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Stereoselective Chemical Defense in the *Drosophila* Parasitoid *Leptopilina heterotoma* is Mediated by (−)-Iridomyrmecin and (+)-Isoiridomyrmecin

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Abstract Chemical defense mechanisms are widespread among insects but have rarely been demonstrated in parasitoid wasps. Here, we show that the *Drosophila* parasitoid *Leptopilina heterotoma* (Hymenoptera, Figitidae) produces (−)-iridomyrmecin and (+)-isoiridomyrmecin in a cephalic gland, and that these chemicals have a highly repellent effect on ants. Stereoselective synthesis of 4 stereoisomers of iridomyrmecin allowed us to demonstrate that the repellent effect of iridomyrmecins depends on the stereochemistry. Potential food items impregnated with natural doses of (−)-iridomyrmecin were avoided by ants much longer than those impregnated with (+)-iridomyrmecin, (+)-isoiridomyrmecin, or (−)-isoiridomyrmecin, respectively. Quantitative headspace analyses revealed furthermore that females and males of *L. heterotoma* released iridomyrmecins in higher amounts when confronted with ants. This is the first time, that (−)-iridomyrmecin and (+)-isoiridomyrmecin are reported as natural products. Females synthesize more iridomyrmecins than males, and the most active (−)-iridomyrmecin is produced by females only. We, therefore, hypothesize that this defense mechanism is used mainly by female wasps when foraging for *Drosophila* larvae on rotten fruits, but also may protect male wasps during dispersal.

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Introduction

Chemical warfare is widespread among insects, especially for defense against predators. Not all insect orders, however, use chemical defense to the same degree. Coleoptera, for instance, have evolved a plethora of defense chemicals that are produced in various exocrine glands (Pasteels et al., 1988; Francke and Dettner, 2005), whereas only few examples exist of the use of defensive compounds in Ephemeroptera or Diptera (Pasteels et al., 1983). Among the Hymenoptera, chemical defense is common in social species such as ants, which use irritant chemicals like formic acid, often in combination with alarm pheromones, to defend their colonies against natural enemies. In contrast, little is known about the chemical defense mechanisms of solitary Hymenoptera. Out of the thousands of parasitoid Hymenoptera only a few have been investigated for chemical defense mechanisms:

Females of *Goniozus nephantidis* (Bethylidae), for example, release a spiroacetal from mandibular glands during aggressive interaction with competing females (Goubault et al., 2008). Other bethylid wasps, e.g., *Cephalonomia gallicola*, produce skatole in their head and probably use it as a defensive allomone (Kuwahara, 1984; Goubault et al., 2008). Another example is the braconid wasp *Leiophron uniformis*. Both sexes produce (S)-4-methyl-3-heptanone in their head. A defensive function of the compounds has been suggested, but has not been experimentally tested (Byers and Levi-Zada, 2011).

The best investigated case of chemical defense in a parasitic wasp is the genus *Alloxysta* (Hymenoptera, Charipidae). These wasps are obligate hyperparasitoids of aphid parasitoids and use a mandibular secretion that repels ants while foraging

in ant-attended aphid colonies (Völkl et al., 1994). This defense mechanism ensures survival of the females and allows them to forage without ant interference for a period of time, hence increasing their oviposition rate and reproductive success. The secretion contains a mixture of 6-methyl-5-heptene-2-one, actinidine, and a few other iridoid compounds, two of which have been identified as *trans*-fused isomers of iridomyrmecin (Fig. 1e, f, Völkl et al., 1994; Petersen, 2000). However, these compounds also are produced by *Alloxysta* species foraging in ant-free aphid colonies (Hübner et al., 2002) and, in this context, have been shown to deter jumping spiders (Hübner and Dettner, 2000). Moreover, aphid colonies are not the only environment where parasitoids encounter ants. The omnipresence of ants in most ecosystems makes them, along with spiders, the top predators of parasitoid wasps (Völkl, 1992; Heimpel et al., 1997). This suggests that any parasitoid might benefit from repellents against ants, spiders, and other predators by increasing the parasitoid's overall lifetime as well as its patch allocation time and consequently its oviposition rate.

Leptopilina heterotoma THOMSON 1862 (Hymenoptera, Figitidae) is a solitary larval parasitoid of some fruit breeding *Drosophila* species, including *D. melanogaster* (Jenni, 1951; Hedlund et al., 1996) and is widespread in the Holarctic region (Nordlander, 1980). Thus, *Leptopilina* females search for host larvae on overripe, rotting fruits, a resource also exploited by ants. Thus, *L. heterotoma* wasps also may benefit from an ant-repelling defense mechanism. In the present study, we investigated whether *L. heterotoma* is chemically defended against natural enemies and asked the following questions: 1) Which compounds are produced by males and females of *L. heterotoma*? 2) Where are these compounds produced? 3) Do the compounds repel ants? 4) Are the compounds released on encounter with ants?

Methods and Materials

Insects We used *D. melanogaster* as host species to rear *L. heterotoma*. *Drosophila melanogaster* was reared on a standard corn-based diet (504 ml water, 66 g sugar, 6 g baker's yeast, 2.3 g agar, 52 g cornmeal, 1.3 ml propanoic acid, 0.8 g nipagin) and kept at 25 °C, ~75 % humidity, and a 16:8 hL:D cycle. For a new rearing, about 30 flies (mixed sexes) were placed into a jar containing fresh fly food for 48 h. The flies were removed and about 10 mated *L. heterotoma* females were put into the jar. Parasitized pupae were removed from the rearing jars a few days before emergence and put singly into 1.5 ml microcentrifuge tubes. All experiments were performed with 1–3 d-old wasps.

A queen-right nest of *Myrmica rubra* with several hundred workers was collected together with nesting material in the vicinity of Regensburg, Germany, kept at RT in a plastic box (40×70 cm, 30 cm high), and fed with dead insects and diluted honey.

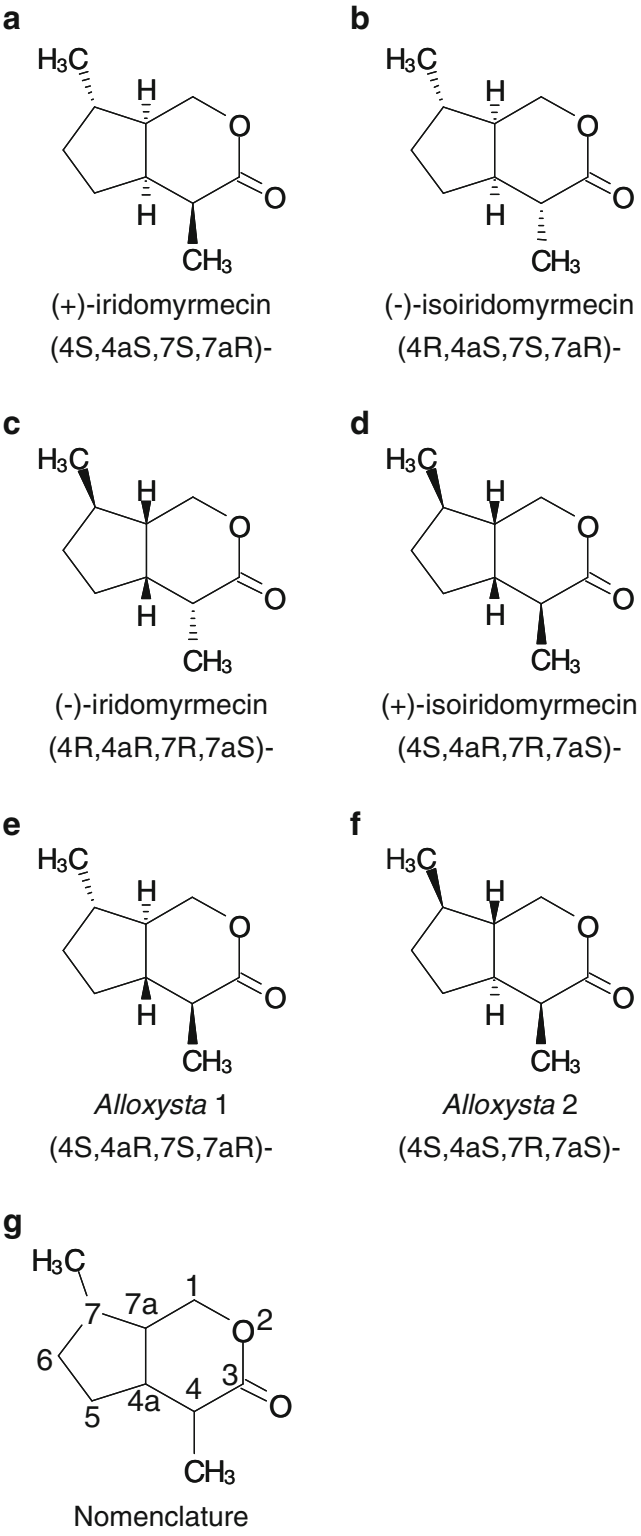

Fig. 1 **a-f** Molecular structure of the six stereoisomers of iridomyrmecin found in nature. **g** Numbering of carbon atoms in the molecule of iridomyrmecin. Numbers 4, 4a, 7, and 7a represent the chiral centers

Chemical Analysis Males and females of *L. heterotoma* were extracted for 10 min with 10 μl dichloromethane (DCM) per wasp. Extracts were fractioned on 50 mg cyanopropyl bonded silica gel columns (DSC-CN, Sigma-Aldrich, Taufkirchen, Germany). For this purpose, the DCM was evaporated completely under a gentle stream of nitrogen, and the sample was redissolved in hexane. Columns were pre-conditioned by rinsing them with 2 ml each of DCM and hexane; subsequently, the sample was applied to the column and eluted successively with 0.3 ml of hexane and 0.3 ml of DCM. The concentration of the fractions was determined by GC and re-adjusted to the concentration of the original extract. These extracts and fractions were used to identify the compounds and for behavioral experiments.

To quantify the iridoids in the wasps, single males and females were extracted in 30 μl DCM containing 5 ng μl^{-1} methyl decanoate as internal standard. Absolute amounts were calculated via a calibration curve obtained by analyzing known amounts of synthetic iridomyrmecin (1–60 ng μl^{-1}) in the presence of the internal standard. We analyzed 25 individuals of each sex.

For a location of the iridomyrmecin producing gland(s), we extracted head, thorax, and abdomen of females and males separately using the same method (5 replicates for each sex).

Samples were analyzed on a Shimadzu GC 2010 gas-chromatograph coupled to a QP2010 plus mass-spectrometer (Shimadzu, Duisburg, Germany). The GC was equipped with a non-polar capillary column (BPX-5, 30 m length, 0.32 mm i.d., 0.25 μm film thickness, SGE Analytical Science, Milton Keynes, UK) and helium was used as carrier gas (50 cm s^{-1} linear velocity). The oven program started at 80 °C and was raised at a rate of 5 °C min^{-1} to 280 °C. All samples were injected splitless. The mass-spectrometer was run in electron impact (EI) mode (70 eV) and set to a scan range from 35 to 600 mz^{-1}.

Additional analyses using a polar (RH-Wax, 30 m length, 0.32 mm i.d., 0.25 μm film thickness, CZT, Kriftel, Germany) GC column were performed on a Fisons 8000 series GC coupled to a Fisons MD800 mass spectrometer (EI mode at 70 eV). Helium was used as carrier gas with a constant inlet pressure of 15 kPa. The oven program started at 80 °C held for 4 min, then raised by 3 °C min^{-1} to 230 °C. GC runs using a cyclodextrin column (Beta-Dex 225, 30 m length, 0.25 mm i.d., 0.25 μm film thickness, Sigma-Aldrich, Taufkirchen, Germany) were performed on a Fisons 8000 series GC equipped with a flame ionization detector (FID). Helium was used as carrier gas with a constant inlet pressure of 120 kPa. The oven program started at 80 °C and was raised by 3 °C min^{-1} to 220 °C.

Compounds were identified by comparing mass spectra and linear retention indices (estimated according to van Den Dool and Kratz, 1963) with those of authentic reference compounds (see below).

Identification of (−)-iridomyrmecin in female extracts was confirmed by epimerizing the female extracts using sodium methoxide. This treatment is known to convert iridomyrmecin to the more stable isoiridomyrmecin by inverting the configuration at carbon 4 (Fig. 1) (Cavill and Locksley, 1957). Given that the identification of (−)-iridomyrmecin in the female extracts was correct, we expected a clear increase of (+)-isoiridomyrmecin after this treatment. Epimerization was accomplished by mixing the polar fractions of female extracts with 200 μl of a 0.1 M solution of sodium methoxide in methanol and heating the resulting mixture at 50 °C for 1 h. Reaction products were extracted with pentane (400 μl) and analyzed by GC-MS. For control, we also epimerized synthetic samples of (−)- and (+)-iridomyrmecin, and we confirmed that the anticipated epimerization actually occurs under our experimental conditions.

Chemical Synthesis Synthetic standards of enantiomerically pure iridomyrmecin and isoiridomyrmecin were prepared in a diasteroselective manner from optically pure citronellal (Fig. 2). Citronellal was first converted to nepetalactol as previously reported (Beckett et al., 2010). Briefly, regioselective oxidation of citronellal (Sigma-Aldrich, St. Louis, MO, USA) with catalytic amounts of selenium dioxide and stoichiometric *tert*-butlyperoxide was followed by 2-iodobenzoic acid (IBX) oxidation of the resulting aldol to produce 2,6-dimethyloct-2-enedial. On exposure to *N*-methylaniline, this enedial underwent intramolecular cycloaddition. The resulting cycloadduct produced nepetalactol (C1 α/β, 1:10) upon hydrolysis with toluenesulfonic acid in wet tetrahydrofuran (THF).

Conversion of nepetalactol into iridomyrmecin and isoiridomyrmecin was accomplished in three synthetic operations: ionic hydrogenation, hydroxymercuration/demercuration, and silver(I) oxidation. First, the carbon-1 position of nepetalactol was reduced by ionic hydrogenation. Nepetalactol (1 eq) and triethylsilane (10 eq) were dissolved in anhydrous dichloromethane (DCM) and cooled to −35 °C under nitrogen. Boron trifluoride ethereate (1.2 eq) was added dropwise, and the resulting reaction mixture was stirred at −35 °C for 30 min. The reaction was quenched with water, and the resulting biphasic mixture was extracted with ether. The combined organic layers were dried over $MgSO_4$ (anhydrous), filtered, and the solvent was removed by careful rotary evaporation. The resulting residue was purified by silica gel column chromatography (0.5 % ether in pentane) to give intermediate 1 as a colorless mobile oil (50 % yield).

Hydroxymercuration/demercuration of intermediate 1 under standard conditions (Bettelli et al., 1998) resulted in the formation of all four possible diastereomers of the desired acetal (intermediates 2). Fortuitously, this mixture of diastereomers could be partially resolved using standard

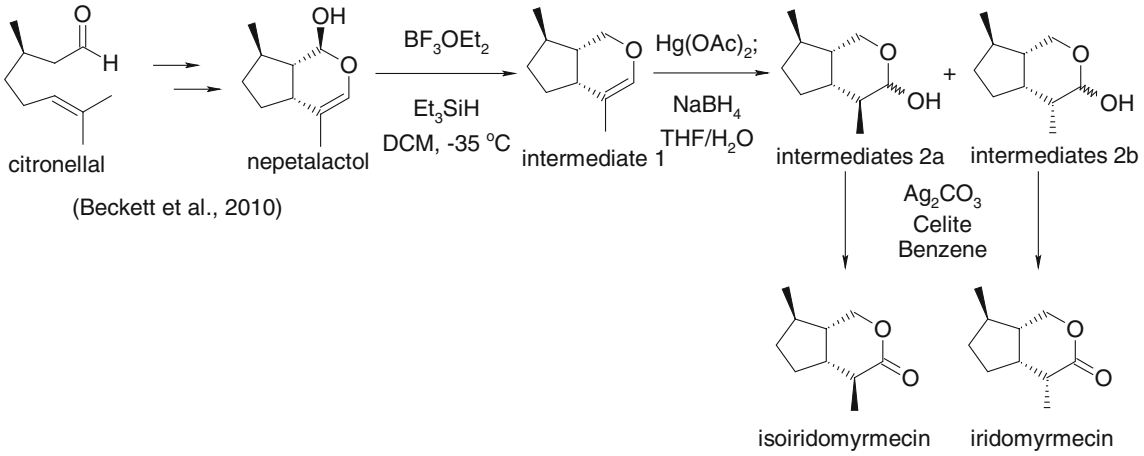

Fig. 2 Diastereoselective synthesis of iridomyrmecin and isoiridomyrmecin from citronellal

silica gel chromatography (DCM as mobile phase) to give two pairs of two diastereomers (Fig. 2), intermediates 2a (30 % yield) and 2b (40 % yield). Separate treatment of 2a and 2b with silver(I) carbonate (1.2 eq) and Celite in refluxing benzene for 30 min generated isoiridomyrmecin (82 % yield from 2a) and iridomyrmecin (92 % yield from 2b), respectively.

This protocol was employed with both antipods of citronellal as starting material to generate enatiomerically pure samples of iridomyrmecin and isoiridomyrmecin. These samples were found to have spectral characteristics entirely consistent with those reported for the natural products (Schöllhorn and Mulzer, 2006; Beckett et al., 2010).

Behavioral Bioassays To test the repellent properties of iridomyrmecins on ants, we put two food items (dead larvae of the green bottle fly, *Lucilia caesar*) at a distance of 20 cm from the entrance of the ants' nests. One food item was impregnated before the test with (a) 10 μl wasp extract (representing one male or female equivalent) or (b) 10 μl of a solution containing one of the four synthetic iridomyrmecins dissolved in DCM at 30 ng μl^{-1}. The dose of synthetic iridomyrmecins was adapted to the amounts of 300 ng found in female wasps. The other food item in each test was impregnated with 10 μl of pure DCM. We observed for 5 min which food item was investigated and touched first by the ants (first choice) and measured the time until each food item was investigated. Each experiment was repeated 20 times. We found iridomyrmecins only in the extracts of heads, not in the extracts of the thorax or the abdomen (see below). Therefore, we allowed the ants to choose in another experiment between two dead (killed by freezing) females or males of *L. heterotoma*, one decapitated, the other one intact. Each experiment was repeated 30 times.

Headspace Analysis To compare the amounts of iridomyrmecins released by *Leptopilina* wasps spontaneously and on encounter with ants, we put ten 2-d-old males or females with or without two workers of *Myrmica rubra* in a 100 ml Erlenmeyer flask equipped with a gas-washing bottle insert. Air was pumped through the flask at a rate of 60 ml min^{-1}. Incoming air was cleaned by an activated charcoal filter; the effluent air stream passed a thermal desorption filter filled with a combined Tenax-TA/Carboxen adsorbent material (Sigma-Aldrich, Taufkirchen, Germany). To check whether the ants produce iridomyrmecin themselves, we also sampled two workers of *M. rubra* alone. For quantification, 5 ng of methyl decanoate (dissolved in methanol) were applied to the adsorbent, and the solvent was removed before volatile sampling by purging the filter for 5 min in a stream of nitrogen at a flow rate of 60 ml min^{-1}. Filters were thermally desorbed (8 min at 250 °C) using a Shimadzu TD20 automated thermal desorption system connected to the Shimadzu GC-MS system, described above, and analyzed with the same GC and MS settings. A calibration curve was created by analyzing known amounts (1–100 ng) of synthetic (−)-iridomyrmecin that were applied to the filter together with the internal standard. Each experiment was repeated 13–15 times.

Statistical Analyses First choices of ants were tested with a one-constraint *chi-square* test against an expected equal distribution. Decision ratios between test groups were compared with a *chi-square* test with sequential Bonferroni correction for ties (Holm, 1979). The time until the first contact with sample and control food items was tested with a *Wilcoxon test*. The difference in time between sample and control was compared between test groups using a *Kruskal-Wallis* non-parametric ANOVA, followed by pairwise *Mann–Whitney U-tests* with sequential Bonferroni correction for ties.

The amount of iridomyrmecin found in the headspace samples were compared between test groups using a *Kruskal-Wallis* non-parametric ANOVA, followed by pairwise

Mann–Whitney U-tests with sequential Bonferroni correction for ties.

All tests were performed in PAST 2.10 (Hammer et al., 2001).

Results

Identification of Iridoids GC-MS analysis and a preliminary comparison of the mass spectra with a library (NIST 8.0) revealed the presence of iridoid compounds and cuticular hydrocarbons in the solvent extracts of *L. heterotoma* (Fig. 3). In the samples of females, we found four iridoid compounds (Fig. 3a), two of which (no. 1 and 3) also were present in the extracts of males (Fig. 3b). Peaks 2 and 3 had identical mass-spectra (Fig. S4) to synthetic reference samples of (−)-iridomyrmecin and (+)-isoiridomyrmecin, respectively, and co-eluted with these compounds on the conventional polar and non-polar GC columns (Fig. S1, S2; Table S1) as well as on the cyclodextrin Beta-Dex 225 stationary phase (Fig. S3). The enantiomers of compounds 2 and 3, i.e., (+)-iridomyrmecin and (−)-isoiridomyrmecin, respectively, were clearly separated by enantioselective GC (Fig. S3) and thus, we were able to rule out the possibility of their presence in the samples. As predicted, treatment of a female extract with sodium methoxide clearly reduced peak 2 and increased peak 3 (Fig. S3c) confirming the identities of these two compounds to be (−)-iridomyrmecin and (+)-isoiridomyrmecin because of the well-known epimerization that occurs under basic conditions (Cavill and Locksley, 1957). Likewise, epimerization of synthetic (−)- and (+)-iridomyrmecin resulted in the formation of the corresponding epimers (+)- and (−)-isoiridomyrmecin, respectively (data not shown). The identities of the two minor iridoids (peaks 1 and 4) remained unknown. The composition of the cuticular hydrocarbons was not investigated in this study.

Production Site of Iridoids The iridoid compounds were found only in the extracts of the wasps' heads, not in extracts of the thorax or the abdomen (same for males and females) (Fig. S5, data for males not shown).

Quantitative Analysis Our quantitative analyses of wasp extracts revealed that females not only produce more complex iridoid blends but also, much higher quantities of iridoids (Table 1).

Behavioral Bioassay Workers of *M. rubra* avoided food items impregnated with extracts of male and female wasps and preferred the solvent treated food items (Fig. 4a, b). This pattern of behavior was observed for both first choice and time until first contact experiments. When they were given the choice between decapitated and intact individuals of *L. heterotoma*, the ants preferred the headless wasps and avoided the intact individuals (Fig. 4a, b). This preference also was observed for both first choice and time until first contact experiments. However, the observed differences were not significant for male wasps.

The experiment using equal amounts of synthetic iridoids revealed that *M. rubra* workers responded to the chemicals in a stereoselective manner (Fig. 5). The preference for the control items was highly significant in all experiments, except for the first choice when (+)-iridomyrmecin was applied (Fig. 5a). Food items impregnated with (-)-iridomyrmecin

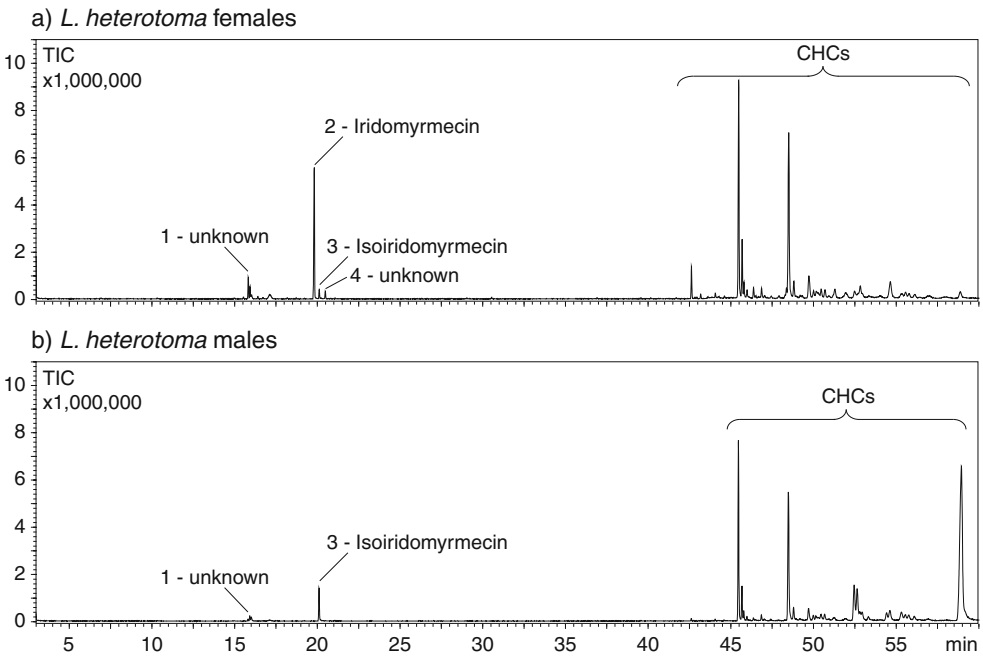

Fig. 3 Total ion current (TIC) chromatograms of dichloromethane extracts of *Leptopilina heterotoma* virgin females (**a**) and males (**b**). Cuticular hydrocarbons (CHCs) have not been identified

Table 1 Mean amounts of iridoid compounds in dichloromethane extracts of females and males of *Leptopilina heterotoma*

No.	Compound	Amount in females ng (mean ± SE)	Amount in males ng (mean ± SE)
1	unidentified iridoid 1	26.1±3.3	trace
2	(−)-iridomyrmecin	236.3±20.6	–
3	(+)-isoiridomyrmecin	22.1±4.8	39.4±3.8
4	unidentified iridoid 2	9.1±0.8	–

were avoided significantly longer than those impregnated with any other stereoisomer (Fig. 5b).

Headspace Analysis Females of *L. heterotoma* released 3.0± 1.2 ng (mean ± SE, collection of 10 females for 2 h) of total iridomyrmecins (sum of (−)-iridomyrmecin and (+)-isoiridomyrmecin) per hour (Fig. 6). If confronted with two workers of *M. rubra,* however, the total release of iridomyrmecins increased significantly (370±70 ng, $U=1$, $P\leq0.001$, Mann–Whitney *U*-test). Males of *L. heterotoma* alone released 5.8± 2.2 ng of (+)-isoiridomyrmecin. Like the females, *L. heterotoma* males significantly increased the emission of isoiridomyrmecins when confronted with the ants (61.8±20.6 ng, $U=30$, $P\leq0.001$, Mann–Whitney *U*-test). Workers of *M. rubra* did not release any iridomyrmecins.

Discussion

The present study clearly demonstrates that *L. heterotoma* wasps release from a cephalic gland increased amounts of (−)-iridomyrmecin and (+)-isoiridomyrmecin upon encounters with natural enemies. Furthermore, extracts from both male and female wasps as well as synthetic iridomyrmecins applied to dead fly larvae prevented ants from contacting these potential food items and transporting them into the nest. Likewise, ants contacted dead parasitoids without heads after a significantly shorter period of time compared to those with heads (the source of the iridoids). We, therefore, conclude that (−)-iridomyrmecin and (+)-isoiridomyrmecin function as defense chemicals in *L. heterotoma* wasps.

Iridomyrmecin (hexahydro-4,7-dimethylcyclopenta[c] pyran-3(1H)-one) has been known since the late 1940s. It was originally identified in the argentine ant *Linepithema humile* (formerly *Iridomyrmex humilis*), which also accounts for its common name (Pavan, 1949). Due to its 4 stereocenters, there are 16 possible stereoisomers of iridomyrmecin, six of which have been found in nature so far (Fig. 1). The argentine ant produces two stereoisomers of iridomyrmecin, namely (+)-iridomyrmecin and (-)-isoiridomyrmecin (Fig. 1a, b) (Cavill and Houghton, 1974; Cavill et al., 1976). In *L. heterotoma*, we found (-)-iridomyrmecin and (+)-isoiridomyrmecin, which are the antipodes of the compounds found in the argentine ant (Fig. 1c, d). The two compounds found in the wasps had identical mass spectra and retention times on non-polar, polar, and chiral GC columns as the synthetic reference compounds. Furthermore, epimerization of female extracts resulted in the predicted decrease of (−)-iridomyrmecin in favor of (+)-isoiridomyrmecin due to the well-known inversion of stereochemistry at carbon atom four that occurs under basic conditions (Fig. 1c, d) (Cavill and Locksley, 1957). This is the first time that (−)-iridomyrmecin and (+)-isoiridomyrmecin have been found as natural products. In contrast to the

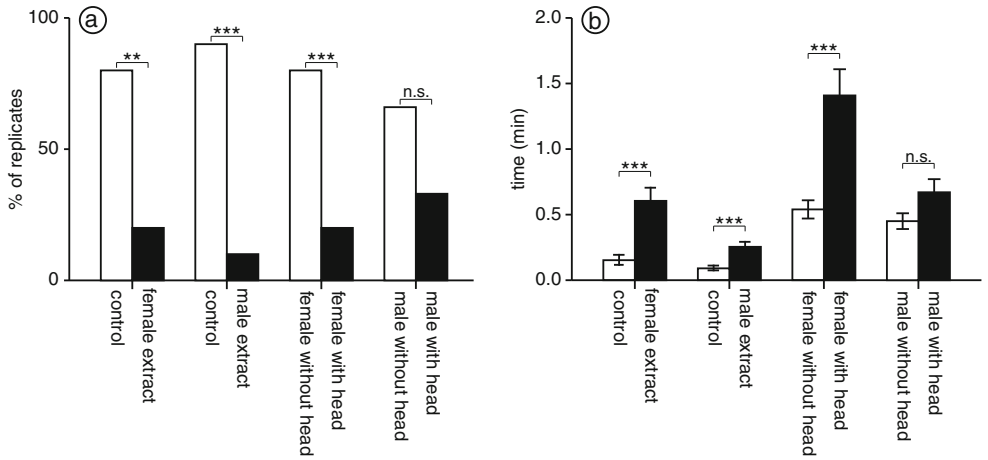

Fig. 4 **a** First choice of *Myrmica rubra* workers (given as percentage of replicates) when choosing between two food items impregnated with an odor and the solvent as control, respectively; or when choosing between a decapitated and an intact individual of *Leptopilina heterotoma. Asterisks* indicated a significant difference between sample and control (one-constraint chi-square test vs. an expected equal distribution, *** $P\leq0.001$, ** $P\leq0.01$). **b** Mean (±SE) time until first investigation by *M. rubra* workers of odor-impregnated and control food items as well as decapitated and intact individuals of *L. heterotoma. Asterisks* indicated a significant difference between sample and control (Wilcoxon test, *** $P\leq0.001$). All statistical values for (**a**) and (**b**) are given in supplementary tables S2 and S3

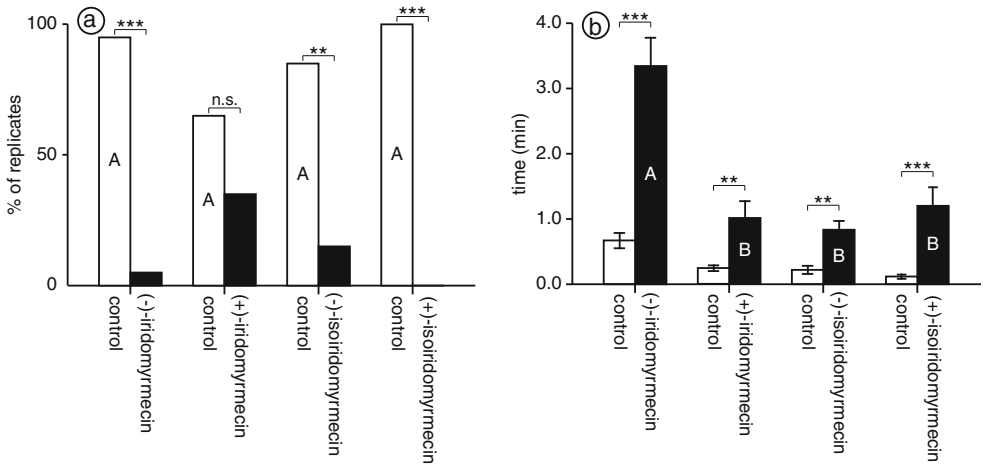

Fig. 5 **a** First choice of *Myrmica rubra* workers (given as percentage of replicates) when choosing between two food items impregnated with enantiopure synthetic iridomyrmecins and the solvent as control, respectively. *Asterisks* indicated a significant difference between sample and control (one-constraint chi-square test vs. an expected equal distribution, *** $P \leq 0.001$, ** $P \leq 0.01$), different *letters* indicate a significant difference between experiments (chi-square test with sequential Bonferroni correction for ties, $P \leq 0.05$). **b** Mean (±SE) time until first investigation by *M. rubra* workers of odor-impregnated and control food items. *Asterisks* indicated a significant difference between sample and control (Wilcoxon test, *** $P \leq 0.001$), different *letters* indicate a significant difference between experiments (Kruskal Wallis test, $H = 19.5$, $P \leq 0.001$, followed by pairwise Mann–Whitney *U*-test with sequential Bonferroni correction for ties, $P \leq 0.01$). All statistical values for (**a**) and (**b**) are given in supplementary tables S2 to S5

four stereoisomers mentioned above, the iridomyrmecins produced by wasps of the genus *Alloxysta* are *trans*-fused stereoisomers (Fig. 1e, f) (Ibarra-Wiltschek, 1995; Hilgraf, 1997). The remaining 10 stereoisomers of iridomyrmecin have, to our knowledge, not been found in nature so far. We found iridomyrmecins only in the extracts of heads of *L. heterotoma* females, but not in extracts of females' thorax and abdomen (Fig. S5). This suggests that the iridomyrmecins are produced in the mandibular gland of *L. heterotoma*, as is the case in *Alloxysta* wasps (Völkl et al., 1994). More detailed morphological and chemical analyses are needed to locate exactly and identify the iridomyrmecin-producing gland(s).

Although iridomyrmecins generally have been supposed to function as a defense compounds in the insects that produce them, the defensive function apparently has never been tested experimentally for *L. humile*. However, iridomyrmecin has a second function in this species: in combination with another iridoid, dolichodial, it signals vitality, thereby controlling necrophoresis of dead ants (Choe et al., 2009). For *Alloxysta*, studies have demonstrated the repellent effect of the mandibular secretion on ants (Völkl et al., 1994) and spiders (Hübner and Dettner, 2000). However, in these studies, only the total secretion was tested. This secretion contained not only iridomyrmecin but also 6-methyl-5-heptene-2-one, actinidine, and other constituents as well. Therefore, the present study is the first to demonstrate a defensive function of isomerically pure iridomyrmecins.

Earlier studies also suggest that iridomyrmecin has insecticidal properties (Pavan, 1952), but it seems to have only a knock-down effect on insects rather than being a real insecticide (Cavill and Clark, 1971). The mode of action of iridomyrmecin as repellent or insecticide and its physiological effects are unknown.

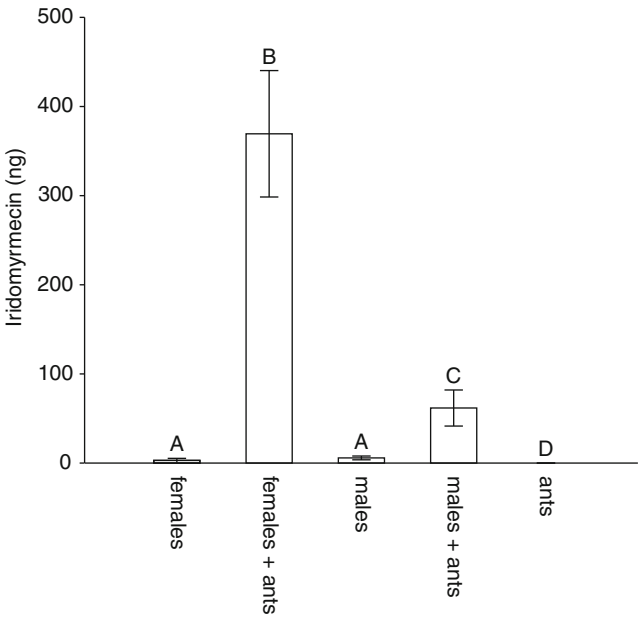

Fig. 6 Mean (±SE) amount of iridomyrmecin in the headspace collections of 10 female and male *Leptopilina heterotoma* without and in the presence of two workers of the ant *Myrmica rubra*. Different *letters* indicate a significant difference between test groups (Kruskal Wallis test, $H = 54$, $P \leq 0.001$, followed by pairwise Bonferroni-corrected *U*-tests, $P \leq 0.01$, $N = 13–15$). All statistical values for (**a**) and (**b**) are given in supplementary table S6

Despite iridomyrmecin being a rather rare natural product, structurally related iridoids such as actinidine, iridodial, and nepetalactone are more widespread and are found in the defensive secretions of a number of insects (e.g., Wheeler et al., 1977; Jefson et al., 1983; Tomalski et al., 1987; Huth and Dettner, 1990) and plants (e.g., catnip *Nepeta cataria*, Schultz et al., 2004). Furthermore, iridoid-glycosides are common secondary plant metabolites, and are as such part of the plants' defense strategy against insect herbivores. Ironically, these compounds are also known to be consumed and sequestered by herbivores to be redeployed as defense compounds (Dobler et al., 2011).

The stereochemistry of natural products may dramatically influence their biological activity. This phenomenon is evident in pheromones (Mori, 2007), hormones (e.g., Kindle et al., 1989; Sakurai et al., 1990), drugs (Reddy and Mehvar, 2004), and odorants (Brenna et al., 2003). Although the defense chemicals of many animals are chiral (Pasteels et al., 1983; Dettner, 2010), examples of stereospecificity in their mode of action are rarely cited in the literature (e.g., Weldon et al., 2006). In the repellent bioassay presented here, all 4 stereoisomers tested showed repellent effects on ants. However, we found a clear and highly significant difference between the stereoisomers in the time ants were deterred from interacting with potential food items. Here, (-)-iridomyrmecin had a stronger deterrent effect compared to the three other stereoisomers, thus demonstrating stereoselectivity in the defensive effect of iridoids in these organisms. Remarkably, females not only possess and release much higher amounts of total iridomyrmecins than males, they also produce exclusively the defense chemical with the highest repellent activity, i.e., (−)-iridomyrmecin. Thus, females not only invest more resources into chemical defense than males in terms of biosynthetic precursors, they also synthesize the less stable but more active diastereomer. This sex specific investment in chemical defense is ecologically plausible. Males do not need to search for host larvae and are, therefore, less vulnerable to predators. So why do males produce iridomyrmecin at all? Predation during dispersion is a major mortality factor for parasitoids (Weisser and Völkl, 1997). Thus, males might also profit from a defensive compounds during their search for females.

Semiochemicals often have more than one function for the releasing organism, a phenomenon referred to in the literature as "semiochemical parsimony" (Blum, 1996). In many insects, for instance, defense chemicals have evolved secondarily to function as sex pheromones (Blum, 1996; Ruther et al., 2001). The clear qualitative and quantitative differences between male and female iridoid profiles suggest that this also is the case in *L. heterotoma* wasps. Current research aims at demonstrating this putative dual function of iridoids in chemical defense and sexual communication of parasitic wasps.

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Host Suitability Affects Odor Association in *Cotesia marginiventris*: Implications in Generalist Parasitoid Host-Finding

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Abstract Insect herbivores often induce plant volatile compounds that can attract natural enemies. *Cotesia marginiventris* (Hymenoptera: Braconidae) is a generalist parasitoid wasp of noctuid caterpillars and is highly attracted to *Spodoptera exigua*-induced plant volatiles. The plasticity of *C. marginiventris* associative learning to volatile blends of various stimuli, such as host presence, also has been shown, but little is known about how this generalist parasitoid distinguishes between host species of varying suitability. *Spodoptera exigua* is an excellent host that yields high parasitoid emergence, while *Trichoplusia ni* serves as a sub-optimal host species due to high pre-imaginal wasp mortality. We have found that *S. exigua* and *T. ni* induce different volatile blends while feeding on cotton. Here, wind tunnel flight assays were used to determine the importance of differentially induced volatiles in host-finding by *C. marginiventris*. We found that, while this generalist parasitoid wasp can distinguish between the two discrete volatile blends when presented concurrently, a positive oviposition experience on the preferred host species (*S. exigua*) is more important than host-specific volatile cues in eliciting flight behavior towards plants damaged by either host species. Furthermore, wasps with oviposition experience on both host species did not exhibit a deterioration in positive flight behavior, suggesting that oviposition in the sub-optimal host species (*T. ni*) does not cause aversive odor association.

Keywords Tri-trophic interactions · Sub-optimal host · Wind tunnel · *Spodoptera exigua* · *Trichoplusia ni* · *Cotesia marginiventris*

Introduction

When damaged by insect feeding, plants release volatile organic compounds that can attract natural enemies of the herbivores (Turlings et al., 1990b). These herbivore-induced plant volatiles (HIPVs) can vary by herbivore and plant species due to differences in insect oral elicitors (Alborn et al., 1997; Schmelz, 2006) and plant physiological responses (Schnee et al., 2006; Schmelz et al., 2009), and have been shown to selectively attract parasitoids of herbivores (Turlings et al., 1995; De Moraes et al., 1998). Parasitoid wasps have the capacity to associatively learn specific odors (Lewis and Tumlinson, 1988; Lewis and Takasu, 1990) or contact cues (Jones et al., 1971) related to host and/or food presence, which may predispose them for more efficient foraging for host and food resources.

Associative learning occurs when an unconditioned stimulus, such as parasitoid oviposition in a host, is combined with a conditioned stimulus, like an odor source, to alter the subsequent behavioral response of an organism (Papaj and Lewis, 1993). The use of sequentially spaced experiences can strongly reinforce this behavior and cause long-term memory formation by synthesizing proteins in the insect

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brain (Smid et al., 2007), and can stimulate searching behavior in the wasp (Bleeker et al., 2006). This behavioral plasticity is useful for parasitic wasps, especially generalists (i.e., wasps that parasitize multiple host species) because the plant and host cues that they learn can vary greatly both spatially and temporally.

Negative associative learning can occur in the absence of reward conditioning or if a low quality reward is provided following the conditioned stimulus. When this occurs, a wasp may learn avoidance behavior in response to an odor source, or switch its odor preference (Takasu and Lewis, 1996). Few studies have explored this form of conditioning, which is probably a widespread occurrence for naturally foraging generalist wasps. Those studies that have examined negative associations have used either hosts unsuitable for oviposition (Takasu and Lewis, 2003; Costa et al., 2010) or a lack of oviposition reward (e.g., no host present) (Papaj et al., 1994). No study thus far has attempted to explain how associative learning occurs during sub-optimal host experiences, which may play a role in shaping realized host range where multiple hosts of varying quality are present in the foraging landscape.

Another area of parasitoid behavior that warrants research is the additive or diminished capacity of associative learning to form memory traces for parasitoids when multiple species are presented in sequence (e.g., a preferred and non-preferred host species). The marginal value theorem predicts that a predator or parasitoid should stay in a patch longer after a positive host encounter (Wajnberg et al., 2008), but no research has determined the effects of the presence of a less desirable host in the patch. The aphid parasitoid *Monoctonus paulensis* (Ashmead) shows a strong preference for the pea aphid [*Acyrthosiphon pisum* (Harris)] over alfalfa aphid [*Macrosiphum creelii* (Davis)] corresponding to increased fitness in the former (Chau and Mackauer, 2001). It can adjust its oviposition rate to maximize parasitization of the preferred host when either host species is presented in a sequential patch (Michaud, 1996). It has yet to be determined if residence time of a parasitoid in a patch that contains both preferred and sub-optimal hosts is different from the length of time spent in a patch with preferred hosts alone. Elucidation of these basic behaviors is necessary to predict how generalist wasps optimally forage when both hosts occur together in nature.

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) is a parasitoid wasp that is considered a "generalist" due to its wide host range (Turlings et al., 1989), which encompasses numerous noctuid species including *Spodoptera exigua* (Hübner) and *Trichoplusia ni* (Hübner), the beet armyworm (BAW) and cabbage looper (CL), respectively (Krombein et al., 1979). The breadth of volatile plant compounds to which *C. marginiventris* is physiologically responsive was established by Gouinguene et al. (2005), and includes as least 38 compounds. The ability of *C. marginiventris* to associate learned volatile cues to specific hosts (Dmoch et al., 1985; Tamo et al., 2006) suggests that there should be flexibility in its host-finding strategy, which has been observed in other parasitoid systems with both aggregated and regularly spaced hosts (Burger, 2006). Here, we used the sub-optimal host CL (Boling and Pitre, 1971), and a preferred host, BAW to evaluate the importance of associative learning related to sub-optimal host experience and its effect on subsequent odor preference by *C. marginiventris*. Specifically, we tested the hypothesis that *C. marginiventris* will associate volatile odors with BAW but not CL.

Methods and Materials

Insects and Plant Rearing BAW and CL were received as eggs from Benzon Research Inc. (Carlisle, PA, USA), and reared on soybean flour-wheat germ diet (Southland Products Inc., Lake Village, AR, USA) at 27±2°C and 40-70 % relative humidity with 16:8 hL:D cycle until late first or early second instar. *Cotesia marginiventris* is a solitary koinobiont endoparasitoid of host larvae. Our data suggest that *C. marginiventris* emergence success from stung caterpillars nears 80 % for BAW hosts, but only 18 % for CL hosts (Harris and Tumlinson, unpublished). A second colony of CL was obtained from Dr. Tom Baker at The Pennsylvania State University (original eggs obtained from K. Haynes, ca. 1995, Kentucky, U.S.A.) to confirm the limited suitability as a host species. Two separate colonies of *C. marginiventris* from different host histories were compared in behavioral bioassays. Colony 1 were reared on late 2nd and early 3rd instar BAW on pinto bean diet (Burton, 1969) and established in 2004 with at least 50 % replacement with field-collected wasps (trapped with sentinel caterpillars) annually. Adults were supplied a 5 % honey/water solution for food and held at 25±4°C and L:D 12:12 h to 14:10 h. Colony 2 originated from individuals collected from fall armyworm [*Spodoptera frugiperda* (J.E. Smith), FAW] hosts on bermudagrass [*Cynodon dactylon* (L.) Persoon], and reared in the laboratory on FAW on corn (*Zea mays* L.). Wasps used in wind tunnel assays were 2–7 d post-pupal emergence.

Cotton (*Gossypium hirsutum* L. var. DPL90) was grown in 10 cm diam plastic pots using Sun Gro® MetroMix 200 Series soil at 27±2°C and 40-70 % relative humidity with a 14:10 hL:D cycle and 200–500 LUX. Autoclaved soil and sterile pots/trays were used to prevent soil bacteria or insect contamination. Plants used for both volatile collection and wind tunnel assays were 28–40 d-old with 5–7 true leaves.

Volatile Collection Plants were grown in an insect-free sealed glasshouse. They were set up at 6:00 p.m. the night

before collections with steel guillotines and cotton surrounding the stem, dividing the pot/soil from leaf tissue. At 5:30 a.m. the following day, twenty second instar BAW or CL were applied to each treatment plant (5 individuals on each of the top four fully expanded leaves, 20 larvae total), and glass bell jars were placed over plants. Volatiles were collected for 3 d from 6:00–10:00 a.m., 10:00 a.m.-2:00 p.m., 2:00–6:00 p.m., and 8:00 p.m.-12:00 a.m. On days 1 and 2 at 5:30 a.m. or 6:15 p.m., caterpillars were removed or added to equalize damage between plant treatments. Plants were base watered with 40 ml at 6:00 p.m. on the first and second day of collections. Following the final volatile collection period, total leaf area for each plant was quantified using UTHSCSA Image Tool® and ranged from 250–500 cm^2. Percent damage was quantified and ranged from 1-5 % of total leaf area.

A push-pull Automated Volatile Collection System (ARS, Inc. Gainesville, FL, USA) was used to collect volatile organic compounds (VOCs) emitted from intact control plants, BAW-damaged, and CL-damaged plants. Charcoal-filtered air was pushed at 1–1.5 l/min into a 3 L bell-glass chamber enclosing a plant, and was vacuumed out through polymeric adsorbent filters (Super-Q®, 30 mg, Alltech Associates) at 1 L/min (Loughrin et al., 1994). Super-Q® filters were subsequently eluted with 100 μl of 1:1 dichloromethane: hexane (J.T. Baker, 95 % purity and Burdick and Jackson High Purity, respectively). For quantification, 25 μl of an internal standard mix (8 ng/μlof nonyl acetate and 5 ng/μl *n*-octane) were added to each sample. Samples were analyzed using a non-polar methyl silicone Equity-1 column (Supelco®) via gas chromatography (GC) with flame ionization detection (FID) and identified using a non-polar HP-1 MS column (Agilent®) on GC-MS (gas chromatography coupled with mass spectrometry) in electron impact (EI) mode. Spectra were compared to known libraries in the National Institute of Standards and Technology (2002 version) for identification, and synthetic compounds were compared for retention times.

Wind Tunnel Bioassays Wind tunnel bioassays were conducted from May-September from 2008–2010. Plants used in behavioral assays were infested with 20–30 1st instar BAW or CL that were allowed to feed freely for 2 d, until they reached the wasp's preferred 2nd instar stage and had similar defoliation amounts as plants used in volatile collections. Two plants (caterpillar-damaged and undamaged control) were placed equidistant from the side walls and from each other, in a 0.61 x 0.61 x 1.83 m acrylic glass wind tunnel, 1 m upwind from a wasp release platform, with a light intensity of 630 LUX and a wind velocity of 0.5 m/s. Damaged plants used in the tunnel contained actively feeding larvae. Air entering the tunnel was passed through charcoal filters, and room and tunnel temperature was maintained at 26-30°C and 50-75 %RH (Steinberg et al., 1992). Plants were used for two wasp subject flights and then replaced with new treatment plants. All flight assays were conducted between 9 a.m. and 4 p.m. Specific details of the parasitoid treatments and assessments are described below.

Parasitoid Oviposition Training Mated 2–7 d-old wasps were given oviposition experiences on three BAW or three CL feeding on cotton in the presence of conspecific-damaged or opposite-species damaged plant tissue, with a 5 min break between each oviposition. Subsequently, they were permitted to fly upwind and to land on a cotton plant damaged by the same or opposite caterpillar species on which they had oviposition experience, and an undamaged cotton plant as a control. To test wasp association of non-plant odors, wasps were given three oviposition experiences on wheatgerm-fed BAW or CL in the presence of host frass and 3 μl diluted vanilla extract (McCormicks®, 1:4 vanilla: distilled water; stored in air-tight flask to prevent evaporation) and permitted to fly upwind to 10 μl diluted vanilla extract on Whatman #1 filter paper or a blank filter paper disc control. One μl of vanilla extract was added to the filter paper every 10 min to compensate for evaporation of the odor stimulus.

Wind tunnel assays were conducted for up to 40 min per wasp, with 25–71 female wasps tested per treatment. A "wall" choice was recorded if the wasp did not land on the odor source or control after three flight platform departures. Positions of odor source and control were alternated between wasps. Each wasp was used only once, and within 30 min of oviposition training.

For sequential experience flight studies, wasps from the fall armyworm-reared colony were experienced as above, and the first flight choice was recorded. Wasps then were removed from the wind tunnel and given an oviposition experience on the opposite host species from which they were initially trained, in the presence of the same odor source they were originally presented with. Within 30 min of the first flight, they were placed back in the tunnel and permitted to fly to damaged plants as before. Because wasps have been shown to remember visual location of hosts (Sheehan et al., 1993), position of damaged plants in tunnel was switched.

Statistical Analyses Plant compounds were analyzed individually using ANOVA for Day 3 2:00–6:00 p.m. (JMP®) when induced volatiles are detectable, and multiple comparisons were analyzed via Tukey HSD. In wind tunnel bioassays, wall-landing (no choice) was affected by treatment type (i.e., host species experience) and was included in statistical analysis. Multinomial Logistic Regression was utilized to analyze flight landing choices (SPSS®), with

significance indicated between the two host species treatments. Significance within treatments was analyzed using likelihood ratio χ^2.

Results

Cotton Volatile Analysis Percent damage calculated for BAW- and CL-damaged plants ranged from 1-5 % and did not vary significantly between treatments (N=9, F=0.020; P=0.887). Cotton volatile induction varied quantitatively for all treatments, and qualitatively for undamaged vs. herbivore-damaged plants, most notably in the absence of sesquiterpenes, indole, and *cis*-jasmone in undamaged control plants (Fig. 1).

Standard Least Squares ANOVA was used to determine treatment effect for BAW, CL, and undamaged (Intact) cotton. Tukey HSD shows BAW and CL (*df* - 2, 29) treatments were significantly different for β-caryophyllene: F - 9.331, P=0.001, (*E,E*)-α-farnesene: F - 6.606, P=0.005, *trans*-bergamotene: F - 5.172, P=0.014, α-humulene: F - 12.846, P<0.001, α-pinene: F - 9.035, P=0.001, β-pinene: F - 8.840, P=0.001, β-myrcene: F - 11.969, P<0.001, limonene: F - 8.468, P=0.002, linalool: F - 9.116, P=0.001, hexanal: F - 6.378, P=0.006, (*E*)-2-hexanal: F - 5.069, P=0.015, (*Z*)-3-hexenol: F - 12.590, P<0.001, (*E*)-2-hexenol: F - 5.586, P=0.011, (*Z*)-2-pentenyl acetate F - 7.468, P=0.003, (*Z*)-3-hexenyl acetate: F - 9.343, P=0.001, and indole: F - 7.536, P=0.003 (Refer to Fig. S1, supplemental material for more detail on how individual volatile compounds varied by type of damage).

