Cashew Volatiles Mediate Short-Range Location Responses in *Pseudotheraptus wayi* (Heteroptera: Coreidae)

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ABSTRACT *Pseudotheraptus wayi* Brown (Heteroptera: Coreidae) is a major pest of cashew in East Africa, but little is known about its chemical ecology. Here, we show by using behavioral assays and chemical analysis that some components of cashew leaf volatiles are attractants for male *P. wayi*. By using a petri dish arena-EthoVision video-tracking assay, males oriented closer to crude cashew leaf odor than females. In coupled gas chromatography-electroantennographic detection analysis, we found that four components of cashew leaf volatiles that were identified by coupled gas chromatography-mass spectrometry and confirmed with authentic standards as (E)- and (Z)-β-ocimene, allo-ocimene, and (Z)-3-hexenyl butyrate appeared to elicit stronger and more consistent responses in male than female antennae. We recorded electroantennograms from antennae of either sex, which confirmed that antennal responses of males to synthetic components of the cashew leaf odor were stronger than those of females. In the petri dish arena-EthoVision video-tracking assay, males oriented closer to the synthetic cashew leaf monoterpenes than females. Our results suggest that specific monoterpenes in cashew leaf volatiles may serve as candidate kairomones for males.

KEY WORDS cashew, kairomone, *Pseudotheraptus wayi*, electrophysiology, monoterpene

The coreid bug, *Pseudotheraptus wayi* Brown (Heteroptera: Coreidae), is a major pest of cashew (*Anacardium occidentale* L.) in Eastern Africa, where it can contribute to 80% cashew nut yield loss (Martin et al. 1997, Mitchell 2000, CABI 2005, Maniania 2009, Nyambo 2009). Damage is caused by both nymphs and adults that feed on the plant, causing wilting and necrosis of young stems, leaves, inflorescences, and fruits, as they suck sap and inject toxins into the host tissues (Mitchell 2000, Hill 2008). Besides cashew, *P. wayi* also feeds on and damages a range of other economically important crops in Eastern and Southern Africa, including coconut (*Cocos nucifera* L.), macadamia (*Macadamia integrifolia* Maiden & Betche), carambola (*Averrhoa carambola* L.), pecan (*Carya illinoinensis* [Wangenheim] K. Koch), cinnamon (*Cinnamomum verum* J. S. Presl), loquat (*Eriobotrya japonica* (Thunberg) Lindley), mango (*Mangifera indica* L.), avocado (*Persea americana* Miller), guava (*Psidium guajava* L.), cocoa (*Theobroma cacao* L.), French beans (*Phaseolus vulgaris* L.), and various wild legumes (Mitchell 2000, CABI 2005, Hill 2008, Egonyu et al. 2013a).

Currently on cashew, *P. wayi* is managed by using the predatory weaver ant, *Oecophylla longinoda* Lattreille, and synthetic chemical insecticides, but both approaches are faced with challenges (Martin et al. 1997, Mitchell 2000, Nyambo et al. 2003, CABI 2005). The predatory weaver ant is not only susceptible to these insecticides, but it also faces competition and predation from other ants such as *Pheidole megacephala* F., *Pheidole punctulata* Mayr, *Anoplolepsis custodiens* (Smith), and *Anoplolepsis longipes* (Smith) (Mitchell 2000, Nyambo et al. 2003). On the other hand, synthetic chemical insecticides can be hazardous to human and environmental health, and are also difficult to apply on tall trees (Nyambo et al. 2003, Steinemann 2004). Alternative methods for protecting cashew from *P. wayi* infestation and damage including use of semiochemicals as a complementary tool can contribute to better management of this pest. To achieve this, knowledge of the chemical ecology of *P. wayi* is essential, but this is largely unknown.

Previous work has shown that terpenes are the most prominent constituents of cashew leaf odor (Maia et al. 2000, Moronkola et al. 2007, Kossouoh et al. 2008). However, the exact function of the leaf odor in the ecology of cashew pests, including *P. wayi*, is unknown. In this article, we report results based on laboratory behavioral assays and chemical analyses demonstrating that some components of cashew leaf volatiles are attractants for male *P. wayi*.

