVs in cotton (*Gossypium hirsutum* L) and other plants include *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate, indole, and various terpenoids such as (*E,E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-ocimene, and linalool (Dicke 1994; Loughrin et al. 1994; McCall et al. 1994; Cortesero et al. 1997).

Although, the emission of volatiles is assumed to represent a generalized response to herbivore damage, the blends of volatile compounds released from herbivore damaged plants differ qualitatively and quantitatively depending on the plant species and variety (Dicke et al. 1990; Loughrin et al. 1994; Hoballah et al. 2002), the herbivore species (De Moraes et al. 1998; Loughrin et al. 1994; McCall et al. 1994), and the stage of the herbivore (Takabayashi et al. 1991; Du et al. 1996). For instance, corn (*Zea mays* L.) plants infested by beet armyworm *Spodoptera exigua* (Hübner) caterpillars emit linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*trans*)-α-bergamotene, and (*E*)-β-farnesene as major compounds, all of which have not been detected in the headspace of soybean (*Glycine max* L.) plants infested by the same species (Turlings et al. 1993). In cotton, feeding by corn earworm *Helicoverpa zea* (Boddie) or *S. exigua* caterpillars induces the production of distinctive volatile blends that are qualitatively and quantitatively different (Loughrin et al. 1994; McCall et al. 1994). McCall et al. (1994) reported that cotton plants damaged by *H. zea* emit several compounds including (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, (*Z*)-3-hexenyl-2-methylbutyrate, (*E*)-2-hexenyl-2-methylbutyrate, and indole. Loughrin et al. (1994) conducted a similar study with cotton plants damaged by *S. exigua* and reported several compounds including some of the above, and many which were not reported by McCall et al. (1994) such as (*Z*)-jasmone, (*E*)-β-farnesene, and (*E,E*)-α-farnesene. Such differences in the composition of volatiles induced by different herbivores may convey herbivore-specific information to parasitoids, and thus shape their foraging strategies (Dicke and Sabelis 1988; Turlings et al. 1990, 1995; McCall et al. 1993). In particular, the volatile blend signature produced by plants in response to different herbivores may be used by specialist parasitoids as signals for host specificity (Du et al. 1996; De Moraes et al. 1998). For instance, the specialist parasitoid *Cardiochiles nigriceps* Viereck can exploit differences in volatile blends produced by cotton or corn plants in response to different herbivores, thus distinguishing infestation by its host *H. virescens* from that of the closely related *H. zea* (De Moraes et al. 1998).

The question of whether specialist and generalist parasitoids show differential responses to different suites of host-related volatiles has been a major focus of evolutionary ecology in recent years (Vet et al. 1993; Geervliet et al. 1996; Bernays 2001; Chen and Fadamiro 2007; Stilmant et al. 2008). It has been predicted that specialist parasitoids that utilize fewer numbers of hosts are likely to possess a more highly sensitive (high olfactory sensitivity to host-related chemical cues) and narrowly-tuned (selective) host detection olfactory system than generalist parasitoids (Vet and Dicke 1992; Cortesero et al. 1997; Smid et al. 2002; Chen and Fadamiro 2007). However, few studies have compared olfactory response and sensitivity to host-related volatiles in specialist and generalist parasitoids, and they have produced contrasting results (Elzen et al. 1987; Vet et al. 1993; Geervliet et al. 1996; Chen and Fadamiro 2007). On the one hand, some studies have reported relatively greater response for specialists compared to generalists (Elzen et al. 1987; Vet et al. 1993). Additionally, Geervliet et al. (1996) recorded no differences in behavioral responses of the specialist, *Cotesia rubecula* Marshall and the generalist, *Cotesia marginiventris* (Cresson) to host-related volatiles, and both species were unable to distinguish between plant volatiles induced by their hosts vs. those induced by non-host species. Similarly, Smid et al. (2002) reported no differences in the receptive range of the specialist, *C. rubecula* and the generalist, *Cotesia glomerata* L. to a wide range of host-related odor compounds. Such discrepancies suggest that diverse species of specialist and generalist parasitoids may respond differently to different types of host-related volatiles. Furthermore, even within a broad category of specialist or generalist parasitoids, differences may exist among species based on the degree of specialization (De Moraes et al. 1998; Tamo et al. 2006).

In this study, we tested the above prediction by using a tritrophic model system consisting of cotton (plant), *H. zea* and *S. exigua* (herbivores), and two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *C. marginiventris*. *Microplitis croceipes* is a relatively specialist parasitoid specific to the caterpillars of *H. zea* and *H. virescens*, while *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *S. exigua*, *H. zea*, *H. virescens* (Jalali et al. 1987; Turlings et al. 1990; Lewis et al. 1991; Röse et al. 1998). Both parasitoids were selected as experimental models for this comparative study because they have served as models in previous studies of parasitoid olfaction, and because several aspects of their responses to host-related volatiles have been characterized (e.g., Dmoch et al. 1985; Li et al. 1992; Cortesero et al. 1997; Röse et al. 1998; Park et al. 2002; Gouinguené et al. 2005). We used the coupled gas chromatography electroantennogram detection (GC-EAD) technique to test for similarities and differences in antennal responses of both parasitoid species to headspace volatiles of cotton plants infested with *H. virescens* (a host species for both parasitoids) vs. *S. exigua* (a host species for *C. marginiventris*

but not for *M. croceipes*). Based on the results of a recent study in which we recorded differences in the electroantennogram (EAG) responses of both parasitoids to various synthetic host-related volatile compounds (Chen and Fadamiro 2007), we hypothesized that *M. croceipes* would show relatively greater GC-EAD responses than *C. marginiventris* (generalist) to the HIPV components of cotton headspaces, whereas the GLV components, which are emitted passively by plants and as a generalized response to herbivore damage, would elicit relatively greater GC-EAD activity in the generalist.