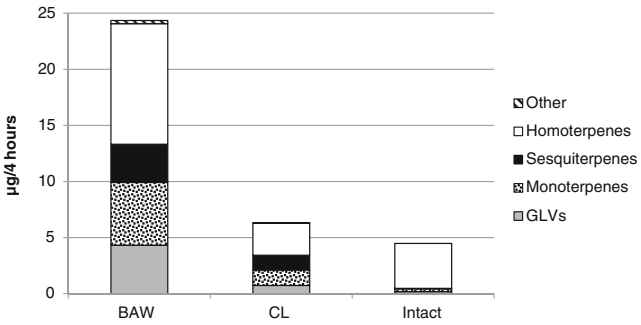


Fig. 1 Ratios of Cotton VOCs Vary Among Damage Treatments. Summed averages for each compound group on Day 2, 2–6 p.m. show different ratios between beet armyworm (BAW)-, cabbage looper (CL)- and undamaged (Intact) cotton plants. Ratios for BAW : CL : Intact are 282:47:0 (Other Compounds), 2:1:1 (Homoterpenes), 23:4:1 (Sesquiterpenes), 23:5:1 (Monoterpenes), and 81:31:1 (GLVs). Standard Least Squares ANOVA: Other Compounds ($F_{2, 29}$ - 9.841; P=0.001), Homoterpenes ($F_{2, 29}$ -10.071; P=0.001), Sesquiterpenes ($F_{2, 29}$ -9.969; P=0.001), Monoterpenes ($F_{2, 29}$ -7.850; P=0.003), GLVs ($F_{2, 29}$ -9.097; P=0.001). Tukey multiple comparison tests show BAW significantly different from CL and Intact for all compound groups, but CL and Intact not different from each other for any compound group

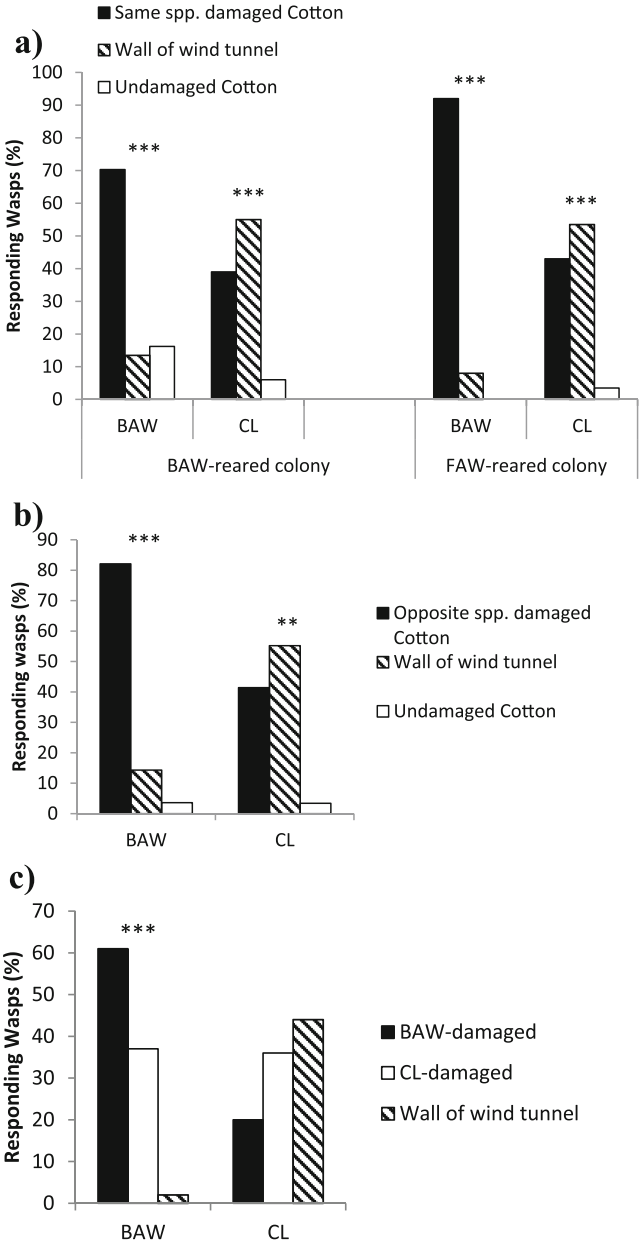
Wind Tunnel Bioassays Wasps were given oviposition experience on three BAW or three CL in the presence of conspecific-damaged cotton tissue (Fig. 2a). Wasps experienced on BAW flew to (i.e., landed on) BAW-damaged cotton 70 % (colony 1) and 92 % (colony 2) of the time, while wasps experienced on CL flew to the wall of the wind tunnel 55 % (colony 1; N=37 (BAW); 31 (CL); χ^2=14.428; P=0.001) and 54 % (colony 2; N=25 (BAW); 28 (CL); χ^2=13.425; P=0.004) of the time. The effect between colony 1 (*C. marginiventris* reared on BAW on wheatgerm diet) and colony 2 (*C. marginiventris* reared on FAW on corn) was not significant; ANOVA-GLM: Choice=Experience, Colony; P=0.285. Likelihood ratio tests for each treatment was as follows: colony 1 (BAW) χ^2=21.106; P<0.001; colony 1 (CL) χ^2=13.946; P=0.001; colony 2 (BAW) χ^2=20.720; P<0.001; colony 2 (CL) χ^2=15.798; P<0.001.

Wasps were given oviposition experience on three BAW or three CL in the presence of cotton tissue damaged by the opposite species (Fig. 2b). Wasps experienced on BAW flew to CL-damaged cotton 82 % of the time, while wasps experienced on CL flew to the wall of the wind tunnel 55 % of the time; N=28 (BAW); 29 (CL); χ^2=11.093; P=0.004. Likelihood ratio tests for each treatment as follows: BAW χ^2=30.242; P<0.001; CL χ^2=16.777; P=0.002. When given a choice between BAW- or CL- damaged cotton, wasps more often flew to plants damaged by the host species on which they were trained (Fig. 2c). BAW-experienced wasps flew to BAW-damaged cotton 61 % of the time, and CL-damaged cotton 37 % of the time (Fig. 3c; N=41; χ^2=28.966; P<0.001); of wasps experienced on CL, 36 % flew to CL-damaged cotton and 20 % flew to BAW-damaged cotton. Likelihood ratio tests for each treatment as follows: BAW χ^2=27.759; P<0.001; CL χ^2=4.139; P=0.126.

Untrained (naïve) *C. marginiventris* tested for attraction toward BAW-damaged cotton in the wind tunnel flew to the wall of the tunnel 96 % of the time (N=25; χ^2=61.438; P<0.001).

Vanilla-experienced wasps were given oviposition experience on three wheat germ-fed BAW or CL in the presence of diluted vanilla extract and host frass (Fig. 3). Wasps experienced on BAW flew to filter discs containing vanilla odor 61 % of the time, while wasps experienced on CL flew to the wall of the wind tunnel 73 % of the time; N=71 (BAW) and 67 (CL); χ^2=17.739; P<0.001. Likelihood ratio tests for each treatment as follows: BAW χ^2=52.141; P<0.001; CL χ^2=14.343; P<0.001.

Sequential Multi-species Oviposition Training Wasps from the FAW-reared colony used in 2a were removed from the tunnel after their first flight choice and given a fourth oviposition experience on the opposite host species from which they were trained (e.g., Experience 1, 2 and 3 on BAW in presence of BAW-damaged plants; Experience 4 on CL in presence of BAW-damaged plants). Wasps experienced



◀**Fig. 2** Effects of host experience on flight response of Cotesia marginiventris to host-associated odors. a) Wasps experienced on beet armyworm (BAW) or cabbage looper (CL) in the presence of conspecific plant tissue, flying to cotton plants damaged by the same host species vs. undamaged plants. Multinomial Logistic Regression comparing choice of BAW- v. CL-experienced wasps as follows: BAW-reared colony: N=37 (BAW); 31 (CL); χ^2=14.428; P=0.001; fall armyworm (FAW)-reared colony: N=25 (BAW); 28 (CL); χ^2=13.425; P=0.004. Test for colony effect (BAW-reared vs. FAW-reared) using GLM: Choice=Experience, Colony; P=0.285. Likelihood ratio tests for each treatment as follows: colony 1 (BAW) χ^2=21.106; P<0.001; colony 1 (CL) χ^2=13.946; P=0.001; colony 2 (BAW) χ^2=20.720; P<0.001; colony 2 (CL) χ^2=15.798; P<0.001. Asterisks denote significance within a host species choice ratio at ***=0.001. **b)** Wasps experienced on BAW or CL in the presence of plants damaged by the opposite host species, flying to cotton plants damaged by the opposite host species vs. undamaged plants: N=28 (BAW); 29 (CL); χ^2=11.093; P=0.004. Likelihood ratio tests for each treatment as follows: BAW χ^2=30.242; P<0.001; CL χ^2=16.777; P=0.002. Asterisks denote significance at **=0.01 and ***=0.001. **c)** Wasps experienced on BAW or CL in the presence of conspecific-damaged plant tissue, flying to BAW- vs. CL-damaged cotton: N=41; χ^2=28.966; P<0.001. Likelihood ratio tests for each treatment as follows: BAW χ^2=27.759; P<0.001; CL χ^2=4.139; P=0.126. Asterisks denote significance at ***=0.001

on BAW that flew to BAW-damaged cotton on the first flight did not have a deterioration of the positive odor stimulus and continued to land on BAW-damaged cotton after a CL-experience on BAW-damaged cotton (Fig. 4). Wasps experienced on CL that flew to the wall on the first flight had a 60 % improvement of flying to CL-damaged cotton after just one BAW experience on CL-damaged cotton.

Discussion

There is a plethora of literature suggesting the importance of differentially induced HIPVs in natural enemy host-finding. Rose et al. (1998) found that BAW and *Helicoverpa zea* (Boddie) induce almost identical volatile blends in cotton plants, and that the specialist parasitoid, *Microplitis croceipes* (Cresson), could not distinguish between the two volatile profiles. Turlings et al. (1990a) found that *C. marginiventris* innately prefers FAW over CL-damaged corn and cotton in olfactometer tests, and that this preference could be modified through prior exposure to CL-damaged plants, but it is unknown if this was due to differentially induced HIPVs. While the role of plant volatiles in the attraction of *C. marginiventris* to BAW has been widely demonstrated (Turlings et al., 1990b; Turlings and Tumlinson, 1992; Rose et al., 1998; Cardoza et al., 2003), its attraction to CL-induced plant volatiles is not as well understood. Furthermore, few of these studies have examined the actual associative learning aspect of volatile cues related to hosts of differing suitability.

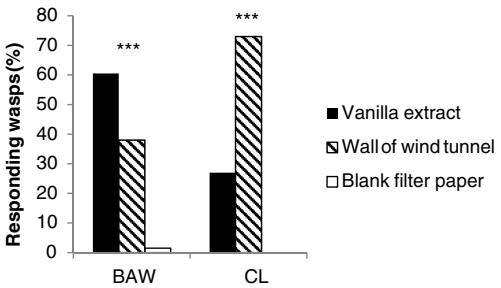


Fig. 3 Wasps flying to vanilla extract following oviposition experience on BAW or CL in the presence of diluted vanilla extract. N=71 (BAW); 67 (CL); χ^2=17.739; P<0.001. Likelihood ratio tests for each treatment as follows: BAW χ^2=52.141; P<0.001; CL χ^2=14.343; P<0.001. Asterisks denote significance at ***=0.001

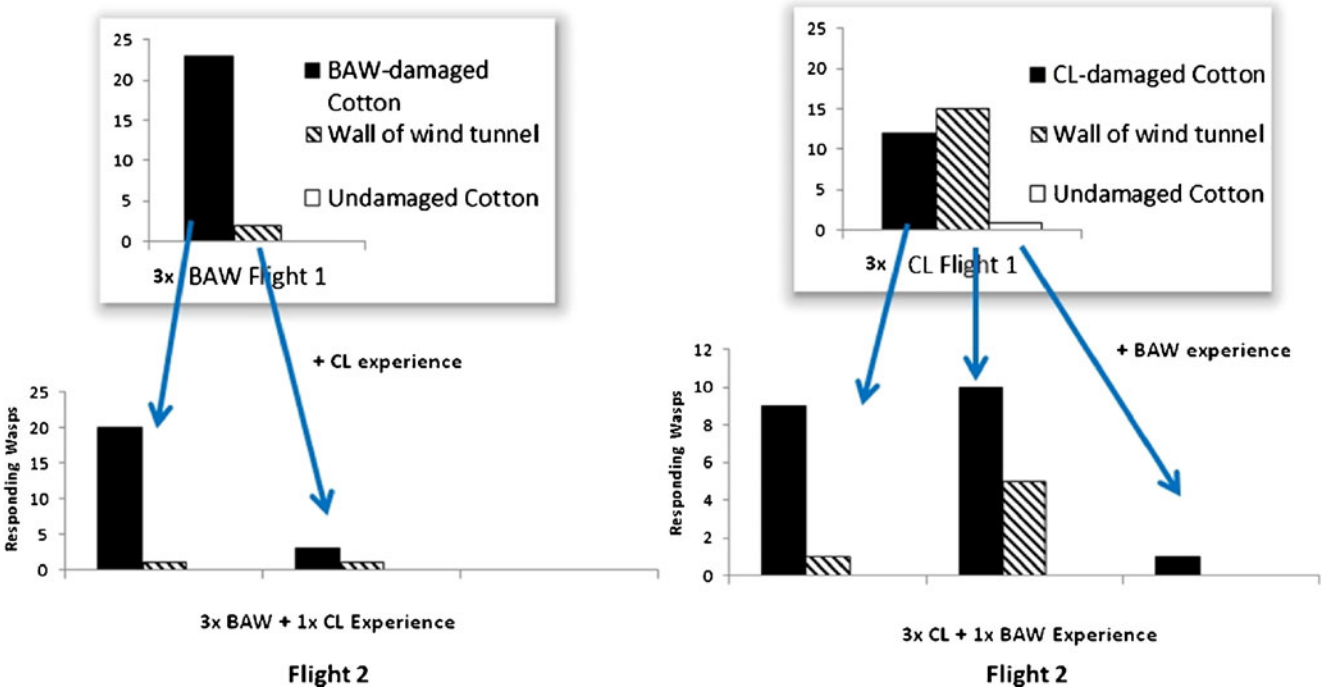


Fig. 4 Second flight of wasps flying to cotton plants after oviposition experiences on both host species. Inset boxes show first flight landing choice after 3 oviposition experiences on beet armyworm (BAW) or cabbage looper (CL) in the presence of conspecific-damaged cotton plants. The bars below show flight choice after an additional experience on the opposite host species in the presence of the original odor source. Wasps from fall armyworm (FAW)-reared colony in 2a, N=41

Costa et al. (2010) explored unrewarding experiences in *C. marginiventris* using the non-host *Pieris rapae* (L.) and found that wasps did not change their response to HIPVs following experience with a non-host. However, their naïve (untrained) wasp control showed an 81 % attraction to plant odors, whereas our untrained wasps flew to a damaged plant only 4 % of the time, suggesting that their control was not as effective as ours at measuring learned responses. Furthermore, by releasing multiple females at the same time, their data often showed a high tendency of positive control wasps to not move toward an odor stimulus. This is not surprising as wasps tend to disperse randomly when foraging in what they perceive to be crowded or highly competitive environments. Preliminary experiments indicated that one or both female wasps would immediately depart the release platform in the wind tunnel after contact with a conspecific. By flying *C. marginiventris* singly, we demonstrated the effects of oviposition training free of foraging pressure by conspecifics.

Our data show that BAW and CL differentially induce cotton volatiles. Differences in BAW- and CL-damaged blends were evidenced by disparities in ratios of GLV, monoterpene, sesquiterpene, and homoterpene emission. Wasps with oviposition experiences on BAW flew to BAW-damaged cotton the majority of the time, but wasps with experience on the semi-permissive host CL flew to the wall of the wind tunnel more often than to CL-damaged plants. By switching these two odor sources, wasps flew to CL-damaged cotton following BAW oviposition experience, but less frequently oriented toward BAW-damaged cotton following CL oviposition experience. This suggests that a positive oviposition experience on the preferred host (BAW) increases associative learning of HIPVs in *C. marginiventris*, which has been previously documented (Turlings et al., 1991). Choice flights where both BAW- and CL-damaged cotton were presented indicate that BAW-experienced wasps fly more often to plants damaged by the host on which they were experienced. This indicates that discrimination of the two odor blends is occurring; however, the relatively lower response of CL-experienced individuals is probably due to the sub-optimal experience. While we considered the possibility that sensitization (i.e., increased responsiveness to an unpaired stimulus when presented repeatedly) could be occurring (Papaj and Prokopy, 1989), associative learning is a more likely explanation. Sensitization, by definition, would have elicited similar flight responses of wasps regardless of host species because it is caused by mere exposure to plant volatiles, which we did not observe.

The ability of braconid wasps to associate a non-plant cue (vanilla extract) to host-finding behavior has been demonstrated previously (Lewis and Tumlinson, 1988). By using vanilla extract in oviposition training and removing all natural plant compounds, we demonstrated that odor association more frequently occurs when wasps have positive oviposition experiences on BAW. By adding an oviposition

experience on the opposite host species from which wasps were originally experienced, we determined that *C. marginiventris* change post-oviposition flight behavior when presented with the preferred BAW hosts, but not with CL. In other words, CL experience does not cause deterioration of the odor-responding individuals trained on BAW, suggesting that the sub-optimal host experience is not causing negative associative learning, rather it is a sub-optimal event. However, no-choice individuals from the CL trained group need a reinforcing positive oviposition experience on BAW to continue foraging.

We tested the hypothesis that *C. marginiventris* would associate volatile odors with BAW but not CL. This was not true. Instead, behavioral analyses indicate that wasp associative learning occurs strongly in BAW, and only moderately in CL, and that the response of CL-experienced wasps can be altered by a single oviposition experience on BAW. This observed preference learning for BAW-associated volatiles may be robust in nature, as wasps reared on both BAW and FAW show the same behaviors. Our studies show that *C. marginiventris* readily sting and oviposit in CL larvae when present, but only 18 % of parasitized hosts yield wasp progeny when hosts are feeding on cotton (Harris and Tumlinson, unpublished data), suggesting that it is a sub-optimal host.

This study demonstrates that the interactive role of HIPVs in parasitoid foraging in nature may be dependent upon host species encounters. The observation of *C. marginiventris* flying to CL-damaged plants following positive BAW oviposition experiences may explain the occurrence of increased parasitism of the semi-permissive host when in the presence of abundant BAW. Conversely, abundant presence of semi-permissive hosts may limit parasitism of more suitable hosts through sub-optimal contact experiences that slow parasitoid foraging behavior or encourage patch abandonment.

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Less is More: Treatment with BTH and Laminarin Reduces Herbivore-Induced Volatile Emissions in Maize but Increases Parasitoid Attraction

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Abstract Chemical plant strengtheners find increasing use in agriculture to enhance resistance against pathogens. In an earlier study, it was found that treatment with one such resistance elicitor, BTH (benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid *S*-methyl ester), increases the attractiveness of maize plants to a parasitic wasp. This surprising additional benefit of treating plants with BTH prompted us to conduct a series of olfactometer tests to find out if BTH and another commercially available plant strengthener, Laminarin, increase the attractiveness of maize to three important parasitic wasps, *Cotesia marginventris*, *Campoletis sonorensis*, and *Microplitis rufiventris*. In each case, plants that were sprayed with the plant strengtheners and subsequently induced to release volatiles by real or mimicked attack by *Spodoptera littoralis* caterpillars became more attractive to the parasitoids than water treated plants. The elicitors alone or in combination with plants that were not induced by herbivory were not attractive to the wasps. Interestingly, plants treated with the plant strengtheners did not show any consistent increase in volatile emissions. On the contrary, treated plants released less herbivore-induced volatiles, most notably indole, which has been reported to interfere with parasitoid attraction. The emission of the sesquiterpenes *(E)*-β-caryophyllene, β-bergamotene, and *(E)*-β-farnesene was similarly reduced by the treatment. Expression profiles of marker genes showed that BTH and Laminarin induced several pathogenesis related (PR) genes. The results support the notion that, as yet undetectable and unidentified compounds, are of major importance for parasitoid attraction, and that these attractants may be masked by some of the major compounds in the volatile blends. This study confirms that elicitors of pathogen resistance are compatible with the biological control of insect pests and may even help to improve it.

Keywords Herbivore-Induced Plant Volatiles (HIPVs) · Parasitoid attraction · Plant enhancers · Defense gene expression · Indole · Sesquiterpene

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Introduction

Upon herbivore attack, plants emit specific blends of volatiles that attract natural enemies of the herbivores (Turlings and Wäckers, 2004; Arimura et al., 2009; Dicke and Baldwin, 2010). This phenomenon has led to the idea that by enhancing these volatile signals in crop plants, biological control of insect pests may be improved (Degenhardt et al., 2003; Turlings and Ton, 2006). Recent attempts to

genetically manipulate the emission of volatile compounds that are involved in the attraction of natural enemies have been successful (Kappers et al., 2005; Schnee et al., 2006), and a first demonstration that this approach can indeed help to enhance crop protection in the field has been shown with transgenic plants that emit (*E*)-β-caryophyllene from their roots and thereby attract entomophagous nematodes, which in turn reduce the abundance of root feeding herbivores (Degenhardt et al., 2009). However, for leaf-feeding pests, the transgenic approach appears to be more complicated, because herbivore induced leaf-volatile blends are more complex, making it difficult to pinpoint the key compounds that are involved in the attraction of predators and parasitoids (D'Alessandro et al., 2006; Dicke, 2009).

An alternative strategy that circumvents this problem is to use elicitors that induce general plant defense responses, including volatile signals (D'Alessandro et al., 2009). The two main plant hormones that could be targeted by such an approach are salicylic acid (SA) and jasmonic acid (JA). Biotrophic pathogens and piercing/sucking insects commonly trigger SA-mediated defenses, whereas necrotrophic pathogens and chewing herbivores induce the JA pathway (Heil and Bostock, 2002; Thaler et al., 2002a; Arimura et al., 2005; Smith et al., 2009). Hence, treatment with these hormones or their analogues may induce resistance against pathogens and insects, respectively (Arimura et al., 2005; Bostock, 2005). Jasmonic acid is also implicated in indirect defense responses, such as the production of extrafloral nectar (Heil, 2004) and the emission of volatile blends that are implicated in the attraction of parasitoids and predators (Turlings et al., 1990; Dicke et al., 1999). Indeed, it has been shown that JA treatment can increase the parasitism of caterpillars in tomato (Thaler, 1999), suggesting that elicitors targeting this pathway could be applied to enhance the attractiveness of crop plants to biological control agents. So far, however, only elicitors of pathogen resistance are commercially used as "plant enhancers" (Gorlach et al., 1996).

Several studies suggest that there is negative cross talk between the SA pathway and the JA pathway, and the induction of pathogen resistance may, therefore, lead to increased susceptibility to insect herbivores (Heil and Bostock, 2002; Thaler et al., 2002b) and perhaps attenuation of volatile emissions. To test the latter, Rostás and Turlings (2008) conducted a series of olfactometer experiments with maize plants that were treated with the plant enhancer BTH (benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid *S*-methyl ester). BTH is a chemical mimic of SA that triggers the expression of a high number of defense genes against microbes (Morris et al., 1998; von Rad et al., 2005), and it has been used successfully to induce resistance to a wide range of diseases on field crops (Friedrich et al., 1996; Inbar et al., 1998; Tally et al., 1999). Against expectations, Rostás and Turlings (2008) found that *Microplitis rufiventris*, a larval endoparasitoid wasp of numerous lepidopteran caterpillars, was much more attracted to BTH-treated plants than to non-treated plants. Compared to non-treated plants, BTH-treated plants released lower amounts of certain volatiles, in particular the aromatic compound indole (Rostás and Turlings, 2008). In another study, it was shown that inhibiting the production of indole indeed enhances the attraction of *M. rufiventris* (D'Alessandro et al., 2006), but other, yet unknown changes induced by BTH treatment may also have contributed to the dramatic increase in attractiveness that was observed by Rostás and Turlings (2008).

The current study was conducted to test whether BTH and another plant enhancer, Laminarin, have a general positive effect on the attractiveness of plants to parasitoids. Laminarin is a water-soluble β -1, 3glucan with an average degree of polymerization of 25 glucose units (Read et al., 1996), and it has been shown to stimulate defense responses in cell suspensions of tobacco (Klarzynski et al., 2000) and grapevine (Aziz et al., 2003; Trouvelot et al., 2008). It induces the accumulation of phytoalexins and expression of a set of pathogenesis related (PR) proteins (Klarzynski et al., 2000; Aziz et al., 2003) through the activation of the SA pathway (Ménard et al., 2004). As both BTH and Laminarin can stimulate the SA pathway, we speculated that they may have the same positive effect on parasitoid attraction. To test this notion, we conducted a series of olfactometer experiments with three generalist parasitoids that attack lepidopteran herbivores, including several *Spodoptera* species. We tested the responses of the endoparasitoids *Microplitis rufiventris* (Kok.), *Cotesia marginiventris* (Cresson) [Hymenoptera: Braconidae], and *Campoletis sonorensis* (Cameron) [Hymenoptera: Ichneumonidae] to odors of maize seedlings that were either attacked by larvae of *Spodoptera littoralis* (Bios.) [Lepidoptera: Noctuidae] or induced by mechanical damage and treatment with regurgitant of the same herbivore. Both these treatments strongly induce the emission of parasitoid attractants (Turlings et al., 1990, 1998; Alborn et al., 1997). Each of the three parasitoid species is known to be attracted to the odor of host-infested maize plants, but they show distinct differences in their odor preferences and response patterns (Hoballah and Turlings, 2005; Tamò et al., 2006; Erb et al., 2010). We, therefore, hypothesized that treatments with BTH or Laminarin may have different effects on the different parasitoids. To correlate parasitoid attraction with the induction of defenses and the emission of plant volatiles, we also measured the expression of a set of defense marker genes, in addition to collecting and analyzing the volatile emissions of treated plants. As BTH and Laminarin are increasingly used as plant strengtheners in agriculture (Vallad and Goodman, 2004), the results of this study will help to estimate their potential impact on biological control of pest insects by parasitoids.

Methods and Materials

Maize Plants Zea mays var. Delprim plants were grown in plastic pots (11 cm high, 4 cm diam) in commercial potting soil (Ricoter, Aussaaterde, Aarberg, Switzerland) and grown in a climate chamber (CLF plant climatics, Percival) at 25± 2°C, 60±5 % r.h., 16:8 hL:D, and 926 μmol/m^2s^1. Maize plants used for the experiments were 10–14 d-old and had three fully developed leaves.

Herbivores Eggs of *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) were supplied by Syngenta™ (Stein, Switzerland). Newly hatched larvae were reared in transparent plastic boxes on a wheat germ-based artificial diet until they had reached the second instar, at which point they were used in the experiments (Turlings et al., 2004).

Parasitoids A colony of the solitary larval endoparasitoids *M. rufiventris* was reared in the laboratory as follows. Twenty-five *S. littoralis* caterpillars (3–4 d-old) were offered to a single mated female (4–7 d-old) for 3 h in a plastic box (5 cm high, 9.5 cm diam). The parasitized caterpillars were kept in an incubator (25°C, 16: 8 hL:D) until the parasitoids formed cocoons. Emerging adults were sexed and kept in plastic cages (30×30×30 cm, Bugdorm I, MegaView Ltd, Taiwan) in incubators (25±2°C, 40±10 % r.h., 16:8 hL:D). Cages were supplied with moist cotton wool and droplets of honey.

To rear *C. marginiventris* and *C. sonorensis*, about 45 *S. littoralis* larvae were offered to three mated females in a plastic box (described above). Wasps were allowed to oviposit for 24 h and were then removed. Herbivore larvae were kept on artificial diet in the boxes until the emergence of the wasp cocoons. Cocoons were removed from the herbivore boxes and transferred to rearing cages. Cages were checked daily for eclosed individuals. Adult parasitoids were provided with water and honey. Cages with adults were transferred to the laboratory 30 min before the bioassays to be acclimatized to the ambient laboratory conditions.

Plant Strengtheners BTH (BION™) was obtained from Syngenta, Switzerland, as a water-dispersible granular formulation containing 50 % active ingredient. Laminarin (IODUS 40®) was obtained from Stähler, Switzerland, as a soluble liquid (SL) formulation containing 3.5 % active ingredient. Both BTH and Laminarin were sprayed on 9–11 d-old plants 48 h before the experiment (Rostás and Turlings, 2008) at concentrations of 0.15 g/L (BTH) and 20 ml/L (Laminarin). These concentrations correspond to the recommended doses by the manufacturers for application in agriculture.

Olfactometer Bioassays A series of experiments that used a six-arm-olfactometer was conducted to evaluate whether the application of the two plant enhancers to maize plants had an effect on the attractiveness of maize plants to parasitic wasps. In all experiments, 2–6 d-old mated female wasps were used (Tamò et al., 2006). The parasitoids were naïve, which means that as adults had no prior contact with host insects or maize plants. For the choice assays, six female wasps were removed from their cage with an aspirator and released into the central choice chamber of the olfactometer (Turlings et al., 2004). Attracted by the diffuse light coming from above, the wasps moved up to the top of the chamber. Depending on the attractiveness of the different odor sources, they then walked into one of the six arms connected to the central chamber. The central choice chamber was connected via a Tygon tube to a water-filled glass U-tube that served as a pressure gauge to balance incoming and outgoing air, thus minimizing pressure differences with the outside (Turlings et al., 2004; Rasmann and Turlings, 2007). Each group of wasps was given 30 min to make a choice. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and considered as individuals that made "no choice" (D'Alessandro et al., 2009). Five groups of six wasps were tested on each experimental day. Each olfactometer experiment was repeated 6 times on different days, each time with a new set of sprayed plants as odor sources and with new wasps. The position of the odor source was changed clock-wise after each day of testing to avoid position effects. All bioassays were carried out between 9:00 A.M. and 5:00 P.M.

Odor Sources

Spodoptera littoralis *Damaged Maize Plants* Two days before the olfactometer experiments, plants were subjected to three treatments: The seedlings were sprayed either with BTH (0.15 g/L), Laminarin (20 ml/L), or distilled water using a spray bottle. The different solutions were distributed equally over the different leaves. To induce maize plants to emit plant volatiles, 10 second instars of *S. Littoralis* larvae were added to each plant. Plants were infested 24 h after elicitor treatment, on the evening before an experimental day. After infestation, plants were kept under laboratory conditions (25±2°C, 16:8 hL:D). The following odor sources then were offered to the parasitoids: (i) a maize plant treated with BTH and damaged by caterpillars; (ii) a maize plant treated with Laminarin and damaged by caterpillars; and (iii) a maize plant treated with distilled water and damaged by caterpillars. The remaining three vessels were left empty. In the olfactometer setup, these empty vessels were alternated with vessels that contained a plant.

Mechanically Damaged Maize Plants The purpose of this experiment was to rule out confounding effects of the plant enhancers on the herbivores that may influence volatile emission. First, plants were sprayed with BTH, Laminarin,

or water as described above. Second, to mimic leaf-herbivore attack, the abaxial side of all fully developed leaves (20 mm^2) was scratched with a scalpel blade without damaging the midrib, and 10 µl of *S. littoralis* larval regurgitant were applied to each wound with a micropipette. Regurgitant had been collected previously with a micropipette from 4th instars that had been feeding on maize leaves for at least 24 h, and was stored at −80°C until use (Turlings et al., 1998). The above mentioned procedure was carried out the evening before and a second time on the morning of each experimental day, about 3 h before the start of the bioassays. The offered odor sources in this experiment were: (i) a maize plant treated with BTH and induced by mechanical damage and regurgitant; (ii) a maize plant treated with Laminarin and induced by mechanical damage and regurgitant; and (iii) a maize plant treated with distilled water and induced by mechanical damage and regurgitant. The three other vessels remained empty and were alternated with the treatment vessels.

Undamaged Maize Plants To test whether BTH and Laminarin change the attractiveness of maize plants in the absence of herbivory, parasitoids were offered a choice between three undamaged maize plants. Plants were treated with plant enhancers as described above and left for 48 h. The offered odor sources consisted of: (i) undamaged maize plant treated with BTH; (ii) undamaged maize plant treated with Laminarin; and (iii) undamaged maize plant treated with distilled water. Again, these the treatment vessels were alternated with three empty vessels.

Plant Enhancers To complete the dataset, we also tested whether the odor of BTH and Laminarin directly affected the behavior of the studied wasps. For this purpose, filter papers (595 Rundfilter 185 mm diam, Schleicher & Schuell GmbH, Dassel, Germany) were sprayed with the tested plant enhancers 48 h before the experiment. Each paper was rolled in a small glass cup (50 ml) and was put in the olfactometer bottles. As a control, filter paper was sprayed with the same amount of distilled water. The remaining three vessels of the six-arm-olfactometer remained empty.

Odor Trapping Volatiles emitted by the various odor sources were trapped on Super Q adsorbent filters (25 mg, 80–100 mesh; Alltech Associates, Deerfield, IL, USA)(Turlings et al., 2004) for 3 h during the bioassays. Before use, traps were washed with 3 ml dichloromethane. In all experiments, a filter was attached to the horizontal port at the top of each odor source vessel. Purified air entered the bottles at a rate of 1.1 L/min, and air carrying the volatiles was pulled through each trap at a rate of 0.7 L/min (Analytical Research System, Gainesville, FL, USA). Traps were extracted with 150 µl dichloromethane (Super solvent; Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane were added to each sample as internal standards (IS). Samples either were analyzed immediately or stored at −80°C before analysis in small vials (Supelco, Amber Vial, 7 ml with solid cap w/PTFE Liner).

Odor samples were analyzed using a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer (Agilent 5975 C VL MSD). After injection of 2 µl of sample, the temperature was maintained at 40°C for 3.5 min, and then increased to 100°C at 8°C/min, and subsequently to 200°C at 5°C/min, followed by a post-run of 5 min at 250°C. Helium at constant flow (0.9 ml/min) was used as carrier gas. Volatiles were identified by comparing their mass spectra with those of the NIST05 library and by comparing their retention times with those of previous analyses (Hoballah-Fritzsche et al., 2002; D'Alessandro and Turlings, 2005; D'Alessandro et al., 2009). The total emission for each compound was calculated as the sum of the amounts for all compounds released during the collection period (3 h).

Effect of Plant Enhancers on Herbivore Leaf Consumption To assess whether the studied plant enhancers affected herbivore growth, experiments were carried out under light benches in a climatized laboratory (25±2°C, 40±10 % r.h., 16:8 hL: D and 148 µmol/m^2s1). First, the plants were treated with plant enhancers as above. Controls were sprayed with water only. After 48 h, individual plants were infested with single second instar *S. littoralis* larvae, and PET-tubes (30 cm high, conal shape, top-diam: 8 cm) were placed over the plants and attached to the pots with Parafilm (N=24) (see Erb et al., 2011). The tubes were open at the top to ensure air-circulation. Larvae were weighed before each assay to determine their initial weight, and larval weight gain was recorded every second day. After 8 d of feeding, the herbivores were removed, and the plants were scored for survival. Plants that had been completely eaten were considered dead.

Quantification of Gene Expression To test whether the studied plant enhancers have an effect on the expression of plant defense marker genes, maize plants with three fully developed leaves were treated with BTH or Laminarin as described above. In a second experiment, the plants additionally were induced by mechanical damage and regurgitant application as described. In total, six plants were used for each treatment and analyzed as individual biological replicates. Total RNA was extracted using Quiagen RNA-Easy extraction kits following the manufacturer's instructions. To remove contaminant genomic DNA, all samples were treated with AmbionDNAse following the manufacturer's protocol. cDNA then was synthesized using Invitrogen Super-Script III reverse transcriptase according to the manufacturer's instructions. Quantitative

reverse transcriptase real time polymerase chain reactions (q-PCR) were carried out using gene-specific primers. The q-PCR mix consisted of 5ul Quantace Sensimix containing Sybr Green I, 3.4ul H_20, 100 nmol of each primer (2x0.3ul H_20), and 1ul of cDNA sample. Q-PCR was carried out using 45 cycles with the following temperature curve: 10s 95°C, 20s 60°, 15 s 72°. The final melt curve was obtained by ramping from 68 to 98°C in 1°C steps every 5 s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4ul solution from every sample was created. Six 10-fold dilution steps were carried out and the standard curve was included into every q-PCR run. The final obtained Ct values (using the automated threshold determination feature of the Rotor-Gene 6000 software) were corrected for the housekeeping gene GapC (Frey et al., 2000) and normalized to control levels to obtain average fold changes of treated plants.

Statistical Analyses The functional relationship between parasitoid responses and the different volatile sources offered in the six-arm olfactometer was examined with a generalized linear model as described earlier (Turlings et al., 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (*R: A language and Environment for Statistical Computing, Version 2.9.0, Zurich, Switzerland, 2009,* http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings et al., 2004). Volatile emission, larval performance, and gene expression data were analyzed by *one-way ANOVAs* and *Tukey's post-hoc comparison* of treatment means when the data were normally distributed and the variances were homogeneous. If assumptions for normally distributed data with homogeneous variances could not be fulfilled, we used the *non-parametric Kruskal–Wallis* or *Mann–Whitney Rank Sum tests* and then compared treatment effects using *Dunn's test*. These analyses were performed with *SigmPlot 12* (SPSS Inc, Chicago, IL, USA).

Results

Wasp Behavior

Spodoptera littoralis *Damaged Maize Plants* In a first test, we investigated whether Laminarin and BTH alter the attractiveness of *S. littoralis* infested plants to parasitoids. Generally, all tested naïve female wasps were more attracted to the odor of *S. littoralis* attacked plants treated with Laminarin and BTH than to *S. littoralis* attacked plants that were not treated. *Cotesia marginiventris* showed a strong preference for both BTH and Laminarin treated plants ($F_{3,\ 176}$=29.64; P<0.001) (Fig. 1a$_1$), while *Campoletis sonorensis* ($F_{3,\ 176}$=14.67; P<0.001) and *Microplitis rufiventris* ($F_{3,\ 176}$=50.04; P<0.001) preferred the odor of Laminarin treated plants, but did not distinguish between untreated and BTH treated plants (Fig. 1a$_2$, 1.a$_3$). In all cases, the wasps chose more often for the arms carrying the odor of plants than control arms.

Mechanically Damaged Maize Plants In a next experiment, we investigated the effect of Laminarin and BTH on the attractiveness of artificially induced maize plants. Again, all tested species were most strongly attracted to induced plants that were treated with Laminarin (Fig. 1b). *Cotesia marginiventris* females showed a strong preference for Laminarin and BTH treated plants ($F_{3,176}$=30.17; P<0.001) (Fig. 1b$_1$), while *C. sonorensis* ($F_{3,176}$=19.67; P<0.001) and *M. rufiventris* were more attracted only by Laminarin treated plants ($F_{3,176}$=51.46; P<0.001) (Fig. 1b$_2$, 1.b$_3$).

Undamaged Maize Plants To test if Laminarin and BTH also increase the attractiveness of undamaged plants, we treated healthy seedlings with the plant enhancers. *Cotesia marginiventris* (Fig. 1c$_1$) did not distinguish among BTH treated, Laminarin treated and untreated control plants, whereas treatment with Laminarin increased the attractiveness of undamaged plants to *C. sonorensis* (Fig. 1c$_2$). Surprisingly, *M. rufiventris* showed a preference for untreated control plants compared to BTH or Laminarin treated plants ($F_{3,\ 176}$=19.67; P<0.001) (Fig. 1c$_3$), implying that the elicitors themselves may have been somewhat repellent to this parasitoid. In all cases, odors coming from vessels containing plants were more attractive than odors from empty control bottles (Fig. 1c).

BTH and Laminarin When we tested BTH and Laminarin on filter paper in the olfactometer, the wasps were not attracted to the compounds themselves (Fig. 1d$_{1,2,3}$). The majority of the wasps remained in the central chamber of the olfactometer, and the few wasps that did make a choice showed no significant preference for any of the arms. (*C. marginiventris*: $F_{3,\ 176}$=1.25, P=0.292; *C. sonorensis*: $F_{3,\ 176}$=2.42, P=0.067; *M. rufiventris*: $F_{3,\ 176}$=2.00, P=0.115).

Volatile Emissions

Spodoptera littoralis *Damaged Maize Plants* Generally, the total amount of volatiles emitted by *S. littoralis* attacked plants treated with Laminarin was significantly less compared to BTH or untreated plants ($F_{2,\ 35}$=3.89; P<0.001), and *trans*-ocimene was the only compound that was released in significantly larger amounts when maize plants were treated with BTH ($H_{2,35}$=8.56; P=0.014).

Indole emission was strongly suppressed by Laminarin and BTH ($H_{2,\ 32}$=7.25; P=0.027). Methyl anthranilate was significantly lower for Laminarin treated plants, but not for BTH treated plants ($H_{2,\ 26}$=7.17; P=0.028). Laminarin and

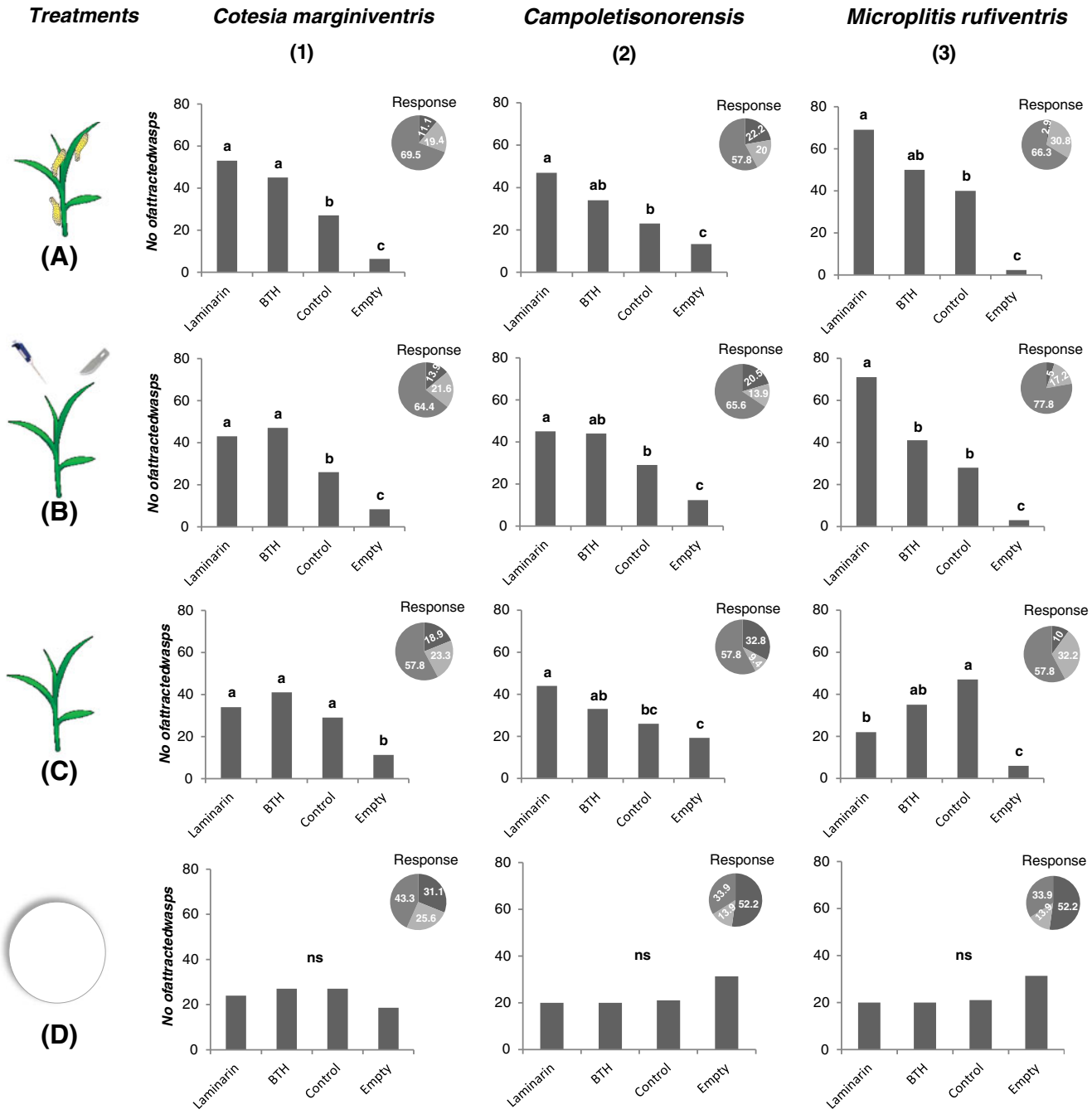

Fig. 1 Responses of naive female wasps tested in six-arm olfactometer. Values shown are number of attracted parasitoid to a particular odor. Wasps were allowed to choose between odors of Laminarin = Laminarin-sprayed maize plants, BTH = BTH-sprayed maize plants, Control = Distilled water-sprayed maize plants, and Empty = Empty control vessel (mean value of three vessels). **a** Ten 2nd instars of *Spodoptera littoralis* were added to plants 24 h before the experiment. **b** Plants were mechanically damaged and treated with *Spodoptera littoralis* regurgitant. **c** Plants were undamaged and carefully inserted in the bottles. **d** Three filter papers (Rundfilter 185 mm diam) were sprayed with Laminarin, BTH, and distilled water. Pie charts indicate percentages of female wasps (dark gray = females choose the empty bottles; light gray = non-responding females; gray = responding females). Thirty wasps were released per experimental day. This assaying was carried out for 6 successive experimental days. Different letters on the same bars indicate significant differences ($P<0.05$)

BTH application led to a decrease in (*E*)-beta-caryophyllene emissions ($F_{2,21}=3.54$; $P=0.047$). Beta-Bergamotene and *(E)*-beta-farnesene were supressed by Laminarin, but not by BTH ($F_{2,\ 34}=3.37$, $P=0.046$; $F_{2,\ 25}=4.24$, $P=0.026$) (Fig. 2).

Mechanically Damaged Maize Plants Similarly to the experiment with *S. littoralis*, the total quantity of volatiles released by artificially induced, Laminarin-treated plants was reduced by 50 % compared to control plants ($H_{2,32}=$

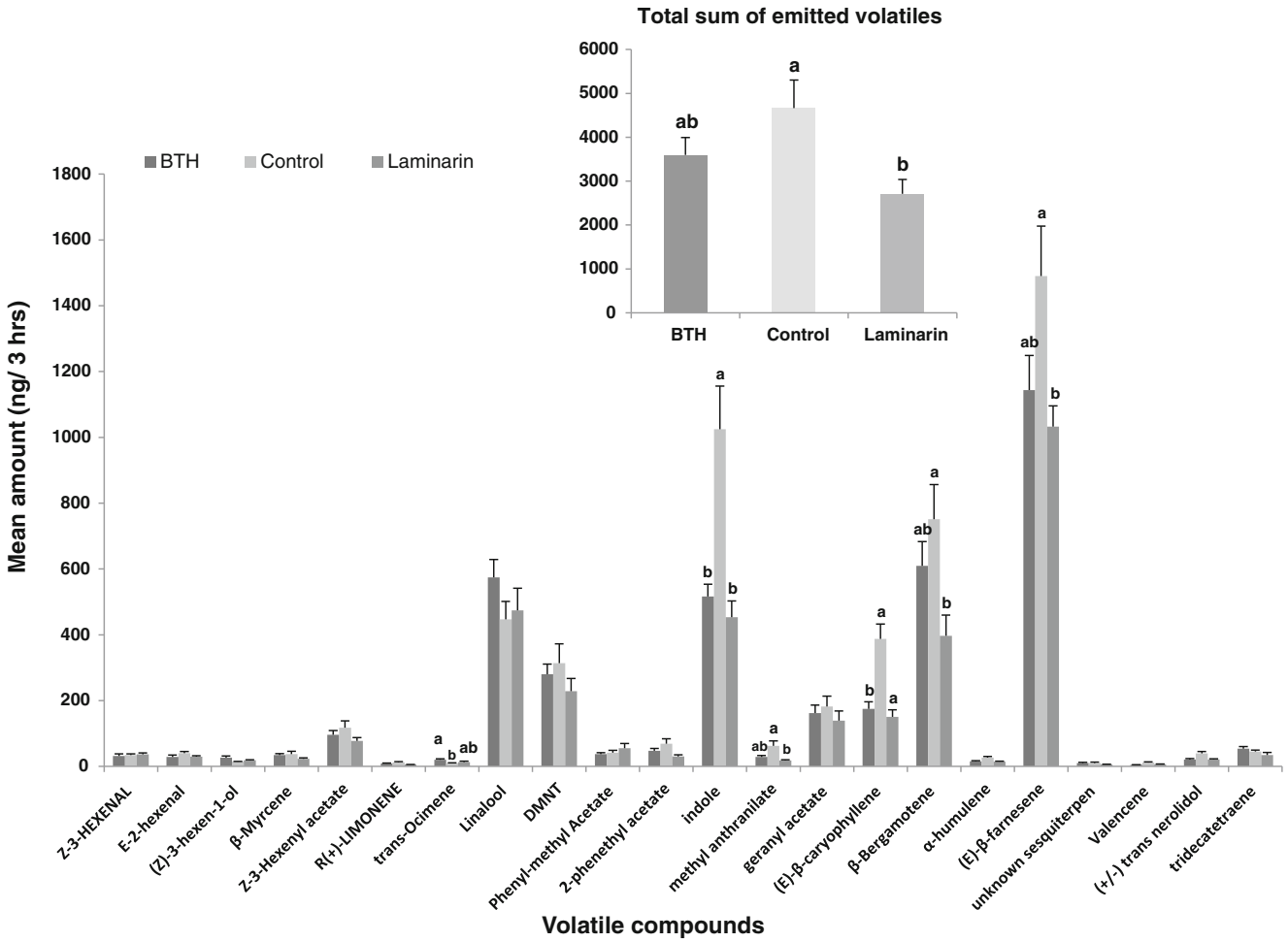

Fig. 2 Mean amount (±SE) (ng) of induced volatiles emitted during 3 h by *Spodoptera littoralis*-damaged maize seedlings (11 d-old). Laminarin = Laminarin-sprayed maize plants, BTH = BTH-sprayed maize plants, Control = distilled water-sprayed maize plants. Different letters on the same bars indicate significant differences ($P<0.05$) in the amount of a specific compound ($N=18$ per treatment). The compounds are ordered in accordance with their retention time in a gas chromatograph

9.34; $P=0.009$). Generally, the release of volatiles following induction with mechanical damage and herbivore regurgitant was 10 times lower than after *S. littoralis* attack (Fig. 3). Therefore, only the 12 most abundant individual volatiles could be quantified. Most quantified compounds showed the same trends as in the previous experiment, with a few exceptions: BTH treatment significantly attenuated the emission of *(Z)*-3-Hexenal acetate ($F_{2,19}=7.46$; $P=0.004$), and (*E*)-beta-caryophyllene ($H_{2,32}=6.20$; $P=0.045$). Also, linalool was significantly suppressed with Laminarin treatment ($F_{2,\ 28}=3.95$; $P=0.031$).

Larval Performance Overall, *S. littoralis* neonate larvae (2nd instar) survived equally well on Laminarin and BTH treated and untreated control plants (Fig. 4). During the first two days after treatment, larval growth was increased on BTH-treated plants ($F_{3,\ 91}=3.19$; $P=0.027$) (Fig. 5). This difference disappeared at later stages of development (4 days: $H_{3,\ 83}=4.83$, $P=0.185$; 6 days: $H_{3,\ 82}=0.42$; $P=0.937$; 8 days: $F_{3,\ 79}=0.69$; $P=0.559$).

Gene Expression Of the 11 defense markers genes that were tested for expression (Erb et al., 2009), two pathogenesis related genes, *Zm-PR1* and *Zm-PR5*, were induced 3-fold by BTH and Laminarin (Fig. 6a). *Zm-CysII*, a herbivore-induced cystatin homologue (Ton et al., 2007), was induced by BTH. Most marker genes were strongly induced by mechanical wounding and *S. littoralis* regurgitant application (Fig. 6b). Neither BTH nor Laminarin treatment changed this pattern: The only differences we observed was a slightly reduced induction of *Zm-PR1* by Laminarin and a reduction of *Zm-Igl*, the maize indole synthase (Frey et al., 2004) following BTH treatment.