Materials and Methods

Insects. A laboratory colony of *P. wayi* was initiated by using adults collected in June 2010 from insecti-

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cide-free cashew nurseries and orchards at the Kenya Agricultural Research Institute (KARI)-Mtwapa Research Centre, located at 3°55′ S, 39°44′ E and 15 m above sea level, Kilifi county, Kenya (Kenya Agricultural Research Institute-Mtwapa 2004). The insects were reared on French beans in the laboratory maintained at 24.6 ± 1°C, 80 ± 1.3% relative humidity (RH), and a photoperiod of 12:12 (L:D) h at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi, Kenya.

Collection of Volatiles. Volatiles were collected onto Super Q traps (30 mg, Alltech, Nicholasville, KY) by passing charcoal-purified air through test and control materials at $\approx$350 ml/min for 12 h from flushing cashew leaves on intact trees at KARI-Mtwapa Research Centre in September 2011 by using a "push-pull" system operated by a portable powered pump (USDA/ARS-CMAVE, Gainesville, FL). Five leaves at the apex of a branch were enclosed in an oven-baked polythene bag (Baco, Wrap Film Systems Ltd., United Kingdom). This was repeated thrice on different trees. The control was an empty oven bag. The traps were each eluted with 200 μl of dichloromethane (99%) (Sigma Aldrich, Chemie, Steinheim, Germany). The leaf and control volatiles from different trees were separately combined and concentrated to 500 μl under a gentle stream of nitrogen. The leaf sample contained 180 cashew hour equivalents (CHEs), where 1 CHE = volatiles emitted by one cashew leaf per h. The crude extracts were stored at -80°C before use.

Responses to Crude Cashew Odor. To test for behavioral responses from *P. wayi*, we used two types of assays: a four-arm olfactometer with an air-flow and a petri dish arena assay in still air with the crude volatile extract at different doses. Based on the preliminary results, we selected the petri dish arena assay for screening both the crude volatile extract and synthetic antennally active compounds for short-range location responses in the insect. Behavioral bioassays were conducted in a petri dish arena comprising a glass base (14-cm-diameter $\times$ 1.5-cm-high) (Yoneya et al. 2010). The base and lid of the petri dish were separated from each other by a cylindrical wire mesh (14-cm-diameter $\times$ 3.5-cm-high) to provide enough vertical space for navigation and ventilation for the insects. Two pieces of filter paper (Whatman No. 1, 1001-090, $\approx$1 cm^2), one loaded with the volatile extract and the other loaded with the solvent only as a control, were placed at opposite sides of the petri dish arena. Doses of 0.1, 0.2, and 0.4 CHE of cashew volatile extracts in 10 μl of dichloromethane were tested against control filter papers loaded with the same volume of solvent only. Before assays, the solvent was allowed to evaporate for 2 min. For each dose, tests were replicated 10 times with fresh samples in a clean petri dish and each insect was used only once. To minimize possible positional effects, locations of odor and control filter papers were switched after five replicates. The petri dishes were washed with Teepol odorless detergent and hot water, rinsed with acetone and distilled water, and heated for 24 h in an oven at 150°C before use.
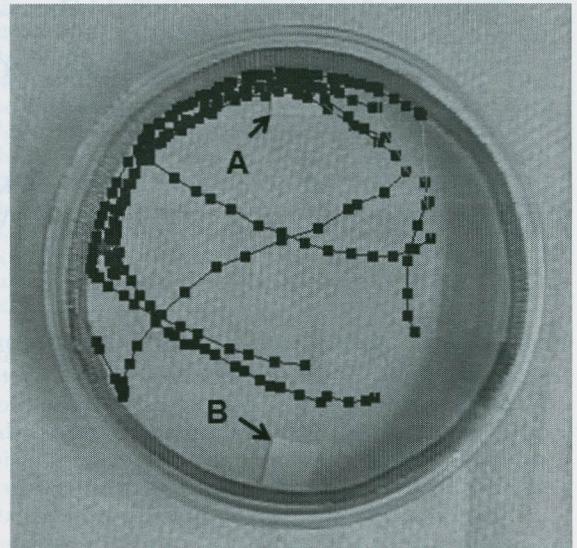

Fig. 1. Representative track of the movement of a test *Pseudotheraptus wayi* in a petri dish arena with filter papers A, containing a preferred odor, and B, serving as a blank control; black squares represent positions of the insect during the trial.