Methods and Materials

Plants

Cotton (*G. hirsutum*, var. max 9) plants were grown in individual pots (9 cm high, 11 cm diam) in a greenhouse (Auburn University Plant Science Greenhouse Facility) at 25°C±10, 15:9 h (L/D) photoperiod and 50±10% relative humidity. Seeds were planted in a top soil/vermiculate/peat moss mixture. Plants used for headspace volatile collections were 4–6 wk-old.

Caterpillars (Parasitoid Hosts)

Two lepidopteran species, *H. virescens* and *S. exigua* were used as parasitoid hosts. Both species are distributed throughout the United States and are important pests of agricultural crops including corn, and cotton. Eggs purchased from Benzon Research (Carlisle, PA, USA) were used to start laboratory colonies. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale 1965) at 25±1°C, 75±5% relative humidity and 14:10-h (L/D) photoperiod.

Parasitoids

The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia) and the University of Georgia, Tifton campus (contact: John Ruberson), respectively. *Microplitis croceipes* was reared on caterpillars of *H. virescens*, its preferred host (Stadelbacher et al. 1984; King et al. 1985), whereas *C. marginiventris* was reared on caterpillars of its main host *S. exigua* (Jalali et al. 1987). The rearing procedures were similar to those of Lewis and Burton (1970), and the rearing conditions were the same as described above for the caterpillar hosts. For each 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 diam 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 4–6 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. Female parasitoids (aged 3–5 d-old) of both species were used.

Collection and GC Analysis of Headspace Volatiles

The methodology and protocols used for volatile collection were similar to those reported by Gouinguené et al. (2005), but with some modifications. Headspace volatiles were collected both from caterpillar damaged and undamaged cotton plants. To induce the production of HIPVs from plants, 30 second instars of *H. virescens* or *S. exigua* were allowed to feed on a potted cotton plant for 12 h prior to volatile collection. The pot with the potting soil was wrapped with aluminum foil to minimize evaporation of water and volatiles. The plant (with the feeding caterpillars) was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL, USA) consisting of a 5 l glass jar. A purified (activated charcoal) air stream of 500 ml/min was passed through the jar at room temperature for 24 h. The results of a pilot test that compared headspace volatile collection for 24 h vs. 12 h showed no noticeable differences in the number or relative proportion of the peaks, however the 24 h duration was selected because it produced consistent profiles in which all key peaks were detected in relatively higher amounts. Headspace volatiles were trapped with a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) and eluted with 200 µl of methylene chloride. The resulting extracts (200 µl) were stored in a freezer (at −20°C) until use. Another container with potting soil without plant was used to check for miscellaneous impurities and background noise. The collection system was checked and controlled for breakthrough of the trap during sampling. One µl of each headspace volatile extract was injected into a Shimadzu GC-17A equipped with a flame ionization detector (FID). The dimension of capillary column used was as follows: Rtx-1MS, 0.25 mm I.D., 0.25 µm film thickness (Restek, Bellefonte, PA, USA). Helium was used as carrier gas at a flow rate of 1 ml/min. The GC oven was programmed as follows: inject at 40°C, hold at 40°C for 2 min, and then increase by 5°C/min to 200°C for a total of 40 min. The temperature of both injector and detector was set at 200°C.

GC-EAD Recordings

The extracts were subjected to coupled gas chromatography-electroantennogram detection (GC-EAD) analyses with

females of both parasitoids to detect biologically active peaks (components). GC-EAD analyses were conducted with samples of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* caterpillars and detected with antennae of *M. croceipes* or *C. marginiventris* females (total of 4 combinations or treatments). The GC-EAD techniques used were similar to those described by Smid et al. (2002). Briefly, the system was based on the above Shimadzu GC-17A equipped with a FID and coupled to an EAG detector. The dimension of the GC capillary column was the same as described above. The column effluent was mixed with 30 ml/min make-up helium and split at a ratio of 1:2 (v/v), with one part going to the FID and the other through a heated (220°C) transfer line (Syntech®, Hilversum, the Netherlands) into a charcoal filtered, humidified airstream (1000 ml/min) directed at the antenna preparation (EAG detector). The GC oven was programmed as above. The antenna preparation and EAG techniques were the same as previously described (Chen and Fadamiro 2007). Glass capillaries (1.1 mm I.D.) filled with Ringer solution were used as electrodes. Parasitoids were first anaesthetized by chilling, and the head was isolated. The reference electrode was connected to the neck of the isolated head, while the recording electrode was connected to the antennal tip (with the last segment of antenna cut off). Chlorinated silver-silver chloride junctions were used to maintain electrical contact between the electrodes and input of a 1× preamplifier (Syntech®). The analog signal was detected through a probe (INR-II, Syntech®), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with software (GcEad 32, Syntech®) on a personal computer. A 3-µl aliquot of each sample was injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each treatment. GC-EAD traces were overlaid on the computer monitor and inspected for significant and consistent qualitative and quantitative differences among treatments.

