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RESEARCH PAPER

Attraction of two larval parasitoids with varying degree of host specificity to single components and a binary mixture of host-related plant volatiles

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Abstract As an indirect defense to herbivore attack, plants release many types of volatile organic compounds (VOCs), which guide parasitoids to their herbivore hosts. In the present study, VOCs were categorized as those released passively from undamaged plants and herbivore-induced plant volatiles (HIPVs). HIPVs were further categorized into: (1) volatiles released by fresh damage plants, and (2) volatiles released by old damage plants. We used as models, two parasitoids with different degree of host specificity, *Microplitis croceipes* (specialist) and *Cotesia marginiventris* (generalist), to address the evolutionary and mechanistic question of whether specialist and generalist parasitoids differ in their use of VOCs for host location. Both species are solitary larval endoparasitoids in the same family (Hymenoptera: Braconidae) and are important parasitoids of caterpillar pests of cotton. Based on the results of previous studies, α-pinene, *(Z)*-3-hexenol, and *(Z)*-3-hexenyl acetate were selected as representatives of the different types of VOCs in cotton. The attraction of both parasitoid species to synthetic components and a binary mixture of the above VOCs was tested in four-choice olfactometer bioassays. Female *M. croceipes* showed the greatest attraction to the HIPVs while female *C. marginiventris* could not discriminate among the three VOCs. Conspecific males showed similar responses with a few exceptions. When presented with the choices; α-pinene, *(Z)*-3-hexenol and a binary mixture (50:50v/v) of the two compounds, the specialist showed the greatest attraction to the mixture. However, the mixture did not elicit such an additive effect on the attraction of the generalist. Overall response latency (time taken to choose VOCs) indicated species and sexual (in the specialist) differences. Using a simple model, this study provides a fundamental insight into odor preferences and discriminatory ability of the test parasitoids. The ecological significance and practical implications of these results are discussed.

Keywords Volatiles organic compounds · Specialist · Generalist · Host location · Four-choice olfactometer · Response latency

Introduction

Natural enemies such as parasitoids; herbivore insects and their host plants interact in a complex tritrophic system in which herbivore infested plants release volatile organic compounds (VOCs) that can attract parasitoids. Host-induced plant volatiles (HIPVs) are released by plants in response to herbivore infestation and may be used for host location by natural enemies such as parasitoids (Turlings et al. 1990; De Moraes et al. 1998; Pare and Tumlinson 1999; Mumm and Hilker 2005; Wei and Kang 2006; Ngumbi and Fadamiro 2012). Plants may release constitutive volatiles or synthesize new ones as an induced response to attack (mechanical/herbivore damage) (Alborn et al. 1997; Pare and Tumlinson 1997; Boland et al. 1998; Rose and Tumlinson 2004). Only certain components of natural volatile blends are attractive or ecologically relevant to parasitic wasps, making the identification of specific VOCs that inform parasitoid behaviors a critical

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task (D'Alessandro and Turlings 2005; Hoballah and Turlings 2005; Schnee et al. 2006; van Dam et al. 2010). Therefore, parasitoids must fine tune their olfactory system to discriminate among several odors to exploit certain VOCs for host location. The degree of host specificity required may determine to what extent a parasitoid species may have to discriminate among plant VOCs. Previous studies have demonstrated the attraction of some parasitoid species to components of plant VOCs both in the laboratory (Wei et al. 2007) and in the field (James and Grasswitz 2005). Others have reported the positive role of synthetic VOCs in recruiting natural enemies for plant defense (Thaler 2002; James and Price 2004). Indeed, most olfactory receptor neurons in insects only respond to one or very few chemical compounds (Kaissling 1986; Meiners et al. 2002; De Bruyne and Baker 2008).

However, natural odors from plants are rarely emitted as single compounds (Bargmann 2006). VOCs that are not attractive to a parasitoid species may still contribute to the olfactory contrast that enhances attraction to other VOCs of interest in the mixture/blend (D'Alessandro et al. 2009). Thus, a mixture of plant VOCs may be more attractive than a single compound because it presents an odor context more similar to what obtains in nature (van Wijk et al. 2011). It is believed that the differences in various VOC blends may serve as important host recognition codes for natural enemies (De Moraes et al. 1998; Smith 1998; De Bruyne and Baker 2008). At the simplest level, the effect of natural plant volatile blends on the attraction of parasitoids can be demonstrated with binary mixtures of synthetic VOCs.