Discussion

One of the main responses of plants to herbivore-attack is the release of a specific blend of volatiles that can attract carnivorous natural enemies of herbivores (Turlings and Wäckers,

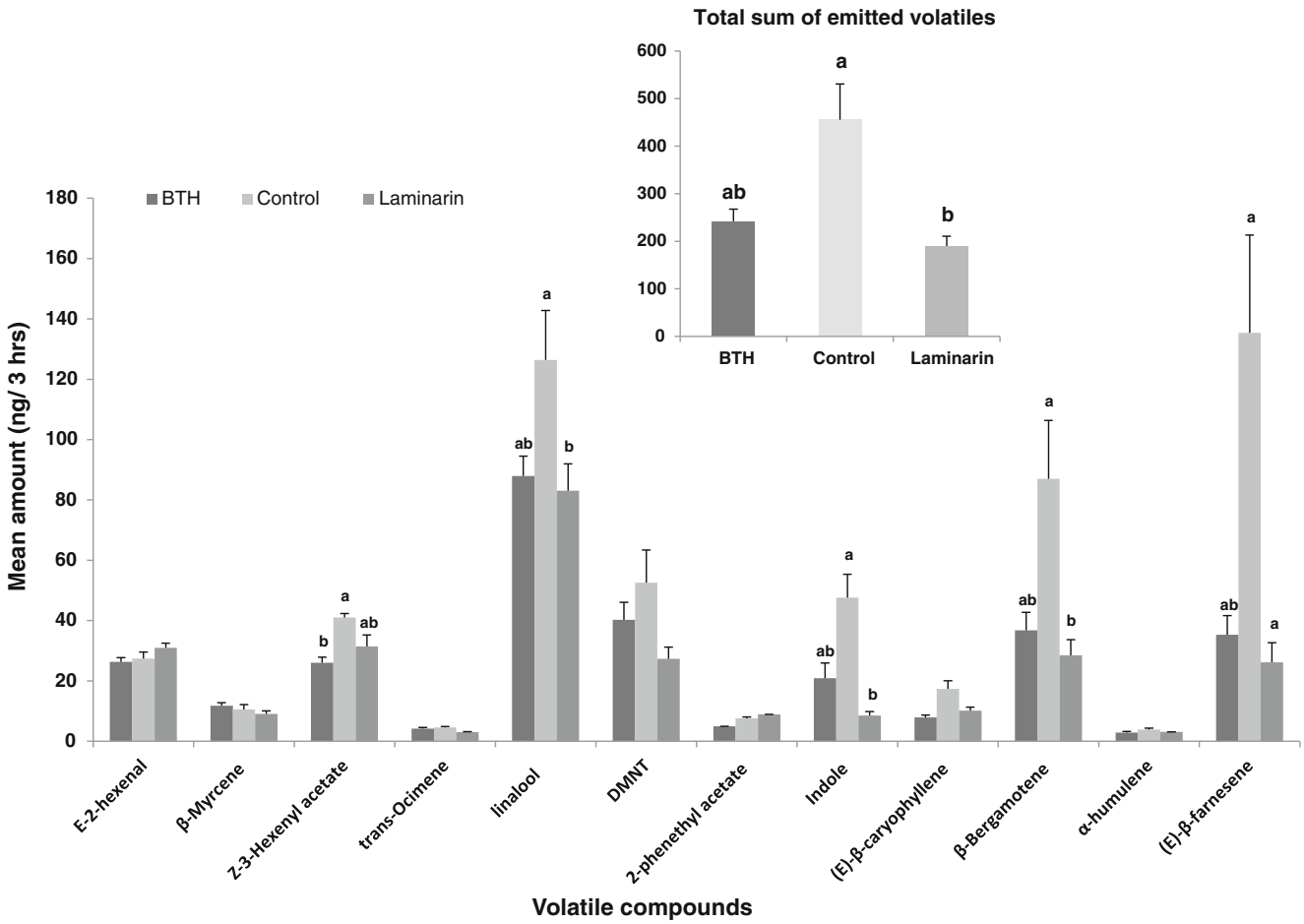

Fig. 3 Mean amount (±SE) (ng) of induced volatiles emitted during 3 h by maize seedlings (11 d-old) that were artificially damaged and treated with regurgitant of *Spodoptera littoralis* caterpillars. Laminarin = Laminarin-sprayed maize plants, BTH = BTH-sprayed maize plants, Control = distilled water-sprayed maize plants. Different letters on the same colored bars indicate significant differences ($P<0.05$) in the amount of a specific compound ($N=18$ per treatment). The compounds are ordered in accordance with their retention time in a gas chromatograph

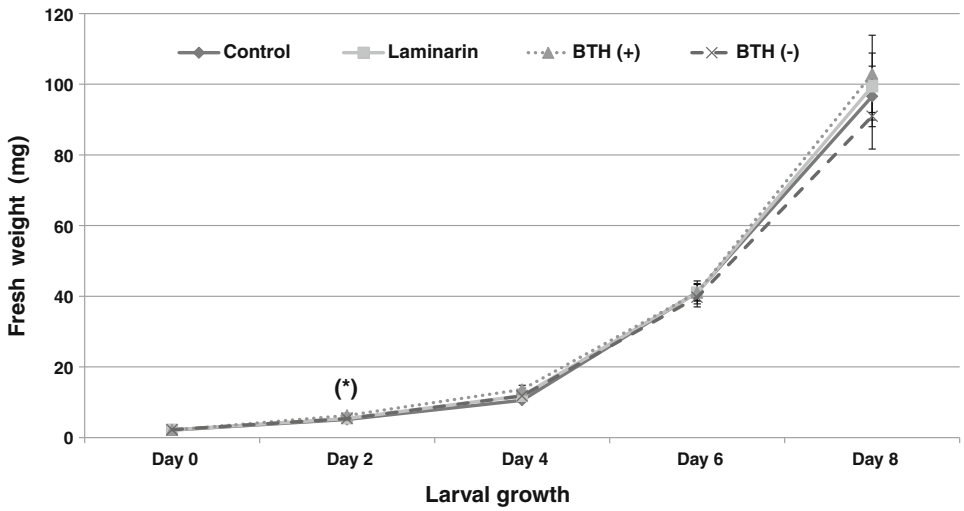

Fig. 4 Performance of *Spodoptera littoralis* larval stage on sprayed maize plants. Laminarin = Laminarin-sprayed maize plants, BTH (+) = BTH-sprayed maize plants, BTH (−) = BTH product without its active ingredient-sprayed maize plants, Control = distilled water-sprayed maize plants. Each caterpillar fed on a single plant. Means±SE are given. Larval weighing was done every second day. No significant differences were found between treatments after the second weighing (Holm-Sidak method, $P<0.05$). (*) There is a significant difference between treatments

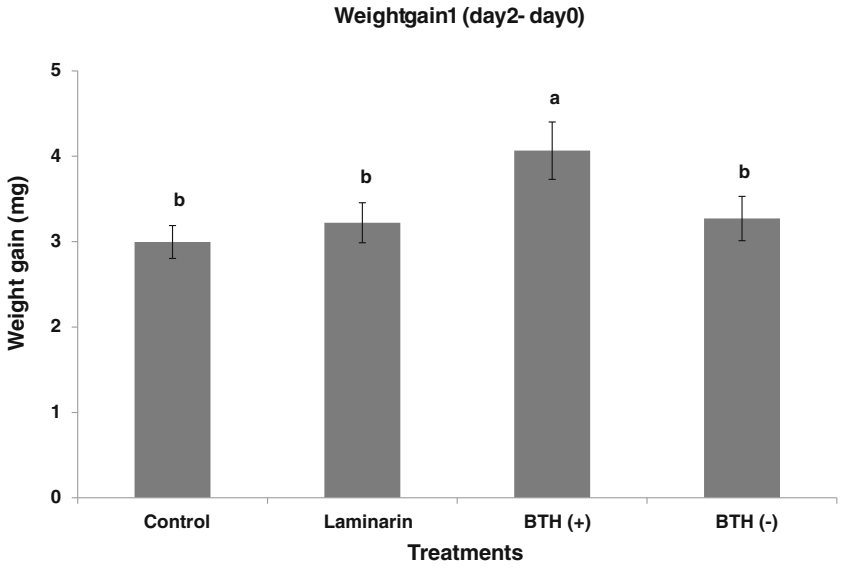

Fig. 5 Weight gain of *Spodoptera littoralis* larvae fed on sprayed maize plants. Laminarin = Laminarin-sprayed maize plants, BTH (+) = BTH-sprayed maize plants, BTH (−) = BTH product without its active ingredient -sprayed maize plants, Control = distilled water-sprayed maize plants. Each caterpillar fed on a single plant. Means±SE are given. A significant difference was found between treatments ($F_{3,91}$= 3.19, P=0.027) (Holm-Sidak method)

2004; Dicke and Baldwin, 2010). It is expected that proper manipulation of the release of such volatiles in crop plants may enhance the effectiveness of biological control agents (Degenhardt et al., 2003; Turlings and Ton, 2006). Yet, in many cases, plant volatile blends are complex, and not all compounds in a mixture contribute equally to herbivore

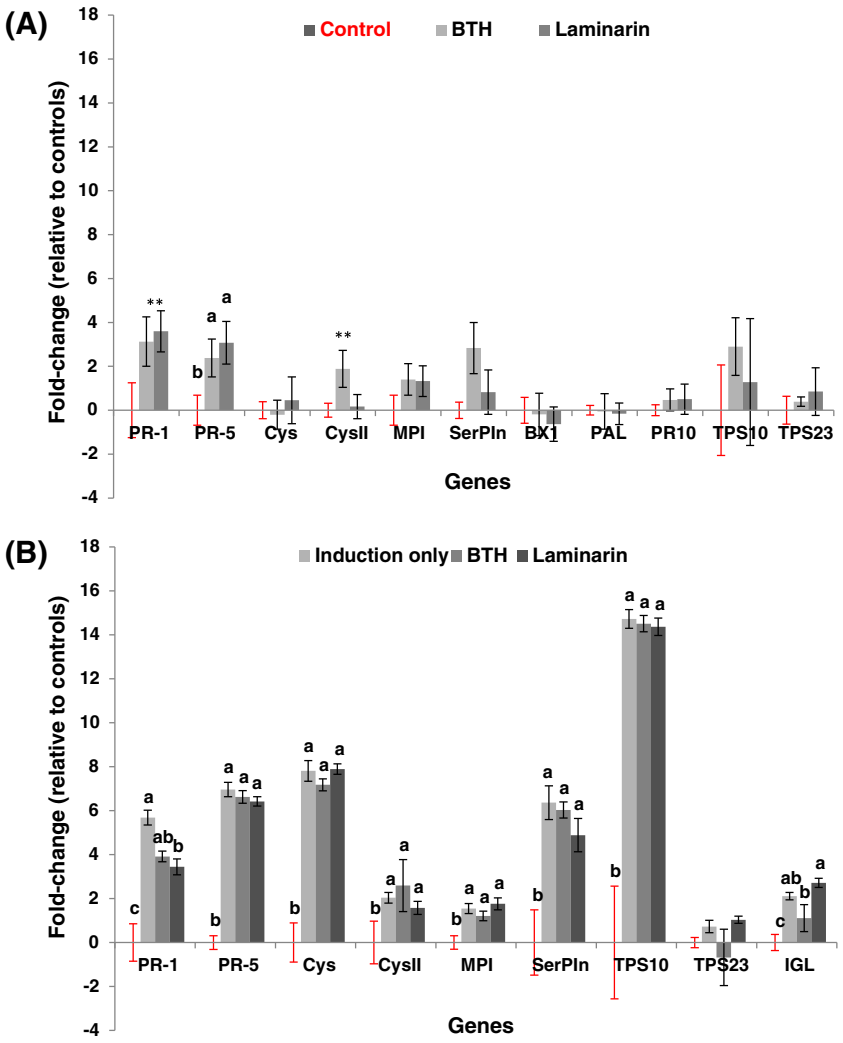

Fig. 6 **a** Fold change (±SE) of defense marker gene expression in plants treated with different elicitors relative to untreated control plants. Asterisks above bars refer to near significant trends ($P_{PR\text{-}1}$=0.051), (P_{CysII}= 0.057). **b** Fold change (±SE) of defense marker gene expression in artificially induced plants treated with different elicitors relative to uninduced, untreated control plants. Different letters denote significant differences between treatments (P<0.05)

attraction, making targeted manipulation for pest control a difficult task.

The results show that pretreating maize seedlings with the plant enhancers Laminarin and BTH increases the attractiveness of herbivore-damaged plants to parasitic wasps. An earlier study (Rostás and Turlings, 2008) found that soil-drenching with BTH enhances the attractiveness of maize plants to *M. rufiventris,* and our data expand upon these results, showing that foliar application has the same effect, also on two other, taxonomically very different wasps. Interestingly and contrary to general expectations, BTH and Laminarin suppressed the emission of several VOCs from *S. littoralis* infested and mechanically damaged plants. Detailed chemical analyses of all volatiles (including minor compounds) did not reveal the release of any additional volatiles that could have been triggered by or emitted from BTH or Laminarin. The compounds alone or applied to undamaged plants were not attractive, with the exception of Laminarin, which increased the attractiveness of undamaged plants to *C. sonorensis*.

We propose two hypotheses to explain why maize plants that emit less HIPVs are more attractive to the parasitoids. First, it is possible that the reduced volatiles may normally mask the attractive signals or even act as repellents. Several studies done on the same system indicate that neither the dominant sesquiterpenes (Schnee et al., 2006) nor aromatic compounds (D'Alessandro et al., 2006) are important for the innate attraction of the studied parasitoids (Rostás and Turlings, 2008). In fact, inhibiting the production of indole has been shown to enhance the attraction of *M. rufiventris* (D'Alessandro et al., 2006). Second, it is possible that BTH and Laminarin treatment enhance the production of yet unknown key attractants. Fractionation-guided bioassays and other earlier studies indeed indicate that the behaviorally active compounds emitted by herbivore-infested maize plants may not be detectable by conventional GC-MS methods (D'Alessandro et al., 2009), and we can, therefore, not exclude that the emissions of these compounds were enhanced by the plant strengtheners. Overall, the presented data suggest that for parasitoid attraction in maize, less may be more. In other words, plants that produce fewer of the common HIPVs are better able at attracting a wide range of parasitoids. Clearly, further studies are required to unravel the mechanisms behind this counter-intuitive phenomenon.

From a mechanistic perspective, the suppressant effect of BTH and Laminarin on the induction of volatile organic compounds may be related to their proposed mode of action. BTH acts downstream of SA (Friedrich et al., 1996) and elicits accumulation of SA responsive genes and pathogenesis-related proteins (PRPs). Our gene expression measurements confirm the accumulation of PR transcripts following BTH treatment. As there is negative cross talk between the SA and the JA pathway (Thaler et al., 2002b; Ozawa et al., 2000; Heil and Bostock, 2002), and HIPVs in maize are thought to depend on JA (Schmelz et al., 2001), it is possible that BTH acts as a suppressor of the herbivore-induced JA response, resulting in a lower production of volatiles. Further evidence hinting at a suppression of defenses comes from our *S. littoralis* performance experiment, which showed that caterpillars grow slightly better on BTH-treated plants. Surprisingly, however, we found no reduced induction of JA-responsive genes following BTH treatment, suggesting that BTH affects a different regulatory mode. As sesquiterpene emissions were reduced, but the corresponding terpene synthases TPS10 and TPS23 were expressed normally, it is possible that BTH changed the available precursor pool or the photosynthetic activity of the plant rather than acting on the genes themselves. In general, it can be expected that the effect of BTH will also depend on the dose of application (Choh et al., 2004). Here, we used the plant strengthener at a relatively low concentration of 0.15 g/L, which corresponds to the recommended dose of the manufacturer for field application.

Similarly to BTH, many studies indicate that oligo- and polysaccharides can act as elicitors of pathogen defense (Kobayashi et al., 1993). Yet, the mechanism by which polysaccharides act in the plant is not fully understood (Mercier et al., 2001). In grapevine, Laminarin elicits a variety of defense reactions, including alkalinization of the extracellular medium, an oxidative burst, activation of two mitogen-activated protein kinases, expression of 10 defense-related genes with different kinetics and intensities, increases in chitinase and 1,3-glucanase activities, and the production of two phytoalexins (Aziz et al., 2003). Obara et al. (2002) also found that chitosan oligomers can trigger the emission of linalool, MeSA, and β-caryophyllene in rice plants (*Oryza sativa*). One study found that after chemical sulfation, Laminarin becomes an inducer of the salicylic acid (SA) signaling pathway in tobacco and *Arabidopsis thaliana.* In tobacco, Laminarin also was shown to induce the expression of ethylene-dependent PR proteins, whereas PS3 (*β*-1, 3-glucan sulfate) triggers the expression of ethylene- and SA-dependent PR proteins (Ménard et al., 2004; Trouvelot et al., 2008). In our experiments, Laminarin had effects similar to BTH, not only in terms of volatile emission and parasitoid attraction, but also regarding the slight induction of PR genes. This suggests that in maize, Laminarin triggers a similar response as BTH. It would be interesting to assess whether the two plant strengtheners act redundantly or in synergy when applied together.

A recent field study conducted in maize plots in subtropical Mexico found only minor effects of BTH application on herbivore and parasitoid recruitment (von Mérey et al., 2012). The severe biotic and abiotic conditions under which these field assays were conducted may have masked the effects of BTH treatment. However, it can be safely

concluded from this field study and the current lab study that treating maize with BTH or Laminarin is compatible with biological control of lepidopteran pests, and their application may, under certain conditions, even improve the control mediated by parasitic wasps.

In the field, pathogens and herbivores often attack an individual plant simultaneously or in sequence (Rostás et al., 2003; Stout et al., 2006). Therefore, protecting crops against diseases by using Laminarin and BTH could lead to a potential conflict, as both elicitors activate defenses against pathogens, but may weaken plant resistance against certain herbivores (Heil and Bostock, 2002; Stout et al., 2002; Taylor et al., 2004; Bostock, 2005; Beckers and Spoel, 2006). Our data suggest, however, that these potential negative effects of plant strengtheners may be outweighed by their positive effect on multitrophic interactions: BTH and Laminarin treated plants are likely to become more attractive to a wide variety of parasitoids after herbivore attack, and furthermore, through the suppression of the dominating plant volatiles, treated plants may be less apparent to herbivores that use HIPVs for host location (Halitschke et al., 2008). The results are encouraging news for those that aim to improve the plants immune system by applying plant strengtheners. Further fieldwork will have to reveal the full potential of this approach.

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Isolation and Identification of Host Cues from Mango, *Mangifera indica*, That Attract Gravid Female Oriental Fruit fly, *Bactrocera dorsalis*

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Abstract The oriental fruit fly, *Bactrocera dorsalis*, is an economically damaging, polyphagous pest of fruit crops in South-East Asia and Hawaii, and a quarantine pest in other parts of the world. The objective of our study was to identify new attractants for *B. dorsalis* from overripe mango fruits. Headspace samples of volatiles were collected from two cultivars of mango, 'Alphonso' and 'Chausa', and a strong positive behavioral response was observed when female *B. dorsalis* were exposed to these volatiles in olfactometer bioassays. Coupled GC-EAG with female *B. dorsalis* revealed 7 compounds from 'Alphonso' headspace and 15 compounds from 'Chausa' headspace that elicited an EAG response. The EAG-active compounds, from 'Alphonso', were identified, using GC-MS, as heptane, myrcene, (*Z*)-ocimene, (*E*)-ocimene, allo-ocimene, (*Z*)-myroxide, and γ-octalactone, with the two ocimene isomers being the dominant compounds. The EAG-active compounds from 'Chausa' were 3-hydroxy-2-butanone, 3-methyl-1-butanol, ethyl butanoate, ethyl methacrylate, ethyl crotonate, ethyl tiglate, 1-octen-3-ol, ethyl hexanoate, 3-carene, *p*-cymene, ethyl sorbate, α-terpinolene, phenyl ethyl alcohol, ethyl octanoate, and benzothiazole. Individual compounds were significantly attractive when a standard dose (1 μg on filter paper) was tested in the olfactometer. Furthermore, synthetic blends with the same concentration and ratio of compounds as in the natural headspace samples were highly attractive ($P<0.001$), and in a choice test, fruit flies did not show any preference for the natural samples over the synthetic blends. Results are discussed in relation to developing a lure for female *B. dorsalis* to bait traps with.

Keywords Fruit volatile · Kairomone · Insect-plant interaction · Attractant · Diptera · Tephritidae · Insect pest

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Introduction

The oriental fruit fly, *Bactrocera* (formerly *Dacus*) *dorsalis* (Hendel) (Tephritidae: Diptera) is a major pest of fruit crops in South East Asia where it is endemic. It has been introduced to Hawaii where it does considerable damage and is a quarantine pest in other countries. *Bactrocera dorsalis* is of major concern to commercial fruit growers, exporters, and government regulatory agencies (Clarke et al., 2005). It is highly polyphagous and causes economic losses to a wide range of fruit crops (Metcalf and Metcalf, 1992). Female fruit flies lay their eggs in mature fruits while they are still on the tree, and the ensuing larvae render the fruit inedible leading to huge pre- and post- harvest spoilage. In India, *B. dorsalis* is a major pest of mango, *Mangifera indica* L., causing losses of up to 31 % (Verghese et al., 2002). Many methods have been investigated for pre- and post- harvest management of fruit flies (Cornelius et al., 1999, 2000a,b; Ishtiaq et al., 1999; Anjum et al., 2000; Verghese and Kamala Jayanthi, 2001; Verghese et al., 2004; Pinero et al., 2009), and recently there has been increasing emphasis

on the development of environmentally friendly techniques for managing *B. dorsalis* at the farm level.

The Male Annihilation Technique (MAT) involves lure-and-kill of males using bait stations that consist of attractants such as methyl eugenol lures combined with an approved insecticide as a killing agent. It is recommended for area wide management of fruit flies (Mumford, 2006). However, the existing methyl eugenol baited traps are effective only in intercepting male *B. dorsalis*, and because oriental fruit flies are highly polygamous a few surviving males can fertilize a substantial number of females (Cunningham, 1989). Therefore, more successful control of oriental fruit fly would be achieved by targeting female *B. dorsalis* because they are responsible for oviposition and subsequent damage to fruits. Crude attractants based on fermenting sugars, hydrolyzed protein, and yeast are available but are limited by lack of potency, short shelf life, and lack of specificity (Siderhurst and Jang, 2006). Identification of kairomones would facilitate the development of lures based on synthetic female attractant blends. The current study focused on mango because it is a preferred host plant and there was not much information previously available on the host cues from mango responsible for attraction of gravid female *B. dorsalis*.

Previous studies have shown that visual and olfactory chemical cues play an important role in the host-finding behavior of fruit flies (Jang and Light, 1991; Vargas et al., 1991; Cornelius et al., 1999, 2000a,b; Alyokhin et al., 2000; Wen-Yen et al., 2007). Host fruit odors have been demonstrated to be attractive for oviposition, feeding and mating of *B. dorsalis* (Drew, 1989; Fletcher and Prokopy, 1991; Landolt et al., 1992; Jang and Light, 1996). Volatile fruit odors have been used successfully as attractants for the other frugivorous tephritids viz., apple maggot fly, *Rhagoletis pomonella* (Walsh) (Reissig et al., 1985; Jones, 1988; Jones and Davis, 1989; Agnello et al., 1990); Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Prokopy and Vargas, 1996; Warthen et al., 1997; Prokopy et al., 1998); Mexican fruit fly, *Anastrepha ludens* Loew (Robacker et al., 1992; Robacker and Heath, 1996), and Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Nigg et al., 1994). However, such studies are limited in the case of oriental fruit fly, *B. dorsalis* with the exception of a study by Siderhurst and Jang (2006) in which attractants from tropical almond, *Terminalia catappa*, were identified. Mango is known to be a preferred host plant of *B. dorsalis*, and therefore, the objective of the present study was to identify host chemical cues involved in attracting gravid female fruit flies to mango fruits.

Methods and Materials

Insects The *B. dorsalis* flies used in this study were obtained as pupae from the mass rearing facility of the Fruit Entomology Laboratory at the Indian Institute of Horticultural Research (Bangalore, India). Emerging adult flies were maintained on yeast, sugar, and moistened cotton swabs. Subsequent generations then were reared on banana (*Musa* spp.) according to the method of Jayanthi and Verghese (2002) in a quarantine facility at Rothamsted Research (25 °C, 65–70 % R.H., 12:12 hL:D). The gravid female fruit flies used were approximately 20–30 days old. According to Jang et al. (1997), 95 % of females from mixed cages are mated by day 7. The gravid female fruit flies were separated from the mixed cages and held in individual glass vials 1 h prior to use in bioassays.

Chemicals Authentic chemical standards (>95 % purity) of heptane, 3-hydroxy-2-butanone, 3-methyl-1-butanol, ethyl butanoate, ethyl methacrylate, ethyl crotonate, (*RS*)-1-octen-3-ol, ethyl hexanoate, 3-carene, *p*-cymene, ethyl sorbate, α-terpinolene, phenylethyl alcohol, ethyl octanoate, (*RS*)-γ-octalactone, benzothiazole, and myrcene (90 % purity) were purchased from Sigma Aldrich (Gillingham, UK). Ethyl tiglate (98 %) was purchased from Alfa Aesar (Heysham, Lancashire, UK). Allo-ocimene (90 % purity) was purchased from Bush Boake Allen (Haverhill, Suffolk, UK). (*Z*)-Ocimene and (*E*)-ocimene were obtained from Givaudan (Ashford, UK). Myroxide (>90 % purity; (*Z*)- and (*E*)-isomers in a 2:1 ratio) was provided by Firmenich (Geneva, Switzerland).

Air Entrainment Headspace samples of volatiles from mango fruits (cv. 'Alphonso' and 'Chausa') were collected by air entrainment. The fruits, imported from India by a local retail outlet, were at full ripe stage at the start of volatile collection. Full ripe was determined as the point at which the fruit started to soften. Ripeness is known to improve the attraction of tephritid fruit flies to host fruit and they prefer overripe (i.e., rotten) fruit to fresh fruit (Liquido et al., 1989; Liquido and Cunningham, 1990; Andrei et al., 2000; Leopoldo et al., 2006). Thus, volatiles were collected from the full ripe fruits at 3 d intervals over a period of 24 d during which the fruit became overripe. Before volatile collection, glassware and aluminum plates were washed with aqueous Teepol detergent (Herts County Supplies, Herts, UK), rinsed with distilled water and acetone, and then dried in an oven at 180 °C for 2 h. The Porapak Q tubes used for collection of volatiles were eluted with redistilled diethyl ether, and heated at 132 °C for 2 h under a stream of purified nitrogen to remove contaminants. Fruits were placed individually inside glass vessels (180 mm high, 100 mm diam), open at the bottom and closed with a collection port at the top along with an inlet port on the side of glass vessel. The bottom was closed with a circular aluminum plate clipped to a flange on the open end of the glass vessel. Air, purified by passage through an activated charcoal filter, was pumped into the vessel through the inlet

port (400 ml/min). Volatiles were collected on Porapak Q (50 mg,60/80 mesh; Supelco, Bellefonte, PA, USA) in a glass tube (5-mm diam; Alltech Associates, Lancashire, UK) inserted into the collection ports on the top of the vessels. Further, pumps drew air (300 ml/min) through these tubes. Rates were controlled so that more purified air was pumped in than was drawn out, ensuring that unfiltered air was not drawn into the vessel from outside. All connections were made with PTFE tubing (Alltech Associates, Lancashire, UK) with brass ferrules and fittings (North London Valve, London, UK) and sealed with PTFE tape (Gibbs and Dandy, Luton, UK). Volatiles were collected from fruits for 3 d and the Porapak Q filters were eluted with 750 μl of redistilled diethyl ether, providing a solution containing the sample of volatile compounds. Samples were stored in glass vials in a freezer (−20 °C) until use.

Olfactometer Bioassays A Perspex four-arm olfactometer (120 mm diam; Pettersson, 1970) was used to measure behavioral responses of gravid *B. dorsalis* females to headspace samples of volatiles, synthetic standards, and synthetic volatile blends. Prior to each experiment, all glassware was washed with Teepol, rinsed with acetone and distilled water, and baked in an oven overnight at 160 °C. Perspex components were washed with Teepol solution, rinsed with 80 % ethanol solution and distilled water, and left to air dry. Experiments were conducted in a controlled environment room (25±2 °C, 60 % RH).The central area was fitted with a filter-paper base (Whatmann No. 1, 12 cm diam) to provide traction for the walking insects. The olfactometer was illuminated from above by uniform lighting from two 18 W/35 white fluorescent light bulbs screened with greaseproof paper to make it diffuse and was surrounded by black paper to remove any external visual stimuli.

Individual gravid female *B. dorsalis* (20–30-d-old mated females) were introduced through a hole in the top of the olfactometer. Each fly was given 2 min to acclimatize in the olfactometer, after which the experiment was run for 16 min for each replicate. The olfactometer was rotated 90 ° every 2 min to eliminate any directional bias in the room. Air was drawn through the central hole at 200 ml min^{-1} and subsequently exhausted from the room. The central arena of the olfactometer was divided into four discrete odor fields corresponding to each of four glass inlet arms (Fig. S1). Of four glass arms, one contained the treatment and the other three served as controls, unless a choice test was performed that used two different treated arms and two control arms. Test samples (10 μl) were pipetted onto filter paper strips, and the solvent was allowed to evaporate prior to placement in the treatment arm. Filter paper strips with respective solvents (diethyl ether and hexane for natural and synthetic blends, respectively) served as controls in the remaining three arms.

Time spent in each olfactometer arm was recorded with Olfa software (F. Nazzi, Udine, Italy). Ten replicates were carried out for each odor source tested. The first two series of bioassays had one treated arm and three solvent control arms in each replicate. In the first series of bioassays, responses of gravid female *B. dorsalis* to the natural air entrainment samples of ripe mango fruit were studied. In the second series, responses to synthetic compounds were studied. Compounds were tested individually (1 μg dose) and as blends that contained identified compounds that elicited an EAG response at the same concentration and ratio as in the headspace sample. For the first and second series of bioassays, the mean time spent in treated and control regions were compared using a paired *t*-test (Genstat version 12, VSN International) after calculating the mean time spent per control arm for each replicate. In the third series, choice tests between the natural sample and synthetic blends were conducted, and each replicate had two treated arms (natural sample, synthetic blend) and two control arms (solvent blank). Bioassay data (time spent in each odour field, natural sample vs. synthetic blend vs. solvent control) were compared by analysis of variance (ANOVA) after conversion of the data into proportions and a logratio transformation. Means were separated using Fisher's LSD test with α set at 0.05 (Genstat version 12, VSN International).

Electrophysiology Coupled Gas Chromatography-Electroantennography (GC-EAG) Electroantennogram (EAG) recordings were made with Ag-AgCl glass microelectrodes filled with saline solution (7.55 gl^{-1} sodium chloride, 0.64 gl^{-1} potassium chloride, 0.22 gl^{-1} calcium chloride, 1.73 gl^{-1} magnesium chloride, 0.86 gl^{-1} sodium bicarbonate, 0.61 gl^{-1} sodium orthophosphate). The head of a gravid female *B.dorsalis*, anaesthetized by chilling, was separated from the body with a microscalpel. The EAG preparations were obtained by cutting the antennae, still joined together at the base, from the head with a microscalpel and placing them in the indifferent electrode and then excising the tips of both antennae and placing the recording electrode over them. The signals were passed through a high impedance amplifier (UN-06, Syntech, Hilversum, The Netherlands) and analyzed using a customized software package (Syntech).

The coupled GC-EAG system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies) equipped with a cold on-column injector and a flame ionization detector (FID), using an HP-1 column. The oven temperature was maintained at 30 °C for 2 min, and then programmed a 15 °C min-1 to 250 °C, and the carrier gas was helium. The outputs from

the EAG amplifier and the FID were monitored simultaneously and analyzed using the Syntech software package. Peaks eluting from the GC column were judged to be active if they elicited EAG activity in three or more of the six coupled runs carried out.

GC Analysis Samples of volatiles (1 μl) were analyzed on an Agilent 6890N GC equipped with a cool on-column injector, flame ionization detector (FID) and a non-polar HP-1 bonded phase fused silica capillary column (50 m× 0.32 mm i.d., film thickness 0.52 μm). The oven temperature was maintained at 30 °C for 1 min, and then programmed at 5 °C/min to 150 °C, and held for 0.1 min, then 10 °C/min, then 10 °C min/min to 250 °C and held for 20 min. Hydrogen was the carrier gas. Data were analyzed using HP Chemstation software. Quantification of volatiles was performed using a single point external standard quantification method using authentic samples of standards (Skelton et al., 2010)

GC-MS Analysis Coupled Gas Chromatography-Mass Spectrometry (GCMS) Samples (1 μl) were analyzed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 μm) directly coupled to a mass spectrometer (Mat 95-XP Thermo Finnigan). Ionization was achieved by electron impact at 70 eV, 250 °C. The oven temperature was maintained at 30 °C for 5 min and then programmed at 5 °C min^{-1} to 250 °C. Tentative identifications of compounds that elicited an EAG response were determined by comparison of spectra with those of authentic samples in a database (NIST data base 2005), and were confirmed by co-injection of the natural headspace sample with authentic standards and showing peak enhancement on two GC columns of different polarity (HP-1 and DB-WAX; conditions as in the GC analysis section).

Results

Behavioral Responses to 'Alphonso' Headspace Samples In an olfactometer bioassay, responses of gravid female *B. dorsalis* to volatiles collected at different stages of fruit over-ripening were investigated. There was no significant response when flies were exposed to volatiles collected at 3 and 5 days after full ripe of mango fruit. However, flies spent significantly more time (P=0.03) in the treated region of the olfactometer (mean time spent (±S.E.)=5.60± 1.44 min) than in the control regions (mean time spent= 2.01±0.40 min) when a 10 μl aliquot of *M. indica* cv. '*Alphonso*' headspace sample, collected 10 days after full ripe, was used. Samples collected as the fruit became more overripe were more attractive, and the most attractive sample was collected 16 days after full ripe. When flies were exposed to this sample, the mean time spent in the treated region was 7.49±1.52 min (response shown in Fig. 1) compared to a mean time spent in the control region of 1.40± 0.43 min which was a significant difference (P=0.004).

Behavioral Responses to 'Chausa' Headspace Samples A sample collected 21 days after full-ripe elicited a positive behavioral response. Gravid female *B. dorsalis* spent more time in the treated region of the olfactometer compared to untreated controls (P=0.001). Mean time spent in the treated region was 6.59±0.77 min, compared to 2.20±0.28 min in the untreated region (Fig. 2).

Identification of Compounds that Elicited an EAG Response Coupled GC-EAG with female *B. dorsalis* revealed seven compounds from 'Alphonso' volatiles that elicited an EAG response (Table 1). EAG-active compounds were identified as heptane, myrcene, (*Z*)-ocimene, (*E*)-ocimene, allo-ocimene, (*Z*)-myroxide, and γ-octalactone, with the two ocimene isomers being the dominant compounds for 'Alphonso'. Similarly 15 compounds were active in coupled GC-EAG analysis of 'Chausa' volatiles (Table 2). These were identified as 3-hydroxy-2-butanone, 3-methyl-1-butanol, ethyl butanoate, ethyl methacrylate, ethyl crotonate, ethyl tiglate, 1-octen-3-ol, ethyl hexanoate, 3-carene, *p*-cymene, ethyl sorbate, α-terpinolene, phenylethyl alcohol, ethyl octanoate, and benzothiazole. A representative GC-EAG trace is shown in Fig. 3. The quantities of the EAG active compounds present in the mango headspace samples that synthetic blends were based on are listed in Tables 1 and 2 for 'Alphonso' and 'Chausa,' respectively.

Behavioral Responses to Synthetic Compounds All seven 'Alphonso' volatiles that elicited an EAG response were behaviorally active when presented individually at a standard dose (1 μg on filter paper), with female *B. dorsalis*

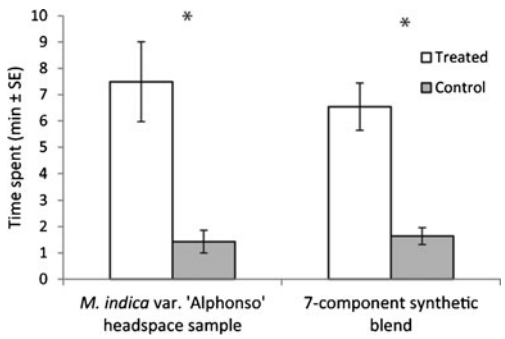

Fig. 1 Behavioral response of female *Bactrocera dorsalis* to mango *Mangifera indica* cv. 'Alphonso' volatiles in the olfactometer: time spent in treated and control arms (N=10). *Significantly different from control (P<0.01). The 7-component synthetic blend contained the compounds listed in Table 1 at the same concentration and ratio as in the natural sample. Volatiles were collected 16 days after full ripe

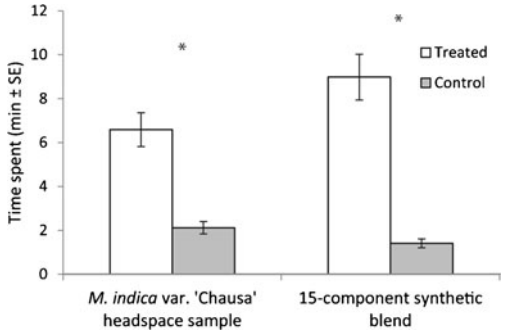

Fig. 2 Behavioral response of female *Bactrocera dorsalis* to mango *Mangifera indica* cv. 'Chausa' headspace sample (collected 21 d after full ripe) and synthetic blend when both odor were sources present together in the same olfactometer (N=11). * Significantly different (P<0.01)

spending significantly longer in the treated region of the olfactometer (Table 3).When presented together as a 7-component blend formulated by using the same ratio and concentration as in the natural sample (Table 1), a similar level of attraction to that observed with the natural sample was achieved (Fig. 1). Furthermore, in a choice test where flies were offered both the natural sample and the synthetic blend, there was no difference in behavioral response (Fig. 4).

Similar results were obtained with the 'Chausa' volatiles, all of which were behaviorally active when tested individually (Table 3).When presented together as a 15-component blend formulated by using the same ratio and concentration as in the natural sample (Table 2), a similar level of attraction to that observed with the natural sample was achieved (P<0.001) (Fig. 2). Furthermore, in a choice test where flies were offered both the natural sample and the synthetic blend, there was no difference in behavioral response (Fig. 5).

Discussion

The current study used GC-EAG to identify compounds from two cultivars of mango, *M. indica*, which were then shown to elicit positive behavioral responses with *B. dorsalis*. Interestingly, the volatile profiles of the two cultivars were quite different, but nevertheless, strong behavioral responses were obtained to headspace samples from both cultivars. The cultivar 'Alphonso' emitted large amounts of (Z)-ocimene, which agrees with the findings of Pandit et al.

Table 1 Electrophysiologically active compounds identified in *M. indica* cv. 'Alphonso' volatiles

Compound	Retention Index (HP-1, non-polar)	Concentration (ng µl^{-1})
Heptane	700	43.4
Myrcene	981	64.5
(*Z*)-Ocimene	1,029	6365.8
(*E*)-Ocimene	1,053	374.4
Allo-ocimene	1,117	5.8
(*Z*)-Myroxide	1,141	11.8
(*RS*)-γ-Octalactone	1,215	81.5

Table 2 Electrophysiologically active compounds identified in *M. indica* cv. 'Chausa' volatiles

Compound	Retention Index (HP-1, non-polar)	Concentration (ng µl^{-1})
3-Hydroxy-2-butanone	700	3.4
3-Methyl-1-butanol	722	0.1
Ethyl butanoate	786	51.4
Ethyl methacrylate	797	0.1
Ethyl crotonate	825	54.1
Ethyl tiglate	922	26.3
(*RS*)-1-Octen-3-ol	968	0.6
Ethyl hexanoate	983	118.6
(*S*)-3-Carene	1,012	99.9
p-Cymene	1,023	0.2
Ethyl sorbate	1,069	16.0
α-Terpinolene	1,086	18.8
Phenylethyl alcohol	1,092	77.3
Ethyl octanoate	1,180	414.9
Benzothiazole	1,193	0.6

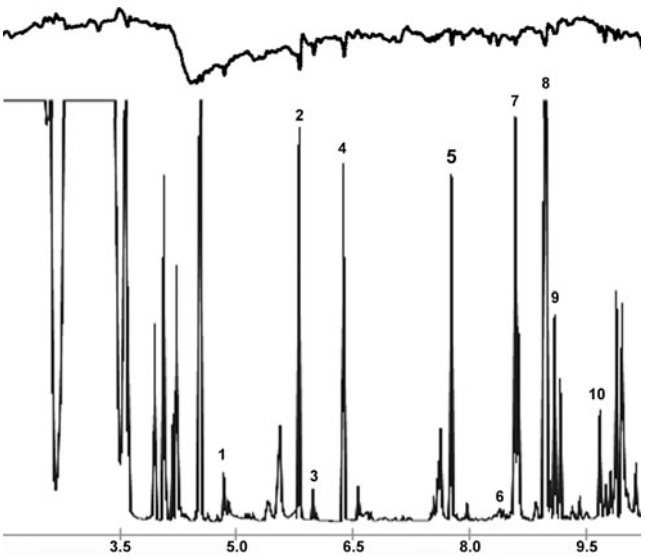

Fig. 3 Representative GC-EAG recording of female *Bactrocera dorsalis* responses to mango *Mangifera indica* cv. 'Chausa' volatiles. The FID peaks marked are those which elicited responses in two or more of the six coupled runs: 1=3-methyl-1-butanol, 2=ethyl butanoate, 3=ethyl methacrylate, 4=ethyl crotonate, 5=ethyl tiglate, 6=1-octen-3-ol, 7=ethyl hexanoate, 8=3-carene, 9=*p*-cymene, 10=ethyl sorbate. Please note other later eluting EAG-active compounds are not shown in this trace

Table 3 Response of gravid female *Bactrocera dorsalis* to compounds (1 μg on filter paper) tested individually in olfactometer bioassay (n=10). Time spent in treated and control regions was compared

Compound	Treated mean (min)	S.E.	Control mean (min)	S.E	*P* value (*t*-test)
'Alphonso' volatiles					
Heptane	4.34	(±0.63)	2.88	(±0.17)	0.045
Myrcene	4.53	(±0.62)	2.91	(±0.30)	0.039
(*Z*)-Ocimene	5.96	(±0.87)	1.74	(±0.40)	0.002
(*E*)-Ocimene	5.31	(±0.64)	2.66	(±0.22)	0.004
Allo-ocimene	4.84	(±0.98)	2.58	(±0.35)	0.049
Myroxide[a]	5.10	(±0.62)	2.02	(±0.33)	0.001
(*RS*)-γ-Octalactone	5.98	(±1.26)	1.87	(±0.30)	0.011
'Chausa' volatiles					
3-Hydroxy-2-butanone	5.58	(±0.82)	2.55	(±0.23)	0.006
3-Methyl-1-butanol	4.80	(±1.02)	2.50	(±0.33)	0.042
Ethyl butanoate	2.76	(±0.48)	1.37	(±0.15)	0.023
Ethyl methacrylate	5.27	(±0.72)	2.44	(±0.34)	0.005
Ethyl crotonate	4.12	(±0.49)	2.02	(±0.26)	< 0.001
Ethyl tiglate	5.83	(±0.59)	2.50	(±0.18)	< 0.001
(*RS*)-1-Octen-3-ol	5.64	(±0.90)	2.28	(±0.31)	0.006
Ethyl hexanoate	5.28	(±0.73)	2.85	(±0.22)	0.011
(*S*)-3-Carene	4.42	(±0.63)	2.86	(±0.31)	0.049
p-Cymene	5.11	(±0.55)	2.59	(±0.20)	0.002
Ethyl sorbate	5.84	(±0.67)	2.56	(±0.30)	0.001
α-Terpinolene	4.68	(±0.67)	2.30	(±0.36)	0.008
Phenylethyl alcohol	4.68	(±0.50)	2.70	(±0.19)	0.002
Ethyl octanoate	4.82	(±0.35)	2.97	(±0.26)	0.003
Benzothiazole	4.92	(±0.46)	2.48	(±0.27)	< 0.001

[a] (*Z*)- and (*E*)- isomers in a 2:1 ratio

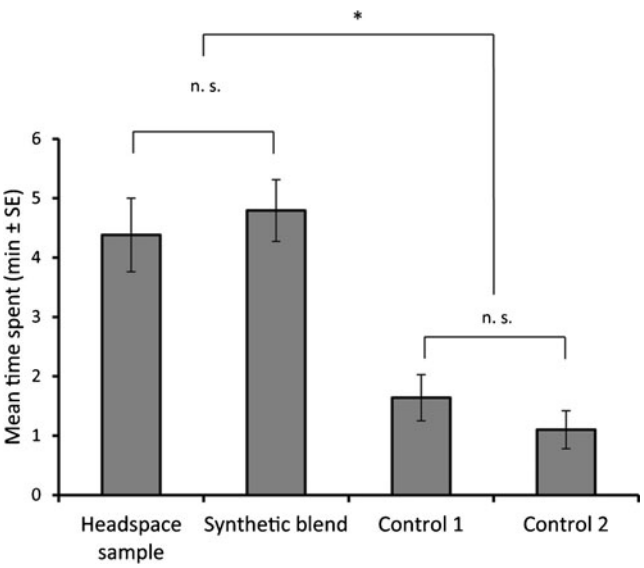

Fig. 4 Behavioral response of female *Bactrocera dorsalis* to mango *Mangifera indica* cv. 'Alphonso' volatiles in the olfactometer: time spent in treated and control arms (N=10). *Significantly different from control (P<0.01). The 7-component synthetic blend contained the compounds listed in Table 1 at the same concentration and ratio as in the natural sample

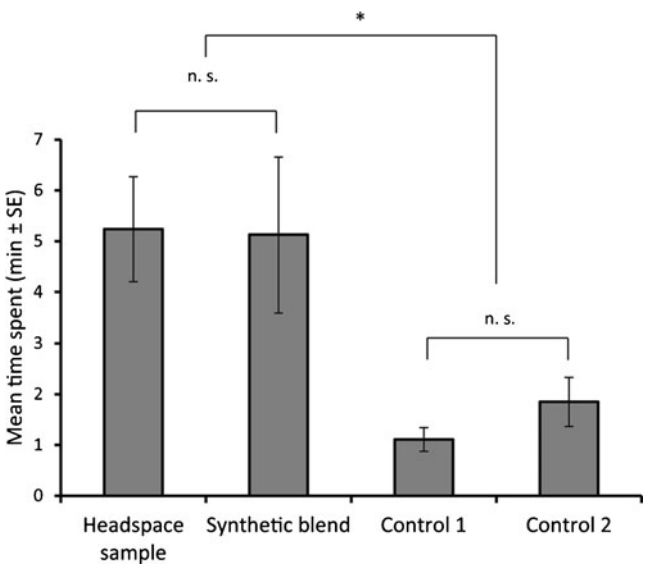

Fig. 5 Behavioral response of female *Bactrocera dorsalis* to mango *Mangifera indica* cv. 'Chausa' headspace sample and synthetic blend when both odor were sources present together in the same olfactometer (N=11). * Significantly different (P<0.01). The 15-component synthetic blend contained the compounds listed in Table 2 at the same concentration and ratio as in the natural sample

(2009). As *B. dorsalis* is highly polyphagous, we would expect it to be attracted to a wider range of host cues than an oligophagous species. Blends of attractants were formulated based on the natural concentration and ratio of compounds in selected headspace samples. These elicited potent attraction in an olfactometer bioassay. Sufficient synthetic compounds were identified to explain the activity of the natural headspace samples because there was no preference in a choice test between the natural sample and the synthetic blend.

Finding a suitable host on which to oviposit is crucial for reproduction of all phytophagous insects (Thompson and Pellmyr, 1991), and olfaction plays an important role in enabling the insect to recognize host plants at distance (Dethier, 1982; Visser, 1986, 1988; Bernays and Chapman, 1994; Pickett et al., 1998). Furthermore, recent studies have suggested that host recognition depends on blends or ratios of volatiles emitted rather just the presence or absence of individual compounds (Bruce et al., 2005; Bruce and Pickett, 2011). However, in the current study, when individual volatiles were offered as a choice against clean air, single compounds did elicit significant attraction. This contrasts with previous studies with *Aphis fabae* where blends were crucial for obtaining positive behavioral responses (Webster et al., 2010). For *B. dorsalis*, blends may be required to elicit attraction outside in the field, and indeed, this will be evaluated in future research.

Chemical cues from the host plant play a major role in the orientation of gravid females to their hosts from a distance, and they determine the probability of alighting on a given host. After landing, the combination of contact chemoreception, visual and physical cues provide further sensory input leading to acceptance or rejection of the oviposition site (Ramaswamy, 1988; Renwick and Chew, 1994). Host plant recognition and selection in insects is determined mainly by the ovipositing female since newly emerged larvae are often limited in their dispersal abilities.

Although gravid female fruit flies can oviposit on physiologically mature green fruits under no choice conditions, several reports have emphasized that fruit ripeness increases the attraction of tephritid fruit flies with overripe, rotten fruit being preferred. Syed et al. (1970) found that *B. dorsalis* adults remained in orchards when there were ripe fruit on the trees. Stark et al. (1991) also reported that the ratio of females to males forging in guava trees increased as the season progressed and guava ripened. This agrees with the observations of Andrei et al. (2000) who suggested that areas with plentiful ripe guava fruit attract females, searching for oviposition sites. Therefore, gravid female *B. dorsalis* may use the odors of overripe fruits as their long-distance orienteering cues while searching for suitable sites for oviposition. These studies suggested that olfactory cues may play a role in attraction of female *B. dorsalis* to host fruit, but the semiochemicals involved were not identified. However, a previous study by Siderhurst and Jang (2006) identified attractants from tropical almond, *Terminalia catappa*, fruit. A preliminary study by Liu and Hwang (2000) reported methyl anthranilate, α-terpineol, ethyl acetate, ethyl butanoate, and cinnamyl alcohol as attractants for female and male *B. dorsalis* from guava (*Psidium guajava*), mango (*M. indica*), citrus (*Citrus grandis*), and carambola (*Averrhoa carambola*) fruits. Of the EAG active compounds identified from mango, *M. indica* (cv. Alphonso) in the present study, myrcene and (*E*)-ocimene were reported to be attractive to both female and male West Indian fruit fly, *Anastrepha obliqua* (Edi et al., 2012). Myrcene, a monoterpene present in the volatiles of white sapote fruit (*Casimiroa edulis*) was attractive to Mexican fruit fly, *Anastrepha ludens* (González et al., 2006). Among the EAG active fractions isolated from cv. Chausa, ethyl butanoate and ethyl hexanoate isolated from *Spondias mombin* fruits were attractive to both sexes of West Indian fruit fly, *A. obliqua* (Jorge et al., 2009).

The current study has shown clearly that mango volatiles elicit attraction of female *B. dorsalis*, and the compounds responsible have been identified. This will make possible future studies where a lure for female *B. dorsalis* may be developed for monitoring and mass trapping applications. Having an attractant for females rather than males greatly increases the scope for using semiochemicals to manage this noxious pest. The next step will be to conduct trapping trials to evaluate the performance of the kairomones identified in the current study under field conditions.

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Pyrethrins Protect Pyrethrum Leaves Against Attack by Western Flower Thrips, *Frankliniella occidentalis*

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Abstract Pyrethrins are active ingredients extracted from pyrethrum flowers (*Tanacetum cinerariifolium*), and are the most widely used botanical insecticide. However, several thrips species are commonly found on pyrethrum flowers in the field, and are the dominant insects found inside the flowers. Up to 80 % of western flower thrips (WFT, *Frankliniella occidentalis*) adults died within 3 days of initiating feeding on leaves of pyrethrum, leading us to evaluate the role of pyrethrins in the defense of pyrethrum leaves against WFT. The effects of pyrethrins on WFT survival, feeding behavior, and reproduction were measured both *in vitro* and *in planta* (infiltrated leaves). The lethal concentration value (LC50) for pyrethrins against WFT adults was 12.9 mg/ml, and pyrethrins at 0.1 % (w/v) and 1 % (w/v) had significantly negative effects on feeding, embryo development, and oviposition. About 20-70 % of WFT were killed within 2 days when they were fed chrysanthemum leaves containing 0.01-1 % pyrethrins. Chrysanthemum leaves containing 0.1 % or 1 % pyrethrins were significantly deterrent to WFT. In a no-choice assay, the reproduction of WFT was reduced significantly when the insects were fed leaves containing 0.1 % pyrethrins, and no eggs were found in leaves containing 1 % pyrethrins. Our results suggest that the natural concentrations of pyrethrins in the leaves may be responsible for the observed high mortality of WFT on pyrethrum.

Keywords Pyrethrum · Pyrethrins · Western flower thrips · *Frankliniella occidentalis* · Natural pesticide · toxicity · *Tanacetum cinerariifolium* · Crop pest

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Introduction

Western flower thrips (WFT), *Frankliniella occidentalis*, is a highly polyphagous insect that causes both direct and indirect effects on plant development and health. The adults and larvae feed on epidermal and subepidermal cells of both meristematic and mature leaf and flower tissues, inhibiting plant growth and development and causing necrotic or light-reflective blotches on the tissue. Furthermore, they indirectly damage plants by transmitting tospoviruses such as tomato spotted wilt virus (Reitz, 2009). As a result, WFT has become the most serious pest in several vegetable and flower crops world-wide (Daughtrey et al., 1997; Reitz, 2009). The widespread use of chemical insecticides to control WFT has led to increasing resistance against the major classes of synthetic insecticides (Broadbent and Pree, 1997; Flanders et al., 2000; Broughton and Herron, 2009). The growing awareness and demand for insecticides that are not environmentally hazardous has stimulated the study of plant-derived compounds for pest control (Boeke et al., 2004). Such compounds could be used as natural pesticides, and in theory, genes responsible for the biosynthesis of those compounds could be isolated and transferred to crops to improve plant defense against WFT (Annadana et al., 2002; Outchkourov et al., 2004).

Among the sources of botanical pesticides, pyrethrins from pyrethrum plants (*Tanacetum cinerariifolium*) represent the economically most important class of compounds with broad

usage both in homes and organic agriculture (Casida, 1973). Pyrethrins are neurotoxins that bind to voltage-gated sodium channels of neuronal cells, causing the channels to remain open (Davies et al., 2007). Pyrethrins comprise a group of six closely related esters, named pyrethrin I and II, cinerin I and II, and jasmolin I and II. They are found in all aboveground parts of the pyrethrum plants, but predominantly in the ovaries of the flower heads (Brewer, 1973). On average, the concentration of pyrethrins is about 0.1 % (dry weight) in leaves and 1-2 % (dry weight) in flowers (Baldwin et al., 1993). Assuming a water content of 90 %, pyrethrins account for around 0.01 % of the fresh weight of leaves and 0.1-0.2 % of the fresh weight of flowers. Pyrethrins are effective against a broad spectrum of insects, while their toxicity for mammals is very low, allowing their use as a preharvest spray (Casida and Quistad, 1995; Schoenig, 1995). Western flower thrips are sensitive to synthetic pyrethroids (Thalavaisundaram et al., 2008), but there is no report on the effect of natural pyrethrins against WFT. Pyrethrins might provide pyrethrum with a broad range protection against many different insect pests, but the role of pyrethrins in pyrethrum defense has not been studied.

In initial experiments, we observed that WFT adults died within one day when fed pyrethrum leaves, but that they were abundant in open flowers. Here, we tested the hypothesis that pyrethrins are responsible for protecting pyrethrum leaves against WFT by assessing adult and embryo toxicity, and by examining feeding and oviposition deterrence both *in vitro* and *in planta*.

Methods and Materials

Field Observation A pyrethrum field close to Luxi, Yunnan province, China, was used for surveying thrips populations (24°27'10.34"N-103°32'21.01"E). The field was 0.5 ha in size, and the presence of insect species was monitored during the flowering period of spring 2010, when the flowers were predominantly in developmental stages 2–5 [numbered according to Casida (1973)]. To assess populations of small resident insects including thrips, flowers at each developmental stage were collected in each one of 3 blocks of the field. Each flower was taken by the stem and turned upside down into a jar containing 75 % alcohol. Flowers were fully immersed and vigorously stirred. The procedure was repeated until each jar contained the insects from 100 flowers from a single block and at a particular stage. The number of insects of each species for each stage was scored. In the case of thrips, the number of adults and larvae were scored separately. Among all collected thrips, 30 were randomly picked and identified, where possible to the species level.

Insects and Plant Material Used in Laboratory Experiments A population of WFT was mass-reared on flowering chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25±2°C. In this study, only adult female thrips were used. The chrysanthemum plants used for bioassays were from the same cultivar, but were grown in an insect-free compartment of the greenhouse under the same light and temperature conditions. All bioassays were conducted in a climate room at 20–22°C with a L16:D8 photo regime.

Insecticide Pyrethrum oil (70 % w/w) had been extracted from dried and ground pyrethrum flower heads with liquid CO_2 leaving no solvent residue (Honghe Senju Biological Co. Ltd., Yunnan, China). Butylated hydroxytoluene (BHT) had been added to the oil (1 %) to prevent oxidation. We confirmed the concentration and composition of the oil by Gas chromatography–mass spectrometry comparison to a pyrethrin standard (Nguyen et al., 1998). Since the major insecticidal compounds in pyrethrum have long been known as pyrethrins (Casida, 1973), the effect of pyrethrum oil was considered to be the effect of pyrethrins. When calculating the concentrations of pyrethrins in different solutions, the percentage of pyrethrins in the oil (70 %) was taken into account. For example, 1 % (w/v) pyrethrins was prepared by dissolving 14.3 mg pyrethrum oil in 1 ml solvent.

In vitro Bioassays-Toxicity Assays The toxicity of pyrethrins was evaluated by topical application to thrips (Robb et al., 1995). Pyrethrum oil was dissolved in acetone to achieve a concentration range of 1 to 30 mg pyrethrins per ml, and the solutions were applied to the thorax with a 10-μl glass syringe at 1 μl per thrips. The droplet briefly covered the thorax of the insect and also the paper support before evaporating in a few seconds, leaving a residue both on the insect and the support. Acetone alone was used as control. After treatment, all thrips were transferred to Petri dishes containing a piece of chrysanthemum leaf embedded in an agar substrate. Mortality was assayed after 24 h by counting the number of insects that did not respond to prodding with a fine brush. Six replicates were used for each concentration, and 10 thrips were used per replicate. Percent mortality was corrected for mortality observed in acetone control using Schneider-Orelli's formula (Schneider-Orelli, 1947). Data were analyzed using probit analysis (Finney, 1977).

In vitro Bioassays-Choice Assays with Topically Applied Pyrethrins A dual-choice leaf disk assay was used to determine the deterrent effect of pyrethrins on WFT. All leaf disks (diam 1.6 cm) were punched from chrysanthemum leaves of similar leaf age. Pyrethrum oil was dissolved in 0.2 % (v/v) aqueous Tween-80 to achieve 3 concentrations of pyrethrins: 0.01, 0.1, and 1 % (w/v). Control leaf disks were sprayed with solvent solution (0.2 % Tween-80), and

Table 1 Frequencies of small insect species living on pyrethrum flowers in the field

Insects[a]	Frequency (%)	Species[b]	Frequency (%)
Thripidae (thrips)	98	*Thrips tabaci*	43
		Frankliniella occidentalis	25
		Thrips flavus	21
		Thrips palmi	3
		Other species	6
Nysius sp.	1.9	n.d.	1.9
Chrysoperla/Chrysopa sp. (lacewing larva)	0.05	n.d.	0.05

[a]A total of 1200 insects were collected to count the frequencies of different insects. [b] A total of 30 thrips were used to identify species. N.d., not determined

test leaf disks were sprayed with the pyrethrin solutions using a Potter Precision Laboratory spray tower, which produced a uniform deposit (3 μl/cm^2) of solution on the leaf disks. After overnight starvation, WFT were anaesthetized on ice. Groups of 10 WFT were positioned between a control and a test leaf disk placed abaxial side up and 2 cm apart on a 1.5 % (w/v) agar-bed in a Petri dish (7 cm diam). After positioning the thrips, the Petri dish was covered by a 120 μm mesh size nylon mesh lid to prevent condensation. The number of WFT on each leaf disk was recorded 0.25, 1, 2, 4, 20, and 28 h after the release of the WFT. Each concentration was replicated with 12 leaf disks. At each time point, a Student's paired *t*-test was used to assess the significance of the differences in the mean number of WFT between test and control.

In vitro Bioassays-Oviposition Assays Oviposition-deterrent effects were assayed with a non-choice method slightly modified from Annadana et al. (2002). The assay was conducted in Perspex ring cages (3 cm in length and 3.5 cm diam), which were closed with a nylon mesh at the bottom. Pollen of Scotch pine (*Pinus sylvestris* L.) was supplied in a small open tube as food source for WFT. After placing 10 WFT in a cage, the top was sealed with two layers of stretched Parafilm, with 300 μl aqueous solution in between the layers. The solutions used were water, 0.2 % Tween-80, or pyrethrins at 0.01, 0.1, or 1 % dissolved in 0.2 % Tween-80. WFT were allowed to adapt to the diet (pollen and water) for 3 d, and then every day for 5 d fresh test solution was provided. All eggs were deposited in the solutions, and were counted daily under a binocular microscope. Each solution was replicated 6 times. Data were analyzed by a one-way ANOVA and a mean separation test was conducted using LSD (α=0.05).