GC-MS Analyses

The GC-EAD active peaks in each treatment were identified by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with an HP-5 ms capillary column (30 m×0.25 mm I.D., 0.25 µm film thickness). One µl of each headspace extract was injected into the GC in splitless mode and under the GC conditions described above for GC-EAD. The chromatographic profiles were similar to those obtained from GC-EAD recordings, thus making it possible to match the peaks. Mass spectra were obtained by using electron impact (EI, 70 eV). Identification of EAD-active peaks was done by using NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparing with published GC profiles of cotton head space volatiles (Thompson et al. 1971; Loughrin et al. 1994; McCall et al. 1994). The structures of the identified compounds were confirmed by using commercially available synthetic standards with purity >97% (as indicated on the labels) obtained from Sigma® Chemical Co. (St. Louis, MO, USA). Significant differences in the amounts of each volatile component emitted by *H. virescens* damaged vs. *S. exigua* damaged cotton plants were established by using the Student's *t*-test ($P<0.05$, JMP® 7.0.1, SAS Institute 2007).

GC-EAD Analyses with Synthetic Blend

In order to confirm the observed differences in the GC-EAD responses of both parasitoids to the headspace extracts, a synthetic blend that mimicked the headspace of caterpillar-infested cotton plants was prepared. This blend was formulated to mimic closely the active components of the headspace of cotton plants infested with *H. virescens*, although the same compounds were detected also in the headspace of cotton plants infested with *S. exigua.* It consisted of 13 synthetic volatile compounds that were identified as key biologically active components in the headspace volatiles of cotton plants infested with *H. virescens*, and blended at an approximate ratio in which they were detected in the headspace. The compounds were purchased from the above source with purity >97% and included *cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, *trans* -2-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, indole, *cis*-jasmone, α-farnesene, α-humulene, and *trans*-nerolidol, blended in the ratio of 4.8, 7.8, 1.9, 19.8, 12.2, 2.2, 13.3, 11.1, 7.2, 0.4, 4.6, 4.3, and 10.2, respectively. Each compound was diluted in hexane and blended at the above ratio to obtain a 100 µg/µl solution. A 3-µl aliquot of the blend (100 µg/µl) was injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each parasitoid species as described above.

Quantification of GC-EAD Responses

GC-EAD responses of both parasitoids to different volatile components were quantified by using a measurement marker tool available with the GC-EAD software (GcEad 32). This tool enabled the quantification of EAD peaks in microvolts (µV). Significant differences in GC-EAD responses of both parasitoid wasps to each volatile component were then established by using the Student's *t*-test ($P<0.05$: JMP® 7.0.1, SAS Institute 2007).

Results

GC and GC-MS Analysis of Headspace Volatiles

The GC profiles of the extracts of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* vs. uninfested (undamaged) plants are shown in Fig. 1. A total of 30 peaks (volatile components) were detected in the headspace of plants infested with *H. virescens* or *S. exigua* (Fig.1a, b). Identical compounds were detected in both extracts, meaning that no qualitative differences were recorded. However, noticeable *quantitative* differences were recorded. In particular, 18 peaks were significantly elevated in the headspace of plants infested with *H. virescens* compared to those infested with *S. exigua* (Table 1). These elevated peaks, as identified by GC-MS, included *cis*-3-hexenal, *cis*-3-hexen-1-ol, α-pinene, β-myrcene, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl-2-methyl butyrate, *cis*-jasmone, α-farnesene, *trans*-nerolidol, and several other HIPV components. No peaks were elevated obviously in the headspace of plants infested with *S. exigua*, relative to those infested with *H. virescens*. Most of the above peaks were not detected or were detected in insignificant amounts in the headspace of undamaged plants (Fig. 1c). Only five peaks (components) were slightly detectable in undamaged plants and were identified by GC-MS as α-pinene, *trans*-2-hexenyl butyrate, linalool, *n*-decanal, and caryophyllene. However, all five components were detected in much greater amounts in the headspace of caterpillar-infested plants.

GC-EAD Responses

Similarities were recorded in the GC-EAD responses of *M. croceipes* and *C. marginiventris* females to volatiles from cotton infested with the two caterpillar species. Sixteen components of the headspace of caterpillar-infested plants elicited consistent GC-EAD responses in both parasitoid species (Figs. 2 and 3). As identified by GC-MS, these volatiles included several GLVs (*cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, and *trans*-2-hexen-1-ol) and HIPVs [(*E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *n*-decanal, *cis*-3-hexenyl-2-methyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, isobutyl tiglate, (*E*)-2-hexenyl tiglate, *cis*-jasmone, caryophyllene, α-*trans* bergamotene, α-farnesene, α-humulene, β-farnesene, β-hemachalene, and *trans*-nerolidol]. More importantly, key differences were recorded in