Behavioral responses of individual virgin *P. wayi* females and males aged 1 mo after molting into adults were tracked by video recording by using EthoVision XT version 8.0 video-tracking system (Grieco et al. 2010). The movements of individual insects released at the center of the petri dish arena were video-recorded for 10 min. Positions of each test insect were monitored every 0.5 s (Fig. 1), and the mean distances of the insect to either odor sources were generated by using the EthoVision computer software.

Analysis of Volatiles. Coupled gas chromatography-electroantennographic detection (GC-EAD) analysis was carried out by using a Hewlett–Packard (HP) 5890 Series II gas chromatograph equipped with an HP-1 column (30 m by 0.32-mm-diameter by 0.25 μm, Agilent, Palo Alto, CA) in the splitless mode. Nitrogen was used as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was programmed at 35°C for 5 min and then increased at 10°C/min to 280°C, and held at this temperature for 5 min. The GC injector temperature was 250°C, with flame ionization detector (FID) at 270°C. The column effluent was combined with nitrogen as make-up gas and then split 1:1 to the FID and EAD by using a splitter (SGE splitter, SGE Incorp., Austin, TX). The reference and recording electrodes were silver-coated wires contained in glass capillaries filled with Ringer's solution (NaCl, 7.5 g; KCl, 0.35 g; CaCl$_2$, 0.21 g/liter of H$_2$O). Antennae of both females and males were cut off at the base of the second antennomere and their tips were clipped off to create an open connection with the internal fluid of the antenna (Tol and Visser 2002). The antennal base was inserted into the reference electrode, and the recording electrode was sleeved over the tip of the antenna. The microelectrodes were connected to an

AC/DC amplifier in DC mode (Syntech, Hilversum, The Netherlands). Aliquots of 5 μl of crude volatile extracts and blends of synthetic EAD-active components of cashew leaf volatiles in the natural ratios were analyzed in six replicates by using fresh antenna of each sex of the insect in each run. A GC-EAD program (Syntech GC-EAD 2000, Hilversum, The Netherlands) was used to simultaneously record and analyze EAD and FID signals on a PC.

Coupled gas chromatography-mass spectrometry (GC-MS) was carried out on an HP-7890A GC (Agilent Technologies, Inc., Beijing, China) linked to an HP-5975 mass spectrometer (EI, 70 eV, Agilent Technologies, Inc., Santa Clara, CA) equipped with an HP-5 MS column (30 m by 0.25-mm-diameter by 0.25 μm) (Restek, Bellefonte, PA). The oven temperature was programmed by using conditions described for the GC-EAD analysis earlier. Aliquots of 1 μl of each sample were analyzed and the components were identified by comparison of their mass spectral data with library data (NIST 2005) and confirmed by comparison with retention times and mass spectra of authentic standards.

Chemicals. A synthetic mixture of ocimene isomers (Z)-β-ocimene, (E)-β-ocimene, and allo-ocimene in the ratio 4:10:1 and butyric acid were purchased from Sigma Aldrich (Milwaukee, WI); and (Z)-3-hexen-1-ol (98%) from Sigma Aldrich (Gillingham, England); whereas (Z)-3-hexenyl butyrate (99%) was synthesized in our laboratory by esterification of (Z)-3-hexen-1-ol and butyric acid.