In vitro Bioassays-Embryo Development Assays Around 200 WFT were kept in a Perspex ring cage (7 cm in length and 9 cm diam) to allow oviposition in a water solution as described above. Eggs laid on the same day were collected with a fine brush under a binocular microscope and then transferred to 2 layers of filter paper in Petri dishes (3.5 cm diam). The filter papers were drenched in 300 μl of assay solution (water, 0.2 % Tween-80 or pyrethrins at 0.01, 0.1, or 1 % in 0.2 % Tween-80) so that each paper was fully wetted but had no excess solution. After transferring the eggs, the Petri dishes were closed with lids and sealed with Parafilm. The developmental status of eggs was monitored every day for 6 d. To facilitate the observations, the bottoms of the Petri dishes were marked with lines that could be seen through the filter paper from the top, and the eggs were placed on filter paper along these lines. This facilitated finding the eggs under the microscope, and the viability of hatched larvae was assessed in terms of their ability to move away (>0.5 cm) from the hatch position. Four replicates of 10 eggs were used for each assay. Data were analyzed by a one-way ANOVA and mean separation test was conducted using LSD (α=0.05).

In planta Bioassays-Mortality Assays on Pyrethrum Leaves Mature pyrethrum leaves were harvested in November from a field in the Netherlands when they were still flowering (51°59'22.08"N-5°39'44.75"E, Wageningen).

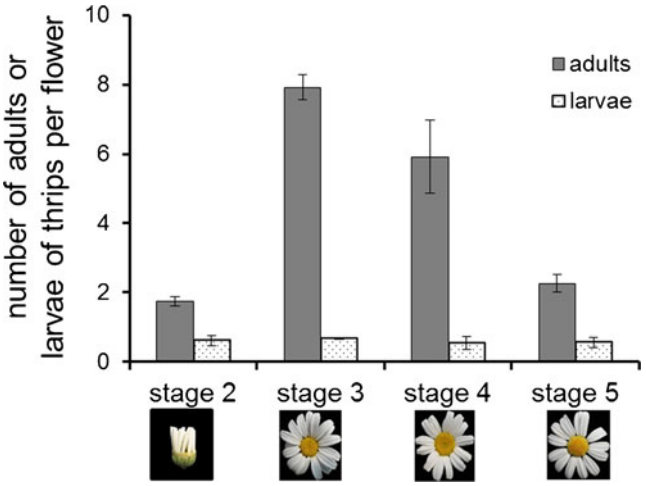

Fig. 1 Distribution of thrips adults and larvae across different developmental stages of pyrethrum flowers in the field. Error bars indicate SE (N=300 per stage). Stage 2, vertical ray florets; stage 3, horizontal ray florets and first row of disk florets open; stage 4, 3 rows of disk florets open; stage 5, all disk florets open

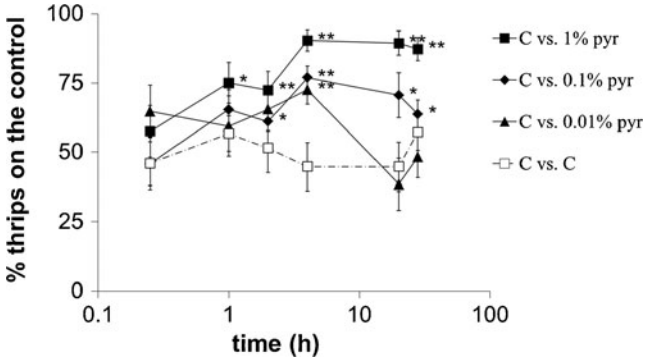

Fig. 2 Dual choice assays of western flower thrips on chrysanthemum leaf disks sprayed with 0.2 % Tween (control) or 0.2 % Tween with 0.01 %, 0.1 % or 1 % pyrethrins. The presence on either leaf disk was visually recorded 0.25, 1, 2, 4, 20 and 28 h after WFT release. The x-axis represents ^{10}log-transformed time data. Asterisks indicate significant differences to the control (*: $P<0.05$; **: $P<0.01$). C, control. Pyr, pyrethrins. Error bars indicate SE ($N=120$ per treatment)

Two or three pieces of leaves were placed, abaxial side up, on 1 % (w/v) agar in a Petri dish (7 cm diam). After transferring 10 WFT to each Petri dish, dishes were covered with lids with gauze. Petri dishes with two leaf disks (1.6 cm diam) of chrysanthemum leaves, with a total mass similar to the mass of the pyrethrum leaf samples, or with only agar were used as controls. Six replicates were carried out for each treatment. The mortality of WFT was recorded daily for 3 d.

In planta Bioassays-Choice Assays To test the *in planta* activity of pyrethrins against WFT, pyrethrins were infiltrated into whole chrysanthemum leaves as described by Ratcliff et al. (2001). Leaf disks (diam 1.6 cm) were punched from the infiltrated leaves, avoiding the infiltration points so that WFT would not contact pyrethrins directly except at the edge of the disk. In the initial experiments, we infiltrated only water into the leaves and determined that on average 29.1 mg (± 2.1 mg) water could be infiltrated into each leaf disk (6 replicates). As the fresh weight of each leaf disk was on average 45.3 mg (± 1.2 mg), we infiltrated 0.025, 0.25, or 2.5 % pyrethrins solution to bring the concentrations to 0.01, 0.1, or 1 % pyrethrins. Leaf disks infiltrated with 0.2 % Tween-80 were used as control. The assay and data analysis were conducted as described above for the choice assays with topically applied pyrethrins. The number of WFT on each leaf disk was recorded 0.25, 1, 2, 4, 20, and 28 h after the release of the WFT.

In planta Bioassays-Reproduction Assays To test the effects of pyrethrins on oviposition and hatching of larvae, WFT were assayed with chrysanthemum leaf disks as described by De Kogel et al. (1997), with slight modifications. Leaf disks were punched from untreated leaves, from leaves infiltrated with 0.2 % Tween-80, or from leaves containing 0.01, 0.1, or 1 % pyrethrins in Tween solution. WFT were placed on leaf disks (1.2 cm diam, 2 WFT/disk), which were embedded, abaxial side up, on agar in wells of 24-well Greiner plates. Plates were covered with Parafilm, and every well was carefully sealed by pressing the Parafilm on the edge of each well. WFT were allowed to oviposit for 48 h and were then removed, with simultaneous assessment of mortality. Subsequently, half of the leaf disks from each plate were used to determine the number of eggs, and the other half of the leaf disks were used to determine the number of hatched larvae. To determine the number of eggs, the leaf disks were boiled in water for 3 min so that the eggs were clearly visible under a binocular microscope with transmitting light. To determine the number of hatched larvae, the leaf disks were transferred to Petri dishes containing water and incubated in a climate chamber (25°C, L16:D8) for 5 d to allow the larvae to hatch. The hatched larvae were counted under a binocular microscope. One plate containing 24 identical leaf disks was used for each treatment. Data were analyzed by a one-way ANOVA and mean separation test was conducted using LSD ($\alpha=0.05$).

Results

Natural Distribution of Insects in Pyrethrum Fields Our field survey in China showed that several thrips species were the most abundant (98 %) insects on pyrethrum flowers (Table 1). In addition, a few *Nysius* species (Heteroptera:

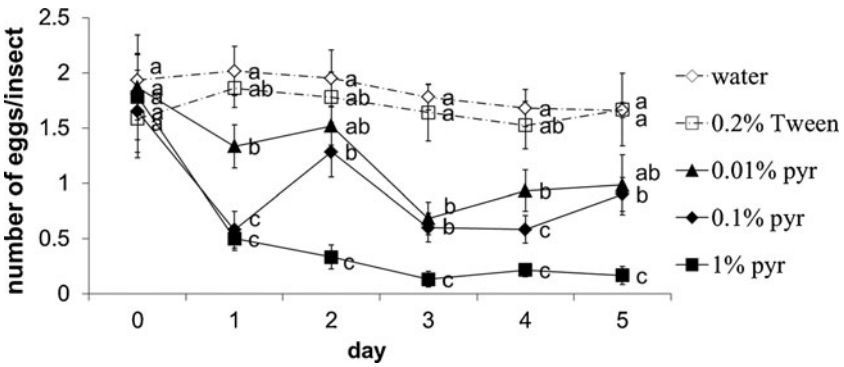

Fig. 3 The number of eggs deposited by western flower thrips when supplied with different concentrations of pyrethrins starting on Day 1. Data points with the same letter within days are not significantly different, $P<0.05$. Pyr, pyrethrins. Error bars indicate SE ($N=60$ per treatment)

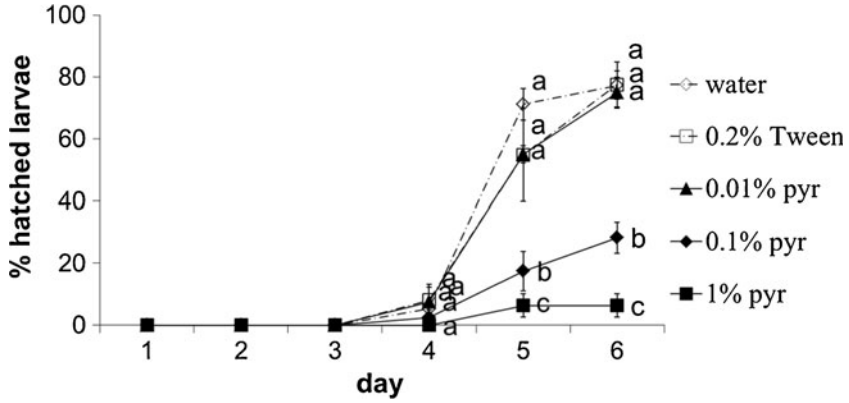

Fig. 4 Percentage of larvae hatching from western flower thrips eggs during incubation with different concentrations of pyrethrins. Data points with the same letter within days are not significantly different, $P<0.05$. Pyr, pyrethrins. Error bars indicate SE ($N=40$ per treatment)

Lygaeidae) (1.9 %) and lacewing larvae (Neuroptera) (0.05 %) were found. A total of 30 individuals were identified to species level; the thrips species found were mainly *Thrips tabaci* (44 %), *Frankliniella occidentalis* (western flower thrips, or WFT, 25 %), and *Thrips flavus* (22 %). The number of thrips in flowers was dependent on the flower's developmental stage (Fig. 1). The number of thrips increased until stage 3 (the first row of disk florets are open), and then decreased in later stages. The thrips found inside flowers were mainly adults. Larvae accounted for 7-26 % of the total number of thrips per flower, depending on the flower developmental stage (Fig. 1).

Effect of Pyrethrum Leaves on Mortality of WFT We assayed the suitability of pyrethrum leaves as a food substrate for WFT. Mortality could be as high as 80 % within 3 days, although the degree of mortality depended on the plant source (data not shown). When only water and agar were provided, with no plant-based food, only 20-30 % WFT died in 3 days. All WFT feeding on control chrysanthemum leaves remained alive during the 3-day-experiment. This showed that the mortality of WFT was caused by a toxic principle of pyrethrum leaves rather than deterrence or starvation.

The toxic principle of pyrethrum plants against insects has long been known to be a group of 6 pyrethrin esters (Casida, 1973). We were, therefore, interested in specifically testing the effect of pyrethrins against WFT.

In vitro Insecticidal and Deterrent Effects To determine the effects of pyrethrins against WFT, pyrethrins were tested *in vitro* at different concentrations on adult mortality, feeding, oviposition, and embryo development.

The mortality of WFT female adults increased with the concentration of topically applied pyrethrins in the range of 1 to 30 mg/ml. Probit analysis showed that the LC_{50} and LC_{90} of pyrethrins was 12.9 mg/ml (with 95 % confidence limit of 10.9-14.8 mg/ml) and 39.0 mg/ml (with 95 % confidence limit from 30.7 to 50.4 mg/ml), respectively.

Thrips were significantly deterred from feeding by 0.1 % and 1 % pyrethrins (Fig. 2). When given a choice between chrysanthemum leaf disks coated with 0.2 % Tween (control) or 0.1 % added pyrethrins, after 2 h significantly more (61-77 % of thrips) settled on control leaf disks. Pyrethrins at 1 % were more highly deterrent. Within 1 h, 72-90 % of thrips chose control leaf disks. For both concentrations of pyrethrins, the maximum deterrent effect was reached at 4 h. Application of 0.01 % pyrethrins on leaf disks did not show significant deterrent effects except at the 4 h time point (Fig. 2).

Pyrethrins negatively affected oviposition by WFT (Fig. 3). The carrier, 0.2 % Tween-80, did not affect the oviposition of thrips compared to water throughout the experiment, but WFT oviposited significantly fewer eggs with increasing pyrethrin concentrations during the 5-day experiment (Fig. 3).

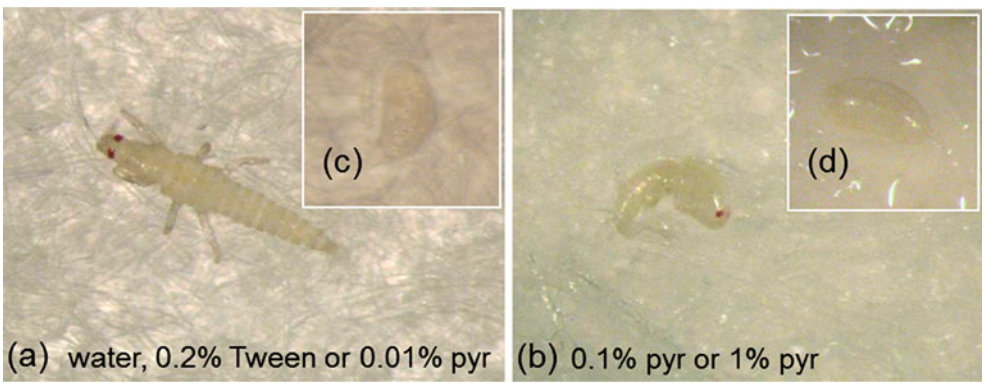

Fig. 5 Effects of pyrethrins on embryo development of western flower thrips at day 5. (a), larva hatched in solutions of water, 0.2 % Tween or 0.01 % pyrethrins at day 5; (b), abnormally developed embryos in solutions of 0.1 % and 1 % pyrethrins at day 5; (c) and (d), embryos before treatment

Table 2 Mortality, number of eggs and hatched western flower thrips larvae per leaf disk on chrysanthemum leaf disks infiltrated with different concentrations of pyrethrins

Treatment of leaf disks	Mortality (%)	Eggs	Hatched larvae
Untreated leaf disks	0 a	2.0±0.4 a	1.4±0.3 a
Leaf disks containing 0.2 % Tween	0 a	1.7±0.5 a	1.3±0.3 ab
Leaf disks containing 0.01 % pyrethrins	25.0±6.7 b	1.3±0.3 ab	0.8±0.2 b
Leaf disks containing 0.1 % pyrethrins	29.2±7.9 b	0.7±0.2 bc	0.1±0.1 c
Leaf disks containing 1 % pyrethrins	68.8±9.3 c	0 c	0 c

Values (mean±SE, N=48 per treatment) followed by the same letter within a column are not significantly different (ANOVA: P>0.05)

The development of eggs was inhibited by 0.1 % and 1 % pyrethrins. About 80 % of larvae hatched when the eggs were incubated with water, 0.2 % Tween, or 0.01 % pyrethrins, while only 28 % or 6 % of the larvae hatched when the eggs were incubated with 0.1 % or 1 % pyrethrins, respectively (Fig. 4). In the latter two treatments, the embryos that did not develop into larvae had severely stunted and abnormal shapes (Fig. 5), and dried out after a few days.

In planta Insecticidal and Deterrent Effects To study *in planta* activity of pyrethrins against WFT, thrips were assayed with chrysanthemum leaves that had been infiltrated with pyrethrins to contain 0.01, 0.1, or 1 % pyrethrins. In this experiment, the pyrethrins could not be contacted directly by thrips except by feeding, and the source of nutrition consisted of leaves instead of pollen.

In the reproduction assay, thrips fed with chrysanthemum leaf disks containing pyrethrins exhibited higher mortality and lower reproduction rates compared to those fed with untreated leaf disks or leaf disks containing 0.2 % Tween (Table 2).

In the dual-choice assay, chrysanthemum leaves containing 0.1 % and 1 % pyrethrins showed significant deterrent effects on thrips within 15 min of release (Fig. 6). A total of 74-93 % of the thrips settled on the control leaf disk when the other leaf disk contained 0.1 % pyrethrins, and 85-95 % thrips settled on the control leaf disk when the other leaf disk contained 1 % pyrethrins. Chrysanthemum leaves containing 0.01 % pyrethrins did not show significant deterrent effects.

Discussion

Pyrethrins, well-known natural insecticidal compounds, are found exclusively in and extracted from the composite flowers of pyrethrum (*Tanacetum cinerariifolium*), which belongs to Anthemideae tribe within the Astaraceae family (Casida and Quistad, 1995). Remarkably, the potential role of pyrethrins in pyrethrum plant defense has not been studied. Here, we report that western flower thrips (WFT) adults thrive on pyrethrum flowers, but die within a few days on pyrethrum leaves. The hypothesis that pyrethrins are responsible for protecting pyrethrum leaves against WFT was tested by spraying or infiltrating pyrethrins to leaves of chrysanthemum, a related pyrethrins-free species belonging to the same tribe. We assessed toxicity to the adult and

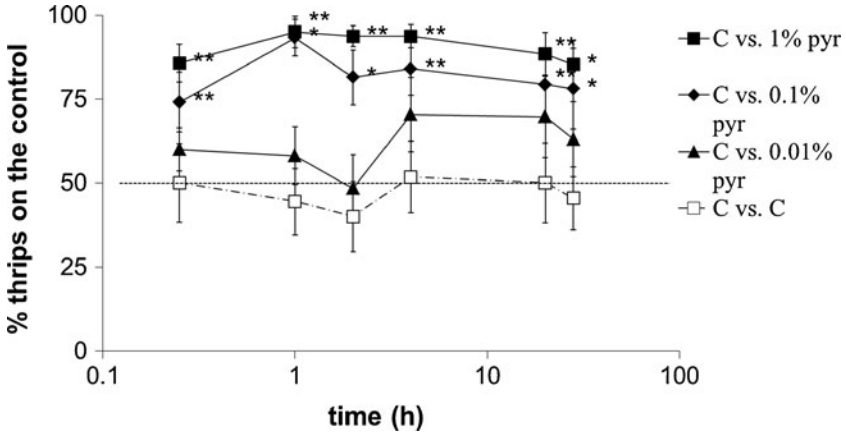

Fig. 6 Percentage of western flower thrips settled on the control chrysanthemum leaf disk in dual choice assays of leaf disks containing 0.2 % Tween with or without 0.01 %, 0.1 %, or 1 % pyrethrins. The solutions were infiltrated into chrysanthemum leaves. The choices were recorded 0.25, 1, 2, 4, 20, and 28 h after WFT release. The x-axis represents ^{10}log-transformed time data. Asterisks indicate significant differences to the control (*: P<0.05; **: P<0.01). C, control. Pyr, pyrethrins. Error bars indicate SE (N=120 per treatment)

embryo stages of WFT, and negative effects on feeding and oviposition both *in vitro* and *in planta,* and found that the natural concentrations of pyrethrins present in leaves have strong negative effects on WFT. We speculate that the thrips found on pyrethrum flowers survive on pollen that is devoid of pyrethrins (T. Yang, unpublished data).

For many populations of WFT, resistance has been reported for some synthetic insecticides (Espinosa et al., 2005). Furthermore, many synthetic insecticides are harmful for human health and the environment. It is relevant, therefore, to find natural insecticides effective against WFT. Previously, several other plant-derived compounds were tested for their insecticidal effects against WFT adults. For example, carvacrol at 1 % and thymol at 0.1 % and 1 % significantly reduced the oviposition rate of WFT when these compounds were sprayed on leaf disks, but neither compound affected the feeding activity of WFT (Sedy and Kosehier, 2003). Salicylaldehyde (0.1 % and 1 %) and methyl salicylate (0.1 % and 1 %) were also tested. Within 24 h of applying 1 % methyl salicylate to bean or cucumber, the feeding and oviposition activities of thrips females were significantly reduced (Koschier et al., 2007). The effect on the insect could be a result of changes in the plant induced by methyl salicylate, since it is a plant hormone involved in induced resistance (Pieterse et al., 2009). A series of commercially available plant-derived essential oils tested at recommended concentrations (0.02-0.5 %), including neem oil, rosemary oil, peppermint oil, garlic oil, and cottonseed oil, caused less than 30 % mortality within 7 days (Cloyd et al., 2009). Compared to other plant-derived compounds, pyrethrins are highly effective against WFT. Our results showed that 0.1 % and 1 % pyrethrin solutions sprayed on leaf disks significantly deterred WFT at 4 h, and topically applied pyrethrins were toxic to adults at an LC_{50} value of 12.9 mg/ml (1.3 %). By mimicking the natural site of pyrethrin accumulation by infiltration of leaves, we found that 1 % pyrethrins caused 69 % mortality and completely inhibited oviposition. Furthermore, 0.1 % pyrethrins was strongly deterrent and resulted in abortion of 95 % of the embryos, while as little as 0.01 % pyrethrins caused 25 % mortality in 2 days. We propose, therefore, that the natural concentrations of pyrethrin in pyrethrum leaves, around 0.01 % by fresh weight, accounts for the observed high mortality of thrips adults on this plant.

Insecticides have not been reported previously to affect the development of WFT embryos. WFT eggs are embedded in plant tissues (Childers, 1997), and as a result they are unlikely to be affected by non-systemic chemicals that are applied on the surface of plants. However, pyrethrins naturally accumulate inside pyrethrum tissues, stored in what appear to be unstructured intercellular cavities (M.A. Jongsma, unpublished observations). Therefore, besides feeding and oviposition deterrence, the embryo-toxic effect of pyrethrins is a third component that contributes to their effect for plant defense against WFT (Figs. 4 and 5).

Compared to some synthetic insecticides, the toxicity of natural pyrethrins against WFT in the absence of synergists was not high. In previous studies using topical application methods, the LC_{50} values of insecticides tested against susceptible WFT strains ranged from 10 to 83 μg/ml for pyrethroids, 20 to 960 μg/ml for carbamates, and 49 to 522 μg/ml for organophosphates (Espinosa et al., 2005; Robb et al., 1995). The LC_{50} value of pyrethrins against WFT by topical application was determined as 12.9 mg/ml, and the action of pyrethrins was, therefore, 10 to 1000-fold weaker than for these synthetic pesticides. On the other hand, pyrethrins did show much stronger negative effects on feeding behavior and reproduction, which may be explained by the action of pyrethrins on the nervous system, resulting in disordered function of excitable (nerve and muscle) cells (Bradberry et al., 2005). At 0.01 % (100 μg/ml), pyrethrins not only caused mortality of adults and embryos, but also significantly reduced oviposition (Table 2). All these factors together cumulatively affect the life history parameters. As a result WFT damage on pyrethrin-containing leaves may be virtually absent, and virus transmission also may be strongly reduced. We hypothesize that if plants such as closely related chrysanthemum species, which do not contain any pyrethrins, were genetically engineered to produce pyrethrins, their resistance to WFT in leaves could be significantly improved.

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Electrophysiological and Behavioral Responses of the Black-Banded Oak Borer, *Coroebus florentinus*, to Conspecific and Host-Plant Volatiles

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Abstract Aspects of the chemical ecology of the black-banded oak borer, (BBOB) *Coroebus florentinus* (Coleoptera: Buprestidae), were studied. Odors produced by males and females were similar, both qualitatively and quantitatively. Nonanal, decanal, and geranylacetone, identified in the headspace of both sexes, elicited strong electroantennographic responses from male antennae, but not from female antennae. In dual-choice olfactometer experiments, a blend of these three compounds was attractive to both sexes; males responded to decanal alone, while females responded to geranylacetone alone, suggesting that these compounds are responsible for activity of the blend to the respective sexes. Antennae of both sexes responded electroantennographically to the green leaf volatiles (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate, all identified from the host plant *Quercus suber*. In behavioral experiments, only females were attracted to host-plant odors, and in tests with synthetic compounds, females were attracted to (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate. It is likely that these compounds play a role in foraging and/or oviposition behavior of BBOB females.

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Introduction

Chemical signals produced by Coleoptera have been the subject of numerous studies on occurrence, biosynthesis, and biological significance (Francke and Dettner, 2005). However, some coleopteran families, notably the Buprestidae, have received little attention, except for the emerald ash borer (EAB), *Agrilus planipennis* (Fairnaire) an invasive species originating from Asia that has caused considerable mortality of ash trees in the US and Canada (Bartelt et al., 2007; De Groot et al., 2008; Francese et al., 2008; Lelito et al., 2008, 2009; Silk et al., 2009, 2011; Crook and Mastro, 2010), and some jewel beetles (Montgomery and Wargo, 1983; McIntosh et al., 2001). The black-banded oak borer (BBOB), *Coroebus florentinus* (Herbst) (Coleoptera: Buprestidae), together with its sister species *C. undatus,* damages cork trees, causing a reduction in the production of high quality cork. The cork oak, (*Quercus suber* L.), is a valuable endemic plant of the Mediterranean region, planted over more than 2.5 million ha worldwide (Institute C.M.C. 1999, http://www.iprocor.org), mainly throughout Portugal and Spain (Soria et al., 1992; Borges et al., 1997). Approximately 340,000 tons of cork, worth some 1.5 billion US dollars, are produced annually (http://www.realcork.org), from which wine stoppers are the most visible and profitable product, with an estimated 13 billion stoppers being produced every year (http://www.corkqc.com).

Following eclosion, BBOB larvae feed inside the bark of young and healthy branches of the tree, making galleries. Adults emerge around the summer solstice, feed in groups on *Quercus spp.* foliage, and live 2–3 weeks before mating and ovipositing. The insect does not kill standing trees, but its attack provokes dryness and yellow leaves, resulting in the death of branches and shoots (Soria and Ocete, 1993; Lombardero et al., 1996). To date, no effective control treatments have been established, as larvae feed underneath the bark, thus making control with insecticides impractical. Moreover, a chemical insecticide would contaminate cork and pose a risk to humans.

Mate location in buprestids is facilitated by host selection, combined with visual or tactile cues (Carlson and Knight, 1969; Gwynne and Rentz, 1983). For example, the EAB is thought to use host-plant volatiles to locate hosts (Rodriguez-Saona et al., 2006; Grant et al., 2010), followed by visual cues to locate mates, rather than long-range sex pheromones (Lelito et al., 2007, 2008). However, other investigations have shown two female-specific hydrocarbons as contact pheromones that are probably involved in mate recognition (Lelito et al., 2009; Silk et al., 2009). A recent study showed that a volatile pheromone produced by EAB, in combination with foliar volatiles, was highly attractive to these beetles (Silk et al., 2011). In the BBOB, male antennae are much longer and contain more olfactory *sensilla basiconica* than do female antennae (Fürstenau, unpublished), which suggests a possible role for a sex pheromone in this species. Herein, we present initial studies on the chemical ecology of the BBOB, with the aim of developing an environmentally friendly approach to control this pest.

Methods and Materials

Chemicals Nonanal (95%), decanal (99%), (*E*)-2-hexenal (98%), (*E*)-2-hexenol (96%), 1-hexanol (98%), (*Z*)-3-hexenyl acetate (98%), *n*-hexyl acetate (99%), and dodecyl acetate (97%), were purchased from Sigma-Aldrich Química (Madrid, Spain). Geranylacetone [(*E*)-6,10-dimethyl-5,9-undecadien-2-one], containing 40% nerylacetone, was obtained from TCI Europe (Zwijndrecht, Belgium). *n*-Hexane [J.T. Baker, Deventer, Holland; analytical purity >95% by gas chromatography (GC)] was used as solvent. Isopropyl *n*-dodecanoate was obtained by esterification of dodecanoic acid with isopropyl alcohol in refluxing sulfuric acid. The product was a colorless oil in 99% yield and 97% purity (by GC), and was characterized by IR, ^{1}H NMR, ^{13}C NMR, and gas chromatography–mass spectrometry (GC-MS).

Insects Live adult BBOB were obtained from woods in the provinces of Girona (NE Spain), Valencia (E Spain), and Madrid (center of Spain). Cork oak trees affected by insect attacks (branches at the treetop with yellowish leaves) were identified at the end of May, from 2005–2010. Collected branches (700–1000 per year, ca. 30 cm long×4 cm diam.) were placed in cardboard boxes (80 x 40 x 40 cm), and half stored at 5–9°C, 50–70% RH for 4 wk so as to retard the emergence of adults. This allowed us to work with insects for a longer period than the usual life expectancy of adult BBOB of around 3 wk. The remaining branches were brought to the laboratory and placed in light-sealed cardboard boxes (50 x 40 x 40 cm), with a perforated lid and a removable glass container (11 cm long×7 cm diam.) fixed in the middle of the front wall. The light passing through the glass attracted emerging adults inside the box. Glass containers were checked daily, and the insects were collected and sexed. The sexes were distinguished based on a) body size (females, at 1.5 cm long, are up to 1.2-fold larger than males), and b) length of antennae (male antennae are 0.4 mm long and typically twice as long as those of females). The emergence of adults kept at room temperature as pupae was around 60%, whereas this value decreased to 40% for pupae previously maintained in the cold. Adults were separated by sex and housed in square glass containers (15 x 15 cm), with a removable plastic lid with wire gauze on top, at 26±2°C, 50±10% RH, and a 14:10 L:D photoperiod. Fresh *Q. suber* leaves were provided to the insects every 2 d. Beetles, aged 8–18 d, were assumed to be "mature" (as described for EAB; Lelito et al., 2009) and used in the bioassays.

Collection and Analysis of Insect Volatiles Charcoal-filtered air (350 ml.min^{-1}) was passed over separated males (N=20) and females (N=20), in a glass, trapping chamber (15 cm length, 3 cm O.D.) for 24 h at ambient temperature. Odor collections from empty chambers were used as blanks. Collections with and without insects were replicated 5 times. Volatiles were trapped on Porapak Q-containing tubes (150/175 mg, 50/80 mesh; Supelco, Bellefonte, PA, USA), placed at the outlet of the trapping chamber. The adsorbent was rinsed with 2 ml of hexane to elute the volatiles. Each extract contained 480 beetle-hour equivalents and was stored at −20°C prior to chemical analysis or electrophysiological assays. For GC-MS analyses, 100 µl of extract were concentrated under a gentle nitrogen stream to 1–2 µl, and 1 µl of a 100 ng.µl^{-1} solution of dodecyl acetate in hexane added as internal standard (IS). The whole (1–3 µl) volume of a sample was injected splitlessly into a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain). Helium (1 ml.min^{-1}) was the carrier gas, and the column used was an HP-5MS (30 m×0.25 mm I.D. × 0.25 µm; Agilent Technologies, Madrid, Spain) temperature programmed from 60°C (held for 5 min.) to 280°C at 5°C.min^{-1}. The MS was used in the electron impact (EI) mode at 70 eV, with a source temperature of 200°C. The mass range scanned was 40–500, at 1.0

scan.sec^{-1}, after a solvent delay of 4.0 min. A C_8–C_{25} hydrocarbon mixture in hexane (100 ng.µl^{-1}), containing a series of odd- and even-numbered *n*-alkanes, was injected to calculate retention indices (RIs) of the compounds, as described previously (van den Dool and Kratz, 1963). Compounds were identified by comparison of their mass spectra and RIs with those of authentic standards, or by comparison with those from a commercial library (NIST Registry of Mass Spectral Data, 2005; Wiley, 2000) or the database published by Adams (2007). Individual compounds were quantified relative to the IS.

Abdominal Extracts The abdomens of three 7-d-old virgin males or females were excised at the thorax and immersed in 500 µl of hexane in 5 ml glass vials. After extraction for 3 h at ambient temperature, abdomens were removed and extracts stored at −20°C until analysis and bioassay. GC-MS analyses were performed by injecting 1 µl of the extracts (not concentrated) and 100 ng of the IS, as described above.

Collection and Analysis of Host-plant Volatiles Volatiles were collected by placing 5–6 freshly cut *Q. suber* branches (ca. 20 cm long, fresh weight ca. 35 g) into a 3-liter Erlenmeyer flask. Charcoal-filtered compressed air (550 ml.min^{-1}) was passed over the branches and the volatiles adsorbed onto a Porapak Q cartridge (50/80 mesh, 150/175 mg). Collection (two replicates) lasted 24 h, after which the adsorbed products were eluted with 2 ml of hexane. One µl of an extract (without concentration) and 1 µl of dodecyl acetate in hexane (100 ng.µl^{-1}) as IS were injected into the GC-MS and analyzed as described above. Linear RIs were estimated by co-injection of a hydrocarbon mixture (C_8-C_{25}), as described previously. The oven temperature was initially 50°C (for 1 min), then increased to 120°C at 3°C.min^{-1}, to 200°C at 5°C.min^{-1}, and finally to 260°C at 10°C.min^{-1}, and held at this temperature for 10 min. Plant compounds were identified by the same approach as for the insect chemicals. For quantification purposes, the percentage of each component was calculated relative to the most abundant compound.

Leaf Extracts Five or six leaves (ca. 500 mg) from fresh *Q. suber* branches were cut into small pieces, placed in 5 ml glass vials, and immersed in hexane (3 ml). After extraction for 3 h, the extract was filtered through Whatman filter paper and then stored at −20°C until subsequent analysis and bioassay. GC-MS analyses of 1 µl of the non-concentrated samples, containing 100 ng of the IS, were performed as described above. The oven temperature program was the same as for the plant volatile analysis.

Electrophysiological Responses Coupled gas chromatography-electroantennographic detection (GC-EAD) analyses were carried out using a Focus GC (Thermo Instruments, Barcelona, Spain), equipped with a flame ionization detector (FID) and a 30 m×0.25 mm ID×0.25 µm HP-5MS capillary column, using helium (1–2 ml.min^{-1}) as carrier and nitrogen as make-up gas. The column effluent was split 1:1 for simultaneous detection by both detectors (FID and EAD). The GC conditions were the same as for the GC-MS analysis (see above), with the transfer tube to the EAD preparation set at 230°C. The outlet for the EAD was delivered to the insect antenna through an L-shaped glass tube (12 cm×6 mm ID) into a humidified airstream. For the antennal preparation (both sexes), an antenna was cut at both ends, and the distal and proximal segments placed in contact with the microelectrodes through a conducting gel (Spectra 360, Parker Lab. Inc., Hellendoorn, The Netherlands). The microelectrodes were connected to an IDAC-2 interface (Syntech, Kirchzarten, Germany), and the antennal and FID signals were amplified (100x), filtered (DC to 1 kHz), and recorded simultaneously using the GC-EAD v4.4 software (Syntech). A 2 µl aliquot of a sample (BBOB headspace volatile extracts, host-plant volatiles, or a mixture of nonanal, decanal, and geranylacetone at 100 ng.µl^{-1} each), was injected into the GC in the splitless mode and the compounds eluting were tested on male and female antennae. A compound was considered electrophysiologically active when it elicited responses at least 3x higher than background noise.

Behavioral Bioassays A vertically posted, dual-choice, Y-shaped glass olfactometer was used to test the responses of BBOB adults to conspecific insect volatiles, synthetic compounds (nonanal, decanal, and geranylacetone, 100 ng and 1 µg each) identified in the insect volatiles, and synthetic compounds [(*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate; 1 µg each] identified in host-plant volatiles. The olfactometer consisted of a main tube (10 cm long×18 mm OD) with two 8-cm arms at 90º to each other. The Y-tube contained an iron wire to facilitate locomotion of the insects up to the far ends of the olfactometer, according to the design used by Sabelis and van de Baan (1983). In odor vs. blank experiments, each arm was connected to a glass adaptor (4.5 cm long× 1.2 mm ID) containing a test sample (3 live insects, abdominal, leaf and host-plant volatile extracts, or synthetic compounds) or control (air or solvent). Males also were tested in competitive experiments, in which 100 ng of two different odorants (3-component blend vs. decanal, and nonanal vs. decanal) were tested against each other in the two arms of the Y-tube. The corresponding amounts of the synthetic compounds or 100 µl of an extract were deposited on a Whatman filter paper (1.5 cm diam.) placed inside the test adaptor. The same amount of solvent was used as control in the other arm. Charcoal-filtered air

(2.5 l.min^{-1}) was passed through the arms to carry the stimuli to the test insects at the entrance of the main tube. The system was lit by a 60 W white light bulb to ensure homogeneous illumination around the olfactometer. All experiments were carried out under similar conditions on 1–2 wk-old adults from 10:00–17:00 h (the time when adults display activities such as feeding and mating; B.F., personal observations), at 26±2°C and 50±10% RH. Insects were removed from their containers 2 h before the tests and kept individually in plastic Petri dishes (3 cm high×5 cm OD), until they were placed at the base of the main arm. Individuals that walked upwind and reached at least the middle of one side arm, without returning to the intersection within 5 min., were recorded as a positive response; those that did not choose either arm were excluded from analysis.

A minimum of 30 insects for each treatment were tested in the bioassay. The number of individuals tested in control assays and in female vs. male experiments were combined from different years, leading to a total of N=50–74. Assays for each treatment were implemented on at least three different days, testing a minimum of 10 individuals per day. After five replicates of the same treatment, the Y-tube and the iron wire were cleaned with ethanol or acetone, left to dry, and the relative positions of the olfactometer arms reversed to account for possible positional effects. Because this insect cannot be reared through its lifecyle in the laboratory, insects were reused, but only after at least 3 d and never to the same treatment.

For statistical analysis, the numbers of insects responding to different stimuli were subjected to a χ^2 goodness-of-fit test, using the Yates correction for continuity (Zar, 1999). Additionally, the number of insects choosing one of the arms was subjected to a binomial test of proportions using the probability mass function to avoid any effect of different sample sizes (N values) of the experiments. The null hypothesis was that the percentages of individuals choosing the odor treatment and the blank were equal. For comparisons between different odor treatments and sexes, the ratios of test/control of the data were subjected to analysis of variance (one-way ANOVA) and means compared by Tukey's Honestly Significant Difference (HSD) tests. In competitive experiments that tested two odors against each other, the resulting ratios of odor treatment I to odor treatment II were analyzed by one-way ANOVA, followed by Student's t-test. In all statistical analyses, a 5% probability level was applied using PASW statistics 18.

Results

Volatile Composition Twenty eight compounds were identified in the abdominal extracts and headspace of adult males and females (Table 1). Most of these compounds were saturated linear or branched hydrocarbons. Several carboxylic acids (entries 7, 13, and 14), 2-phenylethanol (entry 5), and nonanal (entry 4) were also detected in abdominal extracts. Aldehydes (entries 1, 3, 4, and 6), ketones (compounds 2 and 10), and two isopropyl esters (entries 12 and 15) were found mostly in the insect volatiles. No qualitative, and only slight quantitative, differences were observed between the male and female volatiles. The amounts of the active compounds in the volatile collections from 20 individuals over 24 h were: nonanal 249.2±83.8 ng in females and 388.5±167.2 ng in males;

Table 1 Compounds detected in abdominal extracts[A] and volatiles[V] of *Coroebus florentinus* males and females, identified by comparison of their mass spectra (MS) and retention indices (RI) with those of standards and/or reference databases

Entry	Compound	MS/RI[a]	M[b]	RI[c]
1	benzaldehyde[V]	ST/ST	106	963
2	6-methyl-5-heptene-2-one[V]	RD/RD	126	988
3	octanal[V]	ST/ST	128	1005
4	nonanal[A, V]	ST/ST	142	1103
5	2-phenylethanol[A]	ST/ST	122	1112
6	decanal[V]	ST/ST	156	1207
7	nonanoic acid[A]	ST/ST	158	1282
8	isobornyl acetate[V]	ST/ST	196	1289
9	tetradecane[V] ($C_{14}H_{30}$)	ST/ST	198	1399
10	geranylacetone[V]	ST/ST	194	1454
11	hexadecane[V] ($C_{16}H_{34}$)	ST/ST	226	1599
12	isopropyl dodecanoate[V]	ST/ST	242	1635
13	tetradecanoic acid[A]	ST/ST	228	1767
14	hexadecanoic acid[A]	ST/ST	256	1985
15	isopropyl hexadecanoate[V]	ST/ST	298	2025
16	henicosane[V] ($C_{21}H_{44}$)	ST/ST	296	2100
17	docosane[V] ($C_{22}H_{46}$)	ST/ST	310	2200
18	tricosane[A, V] ($C_{23}H_{48}$)	ST/ST	324	2300
19	11-methyltricosane[A, V] ($C_{24}H_{50}$)	RD/RD	338	2330
20	tetracosane[A, V] ($C_{24}H_{50}$)	ST/ST	338	2400
21	pentacosane[A, V] ($C_{25}H_{52}$)	ST/ST	352	2500
22	13-methylpentacosane[A, V] ($C_{26}H_{54}$)	RD/RD	366	2529
23	13-methylhexacosane[A, V] ($C_{27}H_{56}$)	RD/RD	380	2625
24	heptacosane[A, V] ($C_{27}H_{56}$)	ST/ST	380	2700
25	11,14-dimethylhexacosane[A, V] ($C_{28}H_{58}$)	RD/RD	394	2728
26	nonacosane[A, V] ($C_{29}H_{60}$)	ST/ST	408	2900
27	13-methylnonacosane[A, V] ($C_{30}H_{62}$)	RD/RD	422	2943
28	triacontane[A, V] ($C_{30}H_{62}$)	ST/ST	422	3000

[a] Type of identification by comparison of the MS and RI with those of standards (ST) or reference databases (RD).

[b] Molecular mass.

[c] On an HP-5MS (30 m×0.25 mm ID×0.25 μm) fused silica capillary column.

decanal 349.2±107.3 ng in females and 369.1±155.2 ng in males; and geranylacetone 56.2±32.1 ng in females and 122.5±68.6 ng in males. In the blank collections, several phthalates and siloxanes were detected, along with small amounts of nonanal (12.3±5.7 ng) and decanal (18.0± 9.6 ng).

Thirty two compounds were identified from the headspace volatiles of freshly cut branches and leaf extracts of *Q. suber* (Table 2). The host-volatile composition was essentially two groups of compounds, saturated and monounsaturated short-chain aldehydes, alcohols and esters, the so-called green leaf volatiles (GLVs), and monoterpenes. The homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene and some sesquiterpenes, and other esters and aldehydes also were identified. The most abundant compounds were the GLVs (*E*)-2-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, followed by 1-hexanol and (*Z*)-3-hexenol. The relative amounts of all compounds, relative to the most abundant chemical (*E*)-2-hexenol, are given in Table 2.

Table 2 Compounds detected in volatiles of *Quercus suber* branches and leaf extracts, identified by comparison of their mass spectra and retention indices (RI) with those of standards and/or reference databases, and relative amounts to the most abundant compound (*E*)-2-hexenol

Entry	Compound	Rel. amount±SE[a]	M[b]	BP[b]	RI[c]
Green leaf volatiles (GLVs) (C6)					
1	hexanal	10.0±4.1	100	41	813
2	(*E*)-2-hexenal	98.3±14.6	98	41	856
3	(*Z*)-3-hexenol	33.0±2.4	100	67	859
4	(*E*)-2-hexenol	100	100	57	870
5	1-hexanol	59.2±16.3	102	56	872
6	(*Z*)-3-hexenyl acetate	71.9±3.6	142	43	1009
7	*n*-hexyl acetate	11.5±1.5	144	43	1014
8	(*E*)-2-hexenyl acetate	18.6±3.4	142	43	1016
9	*(Z)*-3-hexenyl isobutyrate	0.7±0.2	170	67	1143
10	(*E*)-2-hexenyl isobutyrate	1.3±0.2	170	71	1150
11	(*Z*)-3-hexenyl 2-methylbutyrate	5.7±2.0	184	67	1232
12	(*E*)-2-hexenyl 2-methylbutyrate	1.6±0.5	184	57	1238
Monoterpenes (C10)					
13	α-thujene	0.5±0.1	136	93	925
14	α-pinene	2.0±0.5	136	93	932
15	camphene	0.1±0.1	136	93	946
16	sabinene	1.2±0.3	136	93	972
17	β-pinene	1.3±0.3	136	93	975
18	limonene	0.3±0.1	136	68	1028
19	cineol	0.5±0.1	154	81	1030
20	(*E*)-β-ocimene	0.9±0.3	136	93	1047
21	γ-terpinene	0.5±0.1	136	93	1057
22	linalool	1.9±0.3	154	71	1100
Homoterpenes (C11)					
23	(3*E*)-4,7-dimethyl-1,3,7-nonatriene	8.9±2.0	150	69	1117
Sesquiterpenes (C15)					
24	α-cubebene	0.3±0.1	204	105	1352
25	copaene	0.4±0.1	204	161	1377
Others					
26	nonane	0.1±0.1	128	43	900
27	(*E,E*)-2,4-hexadienal	0.8±0.1	96	81	909
28	*n*-pentyl acetate	0.4±0.1	130	43	912
29	benzaldehyde	0.9±0.1	106	77	959
30	vinyl hexanoate	0.8.0.1	142	60	983
31	γ-hexanolactone	0.9±0.1	114	85	1053
32	nonanal	0.8±0.2	142	41	1104

[a] Based on the relative areas by gas chromatography–mass spectrometry.

[b] M: Molecular mass; BP: Base peak (*m/z)* of electron impact mass spectra.

[c] On an HP-5MS (30 m × 0.25 mm ID×0.25 μm) fused silica capillary column.

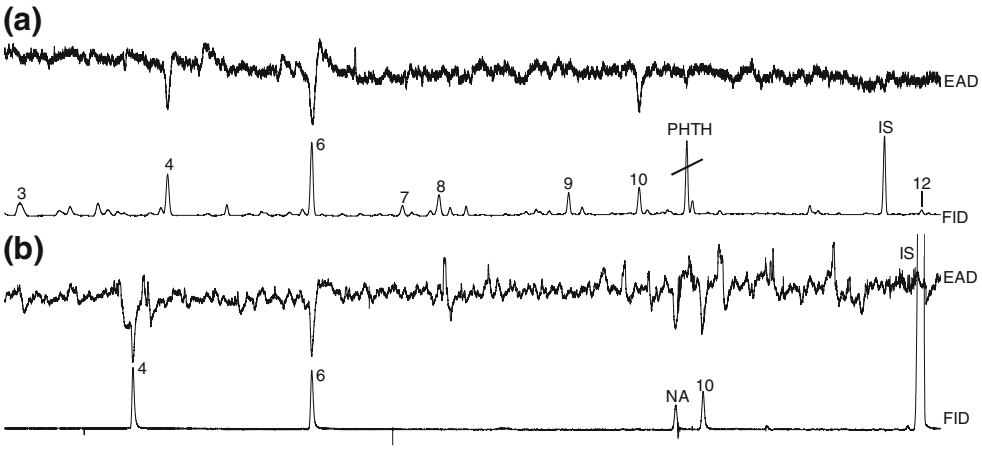

Fig. 1 Representative coupled gas chromatograph-electroantennogram detection (EAD) responses of *Coroebus florentinus* male antennae to: (**a**) female volatiles collected for 24 h, and (**b**) a 3-component blend of synthetic nonanal, decanal, and geranylacetone (100 ng each). 4: nonanal; 6: decanal; NA: nerylacetone; PHTH: phthalate; 10: geranylacetone (Table 1). IS=internal standard, FID=flame ionization detection

GC-EAD Recordings Three compounds in both female and male volatiles elicited EAD responses from male antennae. Because both extracts were similar in composition (see above), only responses to female volatiles are shown in Figure 1a. The EAD-active chemicals (see Table 1 for numbering) were identified as nonanal (entry 4), decanal (entry 6), and geranylacetone (entry 10). EAD activity of these compounds was confirmed using a 3-component blend (100 ng each) of synthetic compounds (Fig. 1b). A fourth EAD-active chemical in the synthetic mixture was identified as nerylacetone (NA), the *cis* isomer of geranylacetone; this originated from the commercial sample of geranylacetone. All four compounds elicited only slight responses from female antennae (data not shown).

GC-EAD analyses of headspace volatiles from *Q. suber* indicated that most of the major GLVs identified, namely (*E*)-2-hexenal (entry 2, Table 2), (*E*)-2-hexenol (entry 4), 1-hexanol (comp. 5), (*Z*)-3-hexenyl acetate (entry 6), and *n*-hexyl acetate (entry 7), elicited responses from the antennae of both sexes (Fig. 2a-b). The homoterpene (3*E*)-4,7-dimethyl-1,3,7-nonatriene (entry 23) elicited responses only from female antennae.

Laboratory Behavioral Bioassays

Live Insects and Abdominal Extracts A preliminary test in the Y-tube olfactometer with no odor (air vs. air) showed that there were no arm preferences by adult males [χ^2= 0.813; *df*=1; $P>0.05$; $P_{\text{binomial}}(x\leq 23)=0.288$] or females [$\chi^2=0.071$; *df*=1; $P>0.05$; $P_{\text{binomial}}(x\leq 23)=0.336$], indicating no positional effect of the experimental setup. The responsiveness of test individuals in the bioassay was almost 100%.

Adult virgin males were attracted to three live females [$\chi^2=13.549$; *df*=1; $P=0.001$; $P_{\text{binomial}}(x\leq 20)=0.001$] and to abdominal extracts of three females [$\chi^2=5.184$; *df*=1; $P=0.023$; $P_{\text{binomial}}(x\leq 12)=0.017$], but not to three live males [$\chi^2=0.567$; *df*=1; $P>0.05$; $P_{\text{binomial}}(x\leq 23)=0.292$]. In contrast, adult virgin females were not attracted to volatiles of either sex or to abdominal extracts of males and females (Fig. 3).

Synthetic Compounds The behavioral responses of males and females to 100 ng of synthetic nonanal, decanal, or geranylacetone, or a blend of the three compounds in a 1:1:1 ratio, are

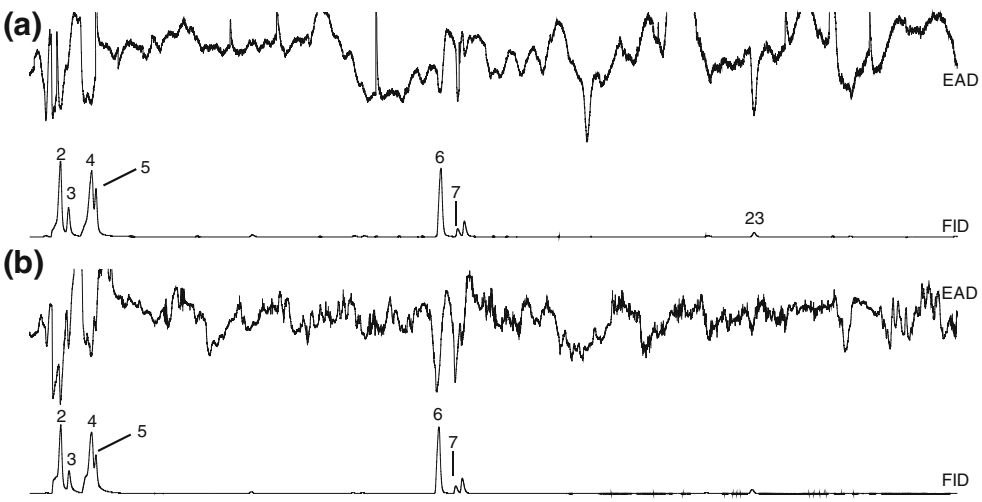

Fig. 2 Representative coupled gas chromatograph-electroantennogram detection (EAD) responses of *Coroebus florentinus* female (a) and male (b) antennae responding to *Quercus suber* volatiles collected for 24 h. 2: (*E*)-2-hexenal; 3: (*Z*)-3-hexenol; 4: (*E*)-2-hexenol; 5: 1-hexanol; 6: (*Z*)-3-hexenyl acetate; 7: *n*-hexyl acetate; 23: (3*E*)-4,7-dimethyl-1,3,7-nonatriene (Table 2). FID=flame ionization detection

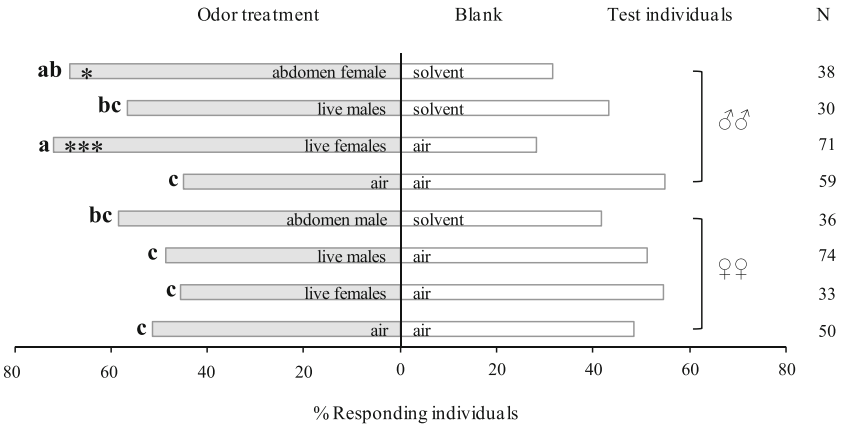

Fig. 3 Y-tube olfactometer responses of virgin *Coroebus florentinus* males and females to volatiles of three live individuals or abdominal extracts (N=3) of both sexes. Number of insects attracted to odors vs. blank (solvent control or air) was analyzed by $\chi 2$ goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; ***$P \leq 0.001$). Bars with different letters indicate differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). *N*=number of responding individuals. Air vs. air=control experiment

shown in Figure 4. Males were attracted to the 3-component blend [χ^2=6.444; *df*=1; *P*=0.011; $P_{binomial}(x \leq 14)$=0.008] and to decanal [χ^2=3.935; *df*=1; *P*=0.047; $P_{binomial}(x \leq 10)$=0.035]. In contrast, virgin females were attracted most strongly to the 3-component blend [χ^2=4.925; *df*=1; *P*=0.027; $P_{binomial}(x \leq 13)$=0.019], and to geranylacetone [χ^2=6.567; *df*=1; *P*=0.01; $P_{binomial}(x \leq 8)$=0.008]. At a dose of 1 μg, males (*N*=30) preferred [χ^2=6.567; *df*=1; *P*=0.01; $P_{binomial}(x \leq 8)$=0.008] the 3-component blend (22 individuals, 73% of response) over the blank (8 individuals, 27% of response), whereas females (*N*=40) preferred [χ^2=4.925; *df*=1; *P*=0.027; $P_{binomial}(x \leq 13)$=0.019] nonanal (27 individuals, 67.5% of response) over the blank (13 individuals, 32.5% of response). The other compounds did not elicit attraction.

In additional experiments (Fig. 5) that tested two odor treatments against each other (decanal vs. 3-component blend and decanal vs. nonanal), males (*N*=30) did not discriminate [χ^2=0.567; *df*=1; *P*> 0.05; $P_{binomial}(x \leq 13)$=0.292] between 100 ng of decanal (13 individuals, 43% of response) and the 3-component blend (17 individuals, 57% of response), whereas they preferred [χ^2=4.833; *df*=1; *P*=0.028; $P_{binomial}(x \leq 9)$=0.021] decanal (21 individuals, 70% of response) over nonanal (9 individuals, 30% of response). The ratio of responses to decanal/nonanal was higher than the ratio of responses to decanal/3-component blend (Student's *t*-test, *F*=12.413; *df*=4; $P \leq 0.001$).

