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Letter from the Editor-in-Chief

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This issue contains three articles that deal with applied research. As stated in our guidelines, "Applied chemical ecology is an appropriate subject matter so long as the work is based on ecological problems or concerns". Two of the papers in this issue, those by Tamirou et al. and Mukabana et al., are African studies that focus on worldwide problems of crop losses (maize) and infectious disease (mosquito control in malaria-infested regions). Some of this research was discussed at SEMIO11, a workshop on "Insect chemical ecology and multilevel pest management towards food security and sustainable development" held in November 2011 at ICIPE in Nairobi, Kenya. Both studies focus on recent developments. The third paper by Alpizar et al. deals with the successful use of mass trapping in the control of pests of banana (a worldwide threatened crop) over an extended time period. All three of these papers explore fundamental ideas in ecology as well as provide insights into important applied problems. We think that these papers will be of interest to our readers.

John T. Romeo
March, 2012

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COMMENTARY

Delineating Compartmentalized Control of Phenylpropanoid Metabolism

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One out of every five photosynthetically fixed carbon atoms is estimated to enter the phenylpropanoid pathway (PPP), ultimately leading to the production of a myriad of secondary metabolites. In plant cells, the initial enzymatic steps of the core PPP are putatively localized to the cytosolic compartment, and can control key features of secondary metabolism. The enzyme PAL (an ammonia-lyase) utilizes the aromatic amino acid phenylalanine to produce (E)-cinnamic acid, and C4H (a monooxygenase) converts (E)-cinnamic acid to *p*-coumaric acid. These three core chemical compounds are precursors to thousands of secondary metabolites, such as lignins, flavonoids, phenolic volatiles, coumarins, tannins, stilbenes, phytoalexins, and at least one phytohormone (Vogt 2010).

A suite of *PAL* genes has been identified in most plant species examined; whereas one or two *C4H* gene copies are common. PAL and C4H can form a heterologous multi-protein association, thereby establishing a metabolic channel that converts phenylalanine to *p*-coumaric acid without allowing exposure of the biosynthetic intermediate, (E)-cinnamic acid, to other cytosolic constituents. Total PAL activity can be negatively regulated by a feed-back mechanism involving (E)-cinnamic acid, which functions as a critical control point in the metabolic flux of the PPP. The dynamic interplay between PAL and C4H appears to be a key feature for directing carbon flux through the core PPP without negatively affecting overall PAL activity, and thus, total carbon flux subsequent of PAL action. However, a problem then exists for biological situations in which elevated quantities of (E)-cinnamic acid are needed for the production of derived compounds such as volatile benzenoids.

A relatively simple plant system that produces considerable levels of benzenoid compounds is *Petunia x hybrida* cv 'Mitchell Diploid'. This petunia cultivar has relatively large, white flowers that when open, emit large amounts of floral volatile benzenoid/phenylpropanoid (FVBP) compounds (~100 ug/gfw/h), and thus can be a suitable biological system for understanding early stages of phenylalanine metabolism. The last common precursor in FVBP biosynthesis is phenylalanine; however, the majority of FVBPs are produced from the PPP intermediates (E)-cinnamic acid and *p*-coumaric acid (i.e. three metabolites from the core PPP—three branches of the FVBP pathway). Metabolite regulation of the core PPP, whether by enzyme kinetics, allosteric regulation, metabolic channeling, and/or compartmentalization may be crucial to the overall volatile blend these flowers emit; but the regulatory complexities remain scientifically under-explored. In short, the petunia petal limb cell must synthesize enough (E)-cinnamic acid to supply the eventual biosynthesis and emission of volatile benzenoids without stimulating a negative feed-back regulatory mechanism. Writ large, a similar challenge may have resulted in the PAL/C4H protein complex 'solution' for supplying *p*-coumaric acid derived compounds without affecting PAL activity.

Benzenoid biosynthesis in petunia petal limb cells follows a β-oxidative chemical strategy in the peroxisomal cellular compartment (Van Moerkercke et al. 2009), and now appears to begin with the activation of (E)-cinnamic acid by a specific enzyme, which produces cinnamoyl-CoA (unpublished data). In retrospect, this is not surprising because fatty acid degradation (occurs in peroxisomes of many plants) utilizes a similar β-oxidative chemical strategy. Curiously, the compartmentalization of (E)-cinnamic acid was suggested over a decade ago without eliciting much enthusiasm. However, this suggestion may have greater traction currently due to advances in technology, and perhaps more importantly, due to changes in scientific perception in general. The spatial strategy discussed could provide the key regulatory mechanism that prevents (E)-cinnamic acid accumulation in the cytoplasmic cellular compartment, where there would be a high probability of feed-back inhibition of phenylpropanoid biosynthesis, reducing, rather than increasing the carbon flux through this metabolic pathway.

Metabolic flux through the core PPP may be 'sensed' through endogenous (E)-cinnamic acid levels. Evidence for this hypothesis is robust but convoluted. Increased (E)-cinnamic acid levels can cause inhibition of PAL transcription and enzyme activity in many plant species. Pharmacological inhibition of PAL activity can result in a 'super-induction' of PAL transcription and enzyme activity. Down-regulation of *C4H* can result in a reduction in PAL activity, and an increase of C4H protein can up-regulate *PAL* transcript levels. Therefore, trafficking (E)-cinnamic acid to peroxisomes, as demonstrated in petunia flowers, may mitigate the negative affects that a pool of (E)-cinnamic acid might have on the overall activity of the core PPP, much like that of the PAL/C4H complex. The molecular mechanisms of peroxisomal import are still unclear, and may represent the frontier of metabolic regulation. Comparative studies of the genetic variation among compartmentalization mechanisms involved in plant metabolism may bring us closer to that frontier.

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Oviposition Induced Volatile Emissions from African Smallholder Farmers' Maize Varieties

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Abstract Maize (corn), *Zea mays*, is a genetically diverse crop, and we have recently shown that certain open pollinated varieties (OPVs) of Latin American origin possess a trait not present in mainstream commercial varieties: they produce volatiles in response to stemborer oviposition that are attractive to stemborer parasitoids. Here, we tested whether a similar tritrophic effect occurs in the African OPVs 'Nyamula' and 'Jowi'. Herbivore induced plant volatiles (HIPVs) were collected from plants exposed to egg deposition by the stemborer *Chilo partellus*. In a four-arm olfactometer bioassay, the parasitic wasp *Cotesia sesamiae* preferred samples containing HIPVs from plants with eggs to samples collected from plants without eggs. EAG-active compounds, including (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), were released in higher amounts from the egg induced headspace samples. Our results suggest that this oviposition trait is not limited to S. American *Z. mays* germplasm, and that it could be used to increase indirect defense against attack by stemborers.

Keywords Insect-plant interaction · Oviposition · Induced defense · Plant volatiles · Multitrophic interaction

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Introduction

Plants have evolved sophisticated defense mechanisms that protect against insect attack. Some of these mechanisms directly affect the herbivore due to their toxic or deterrent properties, while others involve recruitment of the herbivore's natural enemies and are based on plant secondary metabolites. The latter is termed indirect defense (Turlings et al., 1990; Heil, 2008) and involves the plant responding to insect attack by releasing a blend of herbivore induced volatiles (HIPVs) that serve as foraging cues for parasitoids. Production of HIPVs often is triggered on feeding damage by herbivorous larvae. However, a number of recent studies have shown plant responses to an earlier stage of insect attack, i.e., egg deposition (Colazza et al., 2004; Hilker and Meiners, 2006; Bruce et al., 2010; Tamiru et al., 2011). Defenses elicited by the presence of eggs benefit plants because they enable defense to be switched on early, before leaf or stem damage is caused by larvae (Hilker and Meiners, 2006; Bruce et al., 2010).

The spotted stemborer, *Chilo partellus* Swinhoe (Crambidae), is the most damaging pest of maize in eastern and southern Africa and South Asia, causing severe yield losses that can reach 88% (Seshu Reddy and Walker, 1990; Kfir et al., 2002). In sub-Saharan Africa, maize is an important staple and cash crop (Odendo et al., 2001) and is grown mainly by resource-poor farmers under low-input smallholder systems. Effective control of *C. partellus* is difficult, largely due to the protection provided by the plant stem for immature pest stages, and insecticides often are neither practical nor economical for smallholder farmers. The ecology of tritrophic interactions thus presents an opportunity for development of cost effective and environmentally benign pest control approaches that make use of innate plant defenses, which would be relevant for resource-poor African farmers.

Recently, we observed that certain maize open pollinated varieties (OPVs) of Latin American origin had an indirect defense mechanism involving emission of HIPVs in response to stemborer oviposition, which was absent in standard commercial varieties (Tamiru et al., 2011). African maize OPVs, i.e., local varieties adapted to local agroecosystems, are grown by approximately 80% of smallholder farmers in western Kenya (Odendo et al., 2001), but tritrophic interactions following stemborer oviposition have not previously been investigated in these varieties. There is an urgent need to make better use of indirect defense traits when selecting new varieties appropriate for smallholder agriculture. Here, we report responses of African maize OPVs to egg deposition by *C. partellus* and effects on the behavior of the key larval parasitoid, *Cotesia sesamiae*.

Methods and Materials

Insects and Plants. A culture of *Chilo partellus* was initiated from field collected stemborers and reared on a semi-synthetic diet developed by Ochieng et al. (1985). The parasitoid *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) was reared on stemborer larvae according to the method described by Overholt et al. (1994). Maize, *Z. mays*, OPV varieties 'Jowi' and Nyamula', collected from farmers in western Kenya, was grown individually in pots in an insect-proof screen house at ICIPE-Mbita Point field station. All plants were grown under natural conditions (c. 25°C, 65% RH; 12 L: 12D) and used in the experiments when 3–4 weeks old.

Volatile Collection Volatile compounds were collected from whole maize plants, with and without stemborer eggs, using headspace sampling (Agelopoulos et al., 1999). Prior to volatile collection, plants for oviposition were kept overnight inside cages (80×40×40 cm) into which six gravid female stemborer moths were introduced. Volatiles were collected the following day, starting at the last 2 hr of photophase, for 48 hr as described in Tamiru et al. (2011).

Electroantennogram (EAG) Recording GC-EAG analyses were conducted as described in Tamiru et al. (2011). Briefly, an indifferent electrode was placed within the head capsule of a female *C. sesamiae*, and the distal ends of the antennae were inserted into the tip of the recording electrode using micromanipulators. Electrophysiological responses, i.e., negative deflections measured in milliVolts, were recorded using specialized software (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. Recordings were replicated at least five times for each sample used.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) Aliquots of attractive headspace samples were analyzed on a capillary gas chromatography column (EC05, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK). Ionization was performed by electron impact (70 eV, 250°C). The oven temperature was maintained at 50°C for 3 min, and then programmed at 7°C min^{-1} to 200°C and 100°C min^{-1} to 300°C, where it was held for 2 min. Compounds were identified by comparison of retention indices and mass spectra with those of authentic standards. 2-heptanol, decane, (*R*)-limonene, methyl benzoate, (*R*)-linalool, methyl salicylate, decanal, neryl acetate, and eugenol were obtained from Sigma Aldrich. (*E*)-4,8-Dimethyl-1,3,7-nonatriene (>98%) was synthesized from geraniol by oxidation to the aldehyde followed by Wittig methylenation.

Olfactometer Bioassays Responses of parasitoids to plant derived volatiles and authentic standards were tested in a Perspex four-arm olfactometer as described in Tamiru et al., (2011). Air was drawn through the four arms towards the center at 260 ml min^{-1}. Headspace samples (10 μl aliquots) were applied, using a micropipette (Drummond 'microcap', Drummond Scientific Co., Broomall, PA, USA), to a piece of filter paper (4×25 mm) subsequently placed in an inlet port at the end of each olfactometer arm. Mated female parasitoids, without previous exposure to plants or hosts, were transferred individually into the central chamber of the olfactometer using a custom-made piece of glass tubing. Time spent in different regions was compared. A choice test to compare insect responses to headspace samples from oviposition induced and control (unexposed) plants was carried out by placing the test stimuli (10 μl aliquots of headspace sample) in two opposite arms, while the remaining two arms were solvent controls.

Results

Comparison of Volatiles Emitted from Plants With and Without Eggs Coupled gas chromatography-mass spectrometry revealed that the African OPVs 'Nyamula' and 'Jowi' emitted more EAG active compounds when exposed to *C. partellus* egg deposition, compared to unexposed plants (Fig. 1).

Behavioral Responses to Headspace Samples In an olfactometer bioassay, female parasitic wasps, *C. sesamiae*, spent significantly more time in the region with volatiles from

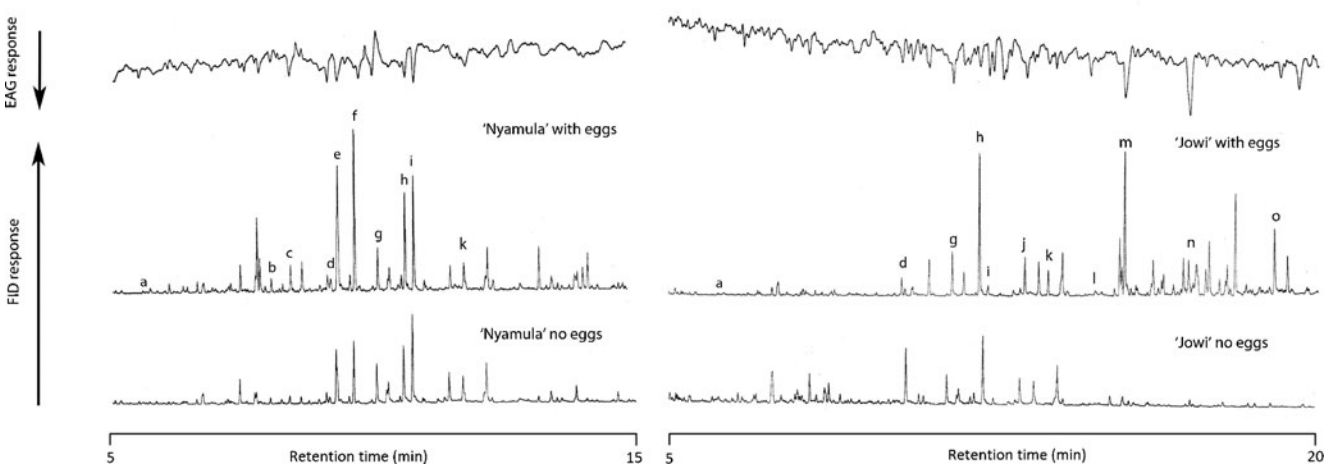

Fig. 1 GC-EAG responses of female *Cotesia sesamiae* to volatiles collected from African maize landraces 'Nyamula' and 'Jowi' (plants with eggs). The FID peaks marked are those which elicited antennal responses in two or more coupled runs: a = 2-heptanol (stereochemistry undefined), b = decane, c = (*R*)-limonene, d = methyl benzoate, e = (*R*)-linalool, f = (*E*)-4,8-dimethyl-1,3,7-nonatriene, g = *cis*-*p*-mentha-2,8-dien-3-ol*, h = methyl salicylate, i = decanal, j = 2,3-dimethylacetophenone*, k = neryl acetate, l = eugenol, m = copaene*, n = germacrene D*, o = unknown. * = tentative identification not confirmed with authentic standard. GC traces of volatiles collected from plants without eggs are shown underneath, for comparison

African maize OPVs exposed to egg deposition by the stemborer *C. partellus*, compared to regions with unexposed and blank controls ('Nyamula' $F=5.72$; $P=0.006$; 'Jowi' $F=18.18$, $P<0.001$). There was no significant difference in time spent between plants without eggs and the blank control (Fig. 2). The increase in time spent is a positive response indicating that attraction and arrestment of parasitoids was increased.

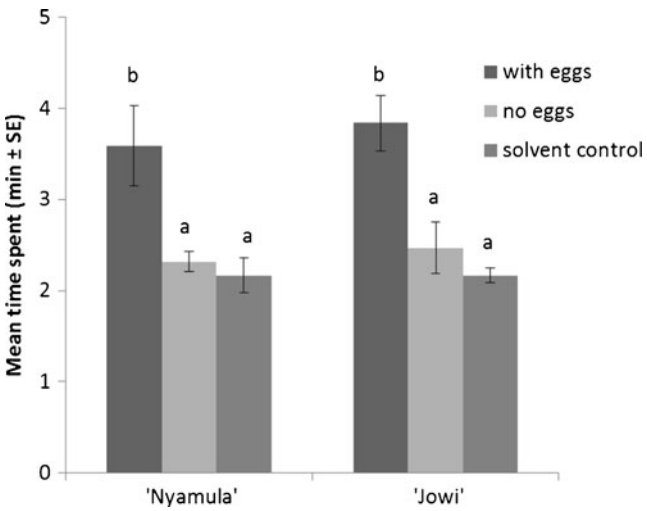

Fig. 2 Behavioral responses of female *Cotesia sesamiae* in a four-arm olfactometer bioassay to volatiles collected from African maize landraces 'Nyamula' and 'Jowi' (plants with and without eggs). Each parasitoid was observed for 12 min ($N=12$). The height of the bar chart is the mean (± s.e.) for time spent (min) in each part of the olfactometer. Parasitoid responses were compared by ANOVA after conversion of the data into proportions and log-ratio transformation. Significant means were separated using Student-Newman-Keuls (SNK) test. Error bars indicate standard error and the different letters above bars show statistically significant differences ($P<0.05$)

Discussion

The present study demonstrated that the locally adapted African maize OPVs 'Jowi' and 'Nyamula' emitted a different blend of volatiles when *C. partellus* eggs were laid on them (Fig. 1). Furthermore, we observed a preference, in an olfactometer bioassay, of *C. sesamiae* parasitoids for volatiles from plants exposed to egg deposition compared to volatiles from unexposed plants (Fig. 2). Recently, we observed similar effects in maize OPVs of Latin American origin, where such early defense responses were not exhibited by standard commercial varieties (Tamiru et al., 2011). Attraction of larval parasitoids as well as egg parasitoids means that natural enemies can also attack newly hatching larvae. Most of the egg induced HIPVs identified in the present study were similar to those found in our earlier study (Tamiru et al., 2011), although qualitative and quantitative variations were observed in the volatile composition. DMNT, a key compound known to attract *C. sesamiae* in a "push-pull" companion cropping system (Khan et al., 1997), was released in larger amounts by oviposition exposed 'Nyamula'.

Previously, studies showed interspecific variation in herbivore induced odor emissions among maize cultivars and inbred lines from temperate regions (Gouinguene et al., 2001; Degen et al., 2004). It is possible that the high selection pressure for other traits, e.g., yield, might have caused the loss of such valuable traits in commercial varieties. Natural enemies such as parasitic wasps and predators exploit herbivore-induced plant odors to locate and parasitize their herbivorous hosts (Dicke et al., 1990; Tumlinson et al., 1993; De Moraes et al., 1998). Identification of important induced defense volatiles in locally adapted African maize

OPVs not only indicates wider occurrence of these traits than in the germplasm studied previously, but also demonstrates possibilities for exploiting indirect maize OPV defenses mediated by HIPVs in African agriculture.

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A Novel Synthetic Odorant Blend for Trapping of Malaria and Other African Mosquito Species

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Abstract Estimating the biting fraction of mosquitoes is of critical importance for risk assessment of malaria transmission. Here, we present a novel odor-based tool that has been rigorously assessed in semi-field assays and traditional African villages for estimating the number of mosquitoes that enter houses in search of a blood meal. A standard synthetic blend (SB) consisting of ammonia, (S)-lactic acid, tetradecanoic acid, and carbon dioxide was complemented with isovaleric acid, 4,5 dimethylthiazole, 2-methyl-1-butanol, and 3-methyl-1-butanol in various combinations and concentrations, and tested for attractiveness to the malaria mosquito *Anopheles gambiae*. Compounds were released through low density polyethylene (LDPE) material or from nylon strips (nylon). Studies were done in a semi-field facility and two traditional villages in western Kenya. The alcohol 3-methyl-1-butanol significantly increased the attraction of SB. The other compounds proved less effective or inhibitory. Tested in a village, 3-methyl-1-butanol, released from LDPE, increased the attraction of SB. Further studies showed a significantly enhanced attraction of adding 3-methyl-1-butanol to SB compared to previously-published attractive blends both under semi-field and village conditions. Other mosquito species with relevance for public health were collected with this blend in significantly higher numbers as well. These results demonstrate the advent of a novel, reliable odor-based sampling tool for the collection of malaria and other mosquitoes. The advantage of this odor-based tool over existing mosquito sampling tools is its reproducibility, objectiveness, and relatively low cost compared to current standards of CDC light traps or the human landing catch.

Keywords Odor-guided behavior · Synthetic odor blend · Mosquito · *Anopheles gambiae* · Sampling · Isovaleric acid · 4,5-dimethylthiazole · 2-methyl-1-butanol · 3-methyl-1-butanol

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Introduction

Blood-questing mosquitoes mainly rely on olfactory cues to locate their hosts (Takken, 1991; Costantini et al., 1996; Takken and Knols, 1999). For the principal African malaria vectors, *Anopheles gambiae* Giles *sensu stricto*, *An. arabiensis* Patton and *An. funestus* Giles, these cues are strongly reminiscent of those released by humans i.e., the principal source of the mosquitoes' blood meals (Gillies and Coetzee, 1987). Odorants from human skin and carbon dioxide from breath are particularly important (Costantini et al., 1996; Mukabana et al., 2004; Spitzen et al., 2008). The anthropophilic malaria mosquito *Anopheles gambiae sensu stricto* (hereafter termed *An. gambiae*) primarily takes blood meals

on humans, whereas its close sibling *An. arabiensis* is more opportunistic, feeding on humans and animals. This difference in host preference is expressed clearly in odor-guided behavior, where *An. arabiensis* responds significantly stronger to carbon dioxide from breath than does *An. gambiae* (Costantini et al., 1996). These behavioral differences also are reflected in the role of these species as malaria vectors, *An. gambiae* being the principal species even though *An. arabiensis* is equally susceptible to the *Plasmodium* parasite but, due to its different feeding behavior, is of less importance as a vector. Host preference, and hence the selection of hosts based on their odorants, is of principal importance to understand the role of these mosquitoes in malaria epidemiology.

Given the strong association of *An. gambiae* with humans, unraveling the odor cues that mediate this behavior is of scientific as well as practical importance. For *An. gambiae* the principal olfactory cues of humans originate from the feet (De Jong and Knols, 1995), and recent work has demonstrated that these cues are partially produced by the microbial flora present on the feet (Verhulst et al., 2010). From these studies, several chemical compounds have been identified that play a critical role in the odor-mediated behavior of *An. gambiae* (Smallegange et al., 2005, 2009; Verhulst et al., 2011a). Detailed information on the role of these compounds allows for the development of synthetic blends that can be used to better understand the host-seeking behavior of this mosquito. Such blends also have the potential to be used for mosquito surveillance or intervention through mass trapping.

Studies on the development of kairomones for malaria vectors have demonstrated strong behavioral responses to synthetic blends of human odorant compounds (Smallegange et al., 2010a). In laboratory and semi-field studies, these blends attract a large proportion of host-seeking mosquitoes, but when tested against a natural human host, or against natural human odorants released from a nylon matrix, these blends demonstrate poor competitive characters compared to the natural odorants. This suggests that either the concentration of the odorants in the blend was insufficient or that one or more compounds to make the blend sufficiently competitive were missing (Smallegange et al., 2005, 2010a; Verhulst et al., 2011a). Recent progress, however, has demonstrated the development of odor blends that approach the attractiveness of natural human skin odorants (Okumu et al., 2010b).

In addition, there is sometimes a mismatch between laboratory behavioral results and field trials, which can be caused by differences in concentration or spatial effects (Verhulst et al., 2009, 2011a). Assessment of existing synthetic attractant compounds under semi-field and field conditions provides a potential for the development of technologies that can be used for sampling and control of malaria mosquitoes (Kline, 2006; Jawara et al., 2009; Okumu et al., 2010b). The current study was designed to evaluate the attractiveness of selected synthetic blends and human hosts to host-seeking mosquitoes in western Kenya, with emphasis on the malaria vectors *An. gambiae sensustricto* and *An. arabiensis*. Thus, the comparative trapping efficacy of the attractant blends and the physiological status of the mosquitoes trapped were investigated.

Methods and Materials

Mosquitoes Behavioral responses of mosquitoes to synthetic attractants were evaluated under field and semi-field conditions. The semi-field experiments utilized a laboratory colony of the Mbita strain of *An. gambiae*. Aquatic stages of the mosquitoes were reared under ambient atmospheric conditions in screen-walled greenhouses at the Thomas Risley Odhiambo (TRO) campus of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita Point township in western Kenya. Mosquito eggs were placed in plastic trays containing filtered water from Lake Victoria. All larval instars were fed on Tetramin® baby fish food supplied three times a day. Pupae were collected daily, transferred to adult holding rooms, and placed in mesh-covered cages (30×30×30 cm) prior to adult emergence. Adult mosquitoes were fed on 6% glucose solution through wicks made from adsorbent tissue paper. Mosquitoes used for semi-field experiments were placed in mosquito-gauze covered plastic cups and starved for 8 hr. They had no prior access to a blood meal. Only water, availed on wet cotton towels placed on top of mosquito holding cups, was provided during starvation. All semi-field experiments were carried out at night (2030–0630 h) inside the screenhouses (Verhulst et al., 2011b). Two hundred, 8-h-starved mosquitoes were used each experimental night.

Compounds Used to Constitute Odorant Blends All chemicals used to constitute the synthetic attractant blends in this study, with the exception of carbon dioxide, water, sugar, and yeast, were purchased from Sigma-Aldrich Chemie GmbH (Germany). The chemicals included propionic acid (99.6%), butanoic acid (≥99.9%), pentanoic acid (>99%), heptanoic acid (98%), octanoic acid (≥99%), tetradecanoic acid (≥99%), ammonia solution (purity 25%), (S)-lactic acid (85%), isovaleric acid (99.8%), 4,5-dimethylthiazole (97%), 2-methyl-1-butanol (99%; a racemic mixture of the R and S isomers of unknown ratio), and 3-methyl-1-butanol (purity≥98.5%). Carbon dioxide was produced by mixing 250 g sucrose (Sony sugar company Limited, Kenya), 17.5 g dry yeast (Angel Yeast company limited, China), and water (2 L) as described in Smallegange et al. (2010b).

Study Sites Field studies were carried out in Lwanda and Kigoche villages of Homa Bay and Kisumu counties of

western Kenya, respectively. Lwanda village is located on the southern shore of the Winam Gulf of Lake Victoria (00°28′28″S, 34°17′22″E) at an altitude of 1,169 m above sea level (Verhulst et al., 2011a). Average rainfall and relative humidity are 1,200 mm and 65%, respectively. The mean temperatures vary between 18°C and 34°C. Hoof prints of cattle and night-grazing hippopotami provide excellent mosquito breeding sites in Lwanda. Fishing and livestock keeping are the main occupation of the local inhabitants. Kigoche village lies adjacent to the Ahero rice irrigation scheme (00°08′19″S, 34°55′50″E) at an altitude of 1,160 m above sea level. Kigoche has an average annual rainfall of 1,000–1,800 mm and an average relative humidity of 65%. Mean annual temperatures in the area vary between 17°C and 32°C. Rice cultivation is the main occupation of the inhabitants. Most houses in the two villages are mud-walled with open eaves, have corrugated iron-sheet roofs, have no ceiling, and are either single- or double-roomed. Eaves, about one foot wide, increase ventilation in the houses and form the predominant entry points for mosquitoes (Snow, 1987; Lindsay and Snow, 1988). Malaria caused by *Plasmodium falciparum* is endemic in the two villages. The villages experience two rainy seasons: between April–June and September–October. During these periods, mosquito breeding grounds proliferate, and mosquito populations rapidly increase in size. Cattle, goats, chicken, dogs, cats, and a few sheep constitute the domestic animal population, with cattle being most abundant. Maize, millet and sorghum are cultivated at subsistence level in Lwanda, whereas rice is a main cash crop in Kigoche.

In both villages, trapped mosquitoes were morphologically identified using the keys published by Gillies and Coetzee (1987), counted, and the data entered in MS Excel spreadsheets. Culicine mosquitoes were identified up to genus level, and anophelines into *An. gambiae* sensu lato, *An. funestus* and other anopheline species. Abdominal statuses of female mosquitoes were categorized as unfed, blood-fed, or gravid. Mosquitoes belonging to the *An. gambiae* complex were identified to species using a ribosomal DNA Polymerase Chain Reaction assay (Scott et al., 1993).

Constituting Prototype Blends and Evaluating Their Attractiveness to Anopheles gambiae Prototype blends were made by adding components to a standard attractive blend (standard blend, SB) consisting of ammonia, (S)-lactic acid, and tetradecanoic acid (Smallegange et al., 2005, 2009). The standard blend was augmented with locally-made carbon dioxide plus individual additional candidate compounds or groups of 2–4 of these compounds. The additional compounds included isovaleric acid, 4,5-dimethylthiazole, 2-methyl-1-butanol, and 3-methyl-1-butanol. These compounds were selected following two studies in which a total of 39 chemical compounds were evaluated for their attractiveness to *An. gambiae* (Verhulst et al., 2011b; Smallegange et al., 2012). All compounds except carbon dioxide were delivered to experimental mosquitoes by using low density polyethylene sachets (LDPE). To prepare a sachet, a tube foil of LDPE material was cut (35×30 mm), thermally sealed at one end before pipetting 1,000 μl of a pure compound through the open end of the sachet. The sachet was subsequently sealed, confining the candidate compounds within an area of 25×25 mm. Only a single candidate compound was placed in an individual sachet. The wall thickness of the LDPE material used was 0.1 mm (isovaleric acid and 3-methyl-1-butanol), 0.2 mm (2-methyl-1-butanol), 0.03 mm (ammonia solution, 4,5 dimethylthiazole, and tetradecanoic acid), and 0.05 mm ((S)-lactic acid). Several sachets, varying in number according to the number of chemical compounds constituting a specific prototype blend, were placed onto a hook (Verhulst et al., 2009), which was subsequently inserted inside the outlet tube of a MM-X trap (American Biophysics Cooperation, RI, USA). Dual-choice tests comparing mosquito behavioral responses to a total of 15 prototype blends, with different combinations of the four candidate compounds+SB vs. SB alone then were conducted in the semi-field screenhouse facility by placing MM-X traps in diagonal corners (Verhulst et al., 2011a). The experiments were replicated four times.

Attraction of Wild Mosquitoes to the Best Prototype Blend against Existing Alternatives The prototype blend attracting the highest numbers of *An. gambiae* mosquitoes in the screenhouse was evaluated in Lwanda village and compared with the attractiveness of other blends, which we had tested in previous field experiments (Okumu et al., 2010b). The number of mosquitoes attracted to each one of three blends including Ifakara blend 1 (IB1; Okumu et al., 2010a), standard blend (Smallegange et al., 2005, 2009) and a best, newly-formulated prototype blend namely Mbita blend (MB) was recorded. The blends were dispensed via LDPE sachets or nylon strips. The nylon fabric was made of 15 denier microfibers. The textile composition was 90% polyamide and 10% spandex (Bata Shoe Company, Kenya). The nylon strips were cut into narrow pieces each measuring 26.5 cm long and 1.0 cm wide. Each strip then was soaked in separate solutions of the attractants until saturation. Treatments included blend IB1 dispensed via nylon strips, IB1 dispensed via LDPE sachets, SB dispensed via nylon strips, SB dispensed via LDPE sachets, and MB dispensed via LDPE sachets. Blend IB1 consisted of propionic acid (0.01%; LDPE thickness 0.2 mm), butanoic acid (1%; LDPE 0.2 mm), pentanoic acid (0.0001%; LDPE 0.2 mm), 3-methyl butanoic acid (0.000001%; LDPE 0.2 mm), heptanoic acid (0.0001%; LDPE 0.1 mm), octanoic acid (0.0001%; LDPE 0.1 mm), tetradecanoic acid (0.00025%; LDPE 0.03 mm), ammonia (2.5%; LDPE 0.03 mm), (S) lactic acid (85%; LDPE 0.05 mm), distilled water (LDPE

0.2 mm), and carbon dioxide (~63.23±2.82 ml/min). An unbaited MM-X trap acted as the negative control. All blends were dispensed from MM-X traps hung outside the bedroom window (for details: see Verhulst et al. (2011b)). A total of eight village houses were selected and experiments carried out from 1830 to 0630 h each night for 40 nights. Treatments were rotated around the eight houses to eliminate positional effects. The selected houses were mud-walled, had open eaves and corrugated iron sheet roofs. The houses, occupied by owners (one person per house) throughout the night, were located at least 25 m. apart (Hill et al., 2007).

Optimizing the Most Attractive Prototype Blend on Nylon Strips by Varying the Concentration of 3-Methyl-1-butanol The blend that attracted most mosquitoes compared to the standard blend was selected and adapted for release on nylon strips, which are known to yield higher mosquito catches than LDPE sachets (Okumu et al., 2010a). The blend, named Mbita blend or MB consisted of 3-methyl-1-butanol, tetradecanoic acid, ammonia solution, (S)-lactic acid, and carbon dioxide. The most effective dilutions for tetradecanoic acid (0.00025%), ammonia (2.5%), (S)-lactic acid (85%) had been determined previously (Okumu et al., 2010b). Carbon dioxide was released at ~63.23± 2.82 ml/min. Thus, the optimal dilution for dispensing 3-methyl-1-butanol on nylon strips was determined experimentally under semi-field conditions using MM-X traps. Binary assays evaluating mosquito behavioral responses to SB with all constituents availed at their optimally attractive concentrations vs. SB plus 3-methyl-1-butanol offered at variable dilutions (100%, 10%, 1%, 0.01% and 0.0001% and 0.000001% *v*/*v*) were run.

Efficacy of Attracting Mosquitoes to Optimized Blend Under Semi-field Conditions The efficacy of attracting host-seeking mosquitoes using MB was evaluated by testing it against Ifakara Blend 1 (IB1), the standard blend (SB), and a control (unbaited trap) under semi-field conditions. A fully replicated 4×4 Latin Square experiment was used. All blends were dispensed on nylon strips by pipetting 1,000 µl of each component of a blend on a separate strip (measuring 26.5×1.0 cm). The 3-methyl-1-butanol component of MB was used at a dilution of 0.000001%.

Capacity of Trapping Wild Mosquitoes Using Optimized Blend Vs. Existing Alternatives In Kigoche village, five houses spaced apart at a distance of at least 25 m and at least 100 m away from rice paddies were selected for the study. A 5×5 Latin square experimental design preceded by a 5 d trial period was run to assess the potential of MB in attracting malaria and other mosquito vectors. The treatments, assigned to each of the five houses on rotational basis per night, included a human host, IB1, SB, MB, and an empty house i.e., the control. All components of the synthetic attractants, with the exception of carbon dioxide, were delivered via nylon strips (Okumu et al., 2010a) and dispensed using MM-X traps. The traps were suspended 15 cm above a bed inside unimpregnated mosquito nets, and were operated using 12 volt batteries. One of the fans on the MM-X trap was disabled to prevent it from trapping mosquitoes. Mosquitoes were trapped by hanging an unlit CDC miniature light trap (Model 512; John W. Hock Company, Gainesville Fl., USA) operated on 6 V batteries (Gaston Battery Industry Ltd, China) beside and at 15 cm (Jawara et al., 2009) above the odor outlet tube of the MM-X trap, outside the bed net. Both MM-X and CDC light traps were hung on the foot end region of the beds in all cases (Mboera et al., 2000). Only one MM-X trap and one CDC light trap were used in houses where mosquito catches were based on synthetic attractants. Only one CDC light trap was used in houses occupied by human subjects. The volunteer slept inside a well tucked-in bed net with a CDC light trap suspended besides it. Whereas a total of five adult men aged 18–25 years volunteered to participate in the study, only one individual participated per night. The volunteers rotated over the treatments according to a random design. Each experimental night lasted from 1900 to 0630 h. After the experiments traps were disconnected from the batteries, the trapped mosquitoes were taken to the laboratory, where they were killed by freezing at −4°C. Traps were cleaned with 30% methanol solution before being reused. During experimental nights, the residents of the study houses were not present. Other houses in the village were occupied normally.

Ethical Considerations Consent for houses to be used in the study was obtained from the household heads and the local administration prior to the start of the study.

Statistical Analysis Dispersion tests, performed to establish whether means equaled variances (Grafen and Rosie, 2005), were employed to determine if the count data indeed assumed a Poisson distribution. Henceforth, the number of mosquitoes attracted to the different sources of behavioral stimuli (human subjects, control, or the synthetic attractants) was modeled as a proportion of the total number of mosquitoes recovered from the different treatments. All statistical analyses were carried out using Generalized Linear Models (Agresti, 1990) in which data were transformed to assume linearity using a logarithmic link function. This approach allowed for differences in attractiveness between treatments to be determined. All fitted models were followed by *post hoc t*-tests, to assess levels of statistical difference between pairs of competing blends assessed

through the binary assays. It was assumed that mosquitoes already trapped did not affect subsequent mosquitoes entering. All data were analyzed using the General statistical software program (GenStat Discovery Edition 3) (Payne, 1986).

Results

Constituting Prototype Blends and Evaluating Their Attractiveness to Anopheles gambiae Adding 3-methyl-1-butanol to the standard blend of ammonia, (S)-lactic acid, tetradecanoic acid, and carbon dioxide either singly or in combination with 2-methyl-1-butanol formed the two most potent attractant blends for host-seeking *An. gambiae* mosquitoes (catching 72% of those released) relative to the standard blend on its own under semi-field conditions (Table 1). Neither adding 2-methyl-1-butanol to the basic blend, nor adding it to 3-methyl-1-butanol enhanced attractiveness. The blend containing 3-methyl-1-butanol plus components of the standard blend was considered the most potent synthetic attractant for *An. gambiae*. The prototype product, termed Mbita blend (MB), consisted of 3-methyl-1-butanol (released in 0.1 mm-LDPE sachets), tetradecanoic acid (0.03 mm-LDPE), ammonia solution (0.03 mm-LDPE), (S)-lactic acid (0.05 mm-LDPE), and carbon dioxide (~130 ml/min). As adding isovaleric acid and 4,5 dimethyl-thiazole to the standard blend diminished the numbers of *An. gambiae* attracted (Table 1), these compounds were excluded from the prototype blend.

Attraction of Wild Mosquitoes to the Best Prototype Blend Tested Against Existing Alternatives A total of 1,286 mosquitoes were trapped in Lwanda village over a period of 40 d from 28 April 2010 to 11 June 2010. The mosquitoes included *An. gambiae* s.l. (n=259), *An. funestus* (n=207), *Culex* species (n=544), *Mansonia* species (n=112), *Aedes* species (n=43), and other mosquito species (n=121) (Table 2). Although all synthetic blends caught significantly more *An. gambiae* s.l. than the unbaited traps, no blend attracted significantly more mosquitoes of this species than the other (Table 2). However, the standard blend dispensed from nylon strips attracted comparatively higher numbers of *Mansonia* spp, *Aedes* spp. and *An. funestus* s.l. than the other blends. Ifakara blend 1 (IB1) dispensed using nylon strips attracted comparatively higher numbers of *Culex* spp. and *An. gambiae* s.l. than all the other blends. As this study was performed before the optimal dilution for releasing MB on nylon strips was determined, it was not possible to fully evaluate its competitiveness against other attractants in Lwanda village. Out of the 107 specimens of *An. gambiae* s.l. that were subjected to PCR analysis, 100 could be identified to species level. Ninety six of these were *An. arabiensis*, whereas four were *An. gambiae s.s.*.

Optimizing the Most Attractive Prototype Blend on Nylon Strips by Varying the Concentration of 3-Methyl-1-butanol The overall combined response of the mosquitoes to the blends ranged from 39% to 68% (Table 3). Dilutions of 3-methyl-1-bunanol from pure compound to 10,000 times were significantly (P<0.001) in favor of the standard

Table 1 Mean (±SE) mosquito catches per night and levels of statistical difference (P-value) between 15 prototype synthetic blends vs. a standard blend (SB) of ammonia, (S)-lactic acid, tetradecanoic acid, and carbon dioxide. Each of the compounds except carbon dioxide was dispensed from a LDPE-sachet in pure form. N is the number of replicates (nights) and n the total number of mosquitoes trapped out of a total of 800 released.% response equals n/800. Compound 1, 2, 3, and 4 are isovaleric acid, 4,5 dimethyl-thiazole, 2-methyl-1-butanol, and 3-methyl-1-butanol, respectively

Description of synthetic blend	N	n	% response	Mosquito trap catches (mean ± SE)		P-value
				Standard blend (SB)	Synthetic blend	
1. SB + compound 1	4	346	43	62.20±3.70	24.25±4.25	0.001
2. SB + compound 2	4	338	42	46.75±8.83	37.75±6.33	0.05
3. SB + compound 3	4	334	42	38.67±20.42	43.00±12.97	0.037
4. SB + compound 4	4	577	72	59.00±7.00	85.20±9.8	0.001
5. SB + compound 2&3	4	508	64	67.67±5.78	66.50±11.2	0.240
6. SB + compound 1&2	4	482	60	70.67±8.35	57.25±4.40	0.025
7. SB + compound 1&3	4	444	56	68.33±12.14	44.75±9.38	0.001
8. SB + compound 1&2&3	4	462	58	49.33±17.53	61.20±12.7	0.008
9. SB + compound 3&4	4	579	72	57.00±6.51	85.00±11.2	0.001
10. SB + compound 1&4	4	450	56	71.00±12.9	31.75±9.72	0.001
11. SB + compound 2&4	4	390	47	45.00±7.21	46.25±9.04	0.748
12. SB + compound 1&2&4	4	253	37	52.00±13.32	11.75±2.87	0.001
13. SB + compound 1&3&4	4	391	49	52.67±4.81	54.00±7.63	0.645
14. SB + compound 2&3&4	4	481	60	63.33±14.77	56.25±16.47	0.388
15. SB + compound 1&2&3&4	4	219	27	28.00±9.81	25.75±13.49	0.350

Table 2 Mean numbers of *Anopheles gambiae* s.l., *An. funestus*, *Culex* spp., *Mansonia* spp., *Aedes* spp. and other mosquito species collected indoors per night in Lwanda village, western Kenya. Mosquitoes were attracted to various synthetic attractants dispensed from nylon strips or LDPE sachets mounted in MM-X traps placed under a bed net. *N*=number of replicates (nights); *n* is total number of mosquitoes per taxon caught over 40 nights. Numbers followed by different letter superscripts in the same column differ significantly ($P<0.05$)

Treatment (delivery)	*N*	*An. gambiae* s.l.	*An. funestus*	*Culex* spp.	*Mansonia* spp.	*Aedes* spp.	Other spp.
Empty (control)	40	0.65^{a}	0.55^{a}	2.28^{a}	0.25^{a}	0.10^{a}	0.43^{a}
IB1 (LDPE)	40	1.30^{b}	0.53^{a}	1.85^{a}	0.53^{b}	0.15^{ab}	0.43^{a}
IB1 (nylon strips)	40	1.43^{b}	1.15^{b}	2.98^{b}	0.55^{b}	0.13^{a}	0.58^{b}
SB (LDPE)	40	1.05^{b}	0.58^{a}	2.48^{a}	0.33^{ab}	0.25^{a}	0.38^{a}
SB (nylon strips)	40	1.03^{b}	1.73^{c}	1.88^{a}	0.60^{b}	0.38^{b}	0.40^{a}
MB (LDPE)	40	1.03^{b}	0.58^{a}	2.15^{a}	0.55^{b}	0.08^{a}	0.83^{c}
Total mosquito catches (*n*)		259	207	544	112	43	121

blend, except for the 1,000-fold dilution, for which no difference in attractiveness between the two blends was found. Only with the highest dilution (100,000) did significantly more ($P<0.05$) mosquitoes respond to the augmented blend than to the standard blend. This blend (MB) subsequently was chosen for further evaluation.

Efficacy of Attracting Mosquitoes to Optimized Blend Under Semi-field Conditions The attraction efficacy of MB vs. IB1, SB, and a control (unbaited trap) was evaluated over a period of 16 nights i.e., from 30 November 2010 to 16 December 2010. Out of the total of 3,200 mosquitoes used for these experiments, 2,152 were captured in response to the various treatments. There was a significant effect (GLM; $P<0.001$) of treatment on mosquito trap catches. Whereas all the synthetic blends attracted significantly more mosquitoes than the unbaited control ($P=0.001$), MB attracted more mosquitoes than blends IB1 ($P=0.001$) and SB ($P=0.001$). Blend IB1 attracted more mosquitoes than SB ($P=0.001$). Results of these experiments are shown in Table 4.

Capacity of Trapping Wild Mosquitoes Using Optimized Blend Vs. Existing Alternatives The competitiveness of MB over other baits in attracting malaria and other wild mosquitoes was evaluated in Kigoche village for 30 d spanning the period 01–30 April, 2011. A total of 2,024 mosquitoes including 1,105 (54.6%) *An. gambiae* s.l., 433 (21.4%) *An. funestus*, 201 (9.9%) other *Anopheles* species and 283 (14%) *Culex* species were collected. The collections also included one *Aedes* and one *Mansonia* mosquito representing 0.1% of the collection. The *An. gambiae* s.l. collected included 34 male and 1,071 female (92.8% unfed; 5.3% blood fed, and 1.9% gravid) mosquitoes. It is worth noting that MB collected higher fractions of blood fed *An. gambiae* s.l. (38.6% i.e., 22/57) and *An. funestus* (75% i.e., 6/8) mosquitoes than IB1 and SB. Polymerase chain reaction analysis revealed that the *An. gambiae* complex mosquitoes were 96.67% *An. arabiensis* and 3.33% *An. gambiae s.s*. The *An. funestus* species of mosquitoes included 56 male and 377 female (96.8% unfed; 2.1% blood fed, and 1.1% gravid) mosquitoes. All *Culex*, *Aedes* and *Mansonia* species collected were female. The *Culex* sub-sample consisted of 196 (97.5%) unfed and 5 (2.5%) blood fed mosquitoes. The other *Anopheles* species were neither classified by sex nor abdominal status.

Table 3 Mean number ± SE of *Anopheles gambiae* caught per night in MM-X traps baited with a synthetic blend containing various dilutions of 3-methyl-1-butanol plus a standard blend vs. the standard blend alone, dispensed from nylon strips, under semi-field conditions. *P*-values give the significance of statistical difference between the catches. *N* is the number of replicates (nights) and *n* the number of mosquitoes trapped

Dilution (%)	*N*	*n*	% response	Mosquito trap catches (mean ± SE)		*P*-value
				SB	Synthetic blend	
Pure compound (99.9)	4	547	68	77.50±16.97	59.25±2.14	0.002
10.0	4	340	43	52.00±18.48	33.00±11.20	0.001
1.0	4	474	59	68.25±13.12	50.25±17.80	0.001
0.1	4	412	52	53.25±4.290	49.75 ±14.42	0.490
0.01	4	307	39	45.25±6.24	31.50±4.05	0.002
0.001	4	379	47	42.25±12.59	52.25±8.09	0.040

Table 4 Mean number ± SE of *Anopheles gambiae* caught per night in MM-X traps baited with different synthetic blends under semi-field conditions. Synthetic odors were dispensed from nylon strips placed in the MM-X trap. Numbers with different letter superscripts in the same column differ significantly (GLM; $P<0.001$). *N* is the number of replicates (nights) and *n* the total number of mosquitoes trapped. The effect of treatment (*P* values) on overall mosquito responses is also shown

Blend (delivery)	*N*	*n*	Mosquito trap catches (mean ± SE)	Treatment (*P*-value)
Control (no odors)	16	58	3.62 ± 0.81^{a}	0.001
IB1	16	691	43.2 ± 4.4^{b}	0.001
SB	16	525	32.8 ± 5.4^{c}	0.001
MB	16	878	54.9 ± 8.1^{d}	0.001

Analysis of the data revealed that houses baited with MB caught 31.5% ($n=637$) of the mosquitoes (males and females) while those with a human being, IB1, SB, or no host cue collected 26.9% ($n=545$), 19.6% ($n=397$), 17.4% ($n=352$), and 4.6% ($n=93$) of the mosquitoes, respectively. The catches of *An. gambiae* s.l. ($P<0.042$), *An. funestus* ($P<0.014$) and *Culex* species ($P<0.041$) were significantly influenced by treatment (Table 5). The mean number of female mosquitoes with the exception of *Culex* species collected from the five traditional houses did not differ significantly. The numbers of mosquitoes of all species collected from the empty house were significantly lower than those collected from houses baited with MB ($P=0.001$), a human being, IB1 ($P=0.001$), or SB ($P=0.001$). Mbita blend attracted significantly higher numbers of *An. funestus* and *Culex* species than a human being ($P=0.002$ and 0.001, respectively) or SB ($P=0.001$ and 0.005, respectively). However, although MB attracted higher numbers of *An. gambiae* s.l. mosquitoes than IB1 ($P=0.001$) and SB ($P=0.001$), these numbers did not differ significantly from those attracted by a human being ($P=0.121$). Furthermore, although MB attracted more *An. funestus* mosquitoes then IB1 ($P=0.001$), the number of *Culex* mosquitoes attracted to MB were not different from those attracted to blend IB1 ($P=0.140$)

Discussion

The mosquito catches in the screenhouse and the village houses consistently showed a higher number of *An. gambiae* when traps were baited with the Mbita blend compared to the Ifakara blend 1 and the standard blend. In Kigoche village, significantly more *An. gambiae* s.l. and *An. funestus*, the main malaria vectors in western Kenya, were caught with MB than with the other two blends, and catches were similar to (*An. gambiae* s.l.) or significantly more than those (*An. funestus*) attracted to a human host. In a previous study in Tanzania, that used experimental huts, Okumu et al. (2010b) reported significantly higher catches of *An. gambiae* s.l. (mostly *An. arabiensis*) with IB1 than with a human host. From both studies, we conclude, therefore, that synthetic blends can be effectively used for sampling African malaria mosquitoes.