Electrophysiology. Because of the sex differential antennal responses found in GC-EAD recordings (see Results), electroantennograms (EAGs) were recorded from excised antennae of both females and males by using the procedure previously described by Njagi et al. (1996) to quantify antennal responses of either sex to synthetic components of cashew volatiles. Filter paper strips ($\approx$1 by 0.5 cm) were inserted into the wider end (0.5-cm-diameter) of labeled 27-cm-long disposable glass Pasteur pipettes (Fisher, United Kingdom). Dichloromethane solutions of the test chemicals were prepared, and aliquots (10 μl) of the ocimene mixture representing 0.005, 0.05, and 0.5 μg, and of (Z)-3-hexenyl butyrate representing 0.0002, 0.002, and 0.02, were applied individually onto the filter paper strips. The differences in amounts of the two compounds per dose represented the natural ratio of 25:1 of (E)-β-ocimene (the major constituent of the ocimene mixture) and (Z)-3-hexenyl butyrate in crude cashew volatiles. The solvent was allowed to evaporate for $\approx$2 min before the pipettes were sealed off at both ends with Parafilm before use. A pulse of air was pumped through the Pasteur pipette into an airstream at 4 ml/s to deliver each of the two test compounds at random into the antennal preparation. A control stimulus (filter paper loaded with 10 μl of dichloromethane only) was applied at the start and end of the two compounds, and each preparation was used only once. Each dose was tested with only one antenna per insect from six females and six males. The stimuli were delivered at 1- to 2-min intervals to allow

for stabilization of the signal. Maximum amplitude (millivolts) depolarization elicited by each stimulus was measured. Net EAG responses to each stimulus were determined by subtracting the mean responses to the solvent controls at the start and end of each dose from the response to each compound (Chinta et al. 1994, Shepherd et al. 2005). Negative net EAG responses, as observed in some instances, were treated as zero responses for statistical analysis (Chinta et al. 1994).

Responses to EAD-Active Synthetic Cashew Volatiles. Dual-choice bioassays were carried out by using the petri dish arena-EthoVision video-tracking assay described earlier for responses to crude cashew odor. Solutions containing 0.025, 0.05, and 0.1 μg/μl of the blend of the ocimene mixture and (Z)-3-hexenyl butyrate in the ratio 25:1 in dichloromethane, representing the natural ratio of (E)-β-ocimene and (Z)-3-hexenyl butyrate in crude cashew volatiles, were prepared. Solutions of the ocimene mixture alone at 0.048 μg/μl and (Z)-3-hexenyl butyrate alone at 0.002 μg/μl, representing the same amounts in 0.05 μg/μl of the blend of the two standards, were also prepared. Aliquots of 10 μl of these solutions representing 0.25, 0.5, and 1 μg of the blend of ocimene mixture and (Z)-3-hexenyl butyrate; 0.48 μg of ocimene mixture alone; and 0.02 μg of (Z)-3-hexenyl butyrate alone were tested. The ocimene mixture alone at 0.48 μg and (Z)-3-hexenyl butyrate alone at 0.02 μg were tested with only males because no significant female responses to the blend of the chemicals were detected.

Data Analysis. Mean distance of insect positions from either odor points in the petri dish arena and EAG responses of either sex to antennally active chemicals were compared by using the two-sample t-test in R-statistical software version 2.15.0 (R Development Core Team 2012).

Results

Responses to Crude Cashew Odor. In this assay, only males recorded detectable responses to the odors. The males responded positively in a dose-dependent manner to crude cashew volatile extracts, orienting significantly closer to 0.2 and 0.4 CHE than to the controls ($t = 6.57$; df $= 18$; $P < 0.001$ and $t = 2.3$; df $= 18$; $P = 0.034$, respectively) (Fig. 2).

Analysis of Volatiles. Four components in the crude cashew volatiles consistently elicited antennal responses in males, whereas the responses of female antennae to these components were rather weak and sometimes not discernible in the background signal (Fig. 3). These chemicals were identified by GC-MS as (Z)-β-ocimene, (E)-β-ocimene, allo-ocimene, and (Z)-3-hexenyl butyrate (ratio 1:25:3:1), and confirmed with authentic standards.

Electrophysiology. In these assays, the detection in antennae of males was significantly stronger to 0.5 μg of the synthetic chemical mixture of ocimene isomers (Z)-β-ocimene, (E)-β-ocimene, and allo-ocimene than the detection in female antennae ($t = 3.35$; df $= 10$; $P = 0.007$), whereas antennal detection of either
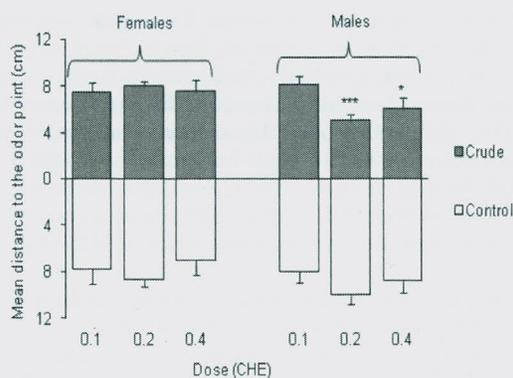
Fig. 2. Behavioral responses of *P. wayi* to crude cashew volatile extracts in the petri dish arena; one and three asterisks indicate significant differences of the treatments from the controls at $P < 0.05$ and $P < 0.001$, respectively; error bars represent SEMs.