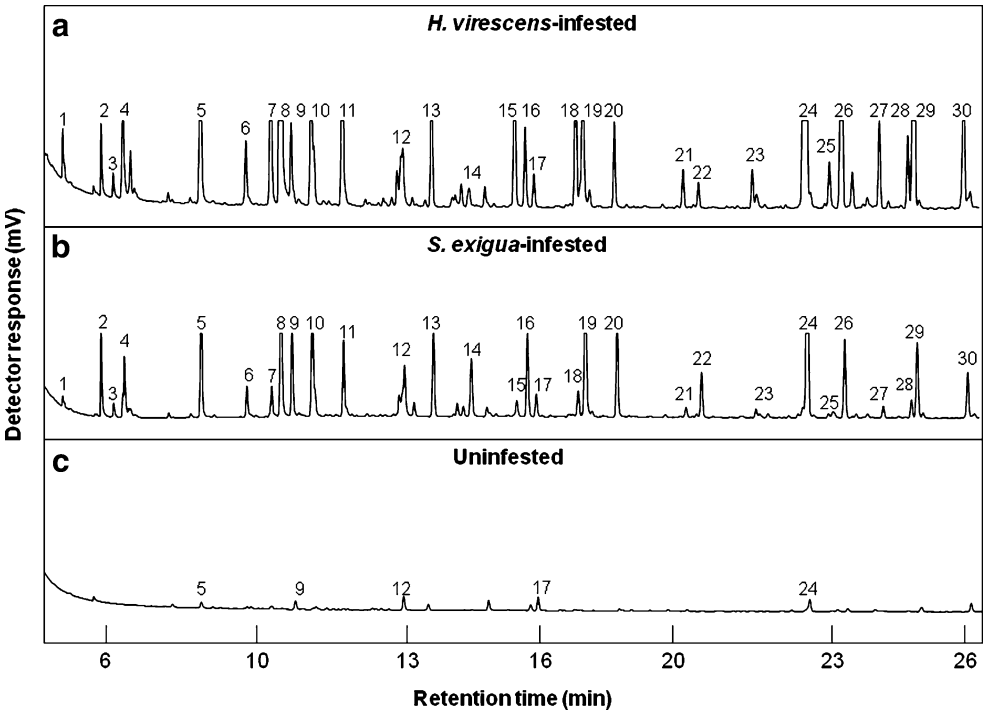

Fig. 1 Chromatographic profiles of headspace volatiles collected from cotton plants infested with *Heliothis virescens* (**a**) or *Spodoptera exigua* (**b**) caterpillars, vs. undamaged control plants (**c**). Identified compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *trans*-2-hexen-1-ol; (5) α-pinene; (6) β-pinene; (7) myrcene; (8) *cis*-3-hexenyl acetate; (9) *trans*-2-hexenyl acetate; (10) limonene; (11) β-ocimene; (12) linalool; (13) unknown; (14) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (15) *cis*-3-hexenyl butyrate; (16) *trans*-2-hexenyl butyrate ; (17) *n*-decanal; (18) *cis*-3-hexenyl-2-methyl butyrate; (19) *trans*-2-hexenyl-2-methyl butyrate; (20) indole; (21) isobutyl tiglate; (22) (*E*)-2-hexenyl tiglate; (23) *cis*-jasmone; (24) caryophyllene; (25) α-*trans* bergamotene; (26) α-farnesene; (27) α-humulene; (28) β-farnesene; (29) β-hemachalene; (30) *trans*-nerolidol

Table 1 Composition of volatiles collected from cotton plants infested for 24 h with *Heliothis virescens* or *Spodoptera exigua* caterpillars and undamaged control plants

ID	Compound[a]	*H. virescens*-infested		*S. exigua*-infested		Uninfested	
		Amount (ng±SE)[b]	Relative %	Amount (ng±SE)[b]	Relative %	Amount (ng±SE)[b]	Relative %
1	*cis*-3-hexenal	39,350±3212[a]	1.9	1,408±238[b]	0.09	0	0
2	*trans*-2-hexenal	63,420±1106	3.0	72,438±2520	5.0	0	0
3	*cis*-3-hexen-1-ol	15,740±670[a]	0.8	8,200±720[b]	0.5	0	0
4	*trans*-2-hexen-1-ol	69,402±2230	3.3	67,120±1340	4.7	0	0
5	α-pinene	98,310±3110[a]	4.5	83,120±2620[b]	5.8	100±25	18.5
6	β-pinene	58,239 ±1939[a]	2.8	42,300±1940[b]	2.9	0	0
7	myrcene	120,259±5920[a]	5.8	15,465±853[b]	1.1	0	0
8	*cis*-3-hexenyl acetate	161,470±2350	7.7	120,475±4860	8.4	0	0
9	*trans*-2-hexenyl acetate	99,214±1074	4.8	111,345±3740	7.8	0	0
10	limonene	110,259±983[a]	5.3	84,330±750[b]	5.9	0	0
11	β-ocimene	120,257±1506[a]	5.8	89,354±2015[b]	6.2	0	0
12	linalool	18,343±939	0.9	18,468±542	1.3	150±38	27.7
13	unknown	59,320±1812	2.8	58,458±2040	4.1	0	0
14	4,8-dimethyl-1,3,7-nonatriene	21,320±1003	1.0	78,800±1296	5.5	0	0
15	*cis*-3-hexenyl butyrate	108,345±1690[a]	5.2	36,900±1165[b]	2.5	0	0
16	*trans*-2-hexenyl butyrate	90,210±4500	4.3	91,356±4300	6.4	135±60	25.0
17	*n*-decanal	5,300±412	0.3	4,800±109	0.3	75±18	13.8
18	*cis*-3-hexenyl-2-methyl butyrate	135,100±3600[a]	6.5	2,800±198[b]	0.2	0	0
19	*trans*-2-hexenyl-2-methyl butyrate	128,950±5300	6.2	115,220±5200	8.0	0	0
20	indole	58,430±1250[a]	2.8	43,200±2700[b]	3.0	0	0
21	isobutyl tiglate	15,900±840[a]	0.8	2,300±350[b]	0.2	0	0
22	2-hexenyl tiglate	6,500±152	0.3	14,999±1650	1.0	0	0
23	*cis*-jasmone	3,200±636[a]	0.2	900±330[b]	0.1	0	0
24	caryophyllene	170,500±6835	8.2	154,230±5300	10.7	80±40	14.8
25	α-*trans* bergamotene	16,378±910[a]	0.8	468±130[b]	0.03	0	0
26	α-farnesene	37,745±2470[a]	1.8	23,300±3564[b]	1.6	0	0
27	α-humulene	35,200±1119[a]	1.7	2,300±745[b]	0.2	0	0
28	β-farnesene	48,239±636a	2.3	1,305±248[b]	0.09	0	0
29	β-hemachalene	94,600±3830[a]	4.5	65,780±3200[b]	4.6	0	0
30	*trans*-nerolidol	83,170±868[a]	4.0	23,450±1950[b]	1.6	0	0