The odor-baited technology has a distinct advantage over the widely-used CDC trap + human-under-a-bed-net method introduced by Garrett-Jones and Magayuka (1975), which has since been used as the standard method for assessing entomological inoculation rates and other relevant epidemiological parameters for malaria (Smith et al., 2006; Bousema et al., 2010). The CDC trap was evaluated by Lines et al. (1991) and found to sample approximately two-thirds of the mosquitoes attracted to a human host. Later assessments of the CDC trap reported a similar efficiency but with local variations (Costantini et al., 1998). Given the natural variation in attractiveness of humans to mosquitoes (Smith, 1956; Brouwer, 1960; Knols et al., 1995; Qiu et al., 2006), caused by the variation in human skin odorants and breath (Mukabana et al., 2004; Verhulst et al., 2012), as well as the need for a human host to be present

Table 5 Total pooled number of female mosquitoes and mean number ±SE per species per night trapped in response to different synthetic attractant blends (dispensed form nylon strips) and a human host in Kigoche village, western Kenya. Numbers with different letter superscripts in the same column differ significantly. The number of replicates (*N*) is shown

Treatment	*N*	Total no. of female mosquitoes collected	Mean (±SE) of female mosquito catches per night					
			An. gambiae s.l.	*An. funestus*	*Culex* spp.	*Aedes* spp.	*Mansonia* spp.	Other anophelines
Control	30	81	1.2 ± 0.41^{a}	0.8 ± 0.28^{a}	0.33 ± 0.15^{a}	0	0	0.37 ± 0.021^{a}
Human	30	527	9.9 ± 2.1^{b}	2.93 ± 0.65^{b}	1.17 ± 0.28^{b}	0	0	3.57 ± 0.96^{b}
IB1	30	375	7.13 ± 2.90^{c}	2.0 ± 0.39^{b}	1.7 ± 0.39^{c}	0.03±0.03	0.03±0.03	1.60 ± 0.49^{c}
SB	30	333	6.27 ± 1.40^{c}	2.03 ± 0.59^{b}	1.27 ± 0.33^{b}	0	0	1.53 ± 0.41^{c}
MB	30	618	11.2 ± 2.1^{b}	4.8 ± 1.26^{c}	2.23 ± 0.72^{c}	0	0	2.37 ± 0.80^{bc}

during the all-night collections, the CDC trap plus bed net combination for mosquito trapping has obvious disadvantages. These disadvantages can be overcome by replacing the human host with synthetic bait (over a time frame of at least 1 week), which produces a consistent and constant blend of odorants and hence avoids the variance caused by the human-dependent method of mosquito sampling. Natural variance in human odorants is likely to affect trap catches, and may potentially lead to stronger attractiveness of the natural host than the synthetic blend, but also to considerably lower attractiveness. Future studies should provide more details on these phenomena. Odor-baited traps also can be used in large numbers simultaneously across a study area, which avoids bias caused by spatial effects.

In this study, we examined the use of LDPE as a material for the slow release of odorants, analogous to the successful use of LDPE for attractants of tsetse flies (Green, 1994; Torr et al., 1995). Odorants attractive to tsetse flies and released through LDPE remain active for many months and have been used widely for the mass trapping of tsetse flies in remote areas. Although odorants released through LDPE used in our study proved attractive to anopheline and other mosquitoes, we found that releasing the odorants from nylon strips caused a significantly higher attraction of several of the mosquito species. Okumu et al. (2010a) also reported this effect. It seems likely that the size of the LDPE sachets we employed was too small to provide a sufficiently large surface to release the quantity of odorants needed for optimal attraction of the mosquitoes. This can be overcome by using larger sachets. It is also possible that the carboxylic acids present in the blend disperse in smaller quantities through LDPE than from the nylon strips. The odor baits used for tsetse flies consist of entirely different chemicals (mostly 1-octen-3-ol, 4-methyl phenol, and 3-n-propyl phenol) (Bursell et al., 1988; Vale, 1993), which may disperse more readily through LDPE than the mosquito kairomones.

From our high throughput work on candidate attractants of *An. gambiae* (Verhulst et al., 2011a; Smallegange et al., 2012), we selected isovaleric acid, 4,5-dimethylthiazole, 2-methyl-1-butanol, and 3-methyl-1-butanol because of the increased attractiveness expressed when these compounds were added to the standard blend. It was, therefore, surprising that in contrast to previous results, here under semi-field conditions both isovaleric acid and 4,5-dimethylthiazole caused an inhibitory effect. Even more so, when two or more of these compounds were added to the standard blend, the effect of each compound individually was cancelled out, or the entire blend seemed repellent, causing most mosquitoes to avoid entering any of the traps. With all four compounds added to the standard blend, the overall response of the mosquitoes was significantly reduced. It is possible that when employing higher dilutions of each of these compounds when studying the effect of multiple compounds, the kairomonal potency of the blend might be further enhanced. We used an industrially-produced batch of 2-methyl-1-butanol. This compound is known to exist of two isomers (R and S). In our study, we used a racemic mixture. It is possible that only one of the isomers would have evoked a behavioral effect in the mosquito, which should be followed up in a future study. The results from the dose-effect study with 3-methyl-1-butanol show that highly diluted concentrations cause attraction of *An. gambiae*, while higher doses cause inhibition. This result is important for the development of attractive odorant blends for mosquitoes. For example, the mosquito repellent DEET is repellent over a wide range of concentrations, but becomes attractive at very low concentrations (Mehr et al., 1990), and this principle of concentration-dependent dual action might apply to many other odorant cues that affect insect behavior (Smallegange and Takken, 2010).

We examined the response of mosquitoes to candidate odorant blends in traps placed outdoors, next to a house, and traps placed indoors. A recent study showed that catches from indoor and outdoor MM-X traps were comparable (Jawara et al., 2009), suggesting that the mosquitoes perceived the odorant cues from a distance, and would either be caught while approaching the house or after house entry. Although Lwanda and Kigoche are approximately 120 km apart, with different ecologies, the mean number of *An. gambiae* s.l., *An. funestus*, and *Culex* spp. was not much different. Odor-baited traps caught significantly more mosquitoes than unbaited traps, thus demonstrating the attractive effect of the blends under investigation. On average, the trap placed next to a human-occupied bed net caught similar numbers of mosquitoes as the trap next to the dispenser baited with the Mbita blend. Unlike in the Tanzania study, where IB1 was 2–3 times as attractive as the human-odor baited trap (Okumu et al., 2010b), here, IB1 was less attractive than the human-baited trap. This difference between these studies may have been caused by different attractiveness of the human volunteers, environmental differences, or both. Each is likely to affect trap catches. The differences were small, though, and the overall result shows that the three synthetic blends approached the attractiveness of the human host (Table 5), with MB being the most attractive blend.

That the majority of the malaria vectors *An. gambiae* s.l. (92.8%) and *An. funestus* (96.8%) collected from Kigoche village was unfed implies that the blends, more so MB that attracted the highest numbers of mosquitoes, mainly target host-seeking mosquitoes. It is not surprising, therefore, that the numbers of mosquitoes attracted to MB were physiologically not significantly different from those attracted to human subjects. However, although MB collected significantly higher fractions of blood fed *An. gambiae* s.l. and *An. funestus* than the trap next to the human-occupied bed net,

the catches of mosquitoes displaying this physiological status as well as gravid ones were small.

Anopheles gambiae s.s. was the species used in the semi-field study, whereas in the field study *An. arabiensis* dominated the *An. gambiae* s.l. collections. In the past decade, populations of *An. gambiae s.s*. have declined dramatically, presumably as a result of widespread bed net use, whereas populations of *An. arabiensis* have remained similar (Meyrowitsch et al., 2011). The latter species is less affected by the bed nets as it is more exophilic, and less anthropophilic. Nevertheless, *An. arabiensis* is attracted strongly to the synthetic blends, as shown by the Okumu et al. (2010b) study as well as by the current study (Tables 2 and 5). Because *An. arabiensis* can be an important malaria vector and is regularly found in human landing catches (Port et al., 1980), the synthetic blends used here can provide a tool for the sampling of this species. Along the same line, the important malaria vector *An. funestus* was also caught in both villages, responding significantly more strongly to the Mbita blend than to the other blends or the human host. As other, non-anopheline mosquitoes were also collected, the synthetic blends, notably MB, should be considered as attractants for a wide range of mosquito species, including other disease vectors.

We conclude that the multiple-component odorant blends described in this study can be considered to replace traditional malaria vector sampling tools such as CDC light traps and the human landing catch. Next to the ease of use, the availability of a "standard" host, used in multiple locations simultaneously over a study area, is a distinct advantage in providing unbiased data on relative mosquito densities. This will be of advantage for malaria epidemiological studies, but also cost effective. Because the baits are also effective outdoors, they open up a novel avenue for sampling of outdoor biting malaria vectors, which have recently been reported to be a major source of malaria transmission (Reddy et al., 2011; Russell et al., 2011).

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Competing interests The authors declare that they have no competing interests.

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Management of *Cosmopolites sordidus* and *Metamasius hemipterus* in Banana by Pheromone-Based Mass Trapping

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Abstract Mass trapping *Cosmopolites sordidus* (Coleoptera, Curculionidae) using a pheromone-baited pitfall trap and *Metamasius hemipterus* (Coleoptera, Curculionidae) using a pheromone-sugarcane-baited open gallon trap was conducted in commercial banana. Four traps for each insect per hectare were placed in each of two 5-hectare plots of banana. Two additional 5-hectare plots were designated as controls and treated according to the plantation protocol. Capture rates of *C. sordidus* and *M. hemipterus* declined by >75 % over 10–12 months. In the banana growing region studied, corm damage was due primarily to *C. sordidus*, while only a minor amount of damage was attributable to *M. hemipterus*. Corm damage reduction in trapping plots was, thus, attributed primarily to *C. sordidus* trapping. In trapping plots, corm damage decreased by 61–64 % during the experiment. Banana bunch weights increased 23 % relative to control plots after 11–12 months of trapping. Fruit diameter did not vary between bunches harvested from trapping plots vs. control plots. Plant vigor, however, as determined by stem circumference at one meter above ground increased in plots with traps compared to control plots. Trapping for *C. sordidus* in two plantations of over 200 hectares each, reduced corm damage 62–86 % relative to pre-trapping levels. Insecticide control measures in place when the experiment commenced resulted in about 20–30 % corm damage, while use of pheromone trapping to manage *C. sordidus* lowered corm damage to 10 % or less. It is estimated that the increase in value of increased yield obtained in this trial (23 %) is about $4,240 USD per year per hectare, while the cost of pheromone trapping is approximately $185 USD per year per hectare. The trapping program becomes revenue neutral if bunch weights increase by an average of 1 % per year of trapping. Approximately 10 % of all plantation area in Costa Rica use the pheromone trapping system described here. The system also is used in Martinique, Guadeloupe, and the Canary Islands.

Keywords Cosmopolites sordidus · Metamasius hemipterus · Mass trapping · Banana · Pheromone · Sordidin

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Introduction

The banana corm weevil (*Cosmopolites sordidus* Germar) is a worldwide pest of banana and plantain (Ostmark, 1974; Valmayer, 1994; Pavis and Lemaire, 1996; Gold et al., 2001). Females prefer to oviposit in the corm. Egg laying is reported to be one to two eggs per week (Cuille, 1950) with females generally laying no more than 50 eggs (Smith, 1982). Damage is caused when larvae burrow into vascular tissue of the corm and is correlated with decreased yields (Vilardebo, 1973; Masanza, et al., 2009). The larval stage of *C. sordidus* is one to two months, while the non-flying adults live up to two years (Jirasurat, 1989; Gold et al., 2001). Within a banana plantation, there is little incentive for *C. sordidus* to migrate from the area from which it emerges (Gold et al., 2001). Bananas propagate by offshoot and because of this characteristic each plant has closely associated with it several other plants growing from the same root system. The groups of plants sharing the same root system are called "mats" that can reach up to one meter

in diameter. Sufficient food for successive generations of *C. sordidus* is provided within a given mat. Since adult *C. sordidus* migrate between mats relatively slowly (i.e., by walking rather than flying), management by techniques such as insecticidal treatment or trapping should be relatively effective because reinfestation of treated areas by immigration would be slow.

A number of techniques have been used to manage weevil populations. The application of synthetic pesticides (Tomlin, 1994; Meister, 1995; Sponagel et al., 1995) has been hampered by the emergence of resistance (Edge, 1974; Collins et al., 1991). Application of botanical pesticides such as neem extract in cake form increases yields in weevil infested plots (Musabyimana et al., 2000). Reduction of weevil populations also has been achieved by application of entomopathogenic nematodes at high baiting density (>1,000 points/Ha/yr, Treverrow, 1994; Nankinga and Moore, 2000) but the high application rate has discouraged this technique. Dissemination of the entomopathogenic fungus *Beauveria bassiana* that infects both larvae and adult Coleoptera, has been reported to be significantly increased when pheromone traps are used to attract weevils to the area in which this pathogen has been applied (Tinzaara et al., 2007b). Field studies have demonstrated the efficacy of ant predation on *C. sordidus* life stages as a management technique (Castineiras and Ponce, 1991; Abera-Kalibata et al., 2008). Several species of myrmicine ants efficiently locate and remove *C. sordidus* eggs and larvae from banana stem (Abera-Kalibata et al., 2007). Cover cropping has been postulated as a method to increase populations of predators (Duyck et al., 2011). A detailed study of the survivorship of *C. sordidus* larvae has shown that banana residues are suitable hosts suggesting that removal of stem after harvest would decrease *C. sordidus* populations (Rukazambuga et al., 2002; Masanza et al., 2005). Within stands of highland bananas in Uganda, Gold and co-workers found some cultivars that exhibited a limited ability to support *C. sordidus* larvae (Kiggundu et al., 2003; Night et al., 2011). The cultivars identified by these researchers offer prospects for breeding *C. sordidus* resistance.

Traps constructed of banana pseudostem have been used for over 50 years to monitor *C. sordidus* populations (Vilardebo, 1973; Arleu and Neto, 1984). Pseudostem traps are made from two pieces of approximately 15 cm long banana plant stem cut from a stem base and placing one piece on top of the other with a small stone or stick to hold the two pieces apart at one side. Arriving weevils are killed by insecticide that is applied to the cut surfaces. These traps usually desiccate or rot over two weeks, after which time they are replaced. A two year study using pseudostem traps in Honduras did not result in declining *C. sordidus* capture rates (Ostmark, 1974). In Kenya, however, it was demonstrated that capture rates in pseudostem traps at ~800 traps/hectare declined by 50 % over 11 months (Koppenhofer et al., 1994). Another Kenyan study over two years showed that capture rates in pseudostem traps declined by 47 % and increased banana yields by 31 % (Seshu-Reddy et al., 1995). In Uganda, at one pseudostem trap/mat, capture rates of *C sordidus* declined 61 % over one year if traps were maintained by researchers, 53 % where traps were maintained by farmers, and 31 % on farms without traps (Gold et al., 2002). A review of banana weevil ecology (Gold et al., 2002) cites additional studies in which reductions in *C. sordidus* capture rates following trapping with pseudostem traps occurred in Kenya (Ngode, 1999) and Uganda, (Masanza, 1995), as well as Tanzania (Ndege et al., 1995).

In 1995, a male produced aggregation pheromone (sordidin) was identified for *C. sordidus* (Beauhaire et al., 1995), and a large scale synthesis allowed evaluation of sordidin in the field (Ndege et al., 1995; Jayaraman et al., 1997). Baiting pseudostem traps with sordidin released at one milligram per day increased capture rates by eight fold, while white pitfall traps containing soapy water and from which sordidin was released at one milligram per day captured about two and a half times more *C. sordidus* than similarly sordidin-baited pseudostem traps (Jayaraman et al., 1997). Several studies have confirmed the efficacy of sordidin-baited pitfall traps (Tinzaara et al., 1999; de Graaf et al., 2005). A more recent study demonstrated that crawl-in ground traps outperform pitfall traps (Reddy et al., 2009), and that traps are more effective in the shade (Reddy et al., 2008, 2009). Reddy's group also has shown that brown or black pitfall traps are more efficacious than other colors (Reddy and Raman, 2011).

Capture rates are higher during rainy periods (Pantoja et al., 2006; Reddy et al., 2008). The addition of pseudostem to pitfall traps does not significantly increase *C. sordidus* capture rates (Tinzaara et al., 2007a).

Several experiments have been reported in which sordidin-baited traps failed to reduce corm damage in relatively unmanaged highland bananas in Uganda (Gold et al., 2001). However, a study in Costa Rica demonstrated that in small holder plantain trapping over 58 weeks, weevil corm damage was reduced by 33 % even though weevil capture rates did not decrease (Polidoro et al., 2008). This is in agreement with an earlier study in commercial plantain (Alpizar et al., 1998).

Metamasius hemipterus (West Indian sugarcane weevil) is a primary pest of sugarcane (Vaurie, 1966), but it is not recorded as a significant pest of plantain and banana (Ostmark, 1974; Nietzen, 1994). In sugarcane, *M. hemipterus* damage is most important in the replant stage when short stalks of sugarcane are planted. From the nodes in these short pieces emerges the next crop. During the rooting process, fermentative decomposition of the non-nodal tissue attracts *M. hemipterus* to mate and oviposit.

Fermentative decomposition of planted sugarcane stalk occurs only in high humidity environments that are those in high elevation (above 1,500 meters). Presoaking planting stalk in insecticide is the preventive measure commonly used, and pheromone trapping in newly planted areas also has been studied, but the reduction in damage did not justify the cost of trapping (Oehlschlager et al., 2002). Surveys have determined that although larva and adults of both *C. sordidus* and *M. hemipterus* are present in corm of plantain and banana in the Atlantic region of Costa Rica (Nietzen, 1994), *M. hemipterus* is not a significant pest of banana in this region (Alpizar et al., 1998).

Studies on one-hectare plots of banana and plantain have shown that pheromone-based trapping of *C. sordidus* and *M. hemipterus* over one year led to significantly lower capture rates, lower corm damage, and higher yields (Alpizar et al., 1998). The purpose of the study reported here was to scale-up the previous work to larger plots. This study was done to determine if trapping *C. sordidus* and *M. hemipterus* in commercial banana was beneficial. The study was conducted by using locally available traps for each species on two plots of five hectares each within a commercial plantation. Then, trapping for *C. sordidus* was conducted on several banana plantations of over 200 ha.

Methods and Materials

Study Sites All study sites were in commercial plantations in the wet tropical (<500 m above sea level) Atlantic region of Costa Rica. The variety of banana in the plantations selected for study was *Musa acuminata* Colla (Cavendish Group), known as Dwarf Cavendish. Planting density was ~437 mats per hectare. Harvesting was conducted on a year-round basis. Trapping in the 5-hectare test plots began September 29, 1997 and ended August 31, 1998.

Trapping Protocol Pheromone lures for *C. sordidus* emitted a mixture of sordidin isomers (Ndiege et al., 1996) at about one milligram per day, and isoamyl acetate at 100 mg/d. Pheromone lures for *M. hemipterus* emitted a mixture of 4-methyl-5-nonanol (8) and 2-methyl-4-heptanol (1) at 7 mg/d (Perez et al., 1997). Lures for both insects were commercially available from ChemTica Internacional, Costa Rica.

Traps for *C. sordidus* were white plastic containers (14 cm ID X 12 cm deep) with four to five 0.2 cm dia. holes located about 3.5 cm from the bottom to prevent filling by rain. Traps were buried with tops at ground level and contained about 3.5 cm detergent laced (3 %) water. Soil was packed firmly against the sides of the buried trap so that arriving weevils could enter easily. Lures were suspended from wires 2–3 cm above trap centers. Because *C. sordidus* prefer areas of high humidity, each trap was covered with part of a freshly cut banana leaf (~ 20 × 20 cm). Traps for *M. hemipterus* were 4 L, yellow, plastic containers with 15 cm wide X 10 cm high windows cut in each side for insect entry (Giblin-Davis et al., 1996). Traps were suspended on sticks 0.5 m above ground and contained 4 or 5 five pieces of 10–12 cm long halved sugarcane stalk presoaked in Sevin (1 % A.I.). Pheromone lures were suspended from wires protruding 5–10 cm below the trap lids.

Pseudostem traps were constructed by cross-cutting the lower portion of the stems of harvested plants in 15 cm sections, and the cut surfaces were sprayed with Furadan (1 %, AI) to runoff. One cut section was placed on the ground near the plants and covered by a leaf section. A second cut section was placed on top of the leaf. Pseudostem traps were renewed every 2 wk. Dead weevils in the trap, clinging to the exterior surface of the trap and under the trap on the ground were counted.

Placement and Movement of Traps Traps for *C. sordidus* were placed initially in a line 10 m from a border and 20 m apart. Each month, traps were moved 20 m along an axis perpendicular to the trap line in a manner that always placed traps within trapping plots. Traps for *M. hemipterus* were placed at 50 m intervals inside the trapping plots and remained in the same location throughout the study.

Trap Servicing Insects were counted and removed from all traps weekly. Water in *C. sordidus* traps was replenished weekly. Lures for *C. sordidus* were renewed monthly, and lures for *M. hemipterus* were renewed when they were exhausted as determined by the absence of liquid in the lure (3–4 mo). Furadan (1 % in water) treated sugarcane in *M. hemipterus* traps was renewed weekly.

Control Plot Treatment Pest management practices for the trapping and control plots were according to standard protocol of the plantation, except that treatment for *C. sordidus* was withheld from the trapping plots. Standard treatment consisted of the application of Terbufos (30 g/mat, ~500 mats/ha) every 3 mo against the banana root nematode, *Radopholus similis,* Furadan (three applications/year, 30 g/mat) against *C. sordidus*, and aerial treatment (triazoles) for black sigatoka (*Mycosphaerella fijiensis*) on a weekly basis.

Assessment When banana plants are cut at ground level, the corm is exposed. An undamaged corm of a freshly cut plant is white throughout. If larval damage has occurred in the corm, air will have entered the larval boring holes, and this causes darkening of the tissue. The method of Vilardebo (1973) involves inspection of the corm of a freshly cut plant to assess visually the proportion of white to dark tissue on the freshly cut surface. Corm damage was by visual inspection of the corms of 50 plants that had been harvested the

same day. Corm tissue was exposed by cutting the stem horizontally at ground level. The same agricultural engineer made all assessments in all studies. This is the currently accepted method of corm damage assessment in commercial plantations in Costa Rica (Vilardebo, 1973).

Banana fruits develop in a large cluster (bunch), made up of 3–20 tiers (hands), with up to 20 fruits (fingers) to a tier. Bunch weights were determined at three dates during the study: October 13–15, 1997, April 13–15, 1998, and August 17–19, 1998. On each date, 50 commercially harvested bunches from the trapping plots and 50 bunches from the control plots were tagged and weighed at the packing station. Fruit diameter was measured (two positions for each bunch) on each weighed bunch. In addition, the number of hands on each weighed bunch was counted. Plants from which tagged bunches were harvested were tagged, and stem circumference at 1 m above ground was measured the same day.

Data Analysis Data were tested for heteroscedasdasticity, and if necessary, transformed to achieve homogeneity. Data were analyzed using Systat 5.2.1, the fully factorial ANOVA analysis routine. Means are presented un-transformed. Means for the same location and date topped or followed by a different letter or topped by an asterisk are significantly different by Bonferonni *t*-test, $P<0.05$ (Zar, 1984).

Results and Discussion

An earlier survey of plantain and banana corm in commercial plantations in the Atlantic region of Costa Rica consistently revealed larvae and adults of both *C. sordidus* and *M. hemipterus*. In plantain corm, about 80 % of larvae were *M. hemipterus*, while in banana, about 80 % were *C. sordidus* (Alpizar et al., 1998).

The previous work demonstrated that in one-hectare plots the use of four traps/hectare for *C. sordidus* and four traps/hectare for *M. hemipterus* over a year led to lower capture rates and lower corm damage in both banana and plantain (Alpizar et al., 1998). In the studies reported here, we examined the efficiency of trapping these insects in larger plots of commercial banana. The 5-hectare plots used the same trap density as in earlier studies of four traps/hectare for each species, while the >200-hectare plots employed four traps/hectare for *C. sordidus* only. According to our earlier protocol, *C. sordidus* traps in the 5-hectare plots were in lines 10 meters from any plot border and at intervals of 20 meters, and each month, traps were moved 20 meters along the axis perpendicular to the trap line. When trap positions approached a plot border, they were then moved back toward the center of the plot. In this fashion, each hectare was trapped with four traps at 20 meter intervals. In plots greater than 200 hectares, trap lines were 100 meters apart and moved monthly by 20 meters along the axis perpendicular to each line.

Capture rates for *C. sordidus* and *M. hemipterus* in the two 5-hectare plots of banana decreased by over 80 % during ten months of trapping (Fig. 1, line graphs). Capture rates for both insects experienced an increase from the previous month during February, which is near the beginning of a period of high rainfall in the region of the experiment. High capture rates for both species during periods of high rainfall have been previously observed (Alpizar et al., 1998). When the equivalent trapping strategy was employed in one-hectare plots, capture rates remained nearly equivalent for eight months (Alpizar et al., 1998). In the present 5-hectare plot experiment, however, capture rates decreased from initial levels in two months. This demonstrates a better performance of the trapping system in the larger plots.

Before commencement of the trial, two 5-hectare plots within the same plantation were selected as controls. The indices of corm damage in these two plots as well as the two 5-hectare plots receiving traps were assessed on 25 plants harvested the same day in each of the four plots (Fig. 1, bar graphs). At the initiation of the trial, corm damage in both trapping and control plots was assessed as 20–22. Based on the established correlation between corm damage and yield (Vilardebo, 1973), this corresponds to reductions in yield of at least 15 % compared with completely corm damage-free plants. Five months after traps were installed, the index of corm damage in trapping plots averaged less than 10, and was 6 at the end of the trial. In control plots, the index rose to 31 after five months and remained significantly higher

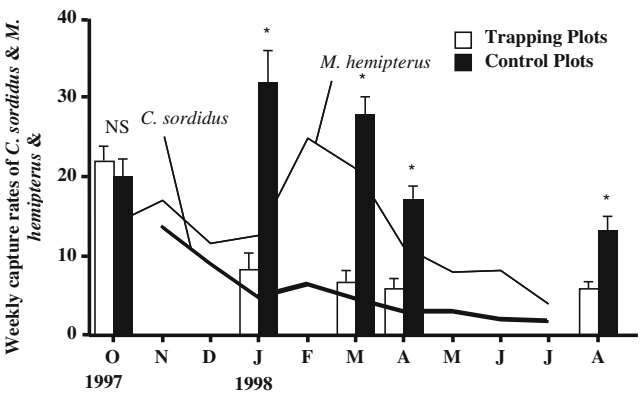

Fig. 1 Mean weekly capture rates for *Cosmopolites sordidus* and *Metamasius hemipterus* in two 5-hectare plots of commercial banana in the Atlantic region of Costa Rica, October 1997 to August 1998. Mean (+SEM) corm damage assessed (Vilardebo, 1973) on 25 harvested plants on each date indicated in each of two five-hectare plots receiving traps (Trapping Plot) and two pre-selected 5-hectare plots not receiving traps (Control Plot). ANOVA on corm damage for 50 harvested plants in trapping plots and 50 harvested plants in control plots gave $P<0.05$ for all dates except October 1997. Bars correspond to corm damage and lines correspond to average trap capture

than in the trapping plots during the remainder of the trial (Fig. 1). At the end of the trial, the average index of corm damage in trapping plots was 6 compared to 13 in control plots.

The weight of harvested bunches in plots receiving traps increased 18 % relative to controls during the first six months of trapping (Fig. 2) and increased by 23 % at the end of the ten month trial. The observed increases in bunch weights are expected based on differences in the index of corm damage between the trapping and control plots during the trial. The index in trapping plots decreased 22→8 during the first five months of the trial and remained near 6 for the remainder of the trial. In the control plots, the index increased from 20→27–31 for several months during the trial before decreasing to 13 near the end of the trial. For last seven months of the trial, the average index of corm damage was about 6.5 in the trapping plots and about 20 in the control plots. This difference in index of corm damage would be expected (Vilardebo, 1973) to give yield increases in the range of 10–20 %.

The diameter of fruit in harvested bunches (of interest to growers) increased in both trapping and control plots during the trial but at any assessment date fruit size in trapping and control plots was statistically equivalent (Fig. 3).

The numbers of hands (tiers) in 50 harvested bunches in trapping and 50 harvested bunches in control plots were statistically equivalent at the beginning of the trial and remained equivalent at each date of assessment (April 1998, August 1998). The numbers of hands per bunch did not change significantly during the trial (data not shown).

Decreasing corm damage would be expected to increase plant vigor. A determinant of plant vigor commonly used in banana is the measurement of stem circumference one meter above ground. Pretrial stem circumference (plants harvested the same day) in trapping and control plots differed by less than 5 % (Fig. 4). After trapping for 10 months, however, stems of harvested plants in trapping plots were significantly larger (15 %) than those in control plots.

Trapping in areas >200 hectares was as successful as in 5-hectare plots in reducing the index of corm damage (Figs. 5 and 6). Trapping in 304 hectares of commercial banana using four pheromone traps per hectare and moving traps each month as indicated in the "Materials and Methods" section gave greater than 60 % reduction in the index of corm damage over five months of trapping (Fig. 5).

In a second plantation of 252 hectares, four pheromone traps per hectare with the same trap movement regime gave >80 % reduction of corm damage over six months (Fig. 6).

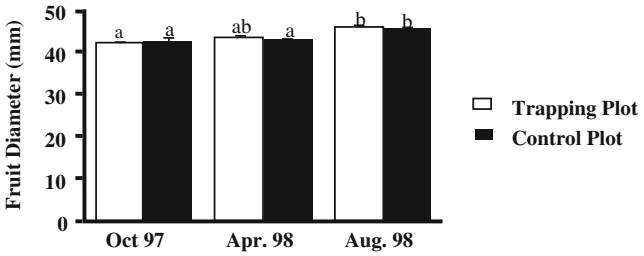

Fig. 3 Mean fruit diameter (mm, +SEM) from 25 bunches harvested from each of two 5-hectare plots (50 bunches total) of commercial banana in Atlantic region of Costa Rica receiving *Cosmopolites sordidus* and *Metamasius hemipterus* traps, October 1997 to August 1998 (Trapping Plots) and 25 bunches harvested from each of two 5-hectare plots (selected pretrial) of commercial banana (50 bunches total) within the same plantation (at the same time, Control Plots). Average fruit diameter for each plot treated as single replicate. ANOVA gave F= 15.97, *df*=5, 6, $P<0.05$. Means topped by a different letter for different dates indicates the means are significantly different between dates by Bonferonni *t*-test, $P<0.05$

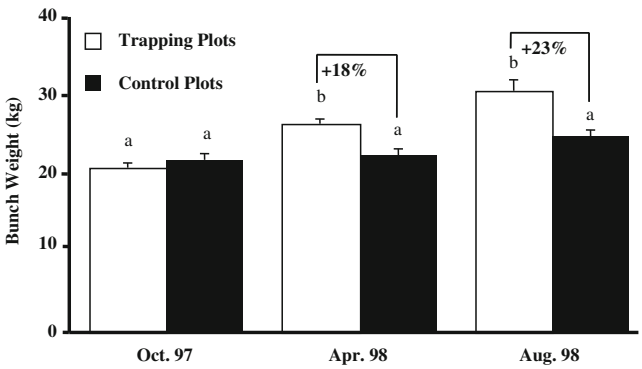

Fig. 2 Mean bunch weights (+SEM) from 25 bunches harvested from each of two 5-hectare plots (50 bunches total) of commercial banana in Atlantic region of Costa Rica, receiving *Cosmopolites sordidus* and *Metamasius hemipterus* traps, October 1997 to August 1998 (Trapping Plots) and 25 bunches harvested from each of two 5-hectare plots (selected pretrial) of commercial banana (50 bunches total) within the same plantation (at the same time (Control Plots). ANOVA for April 1998 and August 1998 gave $P<0.05$. Means topped by a different letter for the same date indicates the means are significantly different by Bonferonni *t*-test, $P<0.05$

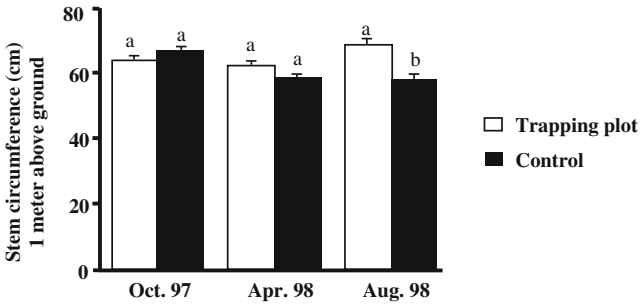

Fig. 4 Mean circumference (cm, +SEM) of 25 harvested plants 1 m above ground level from each of two 5-hectare plots (50 plants total) of commercial banana in Atlantic region of Costa Rica receiving *Cosmopolites sordidus* and *Metamasius hemipterus* traps, October 1997 to August 1998 (Trapping Plots) and 25 harvested plants from each of two 5-hectare plots (selected pretrial) of commercial banana (50 plants total) within the same plantation (at the same time, Control Plots). ANOVA for August 1998 gave $P<0.05$. Means topped by a different letter for the same date indicates the means are significantly different by Bonferonni *t*-test, $P<0.05$

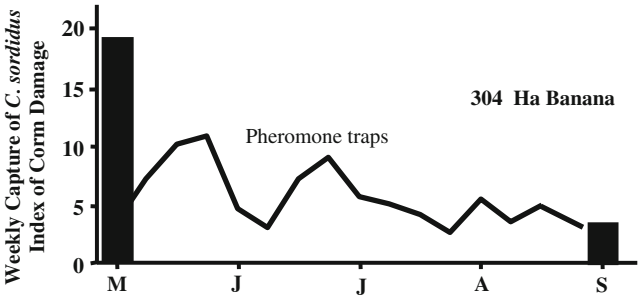

Fig. 5 Mean weekly capture rates (four traps/hectare) for *Cosmopolites sordidus* in 304 hectares of commercial banana in the Atlantic region of Costa Rica during 1998. Mean corm damage assessed (Vilardebo, 1973) on 50 harvested plants in each month indicated. Bars correspond to corm damage and line corresponds to average trap capture

In this trial, we also examined the capture rate in pseudostem traps vs. pheromone-baited buried traps (>50 of each type) over several weeks (Fig. 6). Pseudostem traps consistently captured less than five weevils per trap per week, while captures in pheromone-baited buried traps were initially 15 weevils per week decreasing to five weevils per trap per week after two months of trapping (Fig. 6). These results could be due to the greater efficiency of pheromone traps, which results in lower captures as trapping continues. Alternatively, these results could indicate that pseudostem traps become saturated at higher weevil populations but function as well as pheromone-baited buried traps at low weevil populations. If the latter interpretation were correct, use of pseudostem traps to monitor populations of *C. sordidus* would not give correct indications of high populations but would adequately monitor low weevil populations.

It is noteworthy that at the beginning of all trials, corm damage was usually between 20 and 30 and at the end of each trial trapping plots and trapped plantations had corm damage that was 10 or lower.

Pheromone trapping trials on small holder plots reveals that at four traps/hectare (or even at eight traps/hectare as

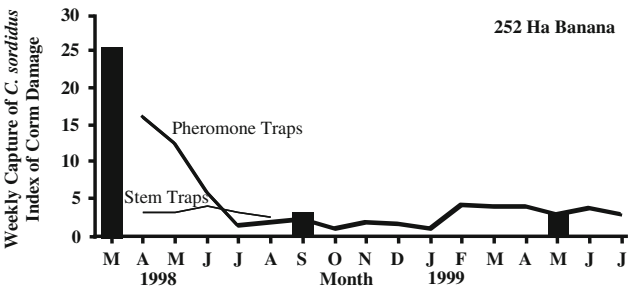

Fig. 6 Mean weekly capture rates (four pheromone and pseudostem traps/hectare) for *Cosmopolites sordidus* in 252 hectares of commercial banana in the Atlantic region of Costa Rica during 1998 and 1999. Mean corm damage assessed (Vilardebo, 1973) on 50 harvested plants in each month indicated. Bars correspond to corm damage and lines correspond to average trap capture

examined in one study) that reduction of *C. sordidus* capture rates and corm damage are often not significantly reduced (Tinzaara, et al., 2005). Failure of this trapping system in small holder plots could be due to less consistent management tactics or to immigration from surrounding areas, or both. It is noteworthy that when one-hectare plots of banana were subject to trapping *C. sordidus* at four traps/hectare capture rates remained near initial levels for eight months suggesting immigration was significant (Alpizar et al., 1998). Thus, the system described in the work reported here clearly is most beneficial in commercial settings.

In previous work, pheromone-baited buried traps were significantly more effective than pheromone-baited pseudostem traps (Jayaraman et al., 1997). During the execution of that study it was found that during periods of high rainfall, buried traps frequently flooded. In dry periods, the ground surrounding the traps often dried creating spaces between the ground and the trap. Both of these problems would be expected to decrease capture efficiency. To offset such problems, several ground level traps have been suggested to be more efficient than buried traps in capture of *C. sordidus* (e.g., Reddy et al., 2009).

Economic Aspects According to the Costa Rican Ministry of Agriculture (personal communication, February 2012) production in the Atlantic Region of Costa Rica was 2,400 boxes (about 18 kg each) of bananas per hectare in 2011. In this banana growing region, there are about 1,750 plants per hectare with about four plants per mat. The government of Costa Rica set a minimum farm gate price of $7.69 USD per box for 2011. Thus, one hectare of bananas produces average revenue of $18,450 per year. Both root nematode, (primarily *Radopholus similis*) and *C. sordidus* are managed by two to three applications rotating between Terbufos, Mucap and Vydate per year at the application rate of 30 g per mat ~26 to 39 kg per hectare per year. As noted here, current treatment against nematode and weevil damage results in an average level of corm damage of 20–30 % in commercial plantations. This level of *C. sordidus* corm damage was evident in seven plantations on which we have conducted corm damage assessments (unpublished data, damage from only three plantations are reported in this study). Addition of a trapping system to the current nematicide/insecticide regime lowers *C. sordidus* corm damage to 10 % or less over one year with a resulting increase in bunch weights of 23 %. Based on the 2011 farm gate price of bananas in Costa Rica, this corresponds to an increase in revenue of about $4,244 (23 % of $18,450) per hectare. The cost of the pheromone-baited trapping system for *C. sordidus* described (in 2012) is approximately $65 per hectare per year for lures. Buried traps are about $0.10 each. Labor to service four traps each month is estimated at no more than $10 per month ($120/year). Thus, the cost of labor and

trapping for one year can be estimated to be $185 per hectare. To be revenue neutral the trapping system would need to increase bunch weights by 1 %.

The above suggests that there is a significant economic incentive for plantation managers to adopt such a mass trapping technique. The National Banana Corporation (Corbana), estimated that in 2010 there were about 43,000 hectares of banana plantations in Costa Rica (Williams, 2010). While the area planted with banana in Costa Rica is known, there is no accurate method to assess the proportion of banana plantations area that have a significant problem with *C. sordidus*. Currently, however, approximately 10 % of the total banana plantation area in Costa Rica employs the pheromone system to manage *C. sordidus*. The system also is used in Martinique, Guadeloupe, and the Canary Islands where the economics of banana production is more lucrative because of subsidies from the European Union.

In summary, pheromone-based trapping of *C. sordidus* in plantations using four traps per hectare effectively lowers the index of corm damage from 20–30 to less than 10 after several months of trapping. Yields of banana are correspondingly increased. The annual cost of pheromone trapping becomes revenue neutral if bunch weights are increased by 1 %.

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In the Nick of Time: Males of the Parasitoid Wasp *Pimpla disparis* Respond to Semiochemicals from Emerging Mates

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Abstract Males of the parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae) aggregate on parasitized gypsy moth, *Lymantria dispar*, host pupae when the emergence of a prospective mate is imminent or under way. We tested the hypotheses that the developing parasitoid ("DePa") inside the host pupal case produces a pheromone that attracts and arrests mate-seeking males, and that the pheromone is most effective during the emergence of the parasitoid from the host. Results obtained in two-choice laboratory experiments, with 4–7-d-old virgin males, indicate that (1) DePa-derived semiochemicals arrest males, (2) the opening of a host pupal case strongly arrests males, and (3) the arrestment cue emanates from oral fluid secreted by both female and male parasitoids while they chew their way out of a host pupal case. This phenomenon implies that emerging females, which are haplodiploid and can reproduce without mating, do not engage in active pheromone signaling to attract males, and that mate-seeking males co-opt chemicals involved in eclosion as a mate-finding cue, taking a 50% chance that the prospective mate is a female.

Keywords *Pimpla disparis* · Mating strategy · Early mate detection · Arrestment behaviour · Mate discrimination · Pheromone evolution

Introduction

Ever since parasitic Hymenoptera (Parasitica) diverged from their ancestral phytophagy, their evolution has been driven, in part, by the natural history of their hosts. This host link spurred an adaptive radiation that resulted in the adoption of diverse mating systems (Quicke, 1997), ranging from staking out a common emergence site, where brothers compete for access to sisters (Somjee et al., 2011), to lekking, where countless "bachelors" swarm around a nuptial site attracting females into the melee (Quicke, 1997).

The spatial and temporal distributions of parasitoids and their hosts are important determinants of parasitoid mating strategies (Alcock, 1978). When hosts are dispersed and parasitoids solitary, males typically seek females near feeding or oviposition sites, whereas gregarious parasitoids, or parasitoids of gregarious hosts, often mate at shared pupation sites (Godfray, 1994). Whether males focus mate searching at sites of female emergence, feeding, or oviposition depends, to a large extent, on when and where receptive females are most abundant (Hölldobler, 1984; Thornhill and Alcock, 1983; Godfray, 1994).

The ichneumon wasp *Pimpla disparis* Viereck is a primary, solitary endoparasitoid of lepidopteran pupae (Fuester et al. 1989; Schaefer et al., 1989; Fuester and Taylor, 1993). Its primary host is pupae of gypsy moth, *Lymantria dispar*, which are commonly found in bark crevices or under leaves of hardwood trees (Leonard, 1981).

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Male *P. disparis* engage in an early mate-detection strategy (Danci et al., 2011), whereby they locate parasitized gypsy moth pupae, each containing a single conspecific developing parasitoid ("DePa"), periodically revisit these pupae, and then aggregate and arrest around them just prior to emergence of the prospective mate (Fig. 1a). This behavior implies that there exists some form of signaling or communication between DePas and adult males searching for them. The signal could be a pheromone, considering that pheromone-mediated communication is common within adult parasitic Hymenoptera (Hölldobler, 1984; Quicke, 1997; Ayasse et al., 2001; Metzger et al., 2010), and that both contact (Steiner et al., 2006; Ruther et al., 2011) and airborne female pheromones have been identified (e.g., Syvertsen et al., 1995, and references therein; DeLury et al., 1999; Krokos et al., 2001; Collatz et al., 2009; Nichols et al., 2010). If the *P. disparis* signal were a pheromone, males would be best able to pinpoint the emergence of a DePa if the pheromone changed in effectiveness over time. In order to emerge from the host pupal case, the adult parasitoid chews an opening while producing an oral fluid that seems to soften the pupal case (personal observation), as has previously been suggested for other ichneumonid species (M.G. Fitton, cited in Quicke, 1997). The timing and brief production of this oral fluid make it an ideal candidate source for an "emergence pheromone".

We tested the hypotheses that: (1) the pre-emergence DePa produces a pheromone that attracts or arrests males; (2) the pheromone is most effective just prior to, or during, the emergence of a parasitoid; and (3) the oral fluid of the emerging parasitoid contains the emergence pheromone.

Methods and Materials

Experimental Insects

Pimpla disparis were field collected near the town of North East, Maryland, USA, and reared on pupae of the laboratory host wax moth, *Galleria mellonella,* in the Global Forest Insect Quarantine Facility of Simon Fraser University (Danci et al., 2011). Host pupae were exposed for 24–72 h to female *P. disparis* adults, isolated in Petri dishes, and kept at 20–25°C, 40–60% RH, and a 16L: 8D photoperiod. Emerged parasitoids were transferred to mesh cages (46×46×46 cm) and provisioned with cotton wicks (1×5 cm; Richmond Dental, Charlotte, NC, USA), imbued with water and honey, on which to feed *ad libitum*. Those to be tested in bioassays were isolated according to age and sex (based on the presence of an ovipositor in females) and housed in separate cages.

General Bioassay Design

Two-choice experiments were conducted in clear Plexiglas cages (9×6×9 cm), lined on the inner right and left sides with single-face corrugated cardboard (Shippers Supply Inc., Delta, B.C., Canada), which served as surrogate tree trunks (Fig. 1b). Unless otherwise stated, bioassay host pupae were frozen ("freeze-killed") on dry ice for 15 min., thawed for 15–20 min., and then attached with elastic thread (1 mm diam., stretch magic bead and jewellery cord, Pepperell Crafts, MA, USA) to small cardboard squares (3×3 cm; Fig. 1c) randomly assigned to the left or right side of the cage. For each replicate, a 4–7-d-old virgin male was introduced into the center of the cage and the time it spent

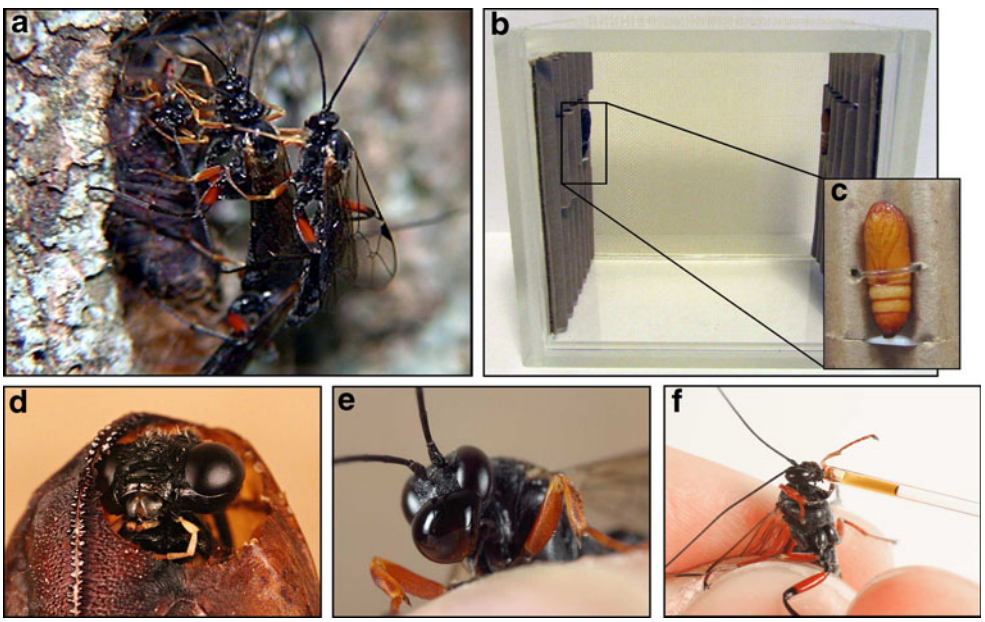

Fig. 1 **a** Photograph of several male *Pimpla disparis* awaiting the emergence of a mate from a gypsy moth, *Lymantria disparis*, host pupa; **b** experimental setup deployed in two-choice experiments 1–10, comprising a clear Plexiglas cage with mesh back, and sides lined with single-face corrugated cardboard; **c** insert: close-up of wax moth, *Galleria mellonella,* host pupa affixed to cardboard with elastic thread, and supported at the base with a small cardboard plate; photographs of *P. disparis* **d** emerging from a host pupal case, **e** secreting oral fluid, and **f** releasing fluid into a microcapillary tube

on, or immediately adjacent to, host pupae recorded over 15 min. Each male was bioassayed only once for each set of test stimuli. In experiments testing candidate pheromone sources (see below), cages were washed, and new cardboard was affixed to the sides between replicates, to prevent cross contamination.