sex to 0.005 and 0.05 μg of this mixture was not significantly different (Fig. 4). Similarly, the detection in male antennae was significantly stronger to 0.02 μg of (Z)-3-hexenyl butyrate than female antennae ($t = 2.36$; df $= 8$; $P = 0.046$), whereas the detection in either sex to 0.0002 and 0.002 μg of the ester was not significantly different.

Responses to Synthetic Cashew Volatiles. As with crude cashew odor, detectable responses to the synthetic chemicals were found in only males. The males responded in a dose-dependent manner, orienting significantly closer to 0.5 and 1 μg of the blend of the ocimene mixture and (Z)-3-hexenyl butyrate than to the controls ($t = 4.08$; df $= 18$; $P = 0.001$ and $t = 3.37$; df $= 18$; $P = 0.003$, respectively) (Fig. 5). The males also oriented significantly closer to 0.48 μg of the ocimene mixture alone, corresponding to the same amount as in the blend at 0.5 μg, than to the control ($t = 5.22$; df $= 18$; $P < 0.001$), whereas (Z)-3-hexenyl

butyrate alone at 0.02 μg elicited no detectable behavioral responses in males.

Discussion

Many insect pests use plant volatiles for host location (Szendrei and Rodriguez-Saona 2010). In some cases, both sexes are attracted to the host volatiles (Pajares et al. 2004, Quiroz et al. 2005), whereas in others, it is the female that is attracted to the host volatiles, mainly to locate oviposition sites (Burguiere et al. 2001, Macel and Vrieling 2003, Hern and Dorn 2004). Males may also use host volatiles as cues to locate females for mating (Landolt and Phillips 1997) or search for hosts and then attract females by using male-produced sex pheromones (Visser 1986, Aldrich et al. 1997). Our results show that crude cashew leaf volatiles contain a chemical signature that attracts only male *P. wayi*. This pattern of sex differential sensitivity to host odors has been reported for related insect species and their host plants. For example, in the mirid, *Trigonotylus caelestialium* (Kirkaldy), males are more attracted to whole rice plant odor than females, although females are more attracted to the plant odor at panicle formation and flowering stages than males (Niiyama et al. 2007, Fujii et al. 2010), probably because of the demand for grains by females for oviposition (Ishimoto and Sato 2006). Our results also concur with reports on bark beetle species, where males primarily select the host tree and females follow, lured by the interplay of pheromones and host odors (Visser 1986). In contrast, in another mirid, *Lygus hesperus* Knight, only females are attracted to alfalfa volatiles, presumably to locate oviposition sites (Blackmer et al. 2004). Furthermore, for *Lygus rugulipennis* Poppius, both sexes are attracted to odors from *Vicia faba* L. and that these responses are dependent on the presence of conspecifics and whether the plant is damaged (Frati et al. 2008, 2009). It is,
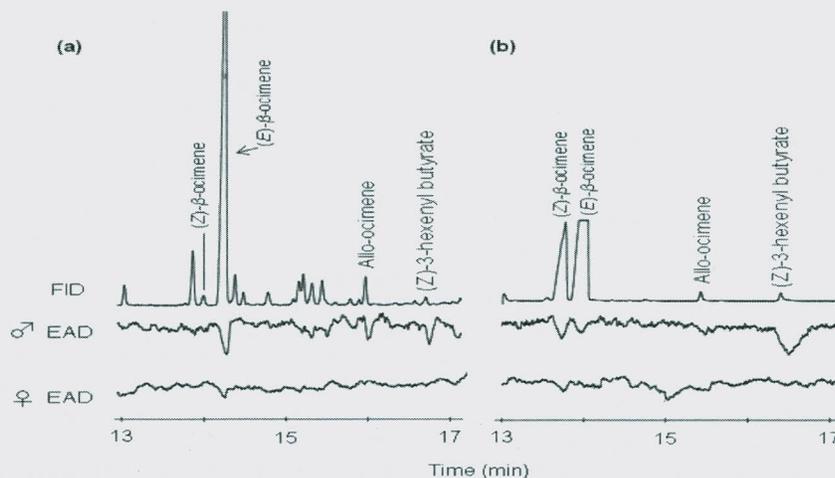