[a] In order of elution during gas chromatography

[b] Values (amount emitted) are mean±SE of five replicate extractions

Means across the same row for the same headspace extract followed by different letters are significantly different ($P<0.05$, *t*-test).

the response patterns of both parasitoids to the different components of the headspace extracts. Quantitatively, *C. marginiventris* (generalist) showed significantly greater GC-EAD responses to the GLV (e.g., *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexen-1-ol) components of the two extracts, compared to *M. croceipes* (specialist) (Table 2, Figs. 2, 3). In contrast, several HIPV components of both extracts (e.g., *cis*-3-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate and *trans*-2-hexenyl butyrate) elicited significantly greater responses in *M. croceipes*, compared to *C. marginiventris*. In addition, α-humulene also elicited greater response in *M. croceipes* than in *C. marginiventris*, but this was significant only for *H. virescens*-infested headspace extract. *Microplitis croceipes* showed relatively greater GC-EAD responses than *C. marginiventris* to indole and *cis*-jasmone, but these differences were significant only for *S. exigua*-infested extract. Note that responses of *C. marginiventris* to some of the HIPV components were very low and barely detectable (Figs. 2, 3). In general, the GC-EAD responses of both parasitoid species to the synthetic blend mimicked their responses to the headspace volatiles of caterpillar-infested plants (Table 2, Fig. 4). A confirmatory test in which the synthetic blend was tested at a reduced amount (i.e., 1 µl of a 0.1 µg/µl solution of the blend was injected for a GC-EAD run) produced results similar to those shown in Fig. 4, suggesting that the amounts tested in the

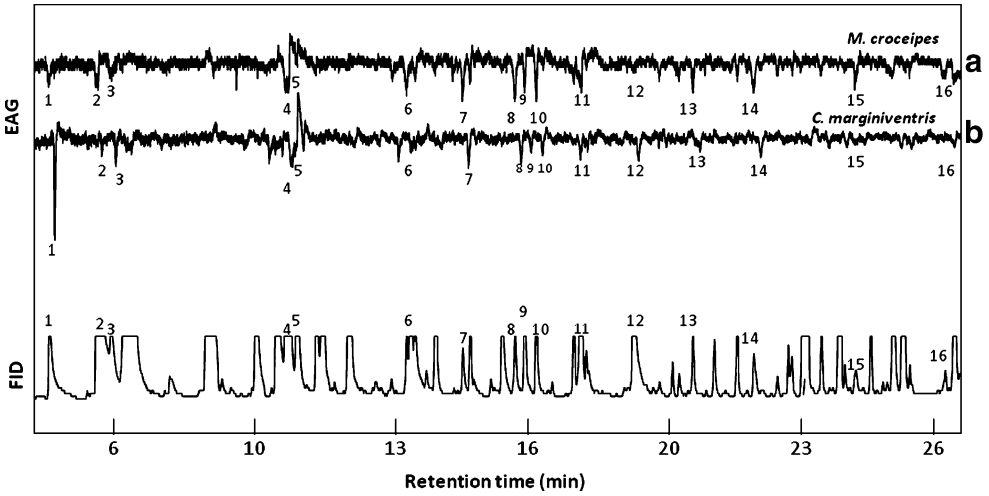

Fig. 2 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to headspace volatiles from *Heliothis virescens* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate ; (11) *trans*-2-hexenyl-2-methyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. Note that responses of *C. marginiventris* to some of the HIPV components were almost too low to be detectable in this and the next two figures. GC-EAD responses of both species to the various compounds are quantified in Table 2

initial experiment with the synthetic blend were neither too high nor physiologically irrelevant.

Discussion

The results show that *M. croceipes* and *C. marginiventris* females were capable of responding antennally to many but not all of the caterpillar-induced cotton volatiles, with both parasitoid species showing differential electrophysiological responses to the different blend components. Compared to undamaged plants, cotton plants emitted detectable amounts of a wide range of volatiles, specifically 30 compounds, in response to damage by *H. virescens* or *S. exigua*. In general, our results are in agreement with those previously reported by other authors on the induction of cotton volatiles (Loughrin et al. 1994; McCall et al. 1994), but with some important differences. Loughrin et al. (1994) and McCall et al. (1994) reported 23 and 22 compounds, respectively, from the headspace of caterpillar-infested cotton plants, most of