Acquisition and Bioassay of Candidate Pheromone Sources

We predicted the presence of at least trace amounts of pheromone on DePa or on the host pupal case. Therefore, moth pupae, 17 d post-parasitism, were opened, the DePa removed and immersed in 3 ml of hexane in a 20-ml vial for 10 min. (without agitation) at room temperature. Intact host pupal cases were immersed in separate hexane–containing vials for 2 min. and agitated gently. This change in protocol for pupal case washes was prompted by concerns that 10 min. immersions may soften the pupal case and allow solvent to diffuse to the DePa inside. Gentle agitation was applied to maximize extraction of chemicals from the pupal case surface. The supernatant of several washes was combined and evaporated under a stream of nitrogen, such that 10 µl equalled 1 host pupa, or 1 DePa, equivalent. For bioassays, 10-µl aliquots of washes, or solvent controls, were uniformly applied to freeze-killed unparasitized host pupae.

Acquisition of Headspace Volatiles

Groups of 20, 16-d-old parasitized pupae were placed into each of three linearly interconnected Pyrex® glass tubes (2×12 cm). Charcoal-filtered air was drawn at 0.5–1 $L.min^{-1}$ through the chambers and a Pyrex® glass tube (4 mm ID×100 mm), containing 250 mg of Porapak-Q (50–80 mesh, Waters Associates, Inc.; Milford, MA, USA) held in place with glass wool. At 24-h intervals, aerations were stopped briefly and volatiles eluted from the Porapak-Q with 2 ml of redistilled pentane, after which aerations were resumed. Extracts were concentrated, as needed, under a stream of nitrogen.

Acquisition of Oral Secretions

Oral secretions of emerging male or female parasitoids were obtained by (*i*) gently grasping the thorax between the thumb and forefinger, (*ii*) wicking the resulting droplet (~ 0.2 µl) of oral fluid (Fig. 1e) into a micro-capillary tube (1×0.58 mm; Fig. 1f; Davies and Madden, 1985), and (*iii*) dispensing the oral fluid into hexane by blowing lightly through the capillary tube.

Specific Bioassay Experiments

Hypothesis 1: Pre-emergence DePa Produces Pheromone that Attracts or Arrests Males Experiment 1 (Table 1) tested whether males arrest for longer periods on or near a freeze-killed host pupa 17 d post-parasitism, than on a pupa that is not parasitized. Freeze-killed, rather than live, pupae were bioassayed to exclude sound and motion of DePa as potentially confounding factors affecting the responses of males. Following bioassays, the treatment pupae were opened to confirm the presence of a DePa, and to note the developmental stage. As males recognized parasitized pupae in experiment 1, experiments 2–4 (Table 1) tested whether pheromone was present on, and can be washed by solvent off, the surface of host pupal cases 17 d post-parasitism (experiment 2), and the integument of ~17-d-old male or female DePa (experiments 3 and 4). Finally, experiments 5–7 (Table 1) tested whether the putative pheromone disseminated from host pupae prior to, as well as during, the emergence of the parasitoid.

Hypothesis 2: Pheromone is Most Effective Just Prior to or During the Emergence of a Parasitoid Experiment 8 (Table 1) tested whether opening of the host pupal case resulted in a release of pheromone, thus giving mate-seeking males a timing signal for emerging parasitoids. In each replicate, the bioassay male was given a choice between a host pupa, either containing a pupal DePa or an eclosed 1–3-d pre-emergence adult male or female parasitoid, and a control unparasitized host pupa of similar age. During the first 15 min. of each bioassay, both pupae were kept intact. The male was then removed, and retained for ~30 sec. under a 30-ml Solo plastic cup, while the treated pupa was either left intact or received a 2-mm incision at the cephalic end to simulate the initial cut a parasitoid makes to initiate emergence (Fig. 1d; supplemental video). During the second 15 min. of each bioassay, the male's preference for treatment or control pupae was again recorded.

Hypothesis 3: Oral Secretion Contains the Emergence Pheromone Experiments 9 and 10 (Table 1) tested whether oral fluid, secreted by male and female adult parasitoids while chewing an opening in the host pupal case, contained the pheromone that attracts and arrests males. For each replicate, the oral secretion was collected from an emerging parasitoid by wicking the liquid into a capillary tube and then immediately dotting it onto a live unparasitized moth pupa that was bioassayed against a live untreated moth pupa.

Statistical Analyses In the two-choice experiments 1–7 and 9–10, the times that males spent on or near the treatment or control host pupal cases were compared using a Wilcoxon signed-rank test. In experiment 8, all possible variables (intact or incised host pupal case; sex of DePa; pupal DePa or eclosed parasitoid inside host pupal case) were subjected to a 3-way analysis of variance (ANOVA). Differences between means for each variable were analyzed by Tukey

Table 1 Stimuli tested in Experiments 1–10

Exp.#	*N*	Stimuli tested	
		Stimulus 1	Stimulus 2
1	19	Freeze-killed wax moth pupa (F-KWMP) 17 days post parasitism	F-KWMP not parasitized
2	15	F-KWMP not parasitized, treated with hexane wash of WMP 17 days post parasitism[a]	F-KWMP not parasitized, treated with hexane (10 µl)
3	21	F-KWMP not parasitized, treated with hexane wash of female *Pimpla disparis* pupa[a]	F-KWMP not parasitized, treated with hexane (10 µl)
4	31	F-KWMP not parasitized, treated with hexane wash of male *P. disparis* pupa[a]	F-KWMP not parasitized, treated with hexane (10 µl)
5	22	F-KWMP not parasitized, treated with headspace volatile extract of WMP 17–18 days post parasitism[b,c]	F-KWMP not parasitized, treated with pentane (10 µl)
6	20	F-KWMP not parasitized, treated with headspace volatile extract of WMP 19–20 days post parasitism[b,d]	F-KWMP not parasitized, treated with pentane (10 µl)
7	13	F-KWMP not parasitized treated with headspace volatile extract of WMP 20–22 days post parasitism[b,e]	F-KWMP not parasitized, treated with pentane (10 µl)
8	22 (T1)[f]	0–15 min	0–15 min
		Intact live WMP containing male *P. disparis* pupa (w)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Intact live WMP containing male *P. disparis* pupa (x)	Intact live WMP not parasitized
	22 (T1)	0–15 min	0–15 min
		Intact live WMP containing male *P. disparis* pupa (y)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Incised live WMP containing male *P. disparis* pupa (z)	Intact live WMP not parasitized
	23 (T2)	0–15 min	0–15 min
		Intact live WMP containing eclosed adult male *P. disparis* (w)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Intact live WMP containing eclosed adult male *P. disparis* (x)	Intact live WMP not parasitized
	23 (T2)	0–15 min	0–15 min
		Intact live WMP containing eclosed adult male *P. disparis* (y)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Incised live WMP containing eclosed adult male *P. disparis* (z)	Intact live WMP not parasitized
	40 (T3)	0–15 min	0–15 min
		Intact live WMP containing female *P. disparis* pupa (w)	Intact live WMP not parasitized
		15–30 min	0–30 min
		Intact live WMP containing female *P. disparis* pupa (x)	Intact live WMP not parasitized
	39 (T3)	0–15 min	0–15 min
		Intact live WMP containing female *P. disparis* pupa (y)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Incised live WMP containing female *P. disparis* pupa (z)	Intact live WMP not parasitized
	20 (T4)	0–15 min	0–15 min
		Intact live WMP containing	Intact live WMP not parasitized

Table 1 (continued)

Exp.#	*N*	Stimuli tested	
		Stimulus 1	Stimulus 2
		eclosed adult female *P. disparis* (w)	
		15–30 min	15–30 min
		Intact live WMP containing eclosed adult female *P. disparis* (x)	Intact live WMP not parasitized
	21 (T4)	0–15 min	0–15 min
		Intact live WMP containing eclosed adult female *P. disparis* (y)	Intact live WMP not parasitized
		15–30 min	15–30 min
		Incised live WMP containing eclosed adult female *P. disparis* (z)	Intact live WMP not parasitized
9	10	Oral secretion[g] of emerging female *P. disparis* applied to live WMP not parasitized	Intact live WMP not parasitized
10	12	Oral secretion[g] of emerging male *P. disparis* applied to live WMP not parasitized	Intact live WMP not parasitized

[a] Aliquots of 0.5 body-wash equivalents of host pupa, or *P. disparis* pupa (chemicals washed off 0.5 host pupa, or developing parasitoid, respectively) were bioassayed in each replicate

[b] Aliquots of 70 insect-hour equivalents (volatiles released by70 parasitized wax moth pupae over one hour) were bioassayed in each replicate

[c] No *P. disparis* were yet emerged

[d] Some male *P. disparis* were emerging

[e] Some female *P. disparis* were emerging

[f] *T* Treatment

[g] One-insect-equivalent of oral secretion of emerging male or female *P. disparis* was bioassayed in each replicate

tests. All data were analyzed with the Statistical Software JMP 7.0.2 (SAS Institute 2009), with α=0.05.

Results

Hypothesis 1: Pre-emergence DePa Produces Pheromone that Attracts or Arrests Males

In experiment 1, males spent more time on or near freeze-killed parasitized pupae than on freeze-killed unparasitized pupae (W=86; P=0.001; Fig. 2). In experiments 2–4, males spent more time on unparasitized host pupae treated with hexane washes of (*i*) parasitized host pupal cases (experiment 2), (*ii*) female DePa pupae (experiment 3), or (*iii*) male DePa pupae (experiment 4) than on host pupae treated with the equivalent amount of hexane (Exp. 2: W=−39, P<0.001; exp. 3: W=−56, P<0.001; exp 4: W=−174, P<0.001; Fig. 3). In experiment 5, males did not discern between unparasitized pupae treated with headspace volatile extract of host pupae containing a pre-emergence DePa (17–18 d post parasitism) or treated with the equivalent amount of pentane (W=−31, P=0.1; Fig. 4). In experiments 6 and 7, males spent more time on unparasitized host pupae, treated with headspace volatile extract of host pupae containing an emerging adult male (19–20 d post parasitism) or adult female (20–22 d post parasitism) parasitoid, than on unparasitized host pupae treated with the equivalent amount of pentane (Exp. 6: W=−102, P<0.001; exp. 7: W=−44, P<0.001; Fig. 4).

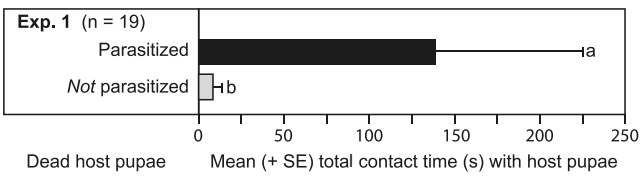

Fig. 2 Mean (+ SE) total time (sec.) spent during experiment 1 (see Table 1) by 4–7-d-old virgin male *Pimpla disparis*, in a 15-min duration bioassay, at or near freeze-killed *Galleria mellonella* pupae, either 17 d post parasitism by female *P. disparis*, or not parasitized. A different letter on bars indicates a difference in response to test stimuli; Wilcoxon paired-sample test; P<0.05

Hypothesis 2: Pheromone is Most Effective During the Emergence of a Parasitoid

In experiment 8, pupae incised for the second period arrested males longer than intact pupae during the first period, irrespective of the sex and developmental stage of the DePa inside (Fig. 5, Table 2). Developmental stage, but not sex, of DePa affected arrestment times of bioassay males. Female parasitoids already eclosed within the incised host pupae elicited longer arrestment by males than did female DePa in the pupal stage. This was not the case for

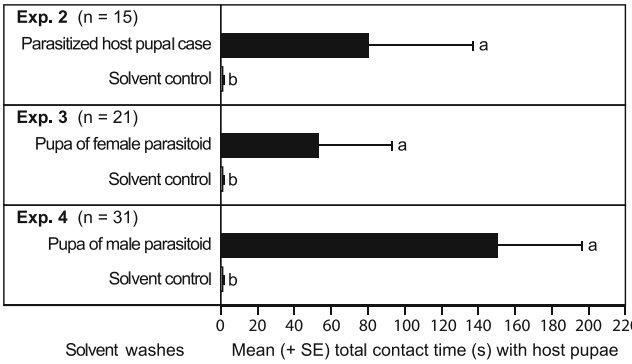

Fig. 3 Mean (+ SE) total time (sec.) spent during experiments 2–4 (see Table 1) by 4–7-d-old virgin male *Pimpla disparis*, in a 15-min duration bioassay, at or near freeze-killed wax moth, *Galleria mellonella,* pupae treated with a 1 insect-equivalent wash of (*i*) intact *G. mellonella* pupae 17 d post parasitism (Exp. 2), (*ii*) 17-d-old female developing parasitoid (DePa) (Exp. 3), or (*iii*) 17-d-old male DePa (Exp. 4). In each experiment, different letters on bars indicate a difference in the response to test stimuli; Wilcoxon paired-sample test; $P<0.05$

eclosed males or male pupal DePa. When parasitized host pupae were kept intact, males spent equal amounts of time on pupae during the first and the second 15-min bioassay periods.

Hypothesis 3: Oral Secretion Contains the Emergence Pheromone

In experiments 9 and 10, males spent more time on live unparasitized moth pupae, treated with oral secretions of emerging female (experiment 9) or male (experiment 10) parasitoids, than on live unparasitized moth pupae not treated (Exp. 9: $W=-28$; $P=0.002$; Exp. 10; $W=-39$, $P<0.001$; Fig. 6).

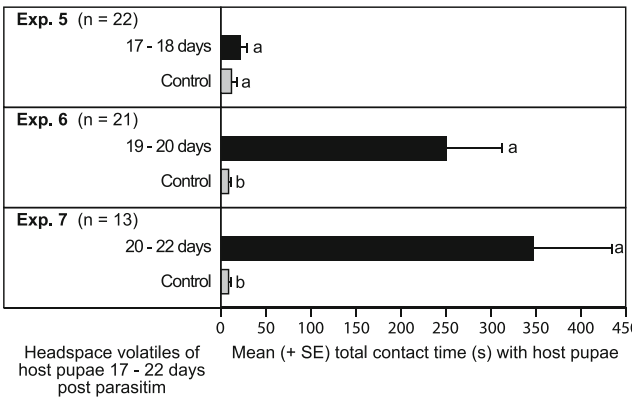

Fig. 4 Mean (+ SE) total time (s) spent during experiments 5–7 (see Table 1) by 4–7-d-old virgin male *Pimpla disparis*, in a 15-min duration bioassay, at or near freeze-killed wax moth, *Galleria mellonella,* pupae treated with headspace volatile extract of live host pupae (*i*) 17–18 d post parasitism (with no parasitoids emerging yet) (Exp. 5), (*ii*) 19–20 d post parasitism (with some males emerging) (Exp. 6), or (*iii*) 20–22 d post parasitism (with some females emerging) (Exp. 7). In each experiment, different letters on bars indicate a difference in response to test stimuli; Wilcoxon paired-sample test; $P<0.05$

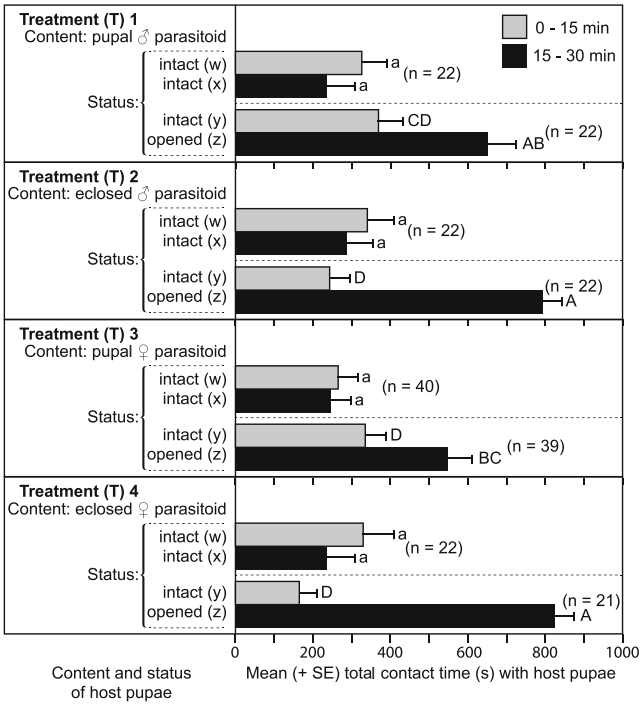

Fig. 5 Mean (+ SE) total time (s) spent during experiment 8 (see Table 1) by 4–7-d-old virgin male *Pimpla disparis* in two consecutive bioassays, each of 15 min duration, at or near wax moth, *Galleria mellonella,* pupae 17–22 d post parasitism, that were kept intact or incised (2 mm opening), and contained a male or female *P. disparis* pupa or a male or female eclosed adult. Paired intact/intact host pupae data were analyzed (and assigned a lower case letter) separate from paired intact/opened host pupae data (which were assigned an upper case letter). Within and between treatments 1–4, all bars with the same lower case letter (a), or the same upper case letter superscript (A, B, C or D), do not differ. All data were analyzed by 3-factor ANOVA (see Table 2 for details). Note: (1) more explanation of test stimuli w, x, y, and z are provided under experiment 8 in Table 1; (2) in treatments 2 and 4, "eclosed" refers to parasitoids that have eclosed within, but not yet emerged from, the host pupal case

Discussion

Our data support the hypothesis that detection of pre-emergence and emerging mates in *P. disparis* is mediated by semiochemicals. Attraction and arrestment of males in response to (*i*) freeze-killed, parasitized host pupae, which could "signal" only with semiochemicals, (*ii*) solvent washes of parasitized host pupae or isolated DePas, and (*iii*) headspace volatiles of parasitized live host pupae with emerging parasitoids, all implicate DePa-derived semiochemicals as a means by which males pinpoint emergence of prospective mates. Experimental opening of host pupal

Table 2 Three-factor ANOVA comparing effects of, and interactions (×) between, parasitoid sex, developmental stage (pupal vs eclosed), and host pupal status (intact vs incised)

Effects tested	*F* Ratio	*P* Value
Experimental design 1: Parasitized pupae (intact/intact)		
parasitoid sex	0.51	0.48
parasitoid developmental stage	0.33	0.57
pupal status	2.1	0.15
parasitoid sex × parasitoid developmental stage	0.0020	0.96
parasitoid sex × pupal status	0.010	0.92
parasitoid developmental stage × pupal status	0.080	0.78
parasitoid sex × parasitoid developmental stage × pupal status	0.50	0.48
Experimental design 2: Parasitized pupae (intact/incised)		
parasitoid sex	0.58	0.45
parasitoid developmental stage	0.98	0.32
pupal status	110	<0.001
parasitoid sex × parasitoid developmental stage	0.048	0.83
parasitoid sex × pupal status	0.016	0.9
parasitoid developmental stage × pupal status	15	<0.001
parasitoid sex × parasitoid developmental stage × pupal status	2.0	0.16

cases containing a pupal DePa, or a recently eclosed parasitoid, induced strong arrestment of males, likely due to a burst of semiochemical release. Parasitoids chewing their way out of the host pupal case (Fig. 1d; supplemental video) release these semiochemicals with the oral fluid, making them a true "emergence cue".

To implicate semiochemicals as the single modality males exploit in order to pinpoint the time of emergence of prospective mates, we needed to exclude visual and vibratory cues. A DePa inside a host pupal case engages in spontaneous spins, which cause vibrations that increase in magnitude but decrease in frequency as DePas mature (Danci et al., unpubl.), thus potentially providing information to a visiting male about the stage of development. As males arrested in response to freeze-killed host pupae and DePas that could no longer spin, they could not have relied on vibrational cues. Moreover, when males responded to body washes of DePas, and to headspace volatiles of host pupae with emerging parasitoids, they could not have relied on visual cues, such as discoloration of the host pupal case. Collectively, these data implicate DePa-derived semiochemicals as the source of information that alerts males to the incipient or ongoing emergence of a prospective mate.

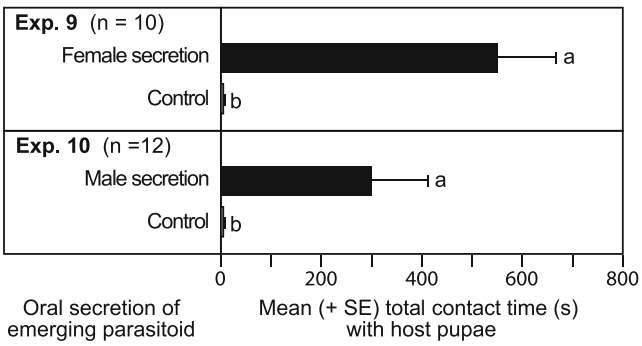

Fig. 6 Mean (+ SE) time (s) spent in experiments 9–10 by 4–7-d-old virgin male *Pimpla disparis*, in 15-min bioassay periods, at or near live wax moth, *Galleria mellonella,* pupae that were either treated with an oral secretion from an emerging female (Exp. 9) or male (Exp. 10) conspecific. The control consisted of a live untreated *G. mellonella* pupa. In each experiment, different letters on bars indicate a difference in response to test stimuli; Wilcoxon paired-sample test; $P<0.05$

Changes over time in the composition and/or abundance of DePa semiochemicals could carry critical information for mate-seeking males. Although we do not know anything about the identity of the chemical(s), and hence cannot discuss qualitative changes, the strong responses of males to freshly incised host pupal cases containing a pupal DePa or an eclosed adult, but not to intact host pupal cases, points to semiochemical concentration as critical information used by males to gauge the emergence of a prospective mate.

While investigating the source of the "emergence semiochemical", we noticed that mate-seeking males pay particular attention to the incision line that parasitoids chew into their host pupal case to open and emerge from. Moreover, macro-videography of this chewing revealed oral fluid (Supplemental video) that appeared to soften the pupal case. These observations made us hypothesize that the semiochemicals are present in the oral fluid released during eclosion. Collecting oral fluids from emerging male and female parasitoids (Fig. 1, e, f), and immediately testing them in bioassays, revealed that they induced strong arrestment, wing fanning, antennation, and even copulatory attempts, by males. Thus, these semiochemicals may originate from mandibular glands, which have been implicated or shown to be the source of sex pheromones in some ichneumonid

(Davies and Madden, 1985) and sphecid wasps (Ayasse et al., 2001). For example, males of the European beewolf, *Philanthus triangulum*, secrete sex pheromone from their mandibular gland that attracts females into their territory, which they cordon off to secure a harem (Herzner et al., 2007).

Insects utilize both signals and cues in their search for prospective mates (Metzger et al., 2010). A cue refers to any kind of sensory information present in the environment (Ruxton and Schaefer, 2011), whereas a signal implies intent on the part of the sender (Wilson, 1975) and requires an active process. Pheromone-based sexual communication with "intentional" signaling is well documented in hymenopteran parasitoids (Syvertsen et al., 1995, and cited references therein; Quicke, 1997), but may not exist in *P. disparis*. The communication systems of some solitary wasp species seem inefficient, in that males are attracted as strongly to emerging males as they are to emerging females (e.g., Heatwole et al., 1962; Robacker and Hendry, 1977; Davies and Madden, 1985; Quicke, 1997; Ayasse et al., 2001; Steiner et al., 2005; King, 2006; Ruther and Steiner, 2008). Interpretations of this rather peculiar phenomenon, in general, include sensory limitations on the part of males (Thornhill and Alcock, 1983; O'Neill, 2001), overeagerness of males to mate (Williams, 1966; Thornhill and Alcock, 1983; O'Neill, 2001), and mimicry of female pheromone by (juvenile) males (Eliyahu et al., 2009). The latter phenomenon has been implicated in some mating systems as playing a role in reducing male aggression (Peschke, 1987; Cremer, et al., 2002), stealing of nuptial gifts (Eliyahu et al., 2009), and in post-copulatory mate guarding (Field and Keller, 1993). In the *P. disparis* mating system, female mimicry has no obvious selective advantage. As female *P. disparis* do not respond to hexane extract of oral secretion (data not shown), our original hypothesis that the oral secretion contains an aggregation pheromone, *sensu* Borden (1985),[1] that attracts males and females and thus facilitates outbreeding and genetic diversity, was not supported. An alternative explanation is that many ichneumon females may not pheromone signal at all, and that males, instead, respond to "pre-existing" information associated with prospective mates (Godfray and Cook, 1997; Ruther and Steiner, 2008), such as metabolites present on the integument (Howard, 1993). This explanation is applicable to *P. disparis.* Males respond to semiochemicals in oral fluids of both male and female conspecifics (Fig. 6), suggesting that emerging females indeed do not signal with long-range sex pheromone.

[1] Aggregation pheromones are defined as substances produced by members of either or both sexes that induce members of both sexes to aggregate.

The metabolites in oral fluids of *P. disparis* may serve a primary function of softening the host pupal case to facilitate the parasitoid's emergence; secondarily, they appear to have been co-opted by males to assist them in locating emerging females which, once fully emerged, do not seem to engage in long-range attraction of searching males, although they do seem to possess a sex-specific cuticular (contact) pheromone that elicits courtship behavior in males (M.H., personal obs.). As these oral fluids are secreted only during emergence, and dissipate thereafter, they are a highly reliable indicator of parasitoid emergence. The strategy of males, of responding to these secretions, may represent a trade-off between a high probability of encountering an emerging conspecific, and a 50% chance of it being a male instead of a mate.

With the emerging female not actively signaling and revealing her location, the onus to find a mate and reproduce is on males. Females, in contrast, are haplodiploid, and thus are capable of reproducing with or without mating (Quicke, 1997; Normark, 2003; Heimpel and de Boer, 2008). Such female parasitoids have reduced evolutionary pressure to allocate resources toward pheromone production and signaling, which may attract predators and, instead, may allocate more resources toward oviposition (Godfray and Cook, 1997; Ayasse et al., 2001; Steiner and Ruther, 2009). This would explain why males ensure reproductive success by co-opting chemicals already present in oral secretions of emerging females (and males) as a mate-finding cue.

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The Social Integration of a Myrmecophilous Spider Does Not Depend Exclusively on Chemical Mimicry

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Abstract Numerous animals have evolved effective mechanisms to integrate into and exploit ant societies. Chemical integration strategies are particularly widespread among ant symbionts (myrmecophiles), probably because social insect nestmate recognition is predominantly mediated by cuticular hydrocarbons (CHCs). The importance of an accurate chemical mimicry of host CHCs for social acceptance recently has been demonstrated in a myrmecophilous silverfish. In the present study, we investigated the role of chemical mimicry in the myrmecophilous spider *Gamasomorpha maschwitzi* that co-occurs in the same host, *Leptogenys distinguenda,* as the silverfish. To test whether spiders acquire mimetic CHCs from their host or not, we transferred a stable isotope-labeled hydrocarbon to the cuticle of workers and analyzed the adoption of this label by the spiders. We also isolated spiders from hosts in order to study whether this affects: 1) their chemical host resemblance, and 2) their social integration. If spiders acquired host CHCs, rather than biosynthesizing them, they would be expected to lose these compounds during isolation. Spiders acquired the labeled CHC from their host, suggesting that they also acquire mimetic CHCs, most likely through physical contact. Furthermore, isolated spiders lost considerable quantities of their CHCs, indicating that they do not biosynthesize them. However, spiders remained socially well integrated despite significantly reduced chemical host similarity. We conclude that *G. maschwitzi* depends less on chemical mimicry to avoid recognition and aggressive rejection than the silverfish previously studied, suggesting that the two myrmecophiles possess different adaptations to achieve social integration.

Keywords Acquired chemical mimicry · Myrmecophile · Social integration · Cuticular hydrocarbons · *Malayatelura ponerophila*

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Introduction

The phenomenon of mimicry was first described by the English naturalist Henry Walter Bates, who discovered that nontoxic species of Neotropical butterflies visually resemble toxic species, thus avoiding predation (Bates, 1862). Since this discovery, visual resemblance and its evolutionary consequences have been studied extensively (Müller, 1878; Fisher, 1927; Wickler, 1968; Brower, 1988; Ruxton et al., 2004). While scientists initially focused solely on visual mimicry, many researchers now also study chemical mimicry (Dettner and Liepert, 1994; Bagnères and Lorenzi, 2010).

Chemical communication is the most widespread form of communication among organisms (Symonds and Elgar, 2008; Steiger, et al., 2011), and social insects base their communication to a great extent on chemicals (Wilson, 1990). This includes a sophisticated chemical recognition system, able to distinguish group members from aliens, which helps protect societies from exploitation. In ants, wasps, bees, and termites, recognition of group members is based mainly on complex blends of cuticular hydrocarbons (CHCs) (van Zweden and

d'Ettorre, 2010). However, various arthropods prevent being recognized as aliens by mimicking the CHCs of social insect workers (chemical mimicry, sensu Dettner and Liepert, 1994) and appearing to be nestmates (Bagnères and Lorenzi, 2010). The origin of the CHCs used by mimics is unknown in the majority of cases; they may acquire CHCs directly from the host ('acquired chemical mimicry', sensu von Beeren et al., 2012), they may actively biosynthesize recognition cues ('innate chemical mimicry', sensu von Beeren et al., 2012), or both mechanisms may occur in combination (Akino, 2008).

Regardless of the origin of mimetic compounds, studies of similarities in CHC profiles between parasites and social insect hosts remain predominantly descriptive. A chemical resemblance alone does not necessarily mean that the host is deceived by the mimic, or that the mimic gains benefits through chemical resemblance. Specific bioassays are necessary to demonstrate whether chemical resemblance indeed affects the behavior of the host, as has been demonstrated in the spider *Cosmophasis bitaeniata* (Allan et al., 2002), the butterfly *Phengaris* (*Maculinea*) *alcon* (Nash et al., 2008), and the silverfish *Malayatelura ponerophila* (von Beeren et al., 2011b).

In this study, we investigated the role of chemical mimicry in the social integration of the kleptoparasitic spider, *Gamasomorpha maschwitzi* (Arachnida: Aranea: Oonipidae), which parasitizes the ponerine army ant *Leptogenys distinguenda.* Many spiders live in close relationship with ants, but little is known about how they specifically adapt to cope with host defenses (reviewed in Cushing, 1997). Species have been shown to be behaviorally adapted (Ceccarelli, 2007), chemically integrated (Allan et al., 2002), or to utilize both strategies together (Witte et al., 2009). Witte et al. (2009) suggested that, in the spider *G. maschwitzi*, additional factors to chemical mimicry might play roles. Although the spiders were chemically less similar to the host than was another myrmecophile, the silverfish *M. ponerophila*, they showed comparable integration and received fewer attacks. Apparently, integration of spiders depended less on chemical cues and more on other mechanisms.

The goals of the present study were twofold. We aimed to answer the question of whether *G. maschwitzi* acquires CHCs from the host or biosynthesizes them. Furthermore, we tested whether the accuracy of chemical host resemblance influences the level of social integration, as previously shown in the silverfish *M. ponerophila*. Silverfish individuals that were chemically more similar to host ants were attacked less often, and thus achieved a higher level of social integration (von Beeren et al., 2011b). We expected that: (1) spiders acquire CHCs from host ants through frequent contact (Witte et al., 2009); and (2) that spiders more closely resembling the host profile would be better integrated socially. To test these predictions, we performed two experiments. First, we applied a stable isotope-labeled hydrocarbon to the cuticle of ants and monitored the transmission of this label to the myrmecophilous spider. We expected that, if *G. maschwitzi* acquired CHCs from its host, the label would also accumulate on their cuticles. Second, we aimed to reduce the chemical resemblance of spiders to the host, so as to study the effects on social integration. For this, we isolated spiders from hosts for 9 days, assuming that spiders acquire host CHCs behaviorally (Witte et al., 2009), and therefore assumed that they would lose host compounds, resulting in reduced chemical similarity to the host. We hypothesized that spiders exhibiting reduced chemical host resemblance would be less socially integrated, as a consequence of being recognized as alien more often. We tested whether isolated spiders (with reduced host similarity) were attacked more frequently than unmanipulated individuals. Finally, we discuss here the integration strategies of the spider *G. maschwitzi* and the silverfish *M. ponerophila*.

Methods and Materials

Field Collections

Animals were collected and observed at the Field Studies Centre of the University of Malaya in Ulu Gombak, Malaysia (03°19.479′ N, 101°45.163′ E, altitude 230 m) and at the Institute of Biodiversity in Bukit Rengit, Malaysia (03° 35.779′ N, 102°10.814′ E, altitude 72 m). A total of 14 mo of fieldwork was carried out from August 2007–April 2011. The two kleptoparasites, *G. maschwitzi* (Wunderlich, 1994) and *M. ponerophila* (Mendes et al., 2011), both parasitize the nocturnal, ponerine army ant *L. distinguenda* (Emery, 1887). We searched for host nest sites during the night, between 8 p.m. and 3 a.m., by backtracking the ants' raiding trails. Subsequently, we checked nest sites every 30 min. for the onset of a colony migration. Host colonies frequently move to new nest sites (on average every 1.5 nights; Steghaus-Kovac, 1994). Under natural conditions, spiders participate in migrations by showing a "tandem-running"-like behavior (Witte et al., 1999; Fig. 1), while silverfish are phoretically transported on ant pupae, as well as running among ant workers (Witte et al., 2008). Using aspirators, we collected host ants during raids, and spiders and silverfish during migrations. Animals that were kept over several days in the laboratory (see below) were fed every day with freshly killed crickets. Crickets are among the natural diet of the host ants (Steghaus-Kovac 1994), the spiders and the silverfish (personal observation). Since *G. maschwitzi* occurs in low numbers, experimental procedures frequently were limited for working with appropriate sample sizes.

Chemical Transfer Experiment

The aims were twofold: first, we tested whether spiders can acquire CHCs from their host; second, we compared the quantity of the transferred label from host to spiders and

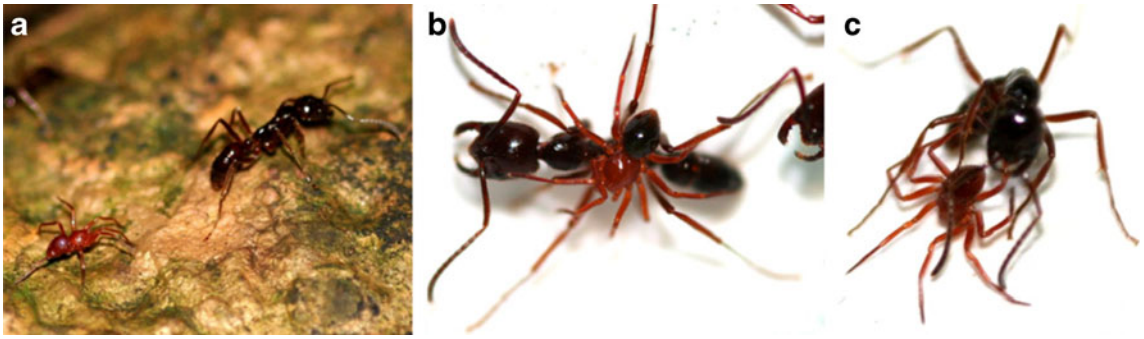

Fig. 1 (**a**) *Gamasomorpha maschwitzi* participates in ant migrations by performing a "tandem-running"–like behavior. The spiders are easily distinguishable from the host ants due to their reddish color. (**b**) In laboratory colonies, spiders frequently interact with ant workers and are often found on top of them. (**c**) Occasionally, a spider is recognized as an intruder and attacked by ant workers

silverfish. All animals were kept in an artificial nest (plastic box: 30×20×15 cm), constructed of soil and leaf litter from the environment; we used 65 ant workers, 65 callows, approx. 40 larvae, 30 pupae, 17 spiders, 12 silverfish, as well as 11 non-myrmecophilous isopods as controls. Workers and callows were treated with a stable isotope-labeled hydrocarbon (eicosane-D_{42}, C/D/N Isotopes Inc., Pointe-Claire Canada). We used eicosane-D_{42}, because of its similar properties (chain length, molecular weight) to the CHCs that occur naturally on the host (Table 1 of supplement). In a clean 20-ml glass vial, 200 µl of a saturated eicosane-D_{42} hexane solution were evaporated so that the hydrocarbon fully covered the bottom of the vial as a solid film. Workers and callows then were placed in the vial, and shaken gently for 30 min. to transfer the labeled compound. Ants did not noticeably suffer from this treatment. Labeled ants, spiders, silverfish, and control isopods were kept together for 3 d in the laboratory nest and, subsequently, 20 ants (10 workers and 10 callows) and all spiders, silverfish, and control isopods were extracted for chemical analysis. Non-myrmecophilous isopods, collected from the same rainforest, were added to test whether eicosane-D_{42} transmits to animals that are not in direct contact with the host. Preliminary studies revealed that isopods rarely had contact with ants and survived well in laboratory nests (von Beeren et al., 2011b). Therefore, they were well suited as controls. Importantly, their cuticle could adsorb the labeled CHC (von Beeren et al., 2011b).

Isolation Treatments

To evaluate the impact of isolation on CHC profiles, and on behavioral interactions with host ants, some spiders were separated from their home colony and kept isolated for 9 d in plastic boxes (21×15×5 cm), with a moistened plaster ground (for sample sizes refer to Table 1). The cuticles of isolated and non-isolated spiders were extracted with hexane to analyze differences in CHCs (colonies 1–4; see section "Comparison of CHCs"). In colony 4, we also tested the social acceptance by hosts of isolated and non-isolated spiders before extraction (see methods, section "Social Acceptance Experiment"). The chemical studies were performed independently (colonies 1–3) of, and in combination with (colony 4), behavioral observations, because the combined experiment is best suited to test whether an individual's chemical host resemblance affects its level of social acceptance. Nevertheless, an increase of CHC quantity through behavioral interactions with host ants (e.g., rubbing behavior) during the social acceptance experiment (see below) cannot be ruled out. Hence, we also extracted the cuticles of spiders that were not subjected to the social acceptance experiment.

Comparison of CHCs between Isolated and Non-isolated Spiders

To analyze whether isolation changed concentration, presence or absence, and/or composition of CHCs in spiders, we extracted the cuticles of individuals directly after collecting them in the field, and after 9 d of separation from the host (colony 1–4) (for sample sizes, see Table 1). Animals were transferred individually into 2-ml vials, with

Table 1 Overview of the number of spider individuals observed within each colony for the analyses of cuticular hydrocarbons (CHCs) and the social acceptance experiments

	Analysis of CHCs		Social Accept. Exp.	
Colony	Sp0d	Sp9d	Sp0d	Sp9d
Colony 1	9	6	-	-
Colony 2	11	5	-	-
Colony 3	9	4	-	-
Colony 4	13	10	13	10
Colony 5	-	-	9	-
Colony 6	-	-	5	-
Colony 7	-	-	5	-
Colony 8	-	-	10	-

Abbreviations: Sp0d=non-isolated spiders; Sp9d=9 d-isolated spiders

a polytetrafluoroethylene (PTFE) septum, and extracted for 10 min. in 200 μl hexane (HPLC grade, Sigma-Aldrich). The solvent was evaporated, and the CHCs were dissolved in 40 μl of hexane, containing an internal standard (methyl stearate, FLUKA Analytics, Sigma-Aldrich). Each sample (1 μl) was analyzed by gas chromatography–mass spectrometry (Agilent Technologies 6890 N-5975), using a Restek Rxi-5MS column (30 m length, 0.25 mm ID, 0.25 μm film thickness), at LMU Munich, Germany. Details of the methods can be found in Witte et al. (2009).

Chemicals were identified by their mass spectra and retention indices (RI), and peak areas calculated using the software AMDIS (version 2.68) (Stein, 1999). A target library of 109 compounds was created, based on compounds found in extracts of host ants and myrmecophiles (a list of identified compounds is given in Witte et al., 2009). Since AMDIS uses the mass spectrum as well as the retention index to identify a compound, it has the advantage of reliably detecting compounds, even at low quantities.

The absolute quantity per surface area of an individual (concentration) of each compound was calculated against the internal standard (20 ng/μl). We divided the total quantity of compounds by the median surface area, in mm^2, for workers, spiders, silverfish, and isopods, to standardize to the presumed concentration of surface compounds at the point of an ant's antennal contact. To determine surface area, a spider's body was divided into geometrical parts, and the relevant body dimensions were measured using a stereomicroscope (Zeiss Stemi 2000-C) with a measuring eyepiece (see supplemental material: Calculation of surface area). We used the calculated surface areas of workers, isopods, and silverfish according to von Beeren et al. (2011b). The median surface areas of animals are: workers (median=78.24 mm^2, range=71.76–83.09 mm^2, N=10), spiders (median=38.19 mm^2, range=32.78–42.03 mm^2, N=15), silverfish (median=13.48 mm^2, range=2.44–19.71 mm^2, N=180), and isopods (median=42.69 mm^2, range=27.04–95.86 mm^2, N=22).

Social Acceptance Experiment

In order to evaluate the social acceptance of non-isolated and isolated spiders, we quantified the aggression of hosts against individual spiders, by performing a contact study in laboratory nests (for sample sizes, see Table 1). The behavioral responses of host workers to spiders were studied under laboratory conditions, using artificial nests, consisting of a transparent plastic container (20×14×1 cm) with a 1.5 cm nest entrance shaded with a plastic cover. The artificial nests were placed in a larger foraging arena (32×25×9 cm), with a moistened plaster ground to maintain humidity. Laboratory nests contained 200 mature host ant workers collected in raids, as workers from raids are more likely to defend the colony compared to young callows that stayed in the nest (CvB, personal observation). Before introducing spiders, host colonies were given 1 h to settle in the artificial nest, because ants tend to be more aggressive in familiar than in unfamiliar areas (Tanner and Adler, 2009). Spiders were tested either within 6 h of collection from the field, or after 9 d of isolation. The interactions of ant workers with one focal spider was observed in approximately 50 consecutive encounters, by recording eight different behaviors (Table 2). Each spider was tested only once. Repeated interactions with the same ants were possible. However, since we were interested in defense at the colony-level against spiders, and since task allocation occurs naturally in ants, repeated interactions do not affect our interpretations.

An aggression index (AI) was calculated for each spider from the observed interactions. The AI focused on the proportion of the aggressive ant reactions, chased, snapped and stung: $\mathbf{AI}=N_A/N_T$; with N_A=number of aggressive interactions and N_T=total number of interactions.

Combined Experiment

To test whether a relationship between chemical similarity and ant aggression exists, we studied both social acceptance and analysis of CHCs for one *L. distinguenda* colony (colony 4; Table 1). Host aggression was first quantified for each spider, via the social acceptance experiment, and then

Table 2 Behavioral interactions between spiders and ants, and categories used for calculating the aggression index

Behavior	Definition	Category
Ignored	An ant worker touches the spider once with its antennae and moves on without any sign of behavioral modification.	-
Groomed	An ant grooms the spider with its mouthparts. The spider remains in position.	-
Avoid	When an ant approaches, the spider avoids contact by quick escape.	-
Antennated	An ant touches a spider repeatedly with its antennae for longer than 2 sec without displaying other behaviors.	-
Unnoticed	An ant comes into, and perhaps stays in contact with a spider, but not with its antennae; the ant does not modify its behavior.	-
Chased	An ant touches the spider with its antennae and quickly lunges in its direction.	Aggressive
Snapped	An ant touches the spider with its antennae and snaps with its mandibles into the direction of the spider.	Aggressive
Stung	An ant touches the spider with its antennae, lunges forward and bends its gaster in direction of the opponent. The attempt does not need to be successful.	Aggressive

cuticular chemicals were extracted. We performed this experiment with non-isolated and nine-days isolated spiders.

Comparison between the Spider and the Silverfish

The experiments were identical to those of a study on the myrmecophilous silverfish *M. ponerophila* (von Beeren et al., 2011b). Consequently, we compared the results of the study on the spider with those on the silverfish (von Beeren et al., 2011b).

Data Analysis

Chemical and behavioral data were evaluated using PRIMER 6 (version 6.1.12, Primer-E Ltd., Ivybridge, U.K.) with the PERMANOVA + add-in (version 1.0.2) (Anderson et al., 2008), using a non-parametric permutational analysis of variance (PERMANOVA) with 9,999 permutations. PERMANOVA Analyses were based on Bray-Curtis similarities (as a semi-metric measure) or simple matching (a presence-absence measure), either calculated from a single response variable (chemical similarity, CHC concentration, aggression index), or from numerous response variables (CHC profiles, presence or absence of CHCs, behavioral interactions). Box plots were created from univariate data with the Microsoft Excel add-in SSC-Stat (version 2.18, Statistical service centre of the University of Reading, Reading, U.K.).

Chemical Analysis

As no spider-specific compounds were detected, only the principal compounds that contributed, in total, to 99% of the chemical profiles of workers (N=49), according to a similarity percentage analysis (SIMPER) on Bray-Curtis similarities, were used in the statistical analysis. These selected data (N= 32 compounds; supplement Table 1) were used to analyze the CHC composition of spiders, the presence or absence of CHCs, the total CHC concentration, and the chemical similarity of spiders to workers. To test whether the chemical similarity of spiders to host colony was influenced by isolation, Bray-Curtis similarities to the average worker CHC profile of the respective host colony were used as a univariate response variable, and a PERMANOVA with a 2-factor nested design [colonies (random), days of isolation (fixed, nested in colony)] was applied. To test for additional differences in the quantity of CHCs, absolute concentrations (per surface area) were analyzed in the same way.

A multivariate approach was used to analyze relative changes in CHC composition (Bray-Curtis similarities) and the presence or absence of compounds, the latter evaluated by calculating resemblances based on "simple matching". A PERMANOVA with a 2-factor nested design, as described above, was applied for both analyses. Chromatograms of chemical profiles of host ants, spiders and silverfish can be found in Witte et al. (2009).

Behavioral Analysis

Aggression indices of isolated and non-isolated spiders were compared using a PERMANOVA, with a two-factor nested design, as described above. The interactions of spiders with host ants were evaluated in a multivariate approach, including all observed behaviors. They were standardized by total and a 2-factor nested design was applied as described above.

Comparison between Silverfish and Spiders

Since spiders and silverfish mostly did not originate from the same colonies, we did not consider colony as a factor for comparison. Accordingly, a PERMANOVA with a 1-factor design [species (fixed)] was applied.

Results

Chemical Transfer Experiment

In line with our expectations, workers extracted directly after the labeling treatment carried high concentrations of eicosane-D_{42} (median=108.87 ng/mm^2; Fig. 2). After the 3 d experimental phase, ant workers (adults and callows) still had the highest eicosane-D_{42} concentrations (median=31.62 ng/mm^2), followed by silverfish (median=7.48 ng/mm^2) and spiders (median=3.40 ng/mm^2). The concentration of eicosane-D_{42} was higher in silverfish than in spiders (PERMANOVA, P=

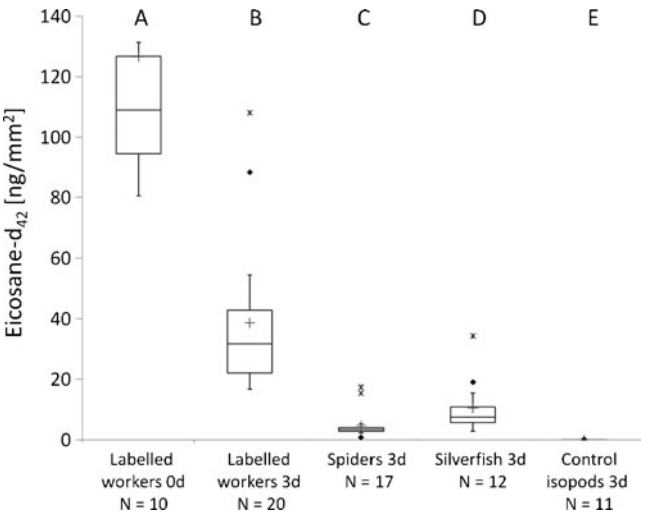

Fig. 2 Concentrations of eicosane-D_{42} in the chemical transfer experiment. Two outliers of labeled workers that were extracted directly after the labeling procedure (0 d) are not shown, allowing for better visibility (worker$_1$=241.51 ng/mm^2; worker$_2$=203.76 ng/mm^2). Different capital letters show differences (P<0.05) between groups evaluated by a PERMANOVA. Median (+=mean), quartiles (*boxes*), 10% and 90% percentiles (whiskers), and outliers (♦=outlier, *=extreme point) are shown. Abbreviations: 0 d=animals extracted directly after labeling; 3 d=animals extracted after the 3 day experimental phase

0.003). Importantly, control isopods had lower eicosane-D_{42} concentrations (median=0 ng/mm^2) than all other animals (PERMANOVA, for all pair-wise comparisons $P<0.001$).

Comparison of CHCs between Isolated and Non-isolated Spiders

For every single CHC, the concentration was lower in spiders after isolation. In addition, the total number of detected compounds decreased after isolation (across colonies: compounds of non-isolated spiders (Sp0d)=35, $N=42$; compounds of 9 d-isolated spiders (Sp9d)=8, $N=25$). There were 78 compounds above detection threshold on individual, non-isolated workers ($N=49$). No spider-specific compound was found.

Non-isolated spiders differed from isolated spiders in CHC composition, in the presence/absence of CHCs and in total CHC concentration (PERMANOVA, for all colonies $P\leq0.015$; Table 3). Nine-days-isolated spiders had lower CHC concentrations than non-isolated spiders (medians across colonies: Sp0d=4.00 ng/mm^2, $N=42$; Sp9d=0.16 ng/mm^2, $N=25$; Fig. 3). Workers had about 30 times greater concentrations of CHCs than non-isolated spiders, and about 700 times higher concentrations of CHCs than isolated spiders (median=112.15 ng/mm^2, $N=49$; Fig. 1 in supplement). Accordingly, workers had greater concentrations than both spider groups (PERMANOVA; $P<0.001$). Non-isolated spiders were chemically closer to host workers than were isolated individuals (Fig. 4).