Host-plant Volatiles and Synthetic GLVs When adults were exposed to headspace volatiles and leaf extracts from *Q. suber*, females were attracted to both odor sources [$\chi_{leaf\ extract}^2$= 3.935; *df*=1; *P*=0.047; $P_{binomial}(x \leq 10)$=0.035; $\chi_{volatiles}^2$= 4.833; *df*=1; *P*=0.028; $P_{binomial}(x \leq 9)$=0.021]. In contrast,

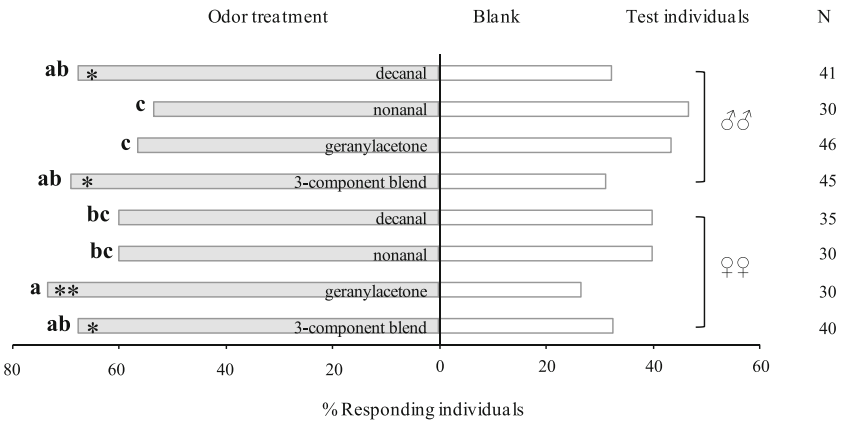

Fig. 4 Y-tube olfactometer responses of virgin *Coroebus florentinus* males and females to nonanal, decanal and geranylacetone, tested individually and as a 3-component blend (1:1:1 ratio), at a dose of 100 ng each. Number of insects attracted to odors vs. blank (solvent) was analyzed by χ^2 goodness-of-fit test with Yates correction for continuity (*$P \leq 0.05$; **$P \leq 0.01$). Bars with different letters indicate differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). *N*=number of responding individuals

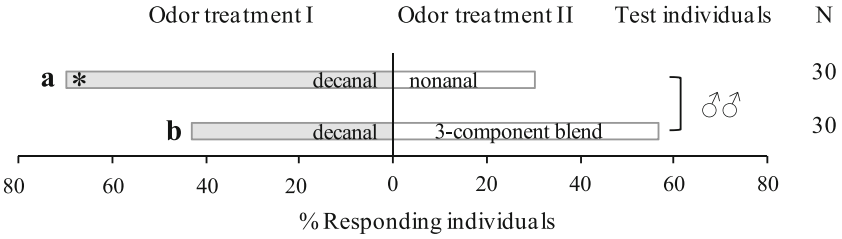

Fig. 5 Y-tube olfactometer responses of virgin male *Coroebus florentinus* to nonanal, decanal and a 3-component blend (geranylacetone, nonanal, decanal, 100 ng each) against each other. Preferences for decanal (odor treatment 1) versus the mixture (blend 3 compounds) or nonanal (odor treatment 2) were analyzed by $\chi 2$ goodness-of-fit tests with Yates correction for continuity (*$P<0.05$). Bars with different letters indicate differences between ratios (odor treatment I/odor treatment II) of the two tests (one-way ANOVA followed by Student's *t*-test; $P\leq 0.05$). *N*=number of responding individuals. The same amount of hexane (10 μl) was applied to the odor treatments

males were not attracted to either source of plant odor (Fig. 6). Furthermore, females, but not males, also were attracted [$\chi_{GLVs}^2=5.484$; $df=1$; $P=0.019$; $P_{binomial}(x\leq 9)=0.015$] to a mixture of synthetic GLVs [(*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate, and *n*-hexyl acetate; 1 μg each; Fig. 6]. Comparison (Tukey's HSD test) of the ratios of responses to odor/blank for the different treatments for each sex revealed no differences, except for the GLV blend, which elicited a lower ratio of response in males. When the GLVs were tested individually, females were attracted to the saturated and unsaturated C_6-alcohols, 1-hexanol and (*E*)-2-hexenol [$\chi_{1\text{-hexanol}}^2=6.567$; $df=1$; $P=0.01$; $P_{binomial}(x\leq 8)=0.008$; $\chi^2_{(E)\text{-2-hexenol}}=3.935$; $df=1$; $P=0.047$; $P_{binomial}(x\leq 10)=0.035$], and to *(Z)*-3-hexenyl acetate [$\chi^2=4.833$; $df=1$; $P=0.028$; $P_{binomial}(x\leq 9)=0.021$], but not to *n*-hexyl acetate or (*E*)-2-hexenal (Fig. 7). Multiple comparisons between responses to the GLVs revealed that 1-hexanol was more attractive than (*E*)-2-hexenal and *n*-hexyl acetate, but attraction to (*E*)-2-hexenol was not different from that of the other odors tested (Fig. 7).

Discussion

There was no qualitative, but a small quantitative, difference between the respective headspace volatiles produced by males and females. In behavioral bioassays, using live insects and abdominal extracts as stimuli, only males responded to female odors, suggesting the presence of a sex pheromone produced by females. The presence of unidentified female-specific compounds in trace amounts would account for this result, but further experiments are needed to test this assumption. Because females were not attracted to corresponding male odors, we might expect BBOB males to display more prominent olfactory structures than do females. Electron micrographs have shown that male and female antennae are similar in structure, but that the male antenna (ca. 4.5 mm long) is roughly twice as long and contains more olfactory related *sensilla basiconica* than the antenna of females (Fürstenau, unpublished). This olfactory specialization is consistent with BBOB males being more sensitive to beetle-produced volatiles than females, as demonstrated in the behavioral assays.

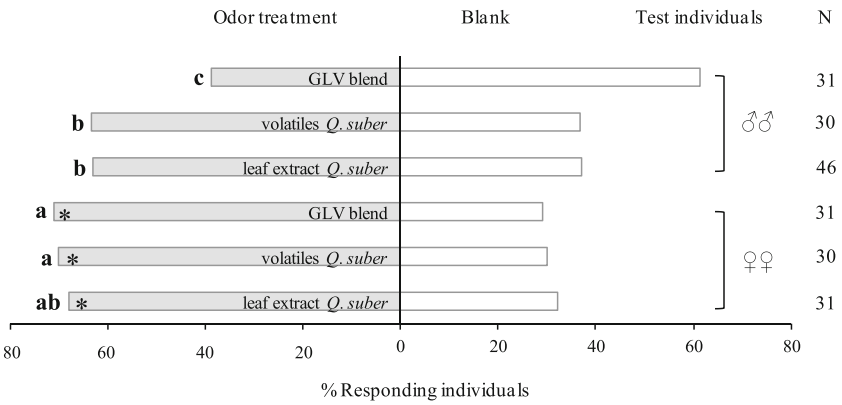

Fig. 6 Y-tube olfactometer responses of *Coroebus florentinus* males and females to a blend of five synthetic green leaf volatiles (GLVs) (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate (1 μg each), and odors from volatile collections and leaf extract of the host plant *Quercus suber*. Numbers of insects attracted to odors were analyzed by $\chi 2$ goodness-of-fit tests with Yates correction for continuity (*$P\leq 0.05$). Bars with different letters indicate differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P\leq 0.05$). *N*=number of responding individuals

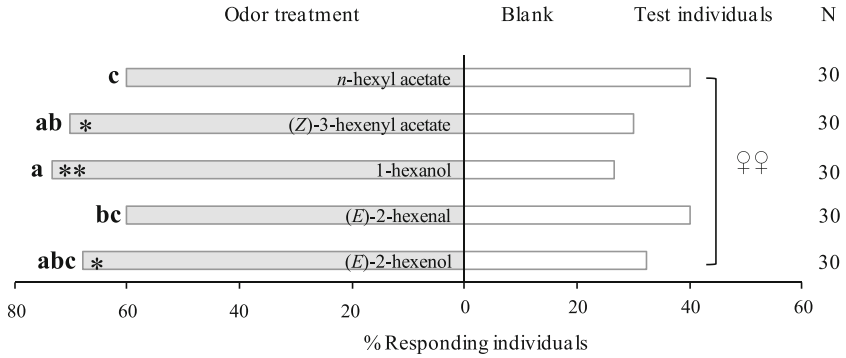

Fig. 7 Y-tube olfactometer responses of virgin *Coroebus florentinus* females to the synthetic green leaf volatiles (GLVs) (*E*)-2-hexenal, (*E*)-2-hexenol, 1-hexanol, (*Z*)-3-hexenyl acetate and *n*-hexyl acetate (1 μg each), found in the host plant *Quercus suber*. Numbers of insects attracted to odors were analyzed by $\chi 2$ goodness-of-fit tests with Yates correction for continuity (*$P \leq 0.05$; **$P \leq 0.01$). Bars with different letters indicate significant differences between ratios (test/control) of the treatments (one-way ANOVA followed by Tukey's HSD test; $P \leq 0.05$). *N*=number of responding individuals

Among the compounds identified in headspace collections, nonanal, decanal, and geranylacetone elicited GC-EAD responses from male antennae, with decanal evoking the strongest response. Female antennae responded only slightly to these compounds. It should be noted that the two aldehydes also were detected in blank odor collections, but only in minute amounts compared to the collections of volatiles of BBOB adults. Nonanal and decanal have been described as contaminants in control volatile collections of male and female flowers of *Breynia vitis-idaea* (Svensson et al., 2010), and as active compounds emitted by insects (Zhang et al., 2003; Torto et al., 2005; Siljander et al., 2008).

In addition to electrophysiological activity, some of the compounds attracted beetles in Y-tube olfactometer experiments. BBOB males were attracted to a mixture of nonanal, decanal, and geranylacetone, and to decanal alone, suggesting that decanal is probably the most attractive compound of the mixture. When testing nonanal against decanal in the olfactometer, males preferred the arm with decanal, indicating that the one carbon difference in chain length is sufficient to allow them to discriminate between the two aldehydes. Females also were attracted to the 3-component blend but, in contrast to males, were attracted to geranylacetone and not to decanal. That females were attracted to synthetic compounds, but not to insect (male or female) volatiles, may be due to the much higher dose of the synthetic compounds tested relative to the amounts of these compounds emitted by beetles. This 'lesser sensitivity' is consistent with the lower number of olfactory sensilla in females, in comparison to males. Although chemically mediated aggregation needs to be shown in the field, our results, combined with the fact that BBOB individuals feed in groups, suggest that these three compounds may promote aggregation in BBOB, with decanal being important for attracting males and geranylacetone for attracting females.

The headspace volatiles of the host-plant *Q. suber* are comprised mainly of saturated and monounsaturated 6-carbon aldehydes, alcohols, and esters, the so-called GLVs that are released by most green plants (Hatanaka et al., 1987; Takabayashi et al., 1996), especially after mechanical or herbivore damage (Paré and Tumlinson, 1996). Other volatile chemicals emitted in minor amounts by freshly cut *Q. suber* branches include mono-, homo-, and sesquiterpenes. These results agree with the findings of Staudt et al. (2004), but contrast with other studies on oaks (Kesselmeier and Staudt, 1999; Loreto, 2002) that considered the holm oak, *Q. ilex*, as the only oak that emits monoterpenes. This inconsistency could be the result of geographic variability in volatile emission by *Quercus spp.*, which may be related to past genetic isolation of populations, adaptations to local growth conditions, and/or hybridization between emitting and non-emitting oak species (Manos et al., 1999; Belahbib et al., 2001).

Most of the major GLVs from the host evoked EAD responses from both sexes. In addition, virgin females, but not males, were attracted to the arm containing the natural blend of GLVs or the individual synthetic compounds, (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate. In previous work, these two alcohols, in combination with (*Z*)-3-hexenol, attracted individuals of the European cockchafer, *Melolontha melolontha*, whereas the corresponding aldehydes and acetates were unattractive (Reinecke et al., 2002). Likewise, several studies have shown that GLVs emitted by foliage of green ash (*Fraxinus pennsylvanica* M.), white ash (*F. americana* L.), and Manchurian ash (*F. mandshurica*), and sesquiterpenes derived from the bark of stressed green ash trees, attract EAB adults (Rodriguez-Saona et al., 2006; Crook et al., 2008; Crook and Mastro, 2010; Grant et al., 2010, 2011). Our results suggest that GLVs from the host, particularly (*E*)-2-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate, may play an important role as

attractants in foraging/oviposition behavior of BBOB adults, particularly females.

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Male-Produced Pheromone of *Spathius agrili*, A Parasitoid Introduced For The Biological Control Of The Invasive Emerald Ash Borer, *Agrilus planipennis*

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Abstract The braconid wasp, *Spathius agrili*, has been released in the U.S. as a biocontrol agent for the invasive emerald ash borer (Coleoptera: Buprestidae: *Agrilus planipennis*), a destructive pest of ash trees (*Fraxinus* spp.). We identified and synthesized seven male-specific volatile compounds. Three of these, dodecanal, (4*R*,11*E*)-tetradecen-4-olide, and (*Z*)-10-heptadecen-2-one, were the key behaviorally active components in flight tunnel bioassays. Male specificity was demonstrated by gas chromatographic comparison of male and female volatile emissions and whole body extracts. Identifications were aided by coupled gas chromatographic-mass spectrometric (GC-MS) analysis, microchemical reactions, NMR, chiral GC analysis, and GC and MS comparison with authentic standards. Both the racemic and chiral forms of the γ-lactone, as well as both *E*- and *Z*-isomers were synthesized. Flight tunnel behavioral tests showed positive male and female *S. agrili* responses to both natural pheromone and synthetic blends, with upwind flight and landing on the source. Large field-cage tests, using yellow sticky traps baited with pheromone, captured approximately 50 % of the released male and female wasps in 24-h periods. The use of pheromone-baited traps in the field could simplify the current detection method for determining parasitoid establishment (i.e., laboriously felling and peeling ash trees for recovery of *S. agrili* from infested EAB larvae).

Keywords Pheromone · (4*R*,11*E*)-tetradecen-4-olide · Flight tunnel behavior · *Spathius agrili* · Hymenoptera · Braconidae · Biocontrol · Invasive

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Introduction

The exotic gregarious larval ectoparasitoid, *Spathius agrili* Yang (Hymenoptera: Braconidae) (Yang et al., 2005, 2010; Bauer et al., 2006), has been released in the U.S. for biological control of the invasive emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Bauer et al., 2009). Both pest and parasitoid are native to China. Since first being detected in 2000 in the Detroit area (Haack et al., 2002), EAB has spread to 15 states and to two Canadian provinces (emeraldashborer.info, 2011) resulting in the loss of millions of ash trees (*Fraxinus* spp). Widespread infestations of EAB make biocontrol with natural enemies one of the most promising control strategies. Successive releases of *S. agrili* in several states have lead to successful establishment of the parasitoid in Michigan, Ohio, and Indiana (Bauer et al., 2011). Current practice for the determination of parasitoid establishment requires a laborious process of felling EAB infested ash trees in the vicinity of parasitoid release sites, removal of EAB larvae from the felled trees, and waiting for the emergence of the adult biological control agent (Bauer et al., 2009). A monitoring system based on traps baited with

semiochemical attractants for *S. agrili* might be a quicker and less laborious alternative to detect newly released populations of this biocontrol agent, and might help in determining population densities and dispersal. We report here the identification, synthesis, and behavioral evaluation of the male-produced pheromone of *Spathius agrili*.

Methods and Materials

Insects Virgin adult *S. agrili* used in this study were shipped from the USDA APHIS PPQ rearing facility (Brighton, MI, USA). Upon arrival in Peoria, Illinois, adults were placed in individual Accuvette II vials (Beckman Coulter), together with a moist filter paper strip and a drop of honey, and kept at 25 °C under a 17L:7D h photoperiod. Male and female insects were housed in different locations within the Peoria facility.

Volatile Collections and Cuticular Extracts Volatiles were collected from individuals and groups of 3-7 male or female insects, which were placed in collection chambers (8×3 cm ID) made from glass thermometer adapters (male 24/40 joint) and glass hose adapters (female 24/40 joint, Ace Glass, Vineland, NJ, USA). Insects had access to a drop of honey, which was also present in the empty control chambers. The collection chamber had a Teflon fitting at each end to hold a volatile trap filter (6×0.4 cm ID) containing 100 mg of HayeSep-Q (80-100 mesh, Restek, Bellefonte, PA, USA), and through which air was drawn (100 ml/min) by vacuum. HayeSep-Q was chosen as an excellent replacement of Super-Q, which is no longer commercially available. Incoming air was cleaned by the inlet filter, and the second filter trapped volatiles emitted within the chamber. Collection duration was 1-3 d, and tapped volatiles were recovered by rinsing the outlet HayeSep-Q filter with 400 μl of hexane into a vial. Collection chambers were kept in an incubator at 27 °C with a relative humidity of about 50 %. Light was provided by eight 40 W fluorescent tubes set about 0.5 m above and behind the collection tubes, and the daily light cycle was a 17L:7D h photoperiod.

Cuticular extracts were obtained from individuals or groups of male or female insects. Insects were killed by freezing, and soaked for 5 min in 0.1-1 ml hexane.

Instrumentation Volatile collections, extracts, liquid chromatography (LC), and HPLC fractions were analyzed by gas chromatography with flame-ionization detection (GC-FID) and coupled GC-mass spectrometry (GC-MS). Samples were injected in splitless mode using a Hewlett Packard 6890 GC, interfaced to a Hewlett Packard 5973 mass selective detector. For most analyses, a 30-m DB-5 capillary column (0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) was used. The temperature program was 50 °C for 1 min, then rising to 280 °C at 10 °C per min, and holding for 5 min at 280 °C. Inlet temperature was 250 °C, and the transfer line temperature was 280 °C. The Wiley MS library (Wiley, 2005) was installed on the data system.

Chiral GC-MS analysis was conducted using a 30-m β-DEX 225 column (0.25 mm ID, 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). The temperature program was 50 °C for 1 min, then 30 °C/min to a final temperature of 190 °C.

A Hewlett Packard 6890 GC equipped with a FID detector was employed for quantitation of the collected volatiles and synthetic compound solutions using a 30-m HP-5 column (0.32 mm ID, 0.25 μm film thickness, Agilent). Temperature program and splitless injection were the same as mentioned for the DB-5 column. Estimation of the amounts of selected compounds in samples was by the internal standard method relative to tridecan-2-one. All GC analyses used He as carrier gas at constant pressure (41.4 kPa).

Representative samples of the volatile collections were submitted to LC on silica gel (70-230 mesh, Fisher Scientific, Pittsburgh, PA, USA) to determine compound polarity. A typical column was 0.5 ID by 1-cm long (in a Pasteur pipet), and was eluted with hexane; 5, 10, 15, 20, 25, and 50 % ether in hexane; and ether. Fractions were stored at -20 °C, and analyzed by GC and GC-MS.

NMR spectra were acquired on a Bruker Avance 500 instrument. ^{1}H and ^{13}C spectra were obtained for male-derived and synthetic **5** (Fig. 1) in $CDCl_3$ and deuterobenzene.

Male-derived **5** was purified by HPLC analysis using a Waters 515 pump (flow rate=1 ml/min), and a Waters R401 differential refractometer detector. The compound was injected onto a Supelcosil LC-SI silica column (25 cm, 0.46 cm ID, 5 μm particle size, Supelco, Bellefonte, PA) with 0.1 % methanol in methylene chloride as solvent.

Microchemical Reactions Hydrogenation of male-derived **3**, **5**, and **6** was used to confirm the number of carbon-carbon double bonds. Samples (100 μl) were stripped to dryness under a stream of nitrogen, and resuspended in 100 μl methylene chloride to which ~0.5 mg of 10 % Pd on charcoal was added. Reduction was accomplished by bubbling a gentle steam of hydrogen through the sample for 5 min at room temperature. The reduced sample was filtered, and analyzed by GC-MS.

Dimethyldisulfide (DMDS) derivatives were prepared for determination of double bond locations of **3**, **5**, and **6** (Attygalle, 1998). Samples (100 μl) were stripped to dryness under a stream of nitrogen, and DMDS and 5 % iodine in ether were added (equal volumes, about 25 μl each). Samples were heated at 45 °C for 1-2 h, then diluted with hexane, and treated with aqueous sodium thiosulfate to destroy the iodine. The organic layer was dried over sodium sulfate, evaporated under nitrogen, resuspended in 10 μl hexane, and analyzed by GC-MS. Oven temperature was

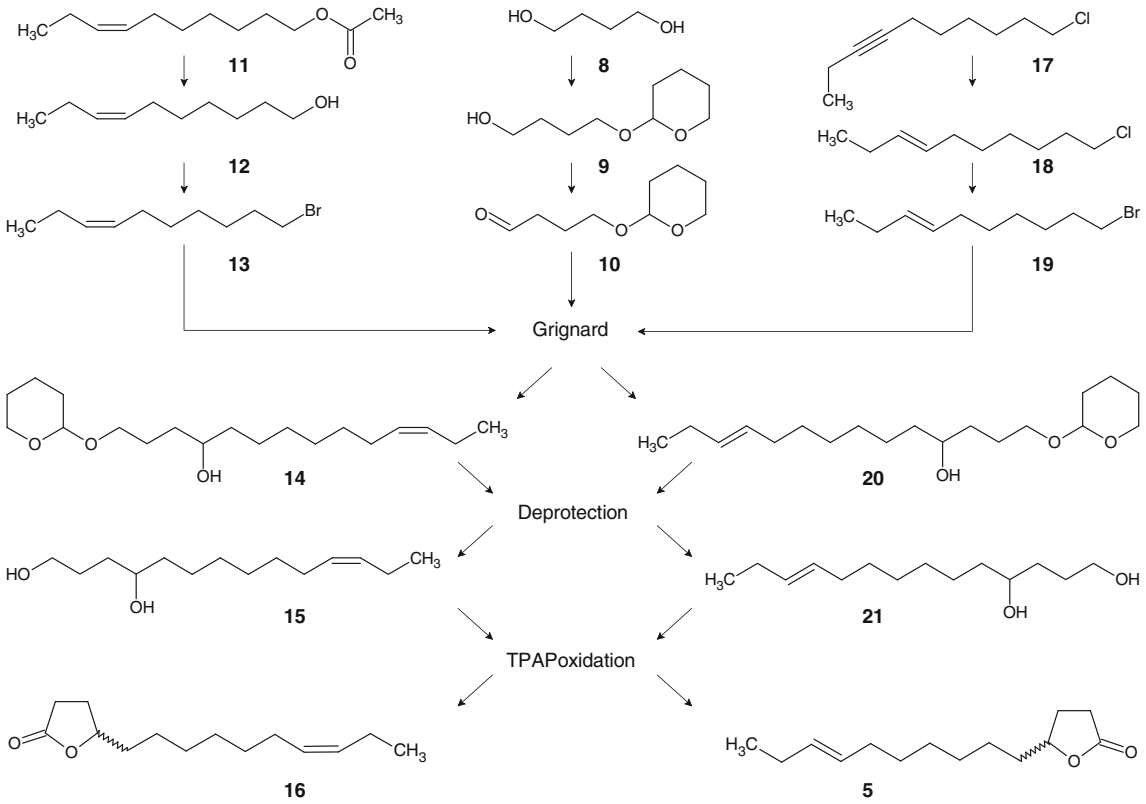

Fig. 1 Synthesis of racemic (*Z*)- and (*E*)-11-tetradecen-4-olide

programmed up to 300 °C and MS scanning range was 40-600 amu.

Chemicals Dodecanal **1**, tetradecanal **2**, pentadecan-2-one **4**, and heptadecan-2-one **7** were obtained from Sigma-Aldrich (St. Louis, MO, USA). (*Z*)-9-Tetradecenoic acid and (*Z*)-9-hexadecenoic acid were obtained from Nu-Chek Prep., Inc. (Elysian, MN, USA), and were the starting materials for one-step synthesis of (*Z*)-10-pentadecen-2-one **3** and (*Z*)-10-heptadecen-2-one **6** according to Bartelt et al. (1989). (*Z*)-7-Tetradecen-1-yl acetate was obtained from Bedoukian Research, Inc. (Danbury, CT, USA), and tetradecyl acetate was obtained by hydrogenation (10 % Pd on charcoal) of (*Z*)-7-tetradecen-1-yl acetate. The synthesis of racemic (*E*)-11-tetradecen-4-olide **5** and (*Z*)-11-tetradecen-4-olide **16** using 10-chloro-3-decyne and (*Z*)-7-decen-1-yl acetate, respectively, as starting materials, are presented in Fig. 1. Chiral synthesis of **5** using commercially available (*R*)- and (*S*)-epichlorohydrins has been reported in summary (Cossé et al., 2011); full details will be reported at a later date.

Preparation of 4-(tetrahydro-pyran-2-yloxy)-butyraldehyde (**10**)

1,4-Butanediol (**8**) was monoprotected to tetrahydropyranyl (THP) ether **9** (Petroski, 2003a), using a biphasic mixture of 3,4-dihydro-2*H*-pyran (DHP), CH_2Cl_2, and a catalytic amount of 0.1N aqueous HCl, and stirring at room temperature for ca. 96 h. The free hydroxyl function of **9** was oxidized with pyridinium dichromate (PDC) in CH_2Cl_2 (Petroski, 2003b) to the four-carbon bifunctional aldehyde **10**.

Preparation of (*Z*)-10-bromodec-3-ene (**13**) and Grignard Reagent

Hydrolysis of (*Z*)-7-decenyl acetate (**11**) to alcohol **12** was accomplished with methanolic KOH (Hijfte et al., 1987). Alcohol **12** was converted to bromide **13**, without isomerization of the *Z*-double bond, by adding triphenylphosphine dibromide to CH_2Cl_2 instead of generating it *in situ* (Kuklev and Smith, 2006). Grignard reagent **13a** was prepared by treatment of the bromide **13** with pre-activated Mg metal (Baker et al., 1991) in refluxing dry THF (distilled from Na and benzophenone).

Preparation of (*Z*)-11-tetradecen-4-olide (**16**)

Bifunctional aldehyde **10** was added dropwise to stirred Grignard reagent **13a**, in dry THF at -20 °C, to afford monoprotected 1,4-diol **14** after workup (James et al., 2003). Cleavage of the THP protective group, to give 1,4-diol **15**, was accomplished with pyridinium *p*-toluenesulfonate (PPTS)

in ethanol at 55 °C (Miyashita et al., 1977). The terminal OH of **15** was selectively oxidized with tetrapropylammonium perruthenate (TPAP) and 4-methylmorpholine-*N*-oxide as co-oxidant (Bloch and Brillet, 1991), to afford racemic (*Z*)-11-tetradecen-4-olide (**16**).

Preparation of (*E*)-10-bromodec-3-ene (**19**) and Grignard Reagent

Alkyne, 10-chlorodec-3-yne (**17**), was reduced to alkene, (*E*)-10-chlorodec-3-ene (**18**), by a two-stage hydrosilylation and subsequent protodesilylation process (Trost et al., 2002). Hydrosilylation was accomplished with triethoxysilane, in the presence of a ruthenium complex, [Cp*Ru $(MeCN)_3PF_6$, and protodesilylation was accomplished with CuI, followed by dropwise addition of tetrabutylammonium fluoride (TBAF). The alkenyl chloride **18** was converted to the alkenyl bromide **19** in the presence of ethyl bromide, *N*-methyl-2-pyrrolidinone, and a catalytic amount of sodium bromide (Willy et al., 1976) Grignard reagent **19a** was prepared by treatment of the bromide **19** with pre-activated Mg metal (Baker et al., 1991) in refluxing dry THF (distilled from Na and benzophenone).

Preparation of (*E*)-11-tetradecen-4-olide (**5**)

Bifunctional aldehyde **10** was added dropwise to stirred Grignard reagent **19a**, in dry THF at -20 °C, to afford monoprotected 1,4-diol **20** after workup (James et al., 2003). Cleavage of the THP protective group, to give 1,4-diol **21**, was accomplished with PPTS in ethanol at 55 °C (Miyashita et al., 1977). The terminal OH of **15** was selectively oxidized with TPAP and 4-methylmorpholine-*N*-oxide as co-oxidant (Bloch and Brillet, 1991), to afford racemic (*E*)-11-tetradecen-4-olide (**5**).

Flight Tunnel Bioassay Olfactory stimuli were released in a flight tunnel (0.6×0.6×1.35 m) at a linear air flow of 0.3 m/s at 25 °C and 40 to 60 % relative humidity (Bartelt et al., 1990). The flight tunnel was illuminated by four 40-W fluorescent tubes (Ecolux Cool White, General Electric) mounted 10 cm from the top. Individually housed males and females were transferred to glass tubes (5×1.2 cm I.D.) covered with wire mesh at one end and a removable cap at the other end.

Volatile collections, body washes, and synthetic compounds were released from filter paper disks (55 mm O.D., Whatman #1) or from a piezo-electric sprayer (El-Sayed et al., 1999) from the upwind portion of the tunnel. Solutions in hexane were fed (10 μl/min) by a motor-driven (CMA/Microdialysis, N. Chelmsford, MA, USA) syringe into a glass capillary suspended 0.5 m above the tunnel floor. Vibration of the capillary by a piezo-ceramic disc at ca. 122 kHz dispersed the solution into micro-droplets, which evaporated within a few centimeters. Male and female wasps were released individually at the downwind end of the tunnel from a platform 0.5 m above the tunnel floor. Batches of 10 - 20 insects were flown on different days. The wasps were scored for plume-oriented upwind flight over at least 50 cm, and for landing on a paper disc surrounding the sprayer capillary.

Field Tests Two field cage tests were conducted: one during May of 2010 to test the attractiveness of the seven component blend, and a second experiment during June of 2011 to test the attractiveness of a three component blend. Tests were conducted on the NCAUR property in Peoria, IL. Batches of 50 virgin males or females were released at ground level into the middle of an outdoor screen cage (3.7×6.1×3.7 m, Sun Mart International Company, Irvine, CA, USA) which contained eight (1.8-m high) potted evergreen ash (*Fraxinus uhdei*) plants evenly spaced on the grass floor of the cage. Yellow sticky traps (15.5×15.5 cm, Sticky Strips, Olson Products, Medina, OH, USA) with glue on both sides were attached 1.4 m off the ground to one branch of each of the plants. *Spathius agrili* adults have shown a preference for the color yellow (Cooperband et al., 2011). Red rubber septa (11 mm, Wheaton, Millville, NJ, USA) were impregnated (Zilkowski et al., 2006) with a CH_2Cl_2 solution of synthetic **1**–**4**, **6** (100 μg), **7**, and natural **5** in ratios presented in Table 1. Solvent-only septa served as controls. Four septa pairs

Table 1 Proportion of male-specific compounds emitted by single and grouped male *Spathius agrili*

Compound	single males ng/male/day (*N*=21, ± SD)	%	3-5 males ng/male/day (*N*=6, ± SD)	6-7 males ng/male/day (*N*=8, ± SD)
Dodecanal (**1**)	780.5±265.7	22.5	20.7±28.4	1.1±1.4
Tetradecanal (**2**)	69.6±18.2	2.0	1.5±1.2	-
(*Z*)-10-Pentadecene-2-one (**3**)	19.1±12.43	0.5	7.2±6.3	1.3±1.6
Pentadecan-2-one (**4**)	372.1±156.3	10.0	21.2±28.9	0.8±1.0
(*E*)-11-Tetradecen-4-olide (**5**)	219.5±104.4	5.8	3.4±6.1	-
(*Z*)-10-Heptadecene-2-one (**6**)	3826.3±1836.1	100	185.4±259.4	5.0±5.3
Heptadecen-2-one (**7**)	113.0±54.67	3.0	1.9±0.6	-

(treatment vs. control) were attached randomly to traps on the ash plants. Insects were released during the middle of the morning, and trap catches were counted the following day. Remaining insects were removed, and the experiments were repeated three times for each sex with fresh septa. The second set of cage release experiments used the same protocols as the first set, but used 6 evergreen ash plants instead of 8 with three septa pairs. Only two releases of 50 virgin females were made. For this experiment, septa were impregnated with a tertiary blend of synthetic **1** (100 μg), racemic **5** (450 μg), and **6** (1 mg). Release rates from septa placed outdoors for 4 wk (N=3) were measured by bringing the septa inside once a week, and placing them into volatiles collectors for 24 h before returning them outside. The volatiles collections were similar to those obtained from live insects. Collections were analyzed by GC-FID, and the release rates were calculated using tridecan-2-one as internal standard.

Statistics Data analyses were performed using Statistix for Windows software (Analytical Software, 1998). Flight tunnel responses were analyzed using 2×2 tables and the Fisher's exact test. Field cage trap data were transformed using log(X + 1) to stabilize variance. The data from the pair-wise experiments were analyzed by paired t-tests.

Results

Male-Specific Compounds GC and GC-MS analysis of volatile collections revealed that males emitted seven compounds that were absent from females or control collections. Mean amounts per male per day are listed in Table 1. Single males emitted approximately 5.4 μg/male/day of the seven component blend with compound **6** as its major constituent. On an individual basis, single males emitted more male-specific compounds than grouped males. Compounds **1**, **2, 4**, and **7** were tentatively identified as dodecanal, tetradecanal, pentadecan-2-one, and heptadecan-2-one, respectively, by a computer library search of their mass spectra. These identifications were confirmed by matching GC retentions and mass spectra with authentic standards. No library EI mass spectra matches were found for compounds **3**, **5**, and **6**.

Compounds **3** and **6** eluted from silica gel with 5 % ether in hexane together with ketones **4** and **7,** and their mass spectra showed some similarity to **4** and **7** although their molecular weights indicated a single degree of unsaturation. Following hydrogenation, **3** and **6** showed matching GC retentions and mass spectra with **4** and **7**, respectively. The readily formed DMDS derivative for **3** showed a molecular ion at *m/z* 318 (=224+94), and the fragment ions (*m/z* 201 and 117) were consistent with a double bond being present at the 10 position. The identity of **3** as (*Z*)-10-pentadecen-2-one was confirmed with the synthetic sample of **3**. The DMDS derivatives of **6** showed a molecular ion at *m/z* 346 (=252+94), and the fragment ions (*m/z* 201 and 145) were consistent with a double bond being present at either the 6 or 10 position. The identity of **6** as (*Z*)-10-heptadecen-2-one was confirmed with the synthetic sample of **6**. The synthetic samples of **3** and **6** contained small amounts (<1 %) of the *E*-isomers, which separated from the *Z*-isomers by GC-MS and were used to determine the isomeric configurations of natural **3** and **6**.

Compound **5** eluted from silica gel with 25 % ether in hexane, suggesting an oxygenated compound. A molecular weight of 224 (Fig. 2a) suggested $C_{15}H_{28}O$ (2 double bonds or ring equivalents) or $C_{14}H_{24}O_2$ (3 double bonds or ring equivalents) as likely molecular formulas. The mass spectrum of **5** after hydrogenation showed a molecular ion at *m/z* 226 (Fig. 2b), indicating the presence of one carbon-carbon double bond. Furthermore, the MS library indicated the possibility of a γ-lactone, which was confirmed by the presence of the fragment ion at *m/z* 85. This would be expected for a γ-lactone (cleavage where side chain joins ring), suggesting that the natural compound was probably a γ-lactone with one carbon-carbon double bond in the side chain; the fragment ion at *m/z* 85 would not be present if the carbon-carbon double bond were residing in a ring. In addition, the mass spectrum of **5** showed a strong resemblance to that of an earlier identified pheromone, (*Z*)-9-octadecen-4-olide, the sex pheromone of the currant stem girdler, *Janus integer* (Cossé et al., 2001; James et al., 2003). The DMDS derivatives of **5** (Fig. 2c) showed a molecular ion at *m/z* 318 (=224+94), and the fragment ions (*m/z* 229 and 89) were consistent with a double bond being present at the 11 position. Approximately 200 μg of the natural γ-lactone were purified for NMR analysis, and the ^{1}H and ^{13}C data are presented in Table 2. The configuration of the double bond could only be determined by NMR simulation of the vicinal coupling constants of the olefinic protons and suggested an *E*-configuration. However, because of this slight ambiguity of the isomeric configuration both the *E*- and *Z*-isomers of 11-tetradecen-4-olide were synthesized. Racemic (*E*)-11-tetradecen-4-olide eluted earlier (19.54 min) from the DB-5 capillary column than the racemic (*Z*)-11-tetradecen-4-olide (19.62 min), and (*E*)-11-tetradecen-4-olide had exactly the same GC retention time and mass spectrum as the natural **5** confirming the *E*-double bond configuration for **5**. The two enantiomers of racemic **5** showed baseline separation on the chiral GC column, and the earlier eluting (*R*)-enantiomer of **5** had the same GC retention time as the natural compound (Fig. 3), establishing the absolute configuration of natural **5** as (4*R*,11*E*)-tetradecen-4-olide.

Female-Specific Compounds GC and GC-MS analysis of volatile collections revealed that virgin females emitted two compounds that were absent from males or control collections. The first compound eluted at 14.14 min, and

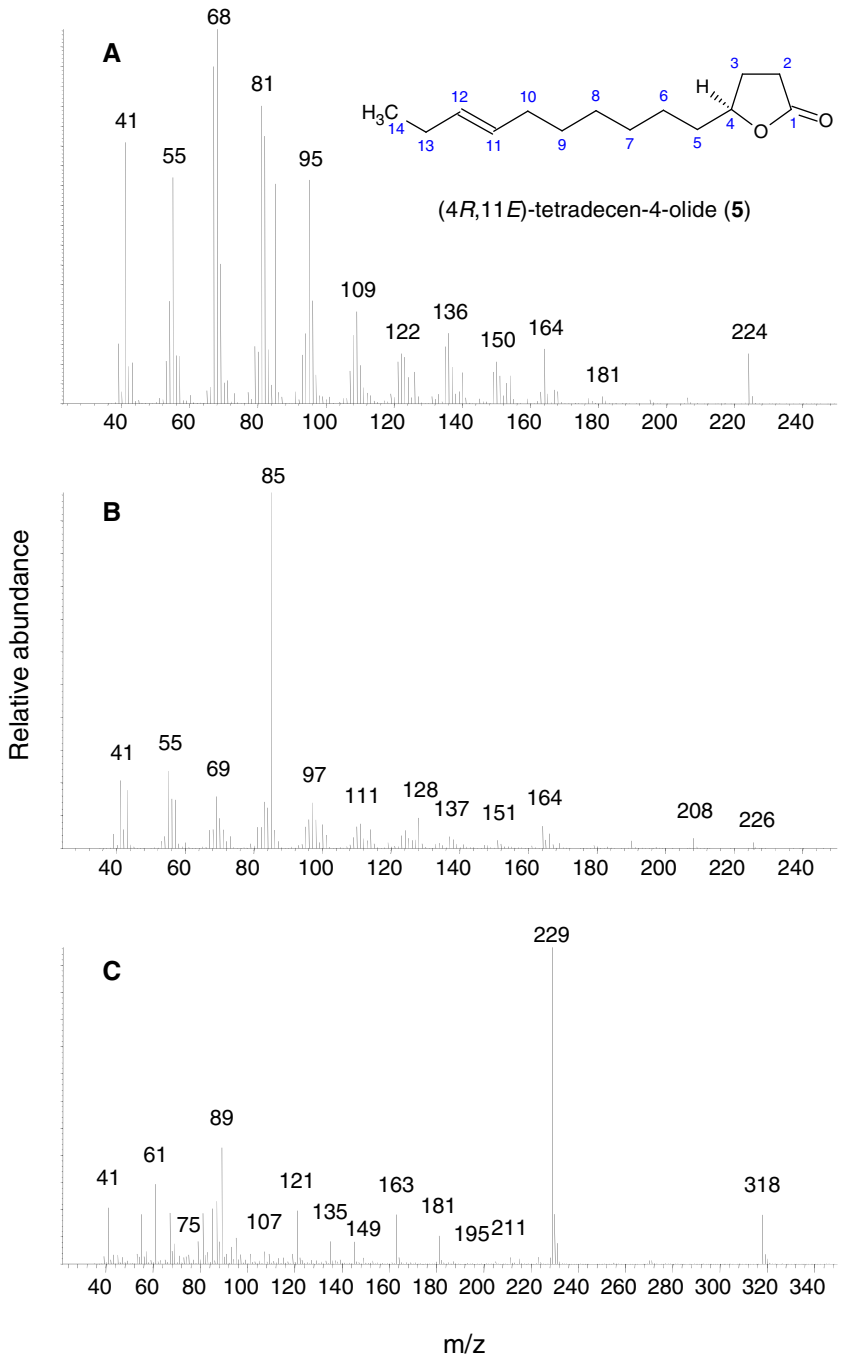

Fig. 2 EI mass spectra (70 eV) of (**a**) natural (4*R*,11*E*)-11-tetradecen-4-olide, (**b**) after catalytic hydrogenation, and (**c**) the dimethyldisulfide adduct

the second eluted at 14.28 min on the DB-5 capillary column. The two compounds were emitted in a 1:10 ratio, respectively. Both the earlier eluting compound (molecular ion *m/z* 254) and later eluting compound (molecular ion *m/z* 256) showed a fragment ion at *m/z* 61, indicative of acetates. A computer library search of their mass spectra tentatively identified them as (*Z*)-7-tetradecen-1-yl acetate and tetradec-1-yl acetate, respectively. The DMDS derivatives of the compound at 14.14 min showed a molecular ion at *m/z* 348 (=254+94), and the fragment ions (*m/z* 203 and 145) were consistent with a double bond being present at the 7 position. The identifications of (*Z*)-7-tetradecen-1-yl acetate and tetradec-1-yl acetate were confirmed by matching GC retentions and mass spectra with authentic standards. The double bond configuration of (*Z*)-7-tetradecen-1-yl acetate was not further investigated; however, *E*- and *Z*-isomers of 7-tetradecen-1-yl acetate do separate on a DB-5 column (De Marques et al., 2000).

Table 2 Assigned NMR shifts (δ) for natural (*E*)-11-tetradecen-4-olide (**5**) and synthetic (*Z*)-11-tetradecen-4-olide (**16**) ($CDCl_3$)

Pos	(*E*)-11-Tetradecen-4-olide (**5**)		Pos	(*Z*)-11-Tetradecen-4-olide (**16**)	
	^{1}H shift (description, splitting)	^{13}C shift		^{1}H shift (description, splitting)	^{13}C shift
1	-	177.2	1	-	177.2
2	H_{2a}:2.55 (d, 9.6)	28.9	2	H_{2a}:2.54 (d, 9.5)	28.9
	H_{2b}:2.54 (dd, 9.6, 1.9)			H_{2b}:2.53 (dd, 9.5, 1.3)	
3	H_{3a}:2.33 (ddt, 12.7, 6.6, 7.4)	28.0	3	H_{3a}:2.33 (m)	28.0
	H_{3b}:1.87 (ddt, 12.7, 8.0, 9.5)			H_{3b}:1.86 (ddt, 12.8, 8.0, 9.5)	
4	4.50 (p, 6.9)	81.0	4	4.49 (p, 6.9)	81.0
5	H_{5a}:1.75 (m, overlap)	35.6	5	H_{5a}:1.74 (m, overlap)	35.6
	H_{5b}:1.61 (m, overlap)			H_{5b}:1.60 (m, overlap)	
6	H_{6a}:1.46 (overlap)	25.2	6	H_{6a}:1.46 (overlap)	25.2
	H_{6b}:1.36 (overlap)			H_{6b}:1.36 (overlap)	
7	1.33 (overlap)	~29	7	~1.33 (overlap)	29-30
8	1.33 (overlap)	~29	8	~1.33 (overlap)	29-30
9	1.33 (overlap)	~29	9	~1.33 (overlap)	29-30
10	1.98 (overlap)	32.5	10	2.02 (overlap)	27.0
11	5.40 (m)	129.1	11	5.32 (m)	129.1
12	5.46 (m)	132.1	12	5.46 (m)	131.7
13	2.00 (overlap)	25.6	13	2.04 (overlap)	20.5
14	0.98 (t, 7.4)	14.0	14	0.98 (t, 7.6)	14.4

Shifts in parts per million from tetramethylsilane, splitting (*J*) in Hertz. Position numbers are given in Fig. 2a

Flight Tunnel Bioassay Preliminary flight tunnel tests with female-specific compounds (natural and synthetic) did not show any long-range attraction to either males or females; therefore, long-range attraction to male-specific compounds became the focus of this study. Females responded in a dose-related manner to the male volatiles when introduced in the flight tunnel by the piezo-electric sprayer (Table 3). A synthetic blend of all seven compounds in ratios stated in Table 1 and sprayed at a rate of 1 ng/μl of the major component **6** showed the same attraction to females compared to the 1 ng/μl and 0.1 ng/μl blends of natural male volatiles (amounts of racemic **5** in the synthetic blend were double the stated ratio in Table 1). Male parasitoids also responded to this synthetic blend, although at lower response levels. Earlier preliminary tests applying natural male-specific volatiles on filter paper disks and hanging them in the flight tunnel showed males responding positively but, again, at lower levels than those found with the female parasitoids. Comparing virgin female flight tunnel responses to the full seven component blend to those of partial blends showed that a blend containing **1**, **5**, and **6,** and the full blend, gave similar results (Table 4). A binary blend of **5** and **6** gave an intermediate response and, when tested as single compounds, responses to **1**, **5**, and **6** did not differ from the responses to the solvent control.

Pheromone Release Rates The mean release rates of the tertiary blend of **1**, racemic **5** and **6** from rubber septa after 4 weeks outdoors is listed in Table 5. Compounds **5** and **6** showed steady release rates during the whole period averaging approximately 0.2 and 1 μg/day, respectively. As expected, the release rate of the more volatile aldehyde **1** decreased over time, but was still present in the release blend after 4 weeks in the field. Daily maximum temperatures ranged from 21 to 34 °C during the collection period.

Field Tests The field cage trapping experiments demonstrated the attractiveness of the 7 component blend of male-specific compounds to male and female *S. agrili* in a more natural setting. Significantly more males (t=5.94, df=23, P< 0.001) and females (t=5.63, df=23, P<0.001) were caught on traps baited with the pheromone blend compared to the control traps. For females, the mean daily trap catch was 6.2±2.3 (± SD) for the pheromone and 0.5±0.7 (± SD) for the control. For males, rates were 5.5±1.5 (± SD) for the pheromone and 1.5±1.1 (± SD) for the control. In total, approximately 45 % of the released males and 50 % of the released females were captured on the pheromone baited traps during the 24 h trapping periods (Fig. 4). Virgin females were also significantly (t=3.37, df=11, P<0.005) attracted to traps baited with the 3 component blend compared to the control traps. For this tertiary blend, the mean daily trap catch was 8.5±1.0 (± SD) compared to 0.5±0.8 (± SD) for the control traps. In total, approximately 50 % of the released females were captured on traps baited with the tertiary blend during the 24 h trapping periods (Fig. 4).

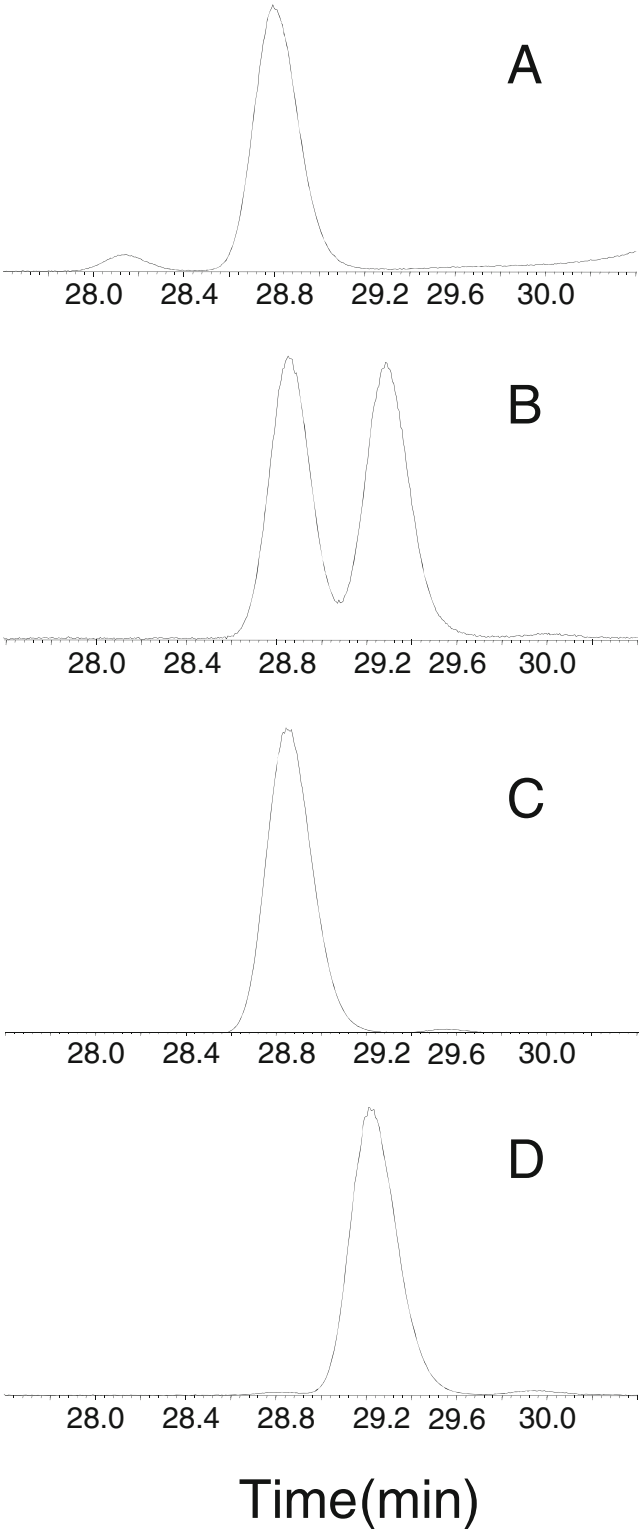

Fig. 3 GC-MS analysis of (**a**) natural (4*R*,11*E*)-tetradecen-4-olide, (**b**) racemic (*E*)-11-tetradecen-4-olide, (**c**) synthetic (4*R*,11*E*)-tetradecen-4-olide, and (**d**) (4*S*,11*E*)-tetradecen-4-olide on a chiral stationary phase β-DEX 225 column (190 °C isothermal)

Table 3 Flight tunnel behavioral responses of virgin male and female *Spathius agrili* to decreasing amounts of natural male-specific volatiles and to its synthetic equivalent

Treatment[a]	Responder	*N*	% Upwind flight[b]	% Landing on source[b]
Male volatiles				
1 ng/μl	♀	20	90.0[a]	85.0[a]
0.1 ng/μl	♀	10	70.0[ac]	60.0[a]
0.01 ng/μl	♀	10	10.0[b]	10.0[b]
Synthetic blend				
1 ng/μl	♀	46	82.6[a]	78.3[a]
	♂	50	52.0[c]	20.0[b]
Control				
	♀	15	6.7[b]	6.7[bc]
	♂	15	0[b]	0[c]

[a] Introduced with a piezo-electric sprayer at ratios stated in Table 1

[b] Mean with different letters indicate significance (Fisher's exact test, a=0.05)

Discussion

The flight tunnel and field cage experiments demonstrated that male *S. agrili* emit a blend of compounds that is attractive to both sexes. We identified dodecanal **1**, (*Z*)-10-heptadecen-2-one **6**, and (4*R*,11*E*)-tetradecen-4-olide **5** as the key behavioral components in this pheromone blend. There was no evidence of a behavioral function for the additional four male-specific compounds. Female *S. agrili* also emit a sex-specific blend comprised of (*Z*)-7-tetradecen-1-yl acetate and tetradec-1-yl acetate, but this blend was not attractive to either males or females in flight tunnel experiments, and its role in the mating behavior of *S. agrili* is still unknown.

Table 4 Flight tunnel behavioral responses of virgin female *Spathius agrili* to full and partial blends of synthetic male-specific volatiles

Treatment[a]	Responder	*N*	% Upwind flight[b]	% Landing on source[b]
1 -7	♀	46	82.6[a]	78.3 [a]
1, 5, 6	♀	29	72.0[ab]	65.0[a]
5, 6	♀	20	60.0[b]	35.0[b]
5	♀	31	16.0[c]	6.5[c]
6	♀	10	20.0[c]	0[c]
1	♀	10	0[c]	0[c]
Control	♀	10	0[c]	0[c]

[a] Introduced with a piezo-electric sprayer at ratios stated in Table 1 and sprayed at 1 ng/μl of **6**

[b] Mean with different letters indicate significance (Fisher's exact test, a=0.05)

Table 5 Mean (± SE) release rates of *Spathius agrili* pheromone components from red rubber septa during June 7 till July 6, 2011

Week	N	Comp. **1** μg/day	Comp. **5** μg/day	Comp. **6** μg/day
0	3	3.58±0.11	0.16±0.02	0.94±0.11
1	3	1.94±0.20	0.19±0.03	0.99±0.13
2	3	1.35±0.18	0.35±0.08	1.28±0.19
3	3	0.69±0.06	0.23±0.06	1.13±0.24
4	3	0.48±0.01	0.19±0.04	1.11±0.16

Female wasps responded equally well to a synthetic blend that contained racemic **5** compared to the natural male-emitted blend, indicating that having the opposite enantiomer in the mix is not detrimental to the observed behavioral responses. The flight tunnel responses showed a higher response level by female wasps to male-specific compounds compared to male wasps, but this difference was absent in the field cage experiments. Female wasps were observed to arrive at the pheromone baited traps throughout the morning until sunset during variable wind conditions in the field cage. Males, however, only responded to the pheromone baited traps during low wind conditions, which could indicate that females wasps are stronger flyers than male wasps and that the wind speed in our flight tunnel might have been set too high for optimal male behavioral responses.

Our flight tunnel studies focused on the behavioral responses of virgin adults. Preliminary behavioral studies indicated that mated males will still respond to the pheromone, but mated females do not (Miriam Cooperband, pers. communication). Female *S. agrili* have an average lifespan of 29 days, during which time they can oviposit up to eight times (Yang et al., 2010). Future studies will examine whether female responses to pheromone are restored during periods between egg laying.

Long-range sex pheromones are known for several parasitic Hymenoptera, and these pheromones are emitted typically by females to attract conspecific males (see reviews by Ayasse et al., 2001; Keeling et al., 2004) with one exception. Ruther et al. (2007) reported identification of a male-produced sex pheromone of the jewel wasp, *Nasonia vitripennis* (Walker) (Hymenoptera:Pteromalidae) as a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide. Male-produced pheromones that attract both sexes have not been reported before for parasitic wasps.

The (4*R*,11*E*)-tetradecen-4-olide of *S. agrili* has not been reported as a pheromone component in Braconidae, but is similar in structure to the hydroxyl lactone in the above mentioned pheromone for *N. vitripennis*. Structurally related lactones have been found in the Hagen's glands of four braconid wasps, *Diachasmimorpha longicaudata* (Ashmead), *D. tryoni* (Cameron), *D. kraussii* (Fullaway), and *Fopius arisanus* (Paddon-Jones et al., 1997), and these authors suggested that the lactones could have a behavioral function during mating and courtship (Williams et al., 1988).

The pheromones of three unrelated species also show a structural similarity to the *S. agrili* lactone: (4*R*,9*Z*)-octadec-9-en-4-olide, the pheromone of the currant stem girdler, *Janus integer* (Norton) (Hymenoptera: Cephidae) (Cossé et al., 2001); (*R*)-(*Z*)-7,15-hexadecadien-4-olide, the sex pheromone of the yellowish elongate chafer, *Heptophylla picea (*Motschulsky) (Coleoptera: Scarabaeidae) (Leal et al., 1996); and (4*R*,9*Z*)-hexadec-9-en-4-olide, the sex pheromone of cerambycid beetle *Desmocerus californicus californicus* (Coleoptera: Cerambycidae) (Ray et al., 2011).