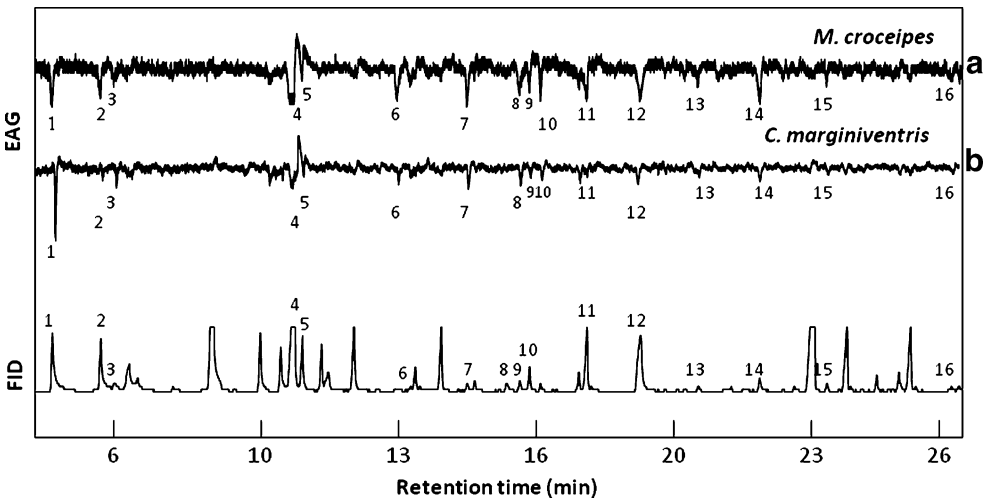

Fig. 3 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to headspace volatiles from *Spodoptera exigua* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate; (11) *trans*-2-hexenyl-2-methyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. GC-EAD responses of both species to the various compounds are quantified in Table 2

Table 2 Quantification of GC-EAD responses of *Microplitis croceipes* and *Cotesia marginiventris* to the different components of headspace extracts of cotton plants infested with *Heliothis virescens* or *Spodoptera exigua*, and a synthetic blend of GC-EAD active components

ID	Compound[a]	*H. virescens*-infested		*S. exigua*-infested		Synthetic Blend	
		Microplitis croceipes (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]	*Microplitis croceipes* (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]	*Microplitis croceipes* (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]
1	*cis*-3-hexenal	72±6.6[b]	192±10[a]	56±4.0[b]	172±12[a]	140±8.9[b]	240±11[a]
2	*trans*-2-hexenal	64±6.3[b]	82±8.4a	56±4.0[b]	88±6.2[a]	62±4.8[b]	96±6.8[a]
3	*cis*-3-hexen-1-ol	44±4.0[b]	72±8.0[a]	48±8.0[b]	80±6.3[a]	76±4.5[b]	98±6.3[a]
4	*cis*-3-hexenyl acetate	144±7.2[a]	92±8.0[b]	176±6.4[a]	72±8.5[b]	136±7.4[a]	84±4.0[b]
5	*trans*-2-hexenyl acetate	52±6.3	48±6.3	54±6.3	46±5.8	96±7.4[a]	28±4.8[b]
6	linalool	72±6.9[a]	24±4.0[b]	80±6.3[a]	24±4.0[b]	80±7.4[a]	64±6.2[b]
7	4,8-dimethyl nonatriene	92±5.0	88±5.0	100±9.0[a]	44±4.0[b]		
8	unknown	108±5.0	88±8.0	100±12	72±4.8		
9	*cis*-3-hexenyl butyrate	104±7.5[a]	60±6.3[b]	172±8.0[a]	56±4.2[b]	240±10[a]	68±4.8[b]
10	*trans*-2-hexenyl butyrate	100±6.3[a]	60±5.3[b]	100±6.3[a]	32±4.8[b]	62±4.8[a]	28±3.6[b]
11	*trans*-2-hexenyl-2-methyl butyrate	60±6.3	40±8.9	88±8.0[a]	24±4.0[b]		
12	indole	24±9.8	36±7.5	80±6.3[a]	32±4.8[b]	28±4.8	16±4.0
13	*cis*-jasmone	52±4.8	38±4.8	48±5.8[a]	12±4.8[b]	88±4.8[a]	52±4.4[b]
14	α-farnesene	60±6.3	48±8.0	42±4.9	12±3.8	88±8.0[a]	24±4.0[b]
15	α-humulene	60±6.3[a]	8±3.8[b]	38±3.7	16±4.2	16±4.0	8±4.8
16	*trans*-nerolidol	16±4.0	12±4.8	12±4.8	9±4.8	20±6.3	20±6.3

[a] In order of elution during gas chromatography

[b] Values (μv) are mean±SE of five replicates

Means across the same row for the same headspace extract or synthetic blend followed by different letters are significantly different ($P<0.05$, *t*-test).

which were identified also in our study. These compounds included GLVs such as *cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol, and HIPVs such as *cis*-3-hexenyl acetate, linalool, (*E,E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, *cis*-jasmone, (*E,E*)-α-farnesene, α-humulene, and *trans*-nerolidol. However, we detected additional volatile compounds that were not reported by Loughrin et al. (1994) and McCall et al. (1994), including *n*-decanal, (*E*)-2-hexenyl tiglate, and β-hemachelene. The differences