Fig. 3. Representative GC-EAD responses of *P. wayi* to (a) crude cashew volatiles and (b) a blend of EAD-active synthetic cashew chemicals; the weak EAD responses to peaks between 15 and 16 min that were also present in the GC-MS profile of the blank control were not considered and were unidentified.
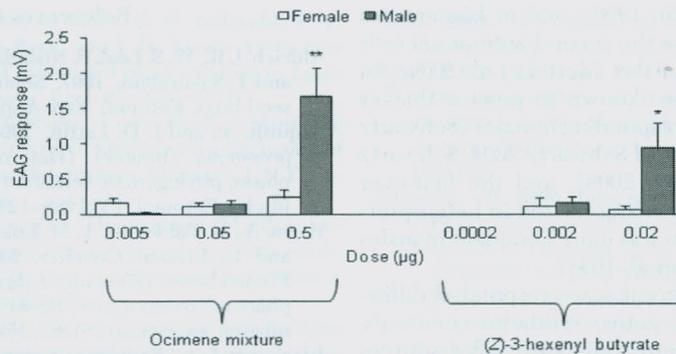
Fig. 4. EAG responses of *P. wayi* to the ocimene mixture and (Z)-3-hexenyl butyrate; one and two asterisks indicate significant differences between females and males at $P < 0.05$ and $P < 0.01$, respectively; error bars represent SEMs.

however, well known that insect age and plant developmental stage or part determine the interaction between an insect herbivore and its host plant (Niiyama et al. 2007; Fujii et al. 2010). In this study, we tested the responses of *P. wayi* aged 1 mo after molting into adults to odors of flushing cashew leaves, which may represent one of the many natural interactive stages between the herbivore and its host. Hence, further research is needed to fully elucidate the chemical interaction between the insect and the different plant stages.

The terpenoids (Z)-β-ocimene, (E)-β-ocimene, and allo-ocimene, and the ester (Z)-3-hexenyl butyrate, in odor of cashew volatiles that elicited electrophysiological antennal detection in *P. wayi* are known components of volatiles of most alternative hosts of the insect, for example, guava (Da Silva et al. 2003, Ogunwande et al. 2003, Rouseff et al. 2008), cocoa (Quijano and Pino 2009), mango (Ollé et al. 1998, Jayanthi et al. 2012), pecan (Corella-Madueño et al. 2011), carambola (Mahattanatawee et al. 2005), and French beans (Barra et al. 2007). This commonality in host volatiles may account for the polyphagous nature of the insect. The use of commonly occurring plant