Social Acceptance Experiment

Unmanipulated spiders were rarely attacked by ants, for all tested colonies (median$_{AI}$=0 for all colonies; for sample sizes see Table 1). The AIs of unmanipulated spiders did not differ among colonies (PERMANOVA, $P=0.232$).

Combined Experiment

Although isolated spiders of colony 4 showed reduced chemical resemblance to host workers (see above), they were rarely attacked (median$_{AI}$=0; Fig. 5). Their AIs did not differ from those of non-isolated spiders (PERMANOVA, $P=0.787$). Considering all behavioral categories, we found no difference between isolated and non-isolated spiders (PERMANOVA; $P=0.142$). However, the low sample size does not allow us to exclude minor differences. When looking at each behavioral

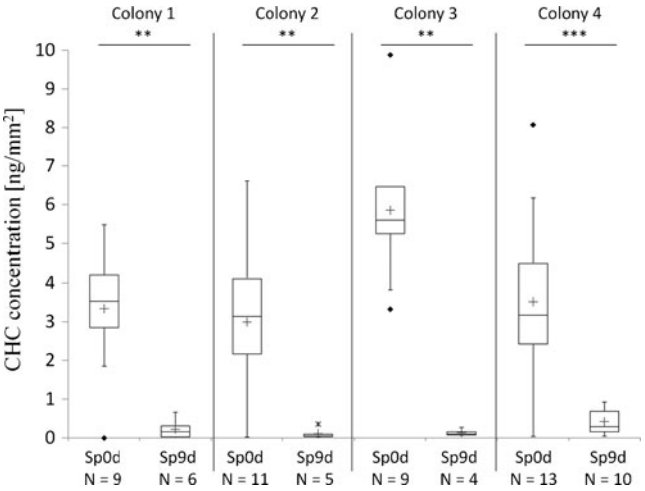

Fig. 3 Cuticular hydrocarbon (CHC) concentrations of non-isolated and 9 d-isolated spiders within colonies. Note that host colonies can differ in CHC concentrations and, therefore, CHC concentrations of spiders may differ as well. Two outliers among non-isolated spiders of colony 4 are not shown for better visibility (15.83 ng/mm^2 and 36.13 ng/mm^2). Median (+=mean), quartiles (*boxes*), 90% and 10% percentiles (whiskers), and outliers (♦=outlier, *=extreme point) are shown. Differences between groups were evaluated by a PERMANOVA (**$P<0.01$, ***$P\leq0.001$). Abbreviations: Sp0d=non-isolated spiders; Sp9d=nine day-isolated spiders

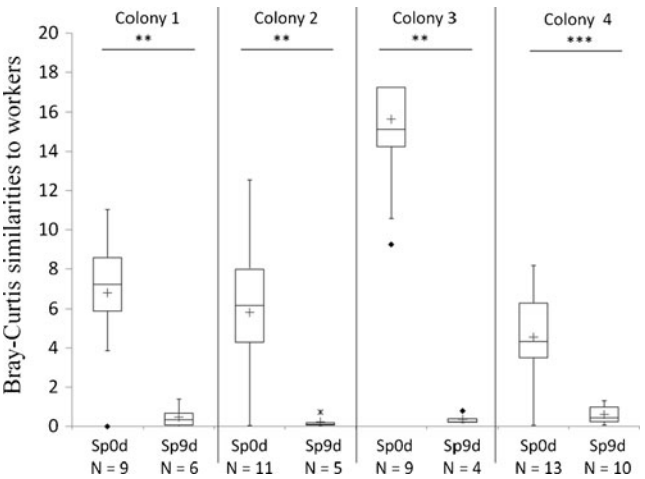

Fig. 4 Bray-Curtis similarities of individual spiders to the average chemical worker profile ($N_{workers}\geq10$) of their native host colony. One outlier of colony 3 is not shown for better visibility (value=25.21). Differences between groups were evaluated by a PERMANOVA (**$P<0.01$, *** $P<0.001$). Median (+=mean), quartiles (*boxes*), 10% and 90% percentiles (whiskers), and outliers (♦) are shown. Abbreviations: Sp0d=non-isolated spiders; Sp9d=nine days isolated spiders

Table 3 Comparison of non-isolated and isolated spiders for cuticular hydrocarbon (CHC) composition, presence or absence of CHCs, and total CHC concentration

Colony	CHC composition	CHC presence/absence	CHC concentration
Colony 1	0.001	0.003	0.001
Colony 2	0.003	0.015	0.001
Colony 3	0.001	0.001	0.001
Colony 4	0.001	0.001	0.001

PERMANOVA *P* values are shown. For sample sizes see Table 1

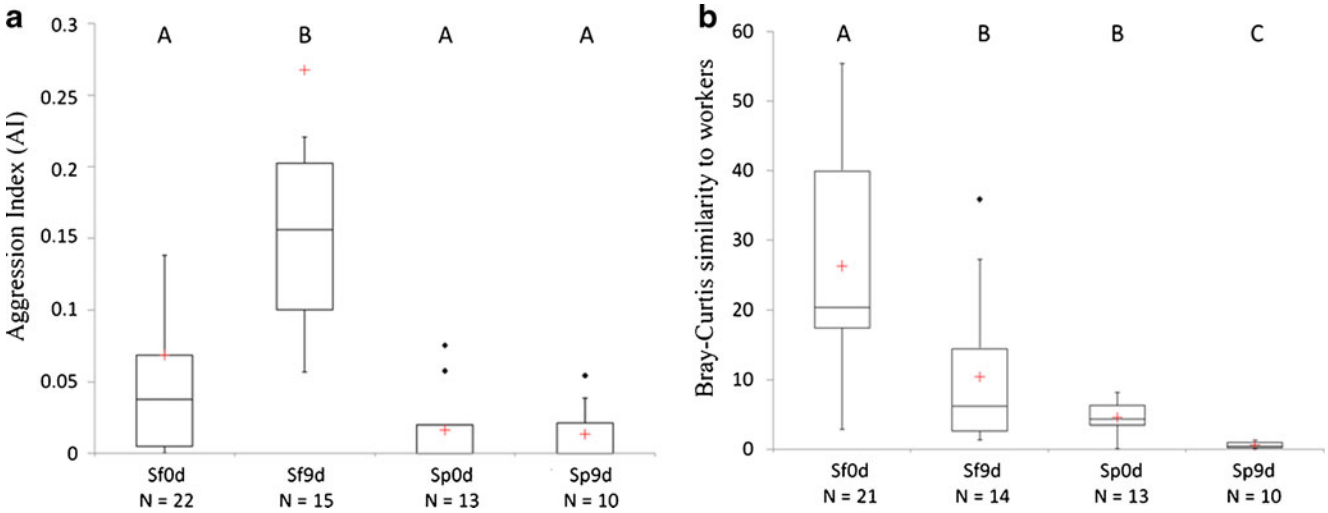

Fig. 5 Aggression indices of two myrmecophiles of *Leptogenys distinguenda*, the silverfish *Malayatelura ponerophila*, and the spider *Gamasomorpha maschwitzi* (**a**), and their chemical (Bray-Curtis) similarities to the average worker profile (**b**). Data originated from the same colony (colony 4). Three outliers of 9 d-isolated silverfish are not shown in the left graph for better visibility (AI=0.5 and two times AI=1). Median (+=mean), quartiles (*boxes*), 90% and 10% percentiles (whiskers), and outliers (♦= outlier, *=extreme point) are shown. Different capital letters depict differences ($P<0.05$) among groups evaluated by PERMANOVA. Abbreviations: Sf0d=non-isolated silverfish, Sf9d=9 d-isolated silverfish, Sp0d=non-isolated spiders; Sp9d=nine day-isolated spiders

category separately, we found a difference in antennation; i.e., isolated spiders were antennated by workers more frequently than non-isolated spiders (supplement Table 2). In addition, ant workers ignored non-isolated spiders more frequently than isolated spiders. For full details of each behavioral category, see supplement Table 2.

Comparison between the Spider and the Silverfish

Non-isolated individuals of both myrmecophilous species were attacked infrequently (silverfish: $median_{AI}=0.02$, $N=67$; spider: $median_{AI}=0$, $N=41$). However, we found differences in the social integration of both myrmecophiles, i.e., non-isolated silverfish were attacked more often in the social-acceptance experiment than were non-isolated spiders (PERMANOVA, $P=0.003$). Interestingly, non-isolated silverfish showed higher chemical similarities to host workers (median=50.85, $N=51$) than did non-isolated spiders (median=6.81, $N=42$; PERMANOVA, $P<0.001$). Furthermore, the concentrations of CHCs per surface area were higher in silverfish than in spiders (PERMANOVA; for all comparisons $P<0.001$; supplement Fig. 1).

Individuals of silverfish and spiders also were tested for the same host colony (colony 4). The AIs of non-isolated silverfish, isolated and non-isolated spiders did not differ (PERMANOVA, for all pairwise comparisons $P\geq0.113$), but the AIs of isolated silverfish were higher (PERMANOVA, $P\leq0.003$; Fig. 5). Non-isolated silverfish showed the highest chemical similarity to host workers (median=17.97), followed by isolated silverfish (median=5.40), non-isolated spiders (median=4.30), and isolated spiders (median=0.44; Fig. 5). Notably, isolated silverfish and non-isolated spiders did not differ in chemical similarity to host workers (PERMANOVA, $P=0.203$).

Discussion

The spider *G. maschwitzi* acquired a chemical label from its host ants and showed reduced chemical resemblance to the host after isolation. Both results indicate that the myrmecophilous spider acquires mimetic CHCs rather than biosynthesizing them. In addition, clear differences between the social integration mechanisms of *G. maschwitzi* and the previously studied silverfish *M. ponerophila* were found. Contrary to expectation, spiders remained socially integrated in host colonies, in spite of experimentally reduced chemical host resemblance. Although spiders apparently do not depend as much as silverfish on high chemical host resemblance, the interactions between spiders and ants suggest that they may benefit from chemical mimicry (see below).

Origin of Mimetic CHCs The transfer of the stable-isotope label to spiders, but not to control isopods, demonstrates that the spiders acquire CHCs from the cuticle of host ants, probably through frequent body contact (Witte et al., 2009; see also video Online Resource 1). During these contacts, spiders often rub their legs, first on ant workers, and then on their own body (Witte et al., 2009) or through their own mouthparts (CvB, personal observation). Further evidence for behavioral acquisition of CHCs is demonstrated by isolation. While the CHC concentration of host workers

did not decrease in isolation (see von Beeren et al., 2011b), the concentrations of mimetic CHCs, as well as qualitative chemical host resemblance, decreased in spiders when contact to host ants was prevented by isolation. The loss of host-specific compounds on the cuticle of a myrmecophile, when separated from its host, has also been shown in several other studies (Vander Meer and Wojcik, 1982; Vander Meer et al., 1989; Akino, 2008; von Beeren et al., 2011b). These results point to an acquisition of mimetic CHCs through host contact ('acquired chemical mimicry', sensu von Beeren et al., 2012) rather than through biosynthesis ('innate chemical mimicry', sensu von Beeren et al., 2012). However, the ability of the spiders to downregulate biosynthesis of mimetic CHCs in the absence of host ants cannot be fully excluded.

Acquisition of CHCs through contact with the host (e.g., rubbing behavior) seems to be a common strategy to facilitate integration (Lenoir et al., 2001), and has been suggested to exist in socially parasitic ants (Lenoir et al., 1997; Bauer et al., 2010), as well as other myrmecophiles (Vander Meer and Wojcik, 1982; Vander Meer et al., 1989). Acquisition, rather than innate biosynthesis of the complex host CHC profile, appears to be a more evolutionarily parsimonious mechanism for taxonomically distant myrmecophiles (von Beeren et al., 2011b). Nevertheless, the time during which previously isolated *G. maschwitzi* individuals interacted with host ants in the social acceptance experiment (median=8 min) was apparently insufficient to re-acquire an amount of CHC similar to that of non-isolated individuals.

The Role of Accuracy in Chemical Mimicry Previous studies on the multi-parasite system of the host ant *L. distinguenda* revealed that certain myrmecophiles are aggressively expelled (Witte et al., 2008; von Beeren et al., 2011a). This raises the question of how other myrmecophiles achieve integration without being attacked and killed. If nestmate recognition in ants is based mainly on a good match of colony-specific CHCs, as commonly seems to be the case (van Zweden and d'Ettorre, 2010), then myrmecophiles should either be driven toward chemical resemblance of hosts ('chemical mimicry', sensu von Beeren et al., 2012) or, alternatively, suppress expression of chemicals able to be detected by the host ('chemical hiding', sensu von Beeren et al., 2012), thus circumventing recognition as aliens. Although all compounds on the cuticles of ants potentially could be involved in recognition, we focused on non-polar compounds, because of the generally accepted role of CHCs in ant nestmate recognition. As hydrocarbons were abundant on the cuticles of the spider, and there were only traces of other compounds (supplement Table 1), we assume that the increased inspection of isolated spiders by host ants (see below) was caused predominantly by a mismatch in CHC signature. Evidence for reliance on chemical mimicry of host CHCs recently was found in the myrmecophilous silverfish *M. ponerophila* (von Beeren et al., 2011b). We expected similar results for *G. maschwitzi*; i.e., a lower resemblance of host CHCs should result in increased recognition and ant rejection. Contrary to our expectation, isolated spiders were not attacked more frequently than unmanipulated spiders, regardless of their reduced accuracy in chemical host resemblance, which was manifest by lower concentrations of CHCs, absence of certain CHCs, and increased chemical distance.

A suppression of chemical recognition cues can be an adaptive strategy of parasites that prevents chemical detection by their hosts ('chemical hiding', sensu von Beeren et al., 2012). Chemical hiding, also referred to as chemical insignificance, has been suggested in various parasites of social insects (Lenoir et al., 2001; Lambardi et al., 2007; Nash and Boomsma, 2008; Baer et al., 2009; Kroiss et al., 2009). Due to the low levels of CHCs on the cuticle of *G. maschwitzi*, chemical hiding could be inferred. However, the spider's chemical appearance likely conveys information to the host, as ant behaviors differed to spiders showing either high or low accuracy of chemical host resemblance. Spiders were "ignored" less often, and "antennated" more often, by ant workers after isolation. Hence, it seems reasonable that workers detect spiders chemically and, therefore, chemical hiding does not apply. Instead, we argue that chemical host resemblance of spiders is best considered as mimicry (sensu von Beeren et al., 2012), because spiders most likely "deceive" the host by chemically resembling an entity of interest, specifically a host worker. This type of deception is likely beneficial for the spider for the following reason. As ant antennae carry mechano- and chemo-receptors, antennation may be a form of inspection that precedes subsequent reactions, including aggression (CvB, personal observation). We assume that unmanipulated spiders reduce inspection through "antennation" because of higher chemical resemblance, which thereby reduces the likelihood of subsequent attacks. Indeed, host attacks against spiders occur occasionally (Fig. 1C; also Witte et al., 2009). As isolated spiders were inspected more intensely, it remains to be explained why they were not attacked subsequently.

An evolutionary explanation for the low levels of host aggression against spiders may be a lower selection pressure on the host to recognize and fend off spiders, compared to silverfish, because of differences in virulence between the two myrmecophiles. Both species are kleptoparasites and do not prey on the host (Witte et al., 2008, 2009). Furthermore, there is no evidence of any benefits to the host from the presence of the two myrmecophiles. That both species are occasionally attacked and killed, points to a parasitic relationship, even if there is little impact on host fitness. Differences in virulence of the two kleptoparasites may arise from the amount of host resources they consume. While body sizes are comparable, silverfish occur in higher numbers (about 4–5 times that of spiders; Witte et al., 2008).

Consequently, silverfish may have a greater negative impact on the host. There is evidence that ants can direct their defense specifically against more costly parasites (von Beeren et al., 2011a). Accordingly, lower aggression against spiders might be explained by lower selection on the host to recognize and fend-off spiders.

Social Integration Mechanisms From the perspective of parasites, decreased host defense against spiders, compared to silverfish, suggests that spiders have evolved additional integration mechanisms that achieve and maintain social integration. Two main differences are obvious between *G. maschwitzi* and *M. ponerophila*, one behavioral and the other morphological. The spider's response to host aggression differs from that of the silverfish. Silverfish attempt to escape, whereas spiders remain stationary until ant aggression ceases (Witte et al., 2009). The stationary behavior apparently causes fewer attacks by the ants, perhaps because escape is a typical behavior of prey items. In laboratory colonies, spiders moved freely among host ants, frequently interacting directly with host workers through their long, thin legs (Witte et al., 1999, 2009). Spiders constantly adjust their position (CvB, personal observation), often resulting in them sitting on top of ants (Witte et al., 2009; see also video of supplement). This may further help spiders avoid confrontation with ant workers. Additionally, our impression is that the movements of spiders resemble those of the ants (see also video Online Resource 1), whereas silverfish appear to move quite differently. This behavioral similarity may make tactile recognition of the spiders as alien difficult. We suspect that these behaviors facilitate peaceful interactions between spiders and host ants.

The formicoid habitus (morphological resemblance of ants) of certain myrmecophiles is likely an adaptation directed at predators (Hölldobler and Wilson, 1990; Nelson, 2011). However, certain morphological traits of myrmecophiles, such as cuticular surface structure, also may serve as an ancillary integration mechanism for host deception (Hölldobler and Wilson, 1990). Tactile mimicry, also called Wasmannian mimicry after its first description (Wasmann, 1895), means that a worker's tactile inspection cannot distinguish the body constitution and the surface structure between a mimic and its model (Gotwald, 1995). Myrmecophilous mites of the genus *Planodiscus*, for example, resemble the surface structure of their host to a high degree (Kistner, 1979). We hypothesize that the spider's body shape and surface, which is covered with setae, sufficiently resembles the host's body, while the silverfish have a completely different body constitution (Fig. 2 supplement). The limuloid, scaled body of the silverfish, with short appendices (antennae, cerci and praecerci) and retractable head, are probably adaptations to escape from ant attacks, rather than adaptations to interact peaceably with them. As spiders mainly interact with workers using their thin, long, setae-covered legs, we suspect that the legs play an important role in deceiving the host, with workers misidentifying spiders as nestmates. Ants frequently groom nestmates (Hölldobler and Carlin, 1989), and grooming behavior can also be observed directed toward spiders (see Table 2 of supplement). Given that the myrmecophile's body parts that frequently interact with the host carry potential recognition cues that need to be mimicked for favorable recognition (Gotwald, 1995), we hypothesize a central role for the spider's legs.

Transmission between Colonies The spider's lower reliance on chemical mimicry also may be beneficial for their dispersal. Invading new host colonies (horizontal transmission) is a difficult task for myrmecophiles that rely on chemical integration mechanisms, because ant colonies possess a colony-specific odor (van Zweden and d'Ettorre, 2010). Since high accuracy of chemical mimicry is necessary for social acceptance in the silverfish, this myrmecophile is likely to face greater difficulties in transmitting horizontally between different colonies than will the spiders. Indeed, spiders have been exchanged successfully between *L. distinguenda* colonies with little or no increased aggression, whereas silverfish were always killed during exchange experiments (Witte et al., 2009). Since new *L. distinguenda* colonies most probably bud from old nests, as is the case in other army ants (Kronauer, 2009), silverfish most likely are mainly limited to vertical transmission from mother to daughter colonies.

In summary, combinations of several integration mechanisms, e.g., chemical strategies, acoustical, behavioral, and morphological adaptations, allow myrmecophiles to integrate with their host ants. As demonstrated here, the degree of dependency on some of these mechanisms may differ between myrmecophilous species. Thus, the more integration mechanisms are studied in combination, the more reliably results will demonstrate which adaptations are most important in the social integration of myrmecophiles.

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Identification of Male-Produced Aggregation Pheromone of the Curculionid Beetle *Sternechus subsignatus*

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Abstract Analyses of the headspace volatiles produced by males and females of *Sternechus subsignatus* Boheman (Coleoptera: Curculionidae) revealed seven male-specific compounds. The major component was (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol, and the minor components were 1-(2′-hydroxyethyl)-1-methyl-2-isopropenylcyclobutane (grandisol), 7-methyl-3-methyleneoct-6-en-1-ol, (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol, (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde, and (*E*)-2-(3,3-dimethylcyclohexylidene) acetic acid. The latter compound is described for the first time as a natural product. Only four of the seven identified compounds showed electrophysiological activity. Enantioselective gas chromatography showed that the natural grandisol is the (1*R*,2*S*)-stereoisomer. The major component, (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol, attracted *S. subsignatus* in olfactometer bioassays. Studies are in progress to evaluate the biological activity of the major component and the EAD-active mixture under field conditions.

Keywords Semiochemicals · Grandlure · Grandisol · GC-EAD · Coleoptera · Curculionidae

Introduction

The soybean stalk weevil, *Sternechus subsignatus* Boheman (Coleoptera: Curculionidae: Molytinae), is native to Brazil where it is broadly distributed (Rosado Neto, 1987). These weevils prefer legumes, such as *Glycine max* (L.) Merrill, as hosts. The adult weevils girdle the main stem cutting the epidermis and cortex of soybean. Larvae penetrate the stem and feed on the medulla forming a gall where the plant was girdled. During early developmental plant stages, the presence of these insects can cause death or eventual decrease in production (Hoffmann-Campo et al., 1990, 1991). Traditional methods of control using contact insecticides are not effective because adults are usually underneath leaves, in the soil or under crop residues. In addition, immature stages develop inside stems limiting the action of the insecticides (Silva et al., 1998).

Pheromones of weevils offer a promising method of direct control through mass trapping (Francke and Dettner, 2005; Tinzaara et al., 2005). Male-produced aggregation pheromones have been reported for many weevils, and generally are used for both host-finding and bringing the sexes together (Bartelt, 1999). Previous research with *S. subsignatus* showed males produce volatiles attractive to both sexes (Ambrogi and Zarbin, 2008); however, the responsible compounds were unknown. Here, we report the isolation, identification, and laboratory bioassay of the male-produced aggregation pheromone of *S. subsignatus*.

Methods and Materials

Source of Insects The colony was established from *Sternechus subsignatus* adults of unknown age and mating status collected from soybean fields located in Fazenda Rio Grande, Parana, Brazil, in January 2010. Sexes were separated based on the sexually dimorphic shape of the fore and mid-tibia (Rosado Neto, 1987), and kept separately in plastic boxes (20×20×20 cm) under a L12:D12 photoperiod at 25±2°C and 60±5% relative humidity. Adults were provided with fresh soybean-stem cuttings.

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Collection of Volatiles Groups of 10 males and 10 females were maintained separately in all-glass aeration chambers (33 cm high×3.5 cm outside diam) with two cuttings of fresh soybean stems (8 cm). Emitted volatiles were collected every 24 hr for 30 d (N=30), and trapped on glass columns (8 cm high×1 cm diam) with 0.8 g of Super Q (Alltech, USA) (Zarbin et al., 1998). Charcoal-filtered, humidified air was pushed though the aeration system (1.0 L/min). Adsorbed aeration volatiles were eluted with 1 ml of distilled hexane, and adsorbent traps were changed after 10 collections. The daily extracts were concentrated to 10 μl (1 insect per 1 μl) under an argon stream.

Analytical Procedures Gas chromatographic (GC) analyses were conducted to detect sex-specific compounds using a Shimadzu GC-2010. The instrument was equipped with a DB-5 column (30 m×0.25 mm i.d. and 0.25 μm film thickness; J&W Scientific Inc., USA), and 1 μl of extract was injected in the splitless mode at an injector temperature of 250°C. The column oven was held at 50°C for 1 min, raised to 250°C at 7°C/min and kept constant for 10 min. Helium was used as the carrier gas at a column head pressure of 170 kPa. The Kovats indexes (KI) of the sex-specific components were calculated using a series of saturated C10-C26 hydrocarbons co-injected with the samples in the same DB-5 GC column (Kovats, 1965). The ratio of the seven male-specific compounds was calculated based on the area of the GC peaks of twenty extracts.

Chiral GC of grandisol was performed using an HP-Chiral 20B (30×0.25 mm i.d. and 0.25 μm film thickness) at a constant temperature of 150°C for 170 min with a gas pressure of 160 kPa.

Extracts were analyzed by GC-electroantennographic detection (GC-EAD) using a Shimadzu GC-2010, and a Syntech electroantennography system (Hilversum, The Netherlands). The same column as described previously was used, and 1 μl of extract was injected in the splitless mode at an injector temperature of 250°C. Temperature was maintained at 70°C for 1 min, raised to 120°C at 4°C/min, elevated to 250°C at 10°C/min, and held for 10 min. The column effluent was split 1:1, with one part going to the FID at a temperature of 270°C and the other through a heated transfer line into a humidified airstream (280 ml/min) directed to antennal preparation of a female or male. Antennae of 30 males and females were excised. The base and distal part of the antenna was fixed between two stainless steel electrodes using electrically conductive gel (Signa gel, Parker Labs., NJ). GC-EAD recordings were analyzed with Syntech GC-EAD32 software (version 4.6 Hilversum, The Netherlands).

GC-mass spectrometry (GC-MS) analyses of extracts from males were conducted using a Shimadzu QP5050A MS system equipped with a DB-5 capillary column as described above was used in the electron impact mode at 70 eV.

Chemicals 7-Methyl-3-methyleneoct-6-en-1-ol (γ-isogeraniol), (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol, (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol, grandisoic acid, and a 1:1 mixture of (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde were obtained from ChemTica Int., Costa Rica. A small amount of (*E*)-2-(3,3-dimethylcyclohexylidene)-acetic acid was obtained by oxidation of the corresponding aldehyde using silver oxide *in situ* in aqueous sodium hydroxide (NaOH, 10% m/v) (Pepperman, 1981). Then, aqueous NaOH was added to an aqueous solution of silver nitrate (10% w/v), agitated until achieving complete precipitation of the silver oxide, filtered, and washed with distilled water. A suspension of silver oxide (20 mg) and NaOH (10% w/v, 30 ml) in 1 ml of water was vigorously stirred with a magnetic stirrer. The mixture of 2-(3,3-dimethylcyclohexylidene)-acetaldehyde (100 μl) isomers was added to the silver oxide suspension in small portions over 30 mins, and then stirred for 3 hr. The colloidal silver was filtered and washed with water. The filtrate was acidified with concentrated HCl, the precipitate was collected, and washed with water. The filtrate was extracted with diethyl ether, and the organic phase was extracted with $NaHCO_3$ solution to remove the carboxylic acid as its salt. The aqueous solution was acidified with HCl solution until it reached pH 3, and then extracted with diethyl ether. The ether solutions were dried over anhydrous Na_2SO_4. These procedures were all performed at room temperature. Pure (1*R*,2*S*)-grandisol was kindly provided by Prof. Kenji Mori, Tokyo University, Japan (Mori and Fukamatsu, 1992).

Microderivatizations The latter extract (100 μl) diluted in 200 μl of hexane was reduced over Pd/C (10%, 1 mg) at room temperature under a hydrogen (25 psi) in a Parr apparatus for 2 hr. After filtration, the filtrate was concentrated and analyzed by GC (Zarbin et al., 2000).

For esterification, crude extract (100 μl) was diluted in hexane (100 μl), and an ethereal solution of diazomethane was added at 0°C until the solution turned yellow. The mixture was stirred at room temperature for 30 min, and directly analyzed by GC (Zarbin et al., 2000)

Laboratory Bioassays Bioassay were performed to determine the attractiveness of the major *S. subsignatus* male-specific component in a glass Y-tube olfactometer 4 cm in diam, 40 cm long with 20-cm long arms operated at 4 L/min flow of humidified and charcoal filtered air (Zarbin et al., 2007a). The behavior of 43 males and 38 females was observed individually for 20 min. The insect was considered to have a chosen a particular arm when the weevil traveled 5 cm past the division of the basal tube. Age and mating status of bioassayed beetles were not controlled. The main component present in the extracts was tested, along with a host plant (HP) extract (Ambrogi and Zarbin, 2008). For each replication, we used a piece of filter paper (2×2 cm)

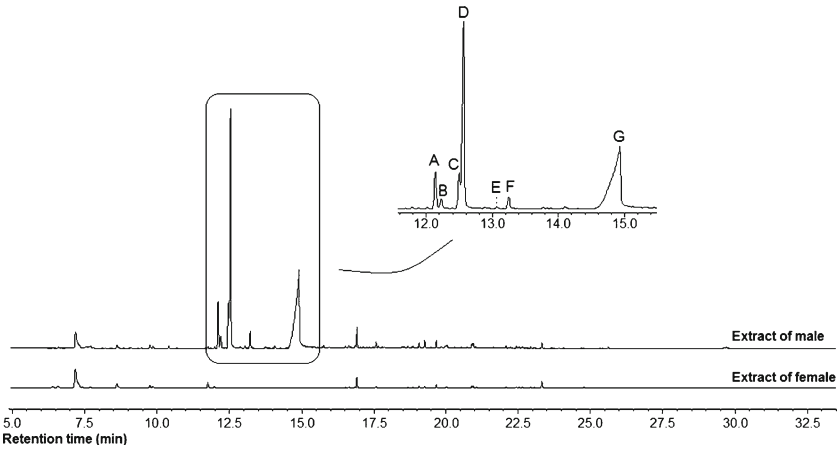

Fig. 1 Gas chromatogram of volatile extracts obtained from aeration of 10 male and females of *Sternechus subsignatus* for 24 h, showing seven male-specific compounds, **A**–**G**

loaded with 5 μl of the main extract component at 1000 ppm. Filter paper containing hexane plus HP was used as a control.

Statistical Analysis The results of the olfactometer bioassays were analyzed with a binomial test using the software R (Vienna, Austria) (R Development Core Team, 2005). Individuals that did not make a choice were excluded from the statistical analysis.

Results and Discussion

Chromatographic profiles of headspace volatiles of male and female *S. subsignatus* revealed the presence of seven male-specific compounds (**A**–**G**) present in a ratio of 10:3:7:41:0.2:2:37 (Fig. 1).

Analysis of the extracts of *S. subsignatus* males via GC-EAD consistently showed that, of the seven male-specific components, four activated the antennae of the male and female weevils. The responses of both sexes were similar for the active components, **A**, **D**, **F**, and **G** (Fig. 2).

The chemical structures of the seven male-specific components were identified based on fragmentation patterns in GC-MS, Kovats indexes, comparison with authentic standards, micro-derivatizations, and synthesis. GC-MS analyses showed that compound **A** (rt: 12.14 min; KI: 1213) generated a molecular ion at *m/z* 154, and a base peak at *m/z* 68, indicating that the compound could be a monoterpenoid. Analysis of the fragmentation pattern [*m/z* (%): 154 (1)M, 139(5), 109(55), 68(100), 41(37)], and comparison of the spectrum of the natural product with one previously reported as a pheromone component from another species in the family Curculionidae studied in our laboratory, *Pseudopiazurus obesus* (Zarbin et al., 2007b), suggested that compound **A** was grandisol. This hypothesis was confirmed after co-injection with an authentic grandisol standard.

To determine the absolute configuration of the isolated grandisol, enantioselective gas chromatography was used with a chiral stationary phase column. Comparison of the retention time of the natural grandisol with those of racemic and chiral synthetic standards confirmed that the natural product is (1*R*,2*S*)-1-(2′-hydroxyethyl)-1-methyl-2-isopropenylcyclobutane; i.e. (1*R*,2 *S*)-grandisol (Fig. 3).

The mass spectrum of compound **B** (rt: 12.22 min; KI: 1217) exhibited the following diagnostic ions: [*m/z* (%): 154 (1) M, 111(42), 109(45), 67(45), 41(100)]. Comparison with the NIST mass spectral library suggested geraniol or nerol as possible structures for this compound. However, synthetic standards of these compounds did not coelute with **B** (Supplemental Material 1), although the mass spectra of these two synthetic compounds were very similar to that of the natural product. Therefore, we considered the isomer, γ-isogeraniol; this compound also seemed plausible because

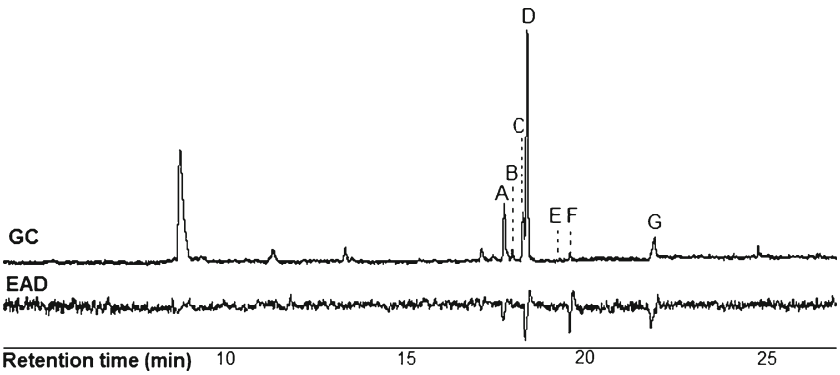

Fig. 2 Simultaneously recorded GC-FID and GC-EAD responses using a *Sternechus subsignatus* antenna stimulated by an extract of volatiles obtained during 24 h from 10 males

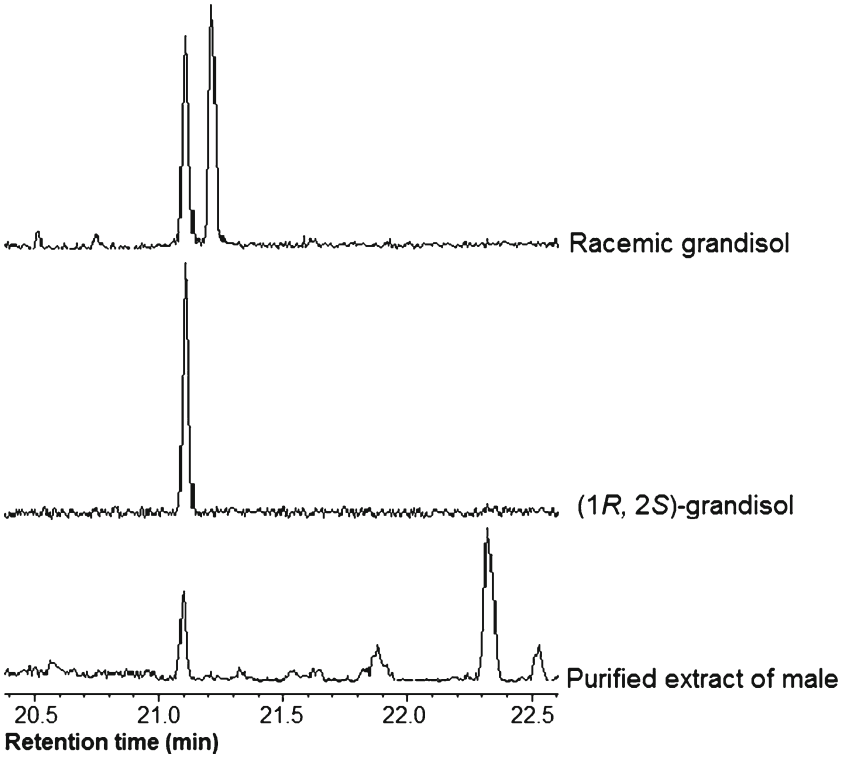

Fig. 3 Determination of the absolute configuration of grandisol released by males of *Sternechus subsignatus* using enantioselective gas chromatographic separation on a β-CD capillary column (HP-Chiral 20B)

it has been postulated to be the biosynthetic precursor for certain pheromone components of the boll weevil, *Anthonomus grandis* (Bedoukian and Wolinsky, 1975). The identification was confirmed by matching the mass spectrum and the retention times of the natural products with those of an authentic γ-isogeraniol.

Compound **C** exhibited a retention time of 12.50 min and a KI of 1231, whereas compound **D**, the major component of the mixture, exhibit a retention time of 12.58 min and a KI of 1235. The mass spectra and the retention times of components **C** and **D** were very similar, suggesting that they were isomers (**C** [*m/z* (%): 154(8)M, 136(75), 121(65), 69 (89), 41(100)]; **D** [*m/z* (%): 154(6)M, 136(62), 121(52), 93 (87), 69(81), 41(100)]). These fragmentation patterns were consistent with those of (*Z*)-2-(3,3-dimethylcyclohexylidene)-ethanol and (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol, and the identification of these compounds was confirmed by co-injection with authentic standards.

Compounds **E** and **F** exhibited retention times of 13.06 min and 13.23 min, and KIs of 1259 and 1268, respectively. The fragmentation patterns of compound **E** [*m/z* (%): 152(56)M, 137(100), 109(58), 41(96)], and compound **F** [*m/z* (%): 152(66)M, 137(27), 109(100), 69(43), 41(96)] were very similar as well. The variation of the mass of the molecular ion by two mass units as compared to the previously identified compounds (**C**, **D** (*m/z* 154) and **E**, **F** (*m/z* 152)), suggested that **E** and **F** were the corresponding aldehydes of **C** and **D**. The structures of compounds **E** and **F** were confirmed to be (*Z*)- and (2*E*)-2-(3,3-dimethylcyclohexylidene)-acetaldehyde, respectively, by co-injection with synthetic standards.

The peak shape associated with compound **G** suggested that it was a carboxylic acid (15.02 min rt, and 1359 KI). We

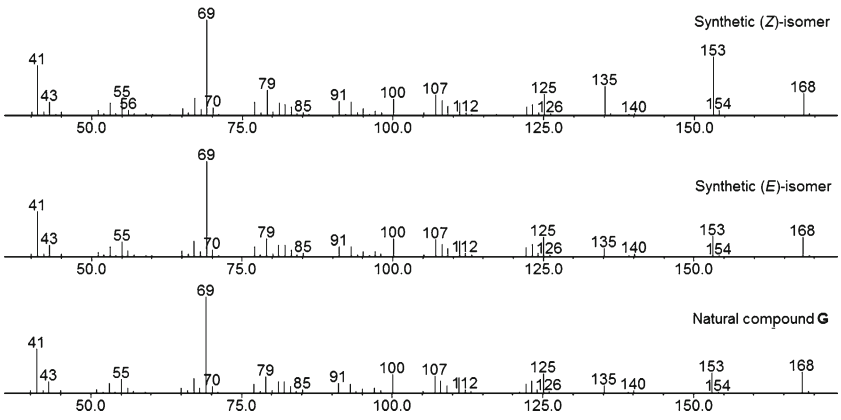

Fig. 4 Mass spectra of synthetic (*Z*)- and (*E*)-2-(3,3-dimethylcyclohexylidene)-acetic acid, and the natural compound **G** of the aggregation pheromone of *Sternechus subsignatus*

hypothesized the compound could be grandisoic acid, as this molecule had been previously reported as a component of the male-produced aggregation pheromone of another weevil, *Conotrachelus nenuphar*, within the same subfamily (Molytinae) as *S. subsignatus* (Eller and Bartelt, 1996). However, **G** did not match the retention time or mass spectrum of grandisoic acid even though the molecular ion at *m/z* 168 was consistent with the natural product.

To unambiguously determine the structure of **G**, microderivatizations of the natural product were carried out. Initially, the potential acid natural product was esterified with diazomethane, forming a product with a molecular ion (*m/z* 182) that was clearly its methyl ester. The resulting ester was further hydrogenated under H_2 pressure in the presence of Pd/C as catalyst, creating a new product with a molecular ion at *m/z* 184. The addition of only two mass units was evidence of the presence of only one double bond, and the base peak observed for the hydrogenated product (*m/z* 74 - McLafferty rearrangement) suggested that the system should be conjugated (Supplemental Material 2). These data led us to suspect that **G** was an acid derived from alcohols **C** or **D**. A synthetic mixture of the (*Z*)- and (*E*)-acids was obtained by the oxidation of the corresponding aldehydes (Pepperman, 1981). The retention time of the natural product was coincident with the second peak of the isomeric mixture of synthetic acids. As the *E*-isomers of the corresponding alcohols (**C** and **D)** and aldehydes (**E** and **F)** consistently had retention times greater than those of the *Z*-isomer, the *E* geometry was most likely for the natural acid. Additionally, the *m/z* 153 (M-15) fragment exhibited greater intensity for the isomer that did not match the retention time of the natural acid. This fact, according to Henson et al. (1976), is explained more facile loss of the CH_3 group of the *Z*- vs. the *E*-isomer (Fig. 4). The greater intensity of this ion also can be clearly seen when comparing the mass spectra of the *Z*- and *E*-isomers of aldehydes **E** and **F** (Supplemental Material 3). Thus, the chemical structure **G** is (*E*)-2-(3,3-dimethylcyclohexylidene)-acetic acid. This is the first time that (*E*)-2-(3,3-dimethylcyclohexylidene)-acetic acid has been isolated as a natural product, although this compound was reported as a decomposition product of the boll weevil pheromone, grandlure (Henson et al., 1976).

The identities of all the *S. subsignatus* pheromone candidates (Fig. 5) were confirmed by the co-injection of authentic standards on two other GC-columns (RTX-wax and HP-1). With the exception of compounds **B** and **G**, the male-specific compounds extracted from *S. subsignatus* have been found to be aggregation pheromone components in several other species in the family Curculionidae, such *Pseudopiazurus obesus* (Zarbin et al., 2007b, 2010), *Anthonomus eugenii* (Eller et al., 1994), *A. grandis* (Tumlinson et al., 1969), *A. rubi* (Innocenzi et al., 2001), *Curculio caryae* (Hedin et al., 1997),

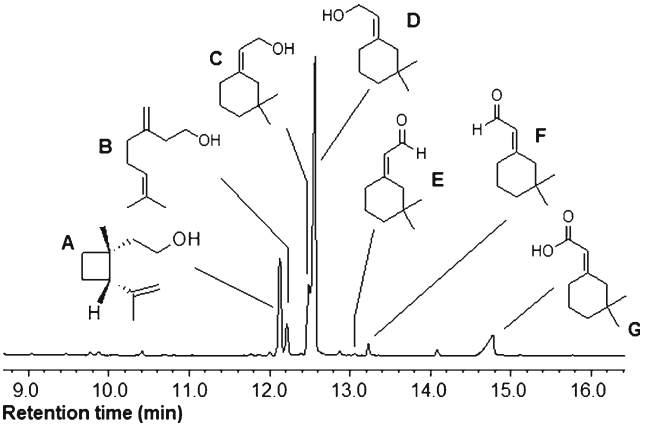

Fig. 5 Chemical structures of the seven male-specific compounds produced by *Sternechus subsigantus*

Bothynoderes punctiventris (Tóth et al., 2007), and *Pissodes* spp. (Booth et al., 1983; Philips et al., 1984).

Table 1 shows the behavioral responses of *S. subsignatus* males and females in a Y-tube olfactometer when stimulated with the major component **D** (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol. The main compound, with host plant volatiles, was more attractive to males (P=0.01) and females (P=0.03) than to the control (hexane plus host plant), indicating that the major component was sufficient to attract *S. subsignatus* in significant numbers. Similar results were obtained in field tests for a longhorned beetle, *Rosalia funebris,* where only the major component of the pheromone blend was necessary for a high level of attraction (Ray et al., 2009).

Even though the major component alone attracted both sexes of *S. subsignatus* in the olfactometer, several insects did not respond to the stimulus, suggesting a blend of all EAD-active components may be needed to produce an optimal response. The seasonality of this weevil and the difficulty of rearing it under laboratory conditions prevented us from performing additional laboratory bioassays to test the importance of the other EAD-active compounds. These complementary laboratory and field studies will be performed during the next period of adult emergence.

Table 1 Responses of male and female *S. subsignatus* adults to the major male-specific volatile, (*E*)-2-(3,3-dimethylcyclohexylidene)-ethanol, in a Y-tube olfactometer

Sex	Main compound+HP	Control	No choice
Male (N=43)	16*	4	23
Female (N=38)	17*	6	15

*Statistically significant differences, *Binomial test,* P<0.05

Individuals that did not made a choice were excluded from the statistical analyses

HP = soybean stem volatiles; Control = Hexane + HP

In summary, our results demonstrated that males of *S. subsignatus* produced seven sex-specific compounds, but only four of these were stimulatory to the insects' antennae. The major component alone was attractive to both sexes in the olfactometer. This pheromone is potentially useful in the field for monitoring and control. Aditional information gleaned in forthcoming field and laboratory tests with the minor EAD-active components should allow us to adjust the blend to increase the attractiveness of the pheromone mixture.

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Defensive Secretions of the Carabid Beetle *Chlaenius cordicollis*: Chemical Components and their Geographic Patterns of Variation

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Abstract The defensive secretion of the ground beetle *Chlaenius cordicollis* is predominantly 3-methylphenol. Adult *C. cordicollis* were collected in Pennsylvania and Manitoba and induced to discharge defensive secretion in a vial. The headspace was sampled by solid phase microextraction, and samples were analyzed by gas chromatography–mass spectrometry. Five alkylphenolic compounds were detected: all beetles secreted 3-methlyphenol, 2,5-dimethylphenol, and 3-ethylphenol, and most beetles from each locality secreted detectable amounts of 2,3-dimethlyphenol and 3,4-dimethylphenol. In about 80% of beetles, we detected small amounts of the alkoxyphenolic compounds 2-methoxy-4-methylphenol and 2-methoxy-5-methylphenol. Multivariate compositional analysis of relative peak areas of alkylphenolic compounds revealed geographic variation and sexual dimorphism in defensive secretions. Compared with samples from Manitoba, relative peak areas of samples from Pennsylvania were lower for 2,3-dimethylphenol and higher for 3-methylphenol. Sexual dimorphism was detected only in Manitoba where, compared with samples from males, relative peak areas for samples from females were higher for 2,5-dimethylphenol and lower for 3-ethylphenol. This is the first report of geographic variation in defensive secretions of carabid beetles, and it demonstrates the need for knowledge of patterns of variation before characterizing the defensive secretions of a species as a whole.

Keywords Phenolics · Solid phase microextraction · Coleoptera · Carabidae · Geographic variation · Sexual dimorphism · Log-ratio analysis

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Introduction

Carabid beetles have paired pygidial glands that produce defensive secretions (Dierckx, 1899; Forsyth, 1972 and references therein). One of the first chemical characterizations of the defensive secretion of a carabid was that for *Chlaenius cordicollis* Kirby by Eisner et al. (1963). These authors reported the secretion to consist of 3-methylphenol (*m*-cresol) and a number of unidentified minor constituents. As an aid to phylogenetic studies, the major components of defensive secretions have since been characterized for several hundred carabid species (Moore and Wallbank, 1968; Schildknecht et al., 1968; Kanehisa and Murase, 1977; Moore, 1979; Will et al., 2000). Within Carabidae, defensive secretions dominated by 3-methylphenol are restricted to the subfamily Panageinae and to four genera of the subfamily Chlaeniinae; these include *Chlaenius* Bonelli (*sensu stricto*), but exclude the quinone-producing genus *Chlaeniellus* Reitter (Bousquet, 1987), which some regard as a subgenus of *Chlaenius*. To date, within the genus *Chlaenius* (*sensu stricto*), 3-methylphenol has been reported as the major defensive component for 22 species (Moore and Wallbank, 1968; Schildknecht et al., 1968; Kanehisa and Murase, 1977; Moore, 1979; Balestrazzi et al., 1985). Several of these studies noted the presence of other compounds, although these were rarely identified. Exceptions are Moore (1979), who reported small proportions of several

unsaturated hydrocarbons from *C. australis* Dejean and *C. greyanus* White, and Balestrazzi et al. (1985), who reported phenolic and aliphatic minor components from *C. velutinus* Duftschmidt.

Although Blum (1981) and Pasteels et al. (1983) drew attention to the potential for intra-specific variation of defensive secretions, studies of such variation in carabid beetles have been few. Attygalle et al. (1991) reported sexually dimorphic defensive secretions in one species of carabid beetle, but Roach et al. (1979) and Davidson et al. (1989) found little within-population variation in the carabids they studied. Geographic variation in defensive secretions has been documented in chrysomelid (Daloze and Pasteels, 1979; Eggenberger and Rowell-Rahier, 1991) and tenebrionid (Tschinkel, 1975) beetles, as well as in termites (e.g., Valterová et al., 1989; Chuah, 2007). There have been no studies on geographic variation in defensive secretions of carabid beetles, or on any form of variation in the defensive secretions of species of the genus *Chlaenius.*

Adults and larvae of *C. cordicollis* live near large lakes and rivers on open shorelines subject to wave action (Bell, 1960), where they feed on dead or injured insects (Larochelle and Larivière, 2003). Frequently the habitat is a stony beach with limestone slabs, under which the nocturnal adults shelter during the day (Larochelle and Larivière, 2003). The peak of larval abundance is July, and newly molted adults are evident from July to September; adults are the overwintering stage (Larochelle and Larivière, 2003). The westernmost limits of *C. cordicollis* are Manitoba, Iowa, and Minnesota, and the range extends east to New Brunswick and the New England States, and south to Arkansas and North Carolina (Bell, 1960; Bousquet and Larochelle, 1993; Majka et al., 2007).