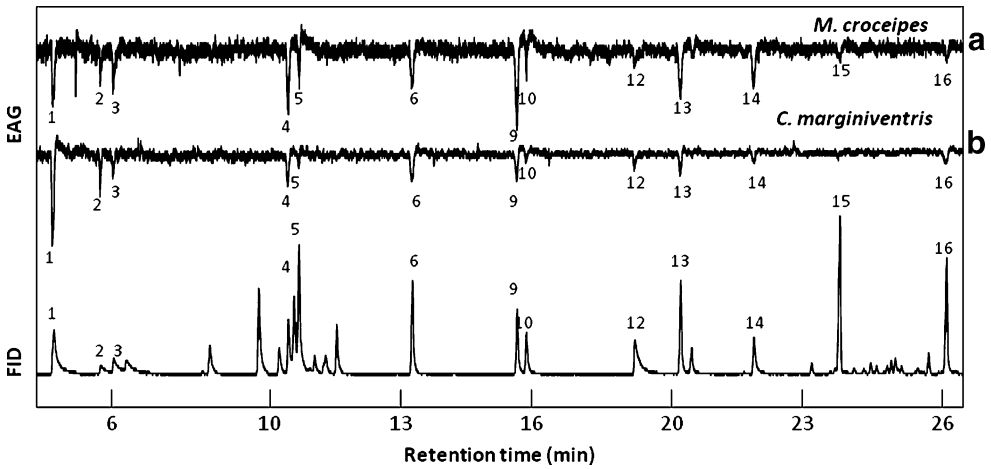

Fig. 4 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to a synthetic blend mimicking the headspace volatiles of caterpillar-infested cotton plants. The blend consisted of 13 compounds (listed below) identified as key biologically active components in the headspace volatiles of cotton plants infested with *Heliothis virescens*, and blended at an approximate ratio in which they were detected in the headspace. Synthetic compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. GC-EAD responses of both species to the various compounds are quantified in Table 2

may be due to several factors, including differences in headspace volatile collection methodology, sensitivity of the analytical system, and cotton cultivar. For instance, we collected cotton volatiles continuously for 24 h beginning 12 h after plants were infested with caterpillars. Loughrin et al. (1994) collected volatiles for 3-h durations in each trap continuously for 60 h, beginning 1 h after plants were infested with caterpillars, while McCall et al. (1994) collected volatiles continuously for 2 h beginning 16–19 h after caterpillar feeding began. Furthermore, differences in the species/strains and stages of caterpillars tested may be important. Loughrin et al. (1994) used *S. exigua* caterpillars, while *H. zea* caterpillars were used by McCall et al. (1994). In the present study, we tested *H. virescens* and *S. exigua* caterpillars.

We recorded major differences in the amounts of volatiles induced by *H. virescens* vs. *S. exigua*. Of the total 30 components identified, 18 were detected in significantly higher amounts in the headspace of *H. virescens* damaged plants, compared to *S. exigua* damaged plants. These results suggest that the essential difference between the volatile blends induced by both caterpillar species is *quantitative*, rather than *qualitative*. Similar differences in the headspace volatile composition of plants infested by different herbivore species have been reported in cotton (McCall et al. 1994; Loughrin et al. 1994; De Moraes et al. 1998), corn (De Moraes et al. 1998; Turlings et al. 1998), cabbage (Agelopoulos and Keller 1994; Geervliet et al. 1997), and tobacco (De Moraes et al. 1998). It has been proposed that herbivore-specific volatile blends that differ significantly and consistently may provide reliable, information-rich signals to foraging parasitoids (De Moraes et al. 1998). Thus, the change in proportions or ratios of volatile compounds in the headspace of *H. virescens* damaged cotton plants, compared to *S. exigua* damaged plants may convey herbivore-specific information to specialist parasitoids, such as *M. croceipes*. On the other hand, generalist parasitoids, such as *C. marginiventris*, which have a wide host range, may not necessarily use herbivore-specific signals for host location. It is important to note that the use of plant volatiles by both parasitoids to locate host-infested plants may suggest that both are generalists in terms of host habitat location.

Only 16 of the 30 volatiles consistently elicited GC-EAD responses in *M. croceipes* and *C. marginiventris*, suggesting that not all components are perceived by both parasitoids, a finding in concert with those previously reported for some other parasitoid wasps (Li et al. 1992; Park et al. 2001; Smid et al. 2002; Gouinguené et al. 2005). The reason why parasitoids do not perceive all components of the headspace volatile of caterpillar-damaged plants is an interesting evolutionary question that deserves to be addressed. It is note worthy that most of the 16 GC-EAD active volatile compounds were among those elevated in *H. virescens* damaged plants. Our results showed no obvious *qualitative* differences in the range of compounds detected by either parasitoid species.

This is the first comparative study of GC-EAD responses of both parasitoid species to herbivore-induced cotton volatiles. In one of the few similar studies on other tritrophic systems, Smid et al. (2002) reported no differences in the GC-EAD responses of the specialist parasitoid, *C. rubecula* and the generalist, *C. glomerulata* to a wide range of volatiles from Brussels sprouts damaged by two species of *Pieris* caterpillars. In contrast, Gouinguené et al. (2005) reported some key differences in the GC-EAD responses of three parasitoid wasps to maize volatiles damaged by *Spodoptera littoralis* Boisduval caterpillars. Relatively more compounds elicited GC-EAD responses in the generalists, *C. marginiventris* and *Campoletis sonorensis* (Cameron), compared to *Microplitis rufiventris* Kok., which is found more often on *S. littoralis* (Gouinguené et al. 2005).