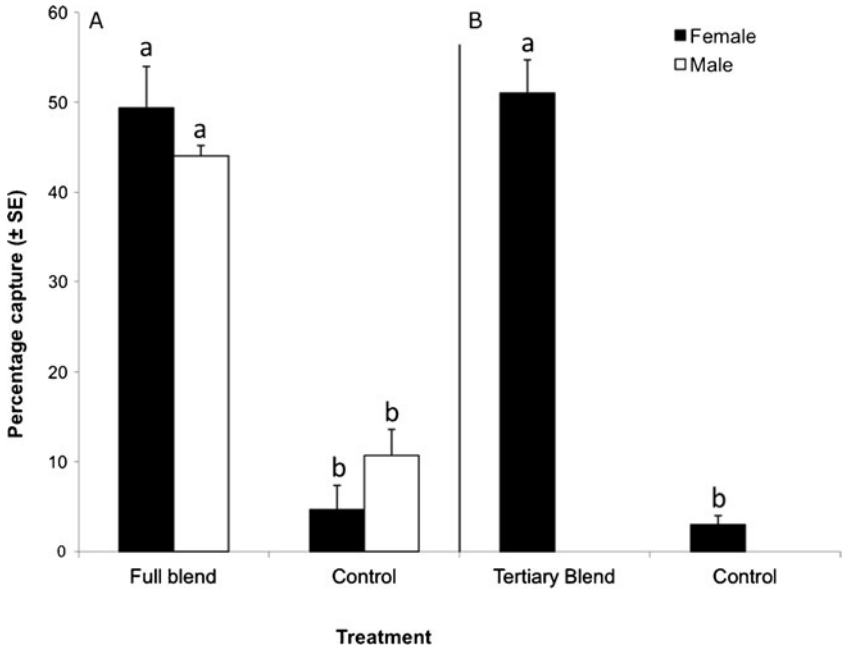

Fig. 4 Percentage (± SE) of captured virgin male and female *Spathius agrili* on yellow sticky traps baited with; (**a**) a blend of synthetic dodecanal (22.5 μg), tetradecanal (2.0 μg), (*Z*)-10-pentadecen-2-one (0.5 μg), pentadecan-2-one (10.0 μg), (*Z*)-10-heptadecen-2-one (100 μg), heptadecan-2-one (3.0 μg), and natural (4*R*,11*E*)-tetradecen-4-olide (5.8 μg) during May 2010; (**b**) a tertiary blend of dodecanal (100 μg), racemic (*E*)-11-tetradecen-4-olide (450 μg), and (*Z*)-10-heptadecen-2-one (1 mg) during June 2011

(*Z*)-10-heptadecen-2-one **6** is not known as a pheromone component in Braconidae, but it has been found in the aggregation pheromones of several *Drosophila* species (Bartelt et al., 1989; Schaner and Jackson, 1992), and dodecanal **1** has been reported as a pheromone component in many different insect species including parasitic wasps (El-Sayed, 2011).

Yang et al. (2010) reported in detail on the biology and behavior of *S. agrili* in its native China. The authors reported that male *S. agrili* typically eclosed prior to the females, and that males waited on the bark outside of the pupation chamber for mating opportunities with female siblings. However, the authors suggested that not all mating occurred between siblings due to the relatively lengthy emergence period for a single brood (5 to 44 days), and they observed mating in aggregations of male and female *S. agrili* wasps on single tree trunks. The identified male aggregation pheromone of *S. agrili* might play a role in attracting conspecific wasps that did not mate immediately at their natal patch. We noticed a relatively strong decrease of male-emitted pheromone when male wasps were placed in groups during our volatile collections. Whether the cessation of pheromone emissions is part of the mating strategy of *S. agrili* during adult aggregations in the field is still unknown. A similar mate finding strategy was reported in the parasitic wasp *Cephalonomia tarsalis* (Ashmead) (Hymenoptera: Bethylidae), in which dodecanal was identified as a female-produced sex pheromone in individuals that failed to mate at the eclosion sites (Collatz et al., 2009). Finally, Quimio and Walter (2000) reported on male mating swarm behavior in the braconid *F. arisanus*, and postulated that males may form aggregations thus amplifying an unidentified male-produced pheromone to attract female wasps at which time female wasps release a pheromone that attracted males.

Future experiments will focus on testing the identified *S. agrili* pheromone in the field, and to assess its potential as a monitoring tool to detect newly established populations of this biocontrol agent. Furthermore, the newly identified pheromone will help us to further elucidate the sexual communication and mating strategy of *S. agrili*.

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Females of the Bumblebee Parasite, *Aphomia sociella*, Excite Males Using a Courtship Pheromone

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Abstract *Aphomia sociella* (Lepidoptera: Pyralidae: Galleriinae) is a parasitic moth of bumblebees. Behavioral experiments show that *A. sociella* females emit semiochemicals that influence male pre-mating behavior and serve as a courtship pheromone. GC/EAD and two-dimensional GC/MS (GCxGC-TOFMS) analyses of extracts of females revealed three antennally active compounds. Comparative GC and GCxGC-TOFMS analyses of extracts and synthetic standards confirmed the identity of the antennally active compounds as hexan-1-ol (**1**), 6,10,14-trimethylpentadecan-2-one (**2**), and 6,10,14-trimethylpentadecan-2-ol (**3**). In laboratory bioassays, alcohol **3** and, at higher doses, ketone **2** initiated male courtship behavior associated with ultrasonic production. Hexan-1-ol (**1**) and ketone **2** enhanced the activity of alcohol **3**. These data suggest that hexan-1-ol, 6,10,14-trimethylpentadecan-2-ol, and 6,10,14-trimethylpentadecan-2-one constitute the female-produced courtship pheromone of *A. sociella*.

Keywords Galleriinae · Courtship pheromone · Ultrasonic signaling · 6,10,14-Trimethylpentadecan-2-ol · 6,10,14-Trimethyl-2-pentadecanone · Lepidoptera · Pyralidae

Introduction

Aphomia sociella is a parasitic moth of bumblebees. This pyralid moth is a member of the subfamily Galleriinae, which are characterized by a unique mating system that differs from that of most other Lepidoptera. Depending on the species, males initiate mating either by releasing sex pheromone, by emitting ultrasonic signals, or a combination of both modalities (Greenfield and Coffelt, 1983; Flint and Merkle, 1983; Spangler et al., 1984; Spangler, 1985, 1987a, b, 1988; Zagatti et al., 1987; Bennett et al., 1991; Conner, 1999; Greenfield, 2002 for reviews). *Aphomia sociella* males attract females using a sex pheromone released from wing glands, and dispersed during stationary-wing-fanning calling behavior (Kalinová et al., 2009). Guided by the male pheromone, females fan their wings as they fly and walk towards the signal source (Kindl et al., 2011). The proximity of a female terminates male calling and triggers male courtship behavior associated with walking-wing-fanning and ultrasonic production (Kalinová et al., 2009; Kindl et al., 2011). This female-induced change in male behavior suggests that an airborne signal(s) exists because direct contact between the two sexes is not necessary to elicit the behavioral switch in males. The signals may involve multiple

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modalities, including chemical, mechanical, and acoustic communication. The only female-produced courtship pheromone known for a galleriine was described for *Corcyra cephalonica* (Hall et al., 1987). Additionally, sound associated with female wing-beating was shown to influence male pre-mating behavior in *Galleria mellonella* (Spangler, 1987b). Apart from producing sound, wing beating also induces air turbulence (Loudon and Koehl, 2000), which may serve as an additional communicative signal either by itself or in combination with other modalities.

The present study addressed the following questions: Do females approaching calling males emit volatiles to communicate with males? If so, what chemicals constitute the pheromone, and, are semiochemicals alone sufficient to induce male courtship behavior?

Methods and Materials

Insects Nests of *Bombus terrestris* (Hymenoptera: Bombidae) were started in the laboratory, and then transferred to outside hives. Bumblebee queens originated from the northern part of Prague (a garden about 100 m^2; 50°08″13‴ N; 14°25″55‴ E; altitude 298 m). The nests were exposed to infestation by *A. sociella*. Both the bumblebee and the moth species were identified by one of us (J.K.).

Aphomia sociella larvae were allowed to develop and overwinter under natural conditions. In the spring, the colonized nests were transferred into net cages in the laboratory. New adult moths were separated by sex, placed in individual plastic cups capped with moisturized cotton plugs, and kept in continuous light at ambient temperature. Virgin males and females (2–4 d-old) were used for experimentation.

Behavioral Experiments Prior to bioassays, males were placed individually into 3-cm diam round wire mesh tea strainers (hereafter referred to as "cages"), and allowed to acclimate in the dark. Stationary-wing-fanning (calling) males were selected for behavioral experiments performed in the dark under red light illumination, at ambient temperature, and ambient humidity. The experiments tested the ability of different stimuli to terminate male stationary-wing-fanning calling behavior, and to initiate walking-wing-fanning courtship associated with ultrasound production. Male behavior was observed visually, and ultrasonic songs were monitored by means of a bat detector (heterodyne Mini 3 Bat Detector, Ultra Sound Advice, UK) set at 80 kHz, the maximum energy at which *A. sociella* ultrasonic calls are transmitted (Kindl et al., 2011). The following stimuli were tested: living females and males, dead (frozen for 15 min) females and males, separate female bodies and wings, hexane extracts of female bodies and wings, and various synthetic compounds and combinations. Living and dead specimens or body parts were placed inside glass tubes (3-cm diam, 5-cm long) covered at both open ends with nylon mesh screens held in place by rubber bands. Extracts were loaded onto filter paper discs (1-cm diam) in amounts equivalent to one moth (Female Equivalent, FE, or Male Equivalent, ME). Synthetic standards were diluted in hexane and applied to filter paper discs in amounts comparable to those found during the chemical analysis of extracts of females. After loading, the solvent was allowed to evaporate from the filter paper discs. Glass tubes and/or loaded filter paper discs were placed on top of cages containing calling males. Male responses were monitored for 2 min after the introduction of a stimulus. The stimulus then was removed, and the males were allowed to become quiescent. Once the male started to call again, another stimulus was applied. Each male was subjected to three stimuli in a series: control (empty glass cartridge or filter paper disc loaded only with hexane), test stimulus (live or frozen specimen, body part, or extract), and a standard (a living female). The control stimuli provided information about background activity, while the standard stimuli indicated the readiness of the male to respond; these treatments were especially important when individual synthetic standards were tested. Each male was exposed to the described three stimuli only once. A total of 24 males were tested for each treatment in experiments with natural stimuli (live/dead specimens or extracts). A total of 60 males were tested in experiments with standards. Males were tested in 5 sessions, each with 12 specimens. Males that did not produce ultrasonic calls within the 2-min testing period were considered non-responsive.

Extraction of Volatiles Virgin 1−2-d-old females were placed individually in cages, and positioned around a caged calling male. Females that responded to the calling male by wing-fanning were cooled to −20 °C for 15 min, and then extracted with hexane for 24 hr (ca 100 μl of hexane per one female or body part). After 24 hr, the female bodies were removed from the extract, the solution was filtered through glass wool, and the samples were stored at −20 °C until two-dimensional GC/MS analysis with time-of-flight mass analyzer (GCxGC/TOFMS) or GC/EAD experiments.

Polydimethylsiloxane (PDMS) fibers for solid phase microextraction (SPME, Supelco, 100 μm coatings) were used for a subset of experiments to determine the possible site of female courtship pheromone production. In these experiments, female wings, the ventral and dorsal parts of a female abdomen, and the intersegmental membrane of the

8-9th segment of a female abdomen were gently dabbed with the fiber. After sampling, the SPME fibers were analyzed using GCxGC/TOFMS. Prior to each sampling, the fibers were conditioned in the injection port of the GC for about 10 min.

Chemical Analyses GC×GC/TOFMS analyses were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with a quad-jet cryomodulator. A non-polar Rxi-5Sil MS column (30 m×250 μm× 0.25 μm, Restek, Bellefonte, PA, USA) was used in the first dimension, while analysis in the second dimension was performed on a polar BPX-50 column (2.5 m×100 μm× 0.1 μm, SGE Inc., Ringwood, Australia). The columns were connected using Press-Tight Connectors (Restek, Bellefonte, PA, USA). Helium at a constant flow rate of 1 ml/min was used as a carrier gas. The temperature program for the primary GC oven was as follows: 60 °C (1 min), 7 °C/min to 250 °C, and then 15 °C/min to 320 °C (5 min). The program in the secondary oven was set to 20 °C above the primary oven temperature, and was operated in an isoramping mode. The modulation period and heating and cooling pulse durations were set at 4.0, 0.6, and 1.1 sec, respectively. The transfer line to the TOFMS detector was held at 260 °C. Detector source temperature was 200 °C with a filament bias voltage of −70 eV. Data were acquired at a rate of 50 Hz (scan/s) for a mass range of 29–600 amu. Detector voltage was set to 1750 V with a solvent delay of 420 sec. One microliter samples were injected in a splitless mode at an inlet temperature of 200 °C. Purge time was 60 sec at a flow rate of 60 ml/min. LECO ChromaTOF software v. 2.32 (LECO corporation, St. Joseph, MI, USA) was used for system control, data acquisition, and data processing. A series of *n*-alkanes (C8–C22; Sigma-Aldrich) was coinjected with samples to determine linear retention indices (LRI) of the analytes. Natural components were identified by comparing MS fragmentation patterns and retention times of natural products to those of synthetic standards. The NIST/EPA/NIH Mass Spectral Library (2008) was used as a source of reference spectra.

Gas Chromatograph/Electroantennogram detector (GC/EAD) Experiments For GC/EAD experiments, isolated antennae of adult males were connected in parallel to a flame ionization detector (FID), and used as biological detectors. Analyzed samples were injected splitless into a 5890A Hewlett-Packard GC equipped with an Rxi-5Sil MS (Restek; 30 m×0.25 μm ID×0.25 μm film) column split at the end by a Graphpack 3D/2 four-arm splitter. The splitter divided the GC eluate sending it both to an FID and an EAD detector. The GC was operated at an initial temperature of 50 °C for 2 min, then increased at a rate of 10 °C/min to 270 °C (held 10 min). Temperatures of the GC inlet and detector were set to 200 °C and 260 °C, respectively. A series of saturated C8-C22 alkanes (Sigma-Aldrich) was coinjected with samples to provide reference retention times for calculating EAD active peak linear retention indices (LRI), and for matching LRI and the antennal activity of authentic compounds with those of synthetic standards.

Chemicals The following synthetic standards were purchased from Sigma-Aldrich: a series of *n*-alkanes, hexan-1-ol (**1**), and phytone (**2,** perhydrofarnesyl acetone; 6,10,14-trimethylpentadecan-2-one). 6,10,14-Trimethylpentadecan-2-ol (**3**) was prepared from phytone by reduction with $LiAlH_4$ in freshly distilled diethyl ether. The reaction was carefully terminated with water, and the identity of the product was confirmed by mass spectral analysis (*m/z* 252 and the *m/z* 45 base-peak are characteristic fragments for the secondary alcohol; for full spectrum see the Supplementary material). All standards were purified by flash chromatography, and dissolved in trace analysis grade hexane (Fluka). In the following text, we use the abbreviations TMPD-one and TMPD-ol for 6,10,14-trimethylpentadecan-2-one (**2**) and 6,10,14-trimethylpentadecan-2-ol (**3**), respectively.

Statistical Analysis Results of behavioral experiments were evaluated using Levene's test for homogeneity of variance, followed by ANOVA and LSD tests at $P=0.05$.

Results

Behavioral experiments showed that females, both dead and alive, are more effective at triggering male courtship and ultrasonic responses than are males. All 24 of the tested males responded to live or dead females; 75 % of the males responded to living males. Dead males elicited ultrasonic signaling in only 10 % of the males tested (Fig. 1). Isolated female bodies and wings triggered ultrasonic calls in males (wings were slightly less effective than bodies). Active compounds from females were successfully extracted with hexane, as evidenced by hexane extracts of females eliciting male ultrasonic calls (Fig. 1 A,B) comparable to responses seen with living females.

GC/EAD analysis consistently revealed 3 EAD-active areas (EAD1, EAD2, and EAD3). The LRI of EAD1 was determined as 864. Its mass spectrum was identical with that of hexan-1-ol obtained from the NIST library. EAD2 and EAD3 eluted closely together with LRI of 1848 and 1852, respectively. The mass spectrum of the EAD2 compound

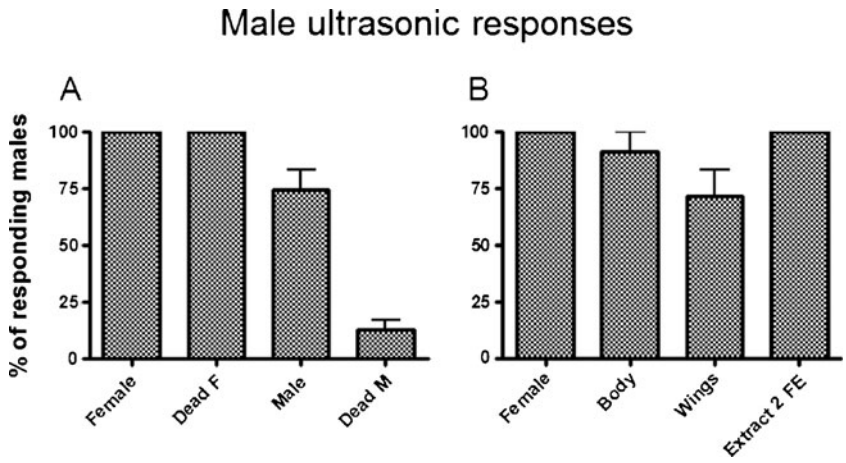

Fig. 1 Percentage of males responding by ultrasound production to (**a**) living or dead conspecifics and (**b**) female body parts and hexane extracts. F, female; M, male; FE, female equivalent. Live females served as a control. A total of 24 males were tested

was identical to that of TMPD-one (**2**) from the NIST library (see Supplementary material). The spectrum of the EAD3 compound contained fragments characteristic for an alcohol (small *m/z* 31 ion and an *m/z* 45 base peak) instead of a ketone (*m/z* 58). Based on the LRI and mass spectrum, the compound was tentatively identified as TMPD-ol (**3**). GC/EAD (Fig. 2) and GCxGC/TOFMS analysis (Fig. 3, Table 1) of synthetic hexan-1-ol, TMPD-ol, and TMPD-one confirmed the identifications. The synthetic standards elicited EAD responses; LRI's of EAD-active areas corresponded to LRI's of those elicited by the hexane extract of females. Mass spectra and RT in 1st and 2nd dimensions in GCxGC/TOFMS analysis of authentic and synthetic compounds were identical (Table 1). Based on an independent analysis of three different extracts, the ratio of hexan-1-ol:TMPD-one:TMPD-ol was 5:1:5. An attempt was made to determine which of the diastereoisomeric pairs of TMPD-ol and TMPD-one were involved, but the quantity of extracted compounds was insufficient for this analysis.

SPME sampling of abdominal tips, thorax, and ventral/dorsal abdomen from females did not provide definitive information about the discrete site of pheromone production. The identified compounds were dispersed relatively evenly over the entire female body, although samples

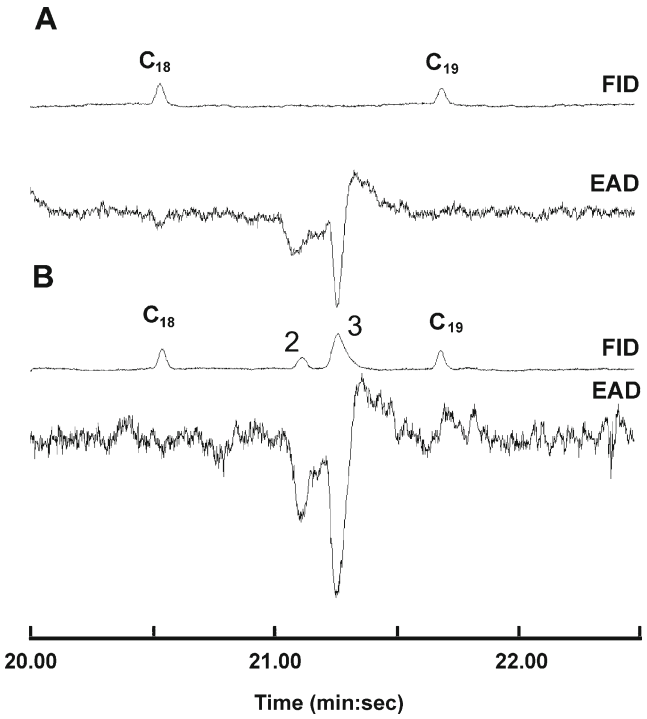

Fig. 2 A section of GC/FID/EAD trace (retention time of 20–22.30 min): (**a**) female body extract co-injected with a series of normal alkanes; two EAD active areas are visible at a retention time of 21.17 min corresponding to LRI 1848 and 1852, respectively; (**b**) synthetic TMPD-one (**2**) and TMPD-ol (**3**) co-injected with alkanes. The retention behavior and antennal activity of synthetic standards match EAD activities elicited by authentic compounds from the female extract (below the FID detection limit)

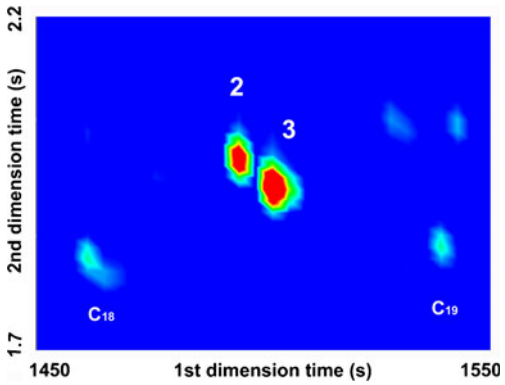

Fig. 3 Detail of a two-dimensional GCxGC-TOFMS chromatogram obtained during the analysis of female body extracts. The selected plot (characterized by 1st retention range from 1450 to 1550 seconds and 2nd retention range from 1.7 to 2.2 sec) corresponds to the GC-EAD area depicted in Fig. 2. Each spot on the plot represents one compound: **2**, TMPD-one; **3**, TMPD-ol. The x-axis represents retention from the 1st column, while the y-axis represents retention from the 2nd column

Table 1 Retention data of pheromonal components

Compound	LRI_{EAD}	LRI_{GCXGC}	GCxGC RT [s] (1st and 2nd dimension) Extract	GCxGC RT [s] (1st and 2nd dimension) Synthetic standards
Hexan-1-ol	864	886	458, 1.740	456, 1.732
TMPD-one	1848	1843	1498, 2.020	1496, 2.012
TMPD-ol	1852	1852	1506, 1.980	1504, 1.974

obtained from wings were less concentrated in comparison with other body parts (Table 2).

Of the three identified compounds, hexan-1-ol and TMPD-one alone were behaviorally inactive, but TMPD-ol elicited ultrasonic signaling in males at doses higher than 1 ng (Fig. 4). A dose of 500 pg of TMPD-one was below the behavioral threshold, but 10 and 100 ng elicited responses in 25 % and 78 % of males, respectively. Higher doses of TMPD-one were not tested. Experiments with binary and ternary blends showed that both hexan-1-ol and TMPD-one enhance the effect of TMPD-ol (Fig. 5). Addition of 10 ng of hexan-1-ol or 2 ng of TMPD-one to 10 ng of TMPD-ol increased the number of responding males from 38 to 45 %, to 58 and 75 %, respectively. A ternary blend of 10 ng of hexan-1-ol, 2 ng of TMPD-one, and 10 ng of TMPD-ol stimulated 85 % of males. The synergistic effects of TMPD-one and hexan-1-ol on TMPD-ol activity were statistically significant (Fig. 5).

Discussion

In the vast majority of lepidopteran species, males produce courtship pheromones. Glands consist of many different types of modified scales that may be located on almost any part of the insect body (Percy-Cunningham and MacDonald 1987). These male-produced courtship pheromones are quite diverse in chemistry, glandular source, and function. They serve as effective reproduction barriers (Phelan and Baker, 1990), may play a role in attracting females (Heath et al., 1992) and/or other males (Takayoshi and Hiroshi, 1999), serve as aphrodisiacs to make females quiescent and more likely to accept a courting male (Teal et al., 1981, Hillier and Vickers, 2004; Phelan et al., 1986), and provide cues for female mate choice (Jacquin et al., 1991; Friberg et al., 2008). Male-produced courtship pheromones can also eliminate competition from conspecific males, either by direct repulsion (e.g., hair-pencil odors) or by inducing termination of female calling (Hendricks and Shaver, 1975; Huang et al., 1996).

In Galleriinae, the roles of searching and signaling are reversed between the sexes in that male moths typically produce long-range signals that initiate searching behavior in females. The males call using either chemical or acoustic signals or a combination of both (Zagatti et al., 1987; Spangler, 1988 and Conner, 1999 for reviews). The reversed roles of signaling and searching in Galleriinae are associated with highly variable species-specific behavioral adaptations in both sexes (Kindl et al., 2011). This also is true for signals required by males to change behavior from calling to courting, which were shown to be auditory in *Eldana saccharina* (Zagatti, 1981). *Galleria mellonella* males respond to female wingbeat sounds and/or substrate vibrations of similar frequencies (Spangler, 1987b).

Table 2 Ratios of pheromonal components in different female body parts

Body part	TMPD-ol/TMPD-one ratio
Thorax	1.66
Abdomen	0.95
Wings	0.45
Ventral abdomen	1.03
Dorsal abdomen	1.11
8th-9th abd. segment	only TMPD-one

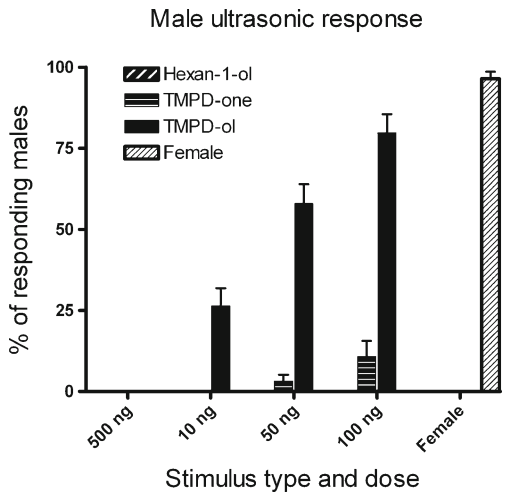

Fig. 4 Percentage of males responding by ultrasound production to various doses of synthetic hexan-1-ol, TMPD-one, and TMPD-ol presented individually. Live females served as a control. Males were tested in 5 sessions, each with 12 specimens (N=60)

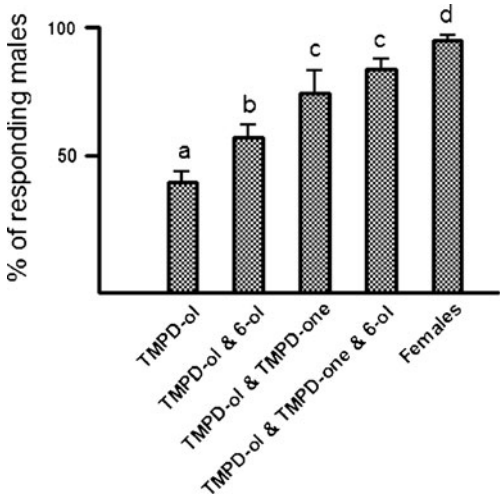

Fig. 5 Percentage of males responding by ultrasound production to TMPD-ol alone, to two-component blends of 10 ng each of TMPD-ol and hexan-1-ol or 10 ng each of TMPD-ol and TMPD-one, and to a three-component blend containing 10 ng of hexan-1-ol, 2 ng of TMPD-one, and 10 ng of TMPD-ol (5:1:5 ratio). Live females served as a control. Males were tested in 5 sessions, each with 12 specimens. Statistics: N= 60, Levene's test for homogeneity of Variances, followed by ANOVA and LSD analysis, P=0.05. (The letters above the error bars represent significant differences of the means)

Corcyra cephalonica female moths emit TMPD-ol (the sole EAD-active compound) as a courtship pheromone that acts over a short distance to attract males, and stimulates them to attempt copulation (Hall et al., 1987). To our knowledge, no other courtship pheromone has been described from females in the Lepidoptera; thus, our data here provide only the second such chemical identification. The *C. cephalonica* female-produced courtship pheromone is active at close-range; beyond 13 cm, the communicative effectiveness is greatly reduced (Hall et al., 1987). Synthetic pheromone was attractive to males when tested in an olfactometer; it terminated calling and initiated mate searching, as well as homosexual copulation attempts. Replacement of live females with dead ones reduced the number of copulation attempts, demonstrating the importance of visual and/or auditory cues in the courtship behavior of this species. The *A. sociella* female-produced courtship pheromone also terminates calling (stationary-wing-fanning), and initiates male courtship behavior (walking-wing-fanning) associated with male ultrasonic signaling. This, in turn, stimulates the female to accept copulation (Kindl et al., 2011). Live and dead females were equally effective at initiating courtship, suggesting that chemical cues dominate in *A. sociella*; however, other modalities, such as female wing-beat sounds and/or substrate vibrations, may also be involved in courtship communication to some extent (e.g., as observed in *G. mellonella*; Spangler, 1987b). Visual stimuli likely play only a negligible role in *A. sociella* since the moths mate during scotophase.

The specific site of courtship pheromone production in *A. sociella* females has not been identified. Likewise, the site of courtship pheromone production in *C. cephalonica* is unknown. For the majority of lepidopteran females, modified intersegmental membranes between abdominal segments VIII and IX serve as pheromone-producing glands (Percy and Weatherston, 1974; Atkinson, 1982, Farine, 1983; Vohringer, 1934), but such glands were not found in *C. cephalonica* (Hall et al., 1987). Our analyses, based on SPME sampling of discreet body surfaces and extraction of different *A. sociella* body parts, also failed to localize the site of production of the female courtship pheromone, but we did find smaller amounts of pheromone on the wings than on the body (for both SPME and extraction sampling). Thus, our data suggest secretory cells excreting courtship pheromone may be evenly distributed over the entire body, as may also be the case for *C. cephalonica* (Hall et al., 1987).

The compounds found in the *A. sociella* female-produced courtship pheromone were previously reported as constituents of plant odors and/or constituents of various insect communication systems. While both sexes of *A.sociella* sexes produce TMPD-2-one, TMPD-ol is a female-specific compound. TMPD-ol has been identified as part of the female courtship pheromone of the closely related galleriine species, *C. cephalonica* (Hall et al., 1987). In other Galleriinae, males often use TMPD-ol for communication: this compound is a component of the male sex pheromone of the African sugarcane borer *Eldana saccharina* (Burger et al., 1985, 1993) and *Tirathaba mundella* (Sasaerila et al. 2003) (Lepidoptera: Pyralidae: Galleriinae). TMPD-ol is also part of the male-produced aphrodisiac pheromone of the small and large cabbage white butterflies *Pieris rapae* and *Pieris brasicae* (Yildizhan et al. 2009), and the African butterfly, *Bicyclus anynana* (Nieberding et al., 2008). *E*-Phytol [(*E*)-3,7,11,15-tetramethyl-2-hexadecen-l-ol], a compound related to TMPD-ol, a constituent in the male-produced wing gland sex pheromone in a related species, *Ephestia elutella* (Lepidoptera: Pyralidae; Phycitinae; Phelan et al., 1986).

TMPD-2-one (phytone) is an abundant floral compound in Orchidaceae, Asteraceae, and Hypericaceae (for a complete list, see http://www.pherobase.com). In insects, phytone is a constituent of the sex pheromones from males of *A. sociella* (Kalinová et al. 2009), *G. mellonella* (Vendilo et al., 1998), and *T. mundella* (Sasaerila et al., 2003) (Lepidoptera: Pyralidae: Galleriinae). The co-production of TMPD-2-one in both sexes of *A. sociella* is interesting and suggestive of a mating complex system. Further research is needed to understand the behavioral roles of this compound in male- and female-produced pheromones.

In summary, our results provide evidence for the existence of a female-produced courtship pheromone in *A. sociella*. This pheromone includes hexan-1-ol, TMPD-ol, and TMPD-one, with TMPD-ol being the dominant component that is

enhanced by the other two compounds. This chemical message delivered by females to males is sufficient to influence male behavior. Our data provide a new facet in the understanding of *A. sociella* unique precopulatory behavior, and provide a basis for broader ecological considerations.

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Identification and Synthesis of a Male-Produced Pheromone for the Neotropical Root Weevil *Diaprepes abbreviatus*

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Abstract An unsaturated hydroxy-ester pheromone was isolated from the headspace and feces of male *Diaprepes abbreviatus*, identified, and synthesized. The pheromone, methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate, was discovered by gas chromatography-coupled electroantennogram detection (GC-EAD), and identified by gas chromatography–mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). The synthesis yielded an 86:14 mixture of methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (active) and methyl (*Z*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (inactive), along with a lactone breakdown product. The activity of the synthetic *E*-isomer was confirmed by GC-EAD, GC-MS, NMR, and bioassays. No antennal response was observed to the *Z*-isomer or the lactone. In a two-choice olfactometer bioassay, female *D. abbreviatus* moved upwind towards the synthetic pheromone or natural pheromone more often compared with clean air. Males showed no clear preference for the synthetic pheromone. This pheromone, alone or in combination with plant volatiles, may play a role in the location of males by female *D. abbreviatus*.

Keywords Citrus root weevil · Pheromone · GC-EAD · Methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate · Coleoptera · Curculionidae · Crop pest

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Introduction

The root weevil, *Diaprepes abbreviatus* (L.) (Curculionidae: Entiminae) (Marvaldi et al., 2002), is a major pest of citrus in the Caribbean and Florida. Prior to the 1960's, *D. abbreviatus* was reported only in the Caribbean; the multiple phenotypic populations that occur in Puerto Rico suggest this as its center of origin (Lapointe, 2004). Since discovery of *D. abbreviatus* near Apopka, Florida in 1964, this weevil has spread to Louisiana, Texas, and California leaving no geographic or climatic barrier to its movement south to Mexico, Mesoamerica, and South America (Lapointe et al., 2007). *Diaprepes abbreviatus* is typical of Entiminae in that the adults do not use the rostrum to create a niche for egg laying, and larvae feed externally on roots (Marvaldi et al., 2002). Adults feed and oviposit on foliage of a wide range

of host plants (Simpson et al., 1996). Neonate larvae fall to the ground and burrow into the soil where they feed on progressively larger roots as they grow. Larvae pupate in the soil and emerge as adults throughout the year.

Within the superfamily Curculionoidea, the majority of known pheromones are long-range, male-produced aggregation pheromones (Seybold and Vanderwel, 2003; Ambrogi et al., 2009). Aggregations of *D. abbreviatus* adults have been observed on so-called "party trees" (Wolcott, 1936). Schroeder (1981) suggested that a *D. abbreviatus* male-produced pheromone attracted females, and a female-produced pheromone attracted males. Jones and Schroeder (1984) demonstrated that a male-produced pheromone in the feces attracted both sexes of *D. abbreviatus*, and there may be a pheromone responsible for arrestment behavior (Lapointe and Hall, 2009). Otálora-Luna et al. (2009) identified plant volatiles from citrus leaves that elicited antennal responses in *D. abbreviatus.* Such kairomones may act in concert with a pheromone to attract conspecifics to a suitable food source (Dickens et al., 1990).

We report here the isolation from headspace and feces of males, identification, and synthesis of a pheromone that attracts *D. abbreviatus* females.

Methods and Materials

Insects Adult weevils were obtained from a laboratory colony maintained at the U. S. Horticultural Research Laboratory, Ft. Pierce, FL, USA, supplemented annually with field-collected adults. Neonate larvae were placed on artificial diet (product no. F1675, Bio-Serv, Inc., Frenchtown, NJ, USA) and reared as described by Lapointe et al. (2008). Adult males and females were held in separate 60 x 60 x 60 cm mesh cages with water-saturated dental wicks, and fed young citrus leaves (*Citrus macrophylla* Wester) until use in aerations or bioassays. Groups of 10–20 adults 4–6 wk-old were held without food for 24 h prior to behavioral assays. After the assay, adults were returned to their respective cages and provided food and water. Individual unmated adults were used in tests no more than once per week over a period of ~3 mo. Cohorts of known age were caged separately.

Gas Chromatography-Electroantennogram Detection (GC-EAD) The GC-EAD system consisted of an Agilent 7890A GC equipped with a split/splitless injector, an HP-1 capillary column (30 m x 0.32 mm x 0.25 μm, Agilent Technologies, Inc., Santa Clara, CA, USA), a post column glass Y-tube (Supelco, Bellefonte, PA, USA) splitter dividing the effluent in a 1:1 ratio between the flame ionization detector (FID) and heated EAD transfer line (200°C). Lengths of deactivated column (0.32 mm ID) were used to carry the effluent to the FID and EAD port after the split. The heated transfer line emptied into to a charcoal-filtered, humidified air stream (200 ml/min at 30 cm/sec) that carried the effluent over an antennal preparation. The air stream was directed past a probe attached to a type PRG-2 amplifier (Universal EAG probe, Syntech, Hilversum, The Netherlands). Signals from the amplifier and the FID were conditioned using a Syntech IDAC-2 interface. EAG and FID signals acquired from the IDAC-2 were displayed and stored on a computer running the GC-EAD 2011 software program (Syntech). An adult weevil antenna was mounted between the leads of the Universal EAG probe. The antennal preparation was made by plucking the antenna from the insect (grasping it firmly at the base of the antenna near the head with fine forceps) and placing it between two metal electrodes on the probe, to which small amounts of salt-free electrode gel (Spectra 360, Parker Laboratories, Fairfield, NJ, USA) had been applied. At the start of GC-EAD runs, the GC oven temperature was held at 35°C for 3 min, increased to 260°C at 15°C/min with a 10 min final hold. Injector and FID temperatures were 220°C and 300°C, respectively. Splitless injection was used with helium as the carrier gas at a flow rate of 2.3 ml/min.

Analytical Methods Coupled GC-mass spectrometry (GC-MS) was conducted using instruments operated in the electron impact (EI) and chemical ionization (CI) modes. EI spectra were obtained using an Agilent 5973 MS interfaced to a 6890 GC equipped with a cool on-column injector. The injector was fitted with a 10 cm length of 0.5 mm id deactivated fused silica tubing connected to 1 m (0. 25 mm id) length of deactivated fused silica tubing as a retention gap. The retention gap was connected to a 30 m x 0.25 mm id with 0.25 μm coating thickness DB5MS® analytical column. The injector and oven temperature were programmed from 30°C for 5 min to 225°C at 10°C/min. Spectra were obtained between 60–300 amu. Chemical ionization spectra were obtained using an Agilent 5975 MS interfaced to a 7890 GC. The GC was equipped with a cool on-column injector fitted with retention gaps as above. The analytical column used was a 30 m x 0.25 mm id, 0.25 μm coating thickness DB1MS®. The GC was operated using the same program as for EI spectra, and the CI spectra were obtained by scanning from m/z 60–300 using isobutane as reagent gas.

Collection and Purification of Volatiles Groups of 20 to 30 male and female *D. abbreviatus*, held separately, were placed in separate glass aeration chambers without plant material, and provided with a continuous flow (500 ml/min) of filtered, humidified air for 24 h at 27°C in an environmental chamber (12:12 hL:D). Volatiles were collected on Super Q filters (Alltech Deerfield, IL, USA) connected to the exit port of the aeration chambers. After collection, the filters were eluted with 500 μl of CH_2Cl_2. The accumulated feces in the aeration chambers at the end of the 24-h collection period were collected by washing the chambers with a minimum amount of CH_2Cl_2;

the resulting extract was filtered and concentrated under nitrogen.

As an initial purification, CH_2Cl_2 eluates (200 µl) from the Super Q filters and washes of the aeration chambers were passed through Supelclean LC-SI solid phase extraction (SPE) columns containing 200 mg of packing (Supelco, Bellefont, PA) previously conditioned with 15 ml of CH_2Cl_2. The CH_2Cl_2 eluates plus a filter wash of 2 ml of CH_2Cl_2 were saved to check for the presence of the EAD-active compound. The SPE column was eluted with 2 ml each of pentane containing 15% ethyl acetate (EtOAc), 30% ETOAc, and 50% ETOAc. The three SPE column fractions, and the saved CH_2Cl_2 eluates, were analyzed by EI GC-MS for the presence of the EAD-active compound suspected to have a molecular weight of 172. Only the 15% EtOAc fraction contained the compound of interest. This fraction was concentrated to ca. 100 µl under a fine stream of N_2 and subjected to fractionation by preparative GC. Initial fractionation was accomplished using an Agilent 6890 GC® with cool-on-column injector fitted with a 20 cm length of deactivated fused silica attached to a 30 m x 0.53 mm id (0.5 µm film thickness) DB1 column. The preparative column was split using a "Y" capillary connector between equal lengths of 0.1 mm id and 0.25 mm id lengths of deactivated fused silica column. The effluent from the 0.1 mm id column (ca. 13.8%) went to the FID, while the 0.25 mm id column (ca 86.2%) exited the wall of the GC and into the heated block (200°C) of a Brownlee-Silverstein collector (Brownlee and Silverstein, 1968). Ten µl samples were injected onto the column at an initial temperature of 30°C, and after 2 min the oven temperature was increased to a final temperature of 225°C at 10°C/min. The fractions were collected in a 30-cm liquid nitrogen-cooled glass capillary (Brownlee and Silverstein, 1968), and were recovered by washing the capillaries with 3 25-µl aliquots of CH_2Cl_2. Fractions were analyzed by GC-MS for the presence of compounds having *m/z* 154, 142, and 140 ions, all of which eluted within 0.01 min of each other. These fractions were combined, concentrated, and re-fractionated using a DB35 column (30 m x 0.53 mm id, 0.5 µm film thickness) as above. The fractions from this separation were eluted from capillaries using deuterated chloroform (99.96% $CDCl_3$, Cambridge Isotope Laboratories, Inc.), analyzed by GC-MS, and the fractions containing the compound having the 172 MW were combined, concentrated under N_2, and submitted for NMR analysis.

NMR Analysis One and two-dimensional NMR spectroscopy, including double-quantum filtered correlation spectroscopy (dqCOSY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and Nuclear Overhauser Enhancement Spectroscopy (NOESY) were used to characterize the EAD-active compound. NMR spectra of the natural product were acquired at 27°C using a 5-mm TXI cryoprobe and a Bruker Avance II 600 console (600 MHz for 1H, 151 MHz for 13 C). The combined fractions containing the EAD-active compound (MW 172) were dissolved in ~150 µl $CDCl_3$ and placed in a 2.5 mm NMR tube (Norell). Residual chloroform ($CHCl_3$) was used to reference chemical shifts to $\delta(CHCl_3)=7.26$ ppm for 1H and $\delta(CHCl_3)=77.36$ ppm for 13 C (Gottlieb et al., 1997). Bruker Topspin 2.0 and Mestrelab MNova NMR (Mestrelab Research SL) software packages were used to process NMR spectra. ^{1}H NMR spectra of synthetic materials, including a NOE difference spectrum for **1***E*, were obtained on a Bruker AVIII-600 MHz spectrometer.

Synthesis of the EAD-Active and Related Compounds (Fig. 1). Unless otherwise specified, all reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). THP protection of 3-butyn-1-ol was conducted following a procedure of Rama Rao et al. (1986). A solution of 3-butyn-1-ol (14.0 g, 0.2 mol) in CH_2Cl_2 (75 ml, dried by distillation from CaH_2) was placed in a flask under N_2. The mixture was cooled to 0°C, and pyridine *p*-toluenesulfonate (0.28 g) was added. 3,4-Dihydro-2H-pyran (20.1 ml) was added via a dropping funnel maintaining the temperature between 0 and 5°C. The mixture was stirred at this temperature for 1 h, then warmed to 25°C, and stirred for an additional 2 h. TLC (silica gel, hexanes/ethyl acetate, 3:1) showed that the reaction was complete. The reaction mixture was taken into cold water (~ 70 ml), and transferred to a separatory funnel. The layers were separated, and the aqueous phase was extracted with CH_2Cl_2. Combined organic extracts were washed with $NaHCO_3$ (aq), brine, and dried with Na_2SO_4 (anh). Distillation produced 23 g of THPO-protected 3-butyn-1-ol with bp 50-55°C/4 mm Hg. GC-MS (*m/z*, relative intensity): 153 (2, M^+-1), 99 (9), 85 (100), 79 (9), 67 (20), 53 (42), 41 (33), a mass spectrum matching that presented in the NIST MS library for this compound.

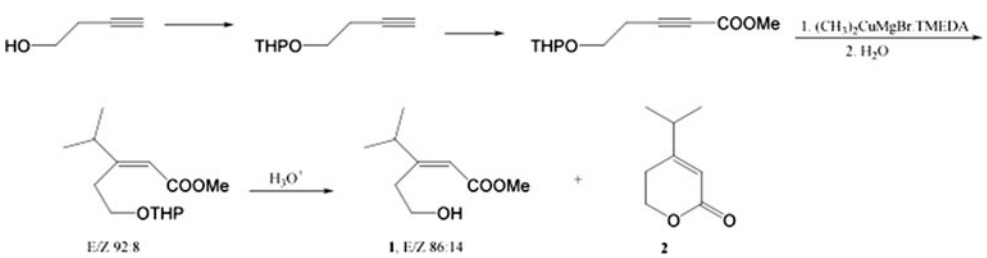


Fig. 1 Synthetic route for methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (**1**), and related compounds. See text for details

A solution of the THPO-ether of 3-butyn-1-ol (2.88 g, 18.7 mmol) in THF (40 ml, dried with sodium benzophenone ketyl) was placed under N_2 into a four-neck flask and cooled to −75°C. Butyl lithium (18.7 mmol, 9.4 ml of 2.0 M in hexanes) was added slowly via a dropping funnel at −75°C. The mixture was stirred at this temperature for 30 min, and then methyl chloroformate (ClCOOMe, 1.4 ml, 18.7 mmol) was added. The resulting mixture was stirred at −75°C for 30 min, then slowly warmed to 25°C in~2 h and poured into a cold saturated NH_4Cl solution. The organic material was extracted with hexanes/ether, 1:1. The combined extracts were washed with brine and dried. After removal of the solvent on a rotary evaporator, the remainder was flash chromatographed on silica gel with hexanes/ethyl acetate, 4:1, to isolate methyl 5-(tetrahydro-2H-pyran-2-yloxy)-2-pentynoate (2.5 g, 65%). GC-MS (*m/z*, relative intensity): 211 (1, M^+-1), 157 (4), 153 (3), 142 (4), 125 (18), 113 (9), 109 (11), 85 (100), 79 (41), 67 (18), 55 (16), 41 (26). ^{1}H NMR ($CDCl_3$): 1.50-1.64 (m, 4H), 1.72 (m, 1H), 1.83 (m, 1H), 2.66 (t, *J*=6.6 Hz, 2H, H-4), 3.53 (m, 1H, H-5A), 3.62 (dt, *J*=9.8, 7.5 Hz, 1H, H-5B). 3.77 (s, 3H, OCH_3), 3.87 (m, 2H), 4.65 (br t, *J*=4.0 Hz, 1H, OCHO). ^{1}H NMR data were in agreement with data obtained for this compound in CCl_4 (Rama Rao et al., 1986).

Freshly purchased copper iodide (CuI, 1.97 g, 12.2 mmol) was placed under N_2 in a four-neck flask. Dry tetrahydrofuran (THF) (35 ml) was added, followed by N,N,N',N'-tetramethylethylenediamine (2.76 ml). The mixture was stirred at room temperature until a green-yellow solution was obtained and then cooled to −70°C, upon which a green suspension was formed. Isopropylmagnesium chloride solution (12.3 mmol; 6.1 ml of 2.0 M in THF) was added slowly, whereupon a green suspension became colorless then turned brown. The mixture was stirred at −70°C for 1 h, then methyl 5-(tetrahydro-2H-pyran-2-yloxy)-2-pentynoate (1.3 g, 6.1 mmol) dissolved in dry THF (5–10 ml) was added. The resultant mixture was stirred at −70°C for 3 h, and quickly poured into an ice-cold mixture of saturated NH_4Cl and hexanes/ether, 5:1. The organic layer was separated, and the aqueous layer was extracted with hexanes/ether, 5:1. The combined organic extracts were washed thoroughly with saturated NH_4Cl solution until no blue color was seen. The organic extract was dried and concentrated. Flash chromatography with hexanes/ethyl acetate, 5:1, afforded methyl 4-methyl-3-[2-(tetrahydro-2H-pyran-2-yloxy)ethyl]-2-pentenoate (1.3 g, 85%) as a 92:8 mixture of *E*- and *Z*-isomers as judged from GC-MS analysis. GC-MS (*E*-isomer, *m/z*, relative intensity): 172 (2), 171 (3), 155 (32), 142 (8), 141 (6), 139 (6), 95 (17), 85 (100), 67 (16), 57 (10), 55 (11), 43 (11), 41 (16). GC-MS (*Z*-isomer, *m/z*, relative intensity): 172 (4), 155 (3), 154 (8), 142 (8), 141 (2), 139 (4), 123 (6), 95 (23), 85 (100), 67 (17), 57 (10), 55 (12), 43 (11), 41 (17). ^{1}H NMR (400 MHz, C_6D_6, δ): 0.86 (d, *J*= 8.0 Hz, *E*-isomer), 0.86 (d, *J*=8.0 Hz, *Z*-isomer), 1,20-1.42 (m, 4H), 1.55-1.62 (m, 2H), 1.70-1.82 (m, 1H), 2.18-2.31 (m, 1H), 3.02-3.18 (m, 2H), 3.41 (s, OCH_3), 3.67-3.73 (m, 1H), 3.81-3.88 (m, 1H), 4.04-4.11 (m, 1H), 4.39 (septet, *J*=8.0 Hz, H-4, *Z*-isomer), 4.52 (t, *J*=4.0 Hz, OCHO, *Z*-isomer), 4.66 (t, *J*=4.0 Hz, OCHO, *E*-isomer), 5.83 (br. s, H-2, *E*-isomer), 5.86 (br. s, H-2, *Z*-isomer). ^{13}C NMR (101 MHz, C_6D_6, δ, *E*-isomer): 20.0, 21.7 (two carbons), 26.3, 31.4, 32.9, 37.3, 50.9, 61.9, 67.3, 98.8, 115.1, 167.2, 167.8.

Methyl 4-methyl-3-[2-(tetrahydro-2H-pyran-2-yloxy)ethyl]-2-pentenoate (256 mg, 1 mmol) was stirred with *p*-toluenesulfonic acid hydrate (9 mg, 0.047 mmol) in a THF-H_2O solution (8+2 ml) at 55-60°C for~1 h, or until TLC analysis (SiO_2 plates; hexanes/ethyl acetate/MeOH, 16:6:1; visualization with $KMnO_4$ solution) showed very little starting ester present. The mixture was cooled to room temperature, treated with 50 µl 1 N NaOH, and concentrated to remove most of the THF. The mixture was extracted with ether/hexanes, 1:1, and the organic extract was dried with Na_2SO_4 (anh). After evaporation of the solvent, the remainder was flash chromatographed with hexanes/ethyl acetate/MeOH, 16:6:1. Two fractions were obtained: a) starting THPO-ester, 16 mg; and b) a mixture of ester **1** and lactone **2** (Fig. 1). The second fraction was chromatographed again with hexanes/ethyl acetate/MeOH, 16:6:1 to furnish **1** (*E/Z* 86:14, 90 mg, 58%) in the less polar fraction. ^{1}H NMR (600 MHz, C_6D_6, δ): 0.79 (d, *J*=6.6 Hz, $(CH_3)_2$, *E*), 0.91 (d, *J*=6.6 Hz, $(CH_3)_2$, *Z*), 2.01-2.08 (m, H-4 *E*, CH_2C=C, *Z*), 2.46 (t, *J*=5.4 Hz, OH, *E*), 2.76 (t, *J*=6.6 Hz, CH_2C=C, *E*), 3.34 (s, OCH_3, *E*), 3.36-3.38 (m, $C\underline{H_2}O\underline{H}$, *Z*), 3.41 (s, OCH_3, *Z*), 3.70 (q, *J*=5.4 Hz, $C\underline{H_2}OH$, *E*), 4.32 (septet, H-4, *Z*), 5.71 (br. s, H-2, *Z*), 5.80 (br. s, H-2, *E*). ^{13}C NMR (151 MHz, C_6D_6, *E*-isomer): 21.7 (two carbons), 35.6, 36.7, 51.1, 62.5, 115.6, 167.7, 168.7; *Z*-isomer: 20.9 (two carbons), 29.8, 35.1, 50.8, 61.6, 116.0, 165.7, 166.8. Lactone **2** (10 mg) was recovered from the more polar (second) fraction. GC-MS (*m/z*, relative intensity): 140 (M^+, 16), 125 (7), 110 (15), 97 (19), 96 (59), 95 (96), 82 (24), 81 (100), 67 (73), 55 (17), 41 (40). ^{1}H NMR (400 MHz, C_6D_6, δ): 0.57 (d, *J*=6.6 Hz, $(CH_3)_2$), 1.37 (br. t, *J*=6.5 Hz, CH_2C=), 1.70 (septet, *J*=6.6 Hz, $C\underline{H}(CH_3)_2$), 3.61 (t, *J*=6.5 Hz, CH_2O), 5.67 (d, *J*=1.0 Hz, CHC=). NMR data are in agreement with those obtained for this compound in $CDCl_3$ (D'Annibale et al., 2007).

NMR signals of the synthetic compound in $CDCl_3$ were: ^{1}H (400 MHz): 1.12 (d, *J*=6.8 Hz, $(CH_3)_2$, *E*), 2.44 (septet, *J*=6.5 Hz, $C\underline{H}(CH_3)_2$), 2.87 (t, *J*=6.4 Hz, CH_2C=), 3.73 (s, OCH_3), 3.81 (br. q, 5.2 Hz, $C\underline{H_2}OH$), 5.85 (br. s, CH=). ^{13}C (101 MHz): 21.5 (two carbons), 34.7, 36.2, 51.3, 62.0, 115.3, 166.8, 168.7.

Confirmation of EAD-Activity and Behavioral Response to Synthetic **1***E* The synthetic methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate was diluted with hexane to approximately 100 ng/µl, and 1 µl of sample were injected on the GC-EAD

system described above. A 1-μl injection of 50 ng of linalool and 100 ng/μl of the synthetic methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate in hexane was used to determine retention times and test antennal responses. Linalool was determined previously to elicit a consistent antennal response, and was co-injected with methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate to confirm that antennae were viable. Antennae from male and female *D. abbreviatus* were tested.

Behavioral response to the pheromone was tested in an olfactometer (Model 4 C, ARS, Inc., Micanopy, FL USA). Individual weevils (starved for 24 h) were placed in a glass inlet, allowed to walk upwards into the center of an arena with a balanced, filtered, and humidified airflow from two arms oriented at 180° to each other and outfitted with glass reservoirs containing an odor source or blank. The assays were conducted in the dark between 9 am and 2 pm because of a strong phototropic response in this insect (Lapointe and Hall, 2009). Each run was terminated when the weevil moved into one of the glass receptacles or after 15 min. Weevil position was scored as no-response (remaining in the inlet), no-choice (moving to, but remaining in the central arena), or choosing one of the two arms. Responses of unmated 4-6-wk-old males and females were recorded to various odor sources: fresh young citrus leaves (flush), flush fed upon for 24 h by male *D. abbreviatus*, 30 μg of methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate in 10:1 hexanes:ethyl acetate pipetted onto a glass slide, and a clean glass slide. All glass components of the olfactometer were washed thoroughly between runs with warm soap and water, rinsed with methanol, and air-dried. Between replications of a given treatment, the arms of the olfactometer used for that treatment were switched to control for bias in the apparatus. The number of weevils choosing a treatment arm was compared with the control arm (clean air) by the *G*-test (Sokal and Rohlf, 1994).