Parasitic wasps have been considered good models for insect olfaction studies (Meiners et al. 2002; Rains et al. 2004; Harris et al. 2012). Based on their relative host range, they can be broadly categorized as specialist or generalist. The question of whether the degree of host specificity affects odor discriminatory ability in parasitoids is yet to be fully answered. This question has serious ecological and evolutionary significance as it concerns the fitness of the two groups of parasitoids. In this study, the specialist parasitoid, *Microplitis croceipes* (Cresson) and the generalist parasitoid, *Cotesia marginiventris* (Cresson) were used as models to test the hypothesis that specialist and generalist parasitoids differ in their use of VOCs for host location. Both wasps are koinobiont, solitary larval endoparasitoids (Hymenoptera: Braconidae) of *Heliothis virescens* (Fab.) (Lepidoptera: Noctuidae), an important pest of cotton. *Microplitis croceipes* and *C. marginiventris*, have been used in many behavioral olfactometer bioassays to study parasitoid attraction to plant VOCs (Navasero and Elzen 1989; Meiners et al. 2002; Olson et al. 2003; Turlings et al. 2004; Sobhy et al. 2012; Ngumbi and Fadamiro 2012).

In the present study, VOCs were categorized as those released passively from undamaged plants and herbivore-induced plant volatiles (HIPVs). HIPVs were further categorized into: (1) volatiles released by fresh damage plants, and (2) volatiles released by old damage plants. In making the selection of test VOCs, results from previous studies (Loughrin et al. 1994; Mc Call et al. 1994; Rose et al. 1996, 1998; De Moraes et al. 1998; Rose and Tumlinson 2004; Ngumbi et al. 2009; Magalhães et al. 2012) that have collected, identified and quantified VOCs from cotton headspace were considered. α-Pinene (undamaged plant volatile), *(Z)*-3-hexenol (fresh damage plant volatile), and *(Z)*-3-hexenyl acetate (old damage plant volatile) were selected as representatives of broader categorizations of plant volatiles. α-Pinene is a monoterpene that is constitutively released by cotton but plant induction via herbivory results in higher emissions (Loughrin et al. 1994). *(Z)*-3-hexenol is generally considered host induced in cotton. Like many green leaf volatiles (GLVs), this VOC is usually released by cotton starting during the early stages (2–6 h) of herbivore damage (Mc Call et al. 1994; Penaflor et al. 2011). *(Z)*-3-hexenyl acetate is also induced by herbivore damage in cotton. Mc Call et al. (1994) reported that *(Z)*-3-hexenyl acetate was the only GLV that was significantly detected in cotton during the late stages (16–24 h) of host infestation. α-Pinene (Lozano et al. 2000; Ozawa et al. 2008), *(Z)*-3-hexenol (Wei et al. 2007; Ngumbi and Fadamiro 2012) and *(Z)*-3-hexenyl acetate (Ozawa et al. 2008; Yu et al. 2010; Uefune et al. 2013) have been associated with the attraction of parasitoids.

In this study, parasitoid attraction to select synthetic VOCs and a binary mixture of cotton volatiles was tested. Based on previous studies from our group (Chen and Fadamiro 2007; Ngumbi et al. 2009, 2010, 2012), it is hypothesized that the two parasitoid species will discriminate among single VOCs to varying extent, and that binary mixtures will generally be more attractive than single VOCs. In addition to testing parasitoid attraction, the time taken to choose different VOCs (response latency) was also recorded in this study. The concept of behavioral response latency to semiochemicals in insects has only been investigated in a few studies (Baker and Vogt 1988; Ngumbi et al. 2012). The ecological significance and practical implications of the results are discussed.