The objectives of this study were to investigate the nature of minor components of the defensive secretion of *C. cordicollis* and to determine whether any geographic or sexual differences in composition of the defensive secretion exist. For this, we stimulated individuals from widely separated populations to release defensive secretions, and sampled and analyzed the headspace by solid phase microextraction (SPME) and gas chromatography–mass spectrometry. Multivariate compositional analysis was used to discern patterns of variation.

Methods and Materials

Beetle Collection and Maintenance In August and September 2011, non-teneral adult *C. cordicollis* were collected by turning over rocks and limestone slabs, as recommended by Larochelle and Larivière (2003). Collections were made on the shores of the Delaware River near Easton, Pennsylvania (12 August: 40°43′N, 75°11′W; 17 September: 40°41′N, 75°12′W) and at two sites on the east coast of Hecla Island, Lake Winnipeg, Manitoba, Canada (1, 11, and 17 September: 51°4′N, 96°41′W and 51°5′N, 96°41′W). The sex of beetles was determined on the basis of the morphology of the basal pro-tarsal segments, which are slender in females and dilated in males (Lindroth, 1969). Voucher specimens from both localities were deposited in the J.B. Wallis/R. E. Roughley Museum of Entomology, University of Manitoba.

In the laboratory, beetles were kept at room temperature and lighting conditions in aquaria set at an angle to simulate a beach. Aquaria contained a mixture of sand, gravel, and limestone pebbles taken from the sites of beetle collections, and had standing water at the lower end. Beetles from Pennsylvania initially were fed crumbled cat treats (Purina Whisker Lickin's Tender Moments salmon flavor, Nestlé Purina PetCare Company, St. Louis, MO, USA); after 17 September 2011, they were fed a mixture of freeze-dried mealworms, crickets and river shrimp (Fluker's Medley Treat for Aquatic Turtles, Fluker Farms, Port Allen, LA, USA). Those from Manitoba were supplied with freeze-dried mealworms (Fluker) and powdered Rollover Premium Cat Treats (High River, AB, Canada).

Chemicals Standards of 3-methylphenol, 3-ethylphenol, 2,3-dimethylphenol, 2,4-dimethyphenol, 2,5-dimethylphenol, 3,4-dimethylphenol, 3,5-dimethylphenol, and 2-methoxy-4-methylphenol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation A Varian (Walnut Creek, CA, USA) Saturn 2100T gas chromatograph-mass spectrometer (GC-MS), equipped with a FactorFour VF-5 ms column (30 m, 0.25 mm i.d., 0.25-μm, Varian, Walnut Creek, CA), was used for analyses**.** Two cm-long dual-layered SPME fibers, coated with highly cross-linked 50/30-μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), were purchased from Supelco (Bellefonte, PA, USA). Before use, fibers were cleaned by leaving them in the GC-MS injector port at 260°C for 30 min.

Sample Collection Headspace SPME sampling mostly was conducted in the laboratory at room temperature. On 17 September 2011, headspace sampling was carried out in the field at the time of beetle collection, both in Manitoba (air temperature 16°C) and Pennsylvania (20°C). To obtain a sample, a beetle was placed into a clean 20 ml glass vial. If a defensive spray was produced during transfer (indicated by a strong smell), the vial was sealed immediately, and the SPME fiber inserted through the septum and exposed to the headspace. If no odor was apparent on transfer, beetles were encouraged to discharge defensive secretions by gently pinching an appendage with forceps (Eisner et al., 1963).

Samples from beetles that did not produce an odor also were taken for comparison. After 5 min of headspace collection, the fiber was removed. SPME fibers used to collect headspace samples for beetles from Manitoba were shipped to Pennsylvania for analysis by GC-MS. Batches of fibers used in Manitoba and in Pennsylvania were compared by simultaneous sampling of the headspace above the same insect in the laboratory in Pennsylvania. Similar simultaneous sampling was used to assess the effect of simulated shipping conditions.

Gas Chromatography–Mass Spectrometry Fibers were desorbed over 30 sec. of a splitless injection at 250°C. Two different column oven temperature programs were used. Before September 10: held at 50°C for 3 min., ramped to 250°C at 10°C.min^{-1}, and held for 7.0 min (total run time 30 min.). This program, however, did not sufficiently resolve peaks of alkoxyphenolic compounds and so, after September 10, a different program was used: held at 50°C for 3 min., ramped to 145°C at 5°C.min^{-1}, then ramped to 250°C at 25°C.min^{-1}, and held for 3.8 min (total run time 30 min). Helium was the carrier gas at a constant flow of 1.2 ml.min^{-1}. Between runs, the SPME fiber was cleaned for 10 min in the injector port at 260°C to ensure that there was no carry-over from one beetle to the next. With the exception of 2-methoxy-5-methylphenol, all chemical compounds were identified based on comparisons of mass spectra and retention times with those of standards. As no standard was available for 2-methoxy-5-methylphenol, its identification was tentative, based on mass spectrum and retention time similarity to that of its isomer, 2-methoxy-4-methylphenol.

Data Analysis Chromatographic peaks from alkylphenol compounds were integrated using the characteristic *m/z* 107 in an extracted ion chromatogram. Alkoxyphenol peaks were integrated using the characteristic *m/z* 138. Absolute amounts of headspace volatiles detected by SPME were variable and so, for each sample, the relative peak area of each alkylphenolic and alkoxyphenolic compound was calculated by dividing the individual peak area by the total peak area of all alkylphenolic compounds, and expressing the result as a percentage. Thus, for alkylphenolic compounds, percentages summed to 100%, regardless of whether GC-MS analysis allowed resolution of alkoxyphenolic compounds. When peaks for alkoxyphenolic compounds were resolved, their relative peak areas were on the same measurement scale as that for alkylphenolic compounds in the same sample.

Multivariate compositional analysis was performed on the relative peak areas of the alkylphenolic compounds only for samples in which the total number of counts of these compounds exceeded 10^4. Percentage composition data sum to 100, and so are not independent of each other, and cannot be formally analyzed by conventional statistical approaches. Therefore, they were subjected to log-ratio transformation for compositional analysis (Aitchison, 1984). As a small number of relative peak areas had a zero value, 0.1 was added to all percentages before transformation. Patterns of compositional variation were investigated by ordination using Canoco 4.5 (Ter Braak and Šmilauer, 2002), in which double centering following log transformation produces a log-ratio transformation, with the ratio being that of each value to the mean of all values. The initial ordination was a principal components analysis. The canonical equivalent, redundancy analysis (also known as reduced rank regression), then was used to assess the significance of the relationship of known independent variables to the patterns of compositional variation (Ter Braak and Looman, 1994). Independent variables were beetle sex, locality of collection, and the interaction of sex with location. These were added by a step-up procedure, with the criterion for inclusion of $\alpha \leq 0.05$, as assessed by Monte Carlo permutations.

Pearson correlation coefficients among relative peak areas were calculated for all pairs of all alkylphenolic and alkoxyphenolic compounds. As correlations could result from differences among or between sexes and locality of collection, partial correlations (Sokal and Rohlf, 1995) also were calculated to assess within-group relationships after controlling for among-group differences.

Results

In almost all samples, five alkylphenolic compounds were detected: 3-methylphenol, 3-ethylphenol, 2,3-dimethylphenol, 2,5-dimethylphenol, and 3,4-dimethylphenol. However, there was no evidence for the presence of 2,4-dimethylphenol or 3,5-dimethylphenol in any sample. In addition, two alkoxyphenols, 2-methoxy-4-methylphenol and 2-methoxy-5-methylphenol, were identified in a majority of samples run with the second temperature program. The maximum total peak area of the alkylphenolic compounds was 1.6×10^8 counts, and a total of 32 headspace samples exceeded the minimum criterion of 10^4 counts. Of these samples, 22 were from Pennsylvania and 10 from Manitoba. Samples below the minimum cut-off either had no detectable volatiles (i.e., from beetles that did not produce odor) or had detectable, but low, amounts of only 3-methylphenol and no detectable minor components.

All samples exceeding the minimum criterion contained detectable concentrations of 3-methylphenol, 2,5-dimethylphenol, and 3-ethylphenol. All but four samples (two from each source locality, 3 females and 1 male) had detectable amounts of 3,4-dimethylphenol. The male from Manitoba for which no 3,4-dimethylphenol was detected also had no detectable 2,3-dimethylphenol. In some cases, failure to

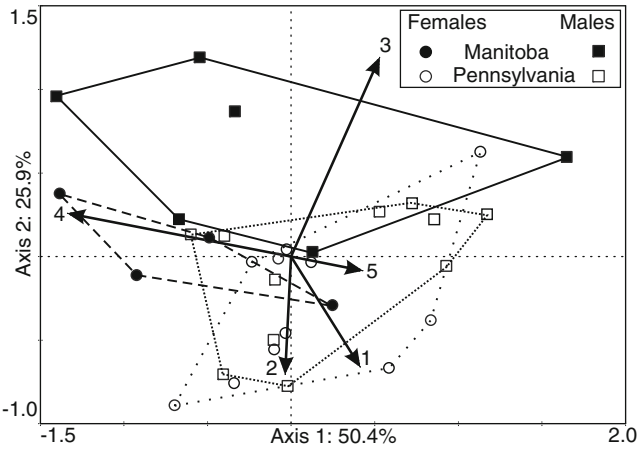


Fig. 1 Principal components analysis of log-ratio transformed relative peak area [%] of alkylphenol compounds in headspace samples from *Chlaenius cordicollis* from Manitoba and Pennsylvania. Points for samples of each sex for each locality are enveloped in a convex hull. 1: 3-methylphenol; 2: 2,5-dimethylphenol; 3: 3-ethylphenol; 4: 2,3-dimethylphenol; 5: 3,4-dimethylphenol

detect compounds was associated with low total volatile capture by SPME: three of the samples with no detectable 3,4-dimethylphenol, including the sample with no detectable 2,3-dimethylphenol, were among the seven with the lowest total peak area and had $<5\times10^5$ counts. However, the remaining individual for which 3,4-dimethylphenol was not detected had a relatively high total peak area (3.1×10^7 counts). Of the 23 samples in which peaks for alkoxyphenols could have been resolved, 19 had detectable levels of both 2-methoxy-4-methylphenol and 2-methoxy-5-methylphenol.

The first two axes of the principal components analysis of alkylphenolic compounds (Fig. 1) explained 76.3% of the variance. The ordination tended to separate samples into three groups in the ordination space: males from Manitoba, females from Manitoba, and both sexes from Pennsylvania. The significance of these groupings was confirmed by redundancy analysis in which two canonical axes were highly significant (Monte Carlo test: $F=4.32$, $P=0.002$); significant independent variables were source locality ($F=5.38$, $P=0.004$) and the interaction of beetle sex with Manitoba ($F=2.90$, $P=0.05$). When simultaneous headspace samples from batches of fibers from Manitoba and Pennsylvania were plotted in the ordination space created by the principal components analysis, they overlapped with each other. Similarly, simulated transportation conditions did not change the location of samples within the ordination space.

The effect of locality in the ordination was most associated with the relative peak area of 2,3-dimethylphenol: samples from Pennsylvania averaged about 36% that of Manitoba samples (Table 1). Also, samples from Pennsylvania had higher relative peak areas of 3-methylphenol than those from Manitoba, a pattern evident in Table 1 and through application of the biplot rule (Ter Braak and Šmilauer, 2002) to the ordination (Fig. 1). The separation of sexes in the samples from Manitoba was associated with 3-ethylphenol and 2,5-dimethylphenol. For Manitoba females, the relative peak area of 2,5-dimethylphenol was about twice that of Manitoba males, and the relative peak area of 3-ethylphenol was 75% of that of Manitoba males.

Correlations between relative peak areas for pairs of compounds were invariably negative for pairs of alkylphenolic

Table 1 Relative peak areas for phenolic compounds in headspace samples from *Chlaenius cordicollis* in relation to source locality and sex of beetles. Data show means and SE values for samples in which total peak area for alkoxyphenol compounds exceeded 10^4 counts

Sample group	Relative peak area (%)[a]								
	Alkylphenol compounds						Alkoxyphenol compounds		
	N[b]	3-methylphenol	2,5-dimethylphenol	3-ethylphenol	2,3-dimethylphenol	3,4-dimethylphenol	N[b]	2-methoxy-5-methylphenol	2-methoxy-4-methylphenol
Pennsylvania									
Females	11	98.092±0.220	0.649±0.092	1.047±0.166	0.170±0.025	0.043±0.012	10	0.025±0.010	0.006±0.002
Males	11	98.051±0.195	0.540±0.064	1.157±0.130	0.185±0.043	0.067±0.010	4	0.045±0.034	0.006±0.004
Pooled sexes	22	98.071±0.143	0.594±0.056	1.102±0.103	0.177±0.024	0.055±0.008	14	0.031±0.011	0.006±0.002
Manitoba									
Females	4	96.615±0.175	1.228±0.156	1.573±0.039	0.521±0.133	0.063±0.021	3	0.063±0.028	0.052±0.043
Males	6	96.807±0.383	0.561±0.035	2.072±0.296	0.489±0.165	0.072±0.019	6	0.015±0.005	0.003±0.002
Pooled sexes	10	96.730±0.232	0.828±0.125	1.873±0.190	0.502±0.107	0.068±0.014	9	0.031±0.012	0.020±0.015

[a] Relative peak area for alkylphenol compounds is the count for each compound divided by the total counts for all five compounds and expressed as a percentage. Relative peak area for alkoxyphenol compounds is expressed on the same scale as for alkylphenol compounds: the count for each alkoxyphenol compound is divided by the total for the alkylphenol compounds and expressed as a percentage

[b] Number of samples

compounds that included 3-methylphenol (Table 2). The high negative values for the partial correlations for these same pairs suggest, as do the scatter plots (Fig. 2), that these associations were not a product solely of differences among source localities or among sexes within source localities, but also occurred within these groupings. Of the remaining correlations, the positive correlations between relative peak areas of 2,3-dimethylphenol and 2,5-dimethylphenol, and between 2,3-dimethylphenol and 3-ethylphenol, disappeared when partialled to account for differences among groups; examination of scatter plots (Fig. 3a, b) suggests that the associations were mostly the results of differences between source localities, rather than representing biochemical processes in individuals within a population. In contrast, the positive correlations of relative peak areas of 3-ethylphenol and 3,4-dimethylphenol, and of 2,5-dimethylphenol and 2-methoxy-5-methylphenol, were robust when partialled, and appeared to represent within-group, as well as among-group, relationships (Fig. 3c, d). There was no evidence of correlation between relative peak areas of 2-methoxy-5-methylphenol and 2-methoxy-4- methylphenol, even though there was a perfect association between their detectability. Either both or neither compound was detected; there were no samples in which one of these compounds was detected without the other.

Discussion

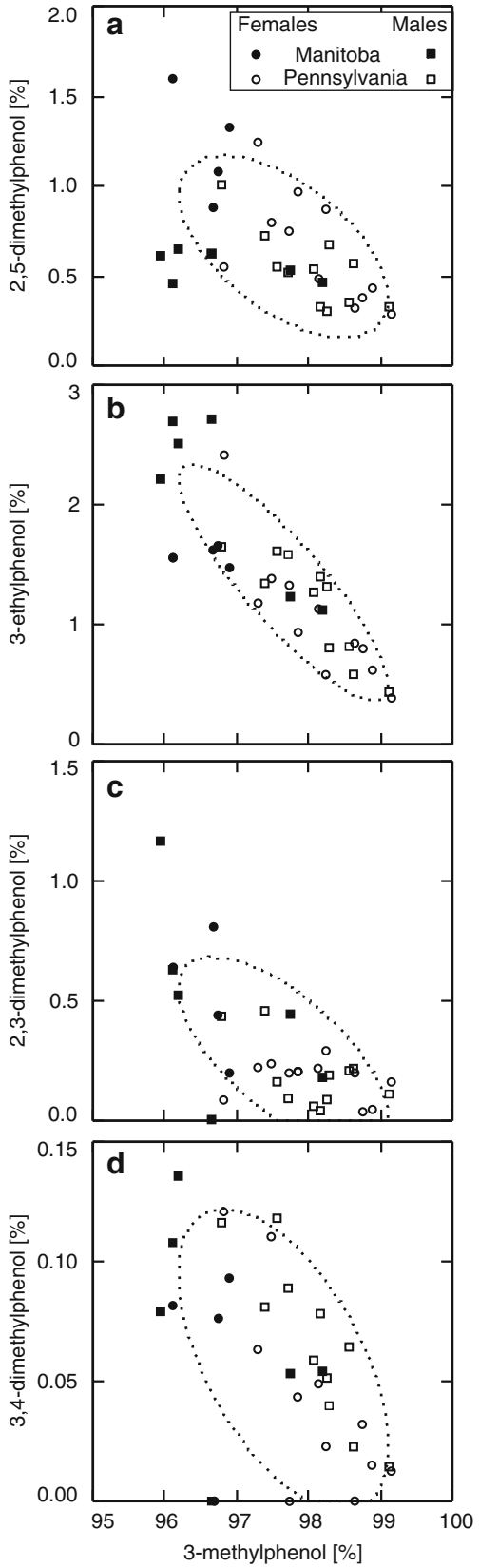
We focused our analysis on relative peak area because the amount of defensive secretion produced depends on the level of stimulus during headspace sampling, and on the frequency and timing of recent discharges (Eisner et al., 1963; Will et al., 2010). Relative peak areas are useful for comparisons among beetles sampled under the same conditions. They do not represent an absolute fractional composition of volatile compounds in the headspace, as SPME of each compound depends both on its concentration and on its equilibrium constant for extraction with the fiber (Zhang and Pawliszyn, 1993). Despite establishing a minimum total peak area criterion, we cannot determine whether 0% values for compounds represent absence of the compound, or levels below detection limits.

Our ordination analyses were restricted to alkylphenolic compounds, as inclusion of alkoxyphenolic compounds was problematic; to avoid variation of biological origin being eclipsed by that from methodological sources, we would need to exclude the many samples run with our first GC-MS temperature program. Also, even using the second temperature program, there were many zeros for alkoxyphenolic compounds. Zeros cannot be log-ratio transformed, and remedies for this problem have unacceptably large

Table 2 Pearson correlation and partial correlation coefficients between pairs of relative peak areas [%] for the phenolic compounds in headspace samples from *Chlaenius cordicollis*

Compound	Number of samples in correlation	3-methylphenol	2,5-dimethylphenol	3-ethylphenol	2,3-dimethylphenol	3,4-dimethylphenol	2-methoxy-5-methylphenol
Correlation coefficient, *r* [a]							
2,5-dimethylphenol	32	**−0.57**	–	–	–	–	–
3-ethylphenol	32	**−0.87**	+0.18	–	–	–	–
2,3-dimethylphenol	32	**−0.67**	**+0.35**	**+0.40**	–	–	–
3,4-dimethylphenol	32	**−0.57**	−0.24	**+0.56**	+0.23	–	–
2-methoxy-5-methylphenol	23	−0.19	**+0.50**	−0.05	+0.10	+0.26	–
2-methoxy-4-methylphenol	23	−0.12	+0.39	−0.03	−0.02	+0.16	−0.12
Partial correlation coefficient, *r*, controlling for source locality [a]							
2,5-dimethylphenol	32	**−0.50**	–	–	–	–	–
3-ethylphenol	32	**−0.80**	−0.02	–	–	–	–
2,3 dimethylphenol	32	**−0.46**	+0.19	+0.08	–	–	–
3,4-dimethylphenol	32	**−0.64**	+0.20	**+0.58**	+0.17	–	–
2-methoxy-5-methylphenol	23	−0.26	**+0.54**	−0.07	+0.12	+0.27	–
2-methoxy-4-methylphenol	23	+0.07	+0.33	−0.21	−0.23	+0.09	−0.13
Partial correlation coefficient, *r*, controlling for source locality and beetle sex [a]							
2,5-dimethylphenol	32	**−0.59**	–	–	–	–	–
3-ethylphenol	32	**−0.86**	+0.21	–	–	–	–
2,3 dimethylphenol	32	**−0.45**	+0.22	+0.09	–	–	–
3,4-dimethylphenol	32	**−0.66**	**+0.37**	**+0.58**	+0.17	–	–
2-methoxy-5-methylphenol	23	−0.26	**+0.61**	−0.04	+0.11	+0.29	–
2-methoxy-4-methylphenol	23	+0.09	+0.16	−0.11	−0.27	+0.14	−0.17

[a] Critical value for *r* (two-tailed, α=0.05) for 32 samples is 0.35 and for 23 samples is 0.41. Coefficients exceeding critical values are in bold



◀ **Fig. 2** Scatter plots showing relationships between relative peak areas [%] in headspace samples from *Chlaenius cordicollis* from Manitoba and Pennsylvania, with an ellipse indicating the approximate standard deviation for the bivariate normal distribution associated with the correlation coefficient. Relationships between 3-methylphenol and **a.** 2,5-dimethylphenol; **b.** 3-ethylphenol; **c.** 2,3-dimethylphenol; and **d.** 3,4-dimethylphenol

influences on results if zeros are numerous (Ter Braak and Šmilauer, 2002). Inclusion of alkoxyphenols in the ordinations would compromise them, and would probably not reveal additional patterns of variation. For alkylphenols, the patterns of influential compounds in ordinations correspond closely to groupings of average relative peak areas in Table 1. However, for alkoxyphenols, Table 1 shows little evidence of geographic or sexual groupings, and within-group variation was large, characteristics that make separation in an ordination unlikely.

Positive correlations in relative abundance of compounds may indicate that such compounds are produced through common biosynthetic pathways (Kusari et al., 2009). Similarly, negative correlations could signify that compounds are synthesized through competing pathways. However, at least some of the negative correlations with 3-methylphenol may be artifacts; when the relative peak area of 3-methylphenol is close to 100%, addition of a small percentage of another alkylphenol must perforce diminish the value for 3-methylphenol and create a negative association. Unlike the negative correlation we observed between 3-methylphenol and 3-ethylphenol in *C. cordicollis*, these compounds are positively correlated in the tenebrionid beetle *Bolitotherus cornutus* (Panzer) (Holliday et al., 2009).

Phenolic minor components have been reported only once previously in defensive secretions of *Chlaenius* species that produce 3-methylphenol: the secretion of *C. velutinus* is 95% 3-methylphenol, 2% 2,5-dimethylphenol, and 1% 3,5-methylphenol (Balestrazzi et al., 1985). Thus, the defensive secretions of *C. cordicollis* and *C. velutinus* have 2,5-dimethylphenol in common, but 3,5-dimethylphenol was not detected from *C. cordicollis*. The co-occurrence of 3-methylphenol and 3-ethylphenol, observed in *C. cordicollis*, also has been reported in defensive secretions of tenebrionid beetles. For example, the prothoracic defensive secretion of *Zophobas rugipes* Kirsch is 97% 3-methylphenol and 2% 3-ethylphenol (Tschinkel, 1969). Smaller quantities (<10%) of these two compounds have been reported in defensive secretions of at least seven other species of tenebrionids (Geiselhardt et al., 2009; Holliday et al., 2009), as well as in the sex pheromones of the tenebrionid, *Parastizopus armaticeps* Peringuey (Geiselhardt et al., 2008).

Geographic variation in defensive secretions has not previously been reported in carabids. Although environmental conditions can affect SPME sampling (Zhang and Pawliszyn,

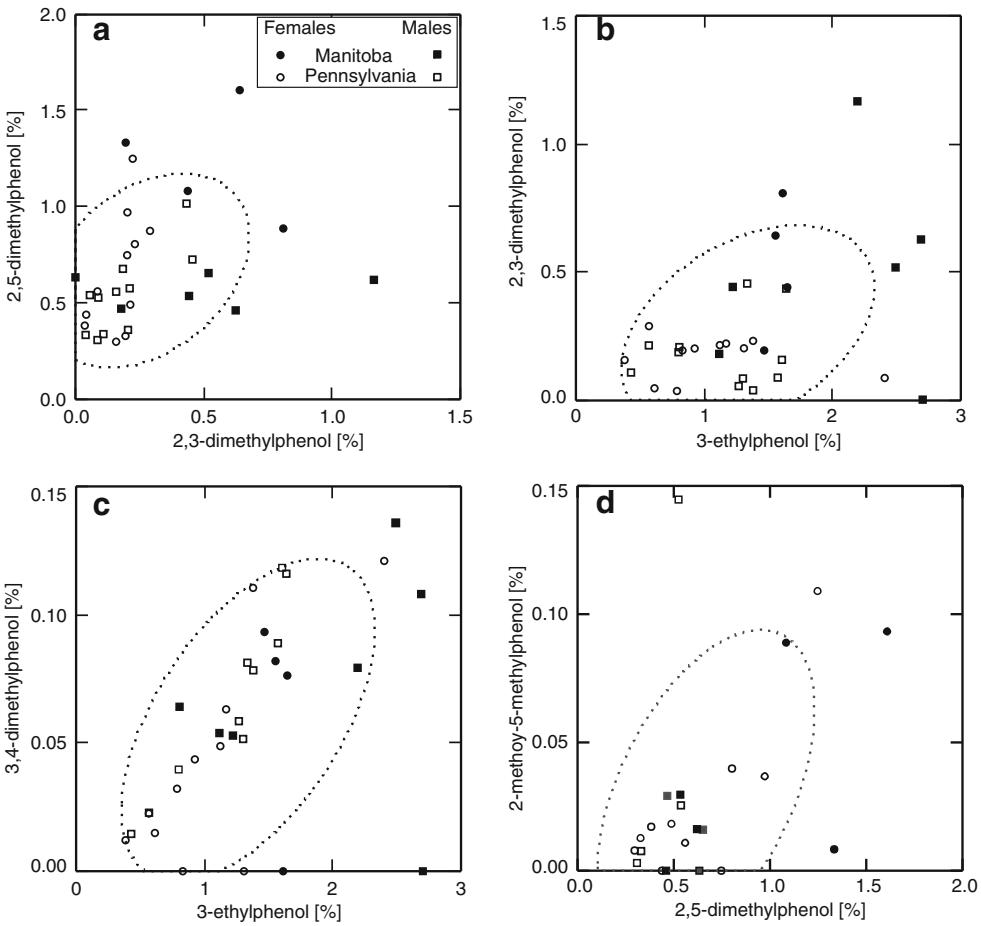


Fig. 3 Scatter plots showing relationships between relative peak areas [%] in headspace samples from *Chlaenius cordicollis* from Manitoba and Pennsylvania, with an ellipse indicating the approximate standard deviation for the bivariate normal distribution associated with the correlation coefficient. Relationships between **a.** 2,5-dimethylphenol and 2,3-dimethylphenol; **b.** 2,3-dimethylphenol and 3-ethylphenol; **c.** 3,4-dimethylphenol and 3-ethylphenol, and **d.** 2-methoxy-5-methylphenol and 2,5-dimethylphenol

1993), most of our SPME samplings for both localities were carried out in laboratory conditions that were similar and thus unlikely to contribute to apparent geographic differences. Proximity in ordination space of points for corresponding laboratory and field samples demonstrated that effects of variable field conditions were small relative to geographic effects. Similar tests eliminated transportation and batch of SPME fibers as sources of variation, leading us to conclude that geographic variation in our study was real, rather than an artifact of method.

Blum (1981) reviewed sources of variation in defensive secretions, and our results exemplified two of these: variations among populations and between sexes. Other sources of variation outlined in Blum (1981) include seasonal, age, and developmental effects. We tried to control for these sources by collecting beetles at the same point in their life cycle in both localities, and on one occasion collecting on the same day in both localities. Pasteels et al. (1983) suggested that geographic differences are probably genetic. Eggenberger and Rowell-Rahier (1991) demonstrated that, among populations of chrysomelid beetles, patterns of variation in defensive secretions are correlated with genetically determined isozyme polymorphisms. However, they, like we, cannot disprove the hypothesis that geographic variation in defensive secretions is attributable to different precursors in food consumed by insects in the field. For example, the composition of defensive secretions of fungus-feeding tenebrionid beetles is food-dependent (Holliday et al., 2009), and the seasonal variation in defensive secretions in a predatory dytiscid beetle may reflect the availability of plant-synthesized precursors acquired by herbivore prey (Miller and Mumma, 1974). The assemblage of insects available for *C. cordicollis* to scavenge no doubt differs between the banks of the Delaware River and the beaches of Lake Winnipeg, and we expect the available dietary precursors of phenolic compounds to differ between our source localities. However, genetic differentiation may also be a factor. When dietary variation is controlled for, variation in defensive secretions of chrysomelid beetles has both genetic and physiological sources (Eggenberger and Rowell-Rahier, 1992, 1993).

Intra-population differences in defensive secretions of carabid beetles have seldom been studied, as secretions of individuals are generally pooled for analysis. Both Roach et al. (1979) and Davidson et al. (1989) analyzed defensive secretions of individual carabid beetles from single

populations and remarked on the low level of qualitative and quantitative variation. Davidson et al. (1989) specifically checked for sexual dimorphism, but failed to find it. In contrast, in *Oodes americanus* Dejean, there are qualitative differences between defensive secretions of the sexes; three of thirteen carboxylic acids in females are not detectable in males (Attygalle et al., 1991). Attygalle et al. (1991) conjectured that these sexually dimorphic defensive secretions may play a role in inter-sexual communication, an example of semiochemical parsimony (Blum, 1996) seen also in some staphylinid beetles (Peschke, 1983). If sexual dimorphism in defensive secretions in *C. cordicollis* were also to have a pheromonal role, it is surprising that dimorphism was detected in Manitoba, but not in Pennsylvania, beetles. At the time of our collections, sexual functions of defensive secretions were likely irrelevant, as mating probably occurs shortly before egg production, which, at least in Vermont, is in late June (Bell, 1960). At other times of year, sexual dimorphism in defensive secretions is, perhaps, maintained only opportunistically if chemical precursors are readily present in food.

Attygalle et al. (1991) cautioned against characterizing defensive secretions for a species without taking into account the sex of the individuals being sampled. Our finding of both intra- and inter-population variation in defensive secretions highlights the need to recognize patterns of variation, rather than to characterize a species' secretions in a typological manner.

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Knockdown of *Microplitis mediator* Odorant Receptor Involved in the Sensitive Detection of Two Chemicals

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Abstract Odorant receptors are thought to play critical roles in the perception of chemosensory stimuli by insects. The primary method to address the functions of odorant receptors in insects is to use *in vitro* binding assays between the receptors and potential chemical stimuli. We injected MmedOrco dsRNA into the abdominal cavity of a braconid wasp, *Microplitis mediator*, and assayed for expression of this gene 72 h after treatment (RNAi). Quantitative real-time PCR demonstrated that the level of mRNA expression in MmedOrco dsRNA-treated *M. mediator* was significantly reduced (>90%) when compared with water-treated controls. Furthermore, electroantennogram (EAG) responses of *M. mediator* to two chemical attractants, nonanal and farnesene, were also reduced significantly (~70%) in RNAi-treated *M. mediator* when compared to controls. RNAi-treated *M. mediator* also responded by walking/flying at a lower rate to both chemicals when compared with controls in a Y-tube olfactometer bioassay, which provides direct evidence that MmedOrco plays an important role in perception of nonanal and farnesene in *M. mediator*.

Keywords Braconidae · Electroantennogram · Hymenoptera · *Microplitis mediator* · Odorant receptor · Quantitative real-time polymerase chain reaction (qPCR) · Reverse transcription PCR (RT-PCR) analysis · RNA interference (RNAi) · Walking/flight behavior · Y-tube bioassay

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Introduction

Olfactory stimuli play a major role in insect behaviors such as host-seeking, enemy-defending, mating, aggregation, and migration. Most of these chemosensory stimuli are recognized by members of two evolutionarily related insect-specific chemosensory receptor families, the odorant receptors (ORs) and gustatory receptors (GRs). Insect odorant receptors are members of a highly divergent multigenic family and expressed in olfactory receptor neurons (ORNs) housed in olfactory sensilla (Clyne et al., 1999; Vosshall et al., 1999). Initially, insects ORs were hypothesized to be G-protein coupled receptors (GPCRs), but they have recently been shown to function as heteromeric ligand-gated ion channels comprised of at least one copy of a variable odorant-binding OR subunit, along with at least one copy of an Or83b-like co-receptor (Larsson et al., 2004; Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008; Nichols and Luetje, 2010). Unlike most other odor receptors, Or83b is highly conserved across insect species, *e.g.*, *Aedes aegypti*, *Anopheles gambiae*, *Calliphora erythrocephala*, and *Ceratitis capitata* (Diptera); *Antheraea pernyi*, *Bombyx mori*, *Helicoverpa zea*, and *Heliothis virescens* (Lepidoptera); *Phyllotreta striolata* and *Tenebrio molitor* (Coleoptera); and *Apis mellifera* (Hymenoptera) (Krieger et al., 2003, 2005; Pitts et al., 2004; Melo et al., 2004; Jones et al., 2005; Zhao et al., 2010). Despite 100 million years of evolutionary divergence, the conserved function of insect olfaction is supported by a 76% similarity of amino acid composition between *D. melanogaster* Or83b and *A. gambiae* AgOr7 (Pitts et al., 2004). Or83b family proteins play important roles in co-expressing with odorant receptors in most olfactory receptor neurons

(Vosshall et al., 2000; Krieger et al., 2003; Larsson et al., 2004; Syed et al., 2010). Or83b is considered to act as a chaperone protein in the transport of ORs to the cell membrane of ORNs (Benton et al., 2006). Furthermore, Or83b also has been shown to form a heterodimer with OR, which configures a ligand-gated cation channel (Sato et al., 2008; Wicher et al., 2008).

RNA interference (RNAi) is a technique whereby sequence-specific double-stranded RNA (dsRNA) is injected into an organism or incubated with the tissues to specifically inactivate the expression of the corresponding gene. This provides an opportunity to investigate the function of the corresponding protein (Carthew, 2001). RNAi was first demonstrated in the nematode, *Caenorhabditis elegans* (Fire et al., 1998). Subsequently, RNAi has been used to knockdown a variety of genes in arthropods including *Anopheles gambiae* (Vlachou et al., 2005; Boisson et al., 2006), *Nilaparvata lugens* (Liu et al., 2010), *Apis mellifera* (Maleszka et al., 2007), *Bemisia tabaci* (Ghanim et al., 2007), *Drosophila melanogaster* (Kennerdell and Carthew, 1998), *Manduca sexta* (Eleftherianos et al., 2006), and *Ixodes scapularis* (Narasimhan et al., 2004). Dicer RNase III type enzymes cleave cytoplasmic dsRNAs into small interfering RNA duplexes (siRNAs) composed of approximately 21–23 nucleotides. The siRNAs duplexes are incorporated into a multiprotein RNA-inducing silencing complex (RISC) where the antisense strand guides RISC to its homologous target mRNA for endonucleolytic cleavage (Dykxhoorn et al., 2003; Meister and Tuschl, 2004).

Microplitis mediator (Haliday) (Hymenoptera: Braconidae) is a polyphagous solitary larval endoparasitoid that attacks approximately 40 species of Lepidoptera. It is widely distributed from Central Europe to China (Shenefelt, 1973; Arthur and Mason, 1986). Zhang et al. (2009) isolated a novel olfactory receptor gene *MmedOrco* from *M. mediator* and demonstrated antennal-specific expression, which reached a maximum level on the day of female emergence, and 2 h prior to male emergence. To further investigate *MmedOrco* gene function in *M. mediator*, we examined whether the corresponding dsRNA would reduce expression of MmedOrco mRNA. We also investigated whether this treatment affected electrophysiological and olfactory responses of *M. mediator* to two potential semiochemicals.

Methods and Materials

Insects A colony of *M. mediator* was obtained from the Institute of Plant Protection, Hebei Academy of Agriculture and Forestry, China. These wasps emerged from the host as mature larvae, and spun a silk cocoon outside the larvae on which they pupated. Emerged adult *M. mediator* were fed on a 30% honey solution in a growth chamber programmed at the temperature of 28±1°C, a photoperiod of 16:8 h, L:D, with 75% relative humidity.

DsRNA Synthesis and Microinjection dsRNA was commercially synthesized by Ribobio (Guangzhou, China). The target sequence used for knocking down MmedOrco (GenBank ID: EF141511) was 5′-GGCTCAATAAGGAAAC TAA-3′. The double-stranded siRNA was dissolved in DEPC (Diethypyrocarbonate)-treated water. Before injection, a 1% agarose plate was made and placed on an ice tray. Under carbon dioxide anaesthesia, newly emerged *M. mediators* were immobilized on the agarose plate with the abdomen directed airward by using manual forceps. Then, 0.05 μl water or siRNA solution (0.1 mol/ml) was injected into the abdomen of each *M. mediator* by using a PLI-100 Pico-Injector (Harvard Apparatus, Holliston, MA, USA) manipulated by a MP-255 Micromanipulator (Sutter, Novato, CA, USA) under a microscope. After injection, *M. mediator* were kept in a cage supplied with 30% honey solution at a temperature of 26±1°C, humidity 70–80%, and 16/8 hL/D to recover. Some specimens were selected at each sampling time and frozen in liquid nitrogen and kept at −80°C for mRNA analysis. The remaining *M. mediator* adults that had recovered for 3 d were used for behavioral and electroantennographic assays (see below).

Reverse Transcription PCR (RT-PCR) Analysis Total RNA was extracted by using Trizol reagent (Invitrogen, Beijing, China) from 3 pairs of (3 females and 3 males) *M. mediator* in 72 h after each treatment of non-injection, water-injection, and siRNA-injection, respectively, and then quantified by using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). One microgram of RNA was reverse transcribed by using the MMLV reverse transcriptase and oligo (dT) primers. Primers used for obtaining *MmedOcro* and 18S rRNA genes by RT-PCR are provided in Table 1. The amplification conditions were 2 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C, and final extension for 10 min at 72°C. RT-PCR products were analyzed on a 1% agarose gel. All RT-PCR reactions were repeated five times.

Quantitative Real-Time PCR (qPCR) Total RNA used for qPCR was extracted at 6, 24, 48, and 72 h after treatments (see above). MmedOcro was amplified by Real-time PCR by using a SybrGreen PCR amplification mix. 18S rRNA also was amplified as a reference gene. Primers for real-time PCR analysis of MmedOcro and 18S rRNA are provided in Table 1. The total 20 μl reaction system consisted of 10 μl 2 x SYBR Green PCR Master Mix, 0.4 μl each primer, 2 μl sample cDNA, and 7.2 μl sterilized ultrapure H_2O. Cycling parameters for real-time PCR were 20 sec at 95°C, followed by 40 cycles of 15 sec at 95°C, 34 sec at 60°C. PCR reactions were performed in triplicate, and data were processed by using the relative quantification $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1 Oligonucleotide primers used for gene expression analysis of *Microplitis mediator* odor receptor

Purpose/Primer Name	Sequence(5′-3′)
Expression analysis (RT-PCR)	
MmedOrco	
Sense	AACATCCGTTTAATGCAA
Antisense	TTATTTAAGTTGTACTAATA
Mmed 18S rRNA	
Sense	CGGAGAGGGAGCCTGAGAA
Antisense	CCGGGAGTGGGTAATTTGC
Expression analysis (real-time PCR)	
MmedOrco	
Sense	AGGTATCTACAGCAATCGCCAAG
Antisense	GCCGTTCGACCCAGTACTTAAT
Mmed 18S rRNA	
Sense	CGGAGAGGGAGCCTGAGAA
Antisense	CCGGGAGTGGGTAATTTGC

Electroantennogram (EAG) Assays To examine antennal sensitivity to two chemicals, nonanal and farnesene, EAGs were used to record the antennal responses of *M. mediators* in water-injected, siRNAi-injected, and non-injected *M. mediator*, respectively. The concentration of the two chemicals was 0.1 μg/μl (dissolved in liquid paraffin), and liquid paraffin was set up as a blank control. The antennae were carefully removed at the base, and a few terminal segments at the distal end were excised. The treated antennae were attached to electrode holders with electrode gel. Filter paper strips (4×15 mm) were loaded with 100 μl of each chemical solution and inserted into a glass Pasteur pipette. The tip of the pipette was inserted about 3 mm into a small hole in the wall of a metal tube (9 mm diam×12 cm long) directed at the antennal preparation. An air stimulus controller (Model CS-55, Syntech, Hilversum, The Netherlands) was used for air and odor delivery. A constant flow (400 ml/min) of activated carbon-filtered air passed over the antenna through the open end of the metal tube positioned 5 mm from the antenna. During odor stimulation, 300 ml/min of air were applied through the Pasteur pipette into the main airflow for 0.2 sec. Antennae were stimulated three times with each substance at 30 sec intervals. EAG recordings were made with an IDAC-2 recording unit with amplifier and computer board (Syntech) and stored on hard disk.

Behavioral Trials Behavioral responses of *M. mediator* were assayed at room temperature (22–25°C) in a Y-tube olfactometer equipped with two-armed glass tubes (2 cm i.d.) with an air flow of 300 ml/min. The system consists of a 15-cm-long central tube and two 15-cm-long lateral arms. The angle between the lateral arms was 120°. One adult *M. mediator* was placed at the end of the central tube. A filter paper strip was immersed to saturation in each of the two chemicals (nonanal and farnesene) to be tested and placed in one of the Y-tube's arms. The other arm was left blank and served as the control. The initial choice of a *M. mediator* that responded by walking/flying into one of the arms and remained there at least 15 sec was recorded. If a *M. mediator* had not made a choice within 2 min of being released, it was removed and discarded. Individuals that did not walk into any arms were not counted. After 5 individual *M. mediator* had been tested, the two arms were exchanged to avoid asymmetric bias effect. To minimize visual distraction for the *M. mediator*, the Y-tube olfactometer was placed inside a white paper box, which was open on the top (for illumination) and on the front side (for observation). Illumination was provided by vertically hanging an office lamp (20 W, 250 Lux) above (50 cm high) the olfactometer tube. After 10 wasps had been observed, the old impregnated filter paper strip was discarded and replaced with a new impregnated filter paper strip, and the olfactometer set-up was rinsed with soap water, and acetone, and then air-dried. Sixty (30 females and 30 males) adult *M. mediator* in each treated group were detected, and *M. mediator* were used only once.

Data Analysis Data from qPCR and EAG tests were analyzed by using SPSS 11.0 for Windows software (SPSS 11.0 for Windows Student Version, 2001, SPSS Inc, Chicago, lL, USA). One-way analysis of variance (ANOVA) and Bonferroni test ($P=0.05$) were used to determine whether differences in MmedOrco mRNA levels or EAG responses were significant among different treatments. Behavioral assay data were analyzed by using a *chi-squared* test with SAS 9.0 software (SAS 9.0 system for windows, 2002, SAS Institute Inc., Cary, NC, USA).

Results

Effect of dsRNA Treatment on MmedOrco Transcript Levels Initial analysis of MmedOrco transcript levels by RT-PCR showed that levels were reduced in dsRNA-injected *M. mediator* relative to controls (Fig. 1). Furthermore, water-injected and non-injected *M. mediator* controls

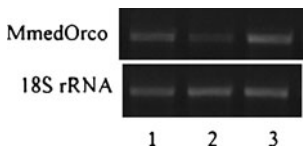

Fig. 1 Analysis (RT-PCR) of MmedOrco transcript levels from *Microplitis mediator* showing reduced levels in dsRNA-injected (*lane 2*) when compared with the transcript levels in water-injected (*lane 1*) and non-injected (*lane 3*) treatment groups. The control gene was *M. mediator* 18 s ribosomal RNA

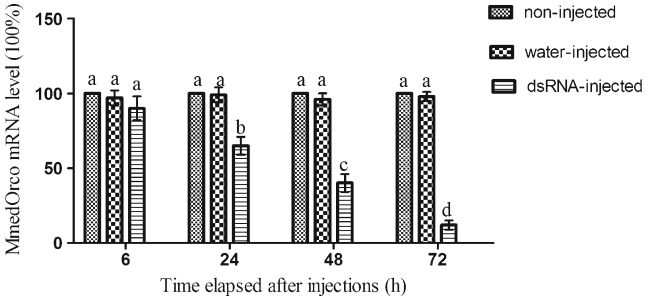

Fig. 2 Analysis (qPCR) of MmedOrco transcript patterns from *Microplitis mediator* in water-injected, non-injected, and dsRNA-injected treatment groups over time following dsRNA injection. The transcript abundance of MmedOrco was normalized to the 18S ribosomal RNA control gene. Values marked with different letters are significantly different based on Bonferroni test and one-way analysis of variance (ANOVA) (F=15.72, $d.f.$=2, 6, P=0.088; F=7.56, $d.f.$=2, 6, P=0.002; F=42.37, $d.f.$=2, 6, P<0.001; and F=86.23, $d.f.$=2, 6, P<0.001 for 6, 24, 48, and 72 h post-injection, respectively)

displayed almost equivalent levels of MmedOrco transcripts. This demonstrated that injection of dsRNA was responsible for the observed reduction in level of MmedOrco mRNA (Fig. 1). Real-time PCR analysis (Fig. 2) confirmed the trend observed in the RT-PCR experiment: there were significant reductions in MmedOrco transcript levels in dsRNA-injected *M. mediator* at 24, 48, and 72 h after injection relative to water-injected or non-injected controls [F=7.56, $d.f.$=2, 6, P=0.002, F=42.37, $d.f.$=2, 6, P<0.001, and F=86.23, $d.f.$=2, 6, P<0.001 for 24, 48, and 72 h post-injection, respectively after injection] (Table 2). MmedOrco transcript levels in non-injected or water-injected *M. mediator* remained unchanged. The amount of MmedOrco transcript declined with time in dsRNA-injected *M. mediator* [F=75.68, $d.f.$=3, 8, P=0.001], leading to significant inhibition during the period between the 24 and 72 h. Although the effect of MmedOrco dsRNA became slightly less significant at hour 6, inhibition on MmedOrco expression could still be detected. This partial silencing of MmedOrco shown by qPCR analysis demonstrated the feasibility of significantly reducing MmedOrco gene by using the RNAi approach.

Effect of dsRNA Treatment on Electrophysiological and Behavioral Responses to Semiochemicals We examined the responses of water-injected, dsRNA-injected, and non-injected *M. mediator* to two potential semiochemicals farnesene and nonanal by using electroantennographic analysis (EAG). The data showed that the voltages measured from dsRNA-treated *M. mediator* responding to the two chemicals were significantly lower than those of the control group, with a reduction of about 80% [female response: F=23.42, $d.f.$=2,6, P=0.002; F=14.76, $d.f.$=2,6, P=0.004 for farnesene and nonanal, respectively; male response: F=57.31, $d.f.$=2,6, P=0.002; F=63.31, $d.f.$=2,6, P=0.003 for farnesene and nonanal, respectively] (Table 3). This indicates that silencing *MmedOrco* leads directly to effects on antennal electrophysiological responses to nonanal or farnesene (Figs. 3 and 4). In addition, there were no differences in EAG responses to the two chemicals between water-injected and non-injected *M. mediator* (Bonferroni test–female response: P=0.826; P=0.944 for farnesene and nonanal, respectively; male response: P=1.000; P=0.233 for farnesene and nonanal, respectively), which illustrated that mechanical

Table 2 Relative quantification by real-time PCR of *MmedOrco* and 18S rRNA gene expression in *Microplitis mediator* following different treatments

Treatment	Time (h)	MmedOcro C_T[a]	18S rRNA C_T[a]	ΔC_T[b]	$\Delta\Delta C_T$[c]	(Range)[d]
Non-injected	6	30.05±0.01	3.31±0.09	6.74±0.03	0.00±0.14	1.00 (0.96–1.03)a
Water-injected	6	30.10±0.04	23.32±0.02	6.78±0.07	0.04±0.11	0.97 (0.91–1.02)a
RNAi	6	30.18±0.02	23.29±0.05	6.89±0.16	0.15±0.07	0.90 (0.81–0.95)a
Non-injected	24	31.47±0.11	24.38±0.09	7.09±0.03	0.00±0.12	1.00 (0.93–1.05)a
Water-injected	24	30.77±0.07	23.67±0.01	7.10±0.16	0.01±0.03	0.99 (0.94–1.02)a
RNAi	24	31.76±0.15	24.06±0.14	7.70±0.27	0.61±0.05	0.65 (0.57–0.72)b
Non-injected	48	30.85±0.03	24.01±0.07	6.84±0.05	0.00±0.13	1.00 (0.96–1.03)a
Water-injected	48	30.75±0.20	23.86±0.05	6.89±0.09	0.05±0.15	0.96 (0.89–1.08)a
RNAi	48	32.14±0.04	23.98±0.19	8.16±0.10	1.32±0.17	0.40 (0.35–0.50)c
Non-injected	72	30.57±0.09	24.54±0.01	6.03±0.08	0.00±0.09	1.00 (0.96–1.06)a
Water-injected	72	30.66±0.12	24.61±0.08	6.05±0.17	0.02±0.06	0.98 (0.91–1.04)a
RNAi	72	33.55±0.06	24.47±0.13	9.08±0.05	3.05±0.11	0.12 (0.08–0.23)d

[a] Mean ± SE

[b] Mean MmedOcro C_T–Mean 18S rRNA C_T±SE

[c] Mean ΔC_T–Mean $\Delta C_{T\ \text{Non-injected}}$±SE

[d] Values (Normalized amount of MmedOcro transcript relative to non-injected wasps) followed by different letters are significantly different based on one-way analysis of variance (ANOVA) and a multiple test

Table 3 Electroantennographic (EAG) responses of treated and control *Microplitis mediator* to farnesene and nonanal

Treatment	Compound	Concentration (μg)	Mean EAG response (mV) ± SE[a]	
			♀	♂
Water-injected wasps	farnesene	10	0.46±0.09 a	0.41±0.07 a
	nonanal	10	0.36±0.06 a	0.38±0.04 a
Non-injected wasps	farnesene	10	0.45±0.05 a	0.40±0.03 a
	nonanal	10	0.37±0.04 a	0.39±0.06 a
ds RNA-injected wasps	farnesene	10	0.03±0.01 b	0.02±0.01 b
	nonanal	10	0.09±0.01 b	0.08±0.01 b

[a]Values (mean EAG response) within a column followed by different letters are significantly different based on one-way analysis of variance (ANOVA) and a multiple comparison test

damage to the body by injection did not affect our experiment results.