Results

One compound from aerations of male weevils consistently elicited antennal responses from both male and female *D. abbreviatus* in GC-EAD experiments (Fig. 2). The amount of this compound in 24-h aerations of ten *D. abbreviatus* males was sufficient to elicit responses from the antennae of males and females, but the compound was barely detectable by GC. The same compound also was recovered in CH_2Cl_2 by washing the frass of males from glass aeration jars. Combining multiple aerations of males provided sufficient material to obtain CI and EI mass spectra (Fig. 3a, b). Initial GC analyses indicated the presence of several compounds eluting in the area of the EAD-active compound (Fig. 2). EI GC-MS analyses indicated that several of these, including the EAD-active compound, had an *m/z* 154 ion and fragmentation patterns

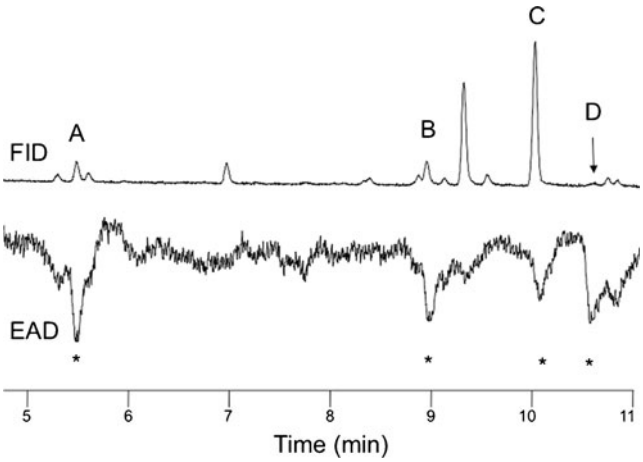


Fig. 2 Simultaneous gas chromatogram (FID) and electroantennogram detection (EAD) of a male *Diaprepes abbreviatus* antenna responding to a hexane extract of headspace volatiles collected from adult males and citrus leaves. Compounds A - C correspond to the plant volatiles: linalool, geraniol and citral, respectively; compound D is male-derived. Asterisks indicate consistent antennal responses

corresponding to monoterpene alcohols. Although the EI mass spectrum of the EAD-active compound showed an *m/z* 154 ion (Fig. 3b), the presence of an *m/z* 142 ion indicated that *m/z* 154 might not be the molecular ion; the latter possibility was verified by SIM analysis showing *m/z* 140, 142, and 154 ions maximized within ±0.01 min of each other. CI-MS analysis (Fig. 3a) exhibited a clear M+1 ion at *m/z* 173, thus supporting a molecular weight of 172 Daltons for the EAD-active compound. In addition, the CI analysis also exhibited a base peak at *m/z* 141 (M+1-CH_3OH), and a prominent *m/z* 155 ion (M+1-18); thus, the *m/z* 154 ion in the EI spectrum is due to loss of H_2O. The CI did not directly indicate the origin of the *m/z* 142 fragment in the EI spectra; however, the loss of 30 amu (*m/z* 172–142) suggested a long range proton transfer to a carbonyl group followed by a neutral loss of CH_2=O, which is consistent with a hydrocarbon chain having a terminal alcohol as well as a carbonyl. This interpretation also was congruent with a methyl ester as suggested by the (M+1-CH_3OH) loss in the CI spectrum. A characteristic neutral loss of 32 amu also was seen in the EI spectra (*m/z* 142–110), thereby supporting the structure of a methyl ester. Furthermore, the neutral loss of 15 amu (*m/z* 142–127 as well as *m/z* 110–95) suggested the presence of a methyl branch. Thus, the fragmentation patterns indicated a formula of $C_9H_{16}O_3$ with 2 degrees of unsaturation, the presence of a methyl ester, and at least one methyl branch, and a terminal C-OH. Further aerations of groups of up to 30 males, and fractionations by preparative GC using both polar and nonpolar capillary columns, resulted in collection of a sufficient amount of the pheromone for NMR analysis (Table 1). These experiments enabled the structure assignment for the EAD-active peak as methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (**1***E*).

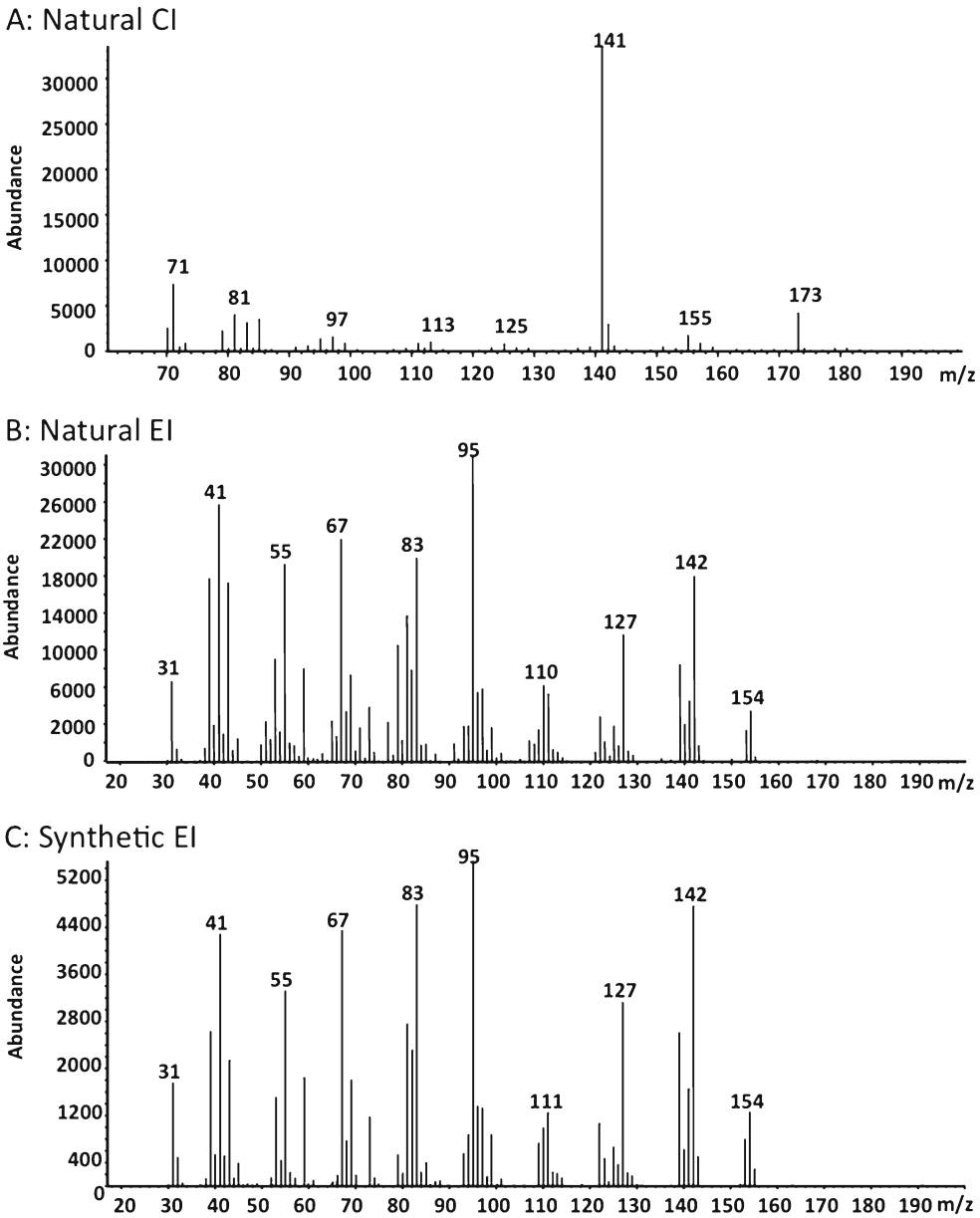


Fig. 3 Chemical ionization **a** and electron impact **b** mass spectra of the EAD-active compound from an aeration extract of *Diaprepes abbreviatus* males, and the EI mass spectrum of synthetic methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate **c**. The CI spectrum shows the M+1 ion at m/z 173, thus supporting a molecular weight of 172 Daltons

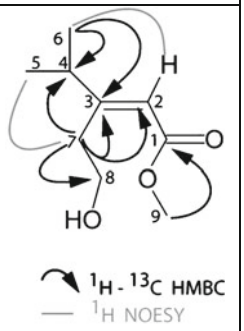
The NMR analysis also revealed an antennally inactive isomer (methyl (*Z*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate), and a related lactone **2** (Table 2) that apparently originated from partial isomerization and lactonization of the putative pheromone catalyzed by $CDCl_3$.

The structure of the synthetic compound was confirmed as identical with the natural EAD-active compound by mass spectral (Fig. 3c) and NMR analyses. As with the natural material, the synthetic material contained a pair of Z/E-isomers of methyl 3-(2-hydroxyethyl)-4-methyl-2-pentenoate, along with the lactone breakdown product. The NMR of the synthetic sample obtained in C_6D_6 showed two doublets at 0.79 and 0.91 ppm (corresponding to the doublets at 1.09 and 1.12 ppm in the isolated material) due to a pair of methyl groups from both stereoisomers split by a single proton on the adjacent carbon. Similarly, there was a pair of singlets at 3.34 and 3.41 ppm due to methyl groups from isomeric esters corresponding to the singlets at 3.73 and 3.71 ppm in the NMR of natural sample recorded in $CDCl_3$. A NOE difference spectrum obtained by irradiating the resonance of the larger doublet at 0.79 ppm resulted in enhancement of the larger singlet from the olefinic proton at 5.80 ppm and vice versa. Therefore, the olefinic proton and the isopropyl group in the major stereoisomer are in *cis*-position, and this stereoisomer and the natural product have the *E*-configuration.

Synthesis and Stability of the EAD-Active Compound The synthesis of methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate **1** is depicted in Fig. 1. The chosen route employs

Table 1 ^{1}H (600 MHz), ^{13}C (151 MHz), HMBC and NOESY NMR spectroscopic data for the putative pheromone of *Diaprepes abbreviatus* in $CDCl_3$. Chemical shifts referenced to $\delta(CHCl_3)$=7.26 ppm for ^{1}H and $\delta(CHCl_3)$=77.36 ppm for ^{13}C. Coupling constants are given in Hertz [Hz]

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	J coupling constants [Hz]	HMBC correlations	NOESY	
1	169.0*	-	-	-	-	
2	115.5	5.83	s		1.10	
3	166.9*	-	-	-	-	
4	36.35*	2.43	m J= 6.7			
5 and 6	21.7	1.10	d, J=6.8	C4, C3	5.83 and 2.84	
7	35.2	2.84	t, J=6.4	C2, C3, C4, C8	1.10	
8	62.5	3.8	br t J= 6.3		-	
9	51.7	3.7	s	C1		

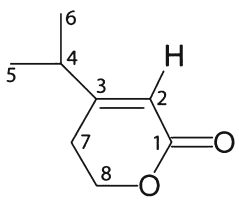


*The ^{13} C chemical shifts are deduced from HMBC; others are deduced from HSQC. ^{1} H chemical shifts are deduced from 1D ^{1} H NMR

stereoselective carbocupration of α,β-acetylenic esters that is exclusively *cis*-stereospecific when the reaction is conducted in THF at low temperatures (Corey and Katzenellenbogen, 1969; Bourque et al., 1999; Drew et al., 1999). However, a conjugate addition of a heterocuprate, formed in situ from isopropylmagnesium bromide and copper(I) iodide in the presence of N,N,N',N'-tetramethylethylenediamine (Crimmins et al., 1984), to the acetylenic ester (Fig. 1) proceeded with some loss of stereoselectivity resulting in a mixture of *E* and *Z* olefinic esters in a 92:8 ratio. During acid-catalyzed removal of the tetrahydropyranyl (THP) protecting group, the ratio of **1***E* and **1***Z* dropped to 86:14, and a considerable amount of lactone **2** was formed, thus indicating instability of the putative pheromone under acidic conditions. A noticeable cyclization of **1***E* to **2** also occurred in the acidic $CDCl_3$ used for recording NMR spectra. Using *t*-butyldimethylsilyl (TBDMS) protecting group (instead of THP) for the acetylenic alcohol provided a similar stereochemistry at the carbocupration (*E*/*Z* 93:7) step but, again, resulted in significant lactonization when the TBDMS group was removed with highly basic tetrabutylammonium fluoride. Lactonization also occurred when a purified sample of the synthetic (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate was injected at 260°C into the split-splitless injection port of the GC- MS, resulting in a total ion chromatogram comprising **1***E*:**1***Z*:**2** in a ratio of 39:8:53; the same sample analyzed by cool-on-column GC-MS injection yielded an 88:9:3 mixture of **1***E*:**1***Z*:**2**. Because of its thermal instability, initial attempts to isolate the putative pheromone by preparative GC equipped with a conventional injection port failed. A purified sample of the synthetic compound could be stored indefinitely at 0-25°C in aprotic solvents, such as benzene, hexane, and ethyl acetate.

Table 2 ^{1}H (600 MHz) and ^{13}C (151 MHz) spectroscopic data for the lactone degradation product present in aeration extracts of *Diaprepes abbreviatus* males. Only HSQC data are reported for the lactone. Chemical shifts referenced to $\delta(CHCl_3)$=7.26 ppm for ^{1}H and $\delta(CHCl_3)$=77.36 ppm for ^{13}C. Numbering is the same as in Table 1

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	
2	114.08	5.80	
4	34.8	2.47	
5 and 6	20.2	1.12	
7	26.4	2.39	
8	66.3	4.36	



Confirmation of EAD-Activity and Behavioral Response to Synthetic **1***E* The antennae of both males and females responded to methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate, and neither responded to the *Z*-isomer or to the lactone (Fig. 4). In two-choice olfactometer tests, female weevils consistently moved upwind more often (α=0.05, *G*-test) to the olfactometer arm containing either 30 μg of methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (**1***E*) or citrus leaves previously fed upon by males (MFUF) than to the arm with clean air (Table 3). Males did not show a clear preference for the **1***E* or MFUF compared with clean air. Neither males nor females showed a preference when offered a choice between clean air and citrus leaves (flush). Results not reported here suggest that males may be attracted

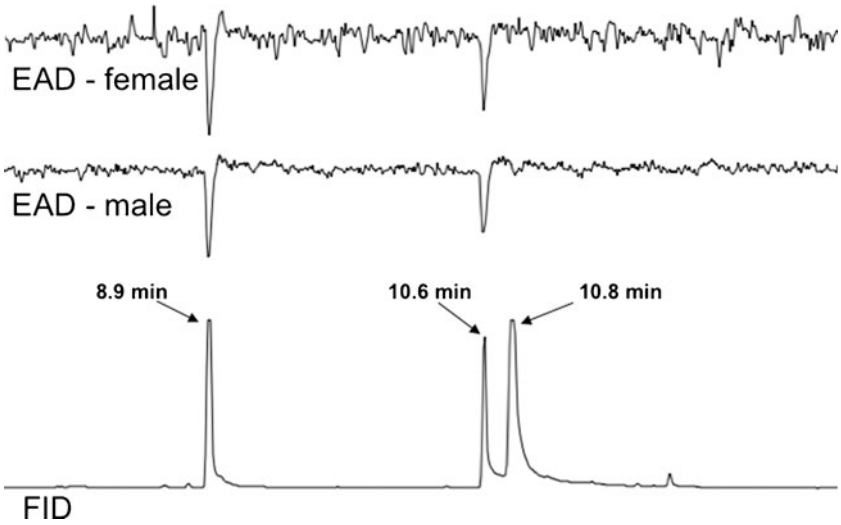


Fig. 4 Simultaneous gas chromatogram (FID) and electroantennogram detection (EAD) of antennae from a female and a male *Diaprepes abbreviatus* responding to 50 ng of linalool (RT 8.9 min) and 100 ng of synthetic methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (10.6 min) in hexane. The peak at 10.8 min corresponds to the lactone degradation product (**2**)

or repelled by the odor of other males due to factors we do not yet understand.

Discussion

An attractant has been sought for *D. abbreviatus* for over 30 years. Our GC-EAD and GC-MS analyses of headspace volatiles and frass collected from male *D. abbreviatus* provided the initial evidence of a putative pheromone consisting of a single, novel compound. GC-MS and NMR analyses enabled us to determine the structure of the EAD-active compound as methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate (***1E***). We then synthesized the putative pheromone compound, and showed that the synthetic compound elicited positive antennal and olfactometer responses verifying that this compound is an attractant pheromone for female *D. abbreviatus*. Whereas the pheromone **1***E* is novel, its lactone degradation product **2** was described as a constituent of tobacco flavor (Pettersson et al., 1993), and synthesized (D'Annibale et al., 2007; Brichacek and Carlson, 2007). As with other curculionids (Ambrogi et al., 2009), the *D. abbreviatus* attractant pheromone is male-produced; however, in the bioassays conducted to date, we have demonstrated a significant attraction of conspecific females but not males. This represents the first progress towards development of semiochemical-based methods for managing *D. abbreviatus*, a serious pest of many crops and ornamental plants that is expanding its geographic range in the Neotropics and subtropics.

Schroeder (1981) conducted field tests suggesting that *D. abbreviatus* weevils were attracted to trees that had been infested overnight by conspecifics of the opposite sex, thereby implying the existence of two sex-specific aggregation pheromones. Subsequently, Beavers et al. (1982) conducted olfactometer bioassays showing *D. abbreviatus* attraction to the frass of the opposite sex, along with same-sex attraction. Both Jones and Schroeder (1984), and Schroeder and Beavers (1985) then conducted field trapping experiments demonstrating that extracts of male *D. abbreviatus* attracted both *D. abbreviatus* males and females; attraction to frass from males was also demonstrated by Harari and Landolt (1997). Lapointe and Hall (2009) found that males responded by arresting their movement on citrus leaves previously fed and defecated upon by males or females,

Table 3 Behavioral responses of *Diaprepes abbreviatus* adults in a two-choice olfactometer to citrus leaves fed upon by males (MFUF; a natural source of pheromone), and the synthetic EAD-active compound, methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate. Tests were conducted in the dark; adults were allowed 15 min to respond; results were scored as no response (weevils remained at the starting point), no choice (weevils moved into the arena but failed to move into one of the two olfactometer arms), or response by moving into one of the arms. Significance is reported for the two-tailed contrast between the number of weevils that chose A or B

	No. choosing				
Sex	A	B	No choice	No response	Pr≥*G*
A: Air; B:30 μg methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate					
Male	30	34	20	69	N.S.
Female	17	48	17	71	< 0.001
A: Air; B: MFUF					
Male	14	15	6	5	N.S.
Female	6	20	13	13	0.009
A: Air, B: Air					
Male	12	18	6	32	N.S.
Female	6	9	13	36	N.S.
A: Air, B: Citrus leaves					
Male	4	4	2	13	N.S.
Female	4	3	7	7	N.S.

thereby suggesting the presence of a female-produced pheromone. From these reports and our own data, we believe there are volatile compounds of insect origin, in addition to methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate, that influence the behavior of *D. abbreviatus*. Our data do not preclude the existence of a female-produced pheromone. The consistent suggestion that female frass also contains an attractant merits further study. Discovery of the male-produced pheromone in *D. abbreviatus* appears to be only the first step towards elucidating the chemical ecology of this highly polyphagous and damaging weevil.

Among the Entiminae (i.e., the broad-nosed weevils), the only one other pheromone known is the aggregation pheromone of *Sitona lineatus* (4-methyl-3,5-heptanedione) (Blight et al., 1984). Blight and Wadhams (1987) suggested that *S. lineatus* produces its aggregation pheromone in the spring, and that pheromonal activity is synergized by host plant volatiles, including (*Z*)-3-hexen-1-ol and linalool. These compounds, and others isolated from headspace over citrus leaves, consistently elicit antennal responses from *D. abbreviatus* (Otálora-Luna et al., 2009). It remains to be seen if these plant volatiles synergize or otherwise augment activity of the *D. abbreviatus* pheromone, and we do not understand the potential influence of rearing conditions, seasonality, age or mating status on production or response to methyl (*E*)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate.

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Analysis of Insect Cuticular Compounds by Non-lethal Solid Phase Micro Extraction with Styrene-Divinylbenzene Copolymers

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Abstract Insect cuticular hydrocarbons including relatively non-volatile chemicals play important roles in cuticle protection and chemical communication. The conventional procedures for extracting cuticular compounds from insects require toxic solvents, or non-destructive techniques that do not allow storage of subsequent samples, such as the use of SPME fibers. In this study, we describe and tested a non-lethal process for extracting cuticular hydrocarbons with styrene-divinylbenzene copolymers, and illustrate the method with two species of bees and one species of beetle. The results demonstrate that these compounds can be efficiently trapped by Chromosorb® (SUPELCO) and that this method can be used as an alternative to existing methods.

Keywords Eusocial bees · Cuticular hydrocarbons · GC-MS · SPME methods · *Melipona marginata* · *Apis mellifera* · *Tenebrio molitor*

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Introduction

Cuticular hydrocarbons, including relatively non-volatile chemicals, contribute to protection of the insect cuticle and chemical communication (reviewed in Howard and Blomquist, 2005; Provost et al., 2008; Blomquist and Bagnères, 2010). In social insects, these compounds are important semiochemicals involved in processes such as recognition of nestmates, castes, and genders (Breed and Bennett, 1987; Blomquist et al., 1998; Singer et al., 1998; Howard and Blomquist, 2005; Le Conte and Hefetz, 2008; Nunes et al., 2009; Tannure-Nascimento et al., 2009). The conventional procedures for extraction of cuticular compounds require toxic solvents. For investigations that require behavioral bioassays, such as the analysis of an individual through various life stages, or the study of reproductive physiology of queens in monogynic colonies, these procedures can be risky (Morgan, 1990; Henderson et al., 1990; Crewe et al., 2004; Roux et al., 2009).

Solid phase microextraction (SPME) is a technique developed by Arthur and Pawliszyn (1990) for application in solid, liquid, or gaseous samples (reviewed in Pawliszyn, 1997). The results obtained by using SPME fibers are similar to those obtained with solvent extraction (Monnin et al., 1998; Turillazzi et al., 1998; Tentschert et al., 2002). The technique involves exposing a fused silica fiber that has been coated with a stationary phase to a sample containing the chemicals of interest. The fiber then is introduced directly into the chromatograph where the compounds are desorbed and analyzed. SPME fibers are made of different materials for the collection of compounds from different chemical classes. For example, polydimethylsiloxane (PDMS) fibers are used to trap non-polar high molecular weight compounds (MW 125–600), volatile compounds (MW 60–275), and non-polar semi-volatiles (MW 80–500), while polydimethylsiloxane/divinylbenzene PDMS/

DVB) fibers are used to trap volatiles, amines and nitro-aromatic compounds (MW 50–300). SPME is an effective tool, but samples must be analyzed rapidly compared to other techniques (Crewe et al., 2004).

Other non-destructive techniques have been proposed for use in living organisms or for successive sampling from the same individual over time. Turillazzi et al. (1998) used a piece of cotton wool or filter paper instead of the SPME fiber to extract cuticular hydrocarbons from live insects. Morgan (1990) described a method in which the samples (glands or wings) were put into micro-capillaries and then injected into a chromatograph with the aid of a holder manufactured and patented by the author. This technique has been used in several studies of cuticular hydrocarbons (Bagnères and Morgan, 1990; Abdalla et al., 2003), but the holder required for the injection of samples is not widely available. Crewe et al. (2004) proposed replacing commercially available SPME fibers with pieces of silicone tubing treated with bis (trimethylsilyl) trifluoroacetamide. Instead of directly heating the silicone tubing, it can be washed with solvents that then can be injected into the GC-MS. The authors proposed that this technique may be generalized for use with semiochemicals of low volatility (such as cuticular hydrocarbons), but the method has been demonstrated only once. Roux et al. (2009) proposed a non-lethal water-based technique for obtaining cuticular compounds. The compounds were extracted from live individuals with tepid water, which formed an emulsion that could subsequently be extracted with solvent for analysis by GC-MS. The authors stated that even non-polar compounds such as hydrocarbons could be removed from the cuticle of the insect by using a polar solvent like water. However, this technique has not been tested in other insects.

Collectively, these studies reinforce the importance of developing techniques for non-lethal extraction of insect cuticular hydrocarbons. Here, we show that a styrene-divinylbenzene polymer (Chromosorb®) can be used to extract cuticular hydrocarbons from live insects, and we compare the results with several other extraction techniques.

Methods and Materials

Insects The study was conducted using two species of social insects and one species of a solitary species. The social insects were *Melipona marginata*, a Neotropical stingless bee found in the southeast of Brazil, and the Africanized honey bee *Apis mellifera,* a hybrid subspecies widely distributed in Brazil. In order to avoid variation in individual chemical profiles related to functional roles of the sampled individuals, we collected only foragers returning to the hives. We also used the solitary insect, *Tenebrio molitor*, which has wide geographical distribution and a typical association with stored grain. For the chemical analyses, we selected only pre-mated females. We used 10 individuals of each species, and extracted cuticular hydrocarbons from each individual successively using four distinct treatments: SPME with PDMS fibers; SPME with PDMS/DVB fibers; solid-phase extraction with styrene divinylbenzene; solvent extraction. All tests were performed at room temperature (about 25°C).

Styrene-Divinylbenzene Copolymer We used Chromosorb® 101 SUPELCO (Celite Corp.) 60/80 mesh, 0.30 g/cc, originally used for chromatography in packed columns. Chromosorb® was designed for analysis of free fatty acids, glycols, alcohols, esters, ketones, hydrocarbons, and ethers. This resin provides the advantages of insolubility in most solvents and chemical resistance, allowing it to be reused. Before use, 10 g of Chromosorb® were washed with 100 ml of chloroform, dried at room temperature, then washed with 100 ml of hexane and again dried at room temperature.

To confirm the effectiveness of styrene-divinylbenzene copolymers for adsorbing chemical compounds, we used a solution containing octacosene and eicosene (100 µl per ml of hexane). A sample comprising 5 ml of this solution was placed in a vial, the solvent was evaporated, and 0.1 mg of Chromosorb® was added. The sample was stirred for 10 min before transferring the polymer to a clean vial, and extracting with 5 ml of hexane. The hexane was allowed to evaporate, and the residue was resuspended in 160 µl of hexane for chromatographic analysis (1 µl injection). The control sample was not sorbed to the beads, but all other steps of evaporation and redisolution were the same.

Cuticular Hydrocarbons Extractions Cuticular hydrocarbons from each insect were extracted first using fibers of PDMS (100 µm) and then with PDMS/DVB (100 µm) (SUPELCO). The fibers were rubbed gently for 10 s against the cuticle of each individual in order to adsorb the chemical compounds. These fibers can be used several times, but we chose to use them no more than four times in our experiments (16 fibers in total) because the contact with the cuticle of the insect and the high temperature of injection in GC-MS (150°C) could result in wear that decreases sorbtive properties. We established that using the fibers for extraction of four insect samples was satisfactory.

After extraction by SPME, each individual was placed in a 5 ml glass vial containing 0.1 g of Chromosorb®. Each vial was sealed and gently agitated for 10 min before removing the insect from the vial. The Chromosorb® was washed in 2 ml of hexane to recover the extracted compounds for analysis.

The final stage of extraction was to freeze-kill the insect and extract with hexane (1 ml/1 min/individual).

Chemical Analysis The analyses were conducted with a SHIMADZU GC-MS QP2010. Separation was achieved on a DB-5MS column (30 m) using helium as the carrier gas at 1.0 ml min^{-1}. The oven temperature was initially 150°C, and increased by 3°C min^{-1} to 280°C. Analyses were performed in the splitless mode. The mass spectra were obtained by 70 eV ionization. The SPME fibers were mounted directly in the CG-MS injection oven port for 4 min to desorb the compounds. The hexane extracts of the Chromosorb® were evaporated under a nitrogen stream, suspended in 50 μl of hexane, and analyzed chromatograpically (1 μl).

The double bond positions of alkenes were identified through derivatization of hexane extracts from 10 individuals of each species (Carlson, 1989). The position of double bonds in alkadienes could not be identified due to the low concentration of these compounds in the extracts. Extracts were dried with nitrogen and re-suspended in 200 μl of hexane using magnetic stirring. Subsequently, 200 μl of dimethyl disulfide (DMDS) (Sigma-Aldrich) and 100 μl of iodine solution (dissolved in diethyl ether, 6% m/v) were added. The vials then were purged with nitrogen, closed, and agitated at ambient temperature for 24 h. The mixtures were diluted in hexane and extracted with 5% sodium thiosulfate solution. The organic phase was subsequently dried with sodium sulfate and analyzed by GC-MS.

Chemical Analyses Data were analyzed with GC-MS Postrun Analysis for Windows (Shimadzu Corporation), and the chemical compounds were identified based on their mass spectra by comparison with the NIST Library data and with an alkane standard solution for compounds with 21 to 40 carbon atoms (Fluka). We obtained the relative abundance of each compound estimated as the proportional peak area from total ion chromatograms. We tested for differences among methods in the relative abundance of classes of hydrocarbon compounds using non-parametric Kruskal-Wallis tests for multiple independent comparisons (Statistica 7.0).

Results

The test to determine the efficiency of recovery showed that the relative abundances obtained after treatment with Chromosorb® were similar to those found in the control sample. The abundances of (*Z*)-1-Octacosene were 49.44% and 53.54% of the total ion chromatograms for the Chromosorb® and control samples, respectively (χ^2=0.25, *P*= 0.61), while for (*Z*)-1-Eicosene the abundances were 50.56% and 46.46% of the total ion chromatograms (χ^2= 0.26, *P*=0.61) (Fig. S-1).

For each insect, all methods of extraction yielded qualitatively similar profiles of cuticular hydrocarbons (Tables 1–3). For all methods, the errors were quite large, obscuring quantitative differences for the profiles.

Melipona marginata Analysis of the cuticular waxes of *M. marginata* workers using Chromosorb® revealed 26 peaks representing hydrocarbons, while the hexane wash, PDMS/DVB and PDMS fibers showed 24, 24, and 23 peaks, respectively (Table 1, Fig. S-2). The compounds were classified as linear alkanes, linear alkenes, and alkadienes. The positions of the double bonds in the 12 alkenes were determined. Subsequent analyses showed that relative proportions of each isomeric alkene were similar for the four methods of extraction (Table 1). Tricosene and hentriacontadiene were not found in the samples collected with PDMS/DVB fibers. Nonacosene and hentriacontadiene were not found in the samples collected with hexane washing or with PDMS fibers.

Apis mellifera Analysis of the cuticular waxes of honey bee workers using Chromosorb® or hexane extraction revealed 42 hydrocarbon peaks, while sorption to the PDMS/DVB or PDMS fibers yielded 39 or 37 hydrocarbons, respectively (Table 2, Fig. 1). Compounds were classified as linear and methyl alkanes, linear alkenes, and alkadienes. These analyses also revealed one alcohol [(*Z*)-9-Eicosanol]. Fourteen alkenes were obtained from honey bee individuals, and double bond positions were determined for all methods. Relative proportions of the isomeric alkenes were not statistically different when the quantitative data of four methods of extraction were compared (Table 2). Triacontane, hentriacontadiene, and tritriacontadiene were not found in samples sorbed to the PDMS and PDMS/DVB fibers. Nonadecane and tritriacontadiene were not found in samples collected with the PDMS fibers.

Tenebrio molitor The analyses presented 23 hydrocarbon peaks when we used Chromosorb®, hexane, or sorption to the PDMS fibers, and 21 peaks with the PDMS/DVB fibers (Table 3, Fig. S-3). Compounds were classified as linear and methyl alkanes, alkenes, and alkadienes. Seven alkenes were found and the double bond position was determined for all methods. There were no differences among the relative proportions of the isomeric alkenes among individuals analyzed with the four methods. Triacontane, hentriacontadiene, and tritriacontadiene were not found in samples collected with PDMS or PMSD/DVB fibers. Nonadecane and tritriacontadiene were not found in samples sorbed to PDMS fibers.

Discussion

This study describes a novel sample extraction technique that can be effectively applied to studies of cuticular

Table 1 Relative proportions (mean±standard deviation) of cuticular hydrocarbons from *Melipona marginata* workers using four different techniques of extraction. $N=10$. RT=Retention time (min). Distinct letters indicate significant differences ($P<0.05$)

RT	Compound	Chromosorb®	Hexane	PDMS/DVB fiber	PDMS fiber
23.07	Tricosene*[1]	0.13±0.21a	0.22±0.2a	-	0.63±0.72a
23.66	Tricosane (*n*-C23)	2.11±1.62a	1.55±1.18a	4.49±4.8a	2.70±2.78a
28.40	Pentacosene*[2]	3.82±2.06a	2.28±1.04a	4.88±4.1a	3.07±3.16a
28.92	Pentacosane (*n*-C25)	3.97±1.75a	3.94±1.77a	9.69±7.82a	5.94±4.15a
33.09	Heptacosene*[3]	2.68±3.48a	1.32±1.02a	0.37±0.51a	2.83±3.66a
33.21	Heptacosene*[3]	1.41±1.57a	2.73±5.51a	3.52±5.05a	1.46±0.85a
33.43	Heptacosene*[3]	6.99±3.35a	4.36±2.01a	6.86±5.01a	6.96±4.59a
33.88	Heptacosane (*n*-C27)	3.23±1.37a	3.67±1.31a	5.52±2.89a	3.70±2.14a
37.46	Nonacosadiene[#]	0.72±0.74a	0.77±0.41a	0.05±0.11a	0.52±0.24a
37.63	Nonacosadiene[#]	1.97±1.5a	1.68±0.97a	0.12±0.29a	1.28±0.94a
37.80	Nonacosadiene[#]	4.26±3.18a	2.00±1.46a	0.50±0.65a	1.74±1.59a
37.94	Nonacosene*[4]	1.44±1.76a	2.91±6.25a	2.47±4.13a	0.59±0.49a
38.03	Nonacosene*[4]	0.43±0.6a	-	1.47±1.94a	-
38.17	Nonacosene*[4]	12.95±6.77a	9.11±5.13a	10.83±6.9a	7.94±3.6a
38.52	Nonacosane (*n*-C29)	2.29±1.32a	3.35±1.49a	3.07±1,69a	2.18±1.15a
41.83	Hentriacontadiene[#]	0.84±0.72a	1.35±0.82a	0.22±0.19a	-
41.94	Hentriacontadiene[#]	1.32±1.01a	1.45±0.95a	0.16±0.25a	0.68±0.17a
42.11	Hentriacontadiene[#]	2.02±1.97a	1.97±1.64a	0.17±0.26a	0.80±0.56a
42.23	Hentriacontadiene[#]	2.88±2.13a	2.13±1.46a	-	-
42.30	Hentriacontene*[5]	0.55±0.77a	-	0.21±0.37a	1.44±0.8a
42.36	Hentriacontene*[5]	1.50±2.2a	1.66±2.36a	0.17±0.27b	0.51±0.2ab
42.57	Hentriacontene*[5]	5.67±2.77a	4.92±3.38a	3.82±2.72a	2.54±1.43a
42.88	Hentriacontene*[5]	2.07±1.23a	4.23±2.09a	3.30±2.18a	2.15±1.21a
45.37	Dotriacontane (*n*-C32)	4.27±3.31a	6.54±3.4a	10.66±5.7a	9.53±4.72a
51.47	Tritriacontane (*n*-C33)	20.92±15.42a	26.37±14.4a	35.76±14.5a	35.25±13.31a
60.02	Pentatriacontane (*n*-C35)	4.64±3.8a	7.42±4.7a	9.83±3.84a	11.02±2.63a

Identification of alkenes isomers and their respective ions obtained in derivatization procedure:

*[1] (*Z*)-4-C23 (103, 313, 416); (*Z*)-5-C23 (117, 299, 416); (*Z*)-6-C23 (131, 285, 416); (*Z*)-7-C23 (145, 271, 416) (majority); (*Z*)-8-C23 (159, 257, 416); (*Z*)-9-C23 (173, 243, 416); (*Z*)-10-C23 (187, 229, 416)

*[2] (*Z*)-7-C25 (145, 299, 444) (majority); (*Z*)-9-C25 (173, 271, 444) (majority); (*Z*)-11-C25 (201, 243, 444); (*Z*)-14-C25 (243, 201, 444)

*[3] (*Z*)-5-C27 (117, 355, 472) (majority); (*Z*)-7-C27 (145, 327, 472); (*Z*)-8-C27 (159, 313, 472); (*Z*)-9-C27 (173, 299, 472); (*Z*)-11-C27 (201, 271, 472); (*Z*)-13-C27 (229, 243, 472)

*[4] (*Z*)-5-C29 (117, 383, 500); (*Z*)-7-C29 (145, 355, 500) (majority); (*Z*)-8-C29 (159, 341, 500); (*Z*)-9-C29 (173, 327, 500); (*Z*)-10-C29 (187, 313, 500); (*Z*)-11-C29 (201, 299, 500) (majority); (*Z*)-12-C29 (215, 285, 500); (*Z*)-13-C29 (229, 271, 500); (*Z*)-14-C29 (243, 257, 500)

*[5] (*Z*)-7-C31 (145, 383, 528) (majority); (*Z*)-8-C31 (159, 369, 528); (*Z*)-9-C31 (173, 355, 528); (*Z*)-10-C31 (187, 341, 528); (*Z*)-11-C31 (201, 327, 528) (majority); (*Z*)-12-C31 (215, 313, 528); (*Z*)-13-C31 (229, 299, 528); (*Z*)-14-C31 (243, 285, 528); (*Z*)-15-C31 (257, 271, 528)

[#] Double bonds not identified

hydrocarbons in live insects. Solid styrene-divinylbenzene copolymer can be used to collect compounds from live individuals, a crucial factor in behavioral analysis (reviewed in Blomquist et al., 1998). As the sorptive polymers are reusable, the method provides an inexpensive alternative to SPME. The copolymers had similar affinities for several classes of compound. Our experiment with two synthetic alkenes with known concentrations demonstrated that adsorption was highly efficient (Fig. S-1).

Qualitative results obtained for the three methods (styrene-divinylbenzene copolymers, hexane and PDMS fiber) showed a higher similarity in the number of compounds compared to those obtained from the PDMS/DVB fiber. Quantitative results showed large amounts of variation of relative proportions in some individual compounds for all studied species. However, these variations were robust for the four extraction methods, and they were most frequently observed in those hydrocarbons with lower relative proportions of the total ion chromatograms (<3%). Large variation

Table 2 Relative proportions (mean±standard deviation) of cuticular hydrocarbons from *Apis mellifera* workers using four different techniques of extraction. *N*=10. RT=Retention time (min). Distinct letters indicate significant differences ($P<0.05$)

Peak	Compound	Chromosorb®	Hexano	PDMS/DVB	PDMS
11.89	Nonadecane (*n*-C19)	0.50±2.08a	0.09±0.06b	2.48±3.46c	-
17.10	Heneicosane (*n*-C21)	2.13±2.11a	0.24±0.21b	0.77±0.71ab	0.60±0.25ab
19.80	Docosane (*n*-C22)	1.40±0.69a	0.18±0.15b	0.35±0.33ab	0.40±0.44ab
21.55	(*Z*)-9-Eicosanol	11.30±7.69a	5.25±4.43a	20.48±28.30a	24.48±30.81a
21.70	Tricosene*[1]	2.55±2.64a	0.41±1.03b	2.42±1.18ab	1.32±1.19ab
22.50	Tricosane	5.57±2.17a	1.91±1.35a	15.20±23.32b	6.39±5.67a
25.10	Tetracosane (*n*-C23)	0.36±0.14a	0.10±0.05a	0.71±0.81a	0.28±0.17a
26.90	Pentacosene*[2]	0.61±0.63a	0.31±0.17b	0.75±0.60b	2.10±1.68ab
27.79	Pentacosene*[2]	0.12±0.01a	0.10±0.01ab	1.75±1.82b	0.47±0.66ab
27.64	Pentacosane (*n*-C25)	2.56±2.26a	5.43±3.49ab	8.78±7.18b	8.61±8.03ab
30.10	Hexacosane (*n*-C26)	4.02±1.09a	0.37±0.24b	0.38±0.29b	0.45±0.32b
31.90	Heptacosene*[3]	0.55±0.65a	0.41±0.32a	1.40±1.12a	1.22±1.06a
32.10	Heptacosene*[3]	0.28±0.22a	0.10±0.06a	0.30±0.15a	0.40±0.11a
32.52	Heptacosane (*n*-C27)	8.03±4.54a	15.17±8.28a	17.60±12.40a	16.73±12.87a
33.27	11- and 13 - Methyl Heptacosane	0.34±0.21a	0.43±0.32a	2.36±3.82b	1.14±0.62b
33.36	9 - Methyl Heptacosane	0.67±0.17a	0.14±0.07b	0.22±0.16a	1.55±2.52a
35.82	Octacosane (*n*-C28)	2.90±1.58a	0.19±0.14b	0.32±0.37ab	0.30±0.19ab
36.54	Nonacosene*[4]	0.87±0.92a	0.84±0.79a	1.48±1.23a	1.16±0.99a
36.72	Nonacosene*[4]	0.55±0.17a	0.10±0.04b	0.34±0.67ab	0.12±1.03ab
37.08	Nonacosane (*n*-C29)	5.08±2.32a	8.40±3.18b	5.36±2.75ab	5.04±2.99ab
37.75	9-, 11-, 13- and 15- Methyl Nonacosane	0.53±0.21a	0.48±0.32a	0.77±0.77a	0.73±0.44a
39.24	Triacontane (*n*-C30)	0.94±0.39a	0.32±0.14b	-	-
40.34	Hentriacontadiene[#]	0.23±0.74a	0.43±1.02a	-	-
40.50	Hentriacontadiene[#]	0.24±0.04a	0.97±2.07a	0.19±0.14a	0.25±0.18a
40.84	Hentriacontene*[5]	1.03±0.47a	1.98±3.02b	0.60±0.61ab	0.80±1.39ab
40.88	Hentriacontene*[5]	0.77±0.55a	0.77±0.40a	0.72±0.62a	0.90±0.84a
40.91	Hentriacontene*[5]	2.29±0.90a	2.78±0.64b	1.82±1.30ab	1.97±1.47ab
41.38	Hentriacontane (*n*-C31)	8.34±4.28a	12.98±5.48b	9.46±14.70abd	3.77±2.62acd
42.96	Dotriacontene *[6]	0.31±0.12a	0.24±0.11a	0.24±0.36a	6.29±3.84b
43.40	Dotriacontane (*n*-C32)	0.10±0.53a	0.20±0.17b	0.15±0.15ab	0.28±0.16ab
44.42	Tritriacontadiene[#]	0.25±0.12a	0.10±0.03a	-	-
44.58	Tritriacontadiene[#]	2.53±0.79a	2.08±1.49a	1.06±0.92a	1.55±1.05a
44.74	Tritriacontadiene[#]	0.63±0.92a	0.47±0.51a	0.57±0.71a	-
44.91	Tritriacontene*[7]	0.31±0.17ab	0.34±0.21a	0.09±0.06b	0.24±0.06ab
45.07	Tritriacontene*[7]	18.37±5.17ab	19.61±8.55a	9.97±8.34b	13.15±7.33b
45.24	Tritriacontene*[7]	2.90±0.96ab	4.00±1.85a	1.37±1.15b	1.51±1.44ab
45.59	Tritriacontane (*n*-C33)	1.55±0.94a	3.05±1.61a	2.79±6.35a	1.51±2.24a
49.80	Pentatriacontadiene[#]	0.94±0.41a	1.23±0.75a	0.70±0.58b	0.40±0.24ab
50.00	Pentatriacontadiene[#]	1.07±0.45a	0.96±0.64a	0.22±0.13b	0.28±0.09b
50.45	Pentatriacontene*[7]	2.08±0.81a	1.91±0.86a	0.26±0.27b	0.68±0.44b
50.66	Pentatriacontene*[7]	2.55±0.91a	4.01±1.95a	0.49±0.63ab	1.07±0.89ab
50.92	Pentatriacontene*[7]	0.91±0.73ab	0.10±0.07a	2.22±3.96ab	18.68±30.47b

Identification of alkenes isomers and their respective ions obtained in derivatization procedure:

*[1] (*Z*)-9-C23 (173, 243, 416)

*[2] (*Z*)-7-C25 (145, 299, 444); (*Z*)-9-C25 (173, 271, 444) (majority); (*Z*)-11-C25 (201, 243, 444)

*[3] (*Z*)-5-C27 (117, 355, 472); (*Z*)-7-C27 (145, 327, 472) (majority); (*Z*)-9-C27 (173, 299, 472) (majority); (*Z*)-10-C27 (187, 285, 472); (*Z*)-11-C27 (201, 271, 472)

*[4] (*Z*)-7-C29 (145, 355, 500); (*Z*)-8-C29 (159, 341, 500); (*Z*)-9-C29 (173, 327, 500) (majority); (*Z*)-10-C29 (187, 313, 500); (*Z*)-11-C29 (201, 299, 500)

*[5] (*Z*)-7-C31 (145, 383, 528); (*Z*)-8-C31 (159, 369, 528) (majority); (*Z*)-9-C31 (173, 355, 528); (*Z*)-10-C31 (187, 341, 528); (*Z*)-11-C31 (201, 327, 528); (*Z*)-12-C31 (215, 313, 528)

*[6] (Z)-9-C32 (173,369,542) (majority), (Z)-10-C32 (187,355,542)

*[7] (*Z*)-8-C33(159, 397, 556); (*Z*)-9-C33 (173, 383, 556); (*Z*)-10-C33 (187, 369, 556) (majority); (*Z*)-12-C33 (215, 341, 556); (*Z*)-14-C33 (243, 313, 556)

*[7] (*Z*)-10-C35 (187,397, 584) (majority); (*Z*)-12-C35 (215, 369, 584); (*Z*)-14-C35 (243, 341, 584)

[#] Double bonds not identified

Table 3 Relative proportions (mean±standard deviation) of cuticular hydrocarbons from *Tenebrio molitor* females using four different techniques of extraction. $N=10$. RT=Retention time (min). Distinct letters indicate significant differences ($P<0.05$)

RT	Compound	Chromosorb®	Hexane	PDMS/DVB	PDMS
23.965	Tricosane (*n*-C23)	5.57±5.84a	6.54±7.16a	12.38±3.95a	7.95±3.82a
23.965	11-Methyl Tricosane	0.61±0.53a	0.48±0.40a	0.85±0.65a	0.91±0.30a
24.975	Tetracosene	0.51±0.75a	1.23±0.26a	0.74±0.27a	0.60±0.46a
25.710	Tetracosane (*n*-C24)	1.10±0.58a	1.64±0.28a	2.42±0.39a	1.19±1.17a
27.530	Pentacosene	2.45±1.19a	2.60±2.05a	3.02±8.18a	3.88±4.39a
27.635	Pentacosene	4.28±2.39a	4.90±1.75a	11.62±6.97a	8.06±6.82a
28.340	Pentacosane (*n*-C25)	19.02±1.07a	20.39±2.56a	16.99±1.56a	16.47±8.90a
29.200	3-, 9- Methyl Pentacosane	0.22±0.09a	0.27±0.12a	0.06±0.10a	5.37±7.57b
30.885	Hexacosane (*n*-C26)	0.15±0.08a	0.43±0.29a	0.08±0.15a	1.89±2.52b
32.575	Heptacosadiene	0.34±0.19a	0.21±0.10a	0.19±0.26a	0.12±0.21a
32.700	Heptacosene	6.29±2.28a	5.89±2.11a	8.20±2.72a	2.07±3.92a
33.390	Heptacosane (*n*-C27)	5.78±4.20ab	8.58±1.84ab	6.33±1.02a	17.11±10.19b
35.750	Octacosane (*n*-C28)	0.27±0.29a	0.93±0.41a	0.62±0.68a	1.73±2.65a
37.550	Nonacosene	17.15±5.13a	16.77±5.86a	25.83±4.97a	9.27±10.65a
38.085	Nonacosane (*n*-C29)	7.25±1.14a	9.21±4.71a	5.22±0.94a	10.93±9.54a
38.770	11-, 13-.Methyl Nonacosane	0.34±0.21a	0.43±0.25a	0.48±0.45a	1.60±2.42a
38.905	11- Methyl Nonacosane	0.41±0.31a	0.45±0.17a	0.39±0.44a	1.40±2.12a
39.020	9 Methyl Nonacosane	0.23±0.26a	0.44±0.26a	0.43±0.34a	0.43±0.35a
42.040	Hentriacontene	2.61±1.78a	2.02±1.88a	4.01±1.15a	2.49±2.38a
42.140	Hentriacontene	0.57±0.51a	0.63±0.74a	0.37±0.63a	1.69±2.55a
42.525	Hentriacontane (*n*-C31)	0.48±0.71a	1.20±0.78a	0.19±0.21a	1.29±1.32a
43.205	5-Methyl Hentriacontane	0.68±0.39a	1.28±1.15a	0.19±0.22a	0.78±0.97a
43.780	Dotriacontadiene	0.55±0.23a	0.92±1.24a	0.43±0.28a	0.72±1.29a

of relative proportions of cuticular compounds was also found previously in *Formica* ants (Martin et al., 2008). Therefore, we assume that cuticular hydrocarbon variation is expected, as they are known to play roles as individual chemical signatures (Ferreira-Caliman et al., 2010).

We speculated at first that glandular products or other undesirable compounds might have contaminated the samples collected with styrene-divinylbenzene copolymers, but with one exception (phthalate) in our preliminary experiments, contaminants were not recorded during our studies. Our results suggest that the risk of contamination may be similar to or lower than that reported for other techniques; especially extraction by hexane washes (Monnin et al., 1998; Turillazzi et al., 1998; Tentschert et al., 2002). Vander Meer and Morel (1998) stated that solvent extracts of dead insects could contain glandular compounds. In another study using non-lethal extraction methods (Roux et al., 2009), cuticular hydrocarbons and alkaloids from the venom gland were obtained through both hexane- and water-based extractions.

The styrene-divinylbenzene copolymers facilitate the collection of samples from various phases of an individual's life, an important point in many studies of chemical ecology. Following collection, the compounds are eluted from the beads with solvent, so the samples can be stored prior to analysis. In some cases, SPME fibers can be saturated by a disproportionate concentration of one or more compounds from a specific body segment (Ferreira-Caliman, personal observation), but we did not observe such saturation with the Chromosorb®. Furthermore, it may be possible to mix Chromosorb® with nest materials in experiments on orientation of ants (Sturgis et al., 2011) or in analyses of bee comb waxes (stingless bees: Pianaro et al., 2007; honeybees: D'Ettorre et al., 2006).

While this work was in review, a similar method for extracting cuticular hydrocarbons from insects was published with the moniker "silica rubbing" (Choe et al., 2012). That study used silica gel to sorb the cuticular hydrocarbons from dead insects. Our study is distinct from that publication because we used styrene-divinylbenzene copolymers and live organisms. Silica gel and styrene-divinylbenzene copolymers are both widely used in column chromatography; however, these materials have different polarities. Silica gel has a higher affinity for polar compounds, while styrene-divinylbenzene binds non-polar compounds. Furthermore, silica gel is toxic to insects because it removes cuticular lipids and desiccates the insect. We did not observe a toxic effect for syrene-divinyl benzene.

Choe et al. (2012) stated that silica-rubbing is suitable for field studies because it does not require several devices for

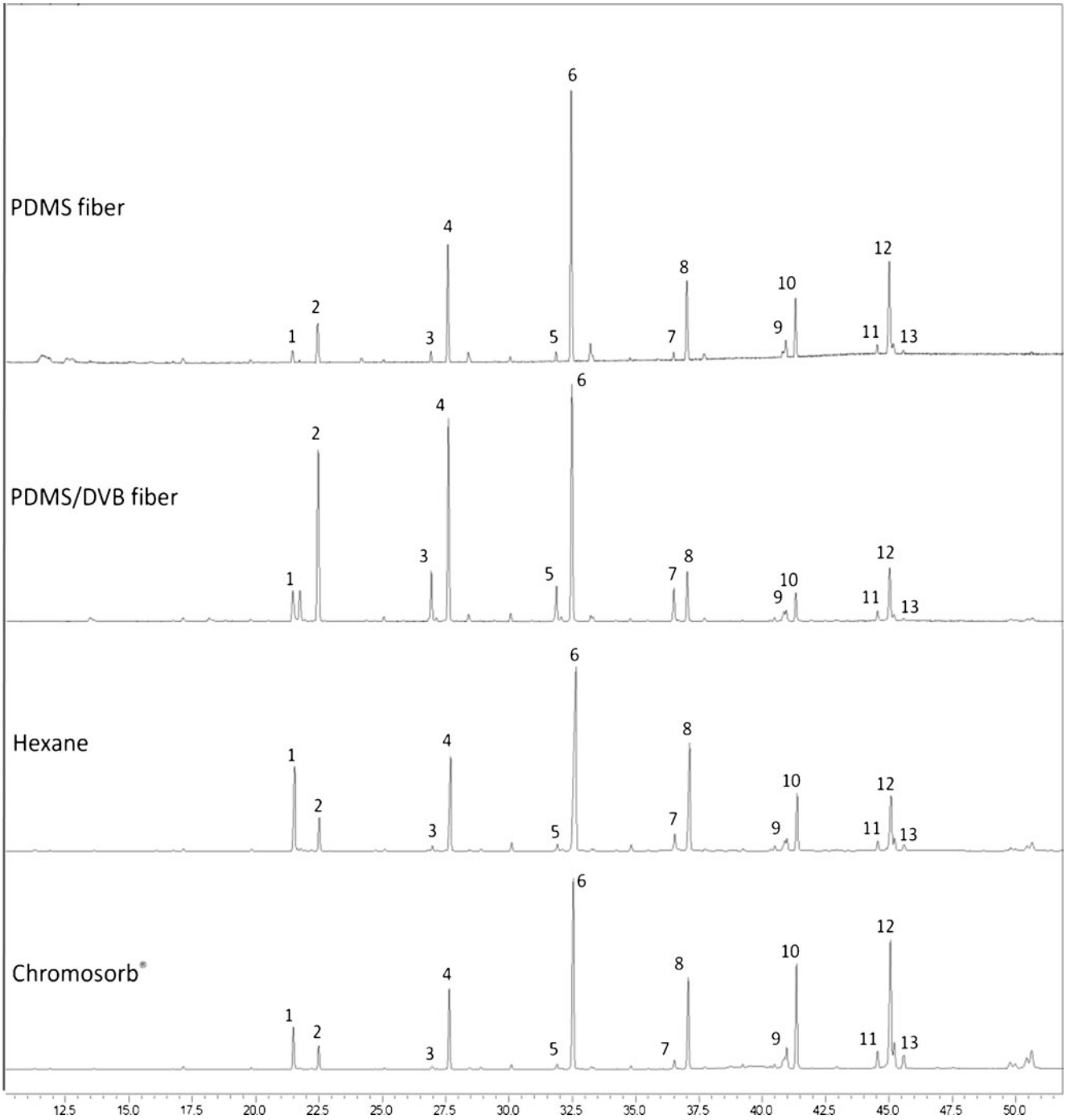

Fig. 1 Chromatograms of cuticular hydrocarbons in a single *Apis mellifera* worker using different techniques of extraction. Main peaks: 1. (*Z*)-9-Eicosanol; 2. Tricosane; 3. Pentacosene; 4. Pentacosane; 5. Heptacosene; 6. Heptacosane; 7. Nonacosene; 8. Nonacosane; 9. Hentriacontene; 10. Hentriacontane; 11. Tritriacontadiene; 12. Tritriacontene and 13. Tritriacontane

extracting multiple samples. However, the steps of freeze killing specimens in order to carry out silica-rubbing are less convenient than our method of extraction directly from the live insect. Thus, the use of styrene-divinylbenzene copolymer may be preferred over the silica gel method because it can be adopted widely for extraction of cuticular compounds from live insects without additional equipment.