Materials and methods

Insects

M. croceipes and *C. marginiventris* were reared in our laboratory (Auburn University AL, USA) on *H. virescens* larvae. The rearing procedures were similar to those described by Lewis and Burton (1970) and Ngumbi et al. (2009). Upon emergence, adult wasps were transferred to aerated plastic cages (~30 × 30 × 30 cm) and supplied with 10 % sugar water. For parasitization, female wasps (2–5 days old) were supplied with 2nd–3rd instar larvae (caterpillars) of *H. virescens* in the ratio 1 female to 20 larvae. Mated, naïve (untrained) parasitoids (aged 2–5 days old) were used in the behavioral bioassays to test innate responses of parasitoids to plant volatiles. Most behavioral studies have utilized mated parasitoids because changes in the physiological state of mated females presumably increase the probability of host searching behavior (Chen and Fadamiro 2007). Larvae of *H. virescens* were reared on pinto bean artificial diet (Shorey and Hale 1965). The general rearing conditions for all insects were 25 ± 1 °C, 75 ± 5 % RH and 14:10 h (L:D) photoperiod.

Four-choice olfactometer

The set-up of the four-choice olfactometer used for behavioral bioassays is as shown in Fig. 1. Consideration for the new design was partly due to studies by Turlings et al. (2004) and Ngumbi and Fadamiro (2012). The olfactometer used was made of glass and supported with a retort stand. The main piece has a spherical bulb 75 mm diameter from which four horizontally inclined arms 10 cm long projected upwards. At the base of the bulb, a central tube 17 cm long extends downwards to form the entry route for insects. A 30 mm diameter hemispherical depression on top of the bulb (decision-making area) created a vantage position from which insects were evenly exposed to odor streams from all four arms. The VOCs tested were placed on filter paper strips (odor source) and inserted into the small connector tubes from which insects were physically excluded to avoid contamination. A white light bulb (20 W, 250 lux) hung about 40 cm above the olfactometer provided illumination. The entire set-up was placed in a white box (80 cm × 60 cm × 60 cm) to minimize visual distraction. An air delivery system (Analytical Research Systems, Gainesville, FL) passed humidified and purified air through Teflon ® tubes into the olfactometer arms.

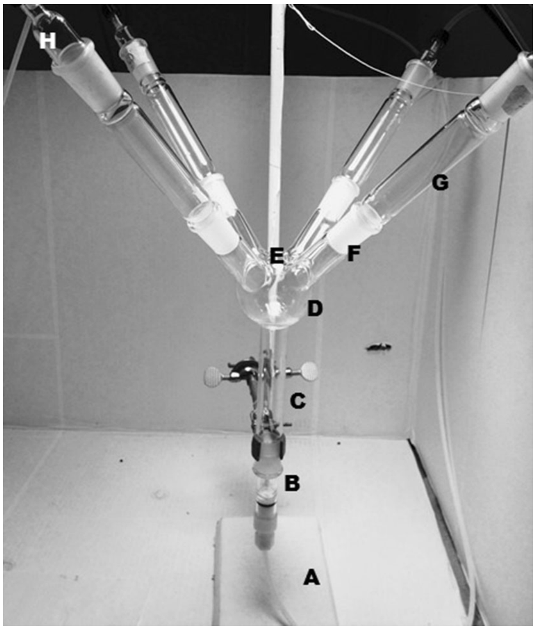

Fig. 1 Major parts of the four-choice olfactometer: Retort stand (*A*), Entry area for insects (*B*), Central tube (*C*), Bulb (*D*), Hemispherical depression (*E*), Olfactometer arm (*F*), Extension tube (*G*), and Connector tube (*H*)

Behavioral bioassays

Humidified and purified (charcoal filtered) air was passed into each of the olfactometer arms at 200 ml/min while the vacuum pump was set at 800 ml/min to avoid a mix-up of volatiles in the chamber. The synthetic VOCs used (purity 95–99 %) were purchased from Sigma® Chemical Co. (St. Louis, Missouri). The compounds were formulated in hexane (HPLC-grade) at 1 μg/μl concentration and delivered as 10 μl samples (10 μg dose) on Whatman No. 1 filter paper strips (25 × 7 mm). This dose was selected based on the results of a preliminary experiment and previous studies by our group (Ngumbi and Fadamiro 2012). The solvent was allowed to evaporate from the filter paper for about 10 s before insertion into the olfactometer arm.