The walking/flight responses of male and female *M. mediator* to the two chemicals in the Y-tube olfactometer experiments revealed that both water-injected and non-injected male or female *M. mediator* were attracted to the two chemicals (water-injected females: $\chi^2_{farnesene}=4.73$, $P=0.016$; $\chi^2_{nonanal}=4.91$, $P=0.033$; non-injected females: $\chi^2_{farnesene}=5.26$, $P=0.012$; $\chi^2_{nonanal}=5.27$, $P=0.032$; water-injected males: $\chi^2_{farnesene}=4.27$, $P=0.036$; $\chi^2_{nonanal}=4.96$, $P=0.046$; non-injected males: $\chi^2_{farnesene}=4.27$, $P=0.036$; $\chi^2_{nonanal}=4.96$, $P=0.046$) (Figs. 5 and 6). In contrast, dsRNA-injected male or female *M. mediator* did not respond to nonanal or farnesene (dsRNA injected females: $\chi^2_{farnesene}=1.03$, $P>0.05$; $\chi^2_{nonanal}=1.91$, $P>0.05$; dsRNA injected males: $\chi^2_{farnesene}=1.57$, $P>0.05$; $\chi^2_{nonanal}=1.68$, $P>0.05$) (Figs. 5 and 6).

Discussion

Our study demonstrates the successful interference of MmedOrco gene expression *in vivo* by injecting dsRNA in the abdominal cavity of *M. mediator*. The knockdown rate of MmedOrco exceeded 90%, which proved that this technology can be applied to *M. mediator* unconventional odorant receptor functional research. In addition, this study further demonstrated that dsRNA-injected *M. mediator* had a weak EAG responses to nonanal or farnesene as compared with those from the control group. In the Y-tube olfactometer bioassay, dsRNA-injected *M. mediator* also had a lower walking/flight response to nonanal or farnesene compared with control animals. These results demonstrated that MmedOrco plays an important role in the insect olfactory system, and that it is consistent with our previous hypothesis (Zhang et al., 2009). To our knowledge, this is the first report that RNAi technology has been applied in parasitic wasps to demonstrate that MmedOrco (the ortholog of the DmOr83) in involved in the olfactory system of *M. mediator*. Our results also suggest that the modulation of this unique receptor family by using RNAi to block host-plant-seeking behavior could represent a novel future biological pest control tool.

Direct injection of dsRNA into target tissues or developmental stages and artificial feeding of dsRNA are still the most effective approach for delivering dsRNA to insects (Misquitta and Paterson, 1999; Bettencourt et al., 2002; Bucher et al., 2002; Rajagopal et al., 2002; Amdam et al., 2003; Gatehouse et al., 2004; Tomoyasu and Denell, 2004). Although artificial feeding is a non-invasive technique that preserves the integrity of the treated animals, the precise amount of the dsRNA taken up by an animal is difficult to monitor, and RNAi efficiency

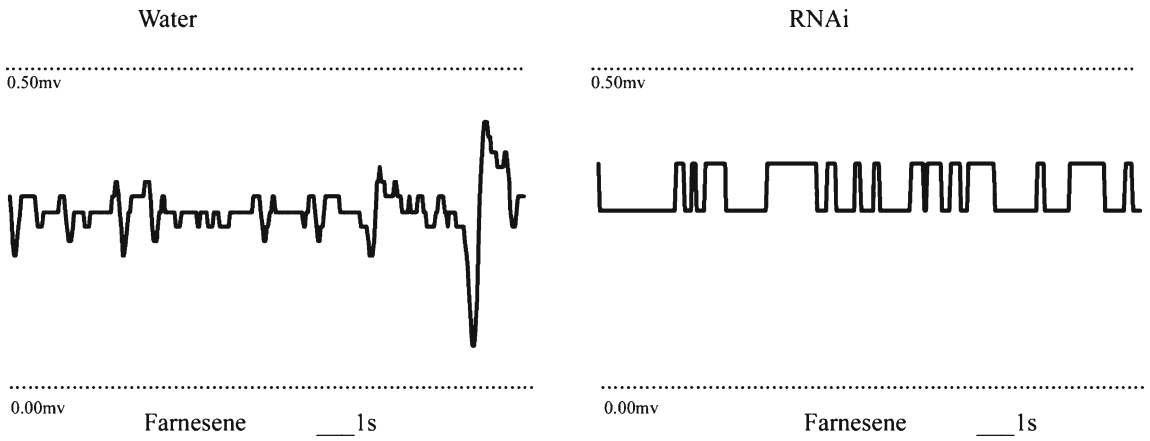

Fig. 3 Electroantennogram traces recorded from antennae of water-injected and dsRNA-injected *Microplitis mediator* challenged with farnesene (10 μg)

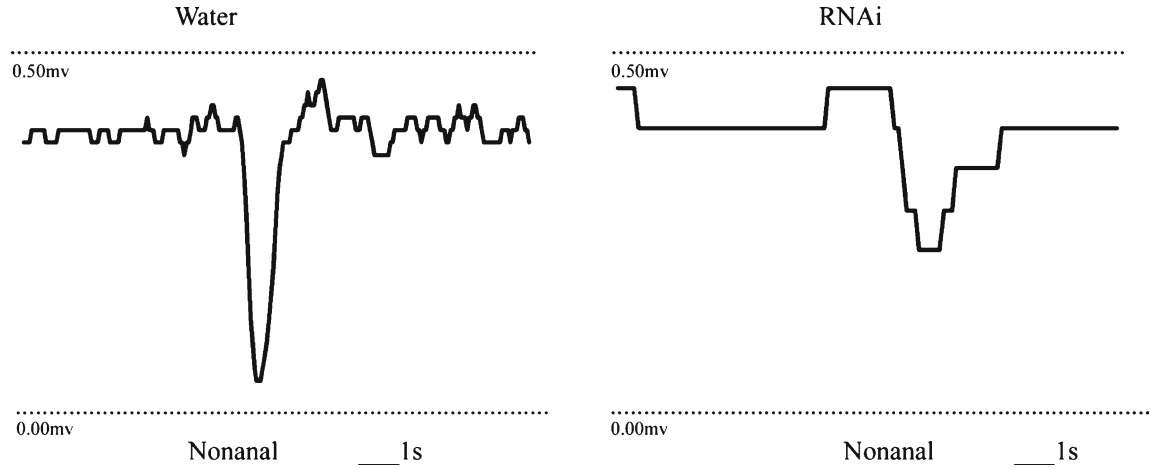

Fig. 4 Electroantennogram traces recorded from antennae of water-injected and dsRNA-injected *Microplitis mediator* challenged with nonanal (10 μg)

among individuals is highly variable (Turner et al., 2006). It is also difficult to perform the artificial feeding technique for insect species if the artificial diet is not successfully constructed (Turner et al., 2006). In contrast, more control of delivery of dsRNA can be exerted through microinjection. RNAi effects can be inconsistent and, in some cases, require large amounts of double-stranded RNA that could cause non-target interference (Boisson et al., 2006). In our study, after dsRNA was injected into the *M. mediator*, we hypothesize that the dsRNA was transported to the target tissue (the antennae) via circulating hemolymph after which it interfered with the target gene. Although, because of imperfect injection technique, (mechanical damage may affect insect survival), there were no significant differences in survival between water-injected and ds RNA-injected wasps. The use of injected water as a control eliminated the possible effects of mechanical injury in the experiment and ensured the reliability of direct injection of dsRNA into the cavity.

Yu et al. (2010) reported that synthetic nonanal or farnesene were both attractive to *M. mediator* in Y-tube olfactometer bioassays where adults were tested in response to volatile compounds from undamaged, mechanically injured, or *H. armigera*-damaged cotton plants. Here, we report that EAG responses of *M. mediator* to nonanal or farnesene were reduced significantly (approx. 70%) in RNAi- treated *M. mediator* when compared to controls. Moreover, RNAi-treated *M. mediator* also responded at a lower rate to either of the two chemicals when compared with controls in Y-tube olfactometer trials. Therefore, nonanal and farnesene might be useful in the search for potential chemical candidates for attractants to control *M. mediator*.

Our RNAi investigation of the role of MmedOrco, the *M. mediator* ortholog of *Drosophila* Or83b, supports the assumption that this highly conserved gene plays a similar role in all insects (Jones et al., 2005). Although we do not have any molecular data to demonstrate how the gene functions in *M. mediator*, MmedOrco knockdown, which also caused an inability in *M. mediator* to respond to farnesene or

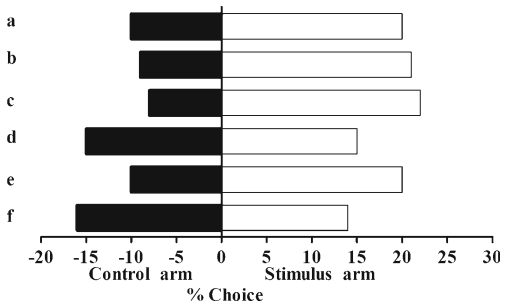

Fig. 5 Response of *Microplitis mediator* in a Y-tube olfactometer bioassay under the laboratory conditions of 16:8 hL:D, 75% RH when given a choice between clean air (control) and farnesene (odor stimulus): (**a**) Water-injected male, (**b**) Water-injected female, (**c**) Non-injected male, (**d**) ds RNA-injected male, (**e**) Non-injected female, and (**f**) ds RNA-injected female. See text of the Results for the statistical analyses of these data

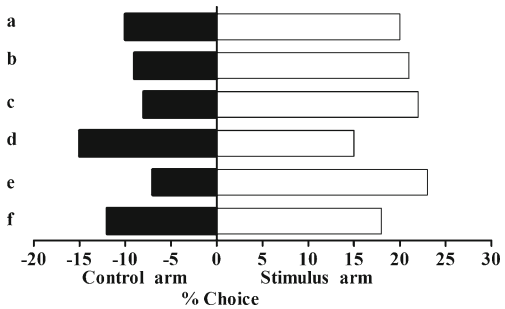

Fig. 6 Response of *Microplitis mediator* in a Y-tube olfactometer bioassay under the laboratory conditions of 16:8 hL:D, 75% RH when given a choice between clean air (control) and nonanal (odor stimulus): (**a**) Water-injected male, (**b**) Water-injected female, (**c**) Non-injected male, (**d**) ds RNA-injected male, (**e**) Non-injected female, and (**f**) ds RNA-injected female. See text of the Results for the statistical analyses of these data

nonanal, suggests that MmedOrco plays a similar role to DmOr83b, which dimerizes with functional OR proteins and permits their integration into the cell membrane (Benton et al., 2006). This research has also confirmed the usefulness of dsRNAi as a technique for investigating the effects of single genes on insect behavior.

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The Effects of Defoliation-Induced Delayed Changes in Silver Birch Foliar Chemistry on Gypsy Moth Fitness, Immune Response, and Resistance to Baculovirus Infection

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Abstract We tested the effects of defoliation-induced changes in silver birch, *Betula pendula*, foliar chemistry (delayed induced resistance, DIR) on the fitness and immune defense of the gypsy moth, *Lymantria dispar*. We measured larval developmental time, pupal weight, rate of survival to the adult stage, and five characteristics of larval immune defense: (1) encapsulation response; (2) phenoloxidase activity; (3) hemocyte concentration and (4) lysozyme-like activity in the hemolymph; and (5) resistance to infection by *L. dispar* nucleopolyhedrovirus (LdMNPV). The latter is an entomopathogenic baculovirus that often causes epizootics during outbreaks of *L. dispar*. We also measured the involvement of foliage non-tannin phenolic compounds in resistance of *B. pendula* to herbivory as well as the relationship between the compounds we identified and *L. dispar* development, growth, and survival. Leaves of *B. pendula* with previous defoliation history contained increased levels of myricetin glycoside, two flavonoid aglycones (acacetin and tetrahydroxy-flavone dimethyl ether), as well as one unidentified simple phenolic. The concentrations of two glycosides of quercetin, as well as the content of one unidentified flavonoid glycoside were significantly decreased under defoliation treatment. DIR of *B. pendula* retarded larval growth rate and increased lysozyme-like activity in the hemolymph, but did not affect encapsulation response, phenoloxidase activity, or hemocyte count. We did not find any DIR-mediated tritrophic interactions among birch, gypsy moth, and LdMNPV. After viral inoculation, the mean hemocyte counts in larvae reared on an individual tree correlated significantly with the survival of larvae reared on that same tree, indicating that hemocyte density in hemolymph might be associated with resistance to viral infection. We found a strong positive correlation between the concentration of 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose and *L. dispar* survival rate, which may indicate an unlikely role of this dominant non-tannin phenolic in *B. pendula* defense against *L. dispar*. Our study also shows that several immune characteristics of insects that function as barriers against different groups of parasites are differently affected by plant induced defenses. This underscores the importance of considering multiple factors when characterizing barriers to insect immunity.

Keywords *Betula pendula* · Delayed induced resistance · Gypsy moth · Hemocyte count · Herbivore performance · Host plant quality · *Lymantria dispar* · Nucleopolyhedrovirus · Phenolic content · Silver birch · Tritrophic interaction, Insect outbreaks

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Introduction

Insects have an important place in the structure of communities owing to their extensive biodiversity and their high

population densities (Price, 1997). Particularly, the swift rise of population densities of outbreak species of herbivorous insects can change the structure of the trophic web of insect communities. Moreover, indirect interactions such as changes in species composition also may occur. Such multitrophic interactions that involve defoliating insects in forests are widely studied (reviewed in Cory and Hoover, 2006). Less studied are how changes in the food quality of an insect herbivore may affect the entire food web and what mechanisms regulate these processes.

The consumption of food where quality has changed as a consequence of either previous or current plant defoliation by herbivores is associated with a change in insect fitness (Larsson, 2002; Haukioja, 2005; Howe and Schaller, 2008). The change in insect fitness is important in assessing food chain dynamics (predators, parasitoids, pathogens). The food that herbivores consume alters the preference of their predators and parasitoids (Cory and Hoover, 2006; Bruinsma and Dicke, 2008) and affects their susceptibility to pathogens (reviewed in Cory and Hoover, 2006). There are studies that show positive (Felton and Duffey, 1990; Hoover et al., 1998; Martemyanov et al., 2006), negative (Cook et al., 2003), and no (Lindroth et al., 1999) associations between the content of secondary compounds in an insect's diet and its resistance to pathogens. However, what happens to insect innate immunity under changing food quality is as yet unknown. One of the factors modifying the interactions among a host and its parasites and pathogens is the host's immune function (Hoffmann et al., 1999; Iwanaga and Lee, 2005). A wide range of barriers to the expression of cellular and humoral components of host innate immunity might effectively prevent the development of pathogenesis. In turn, the development of immune traits in insects might depend upon the quality of consumed food. Both primary metabolites (Lee et al., 2006) and secondary metabolites (Ojala et al., 2005; Haviola et al., 2007) are involved in these relationships.

The gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), is a folivore that is widespread in Asia, Europe, and North America, where it causes large-scale defoliation of host plants during population outbreaks. In contrast to the insect's host plant preferences in Europe and North America (oak and aspen species), the main host of *L. dispar* in Western Siberia (the region in which this study was conducted) is silver birch, *Betula pendula* Roth. The gypsy moth in Siberia (*L. dispar asiatica*) differs in its ecology (host plant, pathogens, dispersal capabilities) from the gypsy moth in Europe and North America (*L. dispar dispar*) (Hajek and Tobin, 2009). The main natural regulatory factors in the population dynamics of *L. dispar asiatica* are parasitic flies (Tachinidae), parasitoids (Braconidae), and viral disease (Baculoviridae) (Koltunov et al., 1998). In addition, bacterial disease caused by *Bacillus thuringiensis* also is common in Western Siberia (Martemyanov, personal observation), as well as in nearby areas of Central Asia (Khodyrev et al., 2010).

Epizootics of *L. dispar* nucleopolyhedrovirus (LdMNPV) (Baculoviridae) often occur during the collapse phase of the herbivore's population cycles, i.e., in years following severe tree defoliation, and may be associated with previous host plant defoliation. The year after larval defoliation, *B. pendula* show a delayed induced resistance response (Haukioja, 2005) marked by the production of phenolic compounds (Valkama et al., 2005). These are dominated by non-tannin phenolics (Mutikainen et al., 2000), which might influence an insect's immune defense (Kapari et al., 2006). Recently McNeil et al. (2010) have shown the involvement of *L. dispar* innate immunity in larval defense to LdMNPV infection. Moreover, the activation of latent baculoviral disease that persists in an insect population (Il'inykh, 2007) might be induced by a weakening of the insect's immune defense as a consequence of the tree's defense (changes in foliar chemistry).

In this study, we used the *B. pendula–L. dispar asiatica*—baculovirus system to test whether or not there are multitrophic interactions occurring among the different components of this food chain. Specifically, we addressed the following questions: (1) does the delayed induced response of *B. pendula* affect the fitness of *L. dispar asiatica* larvae; and (2) do phenolic compounds in leaves play a role in the delayed induced response of *B. pendula*? Additionally, we estimated certain characteristics of *L. dispar asiatica* innate immunity to reveal whether or not immune defense of larvae is involved in plant-mediated tritrophic interactions. We measured the phenoloxidase activity (PO) and encapsulation rate in larval hemolymph based on the knowledge that the outcome of viral challenge of insects may depend on these immune characteristics (McNeil et al., 2010). We also measured lysozyme-like activity in larval hemolymph in order to predict antibacterial resistance (Hetru et al., 1998) in larvae infected with *Bacillus thuringiensis*. Since the immune function of insects is costly to use and maintain (energy-wise), particularly for the elimination of toxic metabolites (Freitak et al., 2003; Valtonen et al., 2010), the maintenance of high levels of immunity may to a large extent depend on the insect's food quality. In this study, we also investigated relationships among the levels of phenolics in leaves, insect fitness, innate immune defense, and resistance to baculovirus.

Methods and Materials

The field experiments were conducted during 2005–2006 in suburban early-successional natural stands of *B. pendula* near Novosibirsk (54°55′ N 83°12′ E), Western Siberia, Russia. The experimental trees were in a stand about 1.5 ha in size and were separated from each other by at least 10 m. During this period and in the previous 3 years, the

natural densities of *L. dispar asiatica* and other insect defoliators in the study area were low. Egg masses used in the experiment were collected in 2004 and 2005 during a natural outbreak in *B. pendula* stands 200 km west of Novosibirsk. Egg masses were held at +4°C until deployment at the beginning of the experiments (Lazarević et al., 2002).

Experimental Design and Field Experiments In 2005, natural defoliation of *B. pendula* by *L. dispar asiatica* larvae was simulated according to the methods described by Hunter and Schultz (1993). Twenty one 9–11-yr-old trees, each about 5 m in height, were chosen randomly for defoliation treatment in a discrete stand. Nineteen similar trees were chosen randomly as controls in the same stand. In mid-June, during which natural defoliation of *B. pendula* by *L. dispar asiatica* occurs in Western Siberia, the entire crown of each tree was covered with a large plastic mesh bag (4×1.5 m). Between 250 and 300 third-instars of *L. dispar asiatica* (reared in the laboratory from eggs collected previously) were released into each bag, i.e., the density of the insects was about 250–300 larvae per young treated tree. Bags in control trees were void of larvae but otherwise were treated similarly. The released larvae severely (no less than 80%) defoliated the experimental *B. pendula* in 2 wk, coincident with defoliation by wild populations of *L. dispar asiatica*, which were in outbreak at the time. After defoliation, the bags were removed together with any late instar larvae. In autumn, all trees involved in the experiment, as well as the neighboring trees, were checked for the presence of egg masses of *L. dispar asiatica*, but none was found.

In the middle of May 2006, *L. dispar asiatica* eggs that had been held at +4°C were transferred to a laboratory insectary. These were allowed to hatch, and the larvae developed at 28°C. Larvae were reared to the third instar in plastic containers (100 larvae per container, one container per tree) and fed with branch tips cut from either control trees or from trees that had been experimentally defoliated in the previous year. To maintain leaf turgidity, freshly cut tips were placed into 10 ml glass vials that were filled with water and then sealed with parafilm. Laboratory rearing was conducted to prevent small instars from escaping. To test the effects of delayed induced response of *B. pendula* on larval fitness, immune defense, and susceptibility to infection by nucleopolyhedrovirus, 100 third-instars were transferred to each of 10 randomly chosen treated trees and to 10 control trees. In other words, to test the plant delayed induced resistance against *L. dispar asiatica* in 2006, only 20 of the 40 trees were involved in the experiment. One control tree from the forementioned trees was excluded from the experiment because of damage that occurred to it in June 2006. Thus, insect data obtained from 19 trees were used in the experiment. After larvae molted into the third instar, we randomly selected three branches from the same tree assigned for this experiment, and enclosed those with mesh bags (80×30 cm). Colonization by third-instars from a corresponding container then followed. In each group of three bags, bag "A" was assigned to estimate fitness traits of the insects; the second, "B", was used to estimate the immune traits of the insects; and the last, "C", was used to estimate the resistance of the larvae to viral disease. If the larvae were about to consume all the leaves in their bag before the end of experiment, the bag was moved to a different branch on the same tree.

Prior to larval deployment, pooled samples of leaves (50 leaves per each tree) were collected from within the entire crown of each of the defoliated, and control trees and were analyzed for phenolic compounds (see below).

Fitness Traits Larval development time, female pupal weight, and survival to the adult stage were recorded. Development time is defined as an average duration of the larval stage from eclosion of the egg to pupal molt (Lazarević et al., 2002). The molting of larvae into pupae in the bags was checked daily, and newly molted pupae were taken to the laboratory and weighed on the same day and kept separately until they became adults. The sex of each insect was determined by morphology, particularly the structure of the antenna (Leonard, 1981). Insect viability was determined as survival to the adult stage.

Immune Traits After group B larvae reached the fourth instar, they were removed from the bags on the trees and taken to the laboratory. Encapsulation rates in their hemolymph, lysozyme-like activity, phenoloxidase (PO) activity in hemolymph, and total hemocyte count (THC) were measured in the laboratory. To collect hemolymph, the cuticle of each caterpillar was pierced with a thin needle under the penultimate proleg. About 25–30 μl of hemolymph from each larva were collected, and then placed into two cooled 1.5 ml Eppendorf tubes. One aliquot (4.5 μl) was mixed with 20 μl of cooled anticoagulant with phenylthiourea to study THC. THC was immediately measured in a hemocytometer and recorded as the number of hemocytes per 1 ml of hemolymph. The other aliquot of hemolymph was centrifuged at 500×*g* and +4°C for 10 min, and the supernatant was used to estimate PO activity, lysozyme-like activity, and protein concentration. PO activity was measured by using L-dopa as a substrate. Ten μl of supernatant were mixed with 500 μl of L-dopa in phosphate buffer, pH 7.2 (concentration 2 mg/ml solution). After incubation for 1 hr at 28°C, PO activity was measured at 490 nm with an Agilent 8453 UV-visible spectroscopy system. Hemolymph protein levels were measured by methods described by Bradford (1976), with a standard curve created from a bovine serum albumin standard. Phenoloxidase activity was measured in units of transmission density (ΔA) of the incubation mixture during

the reaction per 1 min and 1 mg of protein. The remaining hemolymph was mixed with a crystal of phenylthiourea, and 2 μl of the mixture were used to estimate lysozyme-like activity against the bacterium *Micrococcus lysodeikticus* (Lee et al., 2006). Samples were placed into holes in agar medium containing the test microorganism. Samples were incubated for 24 hr at 28°C. Data were recorded as the area of the lytic zones, which were photographed by using a digital camera and then measured with Image Pro software (Media Cybernetics, Silver Spring, MD, USA). Standard curves were obtained from a serial dilution of hen egg-white lysozyme equivalents. The encapsulation response was measured as the degree of melanization of a nylon monofilament implant that was inserted into the hemocoel of the larvae. This is a commonly used technique to measure the strength of immunity in insects (e.g., Ruuhola et al., 2007) and has been associated with resistance against real pathogens in moths (Rantala and Roff, 2007). To measure the encapsulation response, we inserted the monofilament into insects of the same group immediately after hemolymph collection. A 2 mm piece was inserted into the larva's body cavity through the hole made for hemolymph collection. The implants were dissected from the body cavity after 3 hr of exposure and then photographed in a black-and-white format from three perspectives. The degree of the melanization was quantified by using Image Pro software by first measuring the coloration—gray value (g.v.) of all areas on each implant, and then comparing these values with that of an unused implant.

Inoculation with Virus We used fourth instars for insects allocated to the viral inoculation portion of the study. The Tatarskiy strain of LdMNPV, a strain held in the laboratory collection of the Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences, was used. This virus strain was isolated previously from the same general locality from which the insect eggs used in this study were collected. Inoculation was performed in the laboratory to prevent virus contamination of the larvae in the different bags. The tips of each *B. pendula* individual (that corresponded to the source of the insects inside each bag) were washed exhaustively with sterile distilled water to reduce the chance of contamination by any external entomopathogenic agents. The birch tips were then placed onto a wooden frame (0.25 m^2) and arranged so the density of the leaves closely mimicked the natural crown of the tree. The tips were sprayed with a water-virus suspension (10^7 polyhedra ml^{-1}, 50 ml per frame). This dose was chosen according to the results of pilot experiments, and was approximately equal to an LC_{50}. After the leaves were dried at room temperature in the shade, the tips from each tree were presented to the appropriate groups of larvae (corresponding to the trees from whence they came). After the larvae had consumed all leaves, they were placed back into their bags in the field. Then, individual larvae were checked daily, and after they pupated they were transported to the laboratory and individually held until molting to adults. The susceptibility of the larvae to LdMNPV was calculated as the percentage of surviving adults within each bag. Larvae from "A" bags were fed leaves treated with sterile distilled water as a control, and checked as above.

Foliage Chemical Analysis Approximately 50 leaves (leaves had mature stage at the moment of sampling) were collected randomly from the entire canopy of each of the 40 trees involved in the experiment. The freshly collected leaves then were taken to the laboratory and were air-dried at room temperature in the shade for 10 d. The dried leaves were ground to a fine powder for chemical extraction. Three groups of phenolics were extracted: (1) simple phenolics; (2) water-soluble flavonoid glycosides; and (3) lipophilic flavonoid aglycones. To extract simple phenolics and flavonoid glycosides, 20 mg of the powdered leaves were added to 0.5 ml of acetone/water (7/3, V/V, containing 0.1% ascorbic acid, m/V) then shaken for 45 min with a mechanical shaker. Centrifuged pellets of samples were re-extracted twice in the same way. Thus, a final volume of 1.5 ml of supernatant was collected from each sample. Next, acetone was evaporated from the sample supernatant in an Eppendorf concentrator 5301 (Eppendorf AG, Hamburg, Germany). The aqueous mixture remaining was frozen and freeze-dried. The dried extract was dissolved in 1.5 ml of distilled water, filtered, and analyzed with an HPLC system (Merck-Hitachi, Tokyo, Japan). This consisted of a pump L-7100, a diode array detector L-7455, a programmable autosampler L-7250, a D-7000 interface, and a Superspher 100 RP-18 column (75×4 mm i.d., 4 μm particle size, Merck, Germany). Lipophilic surface-bound flavonoids were extracted from 10 mg of leaf powder with 1 ml of 95% ethanol for 1 min, and subsequently filtered and analyzed as described in Lahtinen et al. (2006) with the same HPLC system and column noted above. Simple phenolics were detected at 280 nm, with flavonoid glycosides and lipophilic flavonoid aglycones detected at 349 nm. Additionally, several samples that presented maximal amounts of peaks were analyzed by HPLC-MS according to the methods outlined by Salminen et al. (1999). Compounds were identified on the basis of their UV and mass spectral profiles, and from retention times reported in the literature (Keinänen and Julkunen-Tiitto, 1998). Simple phenolics were quantified as gallic acid equivalents, with water-soluble flavonoid glycosides and lipophilic flavonoid aglycones quantified as quercetin equivalents. Results are presented as the mean of amount of the compound of interest per gram of dried foliage with standard errors (Fig. 1). Compounds 1–6 are simple phenolics, compounds 7–13 are water-soluble flavonoids glycosides, and compounds 14–17 are lipophilic flavonoid aglycones.

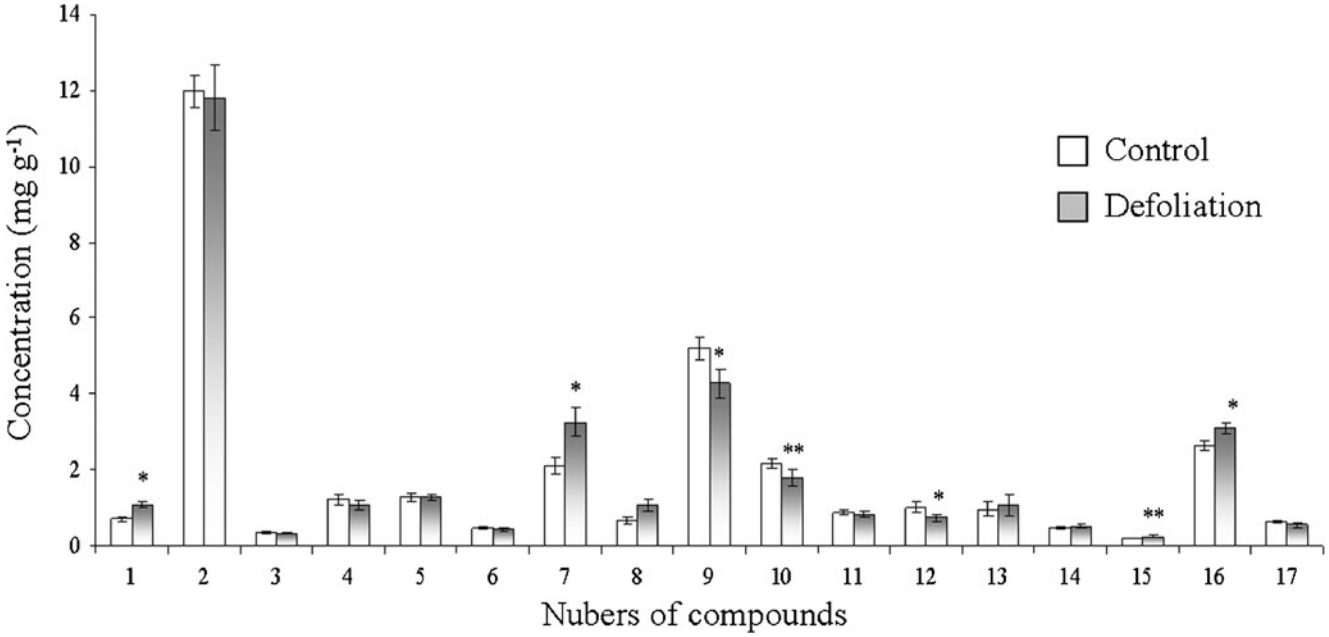

Fig. 1 Concentration (mean+SE) of phenolic compounds in foliage of control and defoliated *Betula pendula* trees. Phenolic compounds are listed in Table 1 with corresponding numbers. *Bars marked with asterisks* indicate a significant difference from controls (* at $P<0.05$, ** at $P<0.001$)

Table 1 One way ANOVA results of comparison of foliage phenolic concentrations between control and defoliated trees of *Betula pendula*

#	Name of compounds	*df* 1	*df* 2	*F*	*P*[a]
	Simple phenolics				
1	Unidentified phenolic #1	1	37	11.68	**0.002**
2	1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose	1	38	0.412	0.525
3	Unidentified phenolic #2	1	38	0.167	0.685
4	Chlorogenic acid	1	38	0.839	0.365
5	Coumaroyl quinic acid derivative # 1	1	37	0.120	0.731
6	Coumaroyl quinic acid derivative # 2	1	37	0.381	0.541
	Water-soluble flavonoids glycosides				
7	Myricetin glycoside	1	37	6.580	**0.014**
8	Quercetin glycoside # 1	1	37	2.638	0.113
9	Quercetin glycoside # 2	1	38	4.702	**0.036**
10	Quercetin glycoside # 3	1	29	7.945	**0.009**
11	Quercetin glycoside # 4	1	37	0.548	0.464
12	Flavonoid glycoside # 1	1	23	4.918	**0.037**
13	Flavonoid glycoside # 2	1	30	0.023	0.880
	Lipophilic flavonoids aglycones				
14	Flavonoid aglycone	1	37	2.285	0.139
15	Acacetin	1	33	8.365	**0.007**
16	Tetrahydroxy-flavone dimethyl ether	1	36	6.692	**0.014**
17	Pentahydroxyflavone	1	38	3.093	0.087

[a] Significant concentrations from defoliated trees have *P*-values in bold-faced text

Statistical Analyses Concentrations of phenolic compounds in leaves collected in 2006 were tested for normality with a Kolmogorov–Smirnov's test. The distribution was not normal, so we $\log_{10}$-transformed our data before analysis, and again checked the normality by using the Kolmogorov–Smirnov's test. Since all transformed data had a normal distribution after $\log_{10}$-transformation, we used a one-way ANOVA with treatment as the fixed factor (Table 1). The analysis was performed with STATISTICA 6.0 statistical software (StatSoft, Tulsa, OK, USA).

The effects of treatment on the pupal weights, development time, and immune parameters were tested with mixed model analysis (SPSS 19.0 for Windows, Chicago, IL, USA) with the following model: treatment, sex, and their interaction were set as fixed factors for pupal weights; treatment was set as fixed factor for development time as well as for larvae immune parameters. Host tree was used in the models as a subject, which means that tree*sex (for pupal weight data only) and tree*treatment interactions were handled as random factors. The tree and its interaction with treatment were redundant items and were omitted from the final model in the case of larval development rate. To study the effects of treatment and sex in more detail, pairwise contrasts were tested with the LSD-method. The distributions of PO and lysozyme-like activity data were not normal, so prior to analysis we $\log_{10}$-transformed the data for both of these measurements as guided by the Kolmogorov-Smirnov procedure.

The significane of the data on insect survival as well as resistance against LdMNPV were analyzed by two-way ANOVA with the *post-hoc* Fisher LSD procedure

(STATISTICA 6.0). Tree herbivory treatment and LdMNPV inoculation treatment were used as fixed factors. Prior to analysis, all data in percentages were arcsine of square root-transformed.

The Pearson Correlation test was used to evaluate the relationship between tree specific means for each immune parameter and the percentage survival for *L. dispar asiatica* per each tree after viral inoculation. Additionally, the Spearman's Rank Correlation was calculated to evaluate the relationship between phenolic concentrations with characteristics of survival, development, and immune defense of *L. dispar asiatica*. Data for control and defoliated trees were handled separately. Bonferroni corrections were made after the correlation analyses.

Results

The Effect of Defoliation of B. pendula on the Content of Phenolics in Leaves We did not register the production of foliage phenolic compound *de novo* in birch foliage during the year after severe defoliation (Fig. 1). However, defoliation history resulted in a significant increase of unidentified phenolic #1, myricetin glycoside, as well as two flavonoid aglycones (acacetin and tetrahydroxy-flavone dimethyl ether) (Fig. 1, Table 1). The concentrations of two glycosides of quercetin, as well as the content of flavonoid glycoside # 1 decreased significantly after defoliation treatment (Fig. 1, Table 1).

The Effect of Defoliation of B. pendula on Fitness of L. dispar asiatica Previous history of herbivory had no significant effect on the pupal weights of *L. dispar asiatica* ($F_{1,15}$=1.646, P=0.219; Fig. 2a). Females were twice as large as males ($F_{1,13}$=48.9, P<0.001), and this difference was not affected by treatments (LSD contrasts for both treatments P <0.001; Fig. 2a). In contrast, development time was prolonged by the DIR treatment ($F_{1,23}$=15.9, P=0.001; Fig. 2b).

The Effect of Defoliation of B. pendula on Immune Characteristics of L. dispar asiatica Tree treatment had an effect on the lysozyme-like activity ($F_{1,92}$=51.4, P<0.001; Fig. 3a) and a near significant effect on the PO activity ($F_{1,14}$=3.63, P=0.077; Fig. 3b). Tree treatment had no significant effect on the numbers of hemocytes ($F_{1,13}$=2.09, P= 0.172; Fig. 3c) or encapsulation rate ($F_{1,12}$=0.025, P=0.878; Fig. 3d).

The Effect of Defoliation of B. pendula on Resistance of L. dispar asiatica Against LdMNPV Virus infection reduced larval survival (F_1=4.835, P=0.035; Fig. 4). Defoliation treatment (F_1=0.520; P=0.476) as well as the defoliation * inoculation treatment interaction ($F_{1,34}$=0.120; P=0.730) did not significantly affect larval survival (Fig. 4). The mean hemocytes counts from larvae reared on each tree correlated significantly with the survival of larvae reared on the same tree in the virus inoculation treatment but not in the control treatment (Table 2).

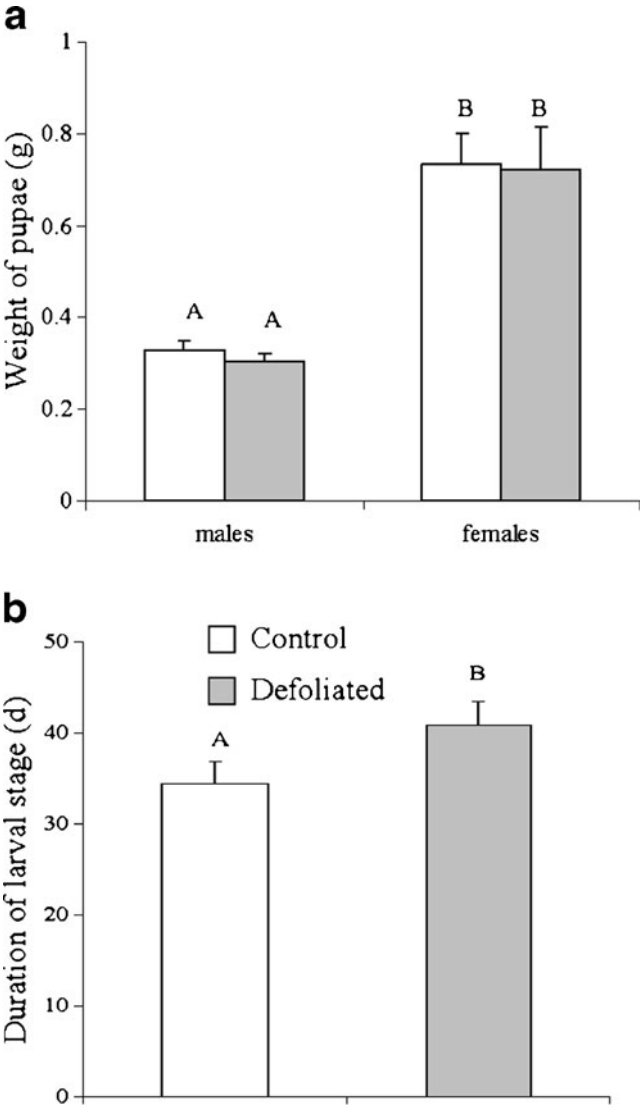

Fig. 2 Weight of pupae (mean+SE) (**a**) and the duration (mean+SE) of the larval stage (**b**) of *Lymantria dispar asiatica* reared on control and defoliated birches, *Betula pendula* (in control N=36 for larval duration, N=25 for pupal weight of males, N=11 for pupal weight of females; in treatment N=44, 28, 15 consequently). *Bars marked with different letters* indicate a significant difference between compared cases

Association of Leaf Phenolics with Characteristics of Survival, Development, and Immune Defense of L. dispar asiatica Among the trends that we examined, we found only a strong positive correlation between the concentration of 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose in leaves of defoliated trees and the survival rate of *L. dispar*

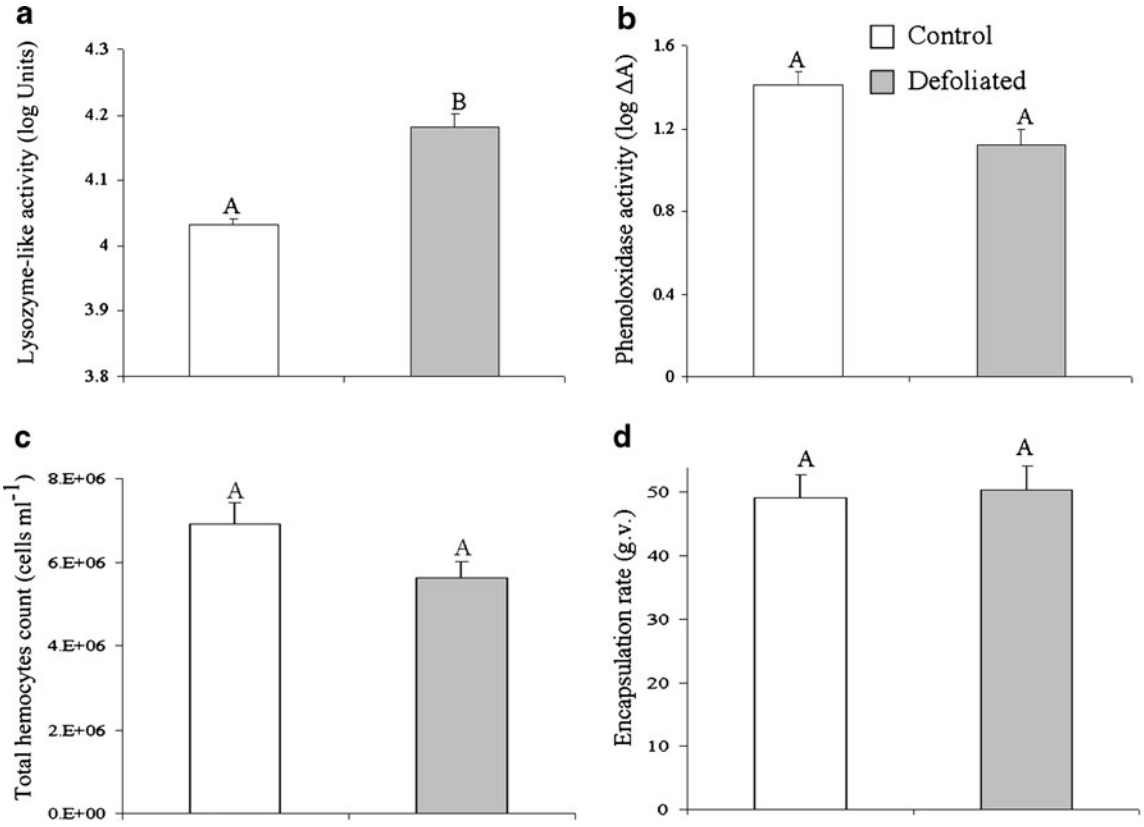

Fig. 3 Lysozyme-like activity (**a**), and phenoloxidase activity (**b**) in the haemolymph, total hemocytes count (**c**), encapsulation rate (**d**) of fourth-instar larvae of *Lymantria dispar asiatica* reared on control and defoliated birches (mean+SE; in control N=48 for (**a**), N=47 for (**b**), N=54 for (**c**), N=43 for (**d**); in treatment N=47, 35, 47, 45 in same order). *Bars marked with a different letter* indicate a significant difference between compared cases

asiatica that consumed those leaves (Table 4). The correlation coefficients of other parameters were not significant (Tables 3 and 4).

Discussion

Although the individual *B. pendula* trees in our study had been planted in the same area under the same environmental conditions and had a similar genotype (vegetative clones), they had considerable variation in their levels of leaf phenolics (Fig. 1). This study has shown that different classes of non-tannin phenolics are affected by defoliation history (Fig. 1, Table 1). The increase in concentrations of such flavonoid aglycones as acacetin and tetrahydroxy-flavone dimethyl ether supports the work of Valkama et al. (2005), who demonstrated that mechanical defoliation of *B. pendula* carried out in the previous season results in a significant increase of surface lipophilic compounds, including flavonoids aglycones. It is noteworthy that concentrations of two glycosides of quercetin declined in the year after defoliation treatment. This might be explained by the higher toxicity of phenolic aglycones than glycosides involved in the effective protection of trees against defoliators (Lahtinen et al., 2004).

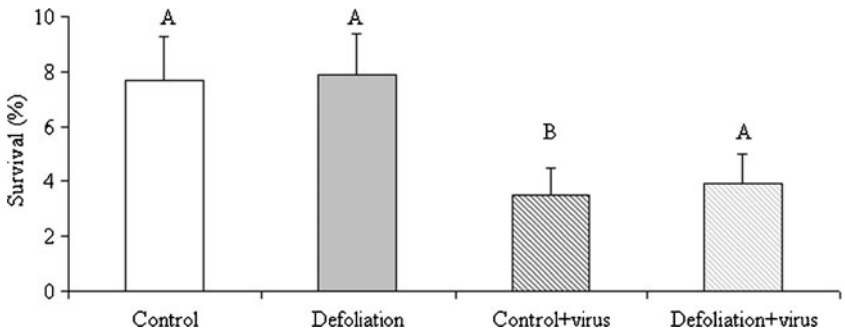

Fig. 4 Survival rates (means+SE) of *Lymantria dispar asiatica* fed on treated and untreated trees after nucleopolyhedrovirus infection (N=9 for "control" group, N=10 for "defoliation" group, N=9 for "virus" group, N=10 for "defoliation+virus" group). *Different letters indicate* significance differences among the means

Table 2 Correlations between immune characteristics of the Asian gypsy moth, *Lymantria dispar asiatica*, and survival to adulthood after infection by *L. dispar* polyhedrosis virus

Immune characteristics	*N*	Survival in virus treatment		Survival in control treatment	
		r	*P*[a]	*r*	*P*
Lysozyme-like activity	16	0.15	0.58	−0.11	0.70
Phenoloxidase activity	16	0.02	0.94	−0.31	0.25
THC[b]	16	**0.57**	**0.02**	0.25	0.35
Encapsulation rate	16	0.20	0.45	−0.07	0.80

[a] Significantly correlated immune characteristics have *P*-values in bold-faced text

[b] *THC* total hemocyte count

Delayed induced response of *B. pendula* prolonged the development time of *L. dispar asiatica* larvae feeding on foliage by more than 5d. This concurs with Ruuhola et al. (2007), who found that delayed induced resistance of mountain birch prolonged larval developmental time in *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) as well. In our study, we did not find a correlation between phenolic concentration after tree defoliation and larval development time (Table 4). Thus, the differences in larval development time likely were induced by other factors. Since *L. dispar dispar* is related to tannin-tolerant species (Barbehenn et al., 2009), it is unlikely that polyphenols were one of the factors. Additionally, we demonstrated previously that primary metabolites such as sugars were not involved in the delayed induced response of *B. pendula* (Bakhvalov et al., 2009). We assume that other classes of plant secondary compounds and/or leaf toughness are important in the rate of larval development. This prolonged larval development could increase the risk of larval parasitism, pathogens and predation. However, other unaffected measures of insect performance (i.e., survival, pupal weight) suggest only a slight contribution of delayed induced response of *B. pendula* to a principal regulation of the population cycle of *L. dispar asiatica*.