volatiles to recognize a large range of plant species may facilitate learning behavior and preference for whichever host is more abundant in the habitat (Webster et al. 2008). However, it would be interesting to study whether *P. wayi* shows any preference for volatiles of a particular host. Interestingly, in coupled GC-EAD and EAG analyses, we found that male antennae detected cashew leaf volatile components more strongly than those of females. Similar observations have been reported in the related family, Miridae, for example, *Lygus lineolaris* (Palisot de Beauvois) (Chinta et al. 1994), *Lygocoris pabulinus* (L.) (Groot et al. 1999), and *Lygus hesperus* Knight (Williams et al. 2010). This suggests that some male phytophagous heteropterans may possess more plant odor receptors than females, a hypothesis that may be supported by previous findings that the distal most hairy antennomere of adult male *P. wayi* is ≈1.5 times longer than that found in females (Egonyu et al. 2013b). Although sexual dimorphism is not apparent in antennae of some heteropteran species (Henry 1989, 2006; Stehlík and Jindra 2008), it is evident in others such as *Nabicula (Limnonabis) propinqua* (Reuter), where the male has disproportionately longer antennae than the fe-
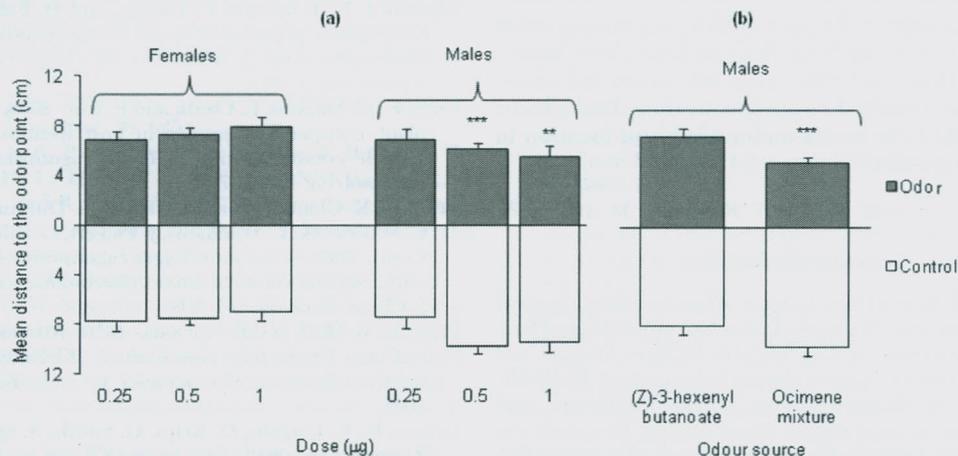

Fig. 5. Behavioral responses of *P. wayi* to (a) a blend of ocimene mixture and (Z)-3-hexenyl butyrate and (b) 0.02 μg of (Z)-3-hexenyl butyrate and 0.48 μg of the ocimene mixture in the petri dish arena; two and three asterisks indicate significant differences of the treatments from the controls at $P < 0.01$ and $P < 0.001$, respectively; error bars represent SEMs.

male (Asquith and Lattin 1990), and in *Limnometra tiomanensis* Mayr, where the second antennomere is longer in males than females (Zettel et al. 2009). In addition, male mirids are known to possess thicker antennae than their corresponding females (Schwartz and Schuh 1999, Schuh and Schwartz 2004, Schwartz 2005, Soto and Weirauch 2009), and the first-ever odorant binding protein characterized in heteropterans from the genus *Lygus* was more abundant in males than females (Dickens et al. 1998).

In bioassays, the different sexes responded differently to the antennally active synthetic chemicals, with males spending more time near odor sources containing the blend of the ocimene mixture and (Z)-3-hexenyl butyrate than females. These results concur with those we found by using crude leaf volatile extracts. Differential responses to host plant volatiles occur in several phytophagous insects for various reasons. For instance, whereas female moths are highly attracted to flowers for oviposition and nectar foraging (Burguiere et al. 2001, Macel and Vrieling 2003, Hern and Dorn 2004), male fruit flies are attracted to and feed on the floral substance methyl eugenol for successful courtship and mating (Landolt and Phillips 1997). The fact that male *P. wayi* were attracted to the ocimene mixture alone than the control, but not to the ester in the natural ratio in cashew leaf odor, suggests that host plant monoterpenes rather than esters contribute more strongly to the kairomonal response of *P. wayi* males. Lack of detectable behavioral response of male *P. wayi* to (Z)-3-hexenyl butyrate, yet their antennae respond strongly to the ester, is however intriguing. Further research to elucidate the role of this ester in the behavior of *P. wayi* would be useful.

In summary, our results suggest that cashew leaf volatiles and specific monoterpenes in the odor attract male *P. wayi*, but not females. These results complement previous research showing differential responses of different sexes of insects to host volatiles. Furthermore, our results suggest that cashew volatiles serve as kairomones for host location by *P. wayi* males and offer prospects for monitoring and management of this pest by using lures derived from these monoterpenes. However, more research is needed to establish how female *P. wayi* locate their host plants, which could help better understand host location in this insect species.

Acknowledgments

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