In the first experiment, each sex of the specialist, *M. croceipes* and the generalist, *C. marginiventris* was presented with α-pinene, *(Z)*-3-hexenol, *(Z)*-3-hexenyl acetate, and hexane (control) in separate tests. α-Pinene elicited the greatest attraction in the generalist while *(Z)*-3-hexenol elicited the greatest attraction in the specialist. Consequently, a second experiment was set up in which the parasitoids were presented with four choices: α-pinene, *(Z)*-3-hexenol, a binary mixture of the both compounds, and hexane (control). The binary mixture tested was made by mixing equal volumes (50:50 v/v) of α-pinene and *(Z)*-3-hexenol at the same concentration (1 μg/μl). Individual insects were tested for odor preference and response latency. Response latency was defined as the duration from the time of insect release to the time insect crosses into the extension tube of an arm. After testing four insects, the odor sources were replaced and the olfactometer was rotated 90° to avoid any error due to position effect. The olfactometer arms were labeled so that the same compound was maintained in each arm after replacements. The entire set-up was cleaned (with acetone) after testing 20 insects. Wasps were used only once and discarded. A wasp that did not make a choice after 15 min of exposure was recorded as 'No choice' and not included in the data analysis (<10 % in all experiments). A parasitoid was recorded to have made a clear choice for the odor offered through an arm when it enters into the extension tube and remains

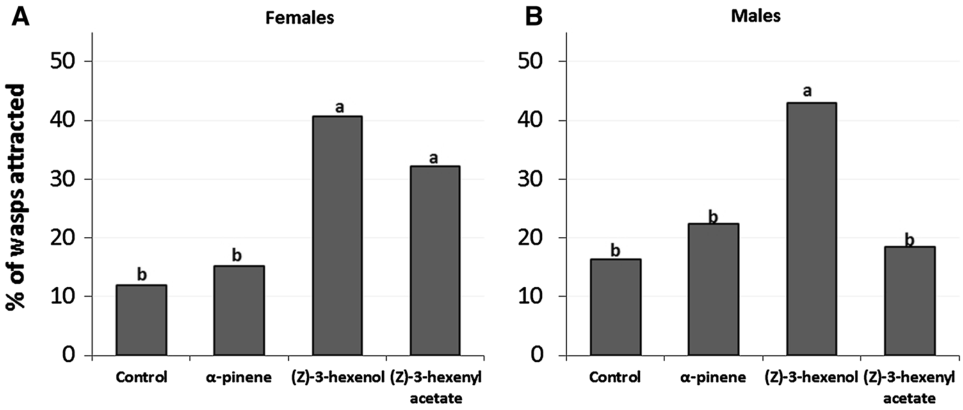

Fig. 2 Attraction of *M. croceipes* to different types of VOCs: females (**a**), and males (**b**). Values (%) having no *letter in common* are significantly different ($P < 0.05$; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps

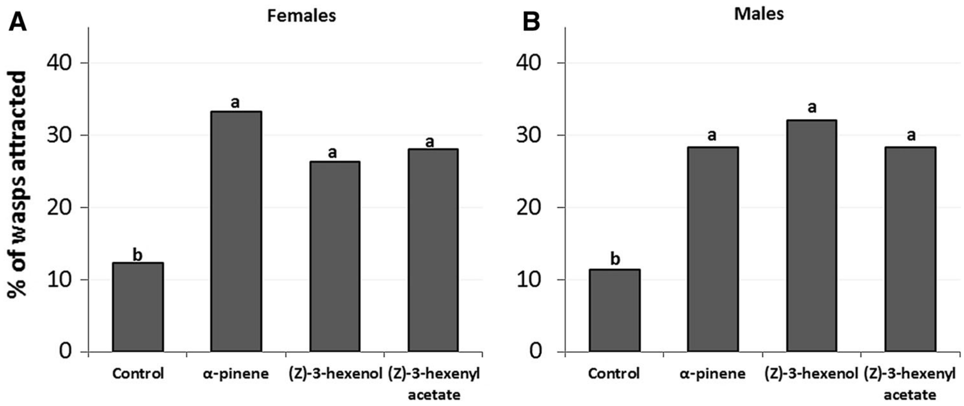

Fig. 3 Attraction of *C. marginiventris* to different types of VOCs: females (**a**), and males (**b**). Values (%) having no *letter in common* are significantly different ($P < 0.05$; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps

there for at least 15 s. Bioassays of different sexes and species were carried out in a randomized block design on different days between 0900 h and 1700 h.

Data analyses

Attraction of parasitoids to each VOC was modeled as a binary response count and treatments were compared using Logistic Regression Analysis. The model adequacy for each set of experiment was confirmed with a Likelihood Ratio (Wajnberg and Haccou 2008). Slopes were separated using Proc Logistic Contrast in SAS. For data presentation, parasitoid attraction to VOCs was represented on charts as percentages of total wasps that responded due to varying sample sizes. Sexual difference in overall response latency was analyzed using two-sided Wilcoxon–Mann–Whitney test. All analyses were performed using SAS 9.2 with 0.05 level of significance.

Results

Attraction to Single VOCs

Female *M. croceipes* (specialist) were significantly ($\chi^2 = 18.17$; $P < 0.0004$; $N = 59$) more attracted to the two HIPVs: *(Z)*-3-hexenol and *(Z)*-3-hexenyl acetate, than to α-pinene and hexane (control) (Fig. 2a). Males were also significantly ($\chi^2 = 10.97$; $P < 0.01$; $N = 49$) more attracted to *(Z)*-3-hexenol than to the other treatments (Fig. 2b). Both sexes of *C. marginiventris* (generalist) could not significantly discriminate among the three VOCs (Fig. 3). These results suggest that the specialist parasitoid showed greater attraction to herbivore-induced VOCs, whereas the generalist did not show preference among the VOCs.

Effect of binary VOC mixture

When females of *M. croceipes* were presented with a choice of α-pinene, *(Z)*-3-hexenol, and a mixture (50:50v/v) of both compounds, the mixture elicited the highest attraction (40 % of wasps) ($\chi^2 = 6.31$; $P < 0.01$; $N = 80$) (Fig. 4a). Similarly, conspecific males showed a significantly ($\chi^2 = 8.99$; $P < 0.0027$; $N = 85$) greater attraction to the mixture, compared to the single VOCs (Fig. 4b). In contrast, female *C. marginiventris* showed no preference among the three treatments (Fig. 5a), while males showed the greatest attraction to α-pinene (Fig. 5b).

Response latency to single VOCs

Overall, a significantly shorter response latency ($Z = 5.91$; $P < 0.0001$; $N = 108$) was recorded for males (68.1 s)

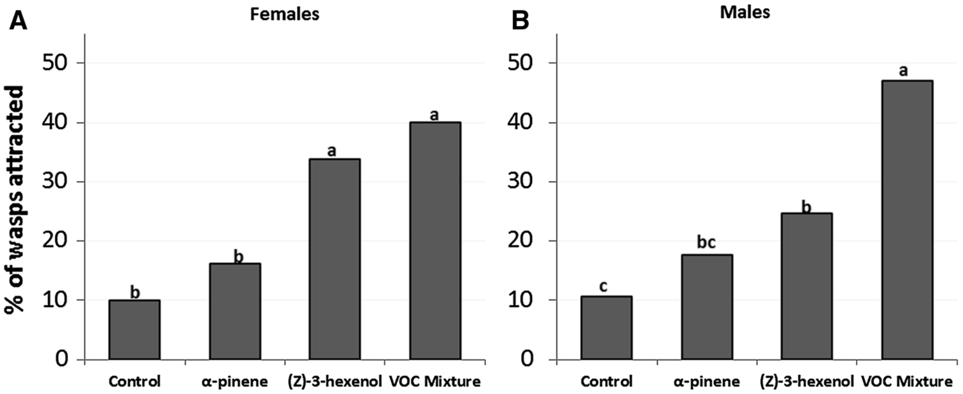

Fig. 4 Attraction of *M. croceipes* to single VOCs and a binary mixture: females (**a**), and males (**b**). Values (%) having no *letter in common* are significantly different ($P < 0.05$; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps

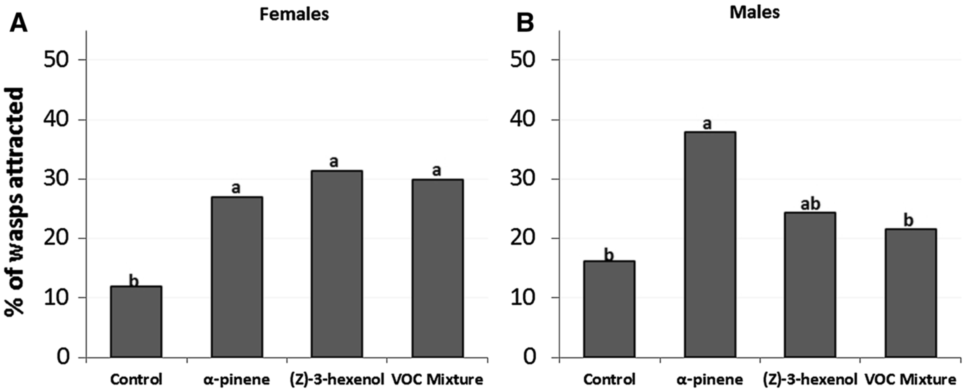

Fig. 5 Attraction of *C. marginiventris* to single VOCs and a binary mixture: females (**a**), and males (**b**). Values (%) having no *letter in common* are significantly different ($P < 0.05$; Proc. Logistic Regression). Attraction to VOCs was modeled as binary response counts and represented on the chart as percentage of total responding wasps

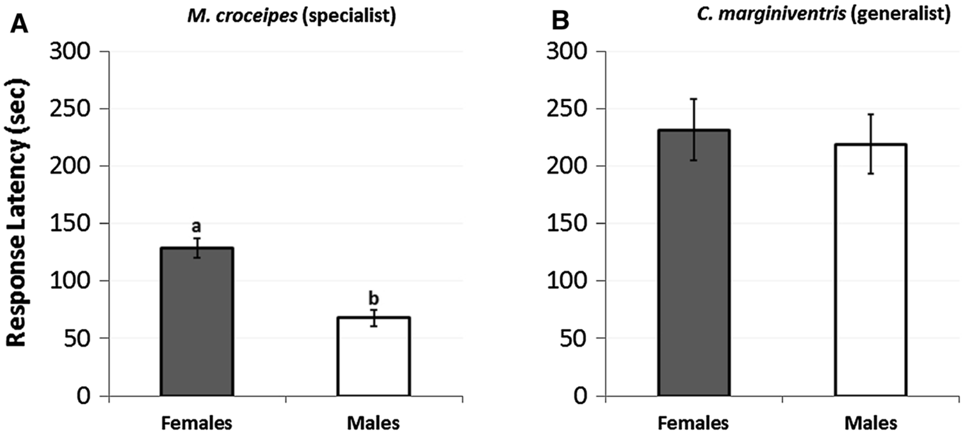

Fig. 6 Overall response latency (time taken to choose all VOCs) of both sexes of *M. croceipes* (**a**) and *C. marginiventris* (**b**). For each parasitoid, mean (± SEM) values for the two sexes having no letter in common are significantly different ($P < 0.05$; Wilcoxon–Mann–Whitney test)

than for females (128.6 s) of *M. croceipes* (Fig. 6a). No significant sexual difference in overall response latency was recorded for *C. marginiventris* (Fig. 6b). Comparing the species, mean response time was significantly ($Z = 2.48$; $P < 0.01$; $N = 116$) shorter for female *M. croceipes* (128.6 s) compared to female *C. marginiventris* (231.2 s).