The major difference recorded in our study was in the intensity of GC-EAD response of both parasitoids to several compounds. We utilized a measurement tool in the GC-EAD software to quantify and then establish significant differences in GC-EAD responses of the two parasitoid species to the various volatile components. The generalist, *C. marginiventris* showed significantly greater GC-EAD responses than the specialist, *M. croceipes* to most GLV components, whereas several HIPV components elicited comparatively greater responses in *M. croceipes*. Similar differences in the intensity of response of parasitoids to host-related compounds also were reported by Gouinguené et al. (2005). The authors reported that the generalist parasitoids, *C. marginiventris* and *C. sonorensis*, showed a greater sensitivity to cotton GLVs *cis*-3-hexanal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol) than the more restricted *M. rufiventris*. Our results in which females of the generalist *C. marginiventris* showed comparatively greater GC-EAD responses to GLVs (*cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol), which are continuously present in the plant and released in freshly damaged plants, support our hypothesis, and they are somewhat in agreement with previous electrophysiological (Gouinguené et al. 2005; Chen and Fadamiro 2007) and behavioral studies (Cortesero et al. 1997; Hoballah et al. 2002; D'Alessandro and Turlings 2005; Hoballah and Turlings 2005). Similar to our results, Gouinguené et al. (2005) also reported that *C. marginiventris* showed little or no antennal response to several HIPVs including β-myrcene, β-caryophyllene, bergamotene, and β-farnesene. In contrast, the specialist *M. croceipes* showed greater GC-EAD responses to the HIPVs, which are more specifically linked to its host. These findings were verified by the results of the GC-EAD tests with the synthetic blend, which also showed the same differences in the intensity of response of both parasitoids.

In general, *M. croceipes* showed slightly greater GC-EAD responses to headspace volatiles collected from cotton damaged by its host species (*H. virescens*) than to headspace volatiles collected from cotton that was damaged by the non-host species (*S. exigua*). Our GC data showed that the essential difference between the volatile blends induced by *H. virescens* vs. *S. exigua* is in the amounts and consequently the ratios of the same compounds. De Moraes et al. (1998) also reported that the main difference in the volatile blends of plants damaged by *H. virescens* vs. *H. zea* was in the ratios of identical compounds. Those authors further reported that the specialist parasitoid *C. nigriceps* could distinguish behaviorally plants damaged by its host, *H. virescens* from those damaged by *H. zea* (a non-host species), possibly by exploring the differences in the ratios of identical compounds in the volatile blends. Thus, the differences recorded in our study in the ratios of the same compounds in the blends induced by the two caterpillar species may be exploited by *M. croceipes* to differentiate plants damaged by its host from non-host species. This proposition is supported by our GC-EAD results which showed greater response of *M. croceipes* to volatiles from *H. virescens* damaged plants, compared to *S. exigua* damaged ones. The need to discriminate hosts from related non-hosts based on subtle differences in the ratios of identical compounds in blends is without doubt a challenging task for specialist parasitoids, such as *M. croceipes*. Thus, it is likely that other unknown minor compounds as well as host-specific volatiles also may play a role in differentiation of host vs. non-host by *M. croceipes*.

In contrast, no obvious differences were observed in the response of *C. marginiventris* to volatile blends induced by either caterpillar species. Our data for *C. marginiventris* are in agreement with the report by Geervliet et al. (1996) that a related generalist species, *C. glomerata* was unable to distinguish between plant volatiles induced by its hosts vs. plant volatiles induced by non-host species. However, *C. glomerata* was able to discriminate between plant volatiles induced by its hosts vs. volatiles induced by non-host species after learning (Geervliet et al. 1998). This suggests that associative learning may improve the overall ability of *C. marginiventris* to respond to the HIPV components of the volatile blends, as has been reported for some other generalist parasitoids (Turlings et al. 1989, 1993; Vet and Groenewold 1990; Vet 1999; Steidle and van Loon 2003; Tamo et al. 2006). Indeed, there is evidence that associative learning may improve response of *C. marginiventris* to induced volatiles (D'Alessandro and Turlings 2005). Furthermore, the results of an ongoing study in our laboratory suggest that associative learning may enhance the behavioral response of *C. marginiventris* to host-related volatiles (unpublished data).

The recorded differences in the antennal sensitivity of *M. croceipes* and *C. marginiventris* to host-related volatiles may be related to differences in the abundance and distribution of olfactory sensilla on the antennae of both parasitoid species. Sensilla placodea has been identified as the main olfactory sensilla responsive to host-related volatiles in *M. croceipes* (Ochieng et al. 2000) and *Cotesia* spp. (Bleeker et al. 2004). A comparative study of antennal morphology of the closely related *C. rubecula* and *C. glomerata* revealed significant differences in the density and distribution of this sensilla type (Bleeker et al. 2004). In an ongoing comparative study of antennal sensilla of *M. croceipes* and *C. marginiventris* in our laboratory, we recorded relatively greater numbers of olfactory sensilla placodea on *M. croceipes* than on *C. marginiventris* antennae (unpublished data). This difference in the density of olfactory sensilla may explain the differences in GC-EAD responses of both parasitoids recorded in this study.

In summary, the results may provide insight into how specialist parasitoids can distinguish between plants damaged by their hosts vs. plants damaged by closely related non-hosts, even though the different hosts may induce the emission of *qualitatively* similar volatile blends. The data suggest that differences between similar blends in the *ratios* of identical volatile compounds may contribute to host specificity in specialist parasitoids, such as *M. croceipes*. Additionally, unknown minor compounds as well as host-specific volatiles also may play a role in differentiation of different host-plant complexes. Further discrimination may be mediated at short range by host contact kairomones (which are typically of relatively lower volatility), such as host feces (Loke and Ashley 1984; Dmoch et al. 1985; Afsheen et al. 2008) and caterpillar chemical footprints on infested plants (Rostas and Wölfling 2009). Future behavioral studies are necessary to confirm whether or not the ability of *M. croceipes* to distinguish between plants damaged by its host and non-host caterpillars (Rosé et al. 1997), is in fact mediated by the subtle quantitative differences in volatile blends recorded in this study. If confirmed, the neurophysiological mechanisms that mediate this fine scale ability for odor discrimination will be addressed in the future by using single sensillum and neuroanatomical techniques.

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