The main premise of immunological ecology is that the maintenance, regulation, and activation of the immune system are costly, and that there is a trade-off between immune defense and other life history traits (Schulenburg et al., 2009). Since we found that induced responses of trees had no effect on pupal mass but did enhance lysozyme-like activity in hemolymph (Fig. 3a), one might conclude that no trade-off occurred. The finding that defoliation treatment prolonged developmental time but led to an enhanced

Table 3 Spearman's rank correlation coefficients between the concentrations of phenolics in leaves of undefoliated *Betula pendula* and characteristics of immune defense, development, and survival of *Lymantria dispar asiatica*

No. of compounds [a]	Lysozyme-like activity	PO [b]	THC [b]	Encapsulation rate	Female pupal weight [c]	Male pupal weight	Larval development time	Survival rate	Survival after virus infection
1	0.43	−0.26	0.33	0.14	–	−0.31	0.66	−0.10	−0.19
2	0.17	−0.38	0.31	−0.19	–	−0.49	0.31	0.53	−0.22
3	0.71	0.36	−0.33	−0.17	–	−0.09	0.09	0.30	−0.27
4	−0.02	0.45	−0.83	0.19	–	0.09	−0.26	−0.29	−0.46
5	0.38	0.24	−0.76	−0.26	–	0.43	−0.09	0.33	−0.55
6	0.24	0.07	−0.62	0.21	–	−0.26	0.26	−0.08	−0.79
7	−0.12	0.38	0.40	−0.45	–	0.26	−0.60	0.20	0.74
8	−0.57	0.19	0.19	0.50	–	−0.54	−0.14	−0.81	0.11
9	0.19	0.40	−0.38	−0.50	–	0.83	−0.49	0.18	−0.11
10	0.44	−0.11	−0.20	−0.68	–	0.46	−0.03	0.81	−0.15
11	0.31	0.67	−0.21	−0.57	–	0.77	−0.77	−0.05	0.08
12	−0.43	−0.30	−0.08	0.81	–	−0.68	0.37	−0.35	−0.70
13	0.33	−0.45	−0.14	−0.14	–	0.14	0.54	0.29	0.00
14	0.57	0.60	0.00	−0.88	–	0.71	−0.71	0.42	0.25
15	0.45	0.02	0.40	−0.38	–	−0.26	−0.09	0.12	−0.19
16	0.31	−0.36	0.55	−0.38	–	−0.14	0.14	0.14	−0.05
17	0.50	−0.24	0.74	−0.45	–	−0.20	0.37	0.25	0.38

[a] The names of the compound is described in Table 1 with corresponding numbering

[b] *PO* phenoloxidase; *THC* total hemocyte count

[c] These data are absent because of small number of trees with surviving females

Table 4 Spearman's rank correlation coefficients between the concentrations of phenolics in leaves of defoliated *Betula pendula* trees and characteristics of immune defense, development, and survival of *Lymantria dispar asiatica*

No. of compounds [a]	Lysozyme-like activity	PO [b]	THC [b]	Encapsulation rate	Female pupal weight	Male pupal weight	Larval development time	Survival rate[c]	Survival after virus infection
1*	0.40	−0.26	0.33	−0.05	0.14	0.40	0.07	0.06	−0.35
2	−0.17	−0.52	0.48	0.14	−0.77	−0.07	−0.81	**0.98**	0.30
3	0.57	0.05	0.00	−0.26	−0.54	−0.26	−0.57	0.40	−0.35
4	0.36	0.40	−0.76	−0.24	−0.54	−0.31	0.12	−0.23	−0.25
5	0.17	0.71	−0.57	−0.29	−0.54	−0.74	−0.14	−0.21	0.14
6	0.31	0.52	−0.64	−0.31	−0.94	−0.71	−0.24	0.04	0.14
7*	−0.64	−0.71	0.31	0.60	0.09	0.19	0.24	0.22	0.87
8	−0.26	−0.40	0.12	0.10	−0.54	−0.17	−0.05	0.42	0.85
9*	0.24	0.21	0.17	−0.05	0.54	0.29	−0.14	−0.19	−0.87
10*	0.00	−0.02	0.14	0.24	0.94	0.57	0.52	−0.57	−0.52
11	−0.24	−0.40	0.12	0.52	0.49	0.29	0.19	−0.10	0.00
12*	0.29	0.64	−0.21	0.07	0.26	−0.07	0.14	−0.44	−0.67
13	−0.07	−0.02	−0.34	0.05	−0.38	0.22	0.29	0.04	0.08
14	−0.14	0.12	0.36	0.02	0.03	0.02	−0.07	0.07	0.22
15	−0.12	−0.24	0.64	0.10	0.49	0.26	0.00	0.11	0.19
16*	−0.21	−0.74	0.81	0.40	0.54	0.67	−0.02	0.30	−0.03
17	0.10	−0.45	0.64	0.24	0.54	0.43	−0.10	0.12	−0.27

[a] The names of the compound is described in Table 1 with corresponding numbering

[b] *PO* phenoloxidase; *THC* total hemocyte count

[c] Significantly correlated characteristics have bold-faced text

Phenolic compounds marked by asterisks in the first column identify the effect of defoliation treatment on their concentrations in *Betula pendula* foliage

immune reaction is, however, consistent with previous studies on other insects. These studies have found that slowly growing individuals have higher lysozyme-like activity in their hemolymph (Rantala and Roff, 2005).

We did not find correlations between leaf phenolics and lysozyme-like activity in the hemolymph of larvae that had consumed leaves from treated trees (Table 4). Thus, other factors besides concentration of flavonoids/simple phenolics in leaves may trigger the increase of a mechanism of antibacterial defense in larvae of *L. dispar asiatica* when they consume leaves from previously defoliated birches. Ecologically, there may be an increased potential for the larvae to resist bacterial diseases (e.g., *Bacillus thuringiensis*), and the frequency of this potential may increase in parallel with the increase in herbivore population density and herbivory. Interestingly, mean hemocyte count in the hemolymph of larvae reared on each tree correlated strongly with the survival of larvae reared on the same tree in the virus inoculation treatment group, but not in the control group (Table 2). Thus, it seems that in larvae of *L. dispar asiatica*, hemocyte levels might be associated with resistance to viral infection. This result supports the work of Trudeau et al. (2001) on two species of noctuids.

We saw no effect of prior year tree defoliation on adult survival after larval infection with LdMNPV (Fig. 4). This is consistent with our previous results, which also failed to find an effect of *B. pendula* response induced by artificial defoliation on *L. dispar asiatica* susceptibility to LdMNPV (Martemyanov et al., 2009). Similarly, D'amico et al. (1998) found no effect of same season defoliation on resistance to viral transmission in larval *L. dispar dispar*. In our study, larval immune traits responsible for resistance to viral disease, i.e., THC, PO, and encapsulation rate (according to Ourth and Renis, 1993; Reeson et al., 1998; Trudeau et al., 2001; McNeil et al., 2010) also were not affected by host plant defoliation. We hypothesize that delayed response of *B. pendula* induced by defoliation was not involved in the resistance of *L. dispar asiatica* to LdMNPV (however, it may be involved in resistance against gram-positive bacteria). It seems that maternal effects of the origin insect population are more important for regulative processes in *L. dispar* populations (low survival rate under rearing on control or treated trees, Fig. 4) than the plant mediated gypsy moth—baculovirus interaction or even direct delayed induced birch resistance to Lepidoptera.

We demonstrated a strong positive correlation between concentration of 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose (DHPPG) in *B. pendula* foliage and survival rate of *L. dispar asiatica* that had consumed leaves of treated trees (Table 4). In contrast, Mori et al. (1992)

demonstrated a weak anti-feedant property of DHPPG against fourth-instar *L. dispar* as well as well as repellent activity against first-instars of the same species. Mutikainen et al. (2000) demonstrated no significant relationship between the content of DHPPG in leaves of *B. pendula* saplings and the relative growth rate of *Epirrita autumnata* larvae that consumed those leaves. Finally, Keinänen et al. (1999) showed that an increased concentration of DHPPG was measured in *B. pendula* leaves after treatment b mechanical defoliation. We hypothesize that advantages given to larvae that feed on leaves with a high concentration of DHPPG are not directly related to the biological activity of this compound because the strong positive correlation occurred only for defoliated trees. It is possible that this phenylpropanoid derivative might interact with other chemicals in the leaves from previously defoliated trees that provide the larvae with some advantages that enhance their survival.

In summary, we demonstrated that the delayed response of *B. pendula* induced by severe *L. dispar asiatica* defoliation is related to the induction of some non-tannin phenolics (Fig. 1). However, despite suppression of larval growth rate when insects consumed leaves of treated trees (Fig. 2b), we did not find an association between the concentration of induced phenolics and various parameters of *L. dispar asiatica* fitness (Table 4). Even tetrahydroxy-flavone dimethyl ether related to highly toxic compounds (i.e., flavonoid aglycones), which had been induced by defoliation treatment, had no negative relationship with *L. dispar* fitness (Table 4). We also found a strong positive correlation between the concentration of DHPPG (the dominant non-tannin phenolic in *B. pendula* foliage) in leaves of defoliated plants and the survival of *L. dispar asiatica*. This suggests an unlikely protective role of this compound in *B. pendula* resistance against larvae of *L. dispar asiatica*. Furthermore, we showed that delayed induced response of silver birch does not have an effect on the insect immune parameters responsible for their resistance against baculoviral disease, as well as finding that there were no DIR-mediated changes in larvae resistance against real baculovirus infection. Perhaps the most interesting finding is the strong increase of lysozyme-like activity when larvae were fed on leaves of defoliated trees. This activity may reflect the provision of potential readiness to the larvae to resist gram-positive bacteria, whose number in turn increases with host population density. The study indicates the importance of considering multiple factors when characterizing barriers to insect immunity in the context of plant-induced defense.

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Genotypic Differences and Prior Defoliation Affect Re-Growth and Phytochemistry after Coppicing in *Populus tremuloides*

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Abstract Although considerable research has explored how tree growth and defense can be influenced by genotype, the biotic environment, and their interaction, little is known about how genotypic differences, prior defoliation, and their interactive effects persist in trees that re-grow after damage that severs their primary stem. To address these issues, we established a common garden consisting of twelve genotypes of potted aspen (*Populus tremuloides*) trees, and subjected half of the trees to defoliation in two successive years. At the beginning of the third year, all trees were severed at the soil surface (coppiced) and allowed to regenerate for five months. Afterwards, we counted the number of root and stump sprouts produced and measured the basal diameter (d) and height (h) of the tallest ramet in each pot. We collected leaves one and two years after the second defoliation and assessed levels of phenolic glycosides, condensed tannins, and nitrogen. In terms of re-growth, we found that the total number of sprouts produced varied by 3.6-fold among genotypes, and that prior defoliation decreased total sprout production by 24%. The size (d^2h) of ramets, however, did not differ significantly among genotypes or defoliation classes. In terms of phytochemistry, we observed genotypic differences in concentrations of all phytochemicals assessed both one and two years after the second defoliation. Two years after defoliation, we observed effects of prior defoliation in a genotype-by-defoliation interaction for condensed tannins. Results from this study demonstrate that genotypic differences and impacts of prior defoliation persist to influence growth and defense traits in trees even after complete removal of above-ground stems, and thus likely influence productivity and plant-herbivore interactions in forests affected by natural disturbances or actively managed through coppicing.

Keywords Aspen · Condensed tannins · Defense · Phenolic glycosides · Root sprout · Stump sprout · Sucker

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Introduction

Plant growth and defense are influenced by genetic and environmental factors, and the interactions between them. Much research has shown how intraspecific genetic variation in plants can present herbivores with a complex and varied array of food choices even within the same tree species (McKey, 1979; Rhoades, 1979; Stamp, 2003; Nyman et al., 2011). Genotypic differences can be modulated further by the environment (Marquis, 1992; Mutikainen et al., 2000; Laitinen et al., 2005; Osier and Lindroth, 2006). One environmental factor that can have a pronounced effect on plant growth and defense is defoliation by herbivores. Although the effects of defoliation on plant growth and

defense have been examined in many studies (Fritz and Simms, 1992), much less is known about how genotypic differences, prior defoliation, and their interactive effects persist in trees that can re-grow after intense damage that severs their primary stem (Agrawal et al., 2002; Frey et al., 2003; Lindroth et al., 2007).

Coppicing is a silvicultural harvest method that involves cutting of mature trees near the base of the stem, resulting in production of new root or stump sprouts (Dickmann et al., 2001). Coppicing also can occur in response to damage by natural factors such as fires, storms, avalanches, and herbivores such as beavers (Basey et al., 1990) and elephants (Jachmann, 1989). Coppicing in certain species, including aspen (*Populus tremuloides*) and other members of its genus, encourages re-growth from the remaining roots and stumps by releasing them from the effects of apical dominance (Schier et al., 1985). One of the primary reasons for the high productivity of *Populus* species in both natural and plantation forests is their prolific growth response to coppicing.

Coppicing can also influence levels of defensive compounds, because leaves produced after coppicing are ontogenetically young (Boege and Marquis, 2005; Barton and Koricheva, 2010). Aspen produce two major groups of phenylpropanoid-derived defense chemicals: phenolic glycosides ["salicinoids" (Boeckler et al., 2011)] and condensed tannins. Donaldson et al. (2006) and Smith et al. (2011) showed ontogenetic variation in aspen defense chemicals with young aspen producing leaves with high levels of phenolic glycosides and low levels of condensed tannins, relative to older trees.

Aspen is a preferred food source for both insect and mammalian herbivores. Insect herbivores include outbreak species such as the forest tent caterpillar, gypsy moth, and large aspen tortrix (Mattson et al., 1991), while mammalian herbivores include beaver, deer, elk, hare, moose, and porcupine (Perala, 1990; Diner et al., 2009). Many studies have shown anti-herbivore properties of phenolic glycosides against both insects (Osier and Lindroth, 2001, 2004; Donaldson and Lindroth, 2007) and mammals (Wooley et al., 2008; Diner et al., 2009). Evidence supporting the anti-herbivore properties of condensed tannins is more limited (Ayres et al., 1997). In aspen, some studies have shown that condensed tannins confer resistance (Bailey et al., 2004; Donaldson and Lindroth, 2004), while many others have not (e.g., Hwang and Lindroth, 1997, 1998; Wooley et al., 2008; Diner et al., 2009).

The objectives of this study were to assess how genotypic differences, prior defoliation, and their interaction affect shoot growth and foliar chemistry following coppicing in aspen (*Populus tremuloides*). We hypothesized that genetic signatures and the effects of previous defoliation events would be evident in terms of both growth and phytochemical metrics even after a disturbance as major as the loss of all aboveground biomass.

Methods and Materials

Experimental Design On the campus of University of Wisconsin-Madison, we established a common garden of potted aspen trees of twelve different genotypes. The trees were either exposed to or protected from severe defoliation for two successive years. The defoliation treatment was applied at the whole plot level, with genotype (12) incorporated at the sub-plot level. The 24 treatment combinations were replicated across five blocks. Missing replicates reduced the total number of trees involved in the study from 120 to 110, such that some treatment combinations had only four replicates. The trees used in this study were a subset of a larger project (Stevens and Lindroth, 2005; Stevens et al., 2007, 2008).

The genotypes used were collected originally as root material from twelve southern Wisconsin aspen trees growing in the wild and represent the natural range of variation in aspen in the region. Microsatellite analysis revealed that each aspen genotype was unique (Cole, Waller, and Lindroth, unpublished data). The root material was replicated into multiple ramets using micropropagation techniques described in Donaldson (2005). Such techniques decrease the non-genetic effects (analogous to maternal effects) from source tissues (Wright, 1976).

In the spring of 2001, the replicated ramets were planted outside into 5-L pots containing a 40:40:20 mix of sand: silt-loam field soil: perlite. Osmocote 3–4 month slow release fertilizer (14:14:14 N-P-K+micronutrients) was added at a rate of 4.5 g/L of soil. In spring 2002, ramets (average height=1.1 m) were transplanted into 80-L pots containing a 70:30 mix of sand and silt-loam field soil and positioned within the common garden. Osmocote 8-9 month slow release fertilizer (18:6:12 N-P-K+micronutrients) was added at a rate of 4.5 g/L of soil in the spring of 2002 and again in the spring of 2003. The trees were grown in pots to facilitate the harvest of roots necessary for the larger project mentioned above. Large (80-L) pots were used to reduce potential pot effects.

Defoliation In early June of both 2002 and 2003, we used forest tent caterpillars (FTC) and scissors to severely defoliate the trees in our defoliation treatment. Our defoliation treatment was designed to mimic a FTC outbreak in both duration and intensity (Mattson et al., 1991; Parry et al., 2003). On defoliated trees, a subset of branches was bagged with mesh. Third and fourth instar FTC were introduced into the bags and allowed to feed for 10 d. The FTC provided saliva and frass that may be important natural cues to trigger tree responses to defoliation (Karban and Baldwin, 1997; Havill and Raffa, 1999; Kim et al., 2011). FTC accomplished only a minor portion of the defoliation treatment. Scissors then were used to complete the bulk of the defoliation

treatment, in which we removed 75% of each leaf. Manual defoliation allowed us to control the severity of the defoliation and ensure that each genotype was damaged similarly regardless of its chemical profile (Stowe et al., 2000; Siemens et al., 2003).

Coppicing and Re-growth Assessment In May 2004, the trees were coppiced (severed near the soil surface with loppers). Trees were not measured immediately prior to being coppiced but averaged 2.8 m tall in August 2003 when they were last measured. At the time they were coppiced, trees had been growing outside for 3 yr and had been defoliated twice in the preceding two Junes (2002 and 2003). The coppiced trees were allowed to regenerate for 5 mo. In October 2004, we counted the number of root sprouts and stump sprouts produced after coppicing. We measured the basal diameter (d) and height (h) of the tallest root sprout and tallest stump sprout produced by each coppiced tree. Diameter was determined at the soil surface by using calipers, and height was measured from the soil surface to the apical meristem. We used d^2h as a non-destructive metric for tree size as is commonly done in studies of *Populus* (Abrahamson et al., 1990; Stevens et al., 2007).

Because only 22.7% of the coppiced trees produced stump sprouts, and the mean number of stump sprouts produced per coppiced tree was low (0.58±0.12) (least-squares mean±SE), we combined the number of root sprouts (9.3±0.7) (least-squares mean±SE) with the number of stump sprouts to produce a metric for re-growth that we refer to as "total sprouts", or simply "sprouts". Of the 1,082 sprouts produced, 94.1% were root sprouts.

Phytochemical Analyses In July 2004 and June 2005, we collected about 15 leaves from the tallest root sprout in each pot. The youngest and oldest leaves on each root sprout were avoided. Leaves were kept under ice in the field and then flash-frozen in liquid N_2 and freeze-dried in the laboratory (Lindroth and Koss, 1996).

We analyzed the leaf tissue for chemicals most likely to affect herbivores, including phenolic glycosides, condensed tannins, and nitrogen, an index of protein. We quantified levels of the phenolic glycosides salicin, salicortin, tremuloidin, and tremulacin by using high performance thin layer chromatography (HPTLC) with purified aspen phenolic glycoside standards (Lindroth et al., 1993). We report levels of phenolic glycosides using concentrations of only salicortin and tremulacin because concentrations of salicin and tremuloidin were very low. Additionally, salicortin and tremulacin are more biologically active than are salicin and tremuloidin (Lindroth et al., 1988). Condensed tannins were extracted from leaf tissue with 70% acetone at 4°C, and quantified via the spectrophotometric acid butanol method of Porter et al. (1986). Condensed tannins purified via adsorption chromatography served as a reference standard. Nitrogen levels were quantified using a LECO elemental analyzer (St. Joseph, MI, USA) with glycine *p*-toluenesulfonic acid (N=5.665%) as a standard.

Statistical Analyses We assessed the effects of genotype, defoliation, and their interaction on re-growth and phytochemistry after coppicing. The total number of sprouts, size (d^2h) of the tallest sprout, and concentrations of phenolic glycosides, condensed tannins, and nitrogen from 2004 and 2005 were analyzed using a mixed-model, two-factor, split-plot ANOVA using JMP Version 8.0.2 (SAS Institute Inc., 2008). In the model, genotype was considered a random effect, and defoliation was considered a fixed effect. Defoliation was analyzed as a whole plot effect with genotype incorporated as a sub-plot factor within the whole plot treatment. All interactions between genotype and defoliation were included as sub-plot interactions. In the split-plot analysis, a whole plot error term (replicates within whole plot error) was used to test the whole plot effect (defoliation), while genotype was tested over of the interaction of genotype and defoliation. A split-plot error term (residual error) was used to test the genotype x defoliation interaction. Block was incorporated into the model and retained when its effect was statistically significant. Initial tree size (d^2h) was also included in the model but was not retained because its effect was never statistically significant. The data displayed normality and uniform variances except for the total sprout data and condensed tannin data from 2004. These data sets were log transformed to normalize their distributions.

Additional analyses included assessments of correlation, heritability, and temporal concordance. We calculated Pearson correlation coefficients to determine the relationships between growth parameters and between chemistry parameters. With regard to growth, we assessed the correlation between d^2h in August 2003 (initial tree size) and the number of sprouts and the d^2h of the tallest sprout produced. With respect to chemistry, we assessed the correlation between levels of chemicals observed in 2004 and 2005 and between the various chemical constituents produced within the same year. We estimated broad-sense (clonal) heritability (Harvell, 1998; Bailey et al., 2004) by calculating the proportion of the total phenotypic variance explained by additive genetic variance in each damage environment for total sprouts and in each damage environment in each of the two years for the phytochemicals (Stevens and Lindroth, 2005). We used Kendall's τ coefficients of concordance to assess the consistency of rank ordering of genotype chemistry across pre-coppice (2003) and post-coppice (2004 and 2005) years; i.e., is genotypic variation in expression of chemistry consistent, despite the impact of coppicing?

Separate analyses were conducted for previously defoliated and undefoliated trees. Pre-coppice data came from a related study that involved the same genotypes and treatments (Stevens et al., 2007). Kendall's τ coefficients range from 0 (no concordance) to 1 (perfect concordance) (Parsons et al., 2008; SAS Institute Inc., 2008).

Results

Re-growth Aspen genotypes responded differently to coppicing in terms of the number of sprouts produced (Fig. 1). Sprout number varied by 3.6-fold among genotypes when averaged across defoliation treatments. Prior defoliation decreased sprout production after coppicing in 75% of the genotypes studied, and by 24% overall. Although production of sprouts in a few genotypes was not adversely affected by prior defoliation, this difference in response was not sufficient to generate a significant genotype x defoliation interaction. The size (d^2h) of the tallest sprout produced after coppicing was not affected by genotype, defoliation, or their interaction. The number of sprouts produced was positively correlated with initial tree size (d^2h in August 2003; r=0.198, P=0.038), but the size (d^2h) of the tallest sprout produced was not (r=0.031, P=0.762). Broad-sense heritability estimates for total sprout production in both damage environments were low (Table 1).

Phytochemistry Phenotypic expression of foliar chemical composition was strongly influenced by genotype and largely unaffected by prior defoliation. In 2004 (one year after the second defoliation), we observed genotypic differences in concentrations of phenolic glycosides, condensed tannins, and nitrogen, but no significant effects of prior defoliation (Fig. 2). In 2005 (two years after the second defoliation), we again observed genotypic differences in concentrations of phenolic glycosides, condensed tannins, and nitrogen (Fig. 3). Effects of prior defoliation were seen in a genotype x defoliation interaction for condensed tannins. When averaged over all genotypes, trees that were previously defoliated had levels of tannins that were 8% higher than undefoliated trees, but one genotype exhibited an 82% increase in tannin levels.

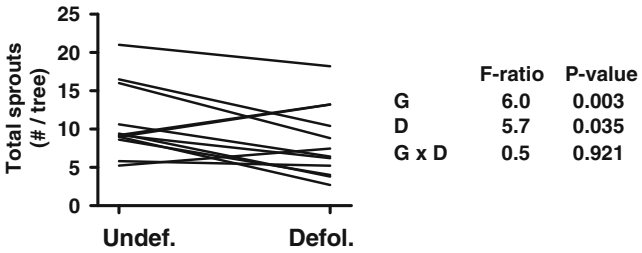

Fig. 1 Norm of reaction plot for production of total sprouts (root sprouts and stump sprouts) in relation to genotype ("G," *df=11*) and defoliation ("D," *df=1*). F-ratios and *P*-values indicate the results of a 2-factor, split-plot ANOVA. Each line represents the mean response (N=5 replicates) of a single aspen genotype in the undefoliated vs. defoliated condition

Phenolic glycosides and condensed tannins exhibited moderate to moderately-high broad-sense heritability estimates, in both damage environments, in 2004 and 2005 (Table 1). Nitrogen had low to moderate broad-sense heritability estimates (Table 1).

When we compared concentrations of phytochemicals in 2004 to concentrations in 2005, we found a 25% decrease in phenolic glycosides (29% and 17% for salicortin and tremulacin, respectively), a 615% increase in condensed tannins, and a 44% decrease in nitrogen concentrations. Levels of both phenolic glycosides (r=0.397, P<0.001) and condensed tannins (r=0.371, P<0.001) were positively correlated across the two years, indicating that patterns of expression within and among genotypes were largely consistent across years. In contrast, levels of nitrogen were not correlated between years (r=0.124, P=0.245).

In 2004, phenolic glycoside concentrations were positively correlated with condensed tannin concentrations, while nitrogen concentrations were negatively correlated with both phenolic glycosides and condensed tannins (Table 2). In 2005, however, phenolic glycosides were negatively correlated with condensed tannins, and the relationship between phenolic glycosides and nitrogen was not statistically significant (Table 2). Similar to 2004, there was a negative correlation between condensed tannins and nitrogen in 2005 (Table 2).

Analyses of concordance in foliar chemical composition between pre- and post-coppice trees revealed moderate to strong concordance for phenolic glycosides and weak to moderate concordance for condensed tannins (Table 3). Interestingly, consistency in expression of phenolic glycosides among genotypes declined from 2004 to 2005 in undefoliated trees, but increased in defoliated trees. Consistency in expression of condensed tannins among genotypes was significant only for undefoliated trees, between pre-coppice and 2005 post-coppice levels.

Discussion

Prolific re-sprouting following coppicing by humans or natural disturbance is a prominent life history trait of *Populus*. This study demonstrates that both the quantity and quality (chemical composition) of re-sprouts can vary among aspen genotypes, as well as in response to prior defoliation of the coppiced trees. Thus, major defoliation events have legacy effects that can extend beyond the lifespan of the tree that sustained damage.

Table 1 Broad-sense heritability estimates with 95% confidence intervals for total sprout production and concentrations of phenolic glycosides, condensed tannins, and nitrogen in both undefoliated and defoliated environments. Estimates for the phytochemicals are provided for both 2004 and 2005

		2004			2005		
	Total sprouts	Phenolic glycosides	Condensed tannins	Nitrogen	Phenolic glycosides	Condensed tannins	Nitrogen
Undefoliated	0.05±0.03	0.42±0.06	0.47±0.06	0.17±0.05	0.48±0.06	0.23±0.05	0.23±0.05
Defoliated	0.13±0.04	0.24±0.05	0.25±0.06	0.17±0.05	0.38±0.06	0.51±0.05	0.12±0.04

Clonal stands of aspen vary in the number of sprouts they produce (Barry and Sachs, 1968; Maini, 1968; Barnes, 1969; Tew, 1970; Schier et al., 1985). Aspen produces root sprouts when the movement of auxin from the shoot to the root is stopped or reduced by disturbance factors such as coppicing, girdling, or burning (Schier et al., 1985). Even though we found genotypic differences in numbers of sprouts produced after coppicing, our estimates of broad-sense heritability for sprout production were quite low. Low estimates of broad-sense heritability indicate that non-genetic factors also play substantial roles in determining the level of re-growth after coppicing.

Our finding that the majority (94%) of sprouts were produced by roots rather than by stumps corroborates the earlier work of Baker (1918), who reported that 92% of the sprouts produced after clearcutting a mature aspen stand in Utah originated from the roots. Horton and Maini (1964) determined that stump sprouts are more commonly produced when young aspen were coppiced but still represented only a small portion of the total regeneration. As we found, Agrawal et al. (2002) reported a positive correlation between number of sprouts after coppicing and tree size (dbh) before coppicing in hybrid poplars.

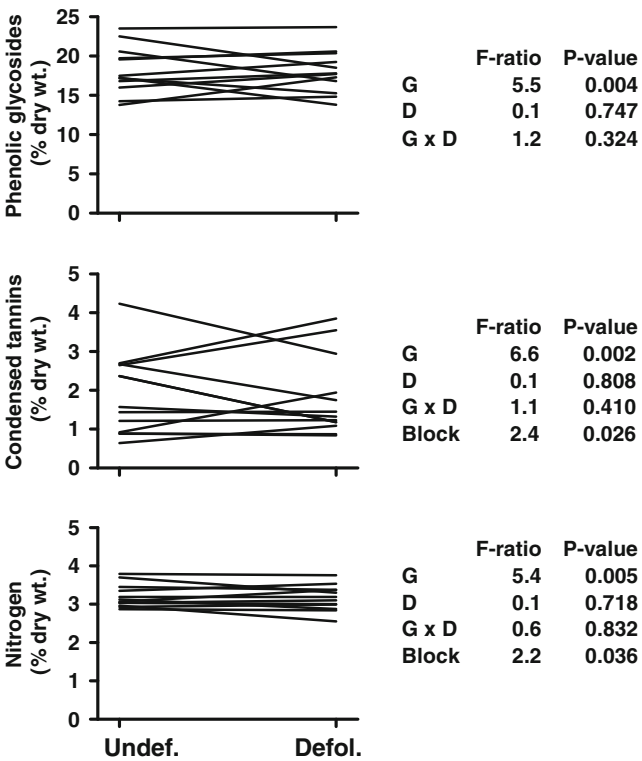

Fig. 2 Norm of reaction plots for foliar concentrations of phenolic glycosides, condensed tannins, and nitrogen in 2004 (one year after the second defoliation) in relation to genotype ("G," *df=11*) and defoliation ("D," *df=1*). *F*-ratios and *P*-values indicate the results of a two-factor, split-plot ANOVA. Each line represents the mean response (*N*=5 replicates) of a single aspen genotype in the undefoliated vs. defoliated condition. Note that the scale for condensed tannins differs from Fig. 3

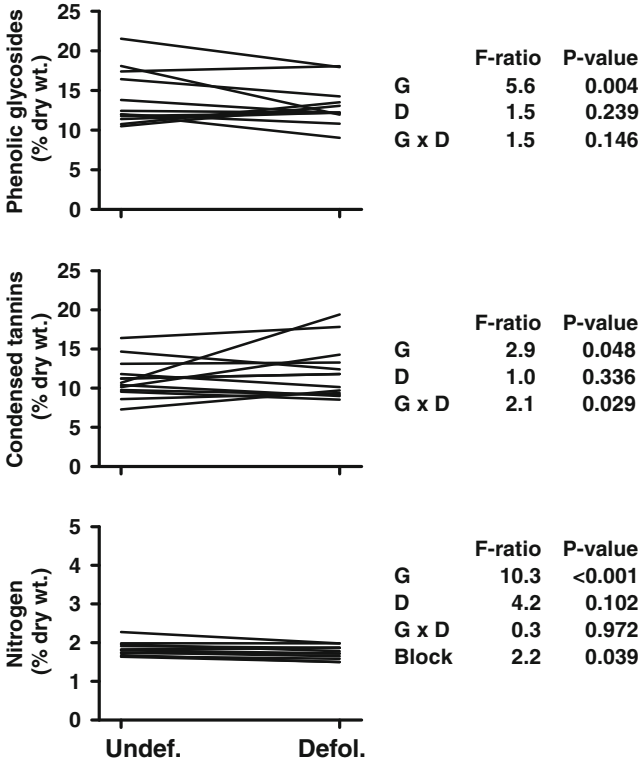

Fig. 3 Norm of reaction plots for foliar concentrations of phenolic glycosides, condensed tannins, and nitrogen in 2005 (two years after the second defoliation) in relation to genotype ("G," *df=11*) and defoliation ("D," *df=1*). *F*-ratios and *P*-values indicate the results of a two-factor, split-plot ANOVA. Each line represents the mean response (*N*=5 replicates) of a single aspen genotype in the undefoliated vs. defoliated condition. Note that the scale for condensed tannins differs from Fig. 2

Table 2 Pearson correlation coefficients (r) and corresponding P-values (in parentheses) for concentrations of phenolic glycosides, condensed tannins, and nitrogen within the years 2004 and 2005

	Phenolic glycosides		Condensed tannins	
	2004	2005	2004	2005
Condensed tannins	0.242 (0.018)	−0.434 (<0.001)		
Nitrogen	−0.272 (0.008)	−0.093 (0.377)	−0.342 (<0.001)	−0.362 <0.001)

We observed negative effects of prior defoliation in terms of the number of sprouts produced after coppicing. The reduction (24%) found in this study was similar to the reduction (25%) reported by Agrawal et al. (2002) in their assessment of stump sprouts in hybrid poplars.

The genotypic differences in re-growth after severe damage observed in our study could mean that selection will favor genotypes that respond more favorably to coppicing in areas where this type of damage occurs frequently. Because previous defoliation reduced the total production of sprouts in a similar way for all genotypes (no genotype x defoliation interaction), the legacy effects of defoliation are less likely to influence the genetic structure of aspen populations than are genotypic differences, when considered in an evolutionary context. That being said, aspen genotypes that avoid defoliation through phytochemical defenses (Donaldson and Lindroth, 2007) could re-grow substantially more after coppicing than genotypes that experience defoliation prior to a major disturbance.

In terms of post-coppicing phytochemistry, we found genotypic differences in concentrations of phenolic glycosides, condensed tannins, and nitrogen in both 2004 and 2005. Lindroth et al. (2007) reported genotypic variation in levels of the same phytochemicals in woody tissue produced after coppicing. That we found genotypic differences in concentrations of phytochemicals among coppiced trees that persisted for more than a year (and across two growing seasons) after the trees' primary stems were severed is evidence for the strong genetic control of these phytochemicals. The moderate to moderately high broad-sense heritability estimates for phenolic glycosides and condensed tannins corroborate the genetic underpinnings of these phytochemicals. Stevens and Lindroth (2005) and Donaldson and Lindroth (2007) reported even higher (>0.70) values of broad-sense heritability for both phenolic glycosides and condensed tannins. In woody tissue, estimates of broad-sense heritability were similar to ours, ranging from low to moderately high depending on the phytochemical examined (Lindroth et al., 2007).

Effects of prior defoliation on phytochemicals can also be lasting in that we observed increased levels of condensed tannins among root sprouts produced by previously defoliated trees in 2005—two years after the second (most recent) defoliation. Although the main effect of defoliation was not statistically significant in 2005, defoliation did increase tannin levels in a few genotypes (but not in others; significant genotype x defoliation interaction). In other studies of aspen leaf chemistry, Osier and Lindroth (2004) reported induction of condensed tannins one year after defoliation, Stevens and Lindroth (2005) described rapid induction of condensed tannins one week after defoliation, and St. Clair et al. (2009) found increased levels of condensed tannins in leaves produced in a second flush after frost defoliation. In woody tissue from root sprouts produced after coppicing, Lindroth et al. (2007) found no effects of prior defoliation on condensed tannin concentrations.

In contrast to the pattern exhibited by condensed tannins, we found no effect of defoliation on phenolic glycoside concentrations. This result also was observed by Osier and Lindroth (2001, 2004). However, other studies of aspen have revealed both increases (Stevens and Lindroth, 2005; Lindroth et al., 2007; St. Clair et al., 2009) and decreases (Lindroth et al., 2007; Stevens et al., 2007) in levels of phenolic glycosides in response to defoliation.

The general lack of genotype x defoliation interactions for all foliar chemicals examined in this study is consistent with previous research, in which similarity in response among genotypes tends to be the norm (Osier and Lindroth, 2001, 2004; Stevens and Lindroth, 2005). We were surprised by the few interactions confirmed in this study,however, as in several panels the norm of reaction plots (Figs. 2 and 3) revealed different slopes among genotypes. The absence of significant interactions could be due to high levels of variation among the 4–5 replicates used to calculate the means depicted in Figs. 2 and 3.

Table 3 Kendall's τ coefficients of concordance and corresponding P-values (in parentheses) for pre- and post-coppice phenolic glycoside and condensed tannin levels in twelve genotypes from undefoliated and defoliated conditions. Pre-coppice data are from 2003 while post-coppice data are from 2004 and 2005. Kendall's τ coefficients range from 0 (no concordance) to 1 (perfect concordance)

	Post-coppice year	Phenolic glycosides	Condensed tannins
Undefoliated	2004	0.758 (<0.001)	0.229 (0.303)
	2005	0.485 (0.028)	0.443 (0.046)
Defoliated	2004	0.273 (0.217)	0.382 (0.086)
	2005	0.455 (0.040)	0.199 (0.372)

When we compared concentrations of phytochemicals in 2004 to those in 2005, we observed a modest (25%) decrease in phenolic glycosides, but a dramatic increase in condensed tannin concentrations (615%). Our finding that the phenolic glycosides decreased, while condensed tannins increased, with age are consistent with the pattern reported by Donaldson et al. (2006) and Smith et al. (2011), who examined concentrations of phenolic glycosides and condensed tannins in aspen across a much wider span of age classes. Moreover, the dramatic increase in tannin concentrations was likely enhanced by a decrease in soil nutrient levels over time in this study, as the last soil fertilization occurred in 2003. Indeed, foliar nitrogen levels, which are strongly influenced by soil nutrient availability, declined 44% from 2004 to 2005. That condensed tannin concentration increased as soil fertility declined is consistent with the carbon-nutrient balance hypothesis (Bryant et al., 1983; Hamilton et al., 2001), and with previous studies of the effects of soil fertility on aspen chemistry (e.g., Osier and Lindroth, 2006; Donaldson and Lindroth, 2007).

Our finding that very young sprouts (produced two months after coppicing in 2004) have leaves with phenolic glycoside concentrations that are 25% higher than those in leaves produced by the same sprouts a year later, is evidence that the selective pressure for defense is especially high for very young sprouts (Donaldson et al., 2006; Smith et al., 2011). This increased pressure is likely driven by mammals rather than by insects because mammals can take advantage of the very young sprouts' short stature during a brief window of opportunity. Previous studies have shown phenolic glycosides to reduce the preference or performance of several mammals (Wooley et al., 2008; Diner et al., 2009) and insects (Hwang and Lindroth, 1998; Osier and Lindroth, 2001, 2004; Donaldson and Lindroth, 2007). Fewer studies have revealed antiherbivore properties of condensed tannins (Ayres et al., 1997), but see Donaldson and Lindroth (2004) and Bailey et al. (2004).

Within the years 2004 and 2005, correlations between the three groups of phytochemicals in the leaves of sprouts produced after coppicing were more commonly negative than positive. The only positive correlation revealed was between phenolic glycosides and condensed tannins in 2004. In contrast, Lindroth et al. (2007), working with these same trees, found positive correlations between salicortin, tremulacin, condensed tannins, and nitrogen in woody tissue produced before coppicing. In other studies involving aspen leaves, Donaldson et al. (2006) and Stevens and Esser (2009) also reported negative correlations between phenolic glycosides and condensed tannins (as we found in 2005), and Osier and Lindroth (2004) reported a negative correlation between phenolic glycosides and nitrogen (as we found in 2004). Just as allocation to phenolic glycosides and condensed tannins can come at a cost to growth (Hwang and Lindroth, 1997; Osier and Lindroth, 2006; Stevens et al., 2007), allocation to one group of phytochemicals, such as phenolic glycosides, may come at a cost to another group of phytochemicals, such as condensed tannins especially if derived from a common metabolic pathway (i.e., phenylpropanoid).

Little work has focused on how pre-disturbance conditions can affect levels of phytochemistry after a disturbance (Frey et al., 2003). When we examined levels of secondary metabolites for concordance between pre- and post-coppice trees, we found generally strong consistency in the ranks of genotype means for phenolic glycosides, but not for condensed tannins. This finding provides evidence that relative genotypic differences in levels of some phytochemicals can persist even after major disturbances. Previous work in aspen indicates that variation in levels of phenolic glycosides is largely a function of genotype, whereas variation in levels of condensed tannins is determined by both genotype and environment (Osier and Lindroth, 2001, 2004, 2006; Stevens and Lindroth, 2005). Our findings are consistent with that earlier work. Our research on legacy effects of genotype and defoliation that persist after major disturbances is analogous to recent work on how effects of prior herbivory can be transmitted across plant generations (Rasmann et al. 2012).

In summary, we found genotypic differences in regrowth after coppicing and a substantial decrease in regrowth after coppicing in trees that were previously defoliated. We also observed genotypic differences in phytochemical concentrations, including phenolic glycosides, condensed tannins, and nitrogen, among coppiced trees and an increase in condensed tannin concentrations in response to defoliation that was especially strong in one aspen genotype. These results confirm that heavy damage to aspen results in initial production of sprouts with juvenile-form chemistry, characterized by very high levels of phenolic glycosides and low levels of condensed tannins. Our analysis of concordance indicates that the effects of genotypic variation and prior defoliation on chemistry that exist prior to a disturbance can persist for multiple years following a disturbance. Thus, aspen forests that re-grow after coppicing may be more similar to the original forests than might be expected. Given the influence of aspen chemistry on ecological and ecosystem processes, genetic control of phytochemistry may contribute to long-term community and ecosystem stability despite substantial changes in forest structure. Such genetic legacies can affect plant-herbivore interactions and secondary succession dynamics in forests that experience major disturbances, both natural and anthropogenic.

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Nocturnal Bees are Attracted by Widespread Floral Scents

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Abstract Flower localization in darkness is a challenging task for nocturnal pollinators. Floral scents often play a crucial role in guiding them towards their hosts. Using common volatile compounds of floral scents, we trapped female nocturnal *Megalopta*-bees (Halictidae), thus uncovering olfactory cues involved in their search for floral resources. Applying a new sampling method hereby described, we offer novel perspectives on the investigation of nocturnal bees.

Keywords Augochlorini · Bee-plant Relationships · Benzenoids · Brazil · Halictidae · *Megalopta* · Synomones · Olfactory Cues

Introduction

Floral fragrances are important signals and attractants to nocturnal pollinators (Knudsen and Tollsten, 1995; Raguso, 2001, 2008; Dobson, 2006). Olfactory cues also are crucial for many bees, in both flower location at a distance (e.g., fragrance-seeking male orchid bees, Apidae, Euglossini; Dressler, 1982) and flower recognition in close proximity (reviewed in Dötterl and Vereecken, 2010).

Excluding the orchid bees, however, no more than ten species among the Apidae, Andrenidae, and Colletidae have been associated to scent-mediated attractiveness, which can be primarily attributed to the scarcity of studies directed to the role of plant volatiles in plant-bee interactions (Burger et al., 2010; Dötterl and Vereecken, 2010; Dötterl et al., 2011). This also is true for pollination systems involving the few night-active bee species.

Nocturnal foraging in bees has been observed among Colletidae (genus *Ptiloglossa*) and Halictidae (*Lasioglossum*, *Megommation*, *Megaloptidia, Reepenia*, and *Megalopta*) in the Neotropics, Apidae (*Xylocopa*) in the Paleotropics, and Andrenidae (*Perdita)* in the Nearctics (Wcislo et al., 2004; Michener, 2007). The genus *Megalopta* Smith, 1853 (27 spp.) (Augochlorini) is distributed from central Mexico to northern Argentina (Michener, 2007). In Brazil, *Megalopta* bees occur in almost all forest habitats, from dry shrublands to tropical rainforests (Silveira et al., 2002). Flying and collecting flower resources under dim light conditions or even in complete darkness, these bees occupy a niche inaccessible to other bee groups (Warrant et al., 2004; Wcislo et al., 2004). Due to their elusive crepuscular/nocturnal habits, *Megalopta* bees are poorly represented in entomological collections and their biology is mostly unknown, with the exception of *M. genalis* (e.g., Warrant et al., 2004; Wcislo and Tierney, 2009).

Some pollination studies involving *Megalopta* bees refer to the strong fragrance of the flowers they visit (e.g., Wolda and Roubik, 1986; Bullock et al., 1987; Hopkins et al., 2000), but the eventual role of these scents as attractive cues for the nocturnal bees has not been assessed. We selected floral scent compounds commonly found in bouquets of night-blooming angiosperms, and known to attract male

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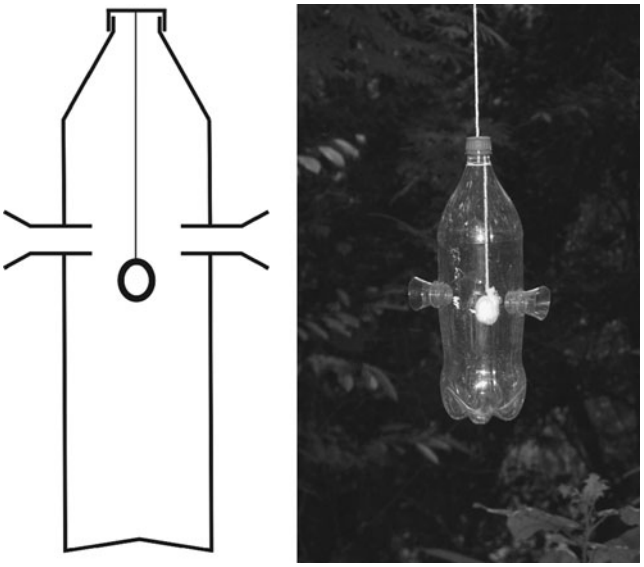

Fig. 1 Scent trap used to capture *Megalopta* bees, scheme and photograph. The trap was made of PET—bottles with two entrances and a scented bait hanging from a thread in the middle of the bottle

orchid bees, and asked: do these floral fragrances also lure nocturnal bees?

Methods and Materials

We conducted scent-mediated experiments in two localities. The first is located between the municipalities of Alto Araguaia, State of Mato Grosso and Santa Rita do Araguaia, State of Goiás (17°18′ S, 53°12′ W) and is characterized by Cerrado vegetation, a savanna complex common to Central Brazil. The second is located in northeastern Brazil (municipality of Camaragibe) (8°02′S 34°59′W) and corresponds to a moderately urbanized patch of original Atlantic Forest, a tropical rainforest found along the Atlantic coast of Brazil.

We assembled custom bait traps with 2 L transparent PET bottles, adapted from models used to sample male euglossine bees. Each unit consisted of a bottle in which two opposite orifices (22 mm diam) were opened up in the middle section. In each orifice, we inserted a screwable funnel, consisting of the cut-up upper section of another bottle. A fragrant bait, consisting of a filter paper ball impregnated with 5 ml scented compound, was hung from the inside of the bottle by a sewing line, tied to the screw cap (Fig. 1). Commercial standards of seven volatile compounds known to occur in night blooming flowers were used as baits: methyl salicylate (Sigma-Aldrich, Brazil ≥99.0%), β-ionone (Sigma-Aldrich, Germany >97%), 1,8-cineole (Merck, Germany >98%), vanillin (Sigma-Aldrich, Germany >97%), benzyl acetate, (Merck, Germany >95%), eugenol (Merck, Germany >98%), and benzyl benzoate (Fluka Buchs, Switzerland >99%). Sets consisting of eight traps, one per tested floral volatile, along with one baitless trap (control), were installed within a 10 m radius in the interior of forest fragments. Traps were installed at 1730 h and retrieved at 0600 h the following day. In the Cerrado locality, 11 sites were sampled between February 21th and March 4th 2010. In the Atlantic Forest locality, samplings were conducted during three nights between March 19th and July 3th 2010. All captured specimens from both localities were pooled for an exact binomial test for goodness-of-fit, used to access the difference in the frequency of bees captured in each of the treatment traps compared to the control. Our null hypothesis was that the same number of bees was trapped in the treatment and control traps. Specimens were deposited in the entomological collections of Universidade Federal da Paraíba, João Pessoa, and Universidade Federal de Minas Gerais—UFMG, Belo Horizonte, Brazil.

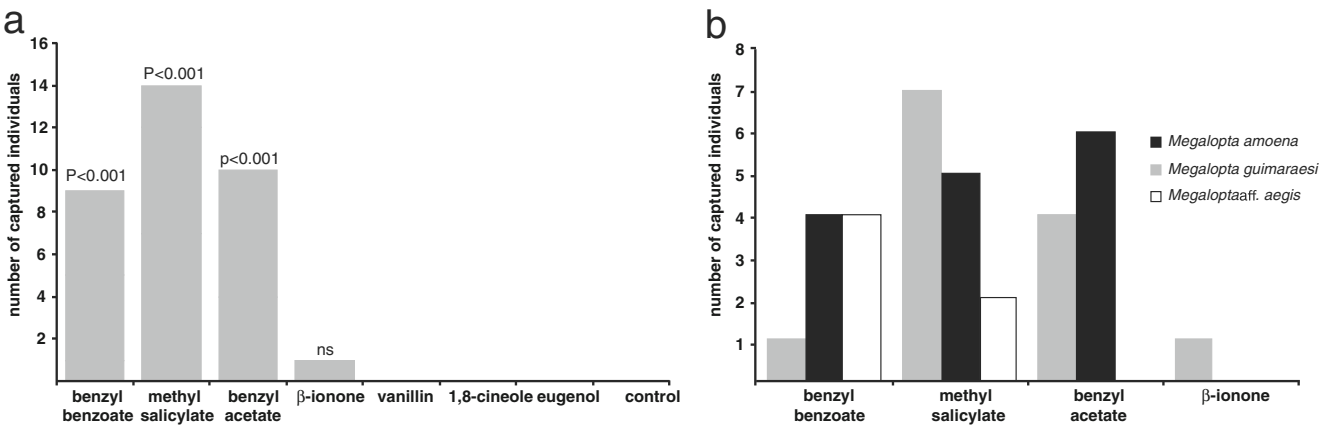

Fig. 2 Number of *Megalopta* females captured in traps with standards of floral volatiles at two localities in Brazil. **a** Number of bees captured per volatile compound. Benzyl benzoate, methyl salicylate, benzyl acetate attracted significantly more bees than the control as indicated by the significant outcome of exact binomial tests. **b** Number of individuals per species of *Megalopta* lured by the volatile compounds; *ns not significant*

Results

Thirty four female bees of three species of *Megalopta* were lured to the scented bait traps: *Megalopta* sp. aff. *aegis* in the Atlantic Forest, *M. guimaraesi* Santos & Silveira, 2009 in the Cerrado, and *M. amoena* (Spinola, 1853) at both sites. Bees were attracted with baits of four out of the seven tested compounds: the benzenoids benzyl acetate, methyl salicylate, and benzyl benzoate, as well as the irregular terpene, ß-ionone. Significant differences between the numbers of bees lured in treatment traps vs. control traps were found only for benzenoids, which attracted 9–14 individuals (Fig. 2a). Only one bee was lured to ß-ionone, and none was recovered in traps baited with 1,8-cineole, vanillin, or eugenol, nor in the baitless control traps. Females of *M. guimaraesi* and *M. amoena* were lured to the three benzenoid bait traps and those of *Megalopta* aff. *aegis* to benzyl benzoate and methyl salicylate (Fig. 2b). In the Cerrado sites, we collected a total of 21 bees, which were more efficiently attracted to benzyl acetate and methyl salicylate. At the Atlantic Forest, benzyl benzoate and methyl salicylate were most attractive to *Megalopta* bees and a total of 13 individuals were recovered in baited traps.