Discussion

In the present study, the attraction of *M. croceipes* (specialist) to *(Z)*-3-hexenol and *(Z)*-3-hexenyl acetate (both HIPVs) was consistent with the findings of van Poecke et al. (2003), Penaflor et al. (2011), and Ngumbi and Fadamiro (2012), which showed that specialist parasitoids were more attracted to induced plant volatiles than to constitutive volatiles. Arguably, there is a greater chance that HIPVs will provide more specific host recognition cues than constitutive plant volatiles. On the other hand, *C. marginiventris* (generalist) showed no preference among the tested constitutive plant volatile (α-pinene) and the two HIPVs [*(Z)*-3-hexenol and *(Z)*-3-hexenyl acetate]. The results are in support of the findings of Fontana et al. (2011) in which *C. marginiventris* was attracted to constitutive volatiles of maize. Although constitutively released in cotton, α-pinene is also released in higher amounts during early stages of herbivore damage (Loughrin et al. 1994). Ozawa et al. (2008) and Uefune et al. (2012, 2013) have also reported the attraction of other parasitoids in the genus *Cotesia* to α-pinene.

In previous electroantennogram studies by our group (Ngumbi et al. 2009, 2010), *M. croceipes* showed greater responses to HIPVs while *C. marginiventris* showed greater responses to GLVs. Furthermore, Ngumbi and Fadamiro (2012) conducted Y-tube olfactometer bioassays to test the attraction of the two parasitoid species to various VOCs using a series of two-choice tests. Since parasitoids are exposed to a wide array of odors in nature, the present study builds on the previous studies by testing preferential attraction of the parasitoids to synthetic VOCs and mixture in multiple-choice tests. In four-choice olfactometer bioassays, three VOCs representing undamaged, fresh damage, and old damage cotton volatiles were tested. Schroder and Hilker (2008) suggested that attraction of insects to specific odors used to locate resources may become enhanced due to the presence of other less attractive odors. Thus, *M. croceipes* showed significantly greater attraction to *(Z)*-3-hexenol in the present study, compared to the previous study (Ngumbi and Fadamiro 2012) probably because the parasitoids experienced the VOC in a more appropriate context. The importance of odor context was previously discussed by Mumm and Hilker (2005) and van Wijk et al. (2011).

Comparing species, *M. croceipes* females were significantly more attracted to *(Z)*-3-hexenol than *C. marginiventris* females, suggesting that the specialist may depend more on induced volatiles for host location. More importantly, the specialist was able to discriminate HIPVs from constitutive VOC of cotton while the generalist could not, possibly indicating a more specialized olfactory mechanism. In contrast, *C. marginiventris* females were significantly more attracted to α-pinene than *M. croceipes* females, suggesting the likelihood of the generalist to frequent plants more. From an ecological perspective, parasitoids that show greater attraction to undamaged plant volatiles may be recruited early, possibly making the first contact with caterpillar hosts on the plant. On the other hand, parasitoids that show greater attraction to HIPVs may arrive much later. Conceivably, the timing of parasitoid recruitment largely dependent on the relative attractiveness of plant VOCs may determine the outcome of interspecific competitions (see De Moraes and Mescher 2005).

A narrowly tuned olfactory mechanism has the advantage of saving valuable energy resources while searching for specific hosts. However, when extrinsic interspecific competition exists, a broadly tuned olfactory mechanism may present an ecological edge. The results corroborate the prediction of previous studies (Smid et al. 2002; Chen and Fadamiro 2007; Ngumbi et al. 2009, 2010, 2012) that the degree of host specificity in parasitoids may affect their use of various plant volatiles for host location. Generally, similar trends were recorded for conspecific males (as their females), suggesting that male parasitoids may be able to exploit certain VOCs as cues to enhance mate location (Chen and Fadamiro 2007; Ngumbi and Fadamiro 2012).