Choe et al. (2012) compared three methods (silica rubbing, hexane, and PDMS/DVB fibers) using dead *Euglossa dillema*. In that paper, only the main peaks in their study (18 peaks on average) were analyzed to establish the efficiency of the methods. However, previous studies have shown that there are many more cuticular hydrocarbons found in two of these three species. For example, *Linepithema humile* had more

than 60 compounds (Liang and Silverman, 2000) *Pogonomyrmex barbatus* had more than 21 compounds (Wagner et al., 2001), and *Camponotus floridanus* had more than 34 compounds (Endler et al., 2004). We used derivatization to identify the positions of the double bonds in the alkenes found in the cuticular hydrocarbons of bees. This procedure allowed us to not only establish that the method effectively extracted general classes of compounds, but also to verify the relative proportion of unsaturated hydrocarbons and the effectiveness of the extraction method for individual isomers.

In summary, our study showed that Chromosorb® is an effective non-lethal method for the extraction of cuticular hydrocarbons from living insects. We demonstrated that it is possible to extract similar amounts of cuticular hydrocarbons from live insects by using copolymers of styrene-divinylbenzene compared to other commonly used techniques.

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Identification and Characterization of Two General Odorant Binding Protein Genes from the Oriental Fruit Moth, *Grapholita molesta* (Busck)

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Abstract Two novel general odorant-binding protein (GOBP) cDNAs (*GmolGOBP1* and *GmolGOBP2*) were cloned and characterized from female antennal tissue of the oriental fruit moth, *Grapholita molesta*. We focused our investigation on this olfactory protein family by using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends-PCR (RACE-PCR). The full-length open reading frames of *GmolGOBP1* and *GmolGOBP2* were 492 and 483 nucleotides long, which encode 164 and 161 amino acid residue peptides, respectively. Protein signature analyses revealed that they each contained six conserved cysteines with an N-terminal signal sequence of 20 amino acids. The alignment of the two deduced protein sequences with other Lepidoptera GOBPs showed high sequence similarity (70–80 %) with other full-length sequences from GenBank. Sequence similarity between the two GOBPs was only 48 %, suggesting that the two proteins belong to different classes of lepidopteran GOBPs. RT-PCR analysis revealed that the two GOBP genes were expressed only in antennae of both sexes. Real-time PCR analysis further indicated that the transcript level of *GmolGOBP1* was higher in males than in females, whereas the transcript level of *GmolGOBP2* was higher in females than in males. Temporally, the two GOBP genes were expressed during the complete photoperiod (15L:9D). The highest transcript levels of *GmolGOBP1* in both sexes and *GmolGOBP2* in females were detected at the end of photophase and during scotophase. The expression of *GmolGOBP2* in males remained at similar levels during the complete photoperiod. Based on these results, the possible physiological functions of GmolGOBPs are discussed.

Keywords Agarose gel electrophoresis · *Grapholita molesta* · Lepidoptera · Molecular cloning · mRNA expression · Rapid amplification of cDNA ends-P polymerase chain reaction (RACE-PCR) · Real-time polymerase chain reaction (PCR) · Reverse transcription-polymerase chain reaction (RT-PCR) · Sequence analysis · Tortricidae · Insect pest

Introduction

The olfactory system is essential for the survival and reproduction of insects. They rely on this system for the reception of environmental chemical signals to locate mates, food sources, and oviposition sites, as well as to avoid predators and other threats. Perception of chemical signals is mediated by low molecular weight, water-soluble proteins, the odorant-binding proteins (OBPs), located mainly in the sensory hairs of the antennae in insects (Leal, 2005). These proteins typically feature six conserved cysteine residues that form three disulfide bridges (Leal et al., 1999; Scaloni et al., 1999). Biochemical data suggest that they solubilize and transport hydrophobic odorants across the aqueous lymph of the chemosensilla to the olfactory receptors (ORs). These receptors are located in the dendritic membranes of olfactory sensory neurons (OSNs) where signal transduction is initiated (Feng and Prestwich, 1997; Krieger and Breer, 1999; Kaissling, 2001). Moreover, there is growing physiological and behavioral evidence that OBPs are indispensable in the recognition

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of odorants in insects. Xu et al. (2005) showed that a *Drosophila* mutant OBP/LUSH has a complete loss of sensitivity to its sex pheromone 11-*cis* vaccenyl acetate (VA), indicating that LUSH is absolutely required for activation of pheromone-sensitive sensory neurons. RNA interference experiments in the southern house mosquito, *Culex quinquefasciatus* (Pelletier et al., 2010), and the malaria mosquito, *Anopheles gambiae* (Biessmann et al., 2010), also demonstrated that reduction in OBP expression leads to lower sensitivity in the detection of specific odorants.

OBPs were first discovered in moth antennae (Vogt and Riddiford, 1981), but they have now been identified from various insect orders, such as Lepidoptera (Picimbon et al., 2001; Wang et al., 2003; Xiu and Dong, 2007; Zhang et al., 2011); Diptera (Ozaki et al., 1995; Biessmann et al., 2002; Vogt et al., 2002); Orthoptera (Ban et al., 2003); Coleoptera (Nagnan-Le Meillour et al., 2004); Isoptera (Ishida et al., 2002); Hymenoptera (Calvello et al., 2003; Lu et al., 2007); and Hemiptera (Vogt et al., 1999; Gu et al., 2011). In the Lepidoptera, the odorant-binding proteins are divided into two classes: Pheromone-binding proteins (PBPs) and general odorant binding proteins (GOBPs) (Vogt et al., 1991a,b). PBPs are enriched or expressed only in male antennae; specifically associated with sex pheromones; and show poor conserved sequences among species (Vogt et al., 1991a). GOBPs, which are divided into the subclasses GOBP1 and GOBP2 based on amino acid sequence homology (Vogt et al., 1991a), seem to play a more general role in olfaction by carrying odorant molecules (Vogt and Lerner, 1989). GOBPs are expressed equally in the antennae of male and female moths, and show a high similarity in amino sequence, which suggests that they are involved in the reception of "general" odorants such as those from plants. To date, several GOBP genes have been cloned from different insects (Vogt et al., 1991b; Maibèche-Coisne et al., 1998; Jacquin-Joly et al., 2000; Wang et al., 2003; Calvello et al., 2005). However, GOBP structures and mechanisms of odor discrimination and signal transduction are not well understood. Adequate descriptions of GOBPs are still needed to study their physiological function.

Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), is a key pest of stone fruit throughout the world (Rothschild and Vickers, 1991). Its host range includes several species of stone and pome fruits in the family Rosaceae (Rothschild and Vickers, 1991). Monitoring of *G. molesta* relies predominantly on pheromone trapping of male moths. However, the flight performance of this species exhibits marked sexual differences. The proportion of long-flying females was three to six times greater than that of males, and gravid females can be considered to be the main colonists (Dorn et al., 2001; Hughes and Dorn, 2002). In the field, female *G. molesta* have the capacity to make inter-orchard flights (Steiner and Yetter, 1933; Hughes and Dorn, 2002). This can pose a serious threat to pear and apple cultivation in orchards in the vicinity of peach crops. Therefore, a strategy for monitoring adult *G. molesta*, based on chemical stimuli derived from host plants, is desirable (Dorn et al., 2001). In addition, host plant odorants that are attractive to adult *G. molesta* also could be used in trap deployment. In this study, we aimed to elucidate the molecular basis of olfactory reception of volatile semiochemical cues and to provide the theoretical basis to facilitate the design and implementation of novel monitoring and control strategies against *G. molesta*. We report the identification of two GOBP genes from *G. molesta* (*GmolGOBP1* and *GmolGOBP2*) and describe their tissue-specific expression pattern. We also evaluated the effect of sex and photoperiod on the expression of *GmolGOBPs*.

Methods and Materials

Insect Rearing and Sample Collection *Grapholita molesta* larvae were reared until pupation under a 15:9 hr (L:D) light cycle at 24±0.5 °C and 70±10 % RH in the laboratory on an artificial diet (Du et al., 2010). Pupae were sexed and kept in separate plastic containers (10 cm diam., 5 cm high). Adult eclosion was assessed during the first 2 hr of each photophase. Emerging males and females were placed in separate glass containers (14.5 cm diam., 18.5 cm high) covered with cheesecloth with a maximum of 20 moths per container. These moths were provided with 10 % honey solution.

To study the tissue distribution of GOBPs in *G. molesta*, antennae and control tissues (heads without antennae, thoraces, abdomens, legs, and wings) from 3-d-old males and females were dissected 2 hr before the onset of scotophase, and immediately transferred into 1.5 ml Eppendorf tubes that were immersed in liquid nitrogen. Tissues then were stored at −75 °C until used. In order to determine the possible variation in *GmolGOBP* expression during the photoperiod, the antennae of 3-d-old males and females were collected at different times during photophase (0900, 1400, and 1900 hr) and during scotophase (000 and 0400 hr) and processed as indicated previously. This photoperiod was the same as that described for rearing. Photophase lasted from 0500 to 2000 hr, whereas scotophase lasted from 2000 to 0500 hr. For each sample, two pools of 50 pairs of antennae were collected and each pool represented a biological sample.

Isolation of Total RNA and Synthesis of First-strand cDNA Total RNA was extracted by homogenizing antennae or other tissues from male and female moths with RNApure Reagent (ZOMANBIO Corporation, Beijing, China) and treated with DNase (MBI Fermentas, Glen Burnie, MD, USA). Single-stranded cDNAs were synthesized from 500 ng total RNA for each sample by using PrimeScript®

RT Reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions.

Degenerate PCR for Amplification of cDNA Fragments The cDNA fragments that encode GOBPs of *G. molesta* were amplified from antennal cDNA of females. A pair of degenerate primers for PCR were designed based on the amino acid consensus regions of several GOBPs from different species of Lepidoptera (Table 1). The amplification conditions were 3 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1 min at 72 °C, and final extension for 10 min at 72 °C. LA Taq polymerase (TaKaRa) was used following manufacturer's instructions. All amplification products were analyzed by 1.5 % agarose gel electrophoresis, and the DNA was purified with a DNA purification kit (Tiangen Biotechnologies, Beijing, China). The purified DNA fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA). After transformation into DH5α-competent cells (Trangen Biotechologies, Beijing, China), the DNA inserts of recombinant clones (on the basis of restriction enzyme cleavage sites and PCR amplification) were isolated by using the plasmid mini kit (Tiangen Biotechnologies), and custom sequenced (Genscript Biotechnologies Nanjing, China).

Table 1 Oligonucleotide primers used for isolation and expression analysis of *Grapholita molesta* general odorant binding proteins

Purpose/Primer Name	Sequence (5′—3′)
cDNA Isolation (RT-PCR)	
Sense	GTBATGAARGAYGTCAC
Antisense	AGVAYHKCRCCRTTNGG
5′ and 3′ cDNA End Isolation (RACE-PCR)	
GmolGOBP1	
5′ Outer GSP	TCGCCATTAGGGAAACTCTTG
3′ Outer GSP	CATCCAGTGCATGTCGAAGT
5′ Inner GSP	TCTCTCCGTCGGTGAGCAGGTTA
3′ Inner GSP	GTCGAAGTACTTTAACCTGCTCACC
GmolGOBP2	
5′ Outer GSP	TATCACCATTGGGGAAGCTCAG
3′ Outer GSP	CGAAGTCTTGGACGAGTTCCACA
5′ Inner GSP	TCGTCCTGCAGCAGCGAGAACTTG
3′ Inner GSP	CATATGCATGTCCAACAAGTTCTCG
Tissue Localization (RT-PCR) and Expression Analysis (Real-time PCR)	
GmolGOBP1	
Sense	CAAGTTCATCAAGAGTTTCCCTAA
Antisense	CAAGCATCAGCCTCCATAATAA
GmolGOBP2	
Sense	GTTGGAGCAGTGTCGGGAGGA
Antisense	AGCGGATAGGACATCACCTTTGG
Actin	
Sense	CTTTCACCACCACCGCTG
Antisense	CGCAAGATTCCATACCCA

Rapid Amplification of cDNA Ends (3'RACE and 5'RACE) A rapid amplification of cDNA ends (RACE) procedure was employed to amplify the 3′ and 5′ ends of the *GmolGOBPs* by using a RACE cDNA Amplification Kit (TaKaRa) following the kit instructions. Gene-specific primers (GSP; Table 1) for 3′ and 5′RACE were designed on the basis of identified GOBP cDNA fragments. By using the 3′- and 5′-RACE cDNA (synthesized according to the RACE Kit protocol) as templates, nested-PCR was carried out to amplify the 3′ and 5′ terminal fragments. Amplification conditions were 3 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 1.5 min at 72 °C, and final extension for 10 min at 72 °C. All RACE products were cloned into a pGEM-T easy vector (Promega) and sequenced by the same methods as described for fragment identification.

Sequence Analysis and Phylogenetic Tree Construction Sequences were identified by using the NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/). Signal peptides were predicted with the SignalP V3.0 program (http://www.cbs.dtu.dk/services/SignalP/). The basic physical features of proteins were determined by using Expasy (http://www.expasy.ch/cgi-bin/pi_tool). Sequence alignment was produced by using ClustalX 1.83 (Thompson et al., 1997). The phylogenetic tree was constructed with the Neighbor Joining method by using MEGA 4.0 software. Bootstrap analysis used 1,000 replications.

Analysis of Patterns of GmolGOBP Tissue-specific Expression An analysis of the patterns of tissue-specific expression was performed by reverse transcription (RT)-PCR. RT-PCR templates were the cDNA from antennae and other tissues of male and female moths. To check for any genomic DNA contamination, all cDNA synthesis reactions were carried out in the absence of reverse transcriptase in parallel for each sample. RT-PCR amplification of cDNAs was performed by using the gene-specific primers that were derived from the cDNA sequences (Table 1). For testing the integrity of the cDNA templates, a control primer pair (Table 1) from the coding region of the *G. molesta* actin gene (GenBank JN857938) was used. PCR began at 94 °C for 3 min, then 35 cycles at 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 1 min, with a final 10 min elongation step at 72 °C. PCR products were analyzed on 1.5 % agarose gels.

Quantitative Real-time PCR of GmolGOBP mRNA Real-time PCR was performed by using iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Benecia, CA, USA) with SYBR green dye bound to double stranded DNA

at the end of each elongation cycle (SYBR® *Premix EX Taq*™ sII; TaKaRa). Specific primer pairs for cloning *GmolGOBP1*, *GmolGOBP2*, and *GmolActin* were the same as the primer pairs for RT-PCR (Table 1), and were expected to amplify 213, 220, and 222 bp fragments, respectively. A 25 μl real-time PCR reaction system contained 12.5 μl of 2×SYBR® *Premix EX Taq*™ II, 1 μl of each primer (10 μM), 2 μl of sample cDNA, and 8.5 μl nuclease free water (TaKaRa). The thermal cycling conditions for real-time PCR were 30 sec at 95 °C, followed by 40 cycles of 60 sec at 95 °C, 30 sec at 55 °C, and 15 sec at 72 °C. A dissociation curve was used to determine primer specificity. A negative control (cDNA reaction without reverse transcriptase enzyme) was used to check for genomic DNA contamination. Three technical replicates were run for each sample. Relative quantification was performed by using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All data were normalized to endogenous *Actin* levels from the same antennal samples. The expression quantity of *GmolGOBPs* of male antennal sample at 0900 hr during photophase, which was one of the experimental samples, was used for calibrating the relative fold changes (Mittapalli et al., 2006). Thus, relative fold changes were assessed by comparing the expression level of *GmolGOBPs* in other antennal samples to those in the male antennal sample at 0900 hr during photophase

An assumption for quantification of gene transcript level in the $\Delta\Delta C_T$ calculation for the comparative $2^{-\Delta\Delta CT}$ method is that the amplification efficiencies of the target and reference are approximately equal. To confirm this, a pilot experiment was conducted to examine the variation of ΔC_T ($C_{T,Target}$–$C_{T,Actin}$) with template dilution (Supplemental Data Fig. S1, Table S1). Briefly, four serial ten-fold dilutions of cDNA from each sample were amplified. For each dilution, amplifications were performed in triplicate with primers for *GOBP* and *Actin*. Mean C_T was calculated for both *GOBP* and *Actin* and $^{\Delta}C_T$ was determined. A plot of log cDNA dilution vs. $^{\Delta}C_T$ was made (Xiu and Dong, 2007) (for background data on real-time PCR analyses see Supplemental Data Table 2).

Statistical Analysis Expression ratios of *GmolGOBP1 and GmolGOBP2* among the different time-groups were subjected to LSD test in analysis of variance (ANOVA) with a critical level of α=0.05. All data were analyzed by SAS (Version 8.2).

Results

Molecular Cloning and cDNA Sequencing of GmolGOBPs The RT-PCR approach with female antennal cDNA yielded two cDNA fragments of 248 bp. We named these fragments *GmolGOBP1* and *GmolGOBP2*, respectively, according to their high sequence similarity with DNA for proteins from these subclasses in BLAST searches. A RACE procedure was further employed to obtain full-length cDNAs for the two genes from *G. molesta.* The sequences of *GmolGOBP1* and *GmolGOBP2* (Figs. 1 and 2) were deposited in GenBank under the accession numbers JN857939 and JN857940, respectively.

The full-length sequence of *GmolGOBP1* was 1090 bp long and contained an open reading frame of 492 nucleotides that encodes 164 amino acids. This putative GmolGOBP1 protein possesses a signal sequence of 20 amino acids. Mature GmolGOBP1 (144 amino acids) has a predicted molecular weight of 16,716 Da with a theoretical pI of 4.77. Full-length *GmolGOBP2* was 637 bp long and comprised the complete GOBP precursor consisting of the 20-amino acid signal peptide followed by the mature 141-amino acid protein with a molecular weight of 15,980 Da and a theoretical pI of 4.85. Thus, the two predicted mature proteins are small, likely soluble, likely secreted, and both have an acidic isoelectric point. In addition, both contained six cysteines in conserved positions when aligned with other known GOBPs: positions C^{19}, C^{50}, C^{54}, C^{97}, C^{108}, and C^{117} (Fig. 3).

The mature amino acid sequences of GmolGOBP1 and GmolGOBP2 were aligned and compared with GOBPs of other Lepidoptera (Fig. 3). The results indicate that all these lepidoptera GOBPs have a 6-conserved cysteine motif and share high overall sequence similarity (69.8 %). GmolGOBP1 exhibits only 48 % sequence similarity with GmolGOBP2. However, GmolGOBP1 displays high similarity with HassGOBP1 (74 %), HvirGOBP1 (73 %), and PxylGOBP1 (72 %), whereas GmolGOBP2 is more similar to EposGOBP2 (85 %), AsegGOBP2 (84 %), and HassGOBP2 (84 %). Sequence similarities of Lepidoptera GOBPs were summarized in a neighbor-joining tree (Fig. 4). This latter analysis revealed two clusters of GOBPs from various species, designated as Group 1 (GmolGOBP1) and Group 2 (GmolGOBP2) (Fig. 4).

Tissue-specificity of GOBP Gene Expression The tissue distribution of GmolGOBPs was examined by RT-PCR with specific primers (Fig. 5). RT-PCR products of the predicted size were observed exclusively in reactions with antennal cDNA from both sexes. No specific product was observed with DNA from head (without antennae), thorax, abdomen, leg, or wing. The integrity of the cDNA templates prepared from different tissues was verified by primers specific for the Actin gene. In all cDNA preparations, an *Actin* amplification product of the correct size was detected. To control for genomic DNA contamination, all cDNA synthesis reactions were carried out in the absence of reverse transcriptase (-RT) in parallel for each sample. In all –RT-PCR reactions, no specific products were observed. Therefore, *GmolGOBP1* and *GmolGOBP2* were expressed only in *G. molesta* antennae.

Diel Periodicity Expression of GmolGOBPs We examined the expression profiles of the two proteins with real-time

Fig. 1 cDNA sequence and deduced amino acid sequence of GmolGOBP1 from antennae of the oriental fruit moth, *Grapholita molesta*. The stop codon is indicated with an asterisk. Putative signal peptide at the N-terminus is underlined

PCR and the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) to compare relative transcript levels of *GmolGOBPs* in both sexes at different times during the photocycle (Fig. 6). To validate the $\Delta\Delta C_T$ calculation, a pilot experiment was conducted to assess how ΔC_T ($\Delta C_{T,\ Target} - \Delta C_{T,\ Actin}$) varies with a ten-fold serial dilution of template cDNA. The results indicated that the absolute value of the slope of the entire dilution curve is close to

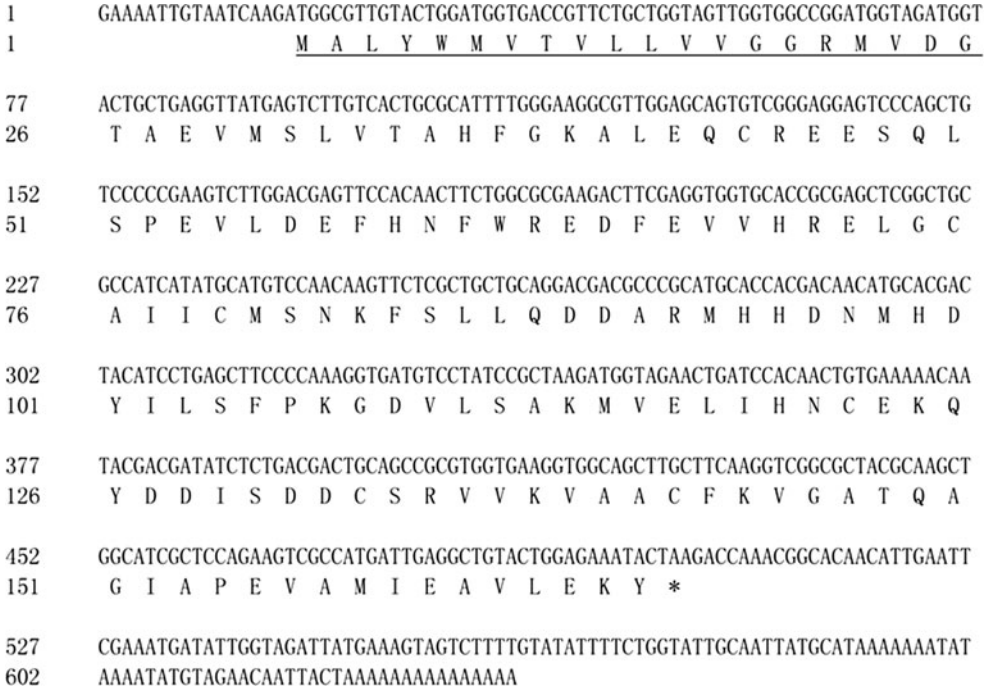

Fig. 2 cDNA sequence and deduced amino acid sequence of GmolGOBP2 from antennae of the oriental fruit moth, *Grapholita molesta*. The stop codon is indicated with an asterisk. Putative signal peptide at the N-terminus is underlined

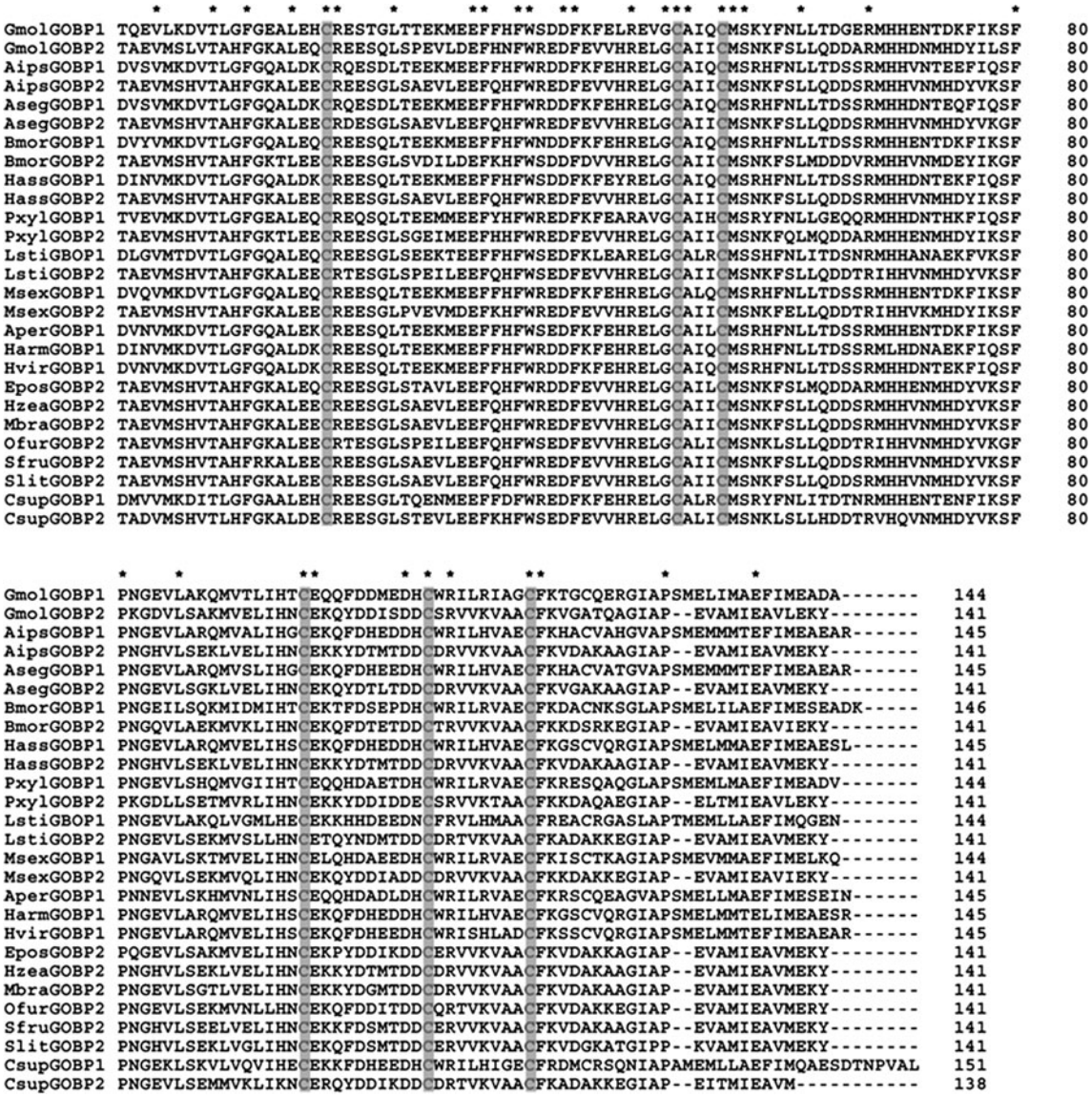

Fig. 3 Alignment of the deduced amino acid sequences of GmolGOBP1 and GmolGOBP2 from antennae of *Grapholita molesta* with GOBPs from other Lepidoptera. Names and GenBank accession numbers of the 27 GOBPs are as follows: GmolGOBP1: JN857939, GmolGOBP2: JN857940 (*Grapholita molesta*); AipsGOBP1: ABI24160, AipsGOBP2: AAP57462 (*Agrotis ipsilon*); AsegGOBP1: ABI24159, AsegGOBP2: ABI24161 (*Agrotis segetum*); BmorGOBP1: CAA64444, BmorGOBP2: NP_001037498 (*Bombyx mori*); HassGOBP1: AAW65076, HassGOBP2: AAQ54909 (*Helicoverpa assulta*); PxylGOBP1: ABY71034, PxylGOBP2: ABY71035 (*Plutella xylostella*); LstiGOBP1: ACB47481, LstiGOBP2: ABY75632 (*Loxostege sticticalis*); MsexGOBP1: AAA29315, MsexGOBP2: AAA29316 (*Manduca sexta*); AperGOBP1: CAA71866 (*Antheraea pernyi*); HarmGOBP1: AAL09821 (*Helicoverpa armigera*); HvirGOBP1: CAA65605 (*Heliothis virescens*); EposGOBP2: AAL05869 (*Epiphyas postvittana*); HzeaGOBP2: AAG54078 (*Helicoverpa zea*); MbraGOBP2: AAC05703 (*Mamestra brassicae*); OfurGOBP2: ABG66419 (*Ostrinia furnacalis*); SfruGOBP2: AAT74555 (*Spodoptera frugiperda*); SlitGOBP2: ABM54824 (*Spodoptera litura*); and CsupGOBP1: ACJ07129, CsupGOBP2: ABD98823 (*Chilo suppressalis*). Residues common to all GOBPs are labeled by asterisks. Six conserved cysteine residues are highlighted with gray background

zero (Supplemental Data Fig. S1, Table S1). Therefore, the efficiencies of the target and reference genes were similar in our analysis, and the $\Delta\Delta C_T$ method was validated for use in relative quantification.

GmolGOBP1 and *GmolGOBP2* transcripts were detected at all times during the photoperiod (Fig. 6). The transcript level of *GmolGOBP1* was consistently higher in males than in females throughout the photoperiod; the transcript level of *GmolGOBP2* was higher in females than in males during scotophase and at the end of photophase (Fig. 6). At 0900 and 1400 hr during photophase, the differences in the transcript levels of *GmolGOBP2* between the sexes were negligible. *GmolGOBP1* expression profiles were similar in both sexes during the photoperiod; the transcript levels of *GmolGOBP1* were low at 0900 and 1400 hr during photophase, increased (approximately 1.7-fold) at the end of the photophase, and

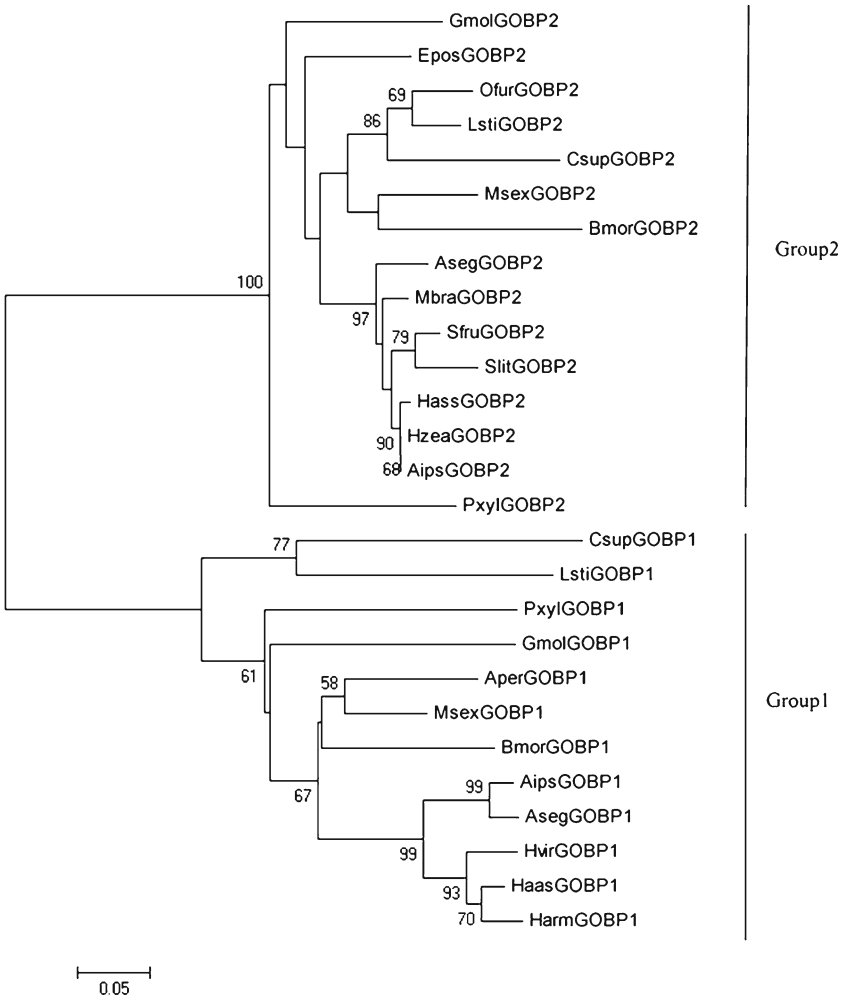

Fig. 4 Phylogenetic analysis of the amino acid sequences of GmolGOBPs in the context of various lepidopteran GOBPs. For the gene abbreviations and the GenBank accession numbers, see Fig. 3. Bootstrap support values (%) based on 1,000 replicates are indicated. The *bar* indicates phylogenetic distance value. Analysis indicates two groups (Group1 and Group 2), as defined by Picimbon and Gadenne (2002)

then high levels were maintained throughout the scotophase. When *GmolGOBP2* expression profiles were analyzed, we observed that the expression differences between the sexes were maintained during the photoperiod; a high transcript level of *GmolGOBP2* in females was detected at the end of the light period (approximately 2.1 times those at 0900 and 1400 hr during light period); and then the high levels were maintained during the dark period. In males, expression of

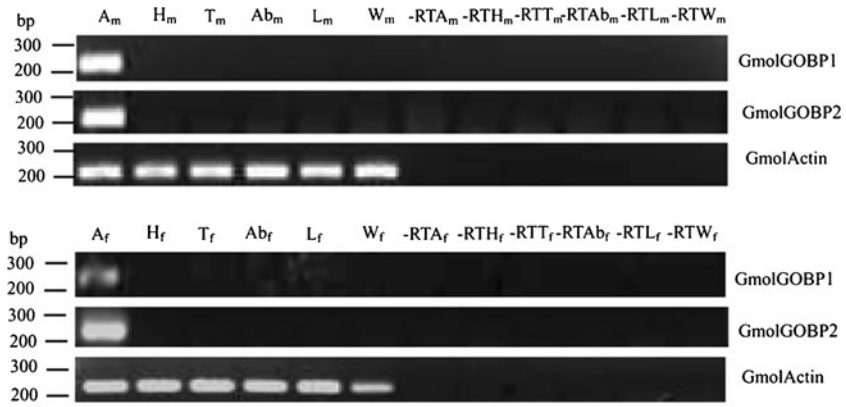

Fig. 5 Nonquantitative RT-PCR showing expression of *GmolGOBP1* and *GmolGOBP2* in different tissues of adult *Grapholita molesta*. A_m: Male antennae, A_f: Female antennae, H_m: male head (without antennae), H_f: female head (without antennae), T_m: male thorax, T_f: female thorax, Ab_m: male abdomen, Ab_f: female abdomen, L_m: male leg, L_f: female leg, W_m: male wing, and W_f: female wing. *Actin* was used as the positive control. Minus reverse transcription (-RT) PCR products (negative control without the use of reverse transcriptase) are also shown for each cDNA sample. The position of molecular weight markers (bp) is indicated on the left side

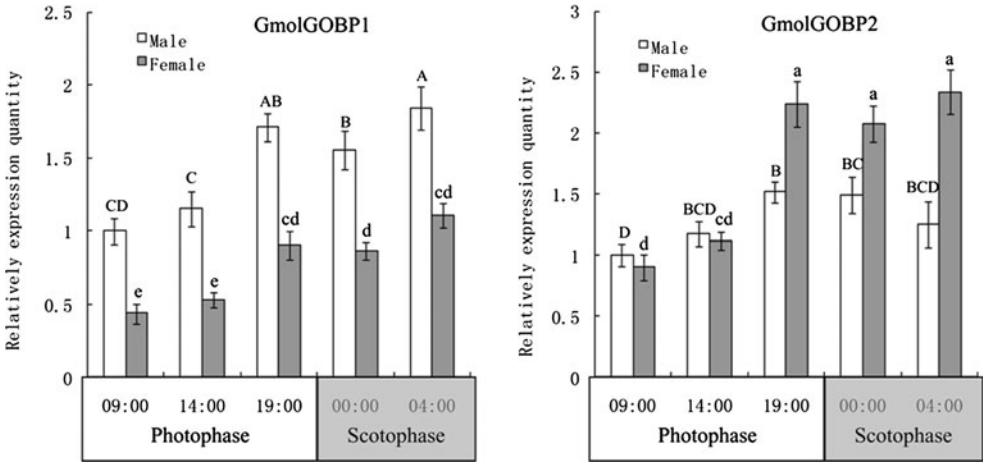

Fig. 6 Real-time PCR analysis of *GmolGOBP1* and *GmolGOBP2* expression levels in the antennae of adult *Grapholita molesta* at different times during the photoperiod. The quantity of expression in *GmolGOBPs* from male antennal samples at 0900 hr during photophase (one of the experimental samples) was taken as the calibrator. Standard error of the mean (N=6. three of each biological sample) is represented by the error bar. Different letters on the error bars indicate significant differences (P<0.05, LSD in ANOVA) among the different times during the photoperiod. We used capital letters for males and lower case letters for females. Photophase lasts from 0500 to 2000 hr; scotophase lasts from 2000 to 0500 hr

GmolGOBP2 was low at the beginning of the light period, increased slightly (approximately 1.3 fold) at the end of the light period, and decreased again at the end of the scotophase.

Discussion

In this study, we cloned and identified two novel genes that encode GmolGOBP1 and GmolGOBP2, respectively, from the antennae of *G. molesta.* The mature predicted proteins contain 144 and 141 amino acids, respectively, and bear all the hallmarks of the OBP family including: (1) they are small hydrophillic proteins with acidic isoelectric points; (2) they are destined for secretion, as demonstrated by the occurrence of a signal peptide at the N-termini; (3) they possess six cysteines in positions conserved across OBPs (Pelosi et al., 2006); and (4) they have considerable amino acid similarity to other GOBPs. The identification of the two GmolGOBPs adds new members to the moth GOBP family, and will enable future studies of structure-function and evolutionary relationships of these GOBPs with those in related taxa.

Phylogenetic analysis has shown that lepidopteran GOBPs are classified into two distinct groups: GmolGOBP1 belongs to group1 and GmolGOBP2 belongs to group 2. Furthermore, all GOBPs from the same family are always in the same clade of the phylogenetic tree (Fig. 4), indicating that lepidopteran GOBPs may be derived from a common ancestor and later diverged into different subfamilies by different selection pressures. Interestingly, almost all lepidopteran GOBP genes have been identified from the Apoditrysia in the Ditrysia. Among identified GOBP genes, only *PxylGOBPs* have been identified from the Ditrysia, excepting the Apoditrysia. Therefore, to construct a more meaningful phylogenetic tree, we need to clone more GOBP genes in the superfamilies Tineoidea, Yponomeutoidea, and Gelechioidea, in the Ditrysia rather than in the Apoditrysia (Kristensen et al., 2007).

In most insect species, the prevailing model is that OBPs are expressed specifically in the antennae. Our RT-PCR data support this because the PCR products of *GmolGOBPs* were present in reactions with antennal cDNA from both sexes, but never in reactions with cDNA from other tissues (e.g., heads without antennae, thoraces, abdomens, wings, or legs) (Fig. 5). However, in some insect species, OBPs have different expression patterns. In the paper wasp, *Polistes dominulus*, OBPs are expressed equally in antennae, wings, and legs of all castes and ages (Calvello et al., 2005). In *Drosophila melanogaster,* most OBPs are expressed in both olfactory and gustatory sensilla (Galindo and Smith, 2001). In the mosquitoes *Anopheles gambiae* and *A. arabiensis*, many of the OBPs are expressed mainly in head tissue and a subset of these show the highest expression in female heads (Li et al., 2005).

The expression profiles of *GmolGOBPs* in both sexes during the photoperiod offer several new insights. First, the transcript level of *GmolGOBP1* was about 2 times higher in males than in females. This result is different from those presented for other insects (Steinbrecht, 1993; Steinbrecht et al., 1995; Wang et al., 2003; Konstantopoulou et al., 2005; Gong et al., 2009), where the expression level of GOBPs is higher in females than in males or similar in both sexes. This implies that *GmolGOBP1* may perform a key role for male *G. molesta* to locate host plants by recognizing odorants. As the quantities of odorants emitted by their hosts are far greater than those of the pheromone itself, males initially attracted to

the host plant increase their chances of subsequently locating a calling female. Second, we observed high transcript levels of *GmolGOBP1* in both sexes and *GmolGOBP2* in females during the scotophase. It is possible that the expression profiles of *GmolGOBPs* are in tune with nocturnal activity of *G. molesta*. In fact, many Lepidoptera are active during the night when high antennal sensitivity is undoubtedly important for them to seek host plants and to detect food sources. However, further electroantennogram (EAG) study is needed to verify the possibilities here.

We detected a relatively high transcript level of *GmolGOBPs* at the end of the light period (1900 hr). This time point coincides with that in previous studies that have reported that sexual activity of *G. molesta* was observed 2–3 hr before sunset in field observation (Gentry et al., 1975) and a few hours before lights-off in a laboratory observation (George, 1965). This is interesting as it suggests the possible involvement of GmolGOBPs in sexual activity of *G. molesta*. There are several studies that support the role of PBP's in pheromone detection (Schneider et al., 1998; Xu et al., 2005), however, the functional role of GOBPs is still unclear. GOBPs were located in the *sensilla basiconica* and presumed to detect interspecific signals such as plant volatiles and other environmental chemicals (Vogt et al., 1991a,b; Prestwich et al., 1995; Pelosi, 1996). Surprisingly, binding affinities of GOBPs have been demonstrated for the major pheromonal compound in several insect species. For example, in *Manduca sexta*, recombinant GOBP2 showed strong affinity for the major pheromonal compound of this species, *trans*-6, *cis*-11-hexadecenyl acetate (Feng and Prestwich, 1997). Recombinant GOBP2 from *Chilo suppressalis* had significant affinity for *cis*-11-hexadecenal, the main component of *C. suppressalis* pheromone (Gong et al., 2009). In *Amyelois transitella*, Liu et al. (2010) reported that AtraGOBPs bind with high affinity to the major component of the sex pheromone (*Z*, *Z*)-(11,13)-hexadecadienal. In the future, we will focus on the binding characteristics of both GmolGOBP1 and GmolGOBP2 and their relationships with host plant volatiles and pheromones of *G. molesta*. This should help us to understand the roles of OBPs in olfactory processing in *G. molesta*.

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RAPID COMMUNICATION

High Conservatism in the Composition of Scent Gland Secretions in Cyphophthalmid Harvestmen: Evidence from Pettalidae

Günther Raspotnig · Julia Schwab · Ivo Karaman

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Abstract The scent gland secretion of *Austropurcellia forsteri* was analyzed by gas chromatography–mass spectrometry, providing the first description of the secretion chemistry in the cyphophthalmid family Pettalidae. The secretion contained a total of 21 compounds: About 60% of the whole secretion consisted of a series of saturated, mono-unsaturated and doubly unsaturated methylketones, from C_{11} to C_{15}, with a cluster of saturated and mono-unsaturated C_{13}-methylketones dominating. A second fraction included several naphthoquinones such as 1,4-naphthoquinone (ca. 20% of secretion), 6-methyl-1,4-naphthoquinone (ca. 17%), and minor amounts of chloronaphthoquinones (ca. 2%). When compared with scent gland compositions of other representatives of cyphophthalmids (e.g. from families Sironidae and Stylocellidae), a highly conservative chemistry of cyphophthalmid secretions is apparent, based on a restricted number of methylketones and naphthoquinones.

Keywords Cyphophthalmi · Chemical defense · Methylketones · Naphthoquinones · Arachnida · Opiliones

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Introduction

The Cyphophthalmi represent the smallest suborder of harvestmen, currently comprising about 190 described species in six families (Giribet et al., 2012). Like all harvestmen, the Cyphophthalmi possess prosomal defensive ("scent") glands, which are strikingly developed across this suborder. When a leg is grasped by forceps, many (if not all) cyphophthalmid species expel droplets of scent gland secretion from dorso-laterally protruding ozophores, dab each droplet with the tip of one leg (mostly leg II), and transfer the secretion to the forceps. This behavior has been described as "leg dabbing," and it clearly identifies cyphophthalmid scent gland secretions as defensive (Juberthie, 1961). The chemistry of cyphophthalmid secretions, however, has been poorly studied. Raspotnig et al. (2005) reported on secretions of two species of the family Sironidae, *Cyphophthalmus duricorius* (from Europe) and *Siro exilis* (from USA), and Jones et al. (2009) studied the chemistry of an undescribed Sulawesian stylocellid. All three species produce multicomponent secretions that, although distinguishable, are based on common chemistry of acyclic methylketones and naphthoquinones. No chemical analyses on the secretions of other cyphophthamid species have been performed.

Here, we investigated the chemistry of the scent gland secretion of a representative of a further cyphophthalmid family, the Pettalidae.

Methods and Materials

Species Collection Twelve adults (5 males, 7 females) and 6 juvenile specimens of *Austropurcellia forsteri* (Juberthie, 2000) (Pettalidae) were collected from leaf litter at Cape Tribulation (Queensland, Australia). This species, originally

included within the genus *Neopurcellia* by Juberthie (2000), has been transferred to the genus *Austropurcellia* on the basis of molecular data (Boyer and Giribet, 2007), and is considered to represent a member of a monophyletic clade of pettalids in the northeast of Australia. Species determination was performed by the original description of Juberthie (2000).

Extraction and Analysis of Secretions Scent gland secretions were obtained either by dabbing secretion from ozopores on filter paper or by whole body extraction of single individuals. Extracts were analyzed by gas chromatography–mass spectrometry (GC-MS), using a Trace GC2000 (with a ZB-5 capillary column, 30 m x 0.25 mm i.d., x 0.25 μm film thickness and helium at 1.2 ml.min^{-1}) coupled to a DSQ MS (ion source at 200°C; transfer line at 310°C). The temperature of the GC oven was programmed from 50°C (1 min. delay) to 300°C at 10°C.min^{-1}, then 5 min at 300°C. Areas of individual peaks were integrated and expressed as % peak area of a whole extract.

Reference Compounds Compounds were identified by comparison of mass spectral and gas chromatographic data to synthetic standards and by comparison to already identified components in *Cyphophthalmus* extracts (Raspotnig et al., 2005). Synthetic 1,4-naphthoquinone, undecan-2-one, dodecan-2-one, and tridecan-2-one were purchased from Sigma (Vienna, Austria), and 4-chloro-1,4-naphthoquinone was synthesized as described in Raspotnig et al. (2005). As a reference source for 6-methyl-1,4-naphthoquinone, we used the scent gland secretion of *Phalangium opilio* (Wiemer et al., 1978).

Results

Chemical Analysis of Scent Gland Secretion Chromatograms of secretion-loaded filter papers or whole body-extracts of individuals showed identical chromatographic profiles. Therefore, further chromatograms were obtained using whole body-extracts of individuals.

In total, 21 compounds were detected (Table 1; Fig. 1), all of which were either methylketones (16 compounds B_1, B, C, D_1, D, F, G. H. I, J, K, M, N, P, Q, U, W) or naphthoquinones (four compounds: E, L, R, and X). Nineteen of the compounds had previously been identified in *Cyphophthalmus duricorius* and *Siro exilis* (Raspotnig et al., 2005). The two remaining compounds (B_1 and D_1) had not been detected in our previous study, but appeared to be mono-unsaturated homologs of undecan-2-one (compound B) and dodecan-2-one (compound C), and thus were tentatively identified as undecen-2-one (B_1) and dodecen-2-one (D_1), respectively. These identifications were supported by molecular ions two mass units lower (M^+ at *m/z* 168 and 182, respectively) than those of the saturated compounds, along with a strong *m/z* 58 (McLafferty-rearrangement, indicating a methylketone).

Chemical profiles of individuals showed only minor intraspecific variation (Table 1). Profiles of males and females were not separable by a principal component analysis (data not shown). Profiles of juveniles did not show major differences from adults, although early instars, in particular, frequently lacked trace components found in adult secretions. Profiles of late instars were indistinguishable from those of adults.

Discussion

Is There a Common Cyphophthalmid Scent Gland Chemistry? Along with the present report, data on the scent gland chemistry of four cyphophthalmid species across three families are now available. The secretions of all four species show very similar composition, based exclusively on methylketones and naphthoquinones, largely comprising even the same compounds. Since these data are derived from different and possibly distantly related families (e.g., Giribet et al., 2012), this suggests a homogeneous chemistry of the scent glands of cyphophthalmids.

Methylketones, especially those ranging from C_{11}-C_{15}, were abundant in all secretions, with tridecan-2-one being the main component in *A. forsteri* (35%) and in the Sulawesian stylocellid analyzed by Jones et al. (2009; 50%), as well as in the two sironids (Raspotnig et al., 2005; 20%). Since methylketones have not been detected in any non-cyphophthalmid opilionid, they may constitute an autapomorphy of cyphophthalmid scent glands. In contrast, naphthoquinones are well-known from the scent gland secretions of both Cyphophthalmi and Palpatores: 1,4-Naphthoquinone and 6-methyl-1,4-naphthoquinone have been reported from the scent glands of phalangiid Eupnoi (Wiemer et al., 1978) and from Dyspnoi (Raspotnig et al., 2010). Although their occurrence in the latter groups is based on single investigations, we know that these components show a wide distribution among the Palpatores (Raspotnig, unpublished). In these terms, naphthoquinones may be regarded as synapomorphic for Cyphophthalmi and Palpatores, possibly indicating a common ancestry of these two suborders.

Interestingly, and as in sironids, *A. forsteri* also produces two highly unusual chloronaphthoquinones in its scent gland secretion, although in low abundance (each less than 1% of secretion). These chloronaphthoquinones were detected previously in both *Cyphophthalmus* and *Siro* (Raspotnig et al., 2005), but were lacking in the Sulawesian stylocellid. Jones et al. (2009) considered these compounds a possible autapomorphy of

Table 1 Scent gland secretion profiles of *Austropurcellia forsteri*

Peak*	RI**	Identified as	Relative abundance (% of whole secretion±SD)		
			males (N=5)	females (N=7)	juvenile (N=6)
B_1	1276	undecenone	0.15±0.13	0.23±0.24	0.02±0.03
B	1293	undecan-2-one	1.34±0.42	1.12±0.58	0.55±0.23
C	1358	dodecan-2-one (branched isomer)	0.43±0.03	0.34±0.16	0.25±0.08
D_1	1383	dodecenone	0.14±0.12	0.37±0.27	0.22±0.18
D	1395	**dodecan-2-one**	**3.58±1.88**	**5.80±2.34**	**6.11±2.77**
E	1421	**1,4-naphthoquinone**	**20.84±1.86**	**19.33±2.81**	**19.47±3.48**
F	1459	tridecan-2-one (branched isomer)	0.46±0.09	0.51±0.17	0.66±0.13
G	1473	**6-tridecen-2-one**	**3.18±0.32**	**3.41±0.80**	**2.41±0.82**
H	1481	**7-tridecen-2-one**	**6.90±1.55**	**6.11±1.20**	**9.83±3.06**
I	1484	**tridecadienone**	**3.70±1.19**	**6.26±2.20**	**4.39±1.39**
J	1498	**tridecan-2-one**	**37.98±1.21**	**34.51±1.58**	**38.55±6.87**
K	1534	tetradecanone (branched isomer 1)	0.16±0.04	0.09±0.05	0.04±0.05
L	1547	**6-methyl-1,4-naphthoquinone**	**17.32±1.37**	**16.90±1.68**	**13.13±1.55**
M	1560	tetradecanone (branched isomer 2)	1.29±0.13	1.67±0.45	1.17±0.28
N	1568	tetradecanone (branched isomer 3)	0.25±0.04	0.24±0.08	0.26±0.10
P	1583	tetradecenone	0.16±0.03	0.18±0.06	0.11±0.09
Q	1597	tetradecan-2-one	0.97±0.23	0.95±0.22	0.77±0.17
R	1604	4-chloro-1,2-naphthoquinone	0.46±0.50	0.97±0.59	1.50±1.19
U	1687	pentadecenone	0.21±0.09	0.40±0.19	0.21±0.12
W	1699	pentadecan-2-one	0.24±0.05	0.21±0.05	0.25±0.05
X	1736	6-methyl-4-chloro-1,2-naphthoquinone	0.24±0.30	0.92±1.09	0.12±0.19

*Peak annotation according to Raspotnig et al. (2005). For completeness, the "missing" compounds in this list (present in *Cyphophthalmus* and *Siro*, but not in *Austropurcellia*) are: A (acetophenone), O (isomeric tetradecenone), S (pentadecadienone), T (pentadecatrienone), and V (unknown). **Retention index, calculated as $RI_x = 100n_0 + (100t_x - -100tn_0)/(tn_1 - tn_0)$, with x= target compound; t_x=retention time of target compound; n_0= number of carbons in the alkane eluting directly before x; tn_0= retention time of alkane directly eluting before x; tn_1=retention time of alkane eluting directly after x. All tetradecanones (K, M, N, and Q; three of these probably methyl-branched) as well as the unsaturated ketones (B_1, D_1, P, and U) are most likely also 2-ketones, as indicated by *m/z* 58. Main compounds (> 3% of whole secretion) in bold

sironids, but their occurrence in *Austropurcellia*, although in low amounts, contradicts this idea.

Are Cyphophthalmid Secretions Ancient? According to Boyer and Giribet (2007) and Giribet et al. (2012), pettalids form a monophyletic clade, and their extant diversity is considered the result of diversification processes that started over 180 million years ago, paralleling the break-up of Gondwana into the present continents and landmasses. The origin of pettalids, however, may be more ancient and may date back to the Carboniferous era. Some authors regard pettalids as basally branching cyphophthalmids, possibly representing the sister group to all other cyphopthalmid families (Giribet et al., 2012). If this is correct, methylketones and naphthoquinones may have already been present in a common ancestor of cyphophthalmids, with this ancestral chemistry being largely unchanged over 300 million years. This presumed chemical conservatism of cyphophthalmid secretions is intriguing, and implies high survival benefits associated with these secretions that, essentially,

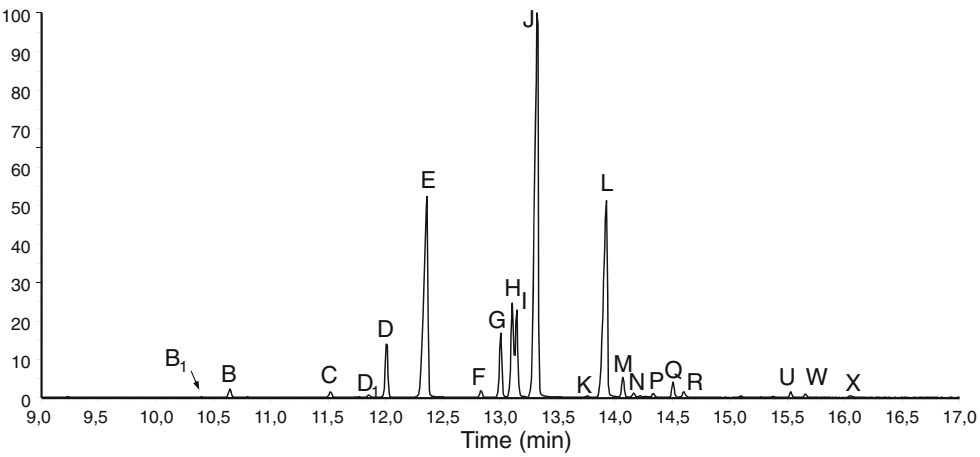

Fig. 1 Total ion chromatogram (y-axis shows relative peak heights) from scent gland secretions of *Austropurcellia forsteri*. Peak identities are listed in Table 1. The profile was obtained by dabbing the secretion from the ozopores of an individual female on filter paper. Note that peaks A, O, S, T, and V (see Raspotnig et al., 2005) are missing in the secretion of *A. forsteri*, but may be present in other cyphophthalmids

could have contributed to the long-term evolutionary success of the group.

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