Discussion

This study shows that night-active female bees of the genus *Megalopta* are attracted to volatile compounds commonly found in flower bouquets (see Knudsen et al., 2006). Because the baited traps were assembled out of transparent PET bottles, influences of visual stimuli (e.g., color, shape) on the insects can be disregarded. Thus, we conclude that olfactory cues alone are capable of guiding these nocturnal bees to potential host flowers. This adds a new group of insects to the list of olfactory-driven nocturnal pollinators, which already includes bats, moths, and beetles (Gottsberger and Silberbauer-Gottsberger, 1991; Knudsen and Tollsten, 1995; Bernhardt, 2000; Raguso, 2001). The compounds that attracted *Megalopta*-females are prominent constituents of the floral bouquets of many angiosperms pollinated by night-active flower visitors (Raguso, 2001; Dobson, 2006; Knudsen et al., 2006). Bat-pollinated *Ochroma* (Malvaceae) and *Gustavia* (Lecythidaceae) whose floral odors include methyl salicylate, benzyl acetate, benzyl benzoate, and β-ionone (Knudsen and Tollsten, 1995; Knudsen and Mori, 1996; Knudsen et al., 2006) also are frequently visited by *Megalopta* bees (Wcislo et al., 2004). These compounds also are known to elicit attractive behavioral responses of male orchid bees (Dressler, 1982).

We are unable to speculate about the details of selective attractiveness of female *Megalopta* bees. Broader test screenings that include a larger assortment of volatile compounds will be an interesting next step. Moreover, the details of the pollination biology of plants visited by *Megalopta* and other nocturnal bees, along with their characterization of floral scent composition, are still needed.

Our results indicate that nocturnal bees, similarly to other night-active pollinators, might strongly rely on olfactory cues when looking for flowers in darkness. We speculate that such bees have come to use chemical signals common to night-blooming flowers to gain access to resources that are poorly exploited by other groups of nocturnal pollinators.

Our findings provide a new sampling method for *Megalopta* bees and open a new field of investigation of the poorly understood relationships of nocturnal bees and their food plants, as well as their behavioral biology and distribution. We expect that the use of these volatile compounds might also induce attractive response of other groups of nocturnal bees.

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Identification of Host Fruit Volatiles from Domestic Apple (*Malus domestica*), Native Black Hawthorn (*Crataegus douglasii*) and Introduced Ornamental Hawthorn (*C. monogyna*) Attractive to *Rhagoletis pomonella* Flies from the Western United States

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Abstract The apple maggot fly, *Rhagoletis pomonella*, infests apple (*Malus domestica*) and hawthorn species (most notably the downy hawthorn, *Crataegus mollis*) in the eastern USA. Evidence suggests that the fly was introduced into the western USA sometime in the last 60 years. In addition to apple, *R. pomonella* also infests two species of hawthorns in the western USA as major hosts: the native black hawthorn (*C. douglasii*) and the introduced ornamental English hawthorn, *C. monogyna*. Apple and downy hawthorn-origin flies in the eastern USA use volatile blends emitted from the surface of their respective ripening fruit to find and discriminate among host trees. To test whether the same is true for western flies, we used coupled gas chromatography and electroantennographic detection (GC-EAD) and developed a 7-component apple fruit blend for western apple-origin flies, an 8-component black hawthorn fruit blend for flies infesting *C. douglasii*, and a 9-component ornamental hawthorn blend for flies from *C. monogyna*. *Crataegus douglasii* and *C. monogyna*-origin flies showed similar levels of upwind directed flight to their respective natal synthetic fruit blends in flight tunnel assays compared to whole fruit adsorbent extracts, indicating that the blends contain all the behaviorally relevant fruit volatiles to induce maximal response levels. The black and ornamental hawthorn blends shared four compounds in common including 3-methylbutan-1-ol, which appears to be a key volatile for *R. pomonella* populations in the eastern, southern, and western USA that show a preference for fruit from different *Crataegus* species. However, the blends also differed from one another and from domesticated apple in several respects that make it possible that western *R. pomonella* flies behaviorally discriminate among fruit volatiles and form ecologically differentiated host races, as is the case for eastern apple and hawthorn flies.

Keywords Apple maggot fly · Olfaction · Fruit odor discrimination · Flight tunnel · Host races

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Introduction

The apple maggot, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) is a model system for sympatric ecologically-based speciation *via* host plant shifting for phytophagous insects (Feder, 1998; Funk et al., 2002). In particular, the shift of the fly from its native host downy hawthorn, *Crataegus mollis* Scheele (Rosales: Rosaceae), to introduced Eurasian apple, *Malus domestica* Mill. (Rosales: Rosaceae) in the northeastern USA

in the mid-1800s is often cited as an example of sympatric host race formation in the absence of geographic isolation and in the face of gene flow (Bush, 1966, 1969; Berlocher et al., 1993; Berlocher and Feder, 2002; Coyne and Orr, 2004). The shift of *R. pomonella* to apple also resulted in the fly becoming a major pest of commercial apple production in the growing regions of the northeast and Michigan.

The geographic range of the apple maggot fly is not limited to the northeastern and mid-western USA, however. In the late 1970s, *R. pomonella* was detected in the Pacific Northwest. Here, the fly costs apple growers tens of millions of dollars annually in monitoring, quarantine, and control, as there is a zero infestation policy for apple fruit export to foreign and domestic markets, a $1.5 billion a year industry in Washington State alone (National Agricultural Statistics Service, 2009). It generally is believed that the apple infesting race of *R. pomonella* was brought to the Pacific Northwest from the eastern USA *via* larval-infested apples sometime in the 1950s (AliNiazee and Westcott, 1987; Brunner, 1987; Tracewski et al., 1987). It is thought that the introduction probably occurred around the Portland, Oregon area where the first confirmed report of apple-infesting flies was made in 1979 (AliNiazee and Penrose, 1981). The fly then spread latitudinally up and down Oregon and Washington along the western side of the Cascade Mountains, reaching as far north as British Columbia, Canada and as far south as northern California. The fly also has encroached into commercial apple growing regions in central Washington, possibly by moving up the Columbia River Gorge.

Rhagoletis pomonella also infests hawthorns in the Pacific Northwest, including native black hawthorn, *C. douglasii*, and the introduced English ornamental hawthorn, *C. monogyna* (Yee and Goughnour, 2008), the latter possibly brought to the region following World War I. If apple flies were introduced from the eastern to the western USA, then these hawthorn-infesting populations represent host shifts of *R. pomonella* from apple back to hawthorn in the Pacific Northwest. Consistent with the introduction and host shift hypothesis, *R. pomonella* was first reported infesting the host *C. douglasii* in central Washington in 2003 (Yee, 2008). Although not an overly abundant plant west of the Cascade Mountains, the range of fruiting black hawthorn is fairly extensive along rivers and streams through the northwest, including central Washington. Thus, if *R. pomonella* has always existed on black hawthorn in the west, then it should have been detected infesting *C. douglasii* in central Washington prior to 2003, given the extensive annual monitoring program to control the fly performed by the Washington State Department of Agriculture begun in 1981 (Yee, 2008; Yee and Goughnour, 2008). Hawthorns would, therefore, appear to represent an alternate host to introduced apple-infesting flies in the West, and black hawthorn flies now in central Washington likely pose a new and serious threat to the apple industry. In addition, it is possible that black hawthorn and ornamental hawthorn populations of *R. pomonella* may also represent new races of the fly in the Pacific Northwest.

In the eastern USA, apple and downy hawthorn-infesting flies use volatile compounds released from the surface of ripening fruit as key olfactory cues to find and discriminate among host plants (Linn et al., 2003, 2004, 2005a,b; Dambroski et al., 2005; Forbes et al., 2005). Host fruit odor discrimination is important because the eastern apple and downy hawthorn-origin flies mate exclusively on or near the fruit of their respective host plants (Prokopy et al., 1971, 1972; Feder et al., 1994). Hence, differences in host plant choice translate directly to mate choice, generating premating reproductive isolation between the apple and downy hawthorn host races (Feder et al., 1994; Linn et al., 2003; Forbes et al., 2005; Forbes and Feder, 2006). In flight tunnel assays and field trapping studies, we found that the eastern apple and downy hawthorn host races of *R. pomonella* preferentially orient to the fruit volatile blends of their respective native host fruit relative to non-natal volatiles (i.e., apple flies positively respond and fly to a synthetic volatile blend developed for apple fruit and are not overly attracted to downy hawthorn fruit volatiles, and the reverse for downy hawthorn flies) (Linn et al., 2003, 2004, 2005a,b; Forbes et al., 2005; Dambroski et al., 2005; Forbes and Feder, 2006; Feder and Forbes, 2007, 2008).

Here, we present results of studies on the chemical ecology of western populations of *R. pomonella* by developing synthetic volatile blends for the black hawthorn, *C. douglasii*, and the introduced ornamental hawthorn, *C. monogyna*, through a combination of gas chromatography/electroantennographic detection (GC-EAD) analysis of both solid phase microextraction (SPME) and adsorbent samples collected from fruit headspace. Our objective was to use these synthetic blends to behaviorally test flies in flight tunnel assays and field trapping studies (the latter to be reported elsewhere) to establish the origins of western populations and to determine whether sympatric host races of *R. pomonella* exist on apple and hawthorns in the Pacific Northwest. We also investigated new formulations of the eastern apple blend by adding additional ester volatiles to the standard 5-component blend of Zhang et al. (1999). We conducted the latter studies based on reports from field trapping trials in Washington that imply that the standard eastern apple blend may not be as attractive to western as eastern flies (Yee et al., 2005).

Methods and Materials

In the following sections, we outline the methods of chemical and behavioral analysis that were used to develop and test synthetic volatile fruit odor blends for western apple, black hawthorn, and ornamental hawthorn-infesting flies. These methods are the same as those that we have successfully used in the past to construct synthetic blends for eastern and southern

R. pomonella populations. Complete details of the procedures can be found in Zhang et al. (1999), Nojima et al. (2003a,b), and Cha et al. (2011a,b).

Insects Flies used in the study were collected as larvae in infested host fruit and reared to the pupal stage in the laboratory at Washington State University in Vancouver, WA using standard *R. pomonella* husbandry techniques (Neilson and McAllan, 1965; Linn et al., 2003; Dambroski and Feder, 2007). Pupae were shipped to the Geneva, NY, lab, and upon eclosion adult flies were kept in a walk-in environmental chamber at 23–24°C, 16L:8 D photoperiod, and 65–70% r.h., on an artificial diet made of water, sugar, vitamins, casein hydrolysate, and salt mixture prior to testing (Fein et al., 1982). Western flies tested in the study came from four different apple sites, three different black hawthorn sites, and one ornamental hawthorn site in Washington (Table 1). Eastern apple-origin *R. pomonella* tested in certain GC-EAD analyses came from a laboratory colony maintained on Red Delicious apples at the Agricultural Experiment Station in Geneva, NY (Neilson and McAllan, 1965; Linn et al., 2003). Eastern apple flies collected and reared from a field site in Grant, MI and eastern downy hawthorn flies from two sites in Grant and Fennville, MI were also tested in certain GC-EAD analyses. Adult flies at 0–7 and 10–21-d-old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruit Black hawthorn fruit (*C. douglasii*) used for the GC-EAD analysis were collected from Wenas, WA, in 2007 (Table 1). Ornamental hawthorn fruit were collected from the Burnt Bridge Creek Greenway site in Vancouver, WA in 2008 (Table 1). Fruit collections were made in the field and shipped overnight to the Cornell Laboratory for volatile characterization. Red delicious fruit were collected from an Experiment Station apple orchard in Geneva, NY in 2007.

Headspace Volatile Sampling Adsorbent samples of fruit headspace volatiles were made from whole fruit using 2.4L closed volatile collection chambers (ARS, Inc., Gainesville, FL, USA; Glass Shop, Cornell University, Ithaca, NY, USA). Field collected fruits (500–800 ml in volume) were gently cleaned with distilled water, thoroughly dried, and then immediately put into a collection chamber. Clean air was pushed into the chamber at 0.7 $Lmin^{-1}$, and volatiles were pushed out through volatile traps (activated charcoal filters, ORBO32-small, Supelco Inc., Bellefonte, PA, USA) on the bottom of the chamber. For each sample of fruit, adsorbent collections were made over a 5-d-period. Volatiles were eluted with 500 µl methylene chloride every 24 h and combined across the five collection days. The combined extract was kept at −20°C and subjected to GC-EAD, GC-MS (mass spectrometry), and flight tunnel analyses.

For the quick evaluation of fruit headspace volatiles, SPME sampling also was performed using a glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, NJ, USA), containing ca. 150 g of fruit. A carboxen–polydimethylsiloxane-coated SPME fiber (film thickness 85 µm; Supelco) was conditioned in the GC injector (280°C) for 5 min, and then passed through the small hole on the cap into the headspace of the jar. After a 10 to 20-min-exposure, collected volatiles were subjected to GC-EAD and GC-MS analysis.

Coupled Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analysis Coupled GC-EAD analysis was performed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a non-polar EC-1 capillary column (30 m×0.25 mm diam, 0.25 µm film thickness; Alltech Associates, Inc., Deerfield, IL, USA) or a Shimadzu GC-17A gas chromatograph equipped with a polar EC-Wax Econo-Cap capillary column (30 m×0.25 mm diam, 0.25 µm film thickness; Alltech) in the splitless mode. Injector and

Table 1 Host plant sites (latitude (Lat.) [N] and longitude (Long.) [W] in deg. and min.) sampled in the study. WA=Washington state, MI=Michigan, NY=New York

Host plant	Location	Lat.	Long.
Western apple	Puyallup, WA	47.11	122.18
	Vancouver, WA (Burnt Bridge Creek Greenway)	45.18	122.36
	Skamania, WA (St. Cloud Park, Hyw. 14)	45.35	122.06
	Wenas, WA	46.87	122.67
Eastern apple	Geneva, NY	42.52	77.00
	Grant, MI	43.35	85.86
Western black hawthorn	Vancouver, WA (Burnt Bridge Creek Greenway)	45.18	122.36
	Skamania, WA (St. Cloud Park, Hyw. 14)	45.35	122.06
	Wenas, WA	46.87	122.67
Western ornamental hawthorn	Puyallup, WA	47.11	122.18
	Vancouver, WA (Burnt Bridge Creek Greenway)	45.18	122.36
Eastern downy hawthorn	Grant, MI	43.35	85.86
	Fennville, MI (62nd St.)	42.36	86.09

detector temperatures were set at 280°C and 270°C, respectively. The oven temperature was programmed for 5 min at 40°C, and 5°C min^{-1} increase to 250°C and then held for 5 min. Whole head preparations were made of individual flies for GC-EAD analysis (Nojima et al. 2003a). The antennal holder was placed inside a humidified condenser and maintained at 5°C. The output signal from the antenna was recorded on an HP 3390A integrator.

Chemical Analysis GC-MS was carried out as previously described (Zhang et al., 1999; Nojima et al., 2003a,b; Cha et al., 2011a,b) with a Shimadzu GCMS-QP5050A quadrupole mass spectrometer in EI (at 70 eV) scan mode coupled with a Shimadzu GC-17A equipped with a nonpolar DB-1 ms capillary column or a polar EC-Wax Econo-Cap capillary column. Helium was the carrier gas (54 kPa at 1.1 ml/min). The GC conditions and temperature program were as for the GC-EAD analyses. The interface temperature was set at 260°C. Volatile compounds were identified by comparison of chromatographic retention times and mass spectra with those of authentic standards analyzed on the same instrument. The identification was further verified by antennal responses in the GC-EAD analyses to the standard compounds. Quantification of the relative ratio of the EAD active compounds was made from the adsorbent collection based on total ion abundances from GC/MS analyses according to the standard curves made from each authentic sample.

Chemicals Pentyl hexanoate, hexyl butanoate, butyl hexanoate, propyl hexanoate, pentyl butanoate, butyl butanoate, 3-methylbutan-1-ol, D-limonene, pentyl acetate, hexyl acetate, ethyl heptanoate, isoamyl butyrate, isoamyl isobutyrate, isoamyl propionate, (*Z*)-3-hexenyl acetate, hexyl isobutyrate (purities≥98%), hexyl propionate, hexyl hexanoate (purities≥97%), nonanal, and linalool (purities≥95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The compound 2-methylbutyl 2-methylbutyrate (≥ 90%) was purchased from TCI America (Portland, OR, USA). The compound (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized by oxidation of geraniol and then by Wittig reaction with methylenetriphenylphosphorane (Greenwald et al., 1963) and purified (>97% with >97% *E*-isomer by GC-MS) using flash chromatography on silica gel. The synthesis product was eluted with hexane and then subjected to Kugelrohr distillation (b.p. ~60–70°C/3.0 mmHg) to remove non-volatile impurities, such as traces of silica.

Synthetic Blends and Flight Tunnel Sources We prepared the black and ornamental hawthorn synthetic blends as listed in Table 2. As in our previous flight tunnel studies (Zhang et al., 1999; Nojima et al., 2003a,b; Linn et al., 2003), we applied 200 μl of synthetic blend (1 μg/μl) to a red rubber septum (hexane-washed; Thomas Scientific, Swedesboro, NJ, Cat. no. 1780J07). The 200 μg dose was selected to provide a comparison with previous studies showing maximal levels of flight behavior with other *Rhagoletis* populations (Zhang et al., 1999; Linn et al., 2003; Nojima et al., 2003a,b). The septum sources were prepared 60 min prior to a test and were clipped onto the bottom of a 7.5 cm red plastic sphere (Gempler's Inc., Mt. Horeb, WI, USA). Fresh sources and red spheres were used for each replicate. Preliminary experiments showed that a 200 μg septum can be used in the tunnel for 6 h without a loss in response levels, a period significantly longer than any single replicate. To test the adsorbent extracts in the flight tunnel, we adjusted (either by diluting with hexane or concentrated under a gentle stream of nitrogen gas) the concentration (GC peak size) of GC-EAD active volatiles to become similar with the GC profiles of the synthetic blend at 1 μg/μl concentration, and applied 200 μl of the extract to a rubber septum.

Modified Synthetic Blends To identify key volatiles involved in attraction or acting as behavioral antagonists to black and ornamental hawthorn-origin flies, we prepared modified blends by subtracting volatile compounds from the candidate blends selected on the basis of consistent EAD responses (Table 2). The modified blends were prepared so that each compound was always in the same amount as in the corresponding complete blends, thus eliminating the potential effect of variation in concentration. For flight tunnel testing we applied 200 μl of the modified blend to a rubber septum. The concentration of each modified blend is listed in Table 2.

Flight Tunnel The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Nojima et al., 2003a). Flight-tunnel conditions were 23–24°C, 50–70% r.h., 35 cm/sec wind speed, 1500 lx light intensity. We used sexually mature adult flies (males and females) that were 10–21-d-old (post-eclosion) and had never been previously exposed to the fruit volatile blends (i.e., fruit odor-naïve flies). These flies were tested during the 3rd to 11th hr of the 16 h photophase period in the flight tunnel. Flies were selected from holding cages located in a separate, environmentally controlled room, placed singly in glass vials, taken to the room housing the flight-tunnel, and then allowed to acclimate for at least 30 min before testing. Individual flies were transferred to a screen holding cage, which was then placed on a release stand such that the open end of the cage faced upwind at a distance of 1 m to the odor source. Flies were given 1 min to respond, and scored by a human observer for the following two behaviors: 1) no response=fly did not take flight, but remained in the release cage, generally walking and grooming or flew for a short distance to the side of the flight tunnel; and 2) upwind-directed flight=fly faced upwind, walked to the edge of the release cage (100 cm from source), and took flight and initiated

Table 2 Relative ratio (%) of volatile compounds in the complete Eastern apple (EA), Western apple (WA1), black hawthorn (BH1), and ornamental hawthorn (OH1) synthetic blends. Differently numbered blends represent different modified blends (e.g., WA2, WA3, WA4, etc.). All the blends were prepared so that the same compound was always in the same amount as in corresponding complete blends (WA1, BH1, and OH1), thus eliminating the potential effect of variation in concentration. For flight tunnel tests of EA, WA1, BH1 or OH1 (prepared at 1.0 μg/μl), 200 μl of the blend, thus 200 μg of total volatile compounds, were loaded to a rubber septum. Ratio and concentration of modified blends (WA2, BH2, OH2, etc.) were formulated as found in the respective complete blends. For flight tunnel tests of the modified blends, we also loaded 200 μl of the modified blend to a rubber septum. Compounds in bold lettering indicate those that were modified in one or more of the blends. DMNT indicates 3(*E*)-4,8-dimethyl-1,3,7-nonatriene, and 2MM indicates 2-methylbutyl 2-methylbutanoate

	Relative ratio of complete and modified synthetic blends (%)																
	Eastern and Western Apple						Black haw							Ornamental haw			
	EA	WA1	WA2	WA3	WA4	WA5	BH1	BH2	BH3	BH4	BH5	BH6	BH7	OH1	OH2	OH3	OH4
3-methylbutan-1-ol							20	20	20	20	20	20		10	10	10	10
Pentyl acetate		**2**	**2**														
Isoamyl propionate														1	1	1	1
Butyl butanoate	10	5	5	5	5	5											
(*Z*)-3-hexenyl acetate							**4**	**4**	**4**	**4**		**4**	**4**				
Hexyl acetate		**20**	**20**	**20**	**20**												
Isoamyl isobutanoate														1	1	1	1
D-limonene							3	3	3	3	3	3	3				
Isoamyl butanoate														1	1	1	1
Pentyl butanoate														2	2	2	2
Propyl hexanoate	4	2	2	2	2	2	**3**										
Ethyl heptanoate							**5**	**5**		**5**	**5**	**5**	**5**				
Nonanal							**12**										
Linalool							**12**										
Hexyl propionate		**4**	**4**	**4**		**4**	5	5	5	5	5	5	5	3	3	3	3
2MM		**4**															
DMNT							**12**	**12**	**12**		**12**	**12**	**12**	**4**	**4**	**4**	
Hexyl isobutanoate														**40**		**40**	**40**
Butyl hexanoate	37	20	20	20	20	20	**12**	**12**	**12**	**12**	**12**		**12**				
Hexyl butanoate	44	20	20	20	20	20	**12**	**12**	**12**	**12**	**12**		**12**	**38**	**38**		**38**
Pentyl hexanoate	5	3	3	3	3	3											
Hexyl hexanoate		**20**	**20**														
Total (%)	100	100	96	74	70	54	100	73	68	61	69	49	53	100	60	62	96
Concentration(μg/μl)	1.0	1.0	0.96	0.74	0.7	0.54	1.0	0.7	0.7	0.6	0.7	0.5	0.5	1.0	0.6	0.62	0.96

upwind oriented flight in the odor plume. An upwind flight response was scored as positive if the fly exhibited plume-oriented flight for at least 50 cm toward the sphere [we previously showed that at this distance flies are not attracted to the red sphere alone with no odor source (Linn et al., 2003)]. In the following sections, 'agonist' volatiles refer to those compounds that contributed to the positive upwind directed flight response to a particular blend, and 'antagonist' refers to a non-natal host volatile that, when added to a blend, resulted in a significant reduction in upwind directed flights.

For each of the host plants, our experimental protocol involved first testing adsorbent extracts in the flight tunnel, with the expectation that response levels should be high, thus indicating that the behaviorally active blend was contained in the extract, and that no potential deterrent compounds were present, such as might occur with over-ripe or rotting fruit. Given a high response level to the extract, we then tested the candidate synthetic blends that were selected from the GC-EAD profiles based on the consistency of the responses over the majority (>75%) of the profiles. Again, the expectation was that response levels should be high and equivalent to those observed with the extracts. We then tested flies to modified blends that had volatiles subtracted or added to the complete blends to determine whether all of the compounds identified from the GC-EAD profiles were required for maximal response (upwind-directed flight). For each extract or synthetic blend, different sets of flies were tested as the flies became available from field collections of infested fruit. For the experiments with apple and black hawthorn-origin flies, each treatment involved a separate group of flies, but for ornamental hawthorn flies, the same group of 25 individuals was tested to all of the treatments. In previous studies, we showed that *R. pomonella* flies can be repeatedly tested without affecting odor preference as displayed in the flight tunnel (Dambroski et al., 2005). Differences in the frequency of upwind flight were compared using Fisher's Exact Test in R (R development core team, Vienna, Austria).

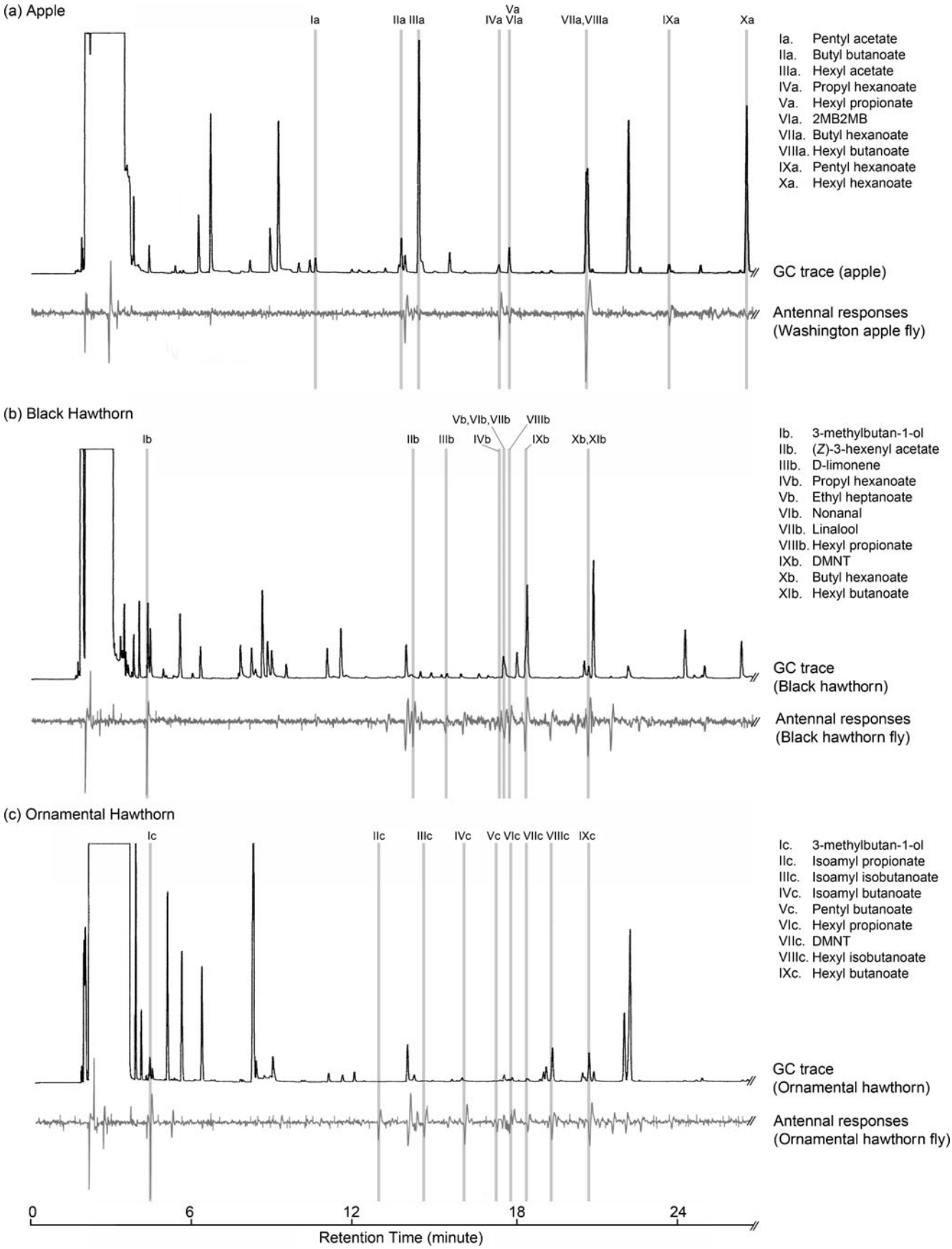
Results

Identification of Fruit Volatiles for Western Apple-Origin Flies Western apple-origin flies were tested for GC-EAD activity using adsorbent extracts from Red Delicious apples from orchards at the Experiment Station in Geneva, NY, the same source of apples used in earlier volatile identifications for eastern apple-origin flies (Zhang et al., 1999). A total of 25 antennal pairs (1–6 replicate runs/pair) were used for the analysis. The flies tested were from Vancouver, Puyallup, Skamania, and Wenas, WA. Figure 1a shows a GC-EAD trace from the antenna of a western apple-origin fly from the Vancouver, WA site to the Red Delicious extract. The corresponding active compounds were identified as (**Ia**) pentyl acetate, (**IIa**) butyl butanoate, (**IIIa**) hexyl acetate, **(IVa**) propyl hexanoate, (**Va**) hexyl propionate, (**VIa**) 2-methylbutyl 2-methylbutanoate (**VIIa**) butyl hexanoate, (**VIIIa**) hexyl butanoate, (**IXa**) pentyl hexanoate, and (**Xa**) hexyl hexanoate. The relative ratio of the EAD active compounds in the red delicious extract estimated with GC-MS are listed in Table 2.

Fig. 1 Simultaneously recorded GC-EAD responses of adult western apple, black hawthorn, and ornamental hawthorn fly antennae to adsorbent samples of volatiles released from apple, black hawthorn and ornamental hawthorn, respectively. Different traces indicate GC and antennal response (EAG) of (**a**) western apple-origin flies to apple fruit adsorbent samples, (**b**) western black hawthorn-origin flies to black hawthorn fruit adsorbent samples, and (**c**) western ornamental hawthorn-origin flies to ornamental hawthorn fruit adsorbent samples. DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene

Identification of Fruit Volatiles from Western Black Hawthorn Western black hawthorn (*C. douglasii*) fruit were analyzed from four samples collected from Wenas, WA in 2007, two in July and two in August. The August samples contained greater quantities of volatiles and were the focus of all our subsequent analyses. A total of 20 antennal pairs (1–4 replicate runs/pair) of black hawthorn-origin flies originating from Vancouver, Skamania, and Wenas, WA were used for the GC-EAD analysis. Figure 1b shows a GC-EAD recording from the antennae of a Vancouver, WA black hawthorn-origin fly exposed to the whole fruit extract at a stage when >90% of the fruit was ripe. We note that whereas the traces indicate the presence of some unidentified peaks with EAD responses, these responses were not present in the majority (>75%) of GC-EAD runs examined. The corresponding active compounds were identified as (**Ib**) 3-methylbutan-1-ol, (**IIb**) (*Z*)-3-hexenyl acetate, (**IIIb**) D-limonene, (**IVb**) propyl hexanoate, (**Vb**) ethyl heptanoate, **(VIb)** nonanal, (**VIIb**) linalool, (**VIIIb**) hexyl propionate, (**IXb**) DMNT, (**Xb**) butyl hexanoate, and (**XIb**) hexyl butanoate. All of the compounds in Fig. 1b were consistently found in the fruit collected from the Wenas, WA location. The relative ratio of the EAD active compounds in *C. douglasii* estimated with GC-FID and GC-MS are listed in Table 2.

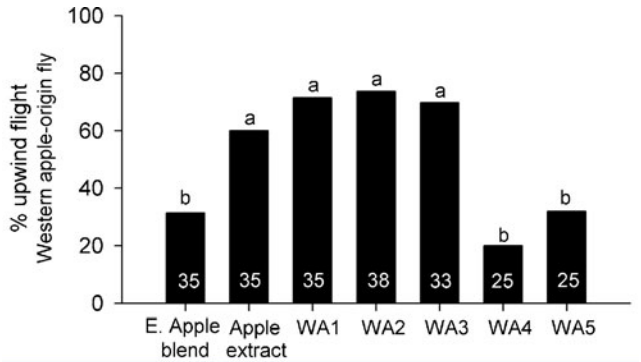
Identification of Fruit Volatiles from Western Ornamental Hawthorn Western ornamental hawthorn fruit (*C. monogyna*) collected in 2008 from the Burnt Bridge Creek Greenway, Vancouver, WA site were analyzed with GC-EAD using a single antennal pair from an ornamental haw-origin fly collected from the same site (5 replicate runs). However, for comparison, apple-origin flies from the Geneva, NY, Lab colony (2 antennal pairs, 3 replicates/pair), apple-origin flies from Grant, MI (1 antennal pair, 3 replicates), downy hawthorn-origin flies from Grant, MI (2 pair, 3 replicates/pair) and Fennville, MI (7 pair, 7 replicates/pair), and black hawthorn-origin flies from Vancouver, WA (2 pair, 4


(a) Apple
Ia
IIa IIIa
Va
IVa VIa
VIIa,VIIIa
IXa
Xa
Ia. Pentyl acetate
IIa. Butyl butanoate
IIIa. Hexyl acetate
IVa. Propyl hexanoate
Va. Hexyl propionate
VIa. 2MB2MB
VIIa. Butyl hexanoate
VIIIa. Hexyl butanoate
IXa. Pentyl hexanoate
Xa. Hexyl hexanoate
GC trace (apple)
Antennal responses
(Washington apple fly)
(b) Black Hawthorn
Ib
IIb
IIIb
Vb,VIb,VIIb
IVb
VIIIb
IXb
Xb,XIb
Ib. 3-methylbutan-1-ol
IIb. (Z)-3-hexenyl acetate
IIIb. D-limonene
IVb. Propyl hexanoate
Vb. Ethyl heptanoate
VIb. Nonanal
VIIb. Linalool
VIIIb. Hexyl propionate
IXb. DMNT
Xb. Butyl hexanoate
XIb. Hexyl butanoate
GC trace
(Black hawthorn)
Antennal responses
(Black hawthorn fly)
(c) Ornamental Hawthorn
Ic
IIc
IIIc
IVc
Vc VIc VIIc VIIIc
IXc
Ic. 3-methylbutan-1-ol
IIc. Isoamyl propionate
IIIc. Isoamyl isobutanoate
IVc. Isoamyl butanoate
Vc. Pentyl butanoate
VIc. Hexyl propionate
VIIc. DMNT
VIIIc. Hexyl isobutanoate
IXc. Hexyl butanoate
GC trace
(Ornamental hawthorn)
Antennal responses
(Ornamental hawthorn fly)
0
6
12
18
24
Retention Time (minute)

replicates/pair) also were used to confirm the ornamental hawthorn fruit volatile identification. Figure 1c shows a GC-EAD recording from the antennae of a western ornamental hawthorn-origin fly from Vancouver, WA exposed to an adsorbent whole fruit extract of *C. monogyna* from the same site. The corresponding active compounds were identified as (**Ic**) 3-methylbutan-1-ol, (**IIc**) isoamyl propionate, (**IIIc**) isoamyl isobutanoate, (**IVc**) isoamyl butanoate, (**Vc**) pentyl butanoate, **(VIc)**, hexyl propionate, (**VIIc**) DMNT, (**VIIIc**) hexyl isobutanoate, and (**IXc**) hexyl butanoate. The relative ratio of the EAD active compounds in *C. monogyna* estimated with GC-FID and GC-MS, are listed in Table 2.

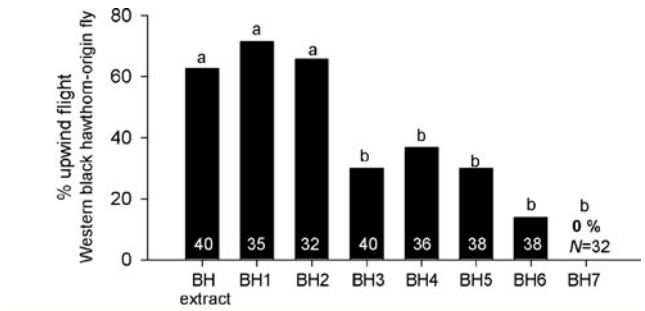
Behavioral Responses of Western Apple-Origin Flies Western apple-origin flies from Skamania, WA were tested in flight tunnel assays to the Red Delicious adsorbent extract, the eastern apple blend, a candidate 10-component blend (WA1) that was based on initial GC-EAD evaluations, as well as to a series of blends (WA2-5) in which certain volatiles were subtracted (Fig. 2). Because all of the volatiles in the previously identified eastern apple blend also displayed EAD activity with western flies, we focused our subtraction tests on volatiles unique to the western flies. Western apple-origin flies displayed only moderate levels of upwind flight to the eastern apple blend in the flight tunnel (31.4%; N=35), significantly lower compared to the adsorbent extract (60.0%, N=35, $P \leq 0.03$ two-tailed Fisher's exact test, 1 *df*) or the 10-component WA1 blend (71.4%, N=35, $P \leq 0.002$, 1 *df*). There was no significant decrease in upwind flight levels when either 2-methylbutyl 2-methylbutanoate (WA2= 73.7%, N=38) or the combination of 2-methylbutyl 2- methylbutanoate+pentyl acetate+hexyl hexanoate (WA3=69.7%, N=33) were removed from the WA1 blend. However, levels of upwind flight were reduced when either hexyl propionate (WA4=20.0%, N=25, $P \leq 0.001$, 1 *df*) or hexyl acetate (WA5=32.0%, N=25, $P \leq 0.004$, 1 *df*) was removed from the active 7-component WA3 blend (Fig. 2). These results indicate that the 7-component WA3 blend provided maximal levels of behavioral activity. We hereafter designate WA3 as the new "western apple" blend.

Behavioral Responses of Western Black Hawthorn-Origin Flies Western black hawthorn-origin flies reared to adulthood from infested *C. douglasii* fruit collected from Skamania, WA were tested in flight tunnel assays to the whole black hawthorn fruit adsorbent extract, to a candidate 11-component synthetic blend (BH1) based on initial GC-EAD evaluations, as well as to a series of blends (BH2-7) in which individual components were subtracted from the 11-component blend (Fig. 3). Western black hawthorn-origin flies displayed similar and non-significantly different levels



Pentyl acetate		**2**	**2**			
Butyl butanoate	10	5	5	5	5	5
Hexyl acetate		**20**	**20**	**20**	**20**	
Propyl hexanoate	4	2	2	2	2	2
Hexyl propionate		**4**	**4**	**4**		**4**
2MB2MB		**4**				
Butyl hexanoate	37	20	20	20	20	20
Hexyl butanoate	44	20	20	20	20	20
Pentyl hexanoate	5	3	3	3	3	3
Hexyl hexanoate		**20**	**20**			

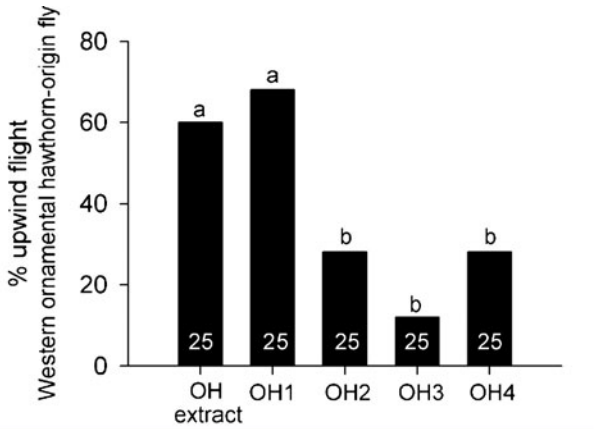
Fig. 2 Upwind flight responses (%) of western apple-origin flies to eastern apple blend, Red Delicious apple adsorbent extract, a candidate 10-component blend (WA1) that was based on initial GC-EAD evaluations, as well as to a series of blends (WA2-WA5) in which certain volatiles were subtracted. Relative ratios (%) of volatile compounds tested are shown under the graph. Bold characters indicate the modified compounds. White letters inside the bars indicate the number of flies tested for each treatment. Different letters on the bars indicate significant differences (P<0.05). 2MB2MB indicates 2-methylbutyl 2-methylbutanoate



3-methylbutan-1-ol	**20**	**20**	**20**	**20**	**20**	**20**	
(*Z*)-3-hexenyl acetate	**4**	**4**	**4**	**4**		**4**	**4**
D-limonene	3	3	3	3	3	3	3
Propyl hexanoate	**3**						
Ethyl heptanoate	**5**	**5**		**5**	**5**	**5**	**5**
Nonanal	**12**						
Linalool	**12**						
Hexyl propionate	5	5	5	5	5	5	5
DMNT	**12**	**12**	**12**		**12**	**12**	**12**
Butyl hexanoate	**12**	**12**	**12**	**12**	**12**		**12**
Hexyl butanoate	**12**	**12**	**12**	**12**	**12**		**12**

Fig. 3 Upwind flight responses (%) of western black hawthorn-origin flies to black hawthorn adsorbent extract, a candidate 11-component blend (BH1) that was based on initial GC-EAD evaluations, as well as to a series of blends (BH2-BH7) in which certain volatiles were subtracted. Relative ratios (%) of volatile compounds tested are shown under the graph. Bold characters indicate the modified compounds. White letters inside the bars indicate the number of flies tested for each treatment. Different letters on the bars indicate significant differences (P<0.05). DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene

of upwind flight to the adsorbent extract (62.5%, N=40) compared with the 11-component BH1 blend (71.4%, N=35). Removal of the compounds propyl hexanoate, nonanal, and linalool resulted in an 8-component blend (BH2) that was not significantly different from the upwind-directed flight levels to the whole fruit extract or BH1 (65.6%, N=32). Removal of ethyl heptanoate (BH3), DMNT (BH4), or (Z)-3-hexenyl acetate (BH5) from blend BH2 resulted in 7-component mixtures that were significantly lower in upwind directed flight response levels compared to BH2 (BH3=30.0%, N=40, $P\leq0.004$, 1 *df*; BH4=36.8%, N=38, $P\leq0.03$, 1 *df*; BH5=30.0%, N=40, $P\leq0.004$, 1 *df*), but were not significantly different from each other. Removal of both butyl hexanoate and hexyl butanoate (BH6) from blend BH2 resulted in significantly lower response levels compared with all other blends (13.9%, N=36). Finally, removal of 3-methylbutan-1-ol (BH7) from blend BH2 resulted in complete loss of upwind flight activity (N=32). These results indicate that the 8-component BH2 blend provided maximal levels of behavioral activity. We hereafter designate BH2 as the new "black hawthorn" blend.



3-methylbutan-1-ol	10	10	10	10
Isoamyl propionate	1	1	1	1
Isoamyl isobutanoate	1	1	1	1
Isoamyl butanoate	1	1	1	1
Pentyl butanoate	2	2	2	2
Hexyl propionate	3	3	3	3
DMNT	**4**	**4**	**4**	
Hexyl isobutanoate	**40**		**40**	**40**
Hexyl butanoate	**38**	**38**		**38**

Fig. 4 Upwind flight responses (%) of western ornamental hawthorn-origin flies to ornamental hawthorn adsorbent extract, a candidate 9-component blend (OH1) that was based on initial GC-EAD evaluations, as well as to a series of blends (OH2-OH4) in which certain volatiles were subtracted. Relative ratios (%) of volatile compounds tested are shown under the graph. Bold characters indicate the modified compounds. White letters inside the bars indicate the number of flies tested for each treatment. Different letters on the bars indicate significant differences (P<0.05). DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene

Behavioral Responses of Western Ornamental Hawthorn-Origin Flies Western ornamental hawthorn-origin flies (N=25) reared to adulthood from infested *C. monogyna* fruit collected from Puyallup, WA, were tested in flight tunnel assays to the ornamental hawthorn whole fruit extract, to a 9-component synthetic blend (OH1) that was based on initial GC-EAD evaluations, as well as a series of three other blends in which one of the compounds in OH1 was removed (Fig. 4). There was no significant difference in upwind-directed flight between the 9-component OH1 blend (68.0%, N=25) and whole ornamental hawthorn fruit adsorbent extract (60.0%, N=25). When hexyl isobutanoate (OH2), hexyl butanoate (OH3), or DMNT (OH4) was removed singly from the 9-component blend, upwind flight levels were lower compared with OH1 (OH2=28.0%, N=25, $P\leq0.01$, 1 *df*; OH3=12%, N=25, $P\leq0.001$, 1 *df*; OH4=28.0%, N=25, $P\leq0.01$, 1 *df*). These results indicate that the 9-component OH1 blend provided maximal levels of behavioral activity. We hereafter designate OH1 as the new "ornamental hawthorn" blend.

Discussion

We have now identified and developed synthetic volatile blends for western black hawthorn (*C. douglasii*) and ornamental hawthorn (*C. monogyna*) fruit using GC-EAD analyses of SPME and adsorbent samples. Western black hawthorn and ornamental hawthorn-origin flies displayed a high level of upwind directed flight to their respective natal fruit volatile blends equivalent to levels observed to their natal whole fruit adsorbent extracts, supporting the conclusion that the blends contain the full complement of compounds inducing oriented flight behavior. The black and ornamental hawthorn blends shared four compounds in common including 3-methylbutan-1-ol, which appears to be a key volatile for all *R. pomonella* populations that show a preference for hawthorn fruit (Linn et al., 2003; Cha et al., 2011a,b). However, the blends also differed from one another and from the eastern and western domesticated apple blends in several respects. These differences make it possible that western *R. pomonella* may behaviorally discriminate among fruit volatiles and form ecologically differentiated host races, as is the case for eastern apple and hawthorn-infesting flies (Linn et al., 2003).

An important finding in the current study bearing on the origin of western *R. pomonella* populations was that apple flies from the western and eastern USA differed in their responses to apple fruit volatiles. A previous field study suggested that the standard five-component eastern apple blend of Zhang et al. (1999) might not be as attractive to western flies as to eastern flies (Yee et al., 2005). Here, we show that western apple-origin flies required the addition of two compounds to the eastern apple blend (hexyl acetate and hexyl propionate) to achieve a similar level of

behavioral response in the flight tunnel as eastern apple-origin flies did to the eastern apple blend. Apple volatile profiles from the eastern and western USA did not appear to differ greatly from one another. Yet flies from these two regions clearly behaved differently.

One possible explanation for the difference is that during the introduction of apple flies from the east, a population founder effect and genetic bottleneck occurred that altered the behavior of western apple flies. A portion of the eastern apple fly population did orient to both the eastern and western apple blends. It is, therefore, conceivable that these flies disproportionately contributed to the establishment of apple flies in the west. An alternative possibility is that western apple-origin flies may not represent an introduction from the east at all, but instead a recent host shift from either black or ornamental hawthorn to apple. In this case, the difference between eastern and western apple flies would result from the independent evolution of behavioral discrimination for apple fruit volatiles from different ancestral hawthorn populations.

Genetic data could help resolve whether eastern and western apple fly populations formed independently in different regions of the country, or represent a single host shift in the East followed by an introduction to the West (McPheron, 1990). For example, it may be that western and eastern fly populations that infest apples and hawthorn possess unique alleles that distinguish them from each other and thus support the independent origins hypothesis. However, it is also possible that black hawthorn-origin flies are native to the west, and apple-origin flies were introduced. In this case, local gene flow from black hawthorn-origin flies could mask the genetic signature of the introduction.

Future behavioral studies of *R. pomonella*'s behavioral responses to natal and non-natal fruit volatile blends also could help clarify the origins of western fly populations, along with their host race status. For example, if western black hawthorn and ornamental hawthorn-origin flies show high levels of upwind flight to the western, but not to the eastern, apple blend comparable to levels they display to their natal hawthorn blends, then this would argue against both western host races and an introduction of apple flies to the Pacific Northwest. Instead, it would suggest that western apple flies arose from a recent host shift from black and/or ornamental hawthorn-origin fly populations. It also is possible that black hawthorn and ornamental hawthorn-origin flies show little interest in both the western and eastern apple blends. Such a finding would be consistent with black hawthorn-origin flies being native and giving rise to the ornamental hawthorn fly population, while western apple flies were introduced. Finally, it is possible that all the western fly populations differ from one another in their response patterns, thus making it difficult to resolve their evolutionary origins, but implying that they represent at least partially ecologically reproductively isolated host races. Regardless, the development of black hawthorn, ornamental hawthorn, and western apple fruit volatile blends now puts us in position to test these hypotheses and further investigate the intriguing story of western *R. pomonella* to assess their evolutionary significance and threat to commercial apple orchards in the Pacific Northwest.

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