In the bioassays with *M. croceipes*, the mixture of α-pinene and *(Z)*-3-hexenol elicited a greater attraction than either compound—an additive effect that was not recorded in bioassays with *C. marginiventris*. There are two general models that may explain how an animal's olfactory system processes odor mixtures, leading to behavioral responses: the elemental and the configural models (Erickson et al. 1990; Alvarado and Rudy 1992; Kay et al. 2005). A classic review of the central processing of odor blends in insects was provided by Lei and Vickers (2008). In the simplest terms, the elemental model holds that responses to odor mixtures resemble that of individual components while the configural model holds that odor blends present an entirely new identity and they elicit responses that are different from those of individual components. In this study, the binary mixture used has highly dissimilar components [α-pinene and *(Z)*-3-hexenol]. The components differ in chemical class, pathway of production (terpenoid and lipoxygenase pathways), and the timing of release by plants. Linster and Cleland (2004) explained that the more dissimilar the components of an odor mixture, the less overlap the signals generated, and the more the response to the mixture becomes a linear summation of the responses to both components (elemental processing). Thus, the greater attraction elicited by the mixture suggests an elemental processing of the binary mixture in the specialist. However, the mixture did not elicit an additive effect in the attraction of the generalist. A possible explanation is that the generalist could not discriminate among the component VOCs of the mixture in the initial bioassays with single compounds. Conceivably, the less apparent the difference in the components, the less likely it is for the odor mixture to elicit an additive effect (Linster and Cleland 2004). It should be noted that the above is considered a possible explanation of the present results from the perspective of neural processing, and that other factors may influence insect behavior. Another plausible explanation is that the specialist may have evolved an olfactory mechanism that is more tuned to VOC mixtures than to single components, as would be expected in nature.

There was no correlation between response latency and attraction of parasitoids to each VOC, suggesting that response latency to VOCs may be more related to a species' olfactory architecture rather than to functional behavioral responses. Furthermore, Ngumbi et al. (2012)

reported no significant differences in the response latencies of trained versus untrained *M. croceipes* and *C. marginiventris* to various host-related plant volatiles, indicating that response latency may be innate in these parasitoids. In the present study, *M. croceipes* generally made choices faster than *C. marginiventris* in the olfactometer, and male *M. croceipes* made choices faster than conspecific females, similar to the report of Ngumbi et al. (2012). Interestingly, Das et al. (2011) reported that sensilla placodea (olfactory sensilla) were significantly more abundant on the antennae of *M. croceipes* than in *C. marginiventris*, and that the same sensilla were significantly more abundant in male *M. croceipes* than in conspecific females. This trend corresponds to the response latency of the parasitoids presently reported, suggesting a possible relationship between the abundance of olfactory sensilla and the response latency of these parasitoids. We are not aware of any previous study relating this morphological characteristic to behavioral responses of parasitoids to host-related odors. It has been shown that adult parasitoids have limited ability to synthesize lipids. Thus, a reduced activity rate in some female parasitoids has been linked to energy conservation (Denis et al. 2013). Further studies with other parasitoids are needed to establish if host specificity affects the response latency of parasitoids to host-related plant volatiles.

In summary, results of the present study showed that key differences exist in the responses (attraction and response latency) of *M. croceipes* and *C. marginiventris* to select synthetic VOCs and mixture. The degree of host specificity is believed to be one of the key factors affecting parasitoids' odor discriminatory ability as well as the use of various VOCs for host location (Smid et al. 2002; Chen and Fadamiro 2007; Ngumbi et al. 2009, 2010, 2012). Since parasitoids are often exposed to blends rather than single VOCs emitted by herbivore-damaged plants (van Wijk et al. 2011), it is reasonable to gain fundamental knowledge of how parasitoids respond to odor mixtures containing attractive VOCs that signal resource location. In the present study, a simple binary mixture was tested so as to gain a fundamental understanding of more complex ratio-specific odor recognition in parasitoids (see Bruce et al. 2009; Uefune et al. 2013). Further behavioral studies comparing the attraction of *M. croceipes* and *C. marginiventris* to more complex VOC blends are needed, as reported for *C. vestalis* by Uefune et al. (2013). In addition, further studies investigating the attraction of various parasitoids to plant VOCs based on other differences in life strategy are needed. These studies are expected to yield results that could inform the identification of attractive VOCs and mixtures that may enhance the performance of the parasitoids as biocontrol agents.

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